Transport of lithium across the lamprey (*Lampetra fluviatilis*) erythrocyte membrane

Gennadii P. Gusev, Natalia I. Agalakova and Tatiana I. Ivanova

Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia

Abstract. Lithium, capable of replacing Na^+ in various membrane transport processes, was used to investigate Na^+ transport pathways across the lamprey erythrocytes membrane. The values of Li^+ influxes have ranged from 8 to 24 mmol/l cells/h. Intracellular accumulation of Li^+ was associated with loss of cellular Na^+, the value of which was less than the value of Li^+ influx. Both Li^+ influx and Na^+ efflux were partially inhibited by amiloride. The amiloride-sensitive Li^+ influx was considerably stimulated by hyperosmotic cell shrinkage. The treatment of lamprey erythrocytes with blockers of protein phosphatases (fluoride and cantharidin) also resulted in a considerable increase in Li^+ accumulation within the cells. No significant difference was observed between the values of Li^+ and Na^+ (^{22}Na) influxes measured in red cells incubated simultaneously in isotonic LiCl and NaCl media (9.2 ± 2.1 and 7.8 ± 1.3 mmol/l cells/h, respectively). In hypo- and hypertonic media, however, the rate of Na^+ influx in lamprey erythrocytes was approximately twice higher as compared to the rate of Li^+ influx, what was determined by the difference in the amiloride-sensitive components. In acidified lamprey erythrocytes (intracellular pH 6.0) Li^+ and Na^+ influxes were considerably increased due to activation of amiloride-sensitive Na^+/H^+ (Li^+/H^+) exchange mechanism, although the activity of Na^+/H^+ exchange was much greater than that of Li^+/H^+ exchange. The data obtained confirm the hypothesis on the presence of two amiloride-sensitive systems of Na^+ transport in the lamprey red blood cells.

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

Introduction

Great interest to the membrane effects of lithium (Li^+) is associated with its capacity to replace Na^+ ions transported through Na^+-K^+ pump, ion carriers and channels of different nature, shown under experimental conditions on the cells of different types (Ehrlich and Diamond 1980; Holstein-Rathlou 1990). Moreover, the recent works (Padan et al. 2001; Orlowski and Grinstein 2004) have established that amongst monovalent cations (K^+, Rb^+, Cs^+, $NH4^+$, choline) only Li^+ possesses a capability to be transported by Na^+/H^+ exchange mechanism. The aforementioned has allowed using lithium in some investigations for determining the Na^+/H^+ exchange activity (Busch et al. 1995) and as a marker for studying Na^+/Na^+ exchange in the plasma membrane of cells of different types including erythrocytes (Semplicini et al. 2003).

Lampreys (Petromyzoniformes, Cyclostomata), the unique animals with peculiar body anatomy and life cycle, diverged from the main stream of vertebrate evolution around 500 millions years ago (Kuraku and Kuratani 2006). Although their developmental and morphological properties are not homologous to other groups of Craniates, they possess a great number of functional commonalities shared with higher vertebrates (Osório and Rétaux 2008). Hence, the lampreys can be considered as a key species to study the evolution of molecular mechanisms throughout the groups of vertebrate animals. Comparing the transport of inorganic ions across erythrocyte plasma membrane in cyclostomes with that reported for representatives of higher vertebrates give information as to which properties are ancient and common to the whole vertebrate lineage and which have evolved during vertebrate evolution. For example, the

Correspondence to: Gennadii Petrovich Gusev, Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Thorez pr. 44, 194223 St. Petersburg, Russia
E-mail: gusev@iephb.ru

erythrocyte membrane of lamprey *Lampetra fluviatilis* is characterized by low anion conductance and the presence of K^+ channels (Nikinmaa and Railo 1987; Virkki and Nikinmaa 1998). Na^+ transport in the lamprey red blood cells exhibits unusual peculiarities as well. While incubated in the standard isotonic medium, lamprey erythrocytes have been revealed to possess an amiloride-sensitive component of Na^+ transport functioning in both directions (Gusev et al. 1992; Gusev and Sherstobitov 1996). Amiloride-sensitive influx and efflux of Na^+ in these cells were stimulated after hyperosmotic shrinkage and under influence of isoproterenol or inhibitors of protein phosphatases (Gusev and Sherstobitov 1996; Gusev and Ivanova 2006). Hyperosmotic shrinkage of lamprey erythrocytes was not accompanied by efflux of H^+, but resulted in decreasing the intracellular concentration of Na^+. On the other hand, acidification of lamprey erythrocytes using nigericin has caused an activation of amiloride-inhibited Na^+/H^+ exchange, the presence of which was confirmed by equivalent fluxes of Na^+ and H^+, as well as by accumulation of Na^+ in erythrocytes (Ivanova et al. 2001). On the basis of obtained data, we have suggested the presence of two amiloride-sensitive pathways of Na^+ transport in lamprey erythrocyte membrane (Gusev and Ivanova 2004). The present work was undertaken to verify this assumption using Li^+ as an analog for Na^+ transport across the lamprey erythrocyte membrane.

Materials and Methods

Cell preparation

Experiments were performed from November to April on erythrocytes of lamprey *Lampetra fluviatilis* weighting 70–80 g. The lampreys were fished out the delta of Neva River in October–November during their pre-spawning migration from Baltic Sea and kept in aquaria with dechlorinated fresh water aerated and cooled to 3–5°C. After decapitation, the blood was immediately collected into heparinized tubes with the standard cold saline containing (in mmol/l): 140 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Tris-HCl (pH 7.4 at 4°C). After rapid centrifugation for 5 min at 4°C and 3000 × *g* the supernatant was aspirated, and the erythrocytes were washed with the same solution three-four times removing the upper layer of white cells. The final suspension of washed erythrocytes was prepared on the same solution.

Li^+ and Na^+ influxes in lamprey erythrocytes

Unidirectional Li^+ influx was measured from the initial velocity of cellular Li^+ uptake in LiCl-medium. The final suspension of erythrocytes was quickly washed twice with cold LiCl solution containing (in mmol/l): 140 LiCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Tris-HCl (pH 7.4 at 4°C). Then the cells were suspended with haematocrit of 30–40% in the standard LiCl-medium of the same ion composition added with 10 mmol/l glucose and buffered to pH 7.4 at 20°C. To prepare hypertonic medium, 100 mmol/l sucrose was added to the standard LiCl-medium. Aliquots of the cell suspension were added to the test tubes with isotonic or hypertonic media in absence or presence of tested chemicals. After incubation, the erythrocytes were injected into 10 ml of ice-cold washing solution containing (in mmol/l) 110 $MgCl_2$ and 10 Tris-HCl (pH 7.4 at 4°C), rapidly sedimented, washed twice in the same solution and lysed in 10 ml of distilled water. Intracellular concentrations of Li^+ and Na^+ were measured using Shimadzu atomic absorbance spectrophotometer AA-6800 and flame photometer Flapho-40, respectively.

In a separate set of experiments, Li^+ and Na^+ influxes were measured in parallel in hypo-, iso- and hypertonic media. Na^+ influx was measured from the uptake of ^{22}Na (~3 μCi/ml) as described earlier (Gusev and Sherstobitov 1996). The final suspension of erythrocytes was divided and rapidly washed in parallel with the NaCl and LiCl media containing (in mmol/l): 100 NaCl or LiCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Tris-HCl (pH 7.4 at 4°C). Hypotonic media contained (in mmol/l): 100 NaCl or LiCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 10 Tris-HCl (pH 7.4 at 20°C). The iso- and hypertonic media were prepared on the basis of hypotonic medium by addition of 100 and 140 mmol/l sucrose, respectively. After incubation, the cell pellets were washed with cold $MgCl_2$-Tris solution (see above) and lysed in 10 ml of distilled water for determination of Li^+ content or in 1 ml of trichloracetic acid for measurement of ^{22}Na accumulation. Li^+ or Na^+ influxes were expressed as millimole per liter packed cells per hour.

Modification of intracellular pH

The final suspension of erythrocytes was washed 2 times with non-buffered KCl solution containing (in mmol/l) 145 KCl and 1 $MgCl_2$, then 2 times with KCl-MES medium containing (in mmol/l) 100 KCl, 30 choline chloride, 20 MES-Tris (pH 6.0 at 20°C). After addition of 1 μmol/l nigericin (K^+/H^+ ionophore), the suspension of erythrocytes was incubated for 10 min at 20°C. Acidified cells were washed twice with non-buffered KCl solution with addition of 1% bovine serum albumin (BSA), and finally with the same solution without BSA. The acidified erythrocytes were resuspended in the same medium in a final haematocrit of 30–40% and used immediately. The cells were incubated in the standard NaCl and LiCl media containing (in mmol/l): 140 NaCl or LiCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Tris (pH 8.0 at 20°C). After incubation, the erythrocytes were washed as described above, lysed in distilled water and subjected to determination of intracellular concentration of Na^+ and Li^+.

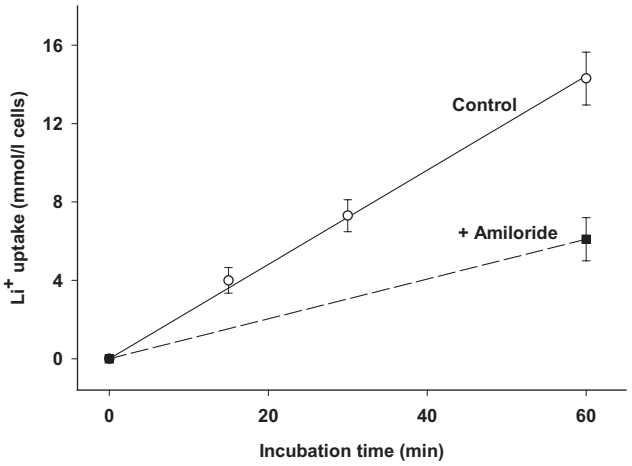

Figure 1. Time course of Li^+ influx into lamprey erythrocytes. Erythrocytes were incubated at 20°C in the standard LiCl medium for 15, 30 and 60 min without amiloride and for 60 min with 1 mmol/l amiloride, then the intracellular concentration of Li^+ was determined in the samples as described in Materials and Methods. Each data point is a mean ± S.E.M. for 9 experiments.

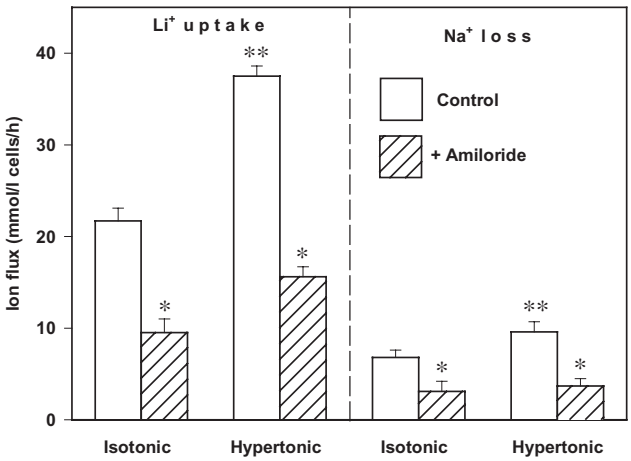

Figure 2. Effect of cell shrinkage on Li^+ influx and Na^+ efflux in lamprey erythrocytes. Red blood cells were incubated for 60 min simultaneously in isotonic and hypertonic LiCl medium in absence and presence of 1 mmol/l amiloride. Intracellular contents of Na^+ and Li^+ were determined in the cell lysates as described in Materials and Methods. Bars represent average values ± S.E.M. for 9 experiments. * $p < 0.001$ as compared to control, ** $p < 0.001$ as compared to isotonic.

Chemicals

All reagents, buffers and salts were analytical grade. Amiloride, nigericin, tris(hydroxymethyl)aminomethane and 2-(N-morpholino)-ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO, USA). Cantharidin was obtained from MP Biomedicals (Irvine, CA, USA). Stock solutions of amiloride and nigericin were prepared on dimethylsulfoxide (DMSO), the same volumes of DMSO were added to the control samples. Stock solution of sodium fluoride (500 μmol/l) was prepared on distilled water. ^{22}Na was obtained from ISOTOP (Russia).

Statistical analysis

All data were processed by SigmaPlot software package version 6.0 (Jandel Scientific). Statistical differences of the measured variables were assessed using Student's *t* test for paired data. Results are mean values with standard errors (± S.E.M.). *p* values less than 0.05 were considered significant.

Results

Influx of Li^+ in lamprey erythrocytes and effect of shrinkage

The Li^+ uptake in the cells incubated in the standard LiCl medium was linear during 60 min of incubation and averaged 14.3 ± 1.3 mmol/l/h (Fig. 1). An exposure of the cells to amiloride resulted in considerable suppression of Li^+ accumulation (6.1 ± 1.1 mmol/l/h). Thus, the transport of Li^+ into lamprey erythrocytes is analogous to transport of Na^+ described earlier (Gusev and Sherstobitov 1996; Gusev and Ivanova 2004, 2006). Therefore, the following experiments were undertaken to study the influx of Li^+ under cell shrinkage, known to affect Na^+ influx into these cells. Parallel incubation of lamprey erythrocytes into isotonic and hypertonic media has shown that hyperosmotic shrinkage of the cell was accompanied by substantial acceleration of Li^+ uptake (37.5 ± 2.4 vs. 21.7 ± 1.4 mmol/l/h) (Fig. 2). Li^+ influx in both media was significantly inhibited in the presence of amiloride, with both amiloride-sensitive and residual components of Li^+ transport elevated under cell shrinkage. Accumulation of Li^+ within the cells was associated with simultaneous loss of Na^+ also partially inhibited by amiloride. The amiloride-sensitive component of Na^+ efflux was significantly increased after cell shrinkage (3.7 ± 0.35 and 5.9 ± 0.44 mmol/l/h in isotonic and hypertonic media, respectively; $p < 0.01$).

Comparison of Na^+ and Li^+ transport in lamprey erythrocytes

To evaluate the ratio between the rates of Na^+ and Li^+ transport, lamprey erythrocytes were incubated in parallel in NaCl and LiCl hypo-, iso- and hypertonic media in absence or presence of amiloride (Figs. 3 and 4). In isotonic medium, no significant difference between Na^+ and Li^+ influxes was observed (9.2 ± 2.1 and 7.8 ± 1.3 mmol/l cells/

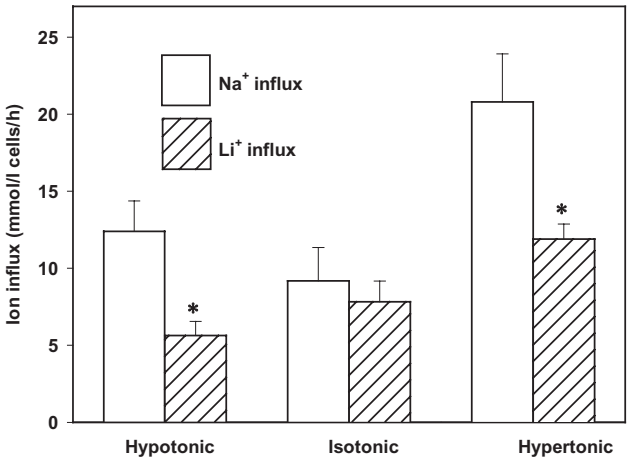

Figure 3. Comparison of Na^+ and Li^+ influxes in lamprey erythrocytes. Erythrocytes were incubated in parallel into hypo-, iso- and hypertonic NaCl and LiCl. Na^+ influx was measured from ^{22}Na uptake for 20 min (see Materials and Methods), Li^+ influx was determined as accumulation of Li^+ in the cells for 20 min. Mean values ± S.E.M. for 6 experiments are given. * $p < 0.01$ as compared to Na^+ influx.

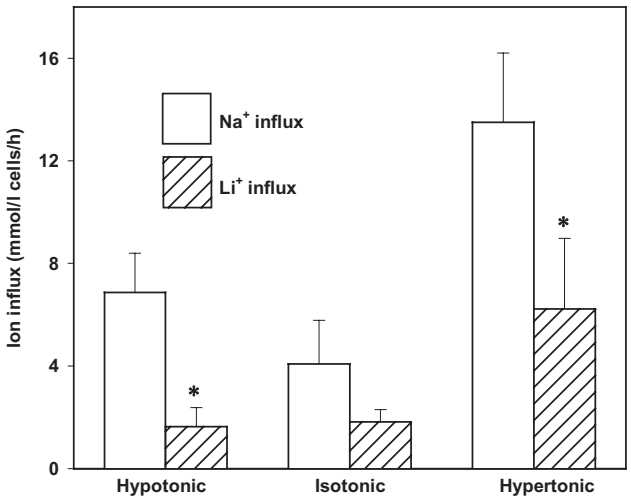

Figure 4. Comparison of amiloride-sensitive Na^+ and Li^+ influxes in lamprey erythrocytes. Erythrocytes were incubated in parallel into hypo-, iso- and hypertonic NaCl and LiCl media with or without 1 mmol/l amiloride. Na^+ and Li^+ influxes were determined as on Fig. 3. Mean values ± S.E.M. for 6 experiments are given. * $p < 0.01$ as compared to Na^+ influx.

h, respectively). However, Na^+ influx was approximately twice higher than Li^+ influx in hypo- and hypertonic media (Fig. 3). Both Na^+ and Li^+ influxes were partially inhibited by addition of amiloride. The values of residual (amiloride-insensitive) components of Na^+ and Li^+ influxes have not differed between incubation media and not depend on the osmolarity of the medium. The calculated residual fluxes of Na^+ and Li^+ in all media were 5.64 ± 0.48 and 5.08 ± 0.43 mmol/l cells/h in average, respectively ($n = 24$). Fig. 4 depicts the calculated amiloride-sensitive components of Li^+ and Na^+ fluxes. It is clear that the higher rate of Na^+ influx relative to Li^+ influx in the cells incubated in hypo- and hypertonic media is accounted for the difference in the amiloride-sensitive components.

Effects of protein phosphatases inhibitors on Li^+ influx in lamprey erythrocytes

In our recent work, an amiloride-sensitive transport of Na^+ in lamprey erythrocytes has been shown to be activated by the inhibitors of types PP-1 and PP-2A protein phosphatases (Gusev and Ivanova 2006). Therefore we have studied the effects of unspecific (fluoride) and specific (cantharidin) inhibitors of protein phosphatases on Li^+ influx in these cells. The treatment of lamprey erythrocytes with 20 mmol/l fluoride resulted in approximately two-fold increase in Li^+ accumulation within the cells (Fig. 5). Under influence of cantharidin, both Li^+ influx (Fig. 6) and Na^+ loss were also considerably elevated.

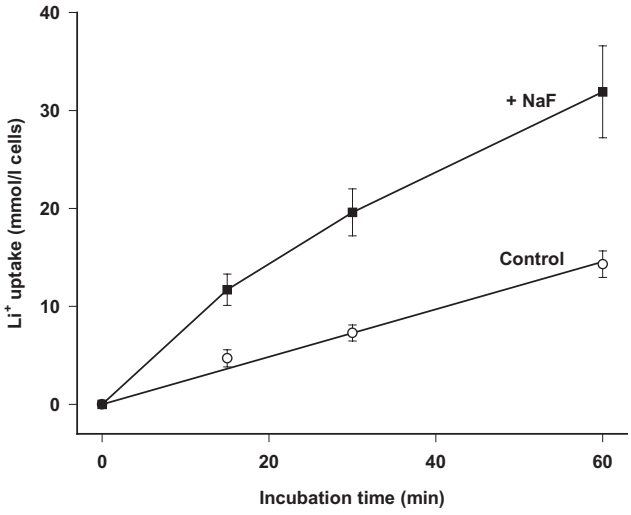

Figure 5. Effect of fluoride on Li^+ influx in the lamprey erythrocytes. Erythrocytes were incubated in the standard LiCl medium for 15, 30 and 60 min in absence or in presence of 20 mmol/l sodium fluoride (NaF), then aliquots of the cell suspensions were taken for determination of intracellular Li^+ content. Presented values are means ± S.E.M. for 9 experiments.

Effect of intracellular acidification on Na^+ and Li^+ influxes

For activation of Na^+/H^+ (Li^+/H^+) exchange, lamprey erythrocytes were acidified to intracellular pH 6.0 and then

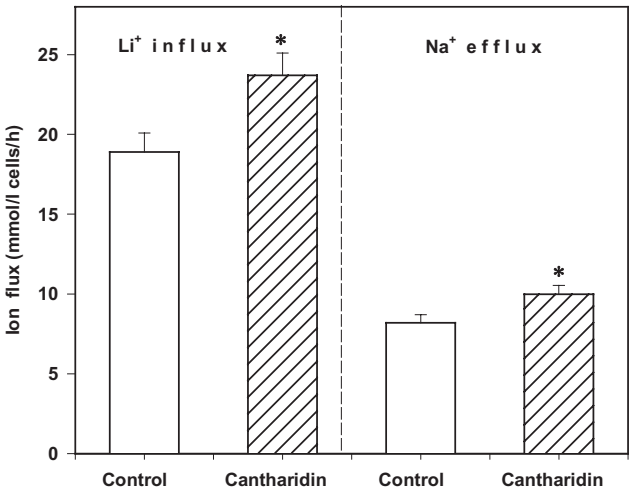

Figure 6. Effect of cantharidin on Li^+ influx and Na^+ efflux in lamprey erythrocytes. Erythrocytes were incubated for 60 min with or without 200 μmol/l cantharidin. Intracellular concentrations of Li^+ and Na^+ were determined in the cell lysates as described in Materials and Methods. Average values ± S.E.M. for 9 experiments are presented. * $p < 0.01$ as compared to control.

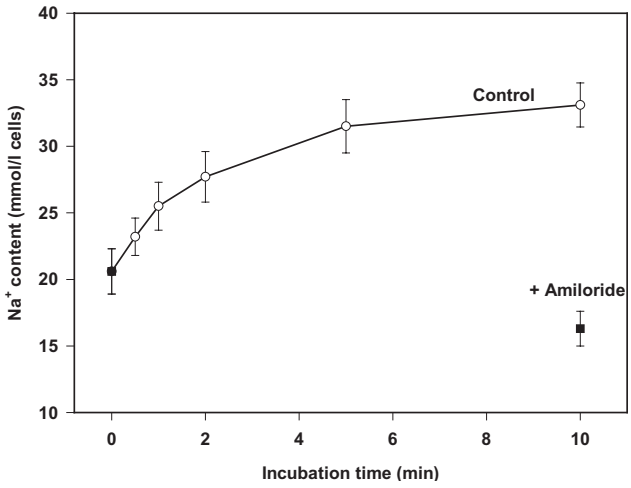

Figure 7. Time course of Na^+ influx in acidified lamprey erythrocytes. Acidified (pH 6.0) lamprey erythrocytes were placed in the standard NaCl medium (pH 8.0) without or with (10 min) 1 mmol/l amiloride and the intracellular Na^+ content was measured for 0.5, 1, 2, 5 and 10 min as described in Materials and Methods. Average data ± S.E.M. for 6 experiments are given.

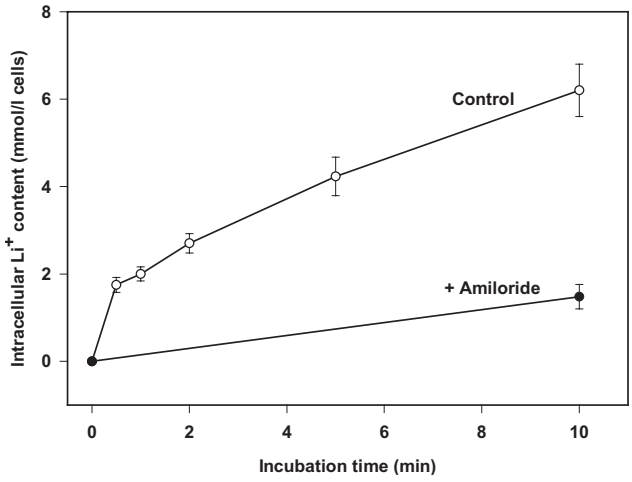

Figure 8. Time course of Li^+ influx in acidified cells. Acidified (pH 6.0) lamprey erythrocytes were placed in the standard LiCl medium (pH 8.0) without or with (10 min) 1 mmol/l amiloride and the uptake of Li^+ was measured for 0.5, 1, 2, 5 and 10 min. Intracellular concentrations of Li^+ in the samples were determined as described in Materials and Methods. Average data ± S.E.M. for 6 experiments are presented.

placed simultaneously in NaCl- and LiCl-media at pH 8.0 to study the accumulation of Na^+ and Li^+ in the cells. Incubation of the cells in NaCl-medium resulted in a rapid rise of intracellular Na^+ concentration with calculated rate of influx of 4.8 mmol/l cells/min (Fig. 7). In LiCl-medium, acidified erythrocytes have accumulated Li^+ with linear rate in the first minutes of incubation (Fig. 8), the calculated rate of Li^+ influx averaged 0.73 mmol/l cells/min. Influxes of both ions have been substantially inhibited in the presence of 0.5 mmol/l amiloride (incubation for 10 min, Figs. 7 and 8). Calculated values of amiloride-sensitive influxes were 16.3 ± 0.60 and 4.7 ± 0.40 mmol/l cells/10 min for Na^+ and Li^+, respectively.

Discussion

Presented results indicate that the transport of Li^+ in lamprey erythrocytes incubated in LiCl-medium considerably resembles that of Na^+ in NaCl-medium. The value of Na^+ influx in *Lampetra fluviatilis* erythrocytes under standard isotonic conditions, obtained in a few studies, was found to be 7–20 mmol/l cells/h (Gusev et al. 1992; Virkki and Nikinmaa 1994; Gusev and Sherstobitov 1996; Gusev and Ivanova 2004). In a few series of present work, accumulation of Li^+ in the cells has varied between separate experiments within 8–24 mmol/l for 60 min. Similarly to Na^+, accumulation of Li^+ in lamprey erythrocytes was partially inhibited by amiloride (Figs. 1, 2) and was activated by cell shrinkage due to stimulation of amiloride-sensitive transport pathway (Figs. 3, 4). We have not observed the statistically significant differences in the values of total (Fig. 3) and amiloride-sensitive (Fig. 4) Li^+ and Na^+ influxes upon simultaneous incubation of the cells in isosmotic LiCl- and NaCl-media. However, while

activated in hyperosmotic medium, amiloride-sensitive Na^+ influx was approximately twice higher as compared to that of Li^+ (Fig. 4). Hence, it is the amiloride-sensitive component of Na^+ transport that determines significant variability of the total influx of this cation in lamprey erythrocytes under standard physiological conditions. The residual amiloride-insensitive component of Na^+ and Li^+ influxes most likely reflects the membrane permeability (leak), which was 4–6 mmol/l cells/h for both cations. The latter value can be characterized by the constant of transport rate (0.03–0.05 h^{-1}), which was not different from the passive permeability of mammalian erythrocytes for monovalent cations (Postnov and Orlov 1985).

The activity of amiloride-sensitive component of Na^+ influx in lamprey erythrocytes was shown to be regulated by protein kinases and protein phosphatases (Gusev and Ivanova 2006). An influx of Li^+ was also stimulated after treatment of the cells with the protein phosphatase inhibitors fluoride and cantharidin (Figs. 5, 6). As shown, acceleration of the Li^+ influx under influence of fluoride (31.9 ± 4.7 mmol/l cells/h vs. 14.3 ± 1.3 in control) was considerably greater than that in the presence of cantharidin (23.7 ± 1.4 mmol/l cells/h vs. 18.9 ± 1.2 in control). Fluoride is known to increase the intracellular Ca^{2+} concentration in cells of various types (Varecka et al. 1998). Apparently, this additional effect of fluoride on Li^+ influx is not related to the inhibition of PPs and can be associated with its influence on intracellular ion homeostasis dependent on the presence of Ca^{2+} in the medium.

Our previous works (Gusev and Sherstobitov 1996; Gusev and Ivanova 2004) presented the definite evidence for existence of a certain "channel type" carrier for Na^+ in the plasma membrane of these cells. The data of present work are in accordance with these findings. Incubation of lamprey erythrocytes in LiCl-medium has revealed an amiloride-sensitive component of Na^+ efflux from the cells (Fig. 2), which was increased as the cell shrunk in hyperosmotic medium, but its value was less than the value of amiloride-sensitive influx of Li^+. Thus, the results of present investigation do not indicate an existence of Na^+/Li^+ exchange mechanism in lamprey erythrocytes. The latter mechanism, reflecting the functioning of Na^+/Na^+ exchange, was described in numerous investigations on erythrocytes of various species. Na^+/Li^+ exchange is known to be an electro neutral, insensitive to amiloride and cell shrinkage (Kahn 1987; van Norren et al. 1997; Semplicini et al. 2003). On the other hand, amiloride-sensitive transport of Na^+ in both directions was observed in erythrocytes of certain mammalian species (Sergeant et al. 1989; Zhao and Willis 1993). It is necessary to mention that amiloride-inhibitable Na^+ channels, found in some tissues, possess similar permeability for Na^+ and Li^+ (Ehrlich and Diamond 1980; Palmer and Frindt 1988; Holstein-Rathlou 1990).

A few studies (Aronson 1985; Bevensee et al. 1997) have shown that different isoforms of Na^+/H^+ exchanger in the tissues of vertebrates and invertebrates are capable of transporting Li^+ ions, i.e. of functioning as Li^+/H^+ exchange mechanism. An existence of amiloride-sensitive Na^+/H^+ exchange in lamprey erythrocytes has been proved by observation of equivalent values of H^+ efflux and Na^+ influx in acidified cells measuring the extracellular pH with ionometer, radioactive tracer (^{22}Na) and spectrophotometric measurement of Na^+ intracellular accumulation (Gusev and Ivanova 2004). In the present work, acidification of erythrocytes caused substantial stimulation of Li^+ influx, which was completely blocked by amiloride (Fig. 8). However, the activity of Li^+/H^+ exchange was approximately 3.5-fold lower as compared to Na^+/H^+ exchange. These data are in good accordance with the results, obtained in investigation of Na^+/H^+ exchange in various mammalian tissues (Ives et al. 1983; Jean et al. 1985; Foster et al. 1986; Simchowitz and Cragoe 1987; Gende and Cingolani 1993; Bevensee et al. 1997), establishing that Li^+/H^+ exchanger possesses considerably greater affinity for Li^+ but lesser maximal rate of transport as compared to Na^+/H^+ exchange.

In conclusion, the transport of Li^+ is realized through the same pathways as Na^+: i) passive influx (leak); ii) amiloride-sensitive Na^+ channel; iii) amiloride-sensitive Na^+/H^+ exchanger. The rate of Li^+ transport through the channels is approximately equal to that of Na^+ transport. The maximal activity of Na^+/H^+ exchange in lamprey erythrocytes, however, is considerably higher than that of Li^+/H^+ exchange.

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