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ACTIVATION AND PHYSIOLOGICAL ROLE OF Na^+/H^+ EXCHANGE IN LAMPREY (*LAMPETRA FLUVIATILIS*) ERYTHROCYTES

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Summary

The effects of intracellular acidification, osmotic shrinkage and β-adrenergic stimulation on sodium transport across the membrane of lamprey (*Lampetra fluviatilis*) erythrocytes were investigated. Unidirectional ouabain-insensitive sodium flux, measured using radioactive ^{22}Na, was increased markedly by intracellular acidification, to a lesser extent by osmotic shrinkage and only modestly by β-adrenergic stimulation. Na^+/H^+ exchange was activated in all of these cases. However, net sodium influx (and cell swelling caused by the influx of osmotically obliged water) was seen only in cells subjected to intracellular acidification. In contrast, practically no changes in red cell pH or in water or ion (Na^+, K^+ and Cl^-) contents were seen after osmotic shrinkage or β-adrenergic stimulation. Calculations of the $[Na^+]_o/[Na^+]_i$ and $[H^+]_o/[H^+]_i$ ratios across the erythrocyte membrane suggest that the virtual lack of net sodium movements in osmotically shrunken erythrocytes is due to the absence of a driving force for net transport of these ions *via* the Na^+/H^+ exchange pathway. It also appears that, in physiological conditions, the increase in the activity of the Na^+/H^+ exchanger by β-adrenergic stimulation is too small to mediate detectable net sodium transport.

Introduction

Lamprey erythrocytes maintain their intracellular pH at a high value using a sodium-dependent amiloride-sensitive acid-extrusion mechanism, probably Na^+/H^+ exchange (Nikinmaa, 1986; Nikinmaa *et al.* 1986; Tufts, 1991*a*). Active regulation of red cell pH by this mechanism is possible because passive equilibration of acid and base equivalents across the membrane of lamprey erythrocytes is slow (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989) in the virtual absence of functional anion-exchange protein (Ohnishi and Asai, 1985; Nikinmaa and Railo, 1987).

Although the sodium-dependent acid-extrusion mechanism of lamprey erythrocytes responds to intracellular acid loads (Nikinmaa *et al.* 1986), the available data are qualitative in nature. For example, there is no information about the effect of intracellular acidification on the apparent sodium permeability of the lamprey erythrocyte membrane.

Key words: lamprey, *Lampetra fluviatilis*, erythrocyte, volume regulation, Na^+/H^+ exchange, sodium permeability, β-adrenergic stimulation, intracellular pH.

Recently, Gusev *et al.* (1992) observed that two other factors, osmotic cell shrinkage and β-adrenergic simulation, increase the sodium permeability of the lamprey red cell membrane. With regard to β-adrenergic stimulation, this finding is in apparent contrast to the data of Tufts (1991*b*), who did not observe any effect of catecholamines on the water content of the erythrocytes of *Petromyzon marinus*. The responses of lamprey erythrocytes to osmotic shrinkage have hitherto not been studied. In dog (Parker and Castranova, 1984), *Amphiuma* (Cala, 1980), rat (Orlov *et al.* 1989) and rabbit (Jennings *et al.* 1986) erythrocytes, however, Na^+/H^+ exchange is activated by osmotic shrinkage and, consequently, volume recovery occurs.

In view of the fragmentary information about Na^+ movements across the lamprey erythrocyte membrane and about the role of Na^+ transport in physiological processes, we have (i) investigated the ouabain-insensitive apparent Na^+ permeabilities of lamprey erythrocytes subjected to intracellular acidification, to osmotic shrinkage and to the permeant cyclic AMP analogue 8-bromoadenosine 3′,5′-cyclic monophosphate (Br-cAMP), calculated from unidirectional ^{22}Na influx, and (ii) determined how the above treatments affect the intracellular ion contents, pH values and water content. The results show that, although all the above treatments increased the sodium permeability of lamprey erythrocytes, the increase was greatest after intracellular acidification and that the sodium permeability and the sodium and proton gradients allowed for large net sodium influx and cell volume increases only after intracellular acidification.

Materials and methods

River lampreys [*Lampetra fluviatilis* (L.); mass 20–70 g] were caught during their spawning run from Perhojoki in western Finland and Kymijoki in southern Finland and maintained in laboratory conditions (dechlorinated Helsinki tap water, 10–13 °C) for at least 2 weeks before they were used in the experiments. Blood samples were taken from anaesthethized (MS-222, 1 g l^{-1}) lampreys by dorsal puncture into heparinized syringes. Red cells and plasma were separated by centrifugation (20 s, 20 000 ***g***) and the red cells were washed twice for at least 30 min with the saline used in the experiments. Blood from 2–6 animals was pooled. The composition of the saline was: 110 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Hepes, 1 g l^{-1} glucose, 1 g l^{-1} sodium pyruvate, pH 7.5, unless stated otherwise. All the experiments were carried out at room temperature (25 °C). This relatively high temperature was chosen to obtain large, easily measurable ion fluxes. An increase in temperature markedly increases Na^+/H^+ exchange activity in trout (Cossins and Kilbey, 1990). In separate experiments we ascertained that the cells were at steady state during the experiments at this temperature: the erythrocytes maintained constant water and ion contents over at least a 4 h incubation period.

Measurement of unidirectional sodium fluxes in cells exposed to acid–base disturbances, shrinkage and adrenergic stimulation

Unidirectional sodium influxes in red cells exposed to intracellular acidification, extracellular alkalization, hyperosmotic shrinkage and adrenergic stimulation were measured using radioactive ^{22}Na. Intracellular acidification was induced by isotonic

addition of 50 mmol l^{-1} sodium propionate to the incubation mixture. The uncharged form of the acid enters the cell and dissociates in the intracellular compartment, imposing an intracellular proton load (see Roos and Boron, 1981). Extracellular alkalization was induced by adding sufficient NaOH to increase the pH of the medium from 7.5 to 8. In the hyperosmotic experiment, the osmolarity of the medium was increased by 90 mosmol l^{-1} by the addition of sucrose. The possible effect of β-adrenergic stimulation was investigated by adding 8-bromoadenosine 3′,5′-cyclic monophosphate (Br-cAMP, dissolved in ethanol; Sigma, MO, USA; 1 mmol l^{-1} final concentration) to the incubation mixture.

The ^{22}Na influx was determined as follows. The cells were allowed to equilibrate for 30 min in the saline described above. Ouabain (Sigma, MO, USA; 0.1 mmol l^{-1} final concentration) was added 10 min prior to the start of the influx measurements. After the initial equilibration, ^{22}Na (Amersham, UK; final concentration 3700 Bq ml^{-1}) was added to the suspension at the same time as the cells were subjected to the different treatments. The suspension was sampled immediately and after a 10 min incubation period. We had previously checked that the influx remained linear for at least 10 min. Red cells and medium were separated immediately after sampling by centrifugation (2 min, 20 000 ***g***) and counted using a γ-counter.

Assuming negligible backflux of the isotope from the cells during the incubation period, the first-order rate constant (k) for entry of the isotope into the red cells was obtained from the formula of Kirk (1991):

$$k = \frac{A_{\mathrm{rbc}}}{A_{\mathrm{m}} \times t}, \tag{1}$$

where A_{RBC} and A_m are the radioactivity of 1 l of packed cells and 1 l of medium, respectively, and t is the incubation time (h). The unidirectional Na^+ influx (J) could be obtained from the rate constant by multiplying it by the concentration of sodium in the medium (120 mmol l^{-1}).

The apparent sodium permeability (P) of the lamprey red cell membrane was calculated from the formula (Parker, 1978):

$$P = \frac{V}{A}\ k\ \frac{e^{-B} - 1}{B \times e^{-B}}, \tag{2}$$

where V (cm^3) is the cell volume, A (cm^2) is the cell surface area and k (s^{-1}) is the rate constant. The quantity B equals $zEF/\boldsymbol{R}T$, where z is the charge, E is the membrane potential (calculated from the chloride distribution ratio to be −18 mV, assuming chloride to be passively distributed at steady state, as appears likely on the basis of the data of Nikinmaa, 1986), and F, $\boldsymbol{R}$ and T (293 K) have their usual meanings.

The red cell volume was estimated by determining the haematocrit values and counting the number of erythrocytes in each sample using a Coulter counter multisizer instrument. The volume of the lamprey red cells in suspension was 288±5.2 μm^3 (mean ± S.E.M., N=12). To estimate the surface area of the cells, the osmolarity of the medium was decreased by 40 %, and the red cell volume was determined as above. The volume of a

swollen cell was 450.0±27 μm^3 (N=5). It was assumed that the swollen cells had become spherical, so that the surface area of the cells could be calculated, giving a value of 283.5 μm^2. Consequently, the volume/surface ratio for lamprey erythrocytes was 1.02×10^{-4} cm.

In the Results, the ^{22}Na influx is given as mmol l^{-1} original cells h^{-1}. The values were corrected for extracellular trapped radioactivity by subtracting the value obtained immediately after the addition of the isotope from the values at 10 min.

Measurement of red cell volume, ion contents and intracellular pH after acid–base disturbances, osmotic shrinkage and adrenergic stimulation

A red cell suspension (2–4 ml, haematocrit 20–25 %) was incubated in a shaking tonometer in the saline described above, unless stated otherwise. When the incubation was started, 10 μl of ^{14}C-labelled DMO (5,5-dimethyl-2,4-oxazolidinedione, 370 000 Bq ml^{-1}; Amersham) was added to the incubation mixture, and the cells were allowed to equilibrate for 30 min.

In the first set of experiments, the effects of intracellular acidification on red cell volume, ion contents and intracellular pH were investigated. After the initial equilibration, the cells were acidified by isotonically adding 50 mmol l^{-1} (final concentration) sodium propionate to the incubation (1 volume of 100 mmol l^{-1} propionate in isotonic solution added to 1 volume of incubation medium). Samples were taken before and 1, 5, 15, 30, 60 and 120 min after the addition of propionate.

To assess the role of the Na^+/H^+ exchanger in the response of the erythrocytes to an internal acid load, one set of incubations was carried out in the presence of amiloride, a known inhibitor of Na^+/H^+ exchange. Amiloride (in dimethylsulphoxide, DMSO, solution; Sigma, MO, USA; final concentration 1 mmol l^{-1}) was added to the incubation mixture 15 min before the addition of propionate. Samples were taken before and 5, 15, 30, 60 and 120 min after the addition of propionate to the medium. Another set of incubations was carried out in nominally sodium-free medium. The composition of this medium was: 110 mmol l^{-1} choline chloride, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Hepes, 1 g l^{-1} glucose, pH 7.5. The cells were washed three times at 30 min intervals with this medium before the incubations were started. After the initial equilibration, isotonic saline containing 50 mmol l^{-1} propionic acid (final concentration) titrated to pH 7.5 with KOH was added to the medium. Samples were taken before and 5, 15, 30, 60 and 120 min after the addition of propionic acid to the medium.

In the second set of these experiments, the effect of extracellular alkalization was investigated. The pH of the incubating medium was increased from 7.5 to 8.0 by adding NaOH to the medium. Samples were taken before and 5, 15, 30, 60 and 120 min after the addition of NaOH.

The effects of osmotic shrinkage on the intracellular ion and water contents and intra- and extracellular pH were determined in the following set of experiments. The incubations were carried out in Hepes-buffered saline as outlined above. After the initial equilibration, the osmolarity of the medium was increased by 90 mosmol l^{-1} by the addition of sucrose or NaCl. Samples were taken before and 1, 5, 15, 30, 60, 120 and 180 min after the medium had been made hyperosmotic. A similar set of experiments on

cells subjected to osmotic shrinkage was carried out in CO_2/HCO_3^--buffered saline. 10 mmol l^{-1} $NaHCO_3$ was substituted for Hepes and the incubation was carried out under an atmosphere of 10 % CO_2 (10 kPa), 90 % air. This gas mixture was obtained using Wösthoff gas-mixing pumps. The elevated CO_2 tension decreased the pH of both cells and medium.

In one set of experiments, the red cell incubation medium was first made 90 mosmol l^{-1} hypertonic by addition of sucrose and, after 30 min of incubation, the cells were acidified by adding 50 mmol l^{-1} propionate at constant osmolarity. Samples were taken before and 1 and 30 min after the osmolarity of the medium had been increased and 5, 15, 30, 60 and 120 min after the addition of propionate.

Since some cells only show a regulatory volume increase after they are first subjected to a hypo-osmotic environment and allowed to undergo a regulatory volume decrease, and then returned to isotonic medium in which they initially shrink below their original volume, in one set of incubations we initially decreased the osmolarity of the medium by one-third by addition of 0.5 volumes of water to the incubation and, after 30 min, resuspended the cells in isotonic medium. Samples were taken before and 1 and 30 min after the addition of water, and 1, 15, 30, 60 and 120 min after resuspension in isotonic medium.

To study the effects of adrenergic stimulation, 10^{-5} mol l^{-1} isoproterenol dissolved in saline or 10^{-3} mol l^{-1} Br-cAMP in ethanol solution (final concentrations) were added to the incubations after the initial equilibration. Samples were taken before and 5, 20 and 60 min after the addition of isoproterenol or Br-cAMP.

Immediately after sampling, the extracellular pH was measured using a Radiometer BMS3 Mk2 and PHM 73 apparatus. The red cells and the medium were then separated by centrifugation in two Eppendorf tubes (2 min, 20 000 ***g***). The supernatants were carefully removed and used for extracellular ion and DMO determinations. The two red cell pellets were used for determinations of red cell ion contents, DMO concentration and water content. The red cell water content was determined by weighing, drying (80 °C, 48 h) and reweighing one cell pellet. The other cell pellet and 100 μl of supernatant were deproteinized in 300 μl of 0.6 mol l^{-1} perchloric acid. Sodium and potassium contents were measured using a flame photometer (Radiometer FLM3, Copenhagen) and chloride content using a coulometric chloride titrator (Radiometer CMT 10, Copenhagen). 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:

$$\mathrm{pHi} = \mathrm{pK_{DMO}} + \log\left\{\frac{[\mathrm{DMO}]_\mathrm{i}}{[\mathrm{DMO}]_\mathrm{e}}(10^{\mathrm{pHe}-\mathrm{pK_{DMO}}}+1)-1\right\}. \quad (3)$$

The samples were analyzed for [^{14}C]DMO using a liquid scintillation counter (LKB Wallac 1211 Minibeta). The pK value for DMO at 25 °C was taken to be 6.23.

In the Results, the ion contents are given in mmol kg^{-1} dry cell mass and the water content is given as percentage water/wet mass. The values are corrected for extracellular trapped ions and water. Extracellular space was measured as polyethylene glycol space (Nikinmaa and Huestis, 1984).

Statistical analyses of the data were carried out using the LSD test with the paired design of the ANOVA/MANOVA module of Statistica Software (StatSoft Inc, Tulsa, USA).

Results

Unidirectional sodium fluxes across the lamprey erythrocyte membrane

The effects of intracellular acidification, extracellular alkalization, osmotic shrinkage and adrenergic stimulation on the unidirectional sodium fluxes, measured with ^{22}Na, across the lamprey erythrocyte membrane are given in Fig. 1. Intracellular acidification (the intracellular pH decreased by about 0.7 units), induced by adding 50 mmol l^{-1} propionate isotonically to the incubation medium, caused a sevenfold increase in the ouabain-resistant unidirectional sodium influx compared with the controls. In contrast, increasing the extracellular pH by 0.5 units by adding NaOH to the medium did not affect the unidirectional sodium flux across the cell membrane.

Osmotic shrinkage in medium made 90 mosmol l^{-1} hypertonic with sucrose doubled the unidirectional sodium influx compared with the controls (Fig. 1). After the addition of 1 mmol l^{-1} Br-cAMP, a 70 % increase in the sodium flux was observed. The apparent

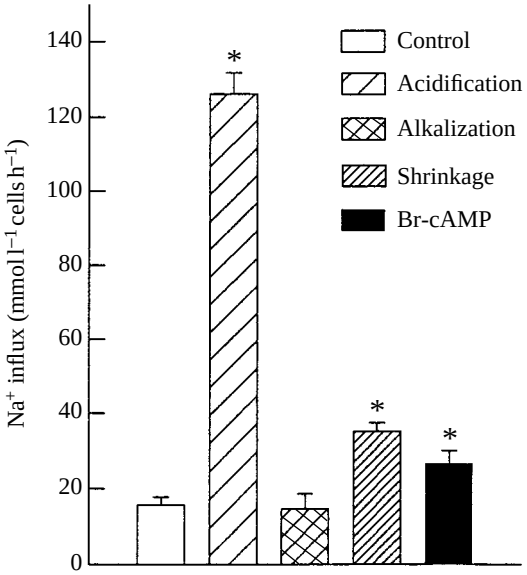

Fig. 1. The effects of intracellular acidification, extracellular alkalization, osmotic shrinkage and 1 mmol l^{-1} Br-cAMP on unidirectional sodium influxes in lamprey erythrocytes, measured with ^{22}Na. Intracellular acidification was caused by isotonic addition of 50 mmol l^{-1} propionate to the incubation medium, extracellular alkalization by increasing the extracellular pH by 0.5 units by adding NaOH to the medium, and osmotic shrinkage was induced by increasing the osmolarity of the medium by 90 mosmol l^{-1} with sucrose. Means ± S.E.M. are given, N=6–9. An asterisk denotes a significant difference from the control value (P<0.01). The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

Table 1. *The apparent ouabain-insensitive Na^+ permeability of lamprey erythrocytes subjected to different treatments*

Treatment	$10^9 \times$ apparent permeability ($cm\,s^{-1}$)	Percentage of control
None	2.66±0.36	100
Intracellular acidification	21.29±0.96	800
Extracellular alkalization	2.49±0.68	94
Hypertonicity	5.96±0.37	224
Adrenergic stimulation	4.53±0.54	170

The permeabilities were calculated from the data of Fig. 1 and from the calculated volume to surface area ratio of 1.02×10^{-4} cm.

Values are mean ± S.E.M., N=6–9.

ouabain-insensitive sodium permeabilities of lamprey erythrocytes subjected to different treatments was calculated from the unidirectional Na^+ fluxes and are given in Table 1. Since the sodium permeability was increased by acidification, osmotic shrinkage and cyclic AMP, net Na^+ movements could potentially occur in all of these cases and, followed by osmotic water fluxes, could cause volume changes.

Red cell pH, volume and ion contents

The addition of propionate to the incubation medium caused a large initial decrease, 0.7 units, in the red cell pH (Fig. 2A), although the extracellular pH remained almost unchanged at 7.5 (maximal change 0.08–0.09 units). After the initial drop, pHi started to recover from 7.36 at 1 min to 7.49 at 120 min. The pH recovery was not complete, however, and the intracellular pH stabilized at a much lower value than before the acidification. The marked initial drop in the intracellular pH was followed by a pronounced net flux of Na^+, as indicated by the increase in the intracellular Na^+ content, and cell swelling, as indicated by the increase in red cell water content (Fig. 2B,C). The calculated initial net sodium influxes were of similar magnitude to the unidirectional flux obtained using ^{22}Na as a tracer. Like the intracellular pH, the red cell Na^+ and water contents reached a steady-state level approximately 30 min after acid loading. Some cell swelling also occurred in the cells treated with amiloride and in the cells equilibrated in Na^+-free medium. This swelling may have been due to an increase in the osmolarity of the cells resulting from the entry of propionate. However, most of the swelling of the red cells was inhibited by amiloride and by the removal of sodium from the incubation medium. Similarly, both the increase in red cell sodium content and the recovery of red cell pH were absent in amiloride-containing and sodium-free incubations. Also, the intracellular pH was significantly lower in the amiloride-treated cells and in the cells incubated in sodium-free medium than in the control cells throughout the experiment.

The red cell K^+ content was not affected by the acidification, although a slight decrease in the Cl^- content of control cells was observed during the incubation (Fig. 3). The reduction in Cl^- content may be a secondary response to cell swelling, since it was not observed in amiloride-treated cells or in cells incubated in Na^+-free medium, in which

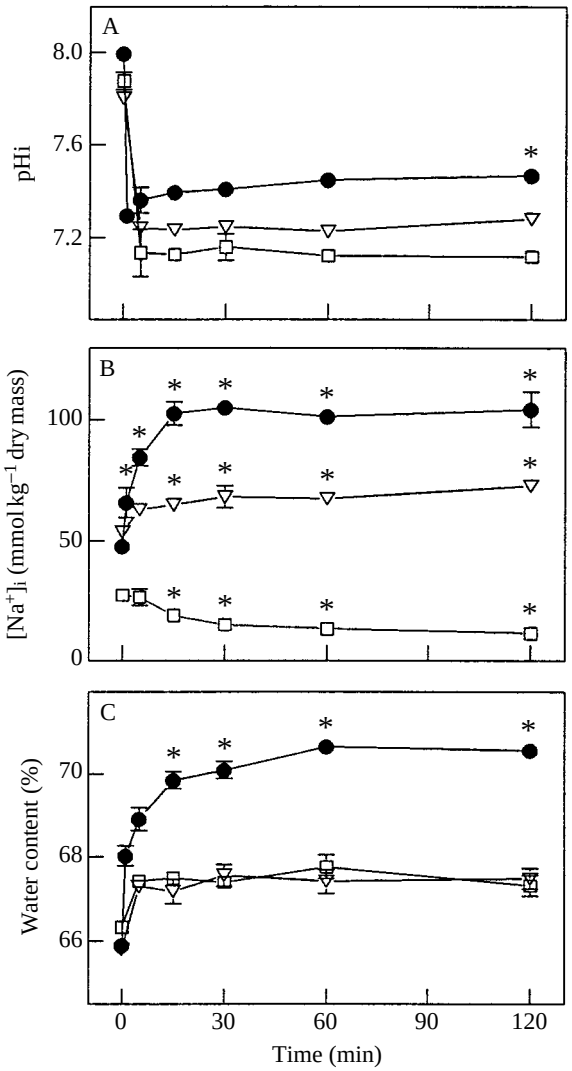

Fig. 2. The effects of intracellular acidification (caused by adding 50 mmol l^{-1} propionate isotonically to the incubation medium) on lamprey erythrocyte intracellular pH (A), intracellular Na^+ content (mmol kg^{-1} dry mass) (B) and cell water content (percentage water/wet mass) (C). (●) control incubation (N=5), (▽) cells incubated in the presence of 1 mmol l^{-1} amiloride (N=5) and (□) cells incubated in sodium-free medium (N=4). Means ± S.E.M. are given. In A, an asterisk denotes a significant difference (P<0.01) from the 5 min time point. In the control incubation, the values at 30, 60 and 120 min differed significantly (P<0.01) from the value at 1 min. In B, an asterisk denotes a significant difference (P<0.01) compared with the value at 0 min. In C, an asterisk denotes a significant difference (P<0.01) compared with the value at 5 min and all values differed significantly (P<0.01) from their respective values at 0 min. The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

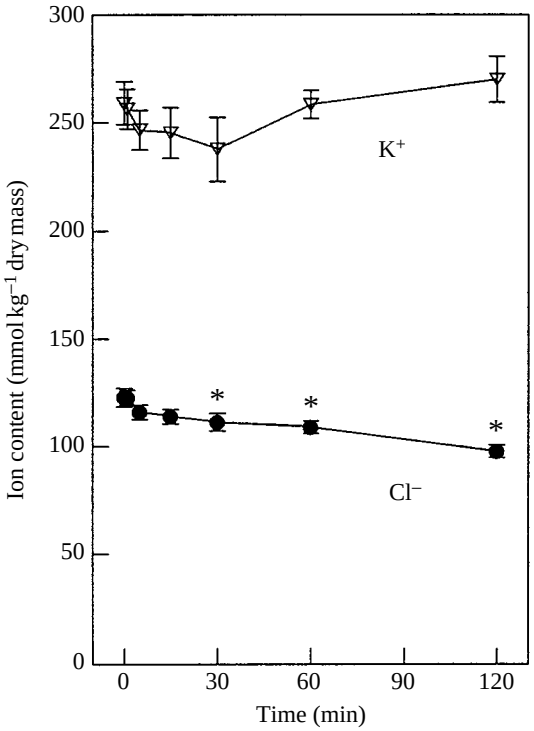

Fig. 3. The effects of intracellular acidification (caused by adding 50 mmol l^{-1} propionate isotonically to the incubation medium) on lamprey erythrocyte K^+ and Cl^- contents (mmol kg^{-1} dry mass). (▽) K^+, (●) Cl^-. Means ± S.E.M. are given (N=5). An asterisk denotes a significant difference (P<0.01) compared with the 0 min value. The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

cell swelling was minimal (not shown). Hypotonic swelling increases the apparent chloride permeability of the lamprey erythrocyte membrane (L. V. Virkki and M. Nikinmaa, unpublished data), resulting in net chloride losses.

When the osmolarity of the medium was increased by 90 mosmol l^{-1} with sucrose, the red cell water content was initially reduced by 10 %; it remained constant throughout the remainder of the experiment (Fig. 4A). Practically no changes in the red cell Na^+, K^+ or Cl^- contents were seen after osmotic shrinkage (Fig. 4B). It is apparent that anion movements are not influenced by osmotic shrinkage: unidirectional chloride fluxes, as measured with radioactive ^{36}Cl, were unaffected by osmotic shrinkage (L. V. Virkki and M. Nikinmaa, unpublished data). The only effect of osmotic shrinkage was an immediate statistically significant increase in the intracellular pH by 0.15 units (Fig. 4C). The increase in red cell pH is probably due to the physicochemical properties of the intracellular haemoglobin solution. It appears that lamprey haemoglobin exists in a monomer–oligomer association–dissociation equilibrium within the erythrocytes (Briehl, 1963; Nikinmaa, 1993; Nikinmaa and Weber, 1993). The equilibrium depends on the

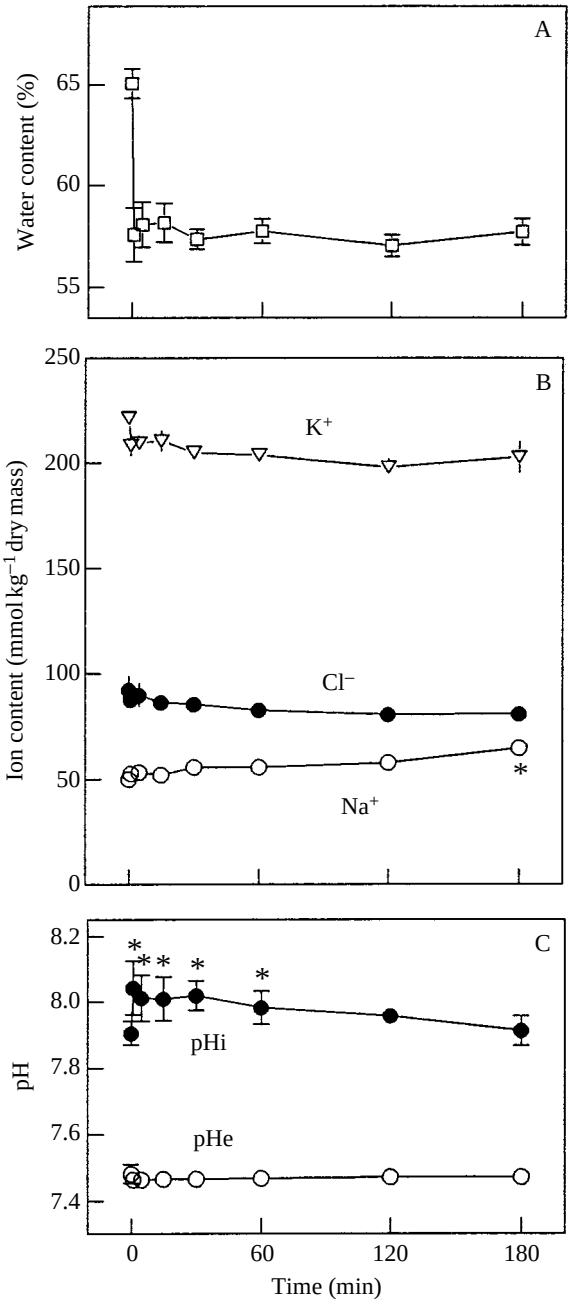

Fig. 4. The effect of increasing the osmolarity of the incubation medium by 90 mmol l^{-1} by addition of sucrose on lamprey erythrocyte water content (percentage water/wet mass) (A), and on intracellular sodium (○), chloride (●) and potassium (▽) content (mmol kg^{-1} dry mass) (B) and intracellular (●) and extracellular (○) pH (C). Means ± S.E.M. are given, N=6. An asterisk denotes a significant difference (P<0.01) compared with the 1 min time point in A and B and compared with the 0 min time point in C. The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

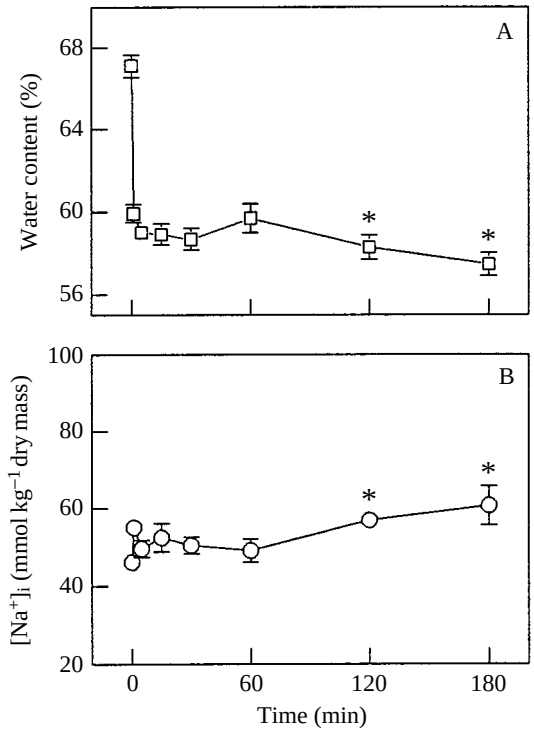

Fig. 5. The effect of increasing the osmolarity of the incubation medium by 90 mmol l^{-1} by addition of sucrose on lamprey erythrocyte water content (percentage water/wet mass) (A) and intracellular sodium content (mmol kg^{-1} dry mass) (B) in medium buffered with CO_2/HCO_3^-. 10 mmol l^{-1} $NaHCO_3$ was substituted for Hepes and the incubation medium was gassed with a mixture of 10 % CO_2 and 90 % air. Means ± S.E.M. are given (N=4–5). An asterisk denotes a significant difference (P<0.01) compared with the 1 min time point in A and the 0 min time point in B. In B, none of the other values differed significantly from the 1 min time point. The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

oxygenation–deoxygenation state, the pH and the concentration of haemoglobin. An increase in the concentration of haemoglobin within the cell, caused by osmotic shrinkage, favours the association of monomers to oligomers (Dohi *et al.* 1973), and one proton is taken up for each haemoglobin chain upon association (see Perutz, 1990), leading to an increase in intracellular pH. Similar results were obtained in a medium made 90 mosmol l^{-1} hyperosmotic by adding NaCl (not shown).

To show that the lack of regulatory volume increase after osmotic shrinkage was not an artefact of omitting bicarbonate ions from the medium, the experiments were repeated in medium buffered with CO_2/HCO_3^-. Initially, a high P_{CO_2} (10 % CO_2) was used to obtain an intracellular pH value similar to that in the acidification experiment in which the Na^+/H^+ exchanger was markedly stimulated (Fig. 5). The intracellular pH was 7.15±0.03 (N=5) before, and increased by 0.1 units immediately after, the addition of sucrose to the medium. During the incubation period, the intracellular pH decreased to the original

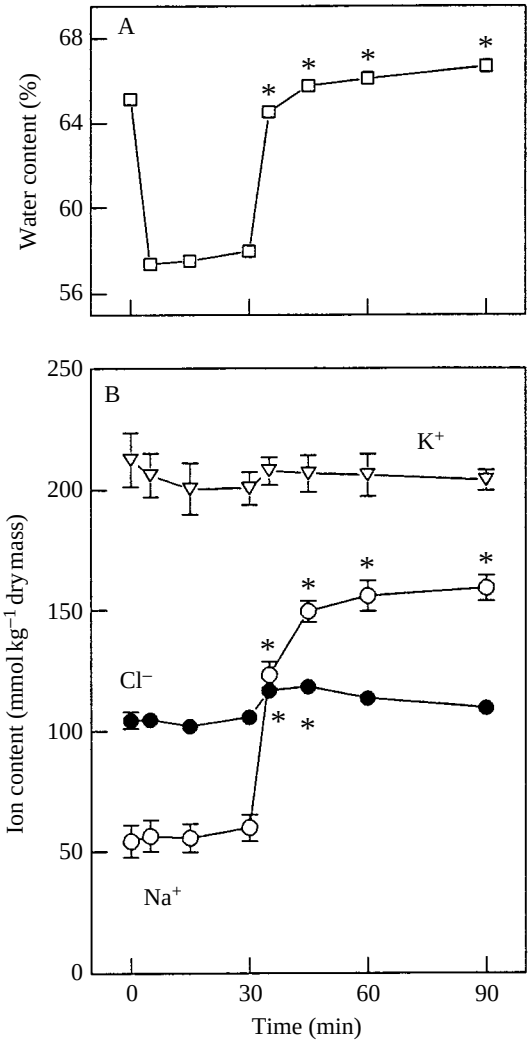

Fig. 6. The effect of intracellular acidification on the water content (percentage water/wet mass) (A) and intracellular sodium (○), potassium (▽) and chloride (●) contents (mmol kg^{-1} dry mass) (B) of osmotically shrunken lamprey erythrocytes. The incubation medium was first made 90 mmol l^{-1} hypertonic by adding sucrose and, after 30 min of incubation, the cells were acidified by adding 50 mmol l^{-1} propionate at constant osmolarity. Means ± S.E.M. are given, N=4. An asterisk denotes a significant difference (P<0.01) compared with the value 1 min after the addition after propionate (31 min time point). The values at 15 and 30 min do not differ significantly from the 1 min value. The statistical significances between means were calculated using the LSD test (within-subject design) of the ANOVA/MANOVA module of Statistica Software.

value. The extracellular pH remained constant at 6.6 throughout the incubation. No volume recovery was observed after the initial osmotic shrinkage at 1 % CO_2 in a separate set of experiments (S. Airaksinen and M. Nikinmaa, unpublished data).

If, however, osmotically shrunken cells were acidified with 50 mmol l^{-1} propionate 30 min after the shrinkage, the red cell sodium content increased, followed by osmotically obliged water, and the cells swelled slightly beyond their original volume (Fig. 6), showing that a net sodium influx may occur in shrunken cells in suitable conditions.

In some cell types, volume regulation after shrinkage only occurs if the cells are first equilibrated in hypotonic medium, in which they undergo a regulatory volume decrease, and thereafter placed in isotonic medium (see review by Hoffmann and Simonsen, 1989). Lamprey red cells exposed to hypo-osmotic medium initially gain osmotic water and swell. This induces net potassium and chloride loss, followed by osmotically obliged water, so that the cell volume decreases towards the original value (Nikinmaa *et al.* 1993). When the red cells are returned to isotonic medium, they shrink to less than their original volume. No significant net ion fluxes were seen and no changes in the red cell water or ion contents were seen after restoration of isotonicity (results not shown).

Adrenergic stimulation of lamprey red cells, either by 10^{-5} mol l^{-1} isoproterenol or by 10^{-3} mol l^{-1} Br-cAMP, did not induce net ion fluxes or cell swelling or affect the red cell pH (results not shown).

Discussion

In control conditions, the unidirectional ouabain-insensitive sodium fluxes of lamprey erythrocytes, measured at 25 °C, appear to be one order of magnitude larger than those of *Amphiuma* (measured at a similar temperature; Siebens and Kregenow, 1985), rat (Orlov *et al.* 1989) and human (Orlov *et al.* 1989) red cells (measured at 37 °C) and 2–3 times larger than those of trout (measured at 15 °C; Garcia-Romeu *et al.* 1988) and dog (Parker *et al.* 1991) red cells (measured at 37 °C). A large fraction (30–50 %) of the basal unidirectional sodium influx in lamprey erythrocytes is mediated *via* the amiloride-sensitive pathway (Gusev *et al.* 1992). The rate of unidirectional sodium influx was not decreased when NaOH was added to the incubation medium, which indicates that the measured flux at an extracellular pH of 8.0 and an intracellular pH of 7.9 represents the basal activity of the transport system.

The present results show that the sodium permeability of lamprey erythrocytes is increased by intracellular acidification and, to a smaller extent, by osmotic shrinkage and adrenergic stimulation. Increasing the osmolarity of the medium by 90 mosmol l^{-1} doubled the unidirectional ouabain-insensitive sodium influx. This result is similar to that obtained by Gusev *et al.* (1992). In their study, a 100 mosmol l^{-1} increase in the osmolarity of the medium doubled the unidirectional Na^+ influx and an increase of 200 mosmol l^{-1} tripled it. In this study, the unidirectional sodium fluxes after osmotic shrinkage were 50–80 % of those in osmotically shrunken *Amphiuma* erythrocytes (Siebens and Kregenow, 1985), larger than those in rat erythrocytes (Orlov *et al.* 1989), and half to one-third of those in dog erythrocytes (Parker *et al.* 1990, 1991). In contrast, the maximal rate of Na^+ influx in rainbow trout erythrocytes after adrenergic stimulation is more than 30 times that of osmotically shrunken lamprey erythrocytes (Garcia-Romeu *et al.* 1988).

Gusev *et al.* (1992) have shown that the increased unidirectional sodium fluxes in

response to osmotic shrinkage and adrenergic stimulation occur largely *via* the Na^+/H^+ exchanger. In the present study, we observed that intracellular acidification induces a large net influx of sodium, and protons are simultaneously extruded from the cell. Both of these effects are inhibited by amiloride and by removal of sodium from the medium. These data and earlier results (Nikinmaa, 1986; Nikinmaa *et al.* 1986; Tufts, 1991*a*) indicate that the Na^+/H^+ exchange is activated by intracellular acidification. Thus, Na^+/H^+ exchange is activated in all of these cases.

However, increasing the activity of the Na^+/H^+ exchanger by osmotic shrinkage or adrenergic activation does not induce large net sodium fluxes. *Amphiuma* and dog red cells, in which the Na^+/H^+ exchange activity after osmotic shrinkage is only slightly greater than in lamprey red cells, show a large net influx of sodium and cell swelling when subjected to osmotic shrinkage. Also, in rat erythrocytes subjected to osmotic shrinkage, in which the unidirectional sodium fluxes are smaller than in osmotically shrunken lamprey erythrocytes, net sodium fluxes and cell swelling occur (Orlov *et al.* 1989).

If the cells are initially at steady state and Na^+/H^+ exchange is then stimulated, net Na^+ influx should occur, provided that $[Na^+]_o/[Na^+]_i$ is greater than $[H^+]_o/[H^+]_i$ (e.g. Cala and Grinstein, 1988). This is the case in lamprey erythrocytes when they are subjected to intracellular acidification. Lowering the intracellular pH greatly increases the activity of the Na^+/H^+ exchanger, and the $[Na^+]_o/[Na^+]_i$ ratio is clearly larger than the $[H^+]_o/[H^+]_i$ ratio in this condition (Table 2). These factors together allow for a large net influx of sodium to occur. Note, however, that the ratios $[Na^+]_o/[Na^+]_i$ and $[H^+]_o/[H^+]_i$ given in Table 2 are only rough estimates of the true ratios of these ions, since measurements of

Table 2. *The Na^+ and H^+ distribution ratios across the membrane of lamprey erythrocytes after different treatments*

Species	$[Na^+]_o/[Na^+]_i$	$[H^+]_o/[H^+]_i$	*N*
Lamprey			
Untreated cells	4.98±0.21	2.42±0.12	33
Hypertonicity	3.32±0.15	3.82±0.31	6
Acidified cells	4.54±0.35	0.52±0.02	5
Hypertonicity+acidification	2.94±0.26	0.91±0.03	4
Extracellular alkalization	3.78±0.44	0.71±0.07	5
Adrenergic stimulation	5.34±0.15	1.99±0.09	10
Trout	4.5[1]	0.89[1]	
Amphiuma	7.70±0.28[2]	0.59±0.08[3]	
Rabbit	6.8[4]	0.74[5]	
Dog	1.2[5,6]	0.63[6]	

For comparison, data from the literature on the distribution ratios for other vertebrate erythrocytes are also given.

Values are mean ± S.E.M.

[1]Nikinmaa *et al.* (1990); [2]Cala (1980); [3]Tufts *et al.* (1987); [4]Bernstein (1954); [5]Calvey (1970); [6]Parker *et al.* (1990).

intracellular Na^+ and H^+ contents by our methods give only the average values for all the intracellular compartments. Since the nucleus is possibly more acidic than the cytoplasm (Bone *et al.* 1976), the estimated $[H^+]_o/[H^+]_i$ ratios across the cell membrane of nucleated erythrocytes given in Table 2 may be smaller than the true ratios. The apparent driving force for net Na^+ entry is thus possibly smaller than indicated in Table 2 in nucleated erythrocytes. However, the data allow for comparison between different treatments and species with nucleated erythrocytes.

In lamprey erythrocytes subjected to osmotic shrinkage, activation of the Na^+/H^+ exchanger does not cause detectable net sodium fluxes. This is probably due to the apparent lack of driving force for net entry of Na^+ *via* this pathway, since the $[Na^+]_o/[Na^+]_i$ ratio is not larger than the $[H^+]_o/[H^+]_i$ ratio in this condition (Table 2). When the shrunken cells are acidified (which both decreases the$[H^+]_o/[H^+]_i$ ratio and further activates the transporter), net ion fluxes were again seen.

The absence of detectable net sodium fluxes in lamprey erythrocytes subjected to adrenergic stimulation might be due to the increase in the activity of the Na^+/H^+ exchanger being too small to mediate detectable net sodium influx. At steady state, sodium influx through the Na^+/H^+ exchanger and the residual transport pathways is matched by sodium efflux through the Na^+/K^+-ATPase (Tosteson and Hoffmann, 1960). A slight activation of Na^+/H^+ exchange would thus be counterbalanced by an increase in the activity of the sodium pump, and net transport of Na^+ would not occur. It is thus possible that net ion transport would occur in catecholamine-stimulated, ouabain-treated cells. However, even if it were to occur, it would not be physiologically relevant.

It is notable that the difference between the estimated $[Na^+]_o/[Na^+]_i$ and $[H^+]_o/[H^+]_i$ ratios in lamprey erythrocytes at steady state is markedly smaller than in the nucleated *Amphiuma* and trout erythrocytes and in the non-nucleated rabbit erythrocytes (see Table 2), all of which show net sodium fluxes in response to moderate increases in the activity of Na^+/H^+ exchange. The smaller difference between the ratios in lamprey erythrocytes compared with these species is due to the high intra-erythrocytic pH. Thus, net transport of sodium and protons *via* the Na^+/H^+ exchanger in lampreys is much more sensitive to small changes in the gradients than in the other species. In the non-nucleated high-sodium dog erythrocytes, the difference between the two ratios in normal conditions is of similar magnitude to that in the lamprey. However, these erythrocytes show net sodium fluxes in response to an increase in the activity of the Na^+/H^+ exchanger induced by osmotic shrinkage, which indicates that the $[Na^+]_o/[Na^+]_i$ ratio must remain greater than the $[H^+]_o/[H^+]_i$ ratio in these erythrocytes when they are subjected to osmotic shrinkage.

The Na^+/H^+ exchanger of lamprey erythrocytes causes net ion, proton and water fluxes mainly in response to internal acid loads. However, full pH recovery is not achieved in cells suspended in a medium containing $50\,mmol\,l^{-1}$ propionate, despite the fact that $[Na^+]_o/[Na^+]_i$ remains greater than $[H^+]_o/[H^+]_i$ throughout the experimental period. A possible explanation for this observation is that the Na^+/H^+ exchanger activity may be rapidly reduced after the initial activation, as happens for the catecholamine-activated Na^+/H^+ exchanger in trout erythrocytes (Garcia-Romeu *et al.* 1988). The pronounced cell volume increase, caused by the Na^+/H^+ exchange, may inhibit the exchanger.

In conclusion, although sodium transport across the lamprey red cell membrane is activated by various treatments, in physiological conditions large net sodium and proton movements only occur in response to internal acid loads. The virtual lack of net sodium movements in osmotically shrunken cells appears to be due to the lack of a driving force for net ion fluxes *via* Na^+/H^+ exchange.

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