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RESPONSES OF THE Na^+/H^+ EXCHANGER OF EUROPEAN FLOUNDER RED BLOOD CELLS TO HYPERTONIC, β-ADRENERGIC AND ACIDOTIC STIMULI

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Summary

The transport pathways mediating regulatory volume increase (RVI) and β-adrenergic responses in red cells of the European flounder *Platichthys flesus* have been investigated. Hypertonic treatment under a low-P_{O_2} atmosphere led to a complete RVI and to a three- to fourfold increase in Na^+ influx. The RVI and the activated Na^+ influx were blocked by the transport inhibitors amiloride and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), both at a concentration of 10^{-4} mol l^{-1}, and the RVI was abolished in a Na^+-free saline, indicating the involvement of a hypertonically induced Na^+/H^+ exchanger and an accompanying Cl^-/HCO_3^- exchanger. Both the hypertonically induced Na^+ influx and the RVI were blocked by oxygenation of shrunk cells. The β-adrenergic agonist isoproterenol also strongly activated a Na^+ influx and caused cell swelling. This response was also inhibited by amiloride and DIDS but was unaffected by oxygenation. Simultaneous application of isoproterenol and hypertonic shrinkage did not lead to additive Na^+ influxes, suggesting that both responses were mediated by the same pool of exchangers. Mild cell acidification activated a Na^+ influx under iso-osmotic conditions; amiloride caused partial inhibition of this influx, but oxygenation had no effect. Acid-induced and isoproterenol-induced Na^+ fluxes were again non-additive. Thus, the Na^+/H^+ exchanger of flounder red cells is strongly activated by three physiological stimuli: hypertonic shrinkage, β-adrenergic hormones and cell acidification. Of these responses, only the first is affected by oxygenation, indicating some differentiation of their respective transduction mechanisms. These characteristics contrast with those of the corresponding exchangers from rainbow trout and eel red cells.

Key words: volume regulation, oxygenation, acid activation, amiloride, Na^+/H^+ exchange, red blood cell, flounder, *Platichthys flesus.*

Introduction

Animal cells are volume osmometers whose volume is subject to aniso-osmotic and solute-driven disturbance. However, disturbance of cell volume generally leads to regulatory responses that, by virtue of an induced net dissipative movement of cell solute and osmotically obliged water, lead to the restoration of cell volume (Hoffmann, 1992; Macknight et al., 1994). Vertebrate red blood cells have been particularly important in characterising the transport systems involved in this important cellular response (Cossins and Gibson, 1997; Sarkadi and Parker, 1991). This is partly because of their relative simplicity, their ease of procurement and the cellular homogeneity of red cell suspensions, but also because they possess a wide variety of transport pathways and the sensitivity of these cells to other modulatory influences, including stress hormones and P_{O_2}.

Volume regulatory solute movements occur through a series of facilitated transport pathways which fall into two functional groups. The first group includes those pathways that promote net solute loss and the regulatory volume decrease response (RVD) following hypotonic swelling. It includes K^+ and Cl^- channels, KCl cotransport and a Na^+-independent taurine pathway. The second group includes the Na^+/H^+ exchanger and the $Na^+/K^+/2Cl^-$ cotransporter, both of which promote net solute uptake and a regulatory volume increase response (RVI) following hypertonic shrinkage. Recent work has suggested that these two groups of transporters are controlled in mutually opposing ways (Cossins, 1991; Parker, 1994). Thus, stimuli that activate RVD effectors tend to inhibit RVI effectors and *vice versa*, giving rise to the concept of ‘reciprocal coordination’. Perhaps the most persuasive evidence in favour of this model comes from studies of dog red cells, which possess a Na^+/H^+ exchanger as the principal RVI effector and a KCl cotransporter as the principal RVD effector (Parker et al., 1990, 1991).

The red cells of lower vertebrates, including teleost fish, have proved to be particularly tractable models for investigating the complex regulation of electroneutral

transporters (Cossins and Gibson, 1997). Trout (*Oncorhynchus mykiss*) red blood cells possess a well-characterised RVD response following osmotic swelling which is mediated by three RVD effectors (Garcia-Romeu et al., 1991; Nielsen et al., 1992). They also possess a powerful Na^+/H^+ exchanger which is stimulated by β-adrenergic agonists (Borgese et al., 1986; Motais and Garcia-Romeu, 1986) but which is unable to mount an effective RVI response (Romero et al., 1996). In contrast, the red blood cells of the winter flounder *Pseudopleuronectes americanus* have long been known to demonstrate a powerful RVI response and a net increase in cellular Na^+ content (Cala, 1977), but the transport pathways involved have not been firmly established. In an attempt to provide a teleost red cell model with a more typical pattern of both RVI and RVD responses, we have therefore re-examined the RVI response in the red cells of the European flounder *Platichthys flesus*, first to identify the transport pathway involved and, second, to determine the controlling effects of other physiological stimuli including P_{O_2}, pH and β-adrenergic stimulation.

Materials and methods

Materials and solutions

Inorganic compounds and D-glucose were purchased from BDH Chemicals Ltd (Poole, Dorset, UK), and *N*-methyl-D-glucamine (NMDG), heparin, amiloride, ouabain, Hepes, furosemide, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), dimethylsulphoxide (DMSO) and (±)-isoproterenol (+)-bitartrate salts were obtained from Sigma Chemical (Poole, Dorset, UK). 5-(*N*-ethyl-*N*-isopropyl)amiloride HCl (EIPA) was obtained from Molecular Probes Inc (Eugene, Oregon, USA).

Standard flounder and trout salines contained 6 $mmol\,l^{-1}$ KCl, 145 $mmol\,l^{-1}$ NaCl, 5 $mmol\,l^{-1}$ glucose, 5 $mmol\,l^{-1}$ $CaCl_2$, 1 $mmol\,l^{-1}$ $MgSO_4$, 10 $mmol\,l^{-1}$ Hepes, pH 7.9 at 20 °C, giving an osmolality of 320 $mosmol\,kg^{-1}\,H_2O$. Preliminary experiments showed that flounder plasma has an osmolality of 323±3.1 $mosmol\,kg^{-1}\,H_2O$ (mean ± S.D., 26 individual fish) and a pH of 7.62±0.02 (19 fish). Eel saline contained 130 $mmol\,l^{-1}$ NaCl with other constituents as for flounder and trout salines; pH was adjusted to 7.85, giving an osmolality of 290 $mosmol\,kg^{-1}\,H_2O$. The isotonic $MgCl_2$ solution used for washing fluxed red cell suspensions contained 110 $mmol\,l^{-1}$ $MgCl_2$, 15 $mmol\,l^{-1}$ Hepes, pH 7.85 at room temperature (20 °C). The low-[Na^+] saline contained 5 $mmol\,l^{-1}$ NaCl, 150 $mmol\,l^{-1}$ NMDG and other components as for normal flounder saline. The Na^+-free saline was made up by replacing NaCl with NMDG. Osmolalities were measured using an Advanced Instruments freezing point depression micro-osmometer (model 3W), and values quoted for red cell suspensions were those determined during each experiment. Stock solutions of isoproterenol (5×10^{-4} $mol\,l^{-1}$), DIDS (0.1 $mol\,l^{-1}$) and amiloride (0.1 $mol\,l^{-1}$) were made up in the appropriate saline immediately before use. The stock solution of ouabain (1 $mol\,l^{-1}$) was made up in DMSO.

Fish and collection of blood

Flounder (*Platichthys flesus*, 0.3–0.4 kg) were trawled from the Dee estuary and maintained in 1000 l thermostatted marine aquaria at 13±1 °C (mean ± range). Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial fish farm (Chirk, North Wales), and eels *Anguilla anguilla* were collected from Lough Neagh, Northern Ireland. Both were maintained in freshwater aquaria, trout at 10±2 °C and eel at 16–22 °C. Blood was obtained from flounder and trout by caudal venipuncture and dilution into the appropriate heparinised saline. Eels were pithed before decapitation, with blood being drained into a beaker containing heparinised eel saline.

The cell suspensions were gently washed three times into more than 10 volumes of the appropriate saline and incubated overnight at 5 °C (Bourne and Cossins, 1983). Immediately before the experiment, the red cells were again washed three times in more than 10 volumes of saline.

Tonometry of cells

As a preliminary to all experiments and to ensure that the cells were in a steady state, suspensions (2–6 ml, haematocrit approximately 30 %) were incubated at 15 °C in Eschweiler tonometers with humidified nitrogen (oxygen-free) for at least 60 min. Except where indicated, a nitrogen atmosphere was rigorously maintained throughout the remainder of the experiment, including during influx determinations. When necessary, oxygenation was achieved by gassing the tonometer with 100 % air. Ouabain was added 5–10 min prior to beginning the experiment at a final concentration of 5×10^{-4} $mol\,l^{-1}$.

Na^+ fluxes

Unidirectional Na^+ influxes were measured at 15 °C using ^{22}Na as a tracer, as described previously (Bourne and Cossins, 1984). Samples (100 μl) of the equilibrated cell suspensions (haematocrit 30 %) were removed from tonometers at appropriate intervals and placed in 900 μl of pre-equilibrated saline, giving a final haematocrit of approximately 3 %. Drugs were also added to these low-haematocrit suspensions. β-Adrenergic activation was achieved by the addition of isoproterenol (0.5×10^{-7} $mol\,l^{-1}$, final concentration unless indicated otherwise), and hypertonic activation was achieved by the addition of 0.1–0.3 volumes of sucrose solution (2 $mol\,l^{-1}$ in the appropriate saline).

Influx measurements were initiated by the addition of ^{22}Na (final activity 0.03–0.05 $MBq\,ml^{-1}$). At the desired times (usually 5 min), triplicate samples (300 μl) were removed to Eppendorf minicentrifuge tubes, washed three times with ice-cold isotonic $MgCl_2$ solution, lysed, deproteinised and the cellular ^{22}Na content was measured by liquid scintillation counting with AquaLuma (Lumac LSC, Groningen, Netherlands) as the scintillation cocktail. For uptake experiments when Na^+ entry was followed for up to 30 min, larger volumes of diluted cell suspensions were used, allowing samples to be drawn at more frequent intervals over the full uptake period. Packed cell volume was determined from the

haematocrit (using the microcapillary method) of the control cell suspension (equilibrated to 100 % nitrogen and the appropriate pH) at the beginning of the experiment. Uptakes over the 5 min period (ouabain-insensitive) were recalculated as mmol $Na^+ l^{-1}$ packed cells h^{-1}, each value being the mean of triplicate samples.

Cell volume determination

Cell volume measurements were obtained from wet and dry mass measurements of packed red cells as described previously (Garcia-Romeu et al., 1991). Cell suspensions with a haematocrit of 3–5 % were concentrated to approximately 30 % by centrifugation at 1000 ***g*** for 1 min. Values are presented as ml g^{-1} dry cell solids. Preliminary experiments demonstrated that flounder red cells behave as near-ideal volume osmometers.

Replication

All experiments reported here have been repeated for at least three separate red cell preparations. Results are reported as means ± S.D., unless stated otherwise, for triplicate determinations within a single representative experiment.

Results

Control cells subjected to the overnight incubation and experimental pretreatment showed sustained Na^+ influxes of 8–30 mmol l^{-1} cells h^{-1} that were substantially inhibited by amiloride, EIPA and furosemide (Table 1). This indicates that the Na^+/H^+ exchanger was active in these cells but at a low level relative to that in activated cells (see below). Oxygenation also inhibited Na^+ influx by approximately 30 % (Table 1), the cells exhibiting a similar degree of inhibition to that induced by amiloride and EIPA under nitrogen.

Table 1. *The effects of oxygenation and transport inhibitors upon the Na^+ influx into control, volume-static flounder red cells*

Condition	Atmosphere	Rate of Na^+ influx (mmol l^{-1} cells h^{-1})	Inhibition (%)
Experiment 1			
Control	N_2	8.1±1.8	
Control	Air	5.6±0.9	29.0±5.1
+Amiloride	Air	3.9±0.1	40.8±0.6 (cf. air control)
Experiment 2			
Control	N_2	7.7±0.8	
+Amiloride	N_2	4.7±0.6	39.2±1.8
+Furosemide	N_2	4.9±1.1	36.6±13.5
+EIPA	N_2	4.3±0.5	44.0±5.0

Values are mean ± S.E.M., *N*=3.

Percentage inhibition was calculated relative to the respective N_2 control value except where specified.

The effect of hypertonic shrinkage upon Na^+ influxes

Fig. 1 shows the behaviour of flounder red cells after a step increase in saline osmolality (from 320 to 750 mosmol kg^{-1} H_2O) following addition of the hypertonic sucrose-containing saline. Fig. 1A shows that after a rapid osmotic shrinkage there was a slower regulatory volume increase (RVI) which restored cell volume to its original, control value after approximately 2 h. Fig. 1B shows, for a different red cell preparation, how unidirectional Na^+ influx varied during this process from measurements taken over 5 min flux periods at each of the indicated times after hypertonic treatment. In control cells held in isotonic saline, the rate of Na^+ influx remained low

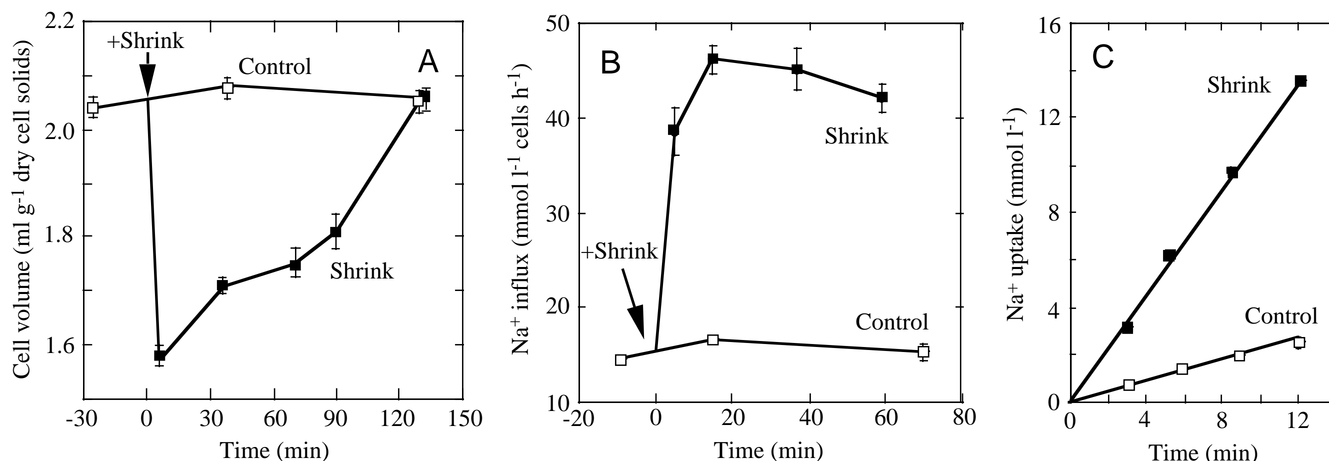

Fig. 1. The effects of hypertonic shrinkage on cell volume (A) and Na^+ influx (B) in flounder red cells. Cells at 30 % haematocrit were pre-equilibrated under a nitrogen atmosphere and ouabain was added (final concentration 5×10^{-4} mol l^{-1}) as described in the Materials and methods section. Samples were then diluted 10-fold into isotonic (control) or hypertonic (Shrink) salines, also under a nitrogen atmosphere. Samples were removed at the indicated intervals for measurements of cell volume (A) and of Na^+ influx (B; 5 min fluxing period). (C) Na^+ uptake experiments for cells suspended in isotonic and hypertonic salines. ^{22}Na was added 2 min after hypertonic treatment, and samples were removed at the times indicated for washing and analysis. Values are means ± S.D. for triplicate determinations for a single experiment that was representative of at least three experiments on different red cell preparations. Where error bars are not shown, they are smaller than the symbols. Control indicates the values for cells held throughout in an isotonic flounder saline.

and constant over the entire duration of the experiment. Hypertonic treatment caused a rapid increase in Na^+ influx that was sustained for the duration of the experiment. Fig. 1C confirms that the kinetics of Na^+ uptake was linear in both control and hypertonically treated cells, justifying the 5 min period for influx determinations used in subsequent experiments.

Variations in hypertonically induced Na^+ influx and RVI

Over the short term (a few weeks), we found reasonable reproducibility in Na^+ fluxes and cell volumes between red cell preparations. Thus, in one series of experiments, the hypertonically induced Na^+ influx was 58.4±14.2 mmol l^{-1} cells h^{-1} (mean ± S.D., 19 experiments) after shrinkage and 15.5±5.8 mmol l^{-1} cells h^{-1} in control cells, an increase of approximately three- to fourfold. The corresponding cell volume of control cells was 1.81±0.22 ml g^{-1} dry cell solids and that of hypertonically treated cells was 1.47±0.17 ml g^{-1} dry cell solids. More recently, we have observed much higher fluxes of up to 250 mmol l^{-1} cells h^{-1}, which might be due to greater levels of oxygenation of the red cell suspensions during the overnight preincubation.

Over the longer term, we have observed some variation in the control influx, in the hypertonically induced influx and in the effectiveness of the RVI response. Fig. 2 summarises the Na^+ influxes measured in flounder caught over the months January to October 1996 and held for up to 4 weeks in the aquarium before sampling of blood. During the winter months, the hypertonically induced influxes were generally high (80–90 mmol l^{-1} cells h^{-1}), whilst during the spring months some individuals demonstrated much lower values. During the summer months, the majority of animals showed low hypertonically induced Na^+ influxes of 20–30 mmol l^{-1} cells h^{-1}. The Na^+ influx measured under isotonic conditions was also reduced such that the increase in Na^+ influx on shrinkage during the month of August (3.31±0.81-fold) was not significantly different from the corresponding value during January (4.05±2.06-fold). These latter red cell preparations showed attenuated RVI responses (data not shown).

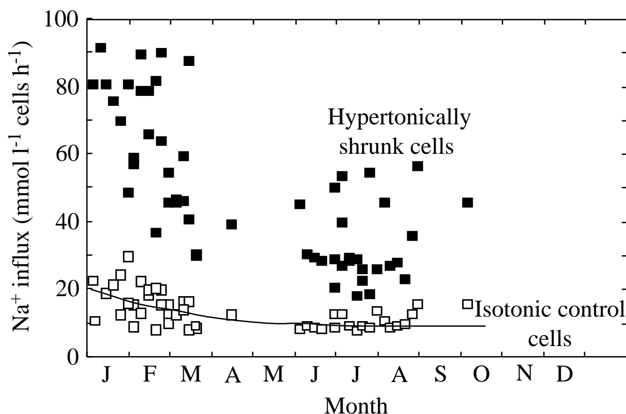

Fig. 2. The seasonal dependence of Na^+ influxes determined under isotonic and hypertonically shrunk conditions. Flounder were caught at intervals from January to October and were held in seawater aquaria for up to 4 weeks. At the indicated times, animals were bled and red cell preparations were prepared as described in the Materials and methods section. Each symbol represents a separate red cell preparation. Other conditions are as for Fig. 1.

Effects of transport inhibitors

The regulatory volume response (RVI) to hypertonic shrinkage was abolished when cells were shrunk in the presence of either amiloride or the anion exchange inhibitor DIDS, both at 10^{-4} mol l^{-1} (final concentration, Fig. 3A). Fig. 3B shows that the addition of either drug 10 min after hypertonic treatment caused a large reduction in the hypertonically induced Na^+ influx. The RVI was also abolished when cells were suspended in Na^+-free saline (Na^+ replaced with *N*-methyl-D-glucamine; data not shown).

Oxygenation-sensitivity of Na^+/H^+ exchange in flounder red cells

Regulatory volume decrease in trout red cells is heavily influenced by altered P_{O_2} as a result of effects on the KCl cotransporter (Nielsen, 1997; Nielsen et al., 1992), and this observation has prompted similar experiments on RVI in flounder red cells. Although flounder red cells maintained continuously under a nitrogen atmosphere displayed an RVI response (Fig. 4A), cells transferred immediately after shrinkage to a separate tonometer and rapidly equilibrated under an air atmosphere were unable to regain cell volume. Fig. 4B shows the corresponding changes in Na^+ influx. Hypertonic treatment under nitrogen resulted in a rapid increase in Na^+ influx, which decreased by only 10 % over 60 min. In contrast, shrunken cells that were oxygenated 10 min after hypertonic treatment showed low Na^+ influxes, below those of control cells. Oxygenation therefore inhibited the hypertonically induced and active Na^+ influxes, and the fact that the RVI is blocked by air indicates that activation of the Na^+/H^+ exchanger is blocked. We have also found that Na^+ influx in control cells was reduced by approximately 30 % on transfer to an air atmosphere (Table 1).

β-Adrenergic stimulation of flounder red cells

Addition of the β-adrenergic agonist isoproterenol to flounder red cells under isotonic conditions caused a substantial and continuing increase in cell volume (Fig. 5A) and a large increase in Na^+ influx (Fig. 5B). Like the hypertonically induced Na^+ influx and RVI response, both responses were inhibited by DIDS and by amiloride. Fig. 5C shows the dose–response curve for the stimulation of Na^+ influx by isoproterenol with an EC_{50} of approximately 8×10^{-8} mol l^{-1} and maximal responses above approximately 5×10^{-7} mol l^{-1}.

Fig. 6A shows that adrenergically induced cell swelling continued under an air atmosphere, albeit at a slightly lower level than under nitrogen. Fig. 6B shows that isoproterenol-stimulated Na^+ influx was only reduced by approximately 20 % on oxygenation. In contrast, the hypertonically induced Na^+ influx was almost completely abolished on oxygenation. Evidently, these two modes of Na^+/H^+ exchanger stimulation have quite different sensitivities to P_{O_2}.

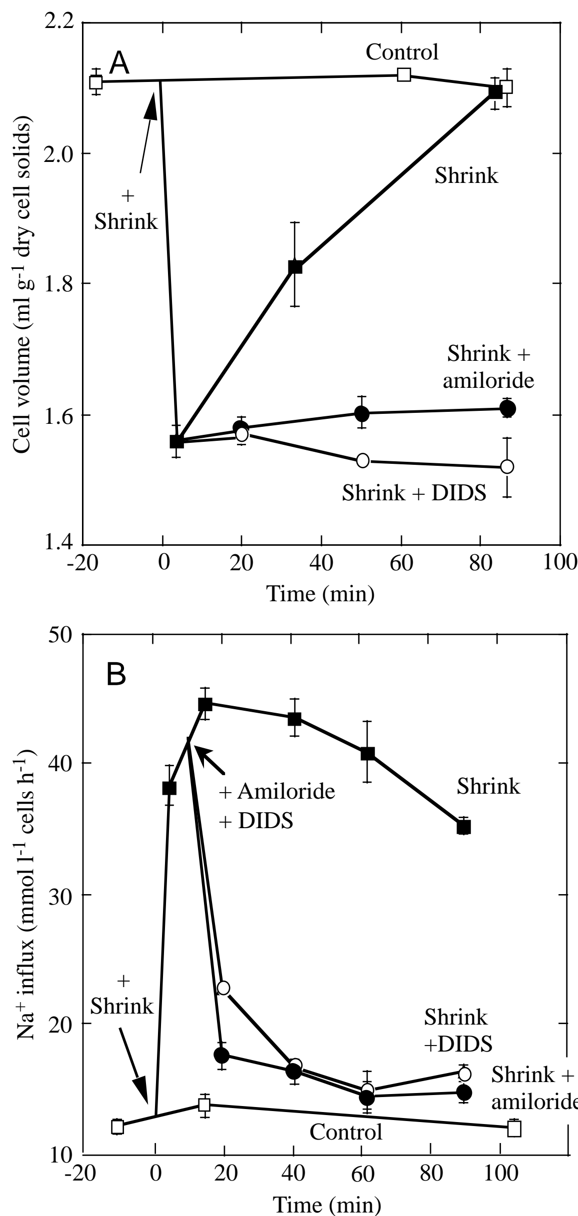

Fig. 3. The effects of DIDS and amiloride upon the regulatory volume increase (RVI) response (A) and the hypertonically induced Na^+ influx (B) of flounder red cells. Drugs (final concentration 10^{-4} mol l^{-1}) were added prior to the 10-fold dilution of cells into isotonic and hypertonic salines. Other conditions were as described in Fig. 1.

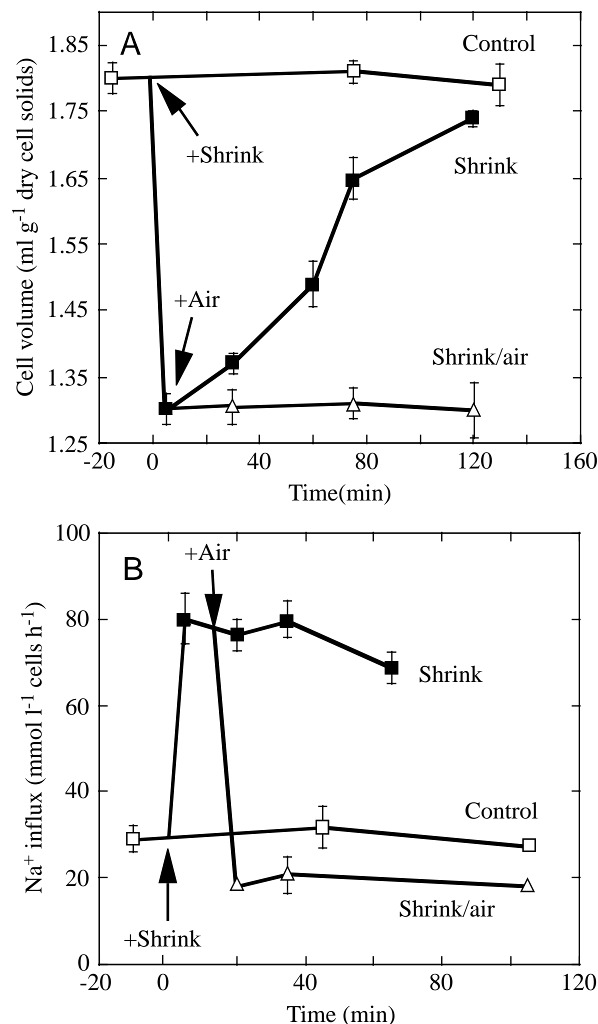

Fig. 4. The effects of oxygenation upon the regulatory volume increase (RVI) and Na^+ influx in hypertonically shrunk flounder red cells. (A) The time-dependence of cell volume in cells that had been hypertonically (480 mosmol kg^{-1}) shrunk. (B) A separate experiment showing the corresponding changes in Na^+ influx. In both A and B, cells were pretreated as described for Fig. 1. Control isotonic and control hypertonically treated cells were maintained under nitrogen throughout. Some of the latter cells were transferred immediately (A) or 10 min (B) after hypertonic treatment to a separate tonometer under an air atmosphere. Samples were removed for cell volume and Na^+ influx determinations at the times indicated. Values are means ± S.D., N=3.

Because the Na^+/H^+ exchanger of flounder red cells is clearly stimulated by both hypertonic shrinkage and β-adrenergic agonists, an obvious question is whether these stimuli activate the same pool of exchanger proteins. Fig. 7A addresses this question by comparing the activity of the exchanger in the presence of both stimuli compared with each acting alone. The maximal exchanger activity immediately after hypertonic stimulation was approximately 60 % of that in the presence of isoproterenol. Activity in the simultaneous presence of both stimuli at this time was slightly less than that in the presence of isoproterenol alone.

Isoproterenol causes a rapid cell swelling in the usual high-[Na^+] saline. This might offset the shrinkage caused by hypertonic treatment and reduce or prevent the shrink-induced response. We have therefore studied the interactions between the two forms of stimulation at low extracellular [Na^+] (5 mmol l^{-1} Na^+, Na^+ replaced with NMDG) when the isoproterenol-induced cell swelling was prevented and the shrinkage caused by hypertonically induced shrinkage was unaffected (data not shown). Fig. 7B shows that co-activation again caused a slight reduction in flux compared with the flux in the presence of isoproterenol alone rather than an additive increase. Taken together, these observations at both high and

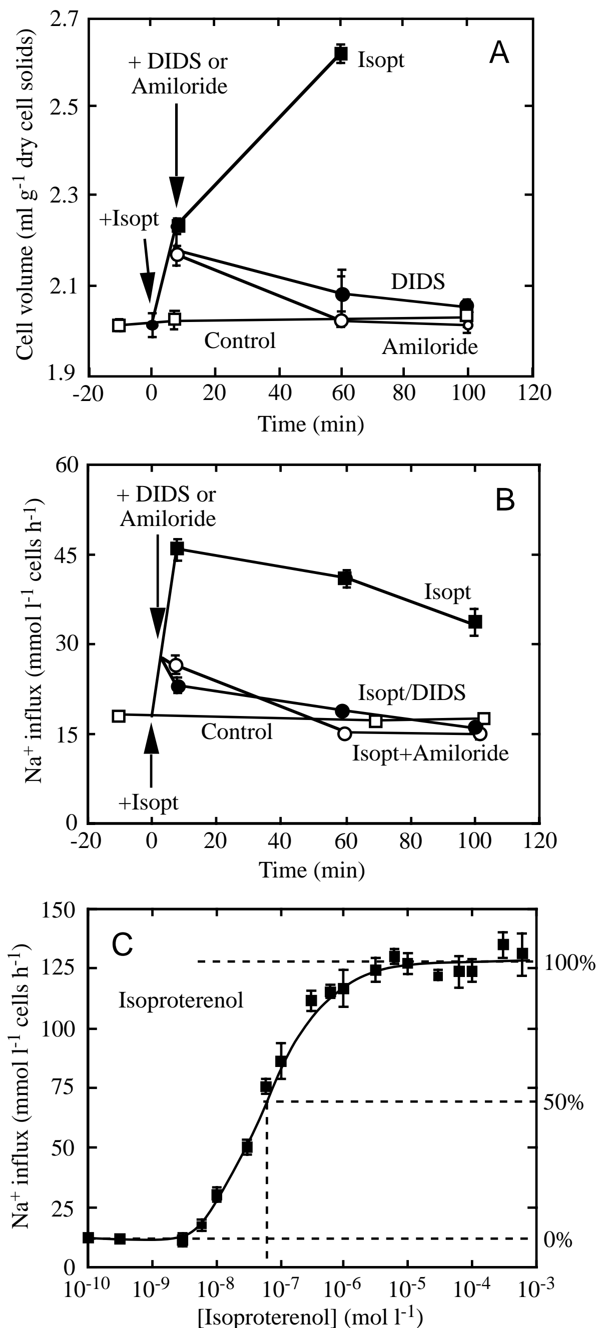

Fig. 5. The effects of the β-adrenergic agonist isoproterenol (Isopt) (5×10^{-7} mol l^{-1}) on cell volume (A) and Na^+ influx (B) in flounder red cells. Cells were equilibrated under a nitrogen atmosphere and pretreated with ouabain as described for Fig. 1. Isoproterenol was added at time zero. After 10 min, samples of the suspension were transferred to separate tonometers and either DIDS or amiloride (final concentrations 10^{-4} mol l^{-1}) was added as indicated. (C) The dose–response relationship for isoproterenol on Na^+ influx. Influxes were determined over 5 min periods initiated exactly 10 min after dilution of pre-equilibrated cells into a saline containing the indicated isoproterenol concentration. The entire experiment was performed under a nitrogen atmosphere. Values are means ± S.D. for triplicate determinations. Data are shown for single experiments that were representative of three similar experiments on different cell preparations.

low [Na^+] demonstrate that the Na^+/H^+ exchange activities observed on co-activation were not additive.

pH-sensitivity of Na^+/H^+ exchange in flounder red cells

Slight acidification of red cells in an isotonic saline and under a nitrogen atmosphere substantially increased the Na^+ influx (Fig. 8A). The activation occurred between pH 7.55 and 7.37 and remained high with further acidification down to pH 7.0. Below this pH, the Na^+ influx decreased to control levels. This acid-induced Na^+ influx (that is, the influx in acid conditions minus the control flux in normal saline) was inhibited by 70±2 % by amiloride (six experiments), by 70±14 % by EIPA (three experiments) and completely by furosemide (Fig. 8B). We have found that acidification of

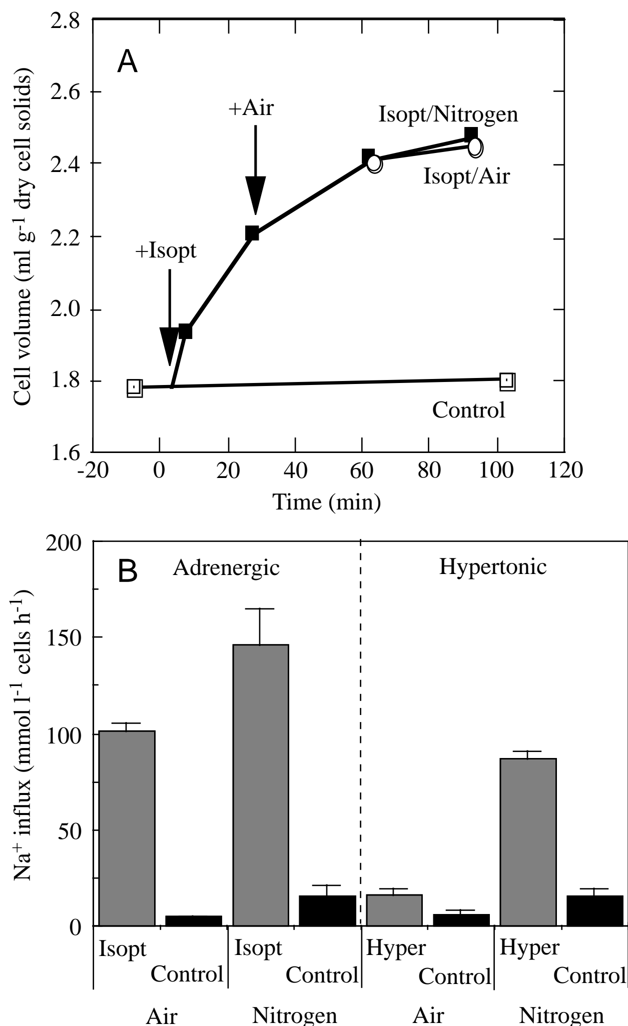

Fig. 6. The effects of oxygenation upon the adrenergically stimulated increase in cell volume (A) and Na^+ influx (B). In A, a portion of the adrenergically stimulated suspension was transferred 30 min after the addition of agonist from a nitrogen atmosphere to an air atmosphere. (B) A comparison of the effects of oxygenation upon the Na^+ influx stimulated by hypertonic treatment (500 mosmol kg^{-1}) and isoproterenol (Isopt) (final concentration 10^{-5} mol l^{-1}). Values are means + S.E.M. of triplicate measurements. Red cells were pretreated as described in Fig. 1.

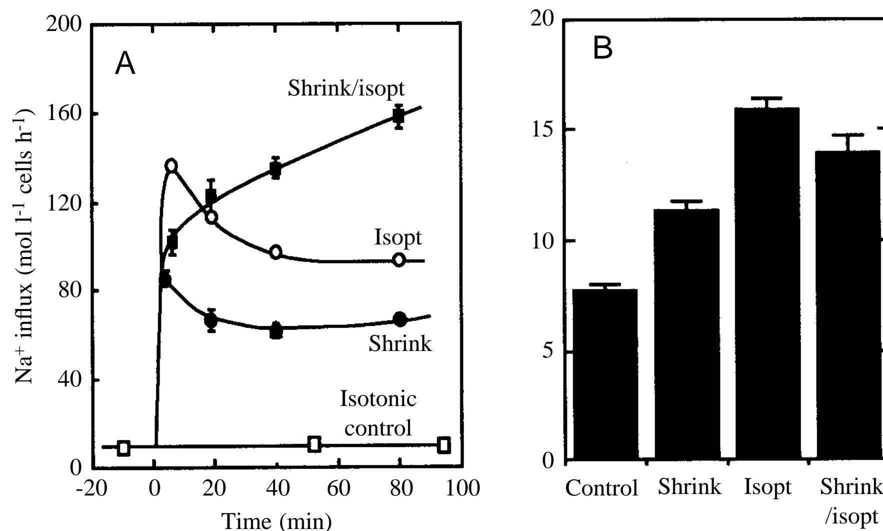

Fig. 7. A comparison of Na^+ influx in flounder red cells following hypertonic and β-adrenergic treatment applied separately and in combination. (A) The time-dependence of Na^+ influx into red cells in normal flounder saline exposed to these different stimuli. The experiment was performed in the standard flounder saline (145 mmol l^{-1} NaCl). (B) A comparison of the effects of hypertonic and β-adrenergic treatment on the rate of Na^+ influx (measured over 5 min) in red cells incubated in a low-[Na^+] saline (5 mmol l^{-1}). Isotope influx was initiated 4 min after the addition of the different stimuli (hypertonic shrink, 500 mosmol kg^{-1}; isoproterenol, 5×10^{-7} mol l^{-1}). Red cell suspensions were pretreated as described in Fig. 1 and maintained under a nitrogen atmosphere throughout the experiment. These results are typical of three other experiments. Data points represent mean ± S.D. of triplicate measurements. Isopt, isoproterenol.

flounder red cells caused an increase in cell volume and an increase in net Na^+ uptake; thus, 15 min after acidification, cell volume had increased from a control value of 1.8 to 2.2 ml g^{-1} dry cell solids and intracellular [Na^+] had increased from 15 to 30 mmol g^{-1} dry cell solids.

The additivity of acid- and isoproterenol-induced Na^+ influx is also addressed in Fig. 8A. At pH values greater than 7.5, isoproterenol caused the expected activation of Na^+ influx above a low control value. Addition of isoproterenol below pH 7.5 caused no further increase in Na^+ influx above the high

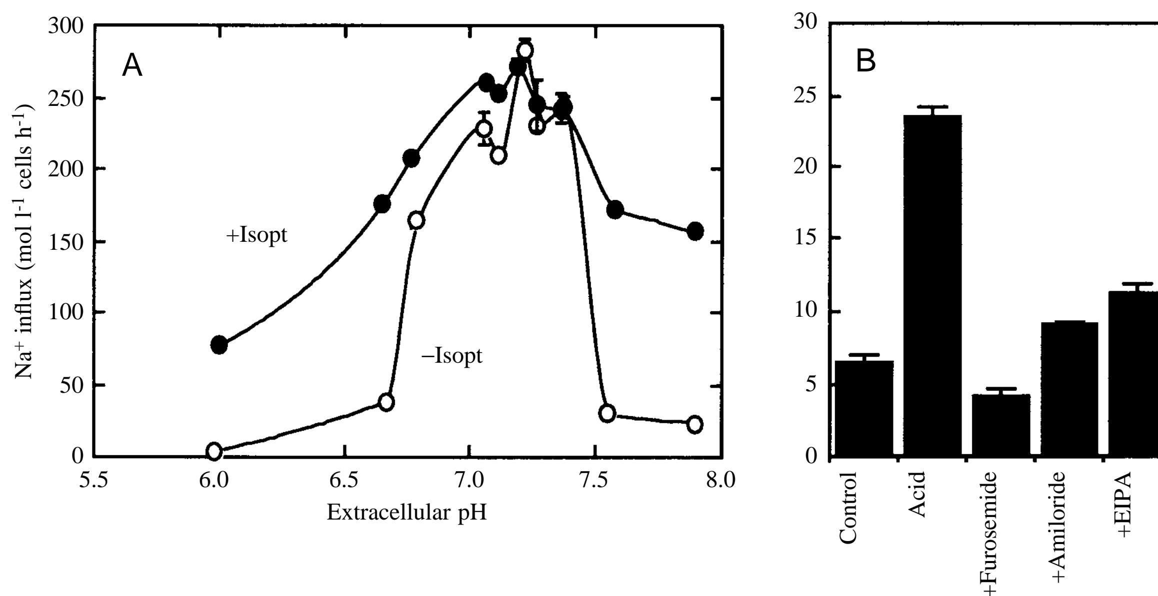

Fig. 8. Acid-induced Na^+ influx into flounder red cells. (A) The pH-dependence of Na^+ influx into flounder red cells in isotonic flounder saline in the presence (filled circles) and absence (open circles) of isoproterenol (Isopt) (10^{-5} mol l^{-1}). Cell suspensions (haematocrit 30 %) were pre-equilibrated under a nitrogen atmosphere and pretreated with ouabain as described in the Materials and methods section and held under nitrogen throughout the experiment. Cells were diluted 10-fold into acidified Na^+-containing salines, and after 4 min the Na^+ influx assay was initiated (5 min flux period). The pH of each diluted suspension was determined prior to the addition of ^{22}Na. (B) The sensitivity of the acid-induced Na^+ influx (pH 7.25) to the membrane transport inhibitors furosemide (10^{-4} mol l^{-1}), amiloride (10^{-5} mol l^{-1}) and EIPA (10^{-5} mol l^{-1}). Cells were washed three times into a saline containing 5 mmol l^{-1} NaCl and 150 mmol l^{-1} NMDG, and preincubated for 10 min in the presence of the respective inhibitor before the addition of isotope (5 min flux period). Values are means ± S.D. for triplicate influx determinations for a single preparation. The low Na^+ concentration in B accounts for the low influx values compared with those in A. The results are typical of three similar experiments using different cell populations.

value recorded in the absence of the agonist. However, below pH 6.8 when the Na^+ influx was low, addition of isoproterenol caused a substantial activation.

Hypertonic and adrenergic responses in trout and eel red cells

Fig. 9 shows the effects of hypertonic treatment on the red cells of trout (Fig. 9A,B) and eel (Fig. 9C,D). Exposure of trout red cells to 480 mosmol kg^{-1} saline produced no perceptible RVI response (Fig. 9A) and Na^+ influx was unaffected (Fig. 9B). However, a substantial increase in Na^+ influx could be stimulated by addition of the β-adrenergic agonist isoproterenol. In contrast, hypertonic treatment of eel red cells led to a substantial RVI response (Fig. 9C) and an increase in Na^+ influx (Fig. 9D), but isoproterenol had no effect (not shown).

Discussion

Hypertonically induced responses in flounder red blood cells

We show here that, like the winter flounder *Pseudopleuronectes americanus* (Cala, 1977), the red cells of the European flounder *Platichthys flesus* respond to hypertonic shrinkage with a restorative increase in cell volume. Cala (1977) originally showed that the RVI in the winter flounder was linked with an increase in cellular Na^+ and Cl^- content and interpreted this as indicating an enhanced influx of Na^+. We confirm in European flounder that the RVI was associated with a three- to fourfold increase in Na^+ influx. This influx remained elevated for up to 90 min, during which time the cells underwent a regulatory volume increase due to the substantial net uptake of Na^+.

The cells used in our experiments were ouabain-treated, obviating any role for the Na^+/K^+ pump in the observed responses. However, the RVI and the hypertonically activated Na^+ influx were inhibited by the transport inhibitors amiloride and DIDS, and the RVI was absent in a Na^+-free saline. These characteristics are consistent with the activation of a Na^+/H^+ exchanger which, in concert with a constitutive anion exchanger, leads to a net uptake of both Na^+ and Cl^-. In these respects, the hypertonic responses of flounder red cells are identical to the well-known β-adrenergic response of trout red cells. However, Romero et al. (1996) have shown that trout red cells have a greatly reduced hypertonic response, and in our experiments we were unable to detect any RVI response or activation of Na^+ influx following hypertonic treatment.

In flounder red cells under volume-static isotonic conditions, the Na^+ flux pathway was larger than that observed in rainbow trout (Borgese et al., 1987) and carp *Cyprinus carpio* (Orlov

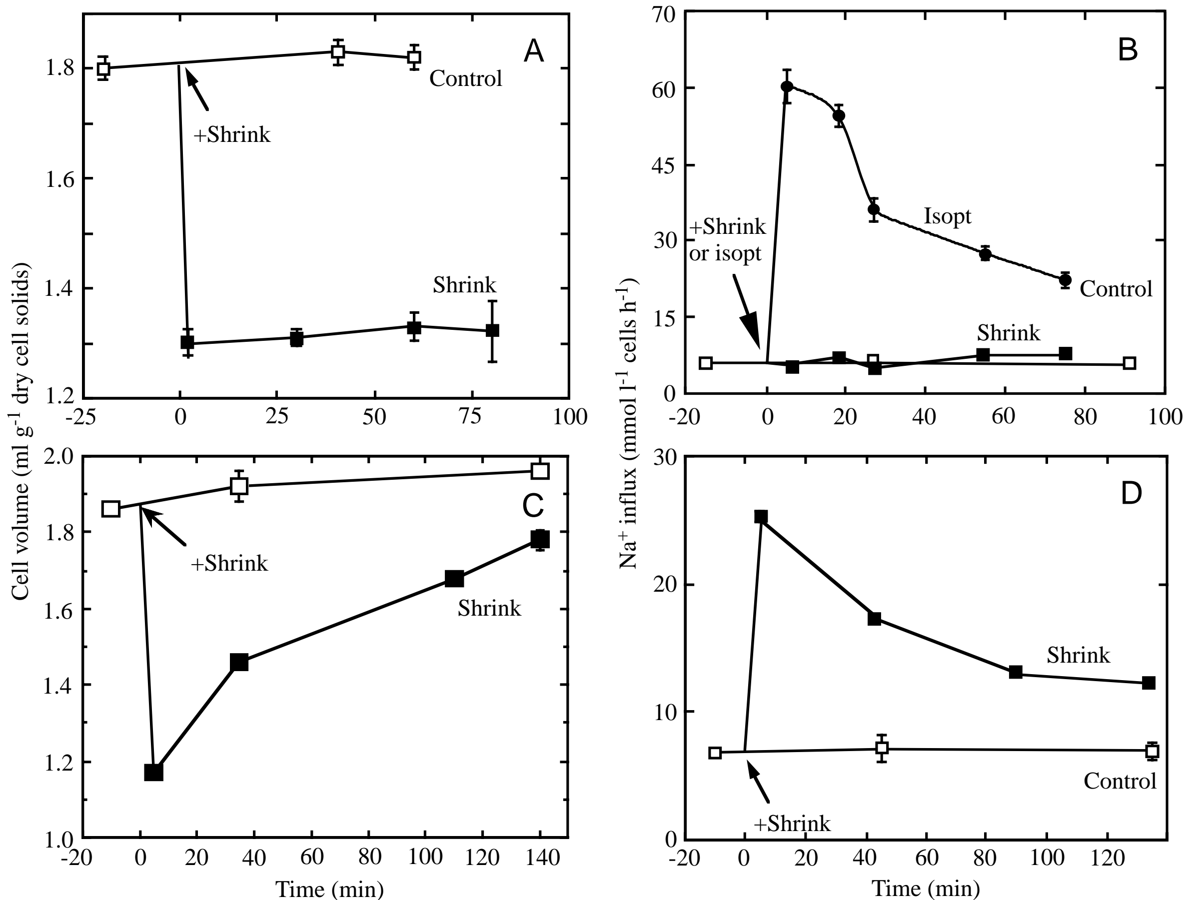

Fig. 9. The effects of hypertonic treatment (480 mosmol kg^{-1}) and β-adrenergic stimulation (5×10^{-7} mol l^{-1} isoproterenol) (Isopt) upon the cell volume and Na^+ influx into red cells of rainbow trout *Oncorhynchus mykiss* (A,B) and eel *Anguilla anguilla* (C,D). Cell suspensions were held under nitrogen throughout. Preincubation and other conditions are as described for Fig. 1. The Na^+ influx was determined over 5 min periods at each of the indicated times.

and Skryabin, 1993). Amiloride inhibited one-third of the isotonic Na^+ influx, which is consistent with a small but measurable Na^+/H^+ exchanger activity. Furosemide also inhibited this flux by approximately 30 %, which may be a result of this drug affecting the anion exchanger and indirectly reducing net movement of NaCl. Alternatively, it may be due to effects on an additional facilitated transport pathway. Indeed, trout red cells possess a swelling-induced and furosemide-sensitive Na^+ influx (Bourne and Cossins, 1984) that appears to be mediated by the Cl^--independent K^+ flux pathway.

The RVI was usually complete in that cell volume was restored to that observed immediately before treatment. However, on some occasions, less than complete responses were observed and in a few cases no response was observed. We also found considerable inter-preparation variation in stimulated Na^+ influxes; fluxes were usually in the range 80–150 $mmol\,l^{-1}\,cells\,h^{-1}$, although fluxes as low as 20–30 $mmol\,l^{-1}\,cells\,h^{-1}$ and as high as 250 $mmol\,l^{-1}\,cells\,h^{-1}$ were measured. Some of this variation might may be accounted for by variations in P_{O_2} during the overnight pre-incubation (K. Kiessling, unpublished observations) or by other unidentified procedural variations. We have not correlated these variations in Na^+ influx with RVI or β-adrenergic responsiveness.

This prompted a more systematic study of seasonally linked variations in shrink-induced Na^+ influxes over the annual cycle. During the winter months, we found that the hypertonically induced Na^+ influxes were substantially greater than during the early summer months, and this was linked to somewhat reduced or absent RVI responses during the summer. This apparently seasonal variation in sensitivity to hypertonic conditions brings to mind the seasonal pattern observed previously in rainbow trout in respect of the β-adrenergically activated Na^+/H^+ exchanger (Cossins and Kilbey, 1989), except that in trout the phase of low exchanger activity was observed during the late winter months. The reasons for this different seasonal pattern are not clear but may be related to the reproductive cycle rather than to seasonal variations in temperature (Cossins and Kilbey, 1989). The Dee estuary flounder used in the present work breed between March and May and are in comparatively poor physiological condition thereafter. In contrast, the rainbow trout used in our previous work breed in November. The decline in Na^+/H^+ exchanger responsiveness in both flounder and trout therefore corresponds more closely with the post-breeding period rather than with a consistent season or with environmental conditions.

β-Adrenergically induced responses in flounder red cells

The β-adrenergic response of trout red cells is characterised by the rapid activation of a powerful Na^+/H^+ exchanger that leads to increases in cell Na^+ and Cl^- content, an increase in intracellular pH (pHi) and a significant iso-osmotic cell swelling (Motais et al., 1992). This response is also fully inhibited by amiloride and DIDS (Borgese et al., 1986, 1987; Cossins and Richardson, 1985), with Na^+ influx showing a slow deactivation immediately after achieving maximal activity (Garcia-Romeu et al., 1988). Motais and colleagues (Borgese et al., 1987; Motais et al., 1992) have demonstrated in trout that this deactivation is caused by the conversion of exchangers to an inactive, refractory state that is no longer sensitive to adrenergic activation.

We show here that the red cells of the European flounder possess an identical β-adrenergic response to that of the trout in that cell enlargement follows the increase in Na^+ influx and these responses are blocked by amiloride, DIDS and a Na^+-free saline and partially by furosemide. Influx of Na^+ was increased rapidly to a maximal value followed by a slower but progressive reduction in influx that corresponded with the deactivation observed in trout red cells. Thus, the responsiveness of the flounder Na^+/H^+ exchanger to hypertonic shrinkage and the expression of the RVI response are not at the expense of its sensitivity to β-adrenergic stimulation. β-Adrenergic stimulation of European flounder red cells causes an increase in intracellular cyclic AMP concentration and proton flux (Thoroed et al., 1995), consistent with the presence of Na^+/H^+ exchange. β-Adrenergic stimulation appears to enhance the RVD response mediated by a Na^+-independent taurine pathway.

The relationship between the β-adrenergically activated and hypertonically activated Na^+/H^+ exchangers has been addressed in co-activation experiments. We show that hypertonic treatment of cells maximally activated with isoproterenol did not lead to any additive increase in Na^+ influx, suggesting that the same pool of exchangers mediated both these responses. Hypertonic treatment did, however, modify the response to β-adrenergic stimulation in two specific respects: (1) the maximal exchanger activity observed soon after the addition of isoproterenol was consistently reduced by 10–20 %, and (2) the progressive slow deactivation of Na^+ influx that is normally observed was prevented. The β-adrenergically and hypertonically activated Na^+ influxes in flounder differ in one other respect, namely in the presence after β-adrenergic stimulation of a noticeable deactivation immediately following activation and the absence of such a deactivation after hypertonic stimulation. This presumably reflects differences in the control of the exchangers under the two forms of stimulation.

Acid-induced activation of the Na^+/H^+ exchanger

One of the most important and widely recognised functions of the Na^+/H^+ exchanger in mammalian cells is the regulation of intracellular pH (pHi) (Pouyssegur, 1994). In non-epithelial cells, the exchanger is quiescent at physiological pHi but becomes rapidly and powerfully activated upon cytosolic acidification. Recovery of pHi after acid-loading of the cytoplasm requires extracellular Na^+ and is amiloride-sensitive, which is consistent with the involvement of the Na^+/H^+ exchanger (Bianchini and Pouyssegur, 1994; Wakabayashi et al., 1997; McKenzie and Pouyssegur, 1996; Pouyssegur, 1994; Tse et al., 1994). Net flux was linearly and negatively related to pHi values that lie just below those normally observed in undisturbed cells, which is thought to represent the influence of an intracellular titratable residue on the exchanger or associated regulatory protein (Grinstein and Rothstein, 1986). This regulation of pHi is regarded as a 'housekeeping' function. In contrast, the β-adrenergically activated Na^+/H^+ exchanger of trout red cells is relatively unresponsive to cytosolic acidification; acid activation

occurs only below an extracellular pH of approximately 6.6, and Na^+ influx was less than 20 % of that observed with adrenergic stimulation (Guizouarn et al., 1993).

We show here that the Na^+ fluxes of flounder red cells are considerably more responsive to extracellular acidification than those of trout red cells. The exchanger was substantially activated by a small reduction in extracellular pH below 7.5, the acid-induced Na^+ influx being somewhat larger than that observed following β-adrenergic stimulation at normal saline pH. This flux had a bell-shaped, biphasic dependence upon extracellular pH similar to that seen in trout red cells under β-adrenergic stimulation (Motais et al., 1987). The acid-induced Na^+ influx was partially inhibited by amiloride but completely inhibited by furosemide, which contrasts with the isoproterenol-induced flux, which is completely inhibited by amiloride and only partially inhibited by furosemide. Furosemide may have effects on several membrane transporters, including the KCl cotransporter (Bourne and Cossins, 1984) and the Cl^-/HCO_3^- exchanger, and the different furosemide sensitivities of the acid- and isoproterenol-activated Na^+ influxes may indicate some differences in the pathways responsible. However, application of isoproterenol under these acid-stimulated conditions failed to increase Na^+ influx further, a lack of additivity that is inconsistent with the existence of separate pools of transporter.

Acid activation leads to a net uptake of Na^+ and a consequent cell volume increase, which is consistent with the involvement of the Na^+/H^+ exchanger. If so, the ability to move protons might endow flounder red cells with the ability to regulate pHi or at least to reduce the dependence of pHi upon extracellular pH. This response would be a novel means of regulating pHi in red cells and, in contrast to the well-known β-adrenergic mechanism, it would be a homeostatic response operating purely at the cellular level.

Controlling effect of P_{O_2} upon activation of the Na^+/H^+ exchanger

Previous work has shown that the RVD response of trout red cells is sensitive to P_{O_2} in that the full response can only be recorded at high P_{O_2} and is abolished at low P_{O_2} (Borgese et al., 1991; Nielsen, 1997; Nielsen et al., 1992). This effect is attributed to an interaction between haemoglobin and the cytosolic domain of the band 3 anion exchange protein (Borgese et al., 1991; Garcia-Romeu et al., 1996), although definitive evidence is lacking. The RVD response is mediated by at least three pathways: by a Na^+-independent taurine transporter, by a KCl cotransporter and by coupled Cl^- and K^+ channels, all of which lead to dissipative net losses of cellular solute together with osmotically obliged water. Of these pathways, only KCl cotransport is altered by variations in P_{O_2} (Borgese et al., 1991; Nielsen et al., 1992), and similar effects have been observed in red cells of carp *Cyprinus carpio* (Jensen, 1995), frog *Rana ridibunda* (Kaloyianni and Rasidaki, 1996), horse (Honess et al., 1996) and humans (Canessa et al., 1987).

The β-adrenergically activated Na^+/H^+ exchanger of trout red cells is moderately affected by P_{O_2} but in the opposite manner to the KCl cotransporter, with approximately twofold greater net Na^+ influxes under a nitrogen atmosphere than under an air atmosphere (Motais et al., 1987). Again this effect has been attributed to a haem-binding protein since carbon monoxide has the same effect as oxygen (Motais et al., 1987). The Na^+/H^+ exchanger of carp red cells is more significantly affected by P_{O_2} since normal adrenergic responses are not recorded at atmospheric oxygen tensions (Salama and Nikinmaa, 1988) unless extracellular pH is lowered to non-physiological values. This may also be true of tench (*Tinca tinca*) red cells (Jensen, 1987).

Table 2. *The stimulus specificities of the Na^+/H^+ exchanger of red cells from teleost fish species*

Feature	Rainbow trout *Oncorhynchus mykiss*	Flounder *Platichthys flesus*	Carp *Cyprinus carpio*	Eel *Anguilla rostrata*
β-Adrenergic activation	+	+	+	−
Hypertonic activation	− (small or none)	+	+	+
Acid activation	−	+	?	?
Oxygenation inhibition	+ (adrenergic)	+++ (hypertonic)	+++	?

+ indicates the presence of a response, − indicates the known absence of response and ? indicates not known.

We show here in flounder red cells that the hypertonically activated Na^+/H^+ exchanger is also expressed only under conditions of low P_{O_2}. An increase in P_{O_2} to atmospheric levels immediately after hypertonic treatment inhibited the exchanger and completely blocked the expression of the normal RVI response. In contrast, the β-adrenergically induced swelling was evident under an air atmosphere as well as under nitrogen, although exchanger activity was slightly reduced. The effect of P_{O_2} therefore differentiates two separate transduction pathways (adrenergic and hypertonic) which activate the same pool of Na^+/H^+ exchangers. Oxygenation causes a cell shrinkage of approximately 10 % due to changes in the Donnan distribution of Cl^- (Borgese et al., 1991) and this, together with a possible change in volume set point, may account for the loss of hypertonic activation. However, this is unlikely since oxygenation block occurs under conditions of extreme hypertonic shrinkage (Y. R. Weaver, unpublished observations).

We have confirmed that the KCl cotransporter of flounder red cells is activated by increases in P_{O_2} (Y. R. Weaver, unpublished observations) as observed in trout red cells (Nielsen et al., 1992). The opposing effects of P_{O_2} upon the RVI and RVD effectors is consistent with growing evidence of linked but opposing regulation of these transporters, as encapsulated within the concept of ‘reciprocal control’ (Cossins, 1991; Cossins et al., 1997; Parker, 1994). However, although the KCl cotransporter is activated simply by a change in P_{O_2}, the Na^+/H^+ exchanger in flounder red cells was unaffected. Thus, in the latter case, deoxygenation acts

permissively in allowing the exchanger to be activated on application of a volume but not an β-adrenergic stimulus.

Comparative aspects of Na^+/H^+ exchanger expression

The growing amount of information on teleost Na^+/H^+ exchangers allows some speculations regarding the phylogenetic origins of transporter responses. Table 2 summarises the principal differences in the responses of trout, carp, flounder and eel exchangers to the principal physiological stimuli and to P_{O_2}. We expect that animal cells would primitively have exhibited both RVI and RVD responses in order to maintain a constant cell volume in the face of a variable osmotic environment. The Na^+/H^+ exchanger is also a major component of the regulation of intracellular pH; this again is likely to be a 'housekeeping' function present in ancestral animal cells. The human NHE1 isoform has these characteristics (Bianchini et al., 1995; Yun et al., 1995) and, of all the teleost species, the red cells of the eel most closely match this condition (Romero et al., 1996). Interestingly, the red cells of the lamprey *Lampetra fluviatilis* possess a Na^+/H^+ exchanger that is strongly stimulated by acidification and to a lesser extent by hyperosmotic shrinkage (Virkki and Nikinmaa, 1994), although only the former leads to net movement of Na^+.

The β-adrenergic response is likely to be a more recent evolutionary development linked to the development of oxygen secretion mechanisms in the swimbladder and choroid retia and of haemoglobins demonstrating a Root effect. In rainbow trout, this response influences red cell pHi during extracellular plasma acidosis or during environmental hypoxia, and in both cases haemoglobin function is conserved or enhanced (Nikinmaa and Boutilier, 1995). Flounder red cells correspond to this condition in that the volume-sensitive Na^+/H^+ exchanger that mediates RVI and is acid-induced is also activated β-adrenergically. Adoption of β-adrenergic responsiveness is evidently not at the expense of hypertonic responsivity; the two responses may co-exist.

The red cells of rainbow trout have a greatly reduced or absent RVI response which, given the proposed primitive status of the hypertonic response and RVI, appears to represent a secondary reduction or loss of volume-sensitivity of the trout β-adrenergically activated Na^+/H^+ exchanger. However, the physiological consequences of reduced hypertonic responsiveness in rainbow trout have not been addressed and, conversely, the presumed physiological benefits of red cell RVI responses in the lives of other species have not been clearly demonstrated. In any case, just how widespread the rainbow-trout-type condition is within teleost fish, or even within salmonid fish, is not clear. The red cells of the brown trout *Salmo trutta* are reported to show significant hypertonically activated Na^+ fluxes (Orlov et al., 1994), suggesting that even closely related species might have different stimulus specificities.

Evolutionary interpretations of Na^+/H^+ exchanger regulation in extant teleosts are thus inextricably linked to the evolutionary development of haemoglobins demonstrating a Root effect and of the retia of the swimbladder and choroid plexus. Yet the eel has a well-developed swimbladder rete and its haemoglobin exhibits a strong Root effect (Forster and Steen, 1969; Krogh, 1924). Eel red cells may therefore have suffered a secondary loss of β-adrenergic sensitivity by the Na^+/H^+ exchanger, despite the puzzling presence of β-adrenoreceptors (Perry and Reid, 1992). Alternatively, red cell adrenoresponsiveness might have evolved in the euteleost groups after they had diverged from the more primitive Anguilliformes. Clearly, a proper understanding of the physiological significance and evolution of red cell adrenoresponsiveness requires a much more complete picture of the phylogenetic distribution of both adreno- and hypertonic-sensitivities across the teleost fish.

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