Amiloride-Sensitive Sodium Transport in Lamprey Red Blood Cells: Evidence for Two Distinct Transport Pathways

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Abstract. To determine Na^+/H^+ exchange in lamprey erythrocyte membranes, the cells were acidified to pH_i 6.0 using the K^+/H^+ ionophore nigericin. Incubation of acidified erythrocytes in a NaCl medium at pH 8.0 caused a considerable rise in $^{22}Na^+$ influx and H^+ efflux during the first 1 min of exposure. In addition, exposure of acidified red cells to NaCl medium was associated with rapid elevation of intracellular Na^+ content. The acid-induced changes in Na^+ influx and H^+ efflux were almost completely inhibited by amiloride and dimethylamiloride. In native lamprey erythrocytes, amiloride-sensitive Na^+ influx progressively increased as the osmolality of incubation medium was increased by addition of 100, 200, or 300 mmol/l sucrose. Unexpectedly, the hypertonic stress induced a small, yet statistically significant decrease in intracellular Na^+ content in these cells. The reduction in the cellular Na^+ content increased with hypertonicity of the medium. The acid- and shrinkage-induced Na^+ influxes were inhibited by both amiloride and 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) in a dose-dependent manner. For both blockers, the half-maximal inhibitory values (IC_{50}) were much greater for the shrinkage-induced (44 and 15 μmol/l for amiloride and EIPA, respectively) than for the acid-induced Na^+ influx (5.1 and 3.3 μmol/l, respectively). The data obtained are the first demonstration of the presence of a Na^+/H^+ exchanger with high activity in acidified (pH_i 6.0) lamprey red blood cells (on average, 512 ± 56 mmol/l cells/h, $n = 13$). The amiloride-sensitive Na^+ influxes produced by hypertonic cell shrinkage and acid load are likely to be mediated by distinct ion transporters in these cells.

Key words: Erythrocytes — Na^+/H^+ exchange — Amiloride — EIPA — Lamprey

Introduction

The Na^+/H^+ exchange (NHE) system is almost ubiquitously present in every cell from prokaryotes to eukaryotes. So far, six mammalian NHE isoforms, termed

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NHE1 to NHE6, and one isoform from trout red cells, termed βNHE, have been cloned and characterized (Noel and Pouyssegur 1995; Yun et al. 1995; Wakabayashi et al. 1997). NHE1, the only ubiquitously expressed and most extensively studied NHE isoform, acts primarily in regulation of intracellular pH (pH_i) and cell volume. All the known NHE isoforms are inhibited by amiloride and its derivatives, though individual NHEs differ in their sensitivity to those compounds. So far, the presence of a NHE mechanism in the plasma membrane of erythrocytes in representatives of agnathans, the most primitive living vertebrates, has not been demonstrated. The red blood cells of hagfish *Mixine glutinosa* were shown to be unable to regulate pH_i after intracellular acidification with NH_4Cl (Nikinmaa et al. 1993). In the most studied agnathan species of river lamprey *Lampetra fluviatilis*, Nikinmaa and collaborators (Nikinmaa et al. 1986; Virkki and Nikinmaa 1994) suggested the existence of NHE in red blood cells. However, the authors have not presented conclusive evidence of a functioning NHE system in lamprey erythrocytes and have not provided quantitative measures of ion flux rates through the carrier. Our previous study (Gusev and Sherstobitov 1996) has documented the presence of amiloride-sensitive Na^+ transport across lamprey erythrocyte membranes in both directions. Thus, in lamprey red blood cells, both Na^+ influx and Na^+ efflux were activated by cell shrinkage and isoproterenol and inhibited in the presence of 1 mmol/l amiloride. Still the presence of NHE in these cells remains an open question.

The present study was undertaken to elucidate the existence of the NHE transport pathway in lamprey erythrocytes. We determined Na^+ influx, H^+ efflux and cellular Na^+ content in the cells after intracellular acidification to pH_i 6.0 using the K^+/H^+ ionophore nigericin. Effects of increasing hypertonicity on Na^+ influx and cellular Na^+ content were also investigated. To characterize these amiloride-sensitive Na^+ transport pathways, concentration-dependent effects of amiloride and its more active analogue, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), were studied for acid- and shrinkage-induced Na^+ transport in the cells. The results obtained demonstrate a marked activation of NHE in acid-loaded lamprey erythrocytes, whereas either NHE or Na^+ channel pathway is activated by cell shrinkage.

Materials and Methods

Cell preparation

The experiments were carried out on river lamprey *Lampetra fluviatilis* from December to March. Animals were kept at 2–4 °C in aquaria with tap water, periodically replaced with dechlorinated fresh water. After rapid decapitation, blood was withdrawn by bleeding into a heparinized tube containing a solution of the following composition (mmol/l): 140 NaCl, 4 KCl, 10 Tris-HCl (pH 7.4 at 20 °C). After immediate centrifugation (2700 × g for 5 min at 2 °C), plasma and the upper cell layer were discarded and the erythrocytes were washed three times and resuspended in standard medium to a final hematocrit of 30–40 %. The standard

medium contained (mmol/l): 140 NaCl, 4 KCl, 10 Tris-HCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose (pH 7.4 at 20°C).

Modification of pH_i

The suspension of washed erythrocytes was added to a KCl medium containing (mmol/l): 100 KCl, 30 choline chloride, 20 MES-Tris, 0.15 $MgCl_2$ (pH 6.0 at 20°C). The cells were then washed in the same medium 4 to 5 times and suspended to a final hematocrit of about 15%. After addition of 10 μmol/l nigericin (K^+/H^+ ionophore), the cell suspension was incubated for 10 min at 20°C. Afterwards, the acidified cells were sedimented by centrifugation and washed twice in a solution containing (mmol/l): 145 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 1% bovine serum albumin (BSA), and finally washed with the same KCl solution without BSA. The washed acidified erythrocytes were resuspended in the same solution in a final hematocrit of 35–40%. The cell suspension was used within 30 min for determination of ^{22}Na influx or H^+ efflux.

Ion flux measurement

All experiments were performed at a temperature of 18–20°C. Na^+ influx was measured from the uptake of ^{22}Na as described previously (Gusev and Sherstobitov 1996). In experiments on intact erythrocytes, the ^{22}Na uptake was linear for 30 min in a standard isotonic medium and within 10–20 min in hypertonic media. The incubation times were used to calculate the unidirectional Na^+ influxes.

Suspension of acidified lamprey erythrocytes in the KCl medium was added to an incubation medium containing (mmol/l): 145 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Tris-HCl (pH 8.0 at 20°C) and ^{22}Na ($\sim$3 μCi/ml). After incubation, 1 ml of the cell suspension was inserted into 10 ml of an ice-cold solution, containing 100 mmol/l $MgCl_2$ and 10 mmol/l Tris-HCl (pH 8.0). The erythrocytes were rapidly sedimented, washed twice in the same solution, and lysed by subsequent addition of 1 ml of distilled water. Radioactivity of incubation media and lysates was measured using a gamma-counter. Na^+ influx was calculated as

$$J_{Na} = A_{RBC}/A_m \cdot V \cdot t$$

where A_{RBC} is the radioactivity of erythrocytes (counts/min), A_m is the specific radioactivity of the medium (counts/min/mmol), V is the volume of erythrocytes (liters) and t is the time of incubation (min or h).

To calculate the unidirectional Na^+ influx, an incubation time was used at which linear isotope uptake occurred (0.5–1 min for control acidified cells, and 5–10 min in the presence of amiloride and EIPA at high concentrations).

To determine H^+ efflux rates, the acidified erythrocytes were incubated in a slightly buffered medium containing (mmol/l): 140 NaCl or KCl and 1 mmol/l Tris-HCl (pH 8.0 at 20°C). After addition of an aliquot of the cell suspension to the medium, extracellular pH (pH_e) was recorded continuously by means of an ionometer and a chart recorder. The initial linear part of the curve was used to

calculate unidirectional H^+ efflux. To determine the amount of H^+ equivalents released by the cells, control titration of the incubation medium with 0.1 mol/l HCl was made in each experiment (scale calibration). The H^+ flux magnitudes (mmol/l cells/min) were calculated as follows:

$$J_H = \Delta H/V \cdot t$$

where ΔH is the amount of acid milliequivalents from the calibration scale, corresponding to the initial pH shift (mmol/l medium), V is cell concentration (l/l, cells : medium) and t is the incubation time (min) corresponding to the pH shift.

To determine the intracellular Na^+ and K^+ content, the lamprey erythrocytes were incubated in the same medium as for the measurement of ^{22}Na influxes. Aliquots of cell suspensions were injected into an ice-cold solution of 100 mmol/l $MgCl_2$ and 10 mmol/l Tris-HCl (pH 7.4 or 8.0), then washed twice with the same solution and lysed in distilled water. Na^+ and K^+ concentration was measured using the flame photometer Flapho-40.

Reagents

Amiloride, 5-(N,N-dimethyl)-amiloride (DMA), nigericin, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), Tris(hydroxymethyl)aminomethane and 2-(N-morpholino)-ethanesulfonic acid (MES) were obtained from Sigma, ^{22}Na from Isotop (Russia). Stock solutions (mmol/l): 1 nigericin, 100 amiloride, 1 or 10 EIPA, and 1 DMA were prepared in dimethyl sulfoxide (DMSO).

Statistics

Statistical analysis was performed using the Student's t-test for paired and unpaired data. Nonlinear regression analysis was carried out using the SigmaPlot software version 5.0 (Jandel Scientific). All data are expressed as mean ± standard error (S.E.).

Results

Effect of cell shrinkage on Na^+ influx and intracellular Na^+ content

Lamprey red blood cells were incubated in isotonic and hypertonic media for 10–20 min in the presence or in the absence of ^{22}Na. Na^+ influx, calculated from ^{22}Na uptake, was significantly higher in the hypertonic medium than in the isotonic medium. As illustrated in Fig. 1A, a progressive increase in the Na^+ influx occurred as the hypertonicity of the incubation medium rose. Thus the Na^+ transport in lamprey erythrocytes in hypertonic medium containing 300 mmol/l sucrose averaged 40.1 ± 2.8 mmol/l cells/h, whereas in the isotonic medium it averaged 4.8 ± 0.4 mmol/l cells/h. Addition of 0.1 mmol/l EIPA or 1 mmol/l amiloride to the hypertonic medium was associated with a significant decrease in the Na^+ influx. However, the hypertonicity-induced Na^+ influx in the presence of either blocker was nearly two-fold higher than that under isotonic conditions.

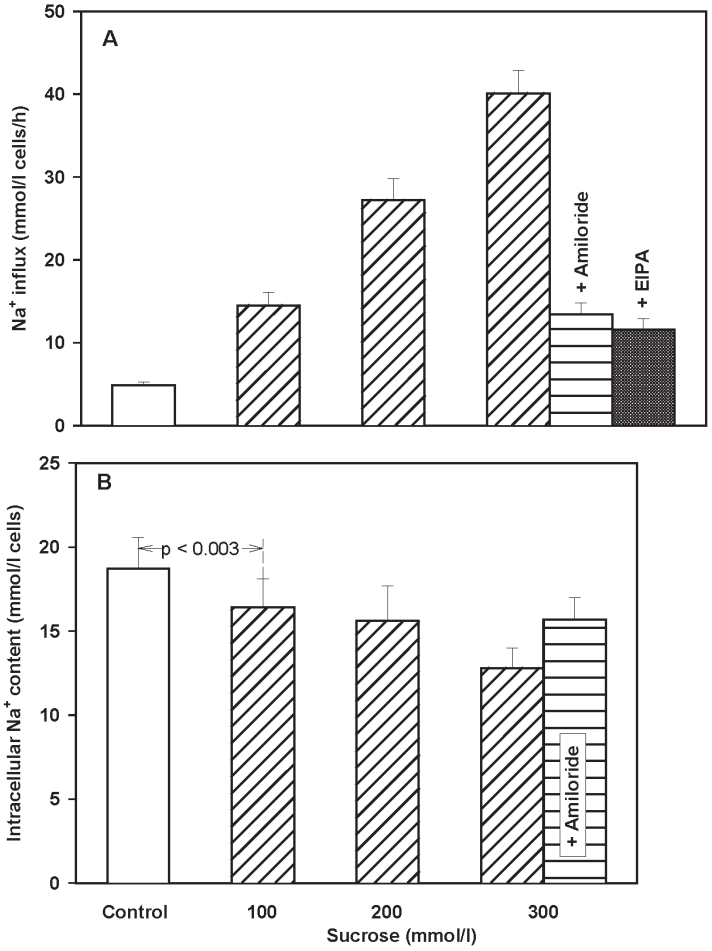

Figure 1. Effect of hyperosmotic stress on Na^+ influx (A) and intracellular Na^+ content (B). Lamprey erythrocytes were incubated in a standard isotonic medium (control) or in the same medium containing additionally 100, 200 and 300 mmol/l sucrose (with or without 1 mmol/l amiloride and 0.1 mmol/l EIPA). Na^+ influxes were determined from ^{22}Na uptakes during 30 min (control) and 10–20 min (hypertonic medium). The same incubation times were used for determination of intracellular Na^+ content. Values are mean ± S.E.M. of 8 experiments. In all cases, there was a significant difference between the control and hypertonic medium ($p < 0.005$, paired t-test).

Despite the stimulation of Na^+ influx in lamprey erythrocytes incubated in hypertonic medium, there was a statistically significant decrease in the intracellular Na^+ content (Fig. 1B). In hypertonic medium with 100 mmol/l sucrose, the cellular Na^+ content was by 3.3 ± 0.4 mmol/l cells lower than in isotonic medium ($n = 6$,

$p < 0.003$, paired t-test). The maximal reduction in intracellular Na^+ content was found in the cells incubated in hypertonic medium with 300 mmol/l sucrose (6.85 ± 0.67 mmol/l cells). The change in the cellular Na^+ content was partially inhibited in the presence of 1 mmol/l amiloride. These data clearly indicate that hypertonic cell shrinkage produces simultaneous stimulation of both Na^+ influx and Na^+ efflux from the cells.

Effect of intracellular acidification on Na^+ influx, H^+ efflux and intracellular Na^+ concentration

To produce intracellular acidification, lamprey erythrocytes were pre-treated with the K^+/H^+ ionophore nigericin in the KCl medium at pH 6.0 (see Materials and Methods). Incubation of the acidified red blood cells in a NaCl medium at pH 8.0 led to a sharp activation of ^{22}Na uptake (Fig. 2). The uptake of ^{22}Na was linear during the initial 1 min period and the interval between 0.5 and 1 min was used to determine the Na^+ influx. The initial rate of unidirectional Na^+ transport in acidified red cells was 12.2 ± 1.0 mmol/l cells/min and it decreased to 0.63 ± 0.20 mmol/l/min in the presence of 1 mmol/l amiloride (Fig. 2). Exposure of the acidified cells to the NaCl medium at pH 8.0 was also accompanied by rapid increase in the intracellular Na^+ content. Maximal elevation of the cellular Na^+ content was achieved during the first 0.5 min of cell incubation. Comparative data on the changes in the unidirectional Na^+ influx and cellular Na^+ content during the first

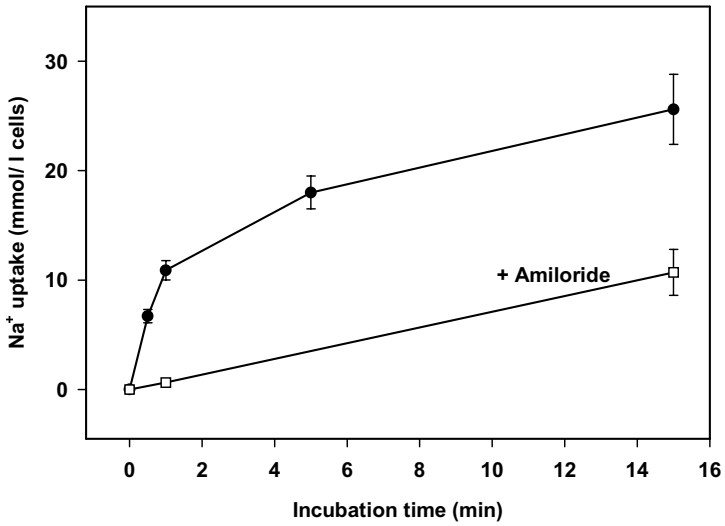

Figure 2. Time course of Na^+ uptake by acidified red cells. Lamprey erythrocytes (pH_i 6.0) in KCl medium were added to NaCl medium (pH 8.0) containing ^{22}Na with or without 1 mmol/l amiloride and radioisotope uptakes were measured (see Materials and Methods). Each data point is mean ± S.E.M. of 6 different experiments.

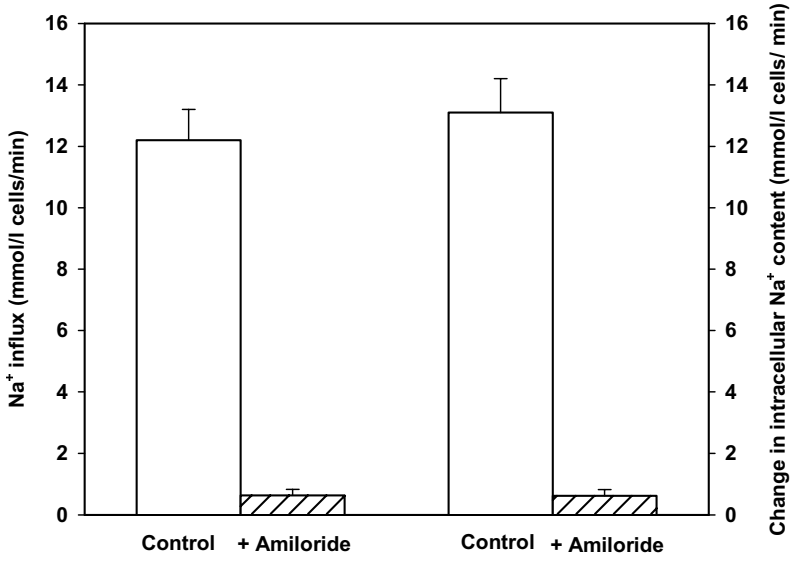

Figure 3. The relationship between Na^+ influx (left) and intracellular Na^+ accumulation (right) in acidified red blood cells or cells treated with 1 mmol/l amiloride. The Na^+ influx was calculated from the initial linear ^{22}Na uptake for 0.5–1.0 min (see Fig. 2). In parallel experiments under the same conditions, the intracellular Na^+ content was measured using a flame photometry method. Changes in cell Na^+ content were calculated during the first 0.5–1.0 min of cell incubation. Mean values ± S.E.M. of 6 experiments are presented.

1 min of incubation are presented in Fig. 3. The increase in Na^+ content was 7.6 ± 0.5 mmol/l within 0.5 min of cell incubation. Changes in cellular Na^+ concentration were significantly inhibited by amiloride. There was no significant alteration in intracellular K^+ content during incubation of acidified erythrocytes in the NaCl medium at pH 8.0. The intracellular K^+ content in acidified cells was 93 ± 3 mmol/l cells before incubation and 88 ± 3.5 mmol/l after 15 min of incubation.

In order to establish conclusively the involvement of the NHE system in the changes of Na^+ transport, H^+ efflux from acidified lamprey erythrocytes was also investigated. The acidified cells were incubated in the NaCl medium at pH 8.0 under continuous registration of pH in the medium (pH_e) using a pH-sensitive electrode (see Materials and Methods). A rapid fall in pH_e by 0.5–0.6 units was found to occur within the first 5 min of cell incubation. The initial linear shift in pH_e during first 1 min reflects a H^+ efflux from these cells. When the acidified red blood cells were incubated in a KCl medium at pH 8.0, the H^+ efflux was much lower compared to the NaCl medium. Fig. 4 shows the calculated mean values of H^+ efflux from lamprey erythrocytes. The treatment of acidified cells with 10 μmol/l DMA caused a marked inhibition of H^+ efflux from the cells in the NaCl medium, but not in the KCl medium. Thus, in contrast to cell shrinkage, acidification of lamprey erythrocytes to pH 6.0 resulted in significant stimulation of NHE.

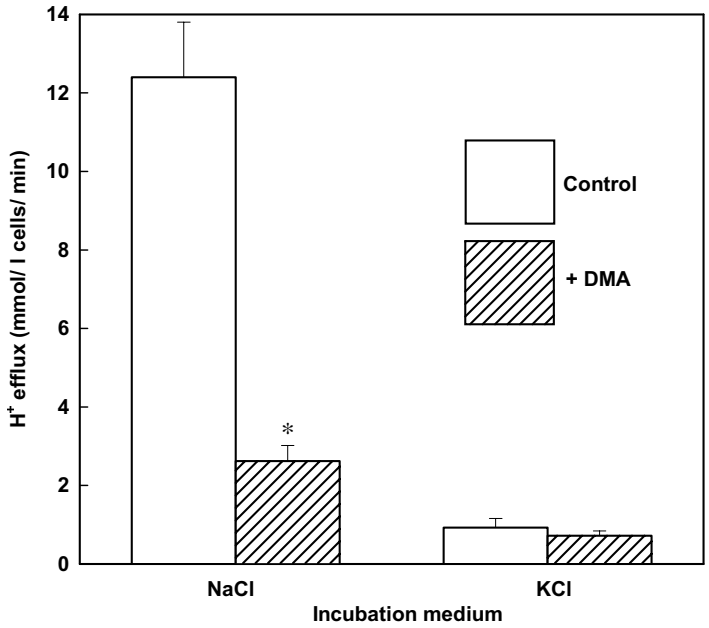

Figure 4. H^+ efflux from acidified red cells incubated in NaCl or KCl medium at pH 8.0. The cells were incubated in low-buffered media with or without 10 μmol/l DMA and H^+ efflux was measured as described in Materials and Methods. Each column represents a mean ± S.E.M. of 6 experiments. Asterisk indicates a significant difference from control ($p < 0.001$).

Amiloride and EIPA dose-dependent inhibition of Na^+ influx induced by cell shrinkage and acidification

Stimulation of Na^+ influx in the lamprey erythrocytes induced both by cell shrinkage and by acidification was significantly inhibited by 1 mmol/l amiloride (Figs. 1–3). To further characterize the Na^+ transport pathway(s) in lamprey erythrocytes, we investigated the inhibitory ability of amiloride and its derivative EIPA, a selective inhibitor of the NHE1 isoform of the Na^+/H^+ antiporter (Noel and Pouyssegur 1995; Yun et al. 1995; Wakabayashi et al. 1997). In two series of experiments, lamprey erythrocytes in hypertonic medium with 100 mmol/l sucrose or acidified erythrocytes in NaCl medium at pH 8.0 were treated with various amiloride (1–1000 μmol/l) and EIPA (0.2–100 μmol/l) concentrations. Both blockers were more efficient in inhibition of Na^+ influx stimulated by cell acidification than by cell shrinkage. Amiloride in a concentration of 20 μmol/l suppressed 78.3 ± 3.3 and $31.4 \pm 12\%$ of the acid- and shrinkage-induced Na^+ influx, respectively. EIPA, a specific inhibitor of NHE, was significantly more potent in inhibiting Na^+ influx in lamprey erythrocytes than amiloride. In concentration of 20 μmol/l, EIPA blocked 87.8 ± 1.9 and $56.2 \pm 4.1\%$ of Na^+ influx induced by cell acidification and

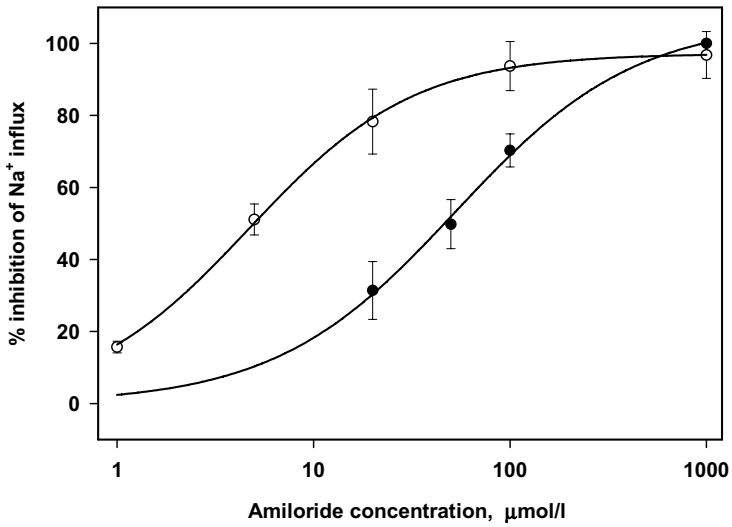

Figure 5. Dose-response curves for inhibition of acid-induced (open circles) and shrinkage-induced (closed circles) Na^+ influx by amiloride. In two series of experiments, Na^+ influxes were determined in acidified erythrocytes incubated in NaCl medium at pH 8.0 or in native erythrocytes incubated in hypertonic medium (+100 mmol/l sucrose) in the presence of various amiloride concentrations. Control values (taken as 100%) in the absence of amiloride were 395 ± 37 and 16.9 ± 1.4 mmol/l cells/h for acid- and shrinkage-induced Na^+ influxes, respectively. Each data point represents mean ± S.E.M. of 6 (acidified cells) and 8 (cell shrinkage) experiments. The data were fitted by hyperbolic curves using nonlinear regression analysis in the SigmaPlot software.

shrinkage, respectively. At maximal concentrations, the two inhibitors produced an almost complete suppression of Na^+ influxes induced by cell acidification and shrinkage.

Figs. 5 and 6 show dose-response curves for inhibition of both acid- and shrinkage-activated Na^+ influx by amiloride and EIPA, respectively. The fit to data obtained for the two inhibitors using a Hill-type equation provided a Hill coefficient close to 1, suggesting inhibition of Na^+ influx at a single site. Therefore, the curves were well described by a hyperbolic function. The hyperbolic lines and apparent inhibition kinetics were obtained from an iterative computer curve-fitting program SigmaPlot. The concentrations of amiloride that inhibit one-half (IC_{50}) of the shrinkage- and acid-induced Na^+ influx were 51 ± 3.7 and 4.7 ± 0.19 μmol/l, respectively. The apparent IC_{50} values for EIPA were 14.6 ± 3.7 and 2.4 ± 0.63 μmol/l, for inhibition of the shrinkage- and acid-activated Na^+ influx, respectively. Apparently, the Na^+ influx activated by cell shrinkage is much less sensitive to both drugs than that stimulated by acid load.

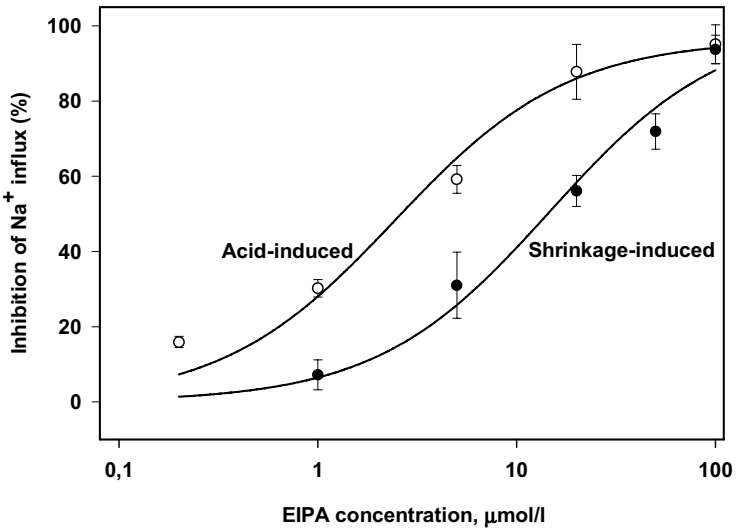

Figure 6. Effects of increasing concentrations of EIPA on acid- and shrinkage-induced Na^+ influxes. The data were obtained in the same experiments as those in Fig. 5.

Discussion

The main finding of this study is the discovery of NHE activity in lamprey red blood cells. We first demonstrated high activity of the NHE system in these cells when they were acidified to pH 6.0 using the K^+/H^+ ionophore nigericin. Activation of Na^+ influx (Fig. 2) and H^+ efflux (Fig. 4), as well as increase in the cellular Na^+ content (Fig. 3) provide a clear evidence for the presence of NHE activity in the acidified lamprey erythrocytes incubated in NaCl medium at pH 8.0. Moreover, these changes in Na^+ and H^+ transport occurred simultaneously, achieving maximum value during the first 0.5–1 min of cell incubation. NHE blockers – amiloride and its analogues – inhibited more than 90% of the acid-induced Na^+ and H^+ fluxes. In the first series of our experiments, the unidirectional fluxes of Na^+ and H^+ (12.2 ± 1.0 and 12.4 ± 1.4 mmol/l/min, respectively) were similar to the calculated rate of the initial increase in cellular Na^+ content (13.1 ± 1.1 mmol/l/min). There was a somewhat lower activity of the NHE in the second series of experiments. For all experiments, calculated as the rate of unidirectional amiloride-sensitive Na^+ influx in acidified cells, the NHE activity averaged 8.53 ± 0.94 mmol/l/min ($n = 13$). The NHE activity in lamprey erythrocytes was considerably greater than that observed in acidified ($pH_i \sim 6.0$) red blood cells of different mammalian species (Jennings et al. 1986; Zhao and Willis 1993; Orlov et al. 1998). Currently, relatively little is known about the effect of intracellular acid load on activation of the NHE in nucleated erythrocytes of lower vertebrates (Gusev 2001). In erythrocytes

of trout, the most extensively studied fish erythrocytes, NHE has been shown to remain inactive even at pH_i as low as 6.3 (Motais et al. 1992). However, stimulation of NHE by cell acidification was observed in nucleated erythrocytes of flounder (Weaver et al. 1999), salamander (Cala and Maldonado 1994), and frog (Jorgensen 1995).

The results obtained in this work confirm our preliminary studies (Gusev and Sherstobitov 1996) showing that hypertonic treatment of lamprey erythrocytes results in an increase of Na^+ influx. In the present study, increasing medium osmolality raised Na^+ influx (Fig. 1A). It is noteworthy that we never observed an increase of cellular Na^+ content in the lamprey red blood cells under these hypertonic conditions (Fig. 1B). On the contrary, the intracellular Na^+ content decreased the more, the greater the medium hypertonicity. The inability of hypertonic shrinkage to increase the cellular Na^+ content cannot be accounted for by activation of the Na^+-K^+ pump, since the Na^+,K^+-ATPase inhibitor ouabain did not affect cellular Na^+ concentration (unpublished data). However, the shrinkage-induced changes in Na^+ influx and Na^+ cell content were inhibited by amiloride and its analogue EIPA (Fig. 1A,B). At relatively low hypertonicity (addition of 100 mmol/l sucrose), the two inhibitors caused an almost complete suppression of the shrinkage-induced Na^+ influx. However, at maximal hypertonicity (300 mmol/l sucrose), 1 mmol/l amiloride and 0.1 mmol/l EIPA suppressed only 79% and 83% of the shrinkage-induced Na^+ influx, respectively, suggesting that other amiloride-insensitive mechanisms could be involved in the hypertonically activated Na^+ transport.

We have argued that osmotically induced changes of amiloride-sensitive Na^+ influx are likely to reflect a Na^+ channel or Na^+/Na^+, but not Na^+/H^+, exchange activities. As has been shown earlier, increasing osmolality of incubation medium did not cause H^+ efflux from lamprey red blood cells (Gusev and Sherstobitov 1996). Evidently, the amiloride-sensitive transport system can mediate net Na^+ movement in either direction in the lamprey erythrocytes in hypertonic medium. The reduction of cellular Na^+ content in lamprey erythrocytes under hypertonic stress (Fig. 1B) indicates that Na^+ efflux increases in a greater extent than does Na^+ influx. As shown in other studies on lamprey erythrocytes (Gusev and Sherstobitov 1992; Nikinmaa and Boutilier 1995; Gusev and Sherstobitov 1996), this transport system is operating at a lower activity under isotonic conditions and may be activated by isoproterenol. A similar activation by cell shrinkage of both Na^+ influx and Na^+ efflux has been documented also in mammalian erythrocytes (Sergeant et al. 1989; Zhao and Willis 1993) and other cell types (Wehner et al. 2000). Recently, Huber et al. (2001) in a patch-clamp study on human erythrocytes have demonstrated the presence of shrinkage-activated, amiloride-sensitive nonselective cation channels (NSC). A similar NSC conductance may mediate the volume-sensitive Na^+ transport in lamprey erythrocytes. The data obtained in the present study are in close agreement with our previous observations indicating that lamprey red cells are unable to restore their volume when incubated in hypertonic medium during 4 h (Gusev and Sherstobitov 1997). In this respect, it should be noted that a similar absence of the hypertonic regulatory volume increase (RVI)

has been demonstrated also for many other cell types (see the review of O'Neill 1999).

Thus, the data obtained provide an obvious evidence for the presence of two amiloride-sensitive Na^+ transport pathways in lamprey red blood cells. Both pathways may operate either *via* the same NHE system operating in two different modes, or by different ion transporters. A useful way to distinguish these Na^+ transport pathways is by their sensitivities to inhibition by amiloride and its analogues. We therefore compared dose-dependent responses to amiloride and EIPA of the acid- and shrinkage-induced Na^+ influxes in lamprey erythrocytes. EIPA has been shown to block individual NHE subtypes in a strictly concentration-dependent manner (Noel and Pouyssegur 1995; Yun et al. 1995; Wakabayashi et al. 1997). The results of the present work show that these two Na^+ transport pathways have quite different sensitivities to amiloride and EIPA (Figs. 5, 6). Since amiloride and its derivates are thought to act competitively near the Na^+-binding site, higher IC_{50} values have to be expected in the presence of high concentrations of extracellular Na^+. In our experiments, the shrinkage- and acid-induced Na^+ influxes were measured at the same external Na^+ concentration (140 mmol/l), but at different medium pH (7.4 and 8.0). Amiloride and its analogues inhibit the NHE in the protonated (positively charged) form (Benos 1982). The protonated fraction of amiloride is about 95 % and 80 % at pH 7.4 and 8.0, respectively (assuming pK = 8.7). Taking into account the pH effects, the difference between the shrinkage- and acid-induced Na^+ transport in sensitivity to the two inhibitors would be expected to be more significant. These data indicate that there may be two different amiloride-sensitive Na^+ transporters in the lamprey erythrocyte membrane, one of which is a Na^+/H^+ antiporter.

Among the known NHE isoforms, only NHE1 is a ubiquitous isoform, which has been found in human erythrocytes (Rutherford et al. 1997; Sarangarajan et al. 1998). Moreover, this isoform is activated by intracellular acidification and cell shrinkage. In mammalian tissues that express NHE1, amiloride inhibits the NHE with IC_{50} values ranging between 3 and 10 μmol/l. For EIPA, the IC_{50} values between 0.02 and 0.3 μmol/l have been reported in different cell types that are known to express the NHE1 subtype of the Na^+/H^+ antiporter (Vigne et al. 1983; Astarie et al. 1990; Escobales et al. 1990; Hasselblatt et al. 2000; Orlov et al. 2000). In the present study, the values of half-maximal inhibition of the lamprey NHE were 4.7 and 2.4 μmol/l for amiloride and EIPA, respectively. Evidently, the NHE in lamprey erythrocytes has sensitivity to amiloride similar to that of NHE1. However, there is at least a tenfold difference in the inhibitory potency of EIPA for these antiporters. Moreover, the NHE in lamprey red blood cells is not, unlike NHE1, activated by hyperosmotic stress. Thus, the lamprey Na^+/H^+ antiporter is likely to be pharmacologically and functionally different from the mammalian NHE1 isoform.

In conclusion, these results have led to the opinion that there are two different amiloride-sensitive Na^+ transport systems in lamprey red blood cells. One of them appears to be mediated by a Na^+ conductance, functions under resting conditions, and is stimulated by cell shrinkage and isoproterenol. The other pathway operates

via a high-capacity NHE, which is activated by cytosolic acidification. Further studies will examine other aspects of this antiporter, including its kinetic properties, physiological role and regulation of its activity in lamprey erythrocytes.

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