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THE TEMPERATURE DEPENDENCE OF THE ADRENERGIC Na^+/H^+ EXCHANGER OF TROUT ERYTHROCYTES

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Summary

The effects of temperature upon the adrenergic Na^+/H^+ exchange of rainbow trout erythrocytes have been studied *in vitro*. The initial rates of H^+ ejection and of increase of intracellular Na^+ ($[Na^+]_i$) in adrenergically stimulated cells were highly temperature-dependent, with apparent Arrhenius activation energies of 112.8±10.0 (mean±S.D., N=4) and 84.1±3.0 kJ mol^{-1} (N=3), respectively. The steady-state $[Na^+]_i$ following stimulation decreased progressively with cooling, whilst the time required for $[Na^+]_i$ to return to control values after removal of agonist was greatly increased. The change in intracellular pH resulting from adrenergic stimulation was reduced by cooling, such that at 4°C adrenergic responses were barely measurable. The effect of temperature upon the steady-state $[Na^+]_i$ and pHi was probably caused by a disparity in the temperature dependence of the transport mechanisms that contribute to the respective steady states.

Introduction

Adrenergic agonists induce a rapid increase in intracellular Na^+ concentration of trout erythrocytes from approximately 10 mmol l^{-1} packed cell volume (pcv) to a new steady state of 50–80 mmol l^{-1}. This is coupled to the ejection of H^+ from the cell and so constitutes a Na^+/H^+ exchange mechanism with a stoichiometry of 1 (Baroin *et al.* 1984; Cossins and Richardson, 1985; Motais and Garcia-Romeu, 1988). The resulting efflux of H^+ disturbs the HCO_3^- distribution away from its electrochemical equilibrium and induces a net flux of HCO_3^-, *via* the fast anion exchange mechanism, in exchange for Cl^-. Thus, there is a net loss of H^+ and HCO_3^- from the cell and a gain of NaCl, which is associated with a swelling. Because the net H^+ loss exceeds the loss of HCO_3^- there is a distinct intracellular alkalinization which alters the oxygen-binding properties of haemoglobin (Cossins and Richardson, 1985; Salama and Nikinmaa, 1988).

Virtually all *in vitro* studies to date have been performed at 15–20°C, which for rainbow trout represent high but sub-lethal temperatures. The effects of acute

Key words: Na^+/H^+ exchanger, trout erythrocyte, temperature, adrenergic responses.

variations in temperature have received little attention, despite the fact that trout regularly experience large seasonal variations in temperature (Cossins and Kilbey, 1989). In the only published study, Nikinmaa *et al.* (1987) observed no difference in the adrenergically induced swelling and increase of intracellular pH (pHi) of trout erythrocytes when measured at 10 or 18°C. They concluded that these adrenergic responses were independent of temperature and, furthermore, that differences in the *in vivo* responses of erythrocytes to adrenaline or strenuous exercise at different temperatures were not caused by the temperature dependence of the adrenergic response.

Intuitively, it is expected that the transport mechanisms involved in the adrenergic response should be dependent upon temperature in the conventional manner (Cossins and Bowler, 1987). However, the resulting steady-state values for intracellular Na^+ ($[Na^+]_i$) and H^+ concentration (pHi) are not easy to predict, because the rate constants for the individual permeability mechanisms and their temperature dependences in trout erythrocytes have not been established. In principle, the temperature independence of adrenergically induced changes in cell volume and pHi can only occur through the equality of the temperature coefficients for those transport processes which contribute to the observed steady states. We present a study of the effects of temperature upon the adrenergic Na^+/H^+ exchange mechanism by direct measurements of the adrenergically induced H^+ efflux and the resulting changes in intracellular Na^+ and pHi.

Materials and methods

Materials

Inorganic salts, D-glucose, trichloroacetic acid, mannitol and imidazole were purchased from BDH Ltd, Poole, Dorset. (−)Isoproterenol bitartrate, heparin, bovine serum albumin (BSA, fraction V) and Triton X-100 were purchased from Sigma Chemical Company Ltd, Poole, Dorset. 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) was purchased from Molecular Probes Inc., Eugene, Oregon.

Fish

Rainbow trout (*Salmo gairdneri*, 0.5–1 kg) were obtained commercially and maintained in the laboratory for up to 4 months in 2000 l fibreglass aquaria (2 m×2 m×0.5 m) at 4–13°C. Aquarium water was treated by vigorous recirculation through a biological filter and with a pressurized mechanical filter. Fish were fed once daily to satiation with expanded trout pellets (BP Nutrition, Northwich, Cheshire, UK).

Bleeding

Fish were netted from the tank and stunned with a blow to the head. Arteriovenous blood was rapidly sampled from the caudal vessels with a 21 gauge hypodermic needle into heparinized plastic containers (approximately 45–60 s

after netting) and was gently shaken for 2 min. Erythrocytes were washed by centrifugation at 300–400 *g* for 2 min into an isotonic trout saline (room temperature) containing 6 mmol l^{-1} KCl, 5 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} D-glucose, 15 mmol l^{-1} imidazole–HCl at pH 7.6 (measured at room temperature) and sufficient NaCl to give an osmolality of 308 mosmol kg^{-1}. Cells were washed four times with greater than 10-fold dilutions in each case and then transferred to a refrigerator. For overnight storage, erythrocytes were washed once into a trout saline containing 1 % (w/v) BSA and kept at a haematocrit of 15–20 % in a refrigerator at 5±1 °C.

Determination of intracellular Na^+ ($[Na^+]_i$)

Erythrocyte suspensions were washed twice in trout saline and resuspended at 8–10 % haematocrit. Portions of the suspension were incubated at the desired temperatures for at least 10 min. Extracellular pH was not adjusted to compensate for the effects of temperature. All temperatures were determined to an accuracy of 0.2 °C referable to a precision mercury-in-glass thermometer. Samples (0.25 ml) of erythrocyte suspension were removed as required, centrifuged at 10 000 *g* for 10 s (Eppendorf Microcentrifuge) and the supernatant was removed by aspiration. The pellet was rapidly resuspended in 1 ml of ice-cold isotonic $MgCl_2$ solution (150 mmol l^{-1} $MgCl_2$, 15 mmol l^{-1} imidazole–HCl at pH 7.6, room temperature) mixed thoroughly and centrifuged again. This was repeated three times. The final pellet was lysed by the addition of 0.5 ml of Triton X-100 solution (0.05 %, w/v) and thoroughly mixed. Protein was precipitated by addition of 0.5 ml of 5 % (w/v) trichloroacetic acid followed by centrifugation at 10 000 *g* for 2 min. A sample of the resulting supernatant was diluted into distilled water and the Na^+ concentration determined using a flame emission spectrometer (model S11, Instrumentation Laboratories, Warrington, Cheshire, UK). Values were normalized to the haematocrit of the erythrocyte suspension. Values for adrenergically stimulated suspensions were normalized to an unstimulated but otherwise identical sample of the suspension.

Measurement of Na^+/H^+ exchange activity

The apparent transport of protons from erythrocytes to the extracellular medium was determined using a Radiometer RTS 822 pH-stat. Before each determination, the electrodes were cleaned by washing for at least 4 min in a buffer at pH 4. Erythrocytes were washed four times in an unbuffered trout saline (308 mosmol kg^{-1}) in which the imidazole in the normal buffered trout saline was replaced by mannitol. The final pellet was suspended at a haematocrit of 5–10 % and 1.5 ml was transferred to a conical plastic cuvette (Radiometer). The stirring rate of the titration unit was reduced to prevent frothing of the erythrocyte suspension. The plastic titration cuvettes were immersed directly in a water bath at the desired temperature and the temperature of the suspension was measured directly using a thermistor. The titrant was 10 mmol l^{-1} NaOH and its strength was routinely calibrated by titration against 10 mmol l^{-1} potassium hydrogen phtha-

late. The red cell suspension was manually titrated with 100 mmol l^{-1} NaOH to a pH of approximately 7.3 and the automatic titration started. Agonists and inhibitors were added as aqueous solutions as indicated in the figures. A few crystals of ascorbic acid were added to isoproterenol solutions to inhibit oxidation. The rate of H^+ production (efflux) was calculated from the slope of the graph of titrant volume added against time. The agonist-stimulated rate was routinely determined as the initial rate following addition of agonist minus the rate immediately before addition. Values were normalized to the haematocrit of the unstimulated suspension and reported as mmol h^{-1} l^{-1} pcv (pcv=packed cell volume). Since it is not possible to distinguish changes in extracellular pH (pHe) caused by transport of HCO_3^- or OH^- from those caused by movements of H^+, values are reported as the efflux of H^+ equivalents.

Results

Fig. 1 illustrates the temperature dependence of isoproterenol-stimulated H^+ efflux from trout erythrocytes. Temperature exerted a powerful influence upon exchanger activity, with the rate falling dramatically with cooling. The rates at temperatures up to 19°C could best be described by Q_{10}=7.9 (see Fig. 1), though

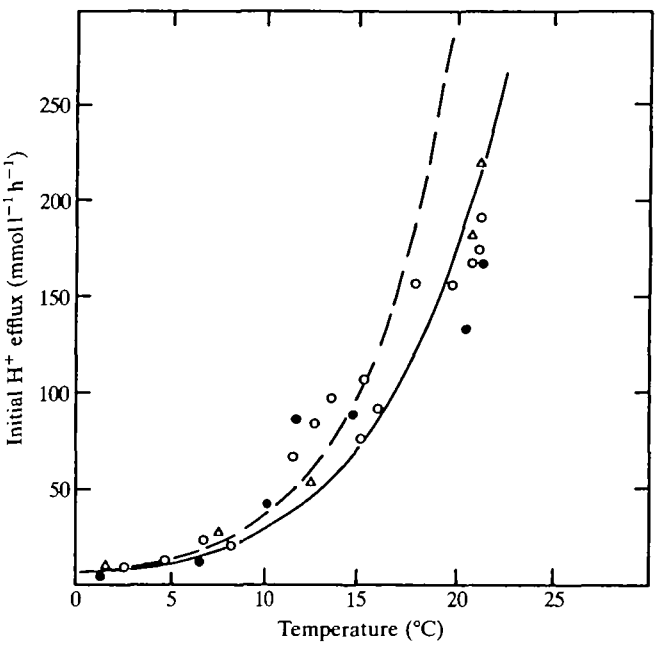

Fig. 1. The temperature dependence of the initial rate of H^+ ejection from trout erythrocytes following stimulation by isoproterenol. Each different kind of symbol represents a separate experiment. The lines were calculated by linear regression of log rate *versus* temperature and are equivalent to Q_{10} values of 5.37 for all values (solid line, log rate=0.072 temperature+0.807, r^2=0.920, N=27) and 7.9 when excluding values above 19°C (dashed line, log rate=0.09 temperature+0.675, r^2=0.925, N=19).

rates above 19°C were, as is commonly observed (Cossins and Bowler, 1987), somewhat below that predicted by this relationship. The Q_{10} recorded in four separate experiments over the full temperature range of 0–22°C was 5.24±0.84 (mean±S.D., N=4) and the corresponding Arrhenius activation energy was 112.8±10.01 kJ mol^{-1}.

Fig. 2A shows the effects of incubation temperature upon the time course of increase in $[Na^+]_i$ following addition of isoproterenol. The rate of increase in $[Na^+]_i$ was highly temperature-dependent, such that at 20°C a plateau was reached within 15 min, whilst at 0°C the corresponding time was 45 min. The time resolution for determining the initial rate of increase in $[Na^+]_i$ is relatively poor compared with that for measurements of H^+ efflux. Nevertheless, calculation of initial rates from the first sampling interval after adrenergic stimulation also showed a high temperature dependence (Fig. 2B). The non-linear logarithmic plot indicates that Q_{10} was temperature-dependent. In a series of four experiments

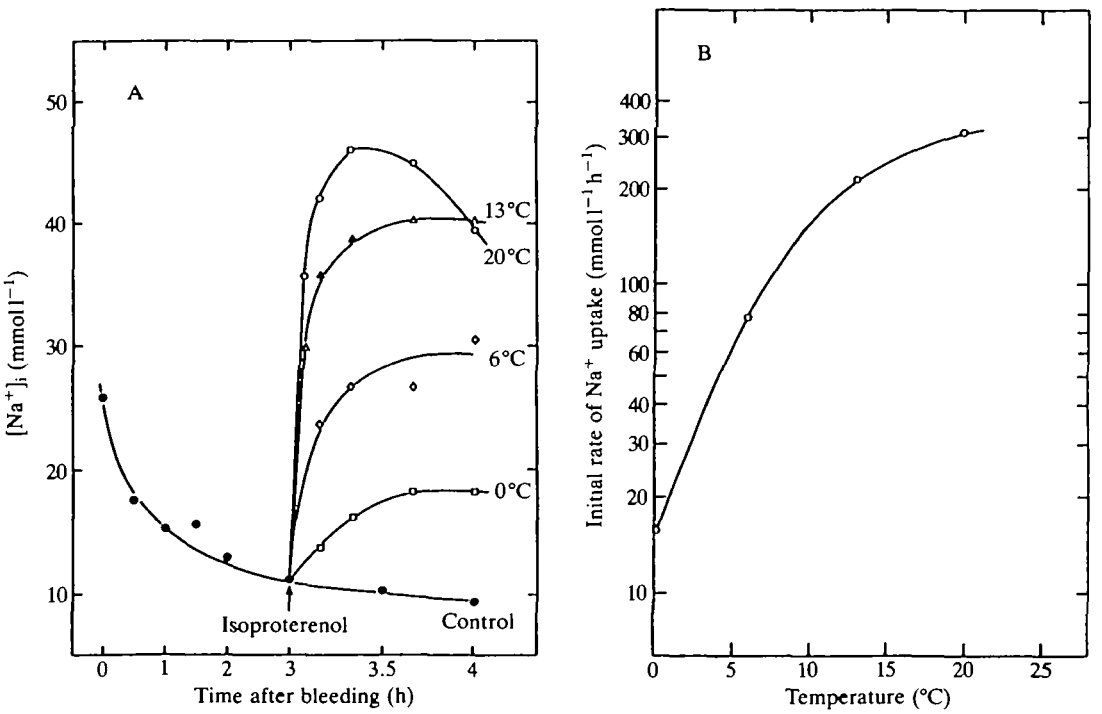

Fig. 2. (A) The effects of temperature upon the intracellular Na^+ concentration of trout erythrocytes following adrenergic stimulation. $[Na^+]_i$ was measured in duplicate in washed erythrocyte suspensions immediately after bleeding into saline at room temperature and during subsequent incubation at 5°C (filled circles: control). Samples of the suspension were transferred to the temperatures indicated and, after equilibration for 10 min, isoproterenol was added to a final concentration of 10^{-5} mol l^{-1}. In B the initial rates determined from the first sampling interval after addition of isoproterenol are plotted as a function of temperature. Similar results were obtained in four other experiments.

(without ouabain) the Q_{10} for 0–6°C was 10.8±1.3 (mean±S.E.M.), dropping to 3.5±0.4 at 6–13°C and 2.7±0.5 at 13–20°C. The overall Q_{10} (0–20°C) was 4.4±0.1, which was equivalent to an Arrhenius activation energy of 84.1±3.0 kJ mol^{-1}.

The plateau values increased with increasing temperature (Fig. 2B). In other experiments (not shown) we found that, although exposure to 26°C led to a more rapid initial rate of increase in $[Na^+]_i$, the maximal $[Na^+]_i$ was lower than at 20°C. At 20°C and above, and in some experiments at 13°C, the plateau was short-lived, there being a noticeable reduction in $[Na^+]_i$ from 15 min onwards. This was a particular feature of higher temperatures since there was no decline below 10°C for up to 3 h after stimulation. Addition of ouabain (10^{-3} mmol l^{-1}) led to a continued increase in $[Na]_i$, albeit at a slower rate than that observed immediately after addition of isoproterenol (data not shown, but see Baroin *et al.* 1984). This indicates the importance of the Na^+ pump in establishing the plateau and enabling the subsequent decline.

Fig. 3 illustrates the effects of temperature upon the rate of return of $[Na^+]_i$ to the lower steady state following a 30 min exposure at 20°C to isoproterenol. The rate of reduction of $[Na^+]_i$ at 20°C was rapid, with a return to control values within 2.5 h. Cooling slowed down the rate of reduction dramatically, such that at 0°C the $[Na^+]_i$ decreased only by approximately 20 % over 3 h. At 13°C approximately 4 h was required to reach control values and at 6°C 6–8 h was necessary.

Fig. 4 shows the effects of temperature upon pHi in control and isoproterenol-

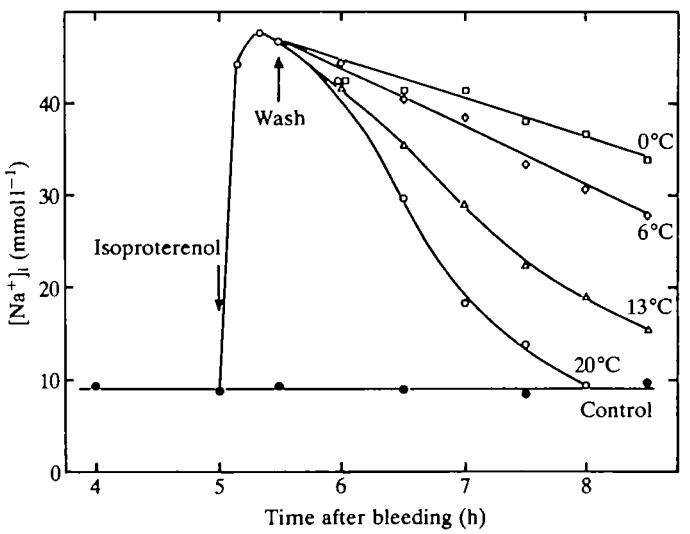

Fig. 3. The effects of temperature upon the reduction in intracellular Na^+ concentration following adrenergic stimulation of trout erythrocytes. Cells were incubated with isoproterenol (10^{-5} mol l^{-1}, final concentration) at 20°C for 30 min and washed three times in saline over a 5 min period and then transferred to water baths at the stated temperatures. Samples were taken for analysis of $[Na^+]_i$ as indicated. Filled symbols represent control, unstimulated erythrocytes maintained at 5°C.

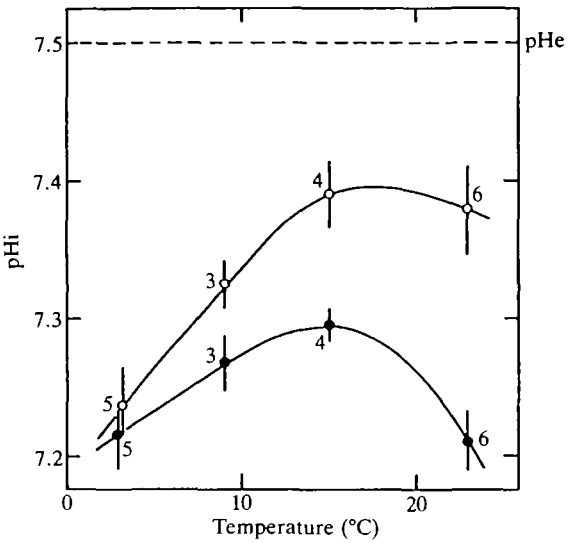

Fig. 4. The effects of temperature upon the isoproterenol-induced increase in intracellular pH in trout erythrocytes. Suspensions (2 ml, haematocrit 8–10%) were vigorously stirred using the titration unit of the pH-stat and pHe was maintained at 7.50±0.01 by continuous titration. Isoproterenol was added to a final concentration of 10^{-5} mol l^{-1} and samples were taken for the determination of pHi at 15 min for 21°C, 25 min for 15°C, 30 min for 9°C and 40 min for 2°C. Values represent the mean±S.E.M. The difference in pHi of control (●) and isoproterenol-stimulated (○) erythrocytes was significant at all temperatures in a paired *t*-test (see text). The numbers of samples are given beside the data points.

stimulated suspensions measured at pHe 7.5. Isoproterenol caused a significant increase in pHi at all temperatures, though the difference between stimulated and control erythrocytes decreased from 0.167±0.018 (mean±S.D., $N=6$, $P<0.001$, paired *t*-test) at 23°C, to 0.095±0.018 ($N=4$, $P<0.01$) at 15°C, to 0.057±0.003 ($N=3$, $P<0.005$) at 9°C and only 0.028±0.006 ($N=5$, $P<0.005$) at 3°C.

Discussion

The present work shows that temperature has an unusually large effect upon the exchange capacity of the Na^+/H^+ mechanism, as shown by the direct measurement of the maximal rate of both H^+ ejection and Na^+ uptake following adrenergic stimulation. The temperature dependence for both was greater than for other constitutive (non-inducible) transport processes. For example, the apparent activation enthalpies for sulphate and chloride exchange by the anion exchange mechanism in brown trout were 58 and 67 kJ mol^{-1}, respectively (Romano and Passow, 1984). The corresponding values for the Na^+ pump in mammalian cells were approximately 60 kJ mol^{-1}, and Raynard (1988) has found in erythrocytes of rainbow trout that ouabain-sensitive K^+ influx, which is mediated by the Na^+ pump, has an Arrhenius activation energy of approximately 57 kJ mol^{-1}.

Temperature influences the pK of commonly used buffers, including imidazole. Given the pronounced pH optimum of Na^+/H^+ exchange in trout erythrocytes (Borgese *et al.* 1987; Cossins and Kilbey, 1989), it is necessary to consider the extent to which the high temperature-dependence of the exchange process was due to an indirect effect of temperature on medium pH. The measurements of $[Na^+]_i$ were carried out in an imidazole-buffered medium in which pH varied with temperature such that, according to the alphastat hypothesis (Reeves, 1977), the ionization state of histidyl imidazole groups on erythrocyte proteins should remain constant. By contrast, measurements of H^+ efflux at all temperatures were performed at pH 7.3, so in these experiments the protein ionization state may have varied with temperature. In that a marked temperature dependence was observed in both sets of measurements, it is clear that it is not due to pH-related phenomena. However, the Q_{10} values for H^+ efflux measurements were somewhat greater than those for Na^+ influx measurements, and this may be due to changes in ionization state of titratable groups on membrane-bound proteins in the former measurements.

It is not clear from the present studies whether cooling has its marked effect upon the exchange mechanism *per se* or upon the means by which the transport mechanism becomes activated. Given the great complexity of the activation process (β-adrenergic agonist–receptor affinity, GTP-binding proteins, adenyl cyclase activity, etc), the system may be particularly sensitive to cooling. Indeed, the adenyl cyclase of rat liver plasma membranes has an apparent activation energy of 50 kJ mol^{-1} at 37 °C which rises to 130 kJ mol^{-1} below 27 °C (Dipple and Houslay, 1978). However, addition of 8-bromo-cyclic AMP to a suspension of erythrocytes at 5 °C (Mahe *et al.* 1985, 1 mmol l^{-1}) did not enhance the isoproterenol-induced efflux of protons (R. V. Kilbey, unpublished observations), which suggests that the low rates were not due to an inadequate production of cyclic AMP.

The effects of cooling upon the steady states that follow adrenergic stimulation are quite clear: a progressive reduction in the resulting changes in both pHi and $[Na^+]_i$ and a considerable increase in the time for transition between the unstimulated and stimulated steady states. Interpretation of these observations requires an appreciation of the dynamics of Na^+ and H^+ transport. $[Na^+]_i$ is a result of at least two processes, active extrusion by the Na^+ pump and dissipative net entry *via* non-specific electrodiffusional permeability or by specific mechanisms including the Na^+/H^+ exchanger. Following stimulation, the active transport of Na^+ increases because of the elevated intracellular Na^+ concentration, whilst Na^+/H^+ exchange, having been greatly increased during the initial phases of stimulation, gradually decreases because of the progressive acute desensitization as described by Garcia-Romeu *et al.* (1988). This eventually equalizes the influx and efflux to produce a pseudo-steady state for $[Na^+]_i$.

If the Na^+ pump and the Na^+/H^+ exchanger possessed identical temperature coefficients, the resulting steady state for $[Na^+]_i$ would be unaffected by cooling although the time taken to reach the steady state would increase. However, if the

coefficients were different, one process would be more depressed by cooling than the other, resulting both in a changed steady-state $[Na^+]_i$ and in an increased time to reach the steady state. Accordingly, the smaller increase in $[Na^+]_i$ observed in the cold is evidence that the activity of the Na^+/H^+ exchanger was reduced more than the activity of the Na^+ pump.

Intracellular pH of mammalian erythrocytes is influenced mainly by intracellular proteinaceous buffers, intracellular carbonic anhydrase and the Jacobs–Stewart cycle (Hladky and Rink, 1977), and similar considerations exist in unstimulated red cells of fish. During adrenergic stimulation this steady state becomes disturbed by the secondary active pumping of protons out of the cell by the Na^+/H^+ exchanger and this induces a net efflux of HCO_3^- which gradually increases in magnitude until it titrates H^+ ejection, resulting in an increased, but steady, pHi. The anion exchange mechanism possesses a lower activation enthalpy than the Na^+/H^+ exchanger, so that cooling will reduce the ejection of protons more than the ability of the anion exchanger to titrate proton movements with HCO_3^-.

Thus, in contrast to the results of Nikinmaa *et al.* (1987), the present experiments *in vitro* indicate that the adrenergic responses of trout erythrocytes are greatly attenuated by cooling, to the extent that responses were barely measurable at temperatures commonly experienced by trout during the winter months. In addition, cooling greatly extends the time taken for the adrenergic response to occur and for the normal steady state to become re-established when agonists are removed. The physiological consequences of these observations may be considerable. The adrenergically induced changes in pHi have been shown to influence the oxygen-binding characteristics of haemoglobin (Nikinmaa, 1983; Cossins and Richardson, 1985) and it has been suggested that this regulation of pHi during stressful episodes is the principal adaptive function of the adrenergic mechanism (Cossins, 1989). The reduced adrenergic responses of trout erythrocytes in the cold presumably limits the ability of cells to offset the effects of plasma acidosis upon haemoglobin oxygen-binding affinity and capacity and this may reduce the resistance of trout to stressful conditions.

The different time course of adrenergic responses observed in the present studies at low and high temperatures indicates perhaps why Nikinmaa *et al.* (1987) originally found no temperature dependence. These authors determined cell volume and pHi after 30 min incubation without following the time course of the changes in detail. Because at higher temperatures $[Na^+]_i$ declines after the initial adrenergically stimulated increase, the difference between $[Na^+]_i$ at low and high temperature gets progressively smaller as the incubation proceeds.

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