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CHLORIDE TRANSPORT IN RED BLOOD CELLS OF LAMPREY *LAMPETRA FLUVIATILIS*: EVIDENCE FOR A NOVEL ANION-EXCHANGE SYSTEM

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Summary

The existence of a furosemide-sensitive Cl^- transport pathway activated by external Ca^{2+} and Mg^{2+} has been demonstrated previously in studies of Cl^- influx across the lamprey erythrocyte membrane. The aim of the present study was to characterize further specific Cl^- transport pathways, especially those involved in Cl^- efflux, in the red blood cell membrane of *Lampetra fluviatilis*. Cl^- efflux was inhibited by 0.05 mmol l^{-1} dihydroindenyloxyalkanoic acid (DIOA) (81 %), 1 mmol l^{-1} furosemide (76 %) and 0.1 mmol l^{-1} niflumic acid (54 %). Bumetanide (100 μmol l^{-1}) and DIDS (100 μmol l^{-1}) had no effect effect on Cl^- efflux. Substitution of external Cl^- by gluconate, but not by NO_3^-, led to a gradual decline of Cl^- efflux. In addition, the removal of external Ca^{2+} resulted in a significant reduction in the rate of Cl^- efflux. Membrane depolarization caused by increasing external K^+ concentration or by inhibiting K^+ channels with 1 mmol l^{-1} Ba^{2+} did not affect Cl^- efflux. The furosemide-sensitive component of Cl^- influx was a saturable function of external $[Cl^-]$ with an apparent K_m of approximately 92 mmol l^{-1} and V_{max} of approximately 17.8 mmol l^{-1} cells^{-1} h^{-1}. Furosemide did not affect intracellular Cl^- concentration (57.6±5.2 mmol l^{-1} cell water), measured using an ion-selective Cl^- electrode, showing that a furosemide-sensitive pathway is not involved in net Cl^- movement. A gradual fall (from 28.1±1.4 to 15.0±1.3 mmol l^{-1} cells^{-1} h^{-1}) in unidirectional Cl^- influx with time was observed within 3 h of cell preincubation in the standard physiological medium. These data provide evidence for the existence for an electroneutral furosemide-sensitive anion-exchange pathway in the lamprey erythrocyte membrane that accepts chloride and nitrate, but not bicarbonate or bromide.

Key words: lamprey, erythrocyte, chloride exchange, *Lampetra fluviatilis*, anion exchange.

Introduction

One of the remarkable features of the red blood cell membrane of agnathans is an almost complete absence of the rapid DIDS-sensitive Cl^-/HCO_3^- exchange mediated by capnophorin (band 3 protein) that is found in the great majority of vertebrates (Ellory *et al.* 1987; Nikinmaa and Railo, 1987; Gusev and Sherstobitov, 1993). Early findings suggested that the low Cl^- flux rates observed for lamprey red blood cells were due to the absence of the band 3 protein in the erythrocyte membranes of lampreys and hagfish (Ohnishi and Asai, 1985; Ellory *et al.* 1987; Tufts and Boutilier,1989). However, recent studies by Kay *et al.* (1995) and Cameron *et al.* (1996) have indicated the presence of genes coding for a protein similar to an anion-transport protein as well as a protein similar to a dimeric form of the human anion exchanger AE1 in the erythrocyte membrane of the sea lamprey *Petromyzon marinus*. The function of these proteins remains unknown.

Little is known about the mechanisms of Cl^- transport in lamprey erythrocytes. Cl^- influx into the cells was shown to be insensitive to stilbene disulphonates (Nikinmaa and Railo, 1987; Gusev and Sherstobitov, 1993), but was significantly inhibited by inhibitors of $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransport systems such as H-74 and furosemide (Kirk, 1991; Gusev and Sherstobitov, 1993). The nature of the furosemide-sensitive Cl^- transport system is unknown. It does not seem to be a conductive pathway since furosemide-sensitive Cl^- influx does not respond to membrane depolarization. Extracellular Ca^{2+} and Mg^{2+} play an important role in modulation of its activity (Gusev and Sherstobitov, 1993). Activation of a furosemide-sensitive component of Cl^- efflux (different from that mediated by the K^+/Cl^- cotransporter) has been demonstrated in response to cell swelling (Virkki and Nikinmaa, 1995).

The aim of the present investigation was to characterize further Cl^- transport pathways across the red blood cell membrane of lamprey, concentrating mainly on Cl^- efflux under physiological conditions. The data obtained for Cl^- (^{36}Cl) fluxes and cell/medium distribution of ^{36}Cl provide evidence for the presence of a furosemide-sensitive anion-exchange system in the lamprey red blood cell membrane which accepts chloride and nitrate, but not bicarbonate or bromide.

Materials and methods

Cell preparation

River lampreys *Lampetra fluviatilis* were trapped in the Neva River and maintained unfed in dechlorinated tap water from October to March. After rapid decapitation, blood was withdrawn by bleeding into a heparinized tube containing a standard solution of the following composition (mmol l^{-1}): 140 NaCl, 4 KCl, 10 Tris/HCl (pH 7.4 at 20 °C). After immediate centrifugation (2700 ***g*** for 5 min at 4 °C), plasma and white cells were discarded and the erythrocytes were washed three times and resuspended in the incubation medium to a final haematocrit of 30–40 %.

Flux measurements

To measure unidirectional Cl^- efflux, the red blood cell suspension at 30–40 % haematocrit was preloaded with ^{36}Cl (final radioactivity 1.85 MBg ml^{-1}) for 2 h at room temperature (approximately 20 °C). The incubation medium had the same composition as the standard solution, containing in addition 10 mmol l^{-1} glucose, 1 mmol l^{-1} $CaCl_2$ and 1 mmol l^{-1} $MgCl_2$. After preloading, the cells were washed four times with ice-cold solution to remove external radioactive tracer and finally resuspended in incubation medium. In the experiments in which external Cl^- was replaced by nitrate, incubation media contained nitrate salts instead of chloride salts. Gluconate-containing incubation medium contained (mmol l^{-1}): 160 sodium gluconate, 1 calcium gluconate, 10 glucose, 4 Hepes/KOH (pH 7.4 at 20 °C). Partial replacement of Cl^- by gluconate was achieved by mixing Cl^--containing and gluconate-containing incubation media to give the extracellular $[Cl^-]$ required. In experiments in which gluconate was partially replaced with HCO_3^- or Br^-, gluconate solution was mixed with a solution containing 145 mmol l^{-1} $NaHCO_3$ (NaBr) and 4 mmol l^{-1} $KHCO_3$ (KBr) to obtain a final HCO_3^- (Br^-) concentration of 25 mmol l^{-1}. The concentration of HCO_3^- during the incubation period was stabilised by equilibration of the incubation medium with carbogen. Samples for measuring ^{36}Cl loss were taken every 10–20 min for 1 h. Flux was stopped by injecting 1 ml of cell suspension into 10 ml of ice-cold standard solution and immediate centrifugation. The radioactivity of cells and media was determined by liquid scintillation counting in dioxane scintillator using a RACBETA (LKB Wallac) counter. Rate coefficients of unidirectional Cl^- efflux were determined by linear regression of semilogarithmic curves, $\ln[1-(A_c-A_c')/A_t]$ *versus* time, where (A_c-A_c') expresses the loss of ^{36}Cl by the cells during the time interval from t to t' and A_t is the total radioactivity of the suspension.

Unidirectional Cl^- influx was measured as described by Gusev and Sherstobitov (1993). The cells were suspended to a final haematocrit of 5–6 % in the incubation medium containing inhibitors, and pre-equilibrated for 10 min (unless stated otherwise). Incubation (5–10 min) was started by the addition of ^{36}Cl to the pre-equilibrated suspension (final radioactivity 1.185 MBq ml^{-1}). Cl^- influx was terminated by 10-fold dilution of the cell suspension with ice-cold standard solution and rapid centrifugation. Cl^- (^{36}Cl) influx (J_{Cl}) was calculated as follows:

$$J_{Cl} = A_{rbc}[Cl^-]_e/A_{med}t,$$

where A_{rbc} is the radioactivity of 1 ml packed cell equivalent, A_{med} is the radioactivity of 1 ml of medium, t is incubation time and $[Cl^-]_e$ is the concentration of Cl^- in the incubation medium. The time course of ^{36}Cl distribution in the cells/medium was assayed in 3 h incubations with the radioactive tracer. A sample of the suspension was taken every 30 min to measure cell uptake of ^{36}Cl. Intracellular Cl^- concentration in cell lysates was determined using an ion-selective Cl^- electrode. After 3 h of incubation in the standard medium with and without furosemide, the cells were washed three times in ice-cold nitrate-containing standard medium. The cell pellet was then lysed in 5 % trichloroacetic acid, and the pH of the supernatant was neutralized by KOH titration. Cl^- concentration was assayed after an approximately 10-fold dilution with 140 mmol l^{-1} $NaNO_3$.

Chemicals

Furosemide, bumetanide, niflumic acid, 4,4′-diisothiocyanostilbene 2,2′-disulphonic acid (DIDS), gluconate salts and Trizma were purchased from Sigma (St Louis, MO, USA), and dihydroindenyloxyalkanoic acid (DIOA) was purchased from Research Biochemicals Incorporated (USA). Stock solutions of furosemide (100 mmol l^{-1}), DIOA (5 mmol l^{-1}), bumetanide (100 mmol l^{-1}) niflumic acid (10 mmol l^{-1} and DIDS (10 mmol l^{-1}) were prepared in dimethyl sulphoxide (DMSO). DMSO was added to the control medium when required. ^{36}Cl was purchased from ISOTOP, Russia. Stock solutions of radioactive tracer were prepared in the standard medium.

Statistical analyses

Results are expressed as means ± S.E.M. Statistically significant differences ($P<0.05$) were assessed using Student's t-test. Computer programs were used for curve fitting (Jandel SigmaPlot, version 3.0).

Results

Effect of external divalent cations and membrane depolarization on Cl^- efflux

We have reported previously (Gusevand Sherstobitov, 1993) that Cl^- influx into lamprey red blood cells depends on the presence of external Ca^{2+} and Mg^{2+}. Unidirectional Cl^- efflux also increased significantly when Ca^{2+} was added to nominally Ca^{2+}-free medium. The rate coefficient for ^{36}Cl efflux increased from 0.20±0.013 h^{-1} in a Ca^{2+}-free medium to 0.42±0.03 h^{-1} in the presence of 1 mmol l^{-1} Ca^{2+} (N=7, $P<0.001$, paired t-test). No additional stimulation of Cl^- transport was observed in medium containing both 1 mmol l^{-1} Mg^{2+} and 1 mmol l^{-1} Ca^{2+} (0.35±0.02 h^{-1} *versus* 0.19±0.01 h^{-1} in a Ca^{2+},Mg^{2+}-free medium, N=9, $P<0.001$). In all other experiments, incubation media contained 1 mmol l^{-1} Ca^{2+} and 1 mmol l^{-1} Mg^{2+}.

The permeability of lamprey erythrocyte membrane to K^+ is

higher than that to other ions owing to the presence of Ba^{2+}-sensitive K^+ channels (Kirk, 1991; Gusev *et al.* 1992*b*; Virkki and Nikinmaa, 1996). The opening time of the K^+ channels was found to be a function of external K^+ concentration. The addition of Ba^{2+} and increasing the extracellular K^+ concentration were used to alter the membrane potential. In paired experiments, exposure of red cells to 1 mmol l^{-1} Ba^{2+} or 20 mmol l^{-1} K^+ replacing 20 mmol l^{-1} external Na^+ did not affect the rate of Cl^- efflux. The Cl^- efflux rate coefficient was 0.21±0.03 h^{-1} for control cells, 0.23±0.04 h^{-1} with 1 mmol l^{-1} Ba^{2+} and 0.22±0.04 h^{-1} with 20 mmol l^{-1} K^+. Thus, like Cl^- influx, Cl^- efflux from lamprey erythrocytes decreased after removal of external Ca^{2+} and Mg^{2+} and seemed to be independent of membrane potential.

Effect of anion transport inhibitors on Cl⁻ efflux

To characterize the nature of the Cl^- transport pathway(s), the effects of a number of anion transport inhibitors on $^{36}Cl^-$ efflux were studied. As one can see from Fig. 1, bumetanide and DIDS at 0.1 mmol l^{-1} had no significant effect on the rate of Cl^- efflux from the cells under the experimental conditions used, whereas in hypotonically swollen cells DIDS blocked Cl^- fluxes (Virkki and Nikinmaa, 1995). Cl^- efflux was significantly inhibited by 1 mmol l^{-1} furosemide, 0.05 mmol l^{-1} DIOA and 0.1 mmol l^{-1} niflumic acid. The inhibitory effects of furosemide and DIOA at the concentrations used were similar, giving a mean inhibition of Cl^- efflux of 76 % for furosemide and 81 % for DIOA. Only 54 % of Cl^- efflux from the red cells was inhibited by 0.1 mmol l^{-1} niflumic acid. Furosemide was used in further experiments to compare the results with those obtained previously for Cl^- influx into lamprey red cells (Gusev and Sherstobitov, 1993).

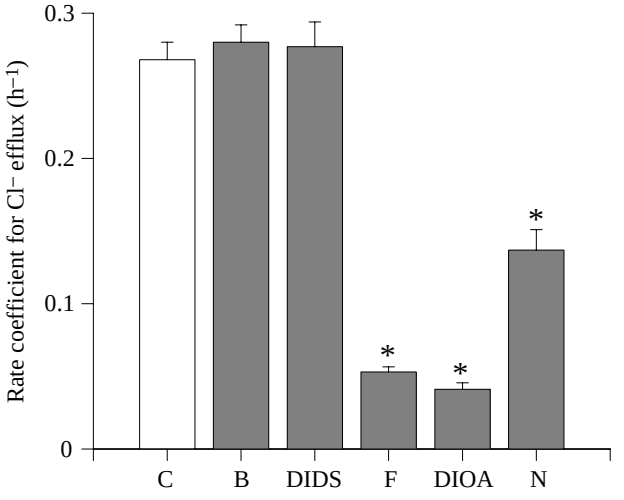

Fig. 1. Effects of anion transport inhibitors on ^{36}Cl efflux. Erythrocytes were preloaded for 2 h with ^{36}Cl. After removal of extracellular radioactive tracer, the cells were resuspended in the incubation medium containing inhibitors: 1 mmol l^{-1} furosemide (F), 100 µmol l^{-1} bumetanide (B), 0.1 mmol l^{-1} niflumic acid (N), 100 µmol l^{-1} DIDS or 50 µmol l^{-1} DIOA and incubated for 1 h with samples taken each 10 min. Values are means + S.E.M. of 4–8 experiments. *Significantly different from the control (C) value (P<0.001).

Effect of replacing external Cl⁻ by NO_3^- and gluconate on Cl⁻ efflux

The inhibition of both influx and efflux of Cl^- by furosemide could be attributed to the presence of an anion-exchange pathway in the lamprey erythrocyte membrane. To elucidate this possibility, the *trans*-effects of extracellular anions on ^{36}Cl efflux were studied. Cl^- efflux was measured in red blood cells incubated in nitrate- or gluconate-containing medium. As can be seen from Table 1, substitution of external Cl^- by NO_3^- did not affect the rate of Cl^- efflux from lamprey erythrocytes. In contrast, complete substitution of external Cl^- by gluconate produced a significant reduction in the Cl^- efflux rate coefficient. The inhibitory effect of 1 mmol l^{-1} furosemide on Cl^- efflux was the same in red cells incubated in standard Cl^- medium and in NO_3^- medium. The rate coefficient of Cl^- efflux in gluconate medium was similar to that in Cl^- medium in the presence of furosemide. However, treatment of the red cells with 1 mmol l^{-1} furosemide in gluconate medium caused a further significant decrease in Cl^- efflux rate coefficient (P<0.05).

The following experiments were performed to study the dependence of Cl^- efflux on external Cl^- concentration. The cells were incubated in medium containing varying Cl^- concentrations at a constant total concentration of Cl^- plus gluconate. (Fig. 2). The rate coefficient of Cl^- efflux increased almost linearly with the external Cl^- concentration. The minimal value of Cl^- efflux in a nominally Cl^--free medium was 0.073±0.011 h^{-1}, and the maximal value of Cl^- efflux was 0.33±0.07 h^{-1} in incubation medium containing 155 mmol l^{-1} external Cl^-. The $[Cl^-]_e$-dependent component of ^{36}Cl efflux was calculated as the difference between the rate coefficients of Cl^- efflux in Cl^--containing and Cl^--free (gluconate) medium. The $[Cl^-]_e$-dependent component displayed

Table 1. *Effect of external anion substitution on unidirectional Cl^- efflux from lamprey red blood cells*

	Rate constants (h^{-1})	
Medium	Control	1 mmol l^{-1} furosemide
Chloride	0.319±0.042	0.070±0.011†
Nitrate	0.300±0.024	0.070±0.014†
Gluconate	0.076±0.012*	0.038±0.018†

The cells were preloaded with ^{36}Cl for 2 h in the Cl^--containing incubation solution, rapidly washed of external radioactivity and resuspended in the incubation solution containing (in mmol l^{-1}): 140 NaCl (NO_3), 4 KCl (NO_3), 1 $CaCl_2$ [$(NO_3)_2$], 1 $MgCl_2$ [$(NO_3)_2$], 10 Tris/HCl (HNO_3), 10 glucose, or 160 sodium-gluconate, 1 calcium-gluconate and 4 Hepes/KOH, and ^{36}Cl efflux was measured as described in Materials and Methods. Values are means ± S.E.M. of four independent experiments.

*Significantly different from the value in the presence of Cl^-; †significantly different from the value in the absence of furosemide (P<0.05, Student's t-test).

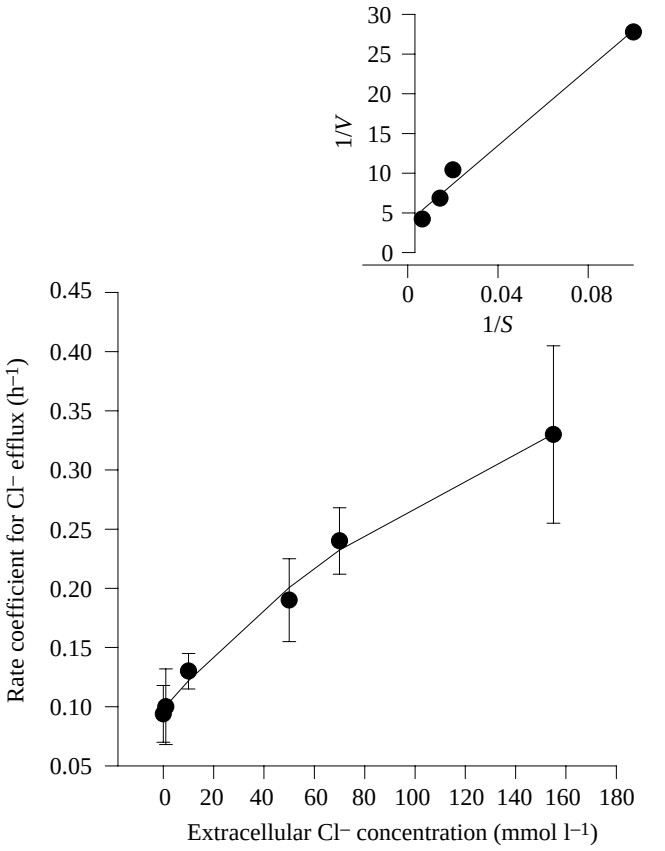

Fig. 2. ^{36}Cl efflux as a function of external Cl^- concentration. The cells were preloaded with ^{36}Cl and resuspended in medium containing various Cl^- concentrations. To adjust some Cl^- concentrations, Cl^--containing medium was mixed with gluconate-containing medium (in mmol l^{-1}: 160 sodium gluconate, 1 calcium gluconate, 10 glucose, 4 Hepes/KOH). The last point represents ^{36}Cl efflux into the standard incubation saline containing (in mmol l^{-1}: 140 NaCl, 4 KCl, 1 $CaCl_2$, 10 Tris/HCl and 10 glucose). Inset: Lineweaver–Burk plot for the Cl^--activated component of ^{36}Cl efflux: y=242x+3.08, r^2=0.99, P<0.01. Values are means ± S.E.M. (N=5). V, rate of Cl^- transport; S, Cl^- concentration.

saturation kinetics and could be described by the Michaelis–Menten equation. Apparent K_m and V_{max} values calculated from a Lineweaver–Burk plot (see Fig. 2, inset) were approximately 80 mmol l^{-1} and 0.32 h^{-1} respectively. The observed phenomenon of *trans*-stimulation of furosemide-sensitive Cl^- efflux by $[Cl^-]_e$ can be easily explained in terms of an anion-exchange mechanism.

Dependence of Cl^- influx on $[Cl^-]_e$

To determine the kinetic parameters of the furosemide-sensitive Cl^- pathway more precisely, unidirectional Cl^- influx was studied for red blood cells incubated in medium in which $[Cl^-]$ varied from 5 to 155 mmol l^{-1} (replacing Cl^- with gluconate). Fig. 3 shows that both total and furosemide-sensitive Cl^- influx in lamprey erythrocytes are saturable functions of $[Cl^-]_e$ (the Lineweaver–Burk plot for the furosemide-sensitive component of Cl^- influx is plotted in the inset to Fig. 3). The kinetic parameters of the furosemide-sensitive Cl^- influx were calculated, giving an apparent K_m of approximately 92 mmol l^{-1} and a V_{max} of approximately 17.8 mmol l^{-1} cells h^{-1} from four separate determinations. The furosemide-sensitive component of Cl^- influx into lamprey erythrocytes was approximately 60 % of the total Cl^- influx at all values of $[Cl^-]_e$. It should be noted that residual furosemide-resistant Cl^- influx into lamprey red blood cells was also saturable, when $[Cl^-]_e$ was increased. This observation is in agreement with the previous finding of Gusev and Sherstobitov (1993) that 1 mmol l^{-1} furosemide does not completely block the Cl^- transport pathway under investigation.

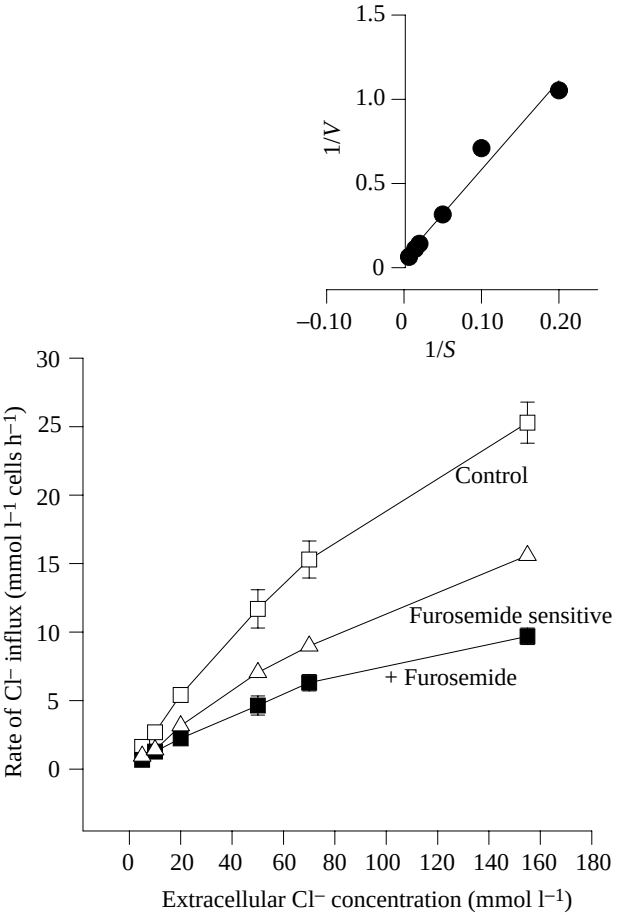

Fig. 3. Rate of Cl^- influx as a function of external Cl^- concentration. The cells were washed three times and resuspended in medium containing (in mmol l^{-1}): 0, 5, 10, 20, 50, 70 and 155 Cl^- with or without 1 mmol l^{-1} furosemide. To adjust certain Cl^- concentrations, standard medium (in mmol l^{-1}: 140 NaCl, 4 KCl, 1 $CaCl_2$, 10 Tris/HCl and 10 glucose) and gluconate-containing medium (in mmol l^{-1}: 160 sodium gluconate, 1 calcium gluconate, 10 glucose and 4 Hepes/KOH) were mixed. Incubation was started after 10 min preincubation by the addition of ^{36}Cl to the suspensions and lasted 5 min for control and 10 min for furosemide-treated cells. Inset: Lineweaver–Burk plot for the furosemide-sensitive component: y=5.20x+0.056, r^2=0.97, P<0.01. Values are means ± S.E.M. of five independent experiments. V, rate of Cl^- transport; S, Cl^- concentration.

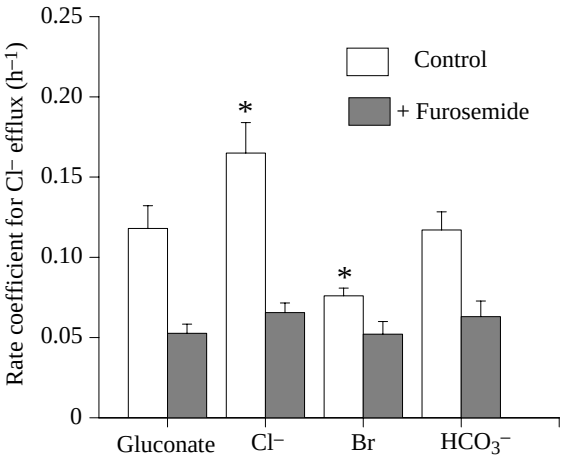

Fig. 4. *Trans*-effects of extracellular bromide and bicarbonate on unidirectional Cl^- efflux. The cells were preloaded for 2 h with ^{36}Cl in the standard incubation solution, washed free of external tracer and resuspended in gluconate-containing medium with 25 mmol l^{-1} NaCl (Cl^-), $NaHCO_3$ (HCO_3^-) or NaBr (Br^-) with and without 1 mmol l^{-1} furosemide. Bicarbonate concentration was kept stable by equilibration of the incubation solution with carbogen (5 % CO_2). Values are means + S.E.M. of five experiments. *Significantly different from the value for gluconate-containing medium (P<0.05).

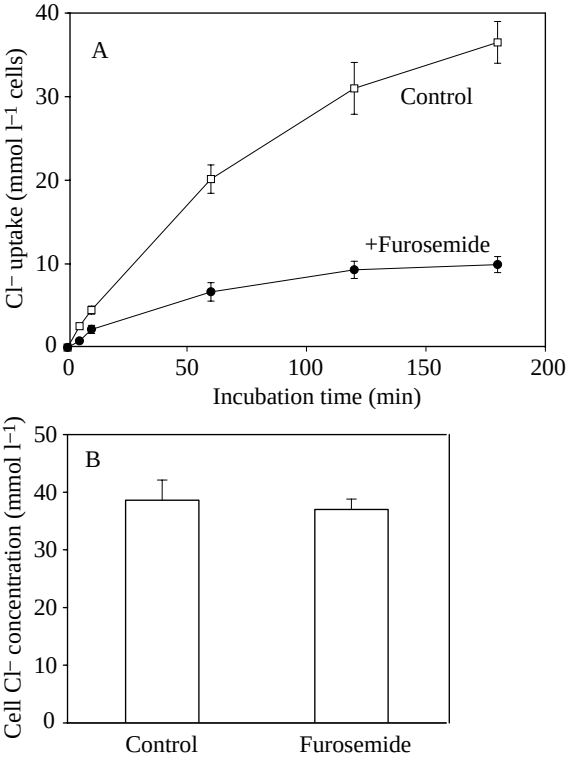

Fig. 5. Time course of ^{36}Cl uptake by lamprey erythrocytes (A) and intracellular Cl^- concentration after 2 h of incubation (B) with and without 1 mmol l^{-1} furosemide. (A) Cells were incubated in the standard incubation medium containing ^{36}Cl with or without 1 mmol l^{-1} furosemide at 20 °C. The curves were fitted as $C_\infty(1-e^{-kt})$ *versus* time (t), where C_∞ represents a steady-state cell/medium ^{36}Cl distribution and k is the rate constant. The values for control cells (open squares) were C_∞=0.285±0.0045, k=0.0105±0.0001 min^{-1}, and for furosemide-treated cells (filled circles) were C_∞=0.066±0.002 and k=0.0184±0.0018 min^{-1}. Values are means ± S.E.M. of five independent experiments. (B) The cell suspension was incubated in Cl^--containing standard medium with (F) and without (C) 1 mmol l^{-1} furosemide. After being washed free of external Cl^-, intracellular Cl^- concentration was assayed ionometrically as described in Materials and methods. Values are means + S.E.M. of 18 experiments.

Trans-*effects of HCO_3^- and Br^- on unidirectional Cl^- efflux*

As can be seen from Fig. 2, a marked activation of ^{36}Cl efflux was observed in the presence of 25 mmol l^{-1} extracellular Cl^-. Are other anions, such as bromide or bicarbonate, capable of *trans*-activation of ^{36}Cl efflux in a similar manner to external Cl^-? ^{36}Cl efflux was studied in gluconate-containing medium with 25 mmol l^{-1} gluconate replaced by 25 mmol l^{-1} Cl^-, by 25 mmol l^{-1} Br^- or by 25 mmol l^{-1} HCO_3^- (Fig. 4). The lowest value of Cl^- efflux was observed for medium containing 25 mmol l^{-1} bromide. Cl^- efflux rate into medium containing 25 mmol l^{-1} bicarbonate did not differ from that into gluconate-containing medium. The only anion able to cause *trans*-stimulation of ^{36}Cl efflux was external Cl^-. Cl^- efflux rates in the presence of 1 mmol l^{-1} furosemide were independent of the anion composition of the extracellular medium.

Time course of ^{36}Cl uptake and intracellular Cl^- concentration

The next set of experiments was undertaken to clarify whether the furosemide-sensitive Cl^- transport pathway is capable of mediating net Cl^- fluxes. The kinetics of ^{36}Cl uptake was assayed with and without 1 mmol l^{-1} furosemide. Unfortunately, true steady-state distribution of ^{36}Cl was not achieved after 3 h of incubation for eihter control or furosemide-treated cells (Fig. 5A). Incubation for more than 3 h resulted in cell lysis. Furosemide induced a significant reduction in Cl^- uptake into the cells compared with untreated cells. However, as can be seen from Fig. 5B, measurements using a Cl^--selective electrode revealed no changes in intracellular Cl^- concentration in furosemide-treated cells (37.0±1.8 mmol l^{-1}) in comparison with control cells (38.6±3.5 mmol l^{-1}).

Effect of preincubation time on ^{36}Cl influx

Rate coefficients obtained for Cl^- efflux from red cells varied from 0.18 to 0.50 h^{-1} among different animals, giving corresponding values of Cl^- fluxes of 6.9–19.4 mmol l^{-1} $cells^{-1}$ h^{-1} at a mean Cl^- concentration of 38.6 mmol l^{-1}. These values were significantly lower than those for Cl^- influx into the red cells calculated from the initial rates of ^{36}Cl uptake (28.8±2.8 mmol l^{-1} $cells^{-1}$ h^{-1}, Fig. 5). The principal difference in measurements of unidirectional fluxes was that Cl^- influx rates were determined in freshly isolated red cells, whilst Cl^- efflux was measured in cells preincubated with ^{36}Cl for 2 h. Hence, the effect of preincubation for 2–3 h on Cl^- influx was investigated in a separate series of experiments. Table

Table 2. *Effect of preincubation in the standard Cl^- medium on the rate of Cl^- influx*

Preincubation time (min)	Cl^- influx (mmol l^{-1} cells h^{-1})		
	Total	+ Furosemide	Furosemide-sensitive
5	28.1±1.4	19.4±0.98	8.70±0.86
120	23.4±2.1*	21.0±1.4	2.47±0.45*
180	15.0±1.3*	13.2±1.8*	1.54±0.23*

The cells were preincubated for 5, 120 or 180 min in the standard saline at room temperature and then unidirectional influx was assayed by measurement of ^{36}Cl uptake for 5 min (control) or 10 min (plus 1 mmol l^{-1} furosemide).

Values are means ± S.E.M. of four independent experiments.

*P<0.01 compared with Cl^- fluxes after 5 min of preincubation.

2 shows that a 3 h preincubation of the red cells in standard medium was associated with a significant decrease in Cl^- influx. Maximal changes in Cl^- influx (by 82 %) were observed for the furosemide-sensitive component, which explains the observed apparent 'steady-state' ^{36}Cl distribution in the presence of furosemide. Thus, under comparable experimental conditions, total Cl^- influx rate was similar to Cl^- efflux rate for lamprey erythrocytes.

Discussion

In our previous work (Gusev and Sherstobitov, 1993), we found that Cl^- influx into the lamprey erythrocytes is mediated by a furosemide-sensitive pathway dependent on external Ca^{2+}. The present study has shown that Cl^- exit from the red cells was also reduced in nominally Ca^{2+}-free medium and significantly inhibited by 1 mmol l^{-1} furosemide. Moreover, both furosemide-sensitive Cl^- influx and efflux were unaffected by cell depolarization induced by 1 mmol l^{-1} Ba^{2+} and increasing external K^+ concentration.

Inhibition of Cl^- efflux in the presence of 1 mmol l^{-1} furosemide, 0.05 mmol l^{-1} DIOA and 0.1 mmol l^{-1} niflumic acid (Fig. 1), known inhibitors of $Na^+/K^+/2Cl^-$ cotransport, K^+/Cl^- cotransport, some types of Cl^- channels and band-3-mediated anion exchange (Hoffmann, 1986; Garay *et al.* 1988; Cabantchik, 1990; Cabantchik and Greger, 1992), does not provide sufficient information on the possible mechanisms of anion transport. It is most likely that $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransport are not involved in Cl^- transport across the lamprey erythrocyte membrane under isotonic conditions. Indeed, bumetanide, a selective inhibitor of $Na^+/K^+/2Cl^-$ cotransport, had no effect on ^{86}Rb influx (our unpublished data) and ^{36}Cl efflux (Fig. 1). The absence of $Na^+/K^+/2Cl^-$ cotransport is also supported by the observation that furosemide did not affect Na^+ influx into lamprey erythrocytes (Gusev *et al.* 1992*a*). Since unidirectional Cl^- influx into lamprey erythrocytes was unaffected by an increase in external K^+ concentration from 0.5 to 10 mmol l^{-1}, coupling between Cl^- and K^+ transport under the experimental conditions used is unlikely (Gusev and Sherstobitov, 1993). DIDS is known to be a potent inhibitor of band-3-mediated anion exchange in the erythrocyte membrane of a variety of species (e.g. Passow, 1986). However, insensitivity to DIDS in isotonic medium of Cl^- efflux, as well as Cl^- uptake (Nikinmaa and Railo, 1987), does not necessarily result from the lack of band-3-mediated anion exchange. Recent studies have revealed the existence of a number of isoforms of anion exchanger that are less sensitive or insensitive to stilbene disulphonate derivatives (Jensen and Brahm, 1995). DIDS was shown to inhibit Cl^- efflux from lamprey erythrocytes under hypotonic conditions, suggesting activation of some other, probably conductive, anion transport pathway by cell swelling (Virkki and Nikinmaa, 1995). Taken together, the data obtained could be interpreted in terms of the presence of an anion exchanger and/or channel in the lamprey erythrocyte membrane. Cl^- efflux from lamprey red cells was markedly reduced when external Cl^- was replaced with poorly permeable gluconate ions. The *trans*-stimulatory effect of external Cl^- on ^{36}Cl efflux appeared to be a saturable function of its concentration in the extracellular medium (Fig. 2) The apparent K_m for external Cl^- was found to be approximately 80–90 mmol l^{-1} for both furosemide-sensitive Cl^- influx and efflux (see Figs 2, 3). Furosemide, which blocked both influx and efflux of Cl^-, had no effect on intracellular Cl^- concentration measured using a Cl^--selective electrode (Fig. 5B). Taken together, the observed saturation kinetics, the *trans*-activation of efflux by external Cl^- and the insensitivity of the furosemide-sensitive components of Cl^- fluxes to membrane depolarization strongly suggest an exchange mechanism for the furosemide-sensitive anion transport pathway. Our data are in good agreement with a patch-clamp study of lamprey erythrocyte membranes in which no conductive anion pathway was found (Virkki and Nikinmaa, 1996).

Kay *et al.* (1995) recently reported the cloning of the cDNA fragment corresponding to the band 3 protein of mammalian red blood cells. They found 73 % homology between that of human red blood cell membrane and that of sea lamprey (*Petromyzon marinus*) red blood cell membrane using the polymerase chain reaction (PCR) technique. The presence of a band-3-like protein in the erythrocyte membrane of this species was confirmed by antibody–antigene investigations. Identification of a band-3-like protein by immunoreaction with polyclonal IgGs against fish (rainbow trout) AE1 provided evidence for the existence of band-3-like protein oligomers (100 and 200 kDa) in the erythrocyte membrane of sea lamprey (Cameron *et al.* 1996). The functional role of this band-3-like protein remains unclear. However, whereas low rates of anion transfer across the erythrocyte membrane of *Lamprey fluviatilis* (Ohnishi and Asai, 1985; Ellory *et al.* 1987; Tufts and Boutilier, 1989; Nikinmaa and Railo, 1987; Gusev and Sherstobitov, 1993; Virkki and Nikinmaa, 1995) could be explained by the small number of protein copies, the anion preference and pharmacological properties of the furosemide-sensitive anion-exchange system described here show substantial differences from those of band-3-mediated anion exchange.

Most of the detailed studies on the anion exchanger have

been performed on human red blood cells, and little is known about anion-exchange mechanisms in erythrocytes of other species. Band-3-mediated anion exchange in mammalian red cell membrane is highly sensitive to stilbene disulphonate derivatives, with an apparent $K_{1/2}$ for DIDS of approximately 1 μmol l^{-1}. In spite of the general similarity between anion exchangers, a significant difference in function was demostrated for the AE isoforms present in human, trout, pigeon and turtle erythrocytes (Stabenau *et al.* 1991; Romano *et al.* 1992; Sieger *et al.* 1994). It is interesting to note that erythroleukaemic cells from both mouse and humans have very low Cl^- exchange rates and that these fluxes are much less sensitive to stilbenes (Hoffmann, 1986) than in mature red cells. In comparative investigations of some fish species, it has been found that the anion exchanger in carp erythrocytes, in contrast to that in trout, eel and cod red blood cell membrane, was not blocked by 0.2 mmol l^{-1} DIDS (Jensen and Brahm, 1995). DIDS-insensitive anion exchange systems have been described in other tissues, such as renal epithelium and Schwann cells (Emmons and Kurtz, 1994; Nakhoul *et al.* 1994). The Cl^- exchanger found in lamprey erythrocytes is insensitive to 0.1 mmol l^{-1} DIDS. Furosemide-sensitive Cl^- fluxes in lamprey erythrocyte membrane differ drastically from band-3-mediated anion exchange described for other cells and tissues. Flux rates are nearly three orders of magnitude lower than those mediated by the band 3 protein in erythrocytes of human and other species (Wieth and Brahm, 1985). The relatively low affinity of the furosemide-sensitive system for Cl^- in comparison with that of band 3 protein suggests that some other substrates (e.g. inorganic phosphate or lactate) may share this transport pathway with Cl^-. The absence of *trans*-stimulation of Cl^- efflux by HCO_3^- and Br^- (Fig. 4) suggests that bicarbonate and bromide are not able to use this pathway, in contrast to the anion exchangers described in red cells of all other species.

Is any other anion transport pathway, other than the furosemide-sensitive exchange, functioning in the lamprey erythrocyte membrane under the experimental conditions used? The rate coefficient of ^{36}Cl efflux from lamprey erythrocytes incubated in gluconate medium did not differ from that in Cl^--containing medium in the presence of furosemide (0.076±0.012 *versus* 0.070±0.011 h^{-1}, P>0.05) (Table 2). However, the addition of 1 mmol l^{-1} furosemide to gluconate-containing medium caused a further reduction in the Cl^- efflux rate to 0.038±0.018 h^{-1} (P<0.01). It is possible that gluconate is transported, albeit with a very low affinity, by the furosemide-sensitive anion-transport pathway. In our previous investigations, 1 mmol l^{-1} furosemide was not capable of causing maximal inhibition of Cl^- influx (Gusev and Sherstobitov, 1993). Since furosemide was shown to be a competitive inhibitior of band-3-mediated anion exchange (Passow *et al.* 1986), replacement of external Cl^- by less-permeable gluconate could result in a more pronounced effect of the inhibitor. As can be seen from Fig. 4, Cl^- efflux into bromide-containing medium is even slower than that into gluconate-containing medium, suggesting that Br^- is unable to share this transport pathway with Cl^-. It is possible that the residual component of Cl^- efflux for furosemide-treated cells incubated in gluconate-containing medium reflects a passive permeability for Cl^-. Assuming that the volume of a lamprey erythrocyte is 250 μm^3 and the volume/area ratio is approximately 10^{-5} cm (Nikinmaa and Railo, 1987), the calculated value of Cl^- permeability is approximately 10^{-10} cm s^{-1} at 20 °C. This value is close to the permeability coefficient for Cl^- calculated from the data on Cl^- influx into lamprey red cells (Gusev and Sherstobitov, 1993) and corresponds to the values of ion permeability for lipid bilayers.

Using a Cl^--selective electrode, the mean intracellular Cl^- concentration in lamprey red cells was found to be 38.6±3.5 mmol l^{-1} under basal conditions. Taking into account the water content of red cells (70 %; Vizkki and Nikinmaa, 1995), the Cl^- concentration is approximately 57.6±5.2 mmol l^{-1} cell water, giving an electrochemical potential, E_{Cl}, of −24 mV at 20 °C. A similar value for intracellular Cl^- concentration can be calculated from the time course of ^{36}Cl uptake by control cells (Fig. 5A). However, after 3 h of incubation of furosemide-treated cells with radioactive tracer, ^{36}Cl uptake is still quite far from electrochemical equilibrium. Moreover, incubation of the cells under isotonic conditions results in a gradual decrease in unidirectional Cl^- influx (both furosemide-sensitive and furosemide-resistant components) during 3 h of incubation (Table 2). Since intracellular Cl^- concentration remains unchanged over a 3 h incubation, it is likely that Cl^- efflux also alters in parallel with Cl^- influx. In our experiments, all efflux measurements were performed between 2 h and 3 h of cell incubation, and the mean rate coefficient of total Cl^- efflux was 0.32±0.016 h^{-1} (N=36). Assuming an intracellular Cl^- concentration of 38.6± 3.5 mmol l^{-1}, the Cl^- efflux amounts to 12.4 mmol l^{-1} cells h^{-1}. The magnitude of Cl^- efflux is comparable to that of Cl^- influx in red cells preincubated in standard medium for 2–3 h (Table 2). The reason for this instability of Cl^- fluxes in the lamprey erythrocytes is not yet understood. This phenomenon of instability in ion fluxes has been confirmed in patch-clamp studies of Na^+ and K^+ transport across the lamprey erythrocyte membrane (Virkki and Nikinmaa, 1996) and also for nucleated erythrocytes of other species (Bourne and Cossins, 1982; Houston *et al.* 1985; Agalakova *et al.* 1997).

In conclusion, our results provide the first evidence that approximately 70 % of the Cl^- transport across the lamprey erythrocyte membrane is mediated by an electrically silent exchange system. The anion exchanger is inhibited by 1 mmol l^{-1} furosemide and a number of other inhibitors, by replacement of external Cl^- with gluconate and by the removal of extracellular Ca^{2+}. Unlike erythrocyte anion exchange mediated *via* the band 3 protein, the lamprey erythrocyte anion exchanger has a 1000-fold smaller exchange rate and a different substrate preference order (Cl^-=NO_3^- ≫gluconate=HCO_3^-; Br^- is not transported). It is also insensitive to 0.1 mmol l^{-1} DIDS. No evidence was obtained for the involvement of $Na^+/K^+/2Cl^-$ or K^+/Cl^- cotransporters in Cl^- transfer across the erythrocyte

membrane of lamprey. The possible physiological role of the anion-exchange mechanism in red blood cells of ancient living vertebrates such as *Lampetra fluviatilis* remains to be established in further investigations.

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