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MECHANISMS OF pH REGULATION IN LAMPREY (*LAMPETRA FLUVIATILIS*) RED BLOOD CELLS

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SUMMARY

Mechanisms regulating the red cell pH in lamprey (*Lampetra fluviatilis*) were studied using the ammonium chloride prepulse technique. The cells were initially incubated in a physiological saline containing 20 mmol l^{-1} ammonium chloride, and intracellular pH measured with the DMO technique. Ammonium chloride was then rapidly removed by centrifugation, and the changes in the intracellular pH followed.

The intraerythrocytic pH is primarily regulated by an amiloride-sensitive sodium/proton exchange. When sodium is present in the incubation medium, the intracellular pH rapidly recovers from the acidification associated with the removal of ammonium chloride from the incubation. When sodium is removed from the incubation medium, intracellular pH does not recover, and when the cells are treated with 10^{-3} mol l^{-1} amiloride in the presence of sodium, carbon dioxide and bicarbonate, the intracellular pH recovery is drastically reduced.

The movements of carbon dioxide, its consecutive catalysed hydration and dissociation to protons and bicarbonate and, possibly, movements of bicarbonate out of the cell acidify the cell contents. This is shown by the observation that the steady-state intracellular pH is higher in a HEPES-buffered medium than in a CO_2/HCO_3^--buffered medium at the same extracellular pH. The acidification is dependent on cellular carbonic anhydrase activity, present in lamprey red cells, which speeds up the hydration reaction. When the action of carbonic anhydrase is inhibited by acetazolamide, removal of ammonium chloride from the incubation medium does not cause intracellular acidification.

INTRODUCTION

The red cell pH of lamprey (*Lampetra fluviatilis*) appears to be actively regulated by a sodium-requiring process (Nikinmaa & Weber, 1984; Nikinmaa, 1986). The intracellular pH, as measured either by the freeze–thaw method (Nikinmaa & Weber, 1984) or by the DMO method (Nikinmaa, 1986), can be higher than the plasma pH, and is far removed from the one calculated on the basis of chloride distribution, supposing that both chloride ions and protons are passively distributed (cf. Hladky & Rink, 1977).

Key words: intracellular pH, sodium/proton exchange, lamprey, erythrocyte.

Sodium is commonly required in cellular pH regulation. Squid axons and snail neurones require extracellular sodium and bicarbonate and intracellular chloride to recover from acid loads (for review see Boron, 1983). Amiloride-sensitive sodium/proton exchange across the cell membrane appears to be an important pH-regulatory pathway, e.g. in fibroblasts (L'Allemain, Paris & Pouyssegur, 1984), chick skeletal muscle (Vigne, Frelin & Lazdunski, 1982), mouse neuroblastoma cells (Moolenaar, Boonstra, van der Saag & de Laat, 1981) and kidney proximal tubule cells (Boron & Boulpaep, 1983). In heart Purkinje fibres, two opposing mechanisms appear to be present: sodium-dependent acid extrusion alkalinizing, and bicarbonate extrusion acidifying, the cells (Vaughan-Jones, 1982).

Membrane-associated rapid chloride/bicarbonate exchange is characteristic of both anucleated (see e.g. Wieth, Brahm & Funder, 1980) and nucleated (see Romano & Passow, 1984) red cells. Bicarbonate movements through this anion exchange pathway bring protons to electrochemical equilibrium across the red cell membrane in mammalian erythrocytes (e.g. Hladky & Rink, 1977). Such chloride/bicarbonate exchange appears to be the most important pathway transporting acid equivalents in fish red cells (cf. Heming, 1984), although beta-adrenergic stimulation additionally activates an amiloride-sensitive sodium/proton exchange (Nikinmaa & Huestis, 1984), which appears to influence the pH gradient across the red cell membrane. Beta-adrenergic stimulation of fish red cells *in vitro* causes an increase in the intracellular pH at constant extracellular pH (Nikinmaa, 1982), and *in vivo* helps maintain constant intracellular pH in stress despite marked extracellular acidosis (Nikinmaa, Cech & McEnroe, 1984; Primmett, Randall, Mazeaud & Boutilier, 1986). In rainbow trout, the intracellular pH can be increased beyond the extracellular one by treating the cells *in vitro* with DIDS, an inhibitor of the chloride/bicarbonate exchange (Nikinmaa & Huestis, 1984). Thus, in the absence of bicarbonate movements across the membrane, the intracellular pH appears to be solely determined by the sodium/proton exchange (Nikinmaa & Huestis, 1984).

The findings about pH regulation in lamprey red cells resemble the results obtained with fish red cells in the absence of bicarbonate movements: pH is regulated by a sodium-requiring process and can be higher than the extracellular pH (see above). Thus, one distinct possibility is that lamprey red cells either lack a functional anion exchange pathway, as suggested by Ohnishi & Asai (1985), or that the anion exchange pathway equilibrates acid equivalents very slowly. On the other hand, the sodium-requiring proton extrusion may be more effective in lamprey red cells than in fish red cells.

We have investigated the mechanisms of pH regulation in lamprey red cells using the ammonium chloride prepulse technique (cf. l'Allemain *et al.* 1984) to acidify cell contents, thereafter following intracellular pH recovery in media with varying ion compositions, in media containing the transport inhibitors DIDS and amiloride, and in media containing acetazolamide, an inhibitor of carbonic anhydrase. Additionally, the carbonic anhydrase activity of intact red cells and of cells treated with DIDS was determined.

MATERIALS AND METHODS

Animals and blood sampling

River lampreys (*Lampetra fluviatilis*; $N = 90$; 25–80 g) were obtained during their spawning run in September 1984 and 1985 from Simojoki river in Northern Finland. They were transported to the Department of Zoology, University of Helsinki, and allowed to acclimate to laboratory conditions (dechlorinated Helsinki city tap water, pH 7·2–7·5, 12–15 °C, $P_{O_2} > 120$ mmHg, natural light rhythm) for at least a month before experimentation. Blood samples were taken from anaesthetized (2 g MS222 l^{-1} water) animals into heparinized syringes (see Nikinmaa, 1986), plasma and red cells separated by centrifugation, and red cells treated thereafter as described below.

Cellular carbonic anhydrase assay

The red cells were washed three times in ice-cold lamprey Ringer (in mmol l^{-1}) Na^+, 110; K^+, 4; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 118; HEPES, 30; pH 7·6, see Nikinmaa, 1986) and suspended in the above buffer to produce a haematocrit value of 10 vol. %. The carbonic anhydrase activity of red cells in suspension was determined using the modified boat technique as described by Haswell & Randall (1976). Although the method used has complications when used with intact red cells suspended in plasma (see Heming & Randall, 1982), it has successfully been used to determine the carbonic anhydrase activity of intact cells in physiological saline (Heming & Randall, 1982). The conversion of bicarbonate to carbon dioxide (in $\mu l\ min^{-1}$) in the reaction was followed manometrically. The reaction was started by adding 1 ml 200 mmol l^{-1} $NaHCO_3$ (in 20 mmol l^{-1} NaOH and 20 mmol l^{-1} NaCl, pH > 8) into 1 ml phosphate buffer (200 mmol l^{-1} Na_2HPO_4 and 200 mmol l^{-1} KH_2PO_4, pH 6·8) and 100 μl sample in the reaction vessel. Carbon dioxide production was followed in the following conditions: A, cells in Ringer; B, cells in Ringer + 10^{-4} mol l^{-1} DIDS (4,4-diisothiocyanostilbene 2,2-disulphonic acid; Sigma; 80–90 % purity); C, cells in Ringer + 1·6 mmol l^{-1} acetazolamide (Sigma); D, Ringer alone. From the CO_2 production in the catalysed and uncatalysed reaction, the enzyme activity (in arbitrary enzyme units) can be calculated: A−C gives the CO_2 production by 10 μl red cells. Thus, the catalysed reaction rate for 1 ml red cells is $(A-C)\times 100$, which, divided by the uncatalysed reaction rate C, gives the effect of cellular carbonic anhydrase on the reaction. Similarly, $(B-C)\times 100/C$ gives the DIDS-insensitive carbonic anhydrase activity which is equal to DIDS-insensitive movement of bicarbonate through the cell membrane. When this value is subtracted from the total carbonic anhydrase activity in intact red cells, and divided by the total carbonic anhydrase activity, the proportion of cellular carbonic anhydrase activity that is due to bicarbonate movement into the cell through the anion exchange pathway can be obtained.

Recovery of intracellular pH after acid–base disturbances

Intracellular acid–base disturbances were created by treatment of red cells with ammonium chloride as described by L'Allemain *et al.* (1984). Cells were washed

Table 1. *The constituents of the incubation media in the ammonium chloride prepulse experiment*

	Na	Choline	HCO_3^-
A	0	110	0
B	10	100	0
C	40	70	0
D	110	0	0
E	0	110	10
F	110	0	10

Concentrations given in $mmol\,l^{-1}$.
Choline, as choline chloride, was obtained from Sigma (99 % purity).

twice with lamprey Ringer containing $20\,mmol\,l^{-1}$ NH_4Cl, or with bicarbonate-containing lamprey Ringer ($10\,mmol\,l^{-1}$ $NaHCO_3 + 1\,\%$ CO_2 substituted for HEPES) containing $20\,mmol\,l^{-1}$ NH_4Cl, and after washes incubated at 15 °C in the same, ammonium chloride-containing, Ringer for 30 min. Ten μl of ^{14}C-labelled DMO (5,5-dimethyloxazolidine-2,4-dione; $1\,\mu Ci\,ml^{-1}$; 98 % purity; Amersham) was added in the incubation medium for intracellular pH determinations. At the end of the incubation the extracellular pH was measured using Radiometer PHM 72 and BMS 3 Mk 2 apparatus thermostatted at 15 °C. The cellular water content was then determined by weighing the cells, drying them to a constant weight and reweighing (e.g. Nikinmaa & Huestis, 1984). The intracellular pH was calculated from the extracellular pH and the distribution of radioactive DMO between the incubation medium and red cells as described earlier (Nikinmaa & Huestis, 1984) by using the formula:

$$pH_i = pK_{DMO} + \log\{DMO_i/DMO_e \times (10^{pH_e - pK_{DMO}} + 1) - 1\},$$

in which pK_{DMO} at 15 °C was taken to be 6·25. Roos & Boron (1981) and Busa & Nuccitelli (1984) in their reviews on intracellular pH have pointed out that the DMO method generally gives results closely similar to the microelectrode, nuclear magnetic resonance or fluorescence methods for measuring intracellular pH.

Ammonium chloride was then removed by rapid centrifugation, and two washes in lamprey Ringer containing no ammonium chloride. Another $10\,\mu l$ DMO was added to the incubation and the cells incubated for 60 min in the incubation media with the properties given in Table 1. To follow the pH recovery, the intra- and extracellular pH were measured after 5, 10, 20, 40 and 60 min or 5, 10, 30 and 60 min incubations. In one set of experiments the cells were incubated in the absence of sodium for 20 min, and thereafter $110\,mmol\,l^{-1}$ Na^+ added to the incubation (final concentration of Na^+ $55\,mmol\,l^{-1}$), and pH changes followed for another 20 min. The effects of the carbonic anhydrase inhibitor, acetazolamide, and the transport inhibitors, DIDS and amiloride (Sigma) on the ammonium chloride-induced pH changes were studied in the following fashion.

(1) $10^{-3}\,mol\,l^{-1}$ acetazolamide was present during all stages of the ammonium chloride prepulse experiment in the medium containing 1 % CO_2 and $10\,mmol\,l^{-1}$ bicarbonate.

(2) 10^{-3} mol l^{-1} amiloride (in dimethylsulphoxide, final concentration 0·5 %) was added to the incubation immediately after the removal of ammonium chloride from the incubation medium both in the HEPES-buffered medium and in the medium containing carbon dioxide and bicarbonate. Simultaneously a control experiment was run to test the effect of dimethylsulphoxide alone on the intracellular pH.

(3) 10^{-4} mol l^{-1} DIDS was present throughout the ammonium chloride prepulse experiment in the medium containing 1 % CO_2 and 10 mmol l^{-1} HCO_3^-.

RESULTS

Carbonic anhydrase activity

Lamprey red cells exhibit carbonic anhydrase activity (Table 2). In intact red cells the carbonic anhydrase activity can be 70 % reduced by treatment of the cells with 10^{-4} mol l^{-1} DIDS, which in mammalian and fish red cells binds specifically to the anion-exchange protein (Wieth *et al.* 1980; Romano & Passow, 1984). Thus, these results suggest that functional anion-exchange protein is present also in lamprey red cells, although the inhibition by DIDS seems to be quite ineffective.

Intracellular pH recovery after ammonium chloride prepulse: effects of Na^+ substitution and amiloride treatment in HEPES-buffered media

Initially, treatment of cells with ammonium chloride causes marked alkalinization, as basic ammonia enters the cell more rapidly than the acidic ammonium ion (cf.

Table 2. *Carbonic anhydrase activity in lamprey red cells measured using the modified boat technique in cells incubated in Ringer (A), in Ringer containing 0·1 mmol l^{-1} DIDS (B), and in Ringer containing 1·6 mmol l^{-1} acetazolamide (C) and the CO_2 production from the bicarbonate solution as such (Ringer without cells; D)*

	A Cells + Ringer	B Cells + DIDS	C Cells + acetazolamide	D Ringer only
CO_2 production				
Mean	18·1	13·5	11·4	11·3
S.E.M.	1·32	1·07	0·60	0·54
N	10	10	10	10
Enzyme activity				
Mean	58·2	19·0	0	0
S.E.M.	7·1	8·7		
N	10	10		

The CO_2 production (in μl CO_2 produced by the 100-μl sample in 1 min) and enzyme activity are given.

For further details see text.

The enzyme activity of cells in Ringer was significantly higher than the enzyme activity of cells treated with DIDS ($P < 0·001$; paired *t*-test was used for comparisons).

DIDS, 4,4-diisothiocyanostilbene-2,2-disulphonic acid.

Vaughan-Jones, 1982). Thus, the intracellular pH at the end of the ammonium chloride prepulse was markedly higher than the extracellular one (Fig. 1). When ammonium chloride is removed from the incubation medium, ammonia leaves the cell rapidly, and ammonium ion much more slowly. Thus, the cell contents are acidified. The degree of acidification 5 min after the removal of ammonium chloride is dependent on the sodium concentration in the incubation medium. At Na^+ concentrations above 40 mmol l^{-1}, the pH gradient ($pH_{medium} - pH_{cell}$) was −0·3 and increased slightly, so that after 60 min incubation it was −0·35. In the absence of sodium, the pH gradient was −0·05 to −0·10 with no sign of recovery during the 1-h incubation. In the presence of 10 mmol l^{-1} sodium, the initial pH gradient was the same as in the absence of sodium, but the intracellular pH increased slowly with time, so that the gradient was −0·15 after 60 min incubation.

These results are opposite to what would be expected if protons were distributed passively and the intracellular pH changes caused by volume-induced changes in the Donnan distribution of protons. When choline was substituted for sodium, the water content of lamprey red cells increased significantly during the 60-min incubation: the water content of cells incubated in normal Ringer was 70·5 ± 1·05 % ($N = 10$), whereas the water content of cells incubated in choline-Ringer was 76·1 ± 0·88 % ($N = 10$; $P < 0{\cdot}01$). The charge on the major intracellular impermeable polyions appears to be negative: the cells contain ATP and GTP and possibly 2,3-DPG

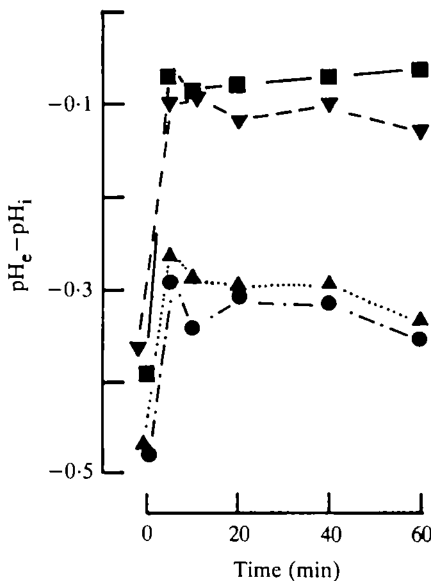

Fig. 1. The effect of sodium concentration on the pH gradient across lamprey red cell membrane ($pH_e - pH_i$) after ammonium chloride prepulse in HEPES-buffered medium. ■ = 0 mmol l^{-1} sodium, $N = 12$; ▼ = 10 mmol l^{-1} sodium, $N = 4$; ▲ = 40 mmol l^{-1} sodium, $N = 4$; ● = 110 mmol l^{-1} sodium, $N = 5$. The pH gradient was significantly ($P < 0{\cdot}05$) different in 40 and 110 mmol l^{-1} sodium concentrations from that in 0 and 10 mmol l^{-1} sodium concentrations. Mann-Whitney *U*-test was used for comparisons.

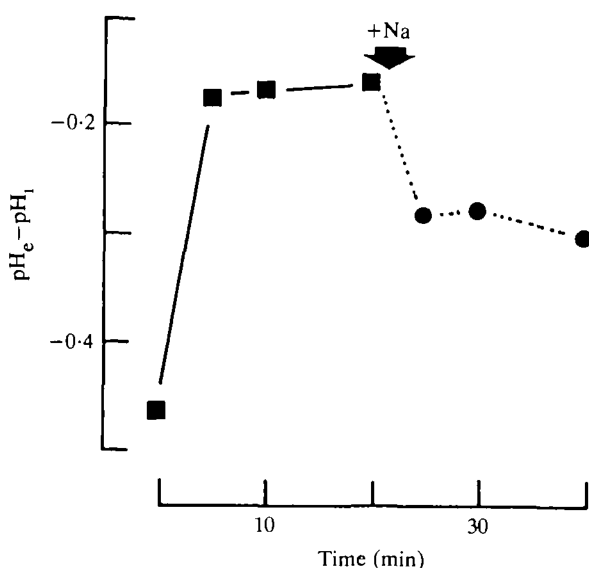

Fig. 2. The effect of sodium addition on the pH gradient across lamprey red cell membrane ($pH_e - pH_i$). The pH gradient after sodium addition was significantly ($P < 0.05$, $N = 9$) different from the pH gradient in Na^+-free medium. Mann-Whitney *U*-test was used for comparisons.

(Bartlett, 1982; Nikinmaa & Weber, 1984), and the isoelectric points of the major haemoglobin components are low (6·6, 5·6, 4·6 and 4·2; R. E. Weber & M. Nikinmaa, in preparation). Dilution of these anions should, if protons were passively distributed, increase intracellular pH at constant extracellular pH (cf. Hladky & Rink, 1977). The experimental data, however, show that choline chloride treatment decreased the intracellular pH. Thus, these results suggest that, in the virtual absence of bicarbonate, the intracellular pH is regulated by a sodium-dependent process. This conclusion is further supported by the data in Fig. 2. When sodium ions (final concentration $55\,mmol\,l^{-1}$) were added to the choline chloride (sodium-free) incubation, the intracellular pH immediately increased, changing the pH gradient from −0·15 to −0·3.

It is notable that even in the virtual absence of sodium, the intracellular pH remains higher than the extracellular pH, and the proton gradient across the red cell membrane is quite different from the chloride distribution gradient (see Nikinmaa, 1986). Additionally, amiloride, which blocks the sodium/proton exchange by competitive inhibition (see e.g. Aronson, Nee & Suhm, 1982) in several cell types (e.g. mouse neuroblastoma cells, Moolenaar *et al.* 1981; chick skeletal muscle cells, Vigne *et al.* 1982; kidney proximal tubule cells, Boron & Boulpaep, 1983) inhibited the acid extrusion of lamprey red cells very poorly in the CO_2/HCO_3^--free medium (Fig. 3). The pH gradient in amiloride-treated cells ($1\,mmol\,l^{-1}$ amiloride) was −0·25 60 min after the removal of ammonium chloride from the incubation, not significantly different from 'control' incubations.

pH recovery after ammonium chloride prepulse: effects of carbon dioxide and bicarbonate concentration

When the CO_2 tension in the incubation was increased from atmospheric to 1 % and the bicarbonate concentration to $10\,mmol\,l^{-1}$, the pH gradient across the red cell membrane ($pH_{medium} - pH_{cell}$) was much smaller at the end of ammonium chloride treatment than in the carbon dioxide and bicarbonate-free medium (−0·15 and −0·47, respectively, $P < 0.001$; Fig. 4). Removal of ammonium chloride further acidified the cells, and the pH gradient became +0·100 after 5 min incubation in the ammonium chloride-free medium. Thereafter the intracellular pH increased, and the pH gradient changed to −0·06 at the end of the 60-min incubation. In the

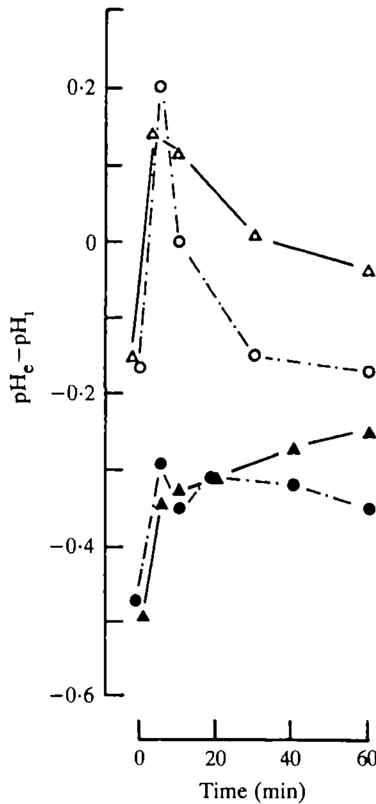

Fig. 3. Effects of $10^{-3}\,mol\,l^{-1}$ amiloride on the intracellular pH recovery after ammonium chloride prepulse. ● = control incubation in HEPES-buffered medium, $N = 5$; ▲ = incubation in HEPES-buffered medium containing $10^{-3}\,mol\,l^{-1}$ amiloride, $N = 8$; ○ = control incubation in medium containing 1 % CO_2 and $10\,mmol\,l^{-1}$ HCO_3^-, $N = 4$; △ = incubation in medium containing 1 % CO_2, $10\,mmol\,l^{-1}$ HCO_3^- and $10^{-3}\,mol\,l^{-1}$ amiloride, $N = 6$. The pH gradient in the presence of carbon dioxide and bicarbonate was significantly different ($P < 0.05$) from that in the absence of carbon dioxide and bicarbonate. In the absence of carbon dioxide and bicarbonate amiloride had no effect on the pH gradient, but in the presence of carbon dioxide and bicarbonate it slowed down the pH recovery. In the presence of carbon dioxide and bicarbonate the pH gradient of amiloride-treated cells was different ($P < 0.05$) from control cells at 10-, 30- and 60-min samples. Mann-Whitney U-test was used for comparisons.

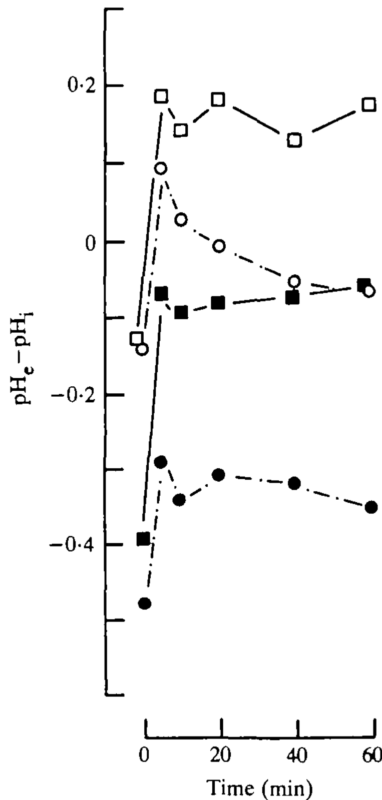

Fig. 4. Effects of carbon dioxide and bicarbonate on the pH gradient across lamprey red cell membrane ($pH_e - pH_i$) after ammonium chloride prepulse. ■ = 0 mmol l^{-1} sodium, atmospheric CO_2 tension and bicarbonate concentration, $N = 12$; ● = 110 mmol l^{-1} sodium, atmospheric CO_2 tension and bicarbonate concentration, $N = 5$; □ = 0 mmol l^{-1} sodium, 1 % CO_2 and 10 mmol l^{-1} bicarbonate, $N = 4$; ○ = 110 mmol l^{-1} sodium, 1 % CO_2 and 10 mmol l^{-1} bicarbonate, $N = 4$. The pH gradient in the presence of 1 % CO_2 and 10 mmol l^{-1} bicarbonate was significantly ($P < 0.05$) different from that in the absence of bicarbonate. In the absence of sodium, no pH_i recovery was observed at 1 % CO_2 and 10 mmol l^{-1} bicarbonate concentration, whereas in the presence of sodium the pH gradient changed significantly ($P < 0.05$) between 5 and 10 min, 10 and 20 min, and 20 and 40 min incubations. Mann-Whitney *U*-test was used for comparisons.

absence of sodium from the incubation, the pH gradient at the end of the ammonium chloride prepulse was similar to that observed in the presence of sodium, but the acidification after ammonium chloride prepulse was much more pronounced than in the medium containing CO_2, bicarbonate and sodium (pH gradient, +0·2). The pH gradient remained at this level throughout the 60-min incubation period with no sign of intracellular pH increase. Furthermore, the intracellular pH measured with the DMO method and the intracellular pH calculated from the chloride distribution ratio were similar when the cells were incubated in the presence of carbon dioxide and bicarbonate but in the absence of sodium. In both the absence and presence of sodium, the steady-state pH gradient across the red cell membrane differed by 0·3 units between the HEPES-buffered, CO_2/HCO_3^--free medium and medium

containing 1 % carbon dioxide and $10\,mmol\,l^{-1}$ bicarbonate (although the extracellular pH was virtually the same). This CO_2/HCO_3^--induced acidification agrees with some earlier reports on other cell types (e.g. Ellis & Thomas, 1976; Vanheel & de Hemptinne, 1985).

In the presence of carbon dioxide and bicarbonate, $10^{-3}\,mol\,l^{-1}$ amiloride effectively slowed down the recovery from the acid load (Fig. 3). It is thus likely that, in the virtual absence of CO_2 and HCO_3^-, even the residual movements of sodium are adequate to affect intracellular pH. When carbon dioxide and bicarbonate are present and acidify the cells, the residual Na^+/H^+ exchange after amiloride treatment is not able to alkalinize the cell in the normal fashion, although some pH recovery occurs.

In addition to the experiments in HEPES-buffered, virtually CO_2/HCO_3^--free medium and in a medium containing 1 % CO_2 and $10\,mmol\,l^{-1}$ HCO_3^-, the effects of carbon dioxide and bicarbonate on the intracellular pH were studied by incubating the cells with acetazolamide, an inhibitor of carbonic anhydrase activity, and DIDS, an inhibitor of anion exchange in mammalian and fish red cells. In the presence of acetazolamide, the removal of ammonium chloride from the incubation medium did not cause any acidification (Fig. 5), indicating that the carbon dioxide movements and consecutive rapid, catalysed hydration reaction to protons and bicarbonate are a major factor behind the acidification phase. Movements of bicarbonate out of the cell

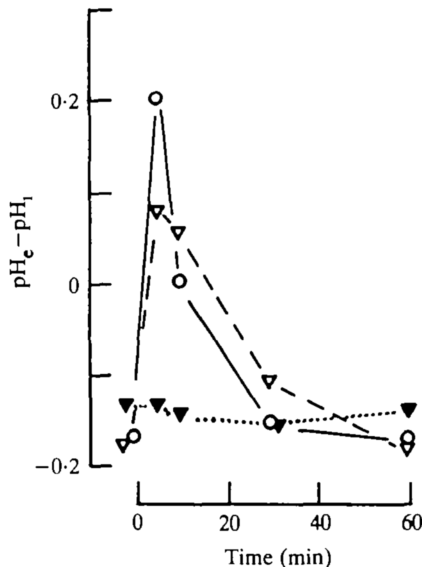

Fig. 5. Effects of $10^{-4}\,mol\,l^{-1}$ DIDS and $10^{-3}\,mol\,l^{-1}$ acetazolamide on the acidification of lamprey red cells, associated with the removal of ammonium chloride from the incubation. ○ = control incubation in medium containing 1 % CO_2 and $10\,mmol\,l^{-1}$ HCO_3^-, $N = 4$; ▽ = incubation in medium containing 1 % CO_2, $10\,mmol\,l^{-1}$ HCO_3^- and $10^{-4}\,mol\,l^{-1}$ DIDS, $N = 4$; ▼ = incubation in medium containing 1 % CO_2, $10\,mmol\,l^{-1}$ HCO_3^- and $10^{-3}\,mol\,l^{-1}$ acetazolamide, $N = 4$. The pH gradient of cells incubated in the presence of acetazolamide was significantly ($P < 0{\cdot}05$) different from controls and from DIDS-treated cells 5 and 10 min after the removal of ammonium chloride. Mann-Whitney *U*-test was used for comparisons.

may also be important. However, treatment of cells with DIDS hardly affected the acidification after the removal of ammonium chloride (Fig. 5), suggesting that bicarbonate efflux through a DIDS-sensitive pathway plays only a minor role in the acidification phase.

DISCUSSION

The present results suggest that two opposing mechanisms are involved in the control of intracellular pH of lamprey red cells: firstly, sodium-dependent acid extrusion, which is an active mechanism and alkalinizes the cells; secondly, carbon dioxide movements, consecutive catalysed hydration and dissociation of carbon dioxide to protons and bicarbonate, and, possibly, bicarbonate efflux, which tend to drive protons towards electrochemical equilibrium thus acidifying the cells. Boron & DeVeer (1976) observed an acidifying effect of carbon dioxide and bicarbonate in the squid axon, and suggested that their passive shuttle movements would be responsible for this acid load. The driving force on the bicarbonate ion is outwardly directed and it may thus leave the cell passively. Efflux of bicarbonate could create an intracellular sink for carbon dioxide, which after hydration and dissociation to protons and bicarbonate, could generate the extra acid load. Working on Purkinje fibres, Vaughan-Jones (1982) observed that much of the acidification after NH_4Cl removal was due to DIDS-inhibitable efflux of bicarbonate. The present results clearly show that the catalysed hydration and dissociation of CO_2 to protons and bicarbonate are of major importance in the acidification. The presence of carbonic anhydrase activity in red cells is required for effective carbon dioxide excretion in fish (cf. Randall & Daxboeck, 1984). Thus, it is likely that carbonic anhydrase activity is also involved in carbon dioxide excretion in lampreys. Therefore, movements of bicarbonate out of the red cell are likely to occur after the hydration reaction in the tissues, and into the cell before the dehydration reaction in the gills. However, a considerable proportion of bicarbonate movements across the cell membrane must occur through a DIDS-insensitive pathway. This is suggested by the observations that even if cells are treated with a high concentration ($10^{-4}\,mol\,l^{-1}$) of DIDS, 30 % of the carbonic anhydrase activity of intact cells remains and that DIDS treatment hardly affected the acidification after the removal of ammonium chloride. Also, as stated in the Introduction, in other red cells studied, movements of bicarbonate are capable of bringing protons to equilibrium, but in lamprey red cells the sodium-dependent acid extrusion markedly affects red cell pH both in the presence and absence of bicarbonate. In view of these apparent dissimilarities between lamprey red cells and red cells of most vertebrates, the movements of bicarbonate and other anions across the lamprey red cell membrane need to be further studied.

Amiloride is a competitive inhibitor of the sodium/proton exchange (e.g. Aronson *et al.* 1982). In the absence of bicarbonate and carbon dioxide which act to acidify the cell, amiloride hardly affected the intraerythrocytic pH of lamprey. In the presence of carbon dioxide and bicarbonate, however, amiloride drastically slowed down the pH recovery after the removal of ammonium chloride. It thus appears that

an amiloride-sensitive, sodium/proton exchange is of major importance in regulating the red cell pH in lamprey, as in many other cell types (e.g. Moolenaar *et al.* 1981; Vigne *et al.* 1982; l'Allemain *et al.* 1984). However, the 'residual' movements of sodium are capable of affecting intracellular pH in the absence of the acidifying effect of carbon dioxide and bicarbonate, and allowing for a slow pH recovery in the presence of CO_2 and HCO_3^-, as indicated by the different responses of the sodium-free medium and amiloride-containing medium with normal sodium concentration. This may reflect either that amiloride, even at a high concentration ($1\,mmol\,l^{-1}$), does not completely inhibit the sodium/proton exchange *via* the amiloride-sensitive pathway, or that there is another sodium-dependent pathway capable of regulating intracellular pH. In thymic lymphocytes, two pathways are capable of regulating cytoplasmic pH (Grinstein, Goetz & Rothstein, 1984*a*,*b*): the amiloride-sensitive, sodium/proton exchange, which is activated by a decrease in intracellular pH and is virtually inactive at intracellular pH values above pH 7·0, and the amiloride-insensitive sodium/sodium exchange which is also capable of transporting protons. Sodium movements *via* this pathway are predominant at intracellular pH values above 7·0. Grinstein *et al.* (1984*a*) suggest that the amiloride-insensitive system may operate continuously to regulate intracellular pH, and that the amiloride-sensitive, sodium/proton antiport would be activated only in response to lowered intracellular pH. Decreased intracellular pH activates the sodium/proton exchange also in renal microvillus membrane vesicles (Aronson *et al.* 1982) and in fibroblasts (Paris & Pouyssegur, 1984). The effects of changes in the intracellular pH on the pH regulatory system of lamprey red cells are not known.

Aggregation lowers the oxygen affinity of lamprey haemoglobins and is favoured by low pH and high haemoglobin concentration (R. E. Weber & M. Nikinmaa, in preparation). Thus, effective regulation of intracellular pH *via* the sodium/proton exchange may be needed to ensure that haemoglobin–oxygen binding is effective. Additionally, the sodium/proton exchange may be involved in the regulation of cell volume, as in *Amphiuma* red cells (see e.g. Cala, 1983). Red cell volume, by affecting the haemoglobin concentration, is also an important determinant of the haemoglobin oxygen affinity of lamprey red cells (cf. Nikinmaa & Weber, 1984).

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