Effect of Metabolic Inhibitors on K^+ Transport Across the Lamprey (*Lampetra fluviatilis*) Erythrocyte Membrane

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Abstract. In order to assess the contribution of oxidative metabolism to K^+($^{86}Rb^+$) transport across the lamprey red cell membrane, the effects of various metabolic inhibitors were examined. The influx of K^+ was reduced markedly in the presence of 20 μmol/l 2,4-dinitrophenol (2,4-DNP) or rotenone, and to a lesser extent by 1 mmol/l cyanide. Rotenone produced complete inhibition of the K^+ active transport and a partial blockade of K^+ channels by 28% on the average. Addition of 2,4-DNP to incubation media resulted in a significant reduction of both active transport of K^+ (by 47 %) and of K^+ movement via channels (by 57%). The inhibitory effect of 2,4-DNP on total K^+ influx was independent on decreasing extracellular pH_e from 7.4 to 6.5. The blocking action of 1 mmol/l Ba^{2+} on K^+ channels was abolished in the red cells incubated at pH_e 6.5. Treatment of the red cells with 1 mmol/l cyanide diminished active transport of K^+ to about 34% of control values but did not affect K^+ channels. The obtained data indicate that in the lamprey red blood cells at least a half of energy needed for the active transport of K^+ is supplied with ATP produced by oxidative phosphorylation. It may be suggested that NADH dehydrogenase is the key enzyme required for active transport of K^+ in the cells, as rotenone, a selective blocker of this enzyme, causes a complete blockade of the Na^+, K^+-pump.

Key words: Erythrocyte — Lamprey — K^+ transport — Metabolic inhibitors

Introduction

In lamprey red cells, as in other nucleated erythrocytes, potassium was found to be the main intracellular cation (Nikinmaa and Weber 1984; Gusev et al. 1992). It is reasonable to suppose that the high concentration of K^+ in lamprey erythrocytes is maintained by the function of the Na^+, K^+-pump. However, in an earlier study Asai et al. (1976) did not reveal any Na^+, K^+-ATPase activity in the erythrocyte membrane of lamprey (*Entosphenus japonicus*). In recent studies

(Kirk 1991; Gusev et al. 1992) the ouabain-inhibitable component of ^{86}Rb influx in the red cells of lamprey (*Lampetra fluviatilis*) was found. Similarly to the erythrocytes of fishes, amphibians and birds, lamprey red blood cells contain nucleus and mitochondria. Nucleated erythrocytes display a relatively high rate of oxygen consumption as compared with non-nucleated red cells of mammals (Hunter and Hunter 1957; Schweiger 1962; Boutilier and Ferguson 1989). It could be expected that the mitochondria oxidative phosphorylation is able to supply ATP for the ion transport through the lamprey red cell membrane. In fish erythrocytes, ATP content was diminished during acclimatization of the animals to hypoxia, or when aerated cells were incubated in the presence of cyanide (Powers 1983; Ferguson and Boutilier 1989; Ferguson et al. 1989). The aim of the present work was to study the possible contribution of oxidative phosphorylation in $K^+(^{86}Rb)$ transport across the lamprey erythrocyte membrane. We examined the effects of some metabolic inhibitors, acting on the different sites of oxidative phosphorylation, on the ^{86}Rb transport in the lamprey red blood cells. Action of the metabolic inhibitors (2,4-DNP, cyanide, rotenone) was compared with the effects of selective inhibitors of active transport (ouabain) and K^+ channel blockers (Ba^{2+}, quinine).

Materials and Methods

Animals

All experiments were carried out on river lampreys (*Lampetra fluviatilis*) of both sexes weighing 40–70 g. Animals were kept in aquaria with aerated tap water at 4 °C for several months (from October to March). The bath water was replaced by fresh dechlorinated tap water every two days.

Cell preparation

After rapid decapitation blood was drawn into tube containing ice-cold heparinized standard saline. The suspension was immediately centrifuged (2700 × *g* for 2 min at 2 °C), supernatant and buffy coat were removed by aspiration. The red cells were then washed twice in ice-cold saline and suspended at 30–40% hematocrit. Washed cells were stored at 4 °C no longer than for one hour before the experiment.

Measurement of $K^+(^{86}Rb)$ *influx*

^{86}Rb influx is usually employed as a substitute for K^+ influx to assay potassium transport in the lamprey erythrocyte membrane (Kirk 1991; Gusev et al. 1992). The standard saline contained (mmol/l): 145 NaCl, 1 KCl and 10 Tris-HCl (pH 7.4 at 20 °C) or 10 mmol/l imidazole buffer (pH 6.5 at 20 °C), when required. Incubation media were supplemented with 10 mmol/l glucose and inhibitors. Our preliminary experiments gave no difference in the rate coefficient of ^{86}Rb influx in the lamprey erythrocytes incubated in media with K^+ concentration 1 and 4 mmol/l. All studies were performed at room temperature (18–19 °C). Suspension of washed cells (about 100 μl) was subsequently added to 4–5 pairs of tubes containing 1 ml of the incubation medium to a 3–4% final hematocrit. The suspension was preincubated for 5 min in the first set of experiments (Table 1) and for 30 min in all the others. Then ^{86}Rb was added to a final activity of 740 kBq/ml, and

Table 1. Effect of metabolic inhibitors, Ba^{2+} and ouabain on the ^{86}Rb influx rate coefficients

Additions (mmol/l)	^{86}Rb influx rate coefficient (h^{-1}) Control	Ouabain-treated	Ouabain-sensitive
None	1.90 ± 0.21	1.67 ± 0.30	0.23 ± 0.09**
DNP (0.02)	1.21 ± 0.24*	1.04 ± 0.13	0.17 ± 0.11
NaCN (1.0)	1.46 ± 0.13*	1.17 ± 0.08	0.29 ± 0.10**
Rotenone (0.02)	1.19 ± 0.15*	1.04 ± 0.08	0.15 ± 0.14
Ba^{2+} (1.0)	0.74 ± 0.09*	0.22 ± 0.01	0.52 ± 0.09**

Washed erythrocytes were preincubated for 30 min with the inhibitors in standard saline. Then ^{86}Rb was added and its uptake for 10 min was measured. Values are means ± S.E.M. for 4 independent experiments.
* indicates a significant difference ($P < 0.01$) compared with corresponding values in the absence of the blockers.
**indicates a significant ($P < 0.05$) effect of ouabain using paired Student's t-test.

the suspension was incubated for 10 min. As found in the preliminary experiments, ^{86}Rb uptake by the lamprey red cells was linear at least for 30–40 min. The uptake of ^{86}Rb was stopped by injection of 1 ml suspension into 10 ml ice-cold standard saline. After centrifugation (2700 × g, 1 min, 2 °C) samples of supernatant were taken to measure radioactivity of the media, and the cells were washed once again. The washed cells were lysed in distilled water. Radioactivity of incubation media (A_m) and cell lysates (A_{cell}) was measured using a gamma-counter. The rate coefficients of ^{86}Rb influx were calculated as follows: $K_{in} = A_{cell}/A_m \cdot t$ (h^{-1}), where A_m and A_{cell} are the radioactivities of 1 ml medium and 1 ml packed cells, t is the time of incubation with ^{86}Rb in hours. The rate coefficients of ^{86}Rb influx correspond the magnitude of K^+ influx through the erythrocyte membrane since the media contain 1 mmol/l K^+ (in mmol/l cells/h).

Chemicals

All the basic chemicals used were of reagent grade. Ouabain, 2,4-DNP and quinine sulfate were obtained from Sigma (St. Louis, Mo.), rotenone was purchased from Serva (FRG). Stock solution of ouabain (10 mmol/l) was prepared in isotonic saline, 2,4-DNP (10 mmol/l) was dissolved in water, quinine (100 mmol/l) in dimethylsulfoxide (DMSO), rotenone (1 mmol/l) was dissolved in ethanol. DMSO and ethanol were added to the control medium when required. ^{86}Rb was obtained from ISOTOP (Russia).

Statistics

Results are presented as mean ±S.E.M. Statistically significant differences ($P < 0.05$) were assessed using Student's two-tailed t-test, paired or unpaired as appropriate to the design of the experiments.

Results

Effects of ouabain, metabolic inhibitors and Ba^{2+} *on* ^{86}Rb *influx.*
In lamprey erythrocytes, in addition to the Na^+, K^+-pump, a larger fraction of K^+ influx is mediated by Ba^{2+}-inhibitable K^+ channels (Kirk 1991; Gusev et al. 1992). Therefore, in the set of experiments the effects of metabolic blocker, Ba^{2+} and ouabain were compared. In the presence of each blocker (Table 1) the potency order was: Ba^{2+} > rotenone = 2,4-DNP > cyanide = ouabain. Ouabain produced statistically significant decrease of ^{86}Rb transport only under control conditions and in the presence of Ba^{2+} and cyanide. As shown in the previous study (Gusev et al. 1992), the blocking effect of ouabain can be masked due to stimulation of ^{86}Rb influx via K^+ channels during 30 min of preincubation of the cells. In these experiments, the Ba^{2+}-sensitive component of ^{86}Rb influx in the red cells was 1.16 ± 0.16 h^{-1} under control conditions and 1.45 ± 0.12 h^{-1} in the presence of ouabain ($P < 0.01$, paired t-test). Therefore, the magnitude of ouabain-sensitive active transport of ^{86}Rb in the lamprey erythrocytes appears to be true when K^+ channels are blocked. The real component for ^{86}Rb active transport is 0.52 ± 0.09 h^{-1}. Thus, elucidation of action of the metabolic blockers on active K^+ transport was complicated by changes in the function of K^+ channels during cell preincubation with ouabain. The following experiments were undertaken to ascertain more correctly the effects of these metabolic inhibitors on active K^+ transport in the lamprey erythrocytes.

Effects of 2,4-DNP, *cyanide and rotenone on* ^{86}Rb *influx in the* Ba^{2+}*-treated erythrocytes.*
In these experiments, we compared the effect of each blocker alone and after inhibiting the K^+ channels in the presence of Ba^{2+}. Again, 2,4-DNP and rotenone were equally effective inhibitors and cyanide was a less potent in untreated red cells (Table 2). Addition of Ba^{2+} produced marked reduction of ^{86}Rb influx in the presence of studied metabolic blockers. 2,4-DNP and rotenone caused a significant decrease of Ba^{2+}-inhibitable component of ^{86}Rb transport in the red cells by 40% and by 28%, respectively. Cyanide had no effect on ^{86}Rb influx via Ba^{2+}-sensitive K^+ channels. Maximal inhibitory effect on ^{86}Rb transport was observed when rotenone and Ba^{2+} were added to the medium together. The residual Ba^{2+} plus rotenone-resistant component of K^+ influx (0.24 ± 0.02 h^{-1}) did not differ from that found in the presence of Ba^{2+} plus ouabain (Table 1). On the basis of these results it is reasonable to conclude that rotenone causes a complete inhibition of ^{86}Rb active transport in the red cells. The average value of rotenone-inhibitable component for Ba^{2+}-treated cells (0.61 ± 0.07 h^{-1}) was similar to ouabain-sensitive component (0.52 ± 0.09 h^{-1}, Table 1). 2,4-DNP and cyanide produced only partial inhibition of active K^+ transport by 54% and 70%, on the average, respectively.

Table 2. Effects of metabolic inhibitors and Ba^{2+} on ^{86}Rb influx rate coefficients

Additions (mmol/l)	^{86}Rb influx rate coefficient (h^{-1})		
	Control	Ba^{2+}-treated	Ba^{2+}-sensitive
None	2.02 ± 0.13	0.85 ± 0.06	1.17 ± 0.15
DNP (0.02)	1.22 ± 0.13*	0.52 ± 0.06	0.70 ± 0.16*
NaCN (1.0)	1.53 ± 0.16*	0.42 ± 0.08	1.11 ± 0.10
Rotenone (0.02)	1.08 ± 0.10*	0.24 ± 0.02	0.84 ± 0.12*

The red cells were preincubated with the inhibitors for 30 min in standard saline, then ^{86}Rb was added and the cells were incubated for 10 min. Values are means ± S.E.M. for 5 independent experiments.
* indicates a significant difference ($P < 0.01$) compared with corresponding values in the absence of the blockers (paired Student's t-test).

Table 3. Effects of 2,4-DNP, Ba^{2+} and quinine on ^{86}Rb influx rate coefficients

Additions (mmol/l)	^{86}Rb influx rate coefficient (h^{-1})	
	pH_e 7.4	pH_e 6.5
Control	1.53 ± 0.15	1.80 ± 0.15
2,4-DNP (0.02)	0.90 ± 0.07*	0.85 ± 0.07*
Ba^{2+} (1.0)	0.75 ± 0.12*	1.84 ± 0.12
Quinine (1.0)	—	0.81 ± 0.19*
2,4-DNP + Ba^{2+}	0.55 ± 0.08**	0.97 ± 0.09*
2,4-DNP + quinine	—	0.51 ± 0.08**

Washed erythrocytes were preincubated for 5 min with the inhibitors in standard saline or in saline buffered with 10 mmol/l imidazole hydrochloride (pH 6.5 at 20 °C). Values are means ± S.E.M. for 6 experiments at pH_e 7.4 and for 5 experiments at pH_e 6.5.
* indicates a significant difference (Student's t-test, $P < 0.001$) form the corresponding control value, ** significantly different ($P < 0.001$) as compared with each blocker alone.

pH dependence of 2,4-DNP *effect on* K^+ *influx*

It is known that 2,4-DNP is a protonophore (McLaughlin and Dilger 1980) and causes intracellular acidification in the lamprey erythrocytes (Nikinmaa 1986). Therefore, the experiments were carried out in standard medium at pH 7.4 and in a saline at pH 6.5. As shown in Table 3, 20 μmol/l 2,4-DNP produced a marked decrease of ^{86}Rb influx in the red cells independent of extracellular pH. Transport of ^{86}Rb in the red cells was significantly reduced in the presence of 1 mmol/l Ba^{2+} in standard saline, but it was the same at pH 6.5. The addition of both blockers to normal saline caused a greater fall in ^{86}Rb influx than for each of the inhibitors. Total influx of ^{86}Rb in the lamprey erythrocytes did not significantly differ at pH

7.4 and 6.5. The blocking effect of Ba^{2+} on K^+ channels completely disappeared when the cells were incubated at pH 6.5. Another inhibitor of K^+ channels, quinine, produced a marked decrease of ^{86}Rb influx in the erythrocytes incubated at pH 6.5. Simultaneous addition of 2,4-DNP and quinine at pH 6.5 led to some additive effect on ^{86}Rb transport in the red cells. There was no difference in the residual component of ^{86}Rb influx in the red cells in the presence of 2,4-DNP plus Ba^{2+} at pH 7.4 and 2,4-DNP plus quinine at pH 6.5 (Table 3). Ba^{2+}-inhibitable component of ^{86}Rb transport under control conditions at pH 7.4 (0.78 ± 0.19 h^{-1}) was significantly diminished ($P < 0.001$) in the presence of 2,4-DNP (0.35 ± 0.04 h^{-1}). The ^{86}Rb influx inhibited by quinine at pH 6.5 was also depressed in the presence of 2,4-DNP to 0.34 ± 0.11 h^{-1} as compared with 0.99 ± 0.23 h^{-1} in control ($P < 0.001$).

Discussion

The present results show that 2,4-DNP, cyanide and rotenone causes a significant reduction of ^{86}Rb influx in the lamprey erythrocytes incubated in standard saline. The metabolic inhibitors used must depress the active transport since they cause an inhibition of aerobic ATP synthesis (Heytler 1979; Singer 1979; Balaban and Mandel 1980; Winkler 1981; Gullans et al. 1982; Tessitore et al. 1986). The value of K^+ active transport in the red cells in all experiments was found to be 0.56 ± 0.06 mmol/l cells/h ($n = 6$) at 1 mmol/l concentration of K^+ in the media. When Ba^{2+} and rotenone were added to the medium together, the rate coefficient of ^{86}Rb influx (Table 2) did not differ from that found in the presence of Ba^{2+} plus ouabain (Table 1). On the basis of these results it is most reasonable to conclude that rotenone causes a complete inhibition of active transport of ^{86}Rb in the red cells. The remaining (Ba^{2+} + ouabain)-resistant and (Ba^{2+} + rotenone)-resistant components of ^{86}Rb influx (0.23 ± 0.01 h^{-1}) probably reflect a movement of K^+ ions via passive diffusion leak. Simultaneous addition to the medium of Ba^{2+} and 2,4-DNP or Ba^{2+} and cyanide produced a smaller inhibitory effect on ^{86}Rb influx than that of Ba^{2+} plus ouabain and Ba^{2+} plus rotenone. The data could be interpreted as indicating that 2,4-DNP and cyanide did not cause a full inhibition of active transport of ^{86}Rb in the red cells. The residual component of active transport in the presence of 1 mmol/l cyanide was 0.18 ± 0.06 h^{-1} or 30% of the total ouabain-sensitive influx of ^{86}Rb. A larger component of active transport was retained in the red cells incubated in the presence of 20 μmol/l 2,4-DNP (0.28 ± 0.05 h^{-1}) or 46% of control value.

The results of our study provide evidence for the partial inhibition of K^+ channels in the lamprey erythrocytes exposed to 2,4-DNP or rotenone. In paired experiments (Tables 2 and 3), the average value of the Ba^{2+}-sensitive component of ^{86}Rb influx was 1.00 ± 0.12 h^{-1} ($n = 11$) as compared with 0.57 ± 0.11 h^{-1} in the

presence of 2,4-DNP. The results are in agreement with the data obtained by Kirk (1990, 1991), who also found a decrease in ouabain-resistant K^+(^{86}Rb) influx in the lamprey erythrocytes in the presence of 20 μmol/l 2,4-DNP. Rotenone produced a two-fold smaller inhibition of ^{86}Rb transport (by 28%) via K^+ channels (Table 2). Cyanide had no effect on the Ba^{2+}-sensitive component of ^{86}Rb influx. In general, 2,4-DNP caused inhibition of ^{86}Rb influx via the Na^+, K^+-pump by 54%, and K^+ channels by 43 %. In the presence of rotenone, there was a complete blockade of active transport and inhibition of K^+ channels by 29%. Only 70% inhibition of ^{86}Rb active transport without change in transport via K^+ channels was observed in the cyanide-treated erythrocytes.

The uncoupling agent 2,4-DNP, cytochrome inhibitor cyanide, and NADH dehydrogenase blocker rotenone causes an inhibition of aerobic ATP synthesis in various types of cells (Singer 1979; Heytler 1979; Balaban and Mandel 1980; Winkler 1981; Gullans et al. 1982). It seems reasonable to propose that the inhibitors act on ^{86}Rb transport in the lamprey red cells only indirectly, by preventing synthesis of ATP needed for the Na^+, K^+-pump. A substantially lesser effect of 2,4-DNP on the ouabain-sensitive ^{86}Rb influx may be attributed to a relatively low concentration of this agent used in our study (20 μmol/l). 2,4-DNP was usually used at 10 μmol/l concentration (Heytler 1979; Weiner 1979) although in some cases a much greater concentration of 2,4-DNP was also used (Lamb and MacKinnon 1971). Another explanation for the relative inefficiency of 2,4-DNP and cyanide may be the existence of the alternative pathways of ATP production which was not blocked by these inhibitors. In the most studied red blood cells of trout, enzymes of glycolysis, tricarbonic cycle, NADH metabolism and pentose phosphate shunt were found (Walsh et al. 1990). It may be suggested that similar alternative pathways of ATP formation also operate in the lamprey red cells. Moreover, the anaerobic synthesis of ATP appears to be associated with NADH metabolism since rotenone, a selective inhibitor of NADH dehydrogenase (Singer 1979), causes a complete blockade of K^+ active transport. The results of our study suggest that in the lamprey erythrocytes both oxidative phosporylation and anaerobic glycolysis play approximately an equal role in generation of ATP for active ion transport. Both pathways are also equally important in some tissues (Van Rossum et al. 1971; Winkler 1981; Paul 1983; Ikehara et al. 1984; Dillingham and Burke 1991).

It should be noted that the effect of 2,4-DNP on ^{86}Rb influx did not differ when the red cells were preincubated with the blocker for 5 min (Table 3) or for 30 min (Table 2). Such rapid action of 2,4-DNP on ^{86}Rb transport appears not to be explained by cellular ATP depletion. In fish erythrocytes of *Fundulus heteroclitus*, ATP content decreased to 68% of the control level during 4 hours incubation in the presence of cyanide (Powers 1983). In human red blood cells, the coupling of glycolysis to Na^+, K^+-pump via membrane-bound glycolytic enzymes was found and the synthesized ATP was proposed to be compartmentalized in the

membrane (Fossel and Solomon 1977; Proverbio and Hoffman 1977; Mercer and Dunham 1981). Such energetic coupling in the lamprey erythrocytes should be considered in further studies. Coupling of glycolysis to Na^+ and K^+ transport via membrane-bound glycolytic enzymes was also postulated in smooth muscle (Paul et al. 1979; Paul 1983), and brain (Lipton and Robacker 1983).

The mechanism by which 2,4-DNP and rotenone caused a partial inhibition of ^{86}Rb transport via K^+ channels in the erythrocyte membrane remains unclear. At present there is no evidence for regulation of K^+ channels in the lamprey erythrocyte membrane. As shown recently by Kirk (1991), the Ba^{2+}-sensitive transport of ^{86}Rb was partially blocked during intracellular Ca^{2+} depletion. The greater effect of 2,4-DNP on K^+ channels as compared with rotenone was probably associated with intracellular acidification induced by the agent (Nikinmaa 1986; Kirk 1991). There is ample evidence for the inactivation of K^+ channels in different types of cells as a result of reduction of the extra- and intracellular pH (Van Driessche and Zeiske 1985; Hagiwara et al. 1978; Wiener et al. 1990). Results of the present study indicate that the shift in extracellular pH from 7.4 to 6.5 was not associated with a significant alteration in both total influx of ^{86}Rb and transport via K^+ channels (Table 3). Similarly to our findings, it was shown that for the lamprey erythrocytes the extracellular acidification (pH 6.6) had a small effect on the Ba^{2+}-sensitive component of ^{86}Rb uptake (Kirk 1991). In our experiments, however, Ba^{2+} induced a complete blockade of K^+ channels at pH_e 7.4, but not at pH_e 6.5.

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