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K^+ TRANSPORT ACROSS THE LAMPREY ERYTHROCYTE MEMBRANE: CHARACTERISTICS OF A Ba^{2+}- AND AMILORIDE-SENSITIVE PATHWAY

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Summary

The characteristics of K^+ transport in erythrocytes from the river lamprey (*Lampetra fluviatilis*) were investigated using standard radioisotope flux techniques. The cells were shown to have a ouabain-sensitive transport pathway that carried $^{43}K^+$ and $^{86}Rb^+$ into the cell at similar rates. Most of the ouabain-resistant $^{43}K^+$ and $^{86}Rb^+$ influx was *via* a pathway that was insensitive to cotransport inhibitors and to the replacement of extracellular Cl^- or Na^+. This pathway showed a strong selectivity for $^{43}K^+$ over $^{86}Rb^+$. It was inhibited fully by Ba^{2+} ($I_{50}\approx2.8\,\mu mol\,l^{-1}$), amiloride ($I_{50}\approx150\,\mu mol\,l^{-1}$) and ethylisopropylamiloride ($I_{50}\approx3.3\,\mu mol\,l^{-1}$) and less effectively by quinine and by the tetraethylammonium ion. Inhibition by Ba^{2+} took full effect within a few minutes whereas the full inhibitory effect of amiloride took more than 1 h to develop. Experiments with the membrane potential probe [^{14}C]tetraphenylphosphonium ion gave results consistent with the lamprey erythrocyte membrane having a Ba^{2+}-sensitive K^+ conductance that was significantly greater than the membrane Na^+ conductance and which gave rise to a marked dependence of the membrane potential on the extracellular K^+ concentration. The rate constants for Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ influx decreased (proportionally) with increasing extracellular K^+ concentration in a manner that was consistent with the transport being *via* a conductive pathway. The decrease was attributed to a depolarisation of the membrane (in response to the increasing extracellular K^+ concentration) and a consequent decrease in the driving force for the conductive movement of $^{43}K^+$ and $^{86}Rb^+$ into the cells. Ba^{2+}-sensitive $^{86}Rb^+$ influx increased significantly with decreasing cell volume and with increasing intracellular pH (at a constant extracellular pH) but increased only slightly with increasing extracellular pH. The pathway operated normally in the complete absence of extracellular Ca^{2+} but its activity decreased in cells pretreated with ionomycin and EGTA; this suggests a role for intracellular Ca^{2+} in the operation of the pathway.

Introduction

Vertebrate erythrocytes are known to possess a variety of transport pathways by

Key words: erythrocyte, lamprey, K^+ transport, K^+ channel, K^+/Cl^- cotransport, *Lampetra fluviatilis*.

which K^+ is able to permeate the cell membrane. In most, though not all, such cells there is a ouabain-sensitive Na^+/K^+ pump that uses energy derived from the hydrolysis of ATP to drive K^+ into, and Na^+ out of, the cell, thereby establishing large and opposing electrochemical gradients for the two cations. Also present in the membrane are a number of 'leak' pathways which, when active, allow the ions to move down their electrochemical gradients. For cells in a steady state the active transport of Na^+ and K^+ is exactly balanced by the passive leak of the two ions in the opposite direction; there is no net movement of either ion across the membrane and the cells thereby maintain a constant volume (Tosteson and Hoffman, 1960). However, under certain conditions the activity of one or more of the pathways may be altered with the result that there is a net movement of ions into or out of the cell and a consequent change in the cell volume (Hoffman and Simonsen, 1989).

In erythrocytes from many mammalian and avian species, much of the ouabain-resistant flux of K^+ across the cell membrane is mediated by the so-called $Na^+/K^+/Cl^-$ cotransport system (see Haas, 1989, for a recent review). In avian and some mammalian red cells this system is activated by cell shrinkage and, under certain extracellular ionic conditions, its activation results in a regulatory volume increase. Erythrocytes from a variety of mammalian, avian and fish species are thought to have a K^+/Cl^- cotransport system which is distinct from the $Na^+/K^+/Cl^-$ cotransporter (see Dunham, 1990, for a recent review). This system is generally activated by cell swelling and it thereby serves as a regulatory volume decrease mechanism. In erythrocytes from the amphibian *Amphiuma*, cell swelling has been shown to activate a K^+/H^+ exchange system which again serves as a regulatory volume decrease mechanism (Cala, 1980). Finally, erythrocytes from a wide variety of mammalian, avian, amphibian and fish species have present in their membrane a Ca^{2+}-activated K^+ channel, commonly referred to as the Gardos channel (Lew and Ferreira, 1978). In all erythrocyte types studied, this pathway is inactive under physiological conditions and its function, if any, remains unknown.

The lampreys are a subclass of the cyclostomes (the only surviving class of agnathans or jawless fish) and are amongst the most primitive vertebrates alive today. Previous studies (Ohnishi and Asai, 1985; Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989) have shown that lamprey erythrocytes lack the band 3 anion exchange protein, which is the principal route for Cl^- transport (as well as that of numerous other anions) in red cells of higher vertebrates. The aim of the work reported here was to investigate the principal K^+ transport pathways in the lamprey erythrocyte and to compare them to those present in erythrocytes from the higher vertebrates.

Materials and methods

Lampreys

River lampreys (*Lampetra fluviatilis* Linnaeus; 25–80 g) were a generous gift

from Dr M. Nikinmaa of the Department of Zoology, University of Helsinki. They were caught during their spawning runs in September 1988, 1989 and 1990 in the Simojoki River in Northern Finland. Following capture they were transported first to Helsinki, where they were allowed 1 month to acclimatise to laboratory conditions, then to Oxford, where they were kept in well-aerated, dechlorinated tap water (maintained at a temperature of approximately 4°C) for several weeks prior to experimentation.

Chemicals

All buffer components were of analytical reagent grade. Amiloride hydrochloride, $BaCl_2$, 2,4-dinitrophenol chloride (DNP), ethyleneglycol-bis-(β-amino-ethyl ether)*N*,*N*′-tetra-acetic acid (EGTA), 3-aminobenzoic acid ethyl ester methanesulphonate salt (MS 222), NH_4Cl, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes), *N*-methyl-D-glucamine ($NMDG^+$), quinine hydrochloride, sodium tetraphenylboron (TPB) and tetraethylammonium chloride (TEA^+) were from Sigma Chemicals (Poole, Dorset). 4-Acetamido-4′-iso-thiocyanato-stilbene-2,2′-disulphonic acid disodium salt (SITS) and ouabain were from BDH Chemicals Ltd (Poole, Dorset). Ionomycin was from Calbiochem Corporation (CA, USA). Bumetanide was a gift from Leo Laboratories (Aylesbury, Bucks). 3-[2-(1-naphthyl)-ethyl]amino-4-phenoxy-5-sulphamoylbenzoid acid (H74), a recently synthesised selective inhibitor of K^+/Cl^- cotransport in human erythrocytes (Ellory *et al.* 1990), was a gift from Dr J. C. Ellory of the Oxford University Laboratory of Physiology. Ethylisopropylamiloride (EIPA) was a gift from Dr W. Fuhrer and Dr K. Scheibli of Ciba-Geigy, Basel, Switzerland.

Ouabain was added to suspensions as a (9 mmol l^{-1}) solution in distilled water. Bumetanide, H74, SITS, quinine and DNP were added as dilute solutions in lamprey Ringer's solution. TEA^+, $BaCl_2$, NH_4Cl and $NaHCO_3$ were added as isosmotic solutions in distilled water. Amiloride, EIPA and ionomycin were added as concentrated solutions in dimethylsulphoxide (DMSO). In those cases in which reagents were added to samples as DMSO solutions, the same volume of DMSO was added to control samples [though it was shown in preliminary experiments that DMSO, at the concentrations used here, had no effect on the transport pathway(s) of interest].

$^{86}RbCl$ was from Amersham International (UK) Ltd. ^{43}KCl was from the MRC Cyclotron Unit (Hammersmith Hospital, London). [^{14}C]Tetraphenylphosphonium bromide ([^{14}C]TPP) was from NEN Research Products (Stevenage, Hertfordshire).

Collection and preparation of erythrocytes

Lampreys were anaesthetised (MS 222, approx. 2 g l^{-1} in water, buffered with $NaHCO_3$) then bled (either from the tail or by decapitation) into a gently agitated ice-cold, heparinised saline solution (130 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes, 0.1 mmol l^{-1} EGTA; pH 7.6). The volume of erythrocytes obtained from a single lamprey varied between 100 and 1000 μl. The suspension was filtered through a

polymer wool column then the cells were washed (by centrifugation for 4 min at 1000 *g* followed by resuspension) twice in the heparinised saline then four times in an ice-cold lamprey Ringer's solution which, unless stated otherwise in the appropriate figure or table legend, was composed of 114 mmol l^{-1} NaCl, 10–30 mmol l^{-1} Hepes, 1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} glucose and was adjusted to pH 7.6 with 10 mol l^{-1} NaOH (Nikinmaa, 1986). When viewed under a microscope (using Nomarski optics) the washed cell suspension appeared to consist of a homogeneous population of nucleated red cells. The suspension was stored on ice and used within 24 h of collection.

$^{86}Rb^+$ and $^{43}K^+$ influx measurements

For all except the short incubation experiments described in the legend to Fig. 3, cells suspended in a lamprey Ringer's solution were combined with the appropriate reagent solutions in an Eppendorf tube at a haematocrit of 1–4 %. The samples were transferred to an 11 °C water bath at least 10 min prior to beginning the influx incubation in order to allow temperature equilibration. Except where specified, experiments were carried out in the presence of ouabain (0.1 mmol l^{-1}), which was added just before transferring the samples to the incubation bath. Influx experiments were commenced by the addition to the cell suspension of $^{86}Rb^+$ or $^{43}K^+$ to give a total suspension volume of 900 μl, an activity of approximately 2 μCi ml^{-1} and an extracellular K^+ concentration ($[K^+]_e$) of 5 mmol l^{-1} (except in those experiments in which $[K^+]_e$ was varied).

Immediately after the addition of the radioisotope, a 200 μl sample was taken from the suspension; a further three 200 μl samples were taken after an appropriate incubation period. The samples were transferred immediately to Eppendorf tubes containing 800 μl of ice-cold lamprey Ringer (to which had been added 1 mmol l^{-1} $BaCl_2$) layered over 250 μl of dibutylphthalate. The tubes were centrifuged for 30 s (10 000 *g*), sedimenting the cells to the bottom of the tube and leaving the diluted extracellular solution above the oil. The supernatant solution and oil were aspirated and all traces of solution on the inner wall of the tube were removed with cotton dental rolls. The cell pellet was lysed with 0.5 % (v/v) Triton X-100 (0.5 ml) then deproteinised by the addition of 5 % (w/v) trichloroacetic acid (0.5 ml) followed by centrifugation (10 000 *g*, 10 min). The activity of $^{86}Rb^+$ in the supernatant solution was measured by Cerenkov counting and the activity of $^{43}K^+$ by scintillation counting.

In preliminary time course experiments $^{86}Rb^+$ uptake appeared to be linear with time for up to 1 h, whether in the presence or absence of ouabain. In the experiments reported here, unless specified otherwise, the radioisotope influx rates were estimated from the radioactivity accumulated during a 20–50 min incubation period. The radioactivity associated with the cells sampled immediately after the addition of radioisotope to each flux sample provided an estimate of the amount of radioisotope trapped in the extracellular space of the cell pellets. For cells pretreated with ouabain (in the absence of other transport inhibitors) the

trapped extracellular radioactivity was always less than 5 % of that taken up by the cells.

The linearity of the time course implies that there was only a negligible loss ('backflux') of the isotope from the cell during the incubation period, in which case the first-order rate constant for entry of the isotope into the cell (k) may be estimated from the expression:

$$k \approx A_i/(A_{total} \cdot t)\,, \tag{1}$$

where A_{total} is the total activity of radioisotope added to the cell suspension and A_i is the activity of radioisotope accumulated by the cells during the incubation time, t. If the radioisotope is to be used as a tracer for K^+, the corresponding initial unidirectional K^+ flux may be calculated from the expression:

$$K^+ \text{ influx} = k \cdot K_e^+/V_{RBC}\,, \tag{2}$$

where K_e^+ denotes the amount of K^+ initially present in the extracellular solution and V_{RBC} denotes the volume of cells into which the uptake occurred.

[^{14}C]tetraphenylphosphonium bromide distribution

[^{14}C]TPP is a lipophilic cation that has been used previously as a probe of the membrane potential (E_m) of erythrocytes (Freedman and Novak, 1989). In this study the uptake and transmembrane distribution of the cation were assessed from its extracellular concentration (Nikinmaa *et al.* 1990). [^{14}C]TPP (0.42 mmol l^{-1} in DMSO, 10 μl) was added to cell suspensions (890 μl; 20 °C) to which had previously been added TPB (0.36 mmol l^{-1} in DMSO, 5 μl), a lipophilic anion that facilitates the rapid equilibration of [^{14}C]TPP across the cell membrane (Freedman and Novak, 1989). The final suspension had a haematocrit of 1–3 % and an activity of approximately 0.1 μCi ml^{-1}. At appropriate times, 200 μl samples of the suspension were transferred to Eppendorf tubes containing 250 μl of dibutylphthalate. Each tube was centrifuged for 15 s and 50 μl of the supernatant solution was sampled for scintillation counting.

Analytical measurements

Intra- and extracellular pH (pHi and pHe) were both measured at 20 °C using a water-jacketed Radiometer pH glass capillary electrode linked to a Radiometer pH meter (PHM 52). pHe was measured in isolated extracellular solution. pHi was measured in packed cell haemolysates obtained by centrifuging the suspension of interest (10 000 g, 1 min), aspirating the supernatant solution and the top-most layer of cells, then thrice freezing then thawing the remaining pellet in liquid N_2. The pH was measured immediately after thawing the haemolysate for the third time.

Relative cell volumes (RCV) were measured using a microhaematocrit centrifuge. Haematocrits of isotonic cell suspensions (from which V_{RBC} of equation 2 was derived) were estimated routinely from the absorbance at 540 nm of the suspension diluted in 'Drabkin's reagent' (Dacie and Lewis, 1975). The theoretical

packed cell absorbance of lamprey erythrocytes under the conditions used in these experiments was found to be 185 (using a 1 cm light path).

Statistics

Unless specified otherwise, the results cited represent the mean value ± the standard error of the mean obtained from N separate experiments, where each experiment was carried out using blood from a different lamprey. Differences between mean values were tested for significance using a paired (two-tailed) t-test. In general, the standard errors for the mean influx values obtained under different experimental conditions (e.g. in the presence and absence of a particular reagent) were approximately proportional to the mean value; the flux values (Y, $mmol\,l^{-1}\,RBC\,h^{-1}$) were therefore subjected to a logarithmic transformation of the form $\log(Y+1)$ prior to application of the t-test in order approximately to equalise the variances for the two populations under comparison (Steel and Torrie, 1981). In figures and tables ** indicates $P<0.01$, * indicates $0.01<P<0.05$, (*) indicates $0.05<P<0.10$ and NS indicates $P>0.10$ for differences between mean values obtained under experimental and control conditions.

In those instances in which the results of a single, representative experiment are presented no errors are given; in most such cases the standard deviation for the triplicate samples taken from each tube at the end of the flux incubation (in the presence of ouabain and in the absence of other inhibitors) was less than 3 % of the mean value.

Results

Effects of transport inhibitors and external ion replacement on $^{86}Rb^+$ and $^{43}K^+$ influx

Fig. 1 shows the effects of different transport inhibitors on the unidirectional rate constants for the entry of $^{43}K^+$ and $^{86}Rb^+$ into lamprey erythrocytes (k^K and k^{Rb}, respectively). In the absence of inhibitors, $^{43}K^+$ moved into the cell much faster than $^{86}Rb^+$. Ouabain caused an almost identical decrease in k^K and k^{Rb} and was maximally effective at a concentration of $0.1\,mmol\,l^{-1}$ (results not shown). The magnitude of the ouabain-sensitive K^+ influx calculated from k^K (using equation 2) was $2.2\pm0.5\,mmol\,l^{-1}\,RBC\,h^{-1}$ ($N=3$). The corresponding value calculated from k^{Rb} was $2.3\pm0.3\,mmol\,l^{-1}\,RBC\,h^{-1}$ ($N=14$). The addition of bumetanide ($0.1\,mmol\,l^{-1}$) caused a further small (and again almost identical) reduction in k^K and k^{Rb}. Pretreatment of cells with either Ba^{2+} ($2\,mmol\,l^{-1}$) or amiloride ($2\,mmol\,l^{-1}$) caused the ouabain-resistant components of k^K and k^{Rb} to decrease dramatically to approximately the same (basal) level. The magnitude of the Ba^{2+}- and amiloride-sensitive K^+ influx (calculated from k^K using equation 2) was $14.8\pm1.9\,mmol\,l^{-1}\,RBC\,h^{-1}$ ($N=3$).

Table 1 shows the effects of different transport inhibitors, as well as the effects of replacing either extracellular Na^+ (with $NMDG^+$) or extracellular Cl^- (with NO_3^-) on $^{86}Rb^+$ influx into cells pretreated with ouabain. The dose–response

curves for the inhibition of the ouabain-resistant $^{86}Rb^+$ influx by Ba^{2+}, amiloride and EIPA are shown in Fig. 2. Ba^{2+} exerted half of its maximal inhibitory effect at a concentration (I_{50}) of approximately 2.8 μmol l^{-1} (Fig. 2A). Amiloride acted with an I_{50} value of approximately 150 μmol l^{-1} and EIPA with an I_{50} value of approximately 3.3 μmol l^{-1} (Fig. 2B).

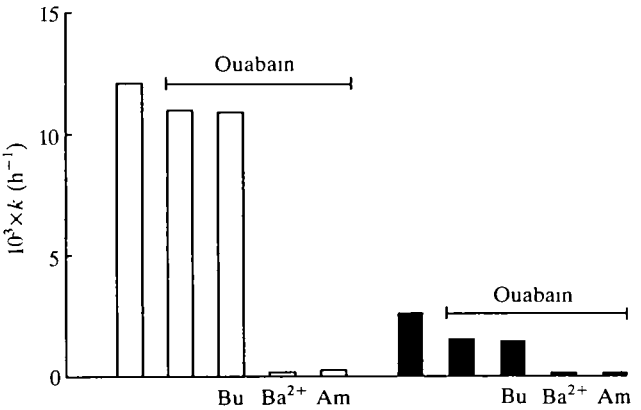

Fig. 1. Rate constants for the unidirectional influx of $^{43}K^+$ (k^K, open bars) and $^{86}Rb^+$ (k^{Rb}, filled bars) into lamprey erythrocytes in the presence of various transport inhibitors. Ouabain (0.1 mmol l^{-1}) was added to the cell suspension approximately 15 min before the addition of radioisotope. Bumetanide (Bu; 0.1 mmol l^{-1}), Ba^{2+} (2 mmol l^{-1}) and amiloride (Am; 2 mmol l^{-1}) were added at least 1 h before the addition of radioisotope to allow the reagents to take full effect. The data shown are from a single experiment and are representative of those obtained in three similar experiments.

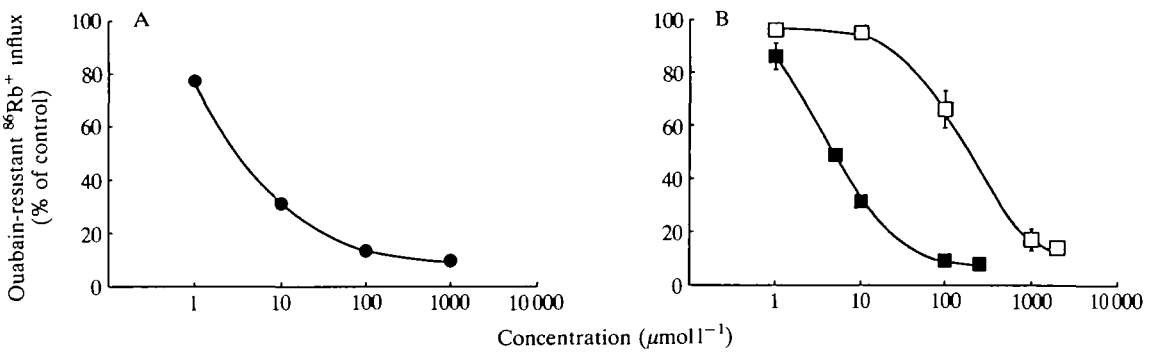

Fig. 2. (A) Dose–response curve for the effect of Ba^{2+} (●; N=3; $I_{50}\approx$2.8 μmol l^{-1}) on ouabain-resistant $^{86}Rb^+$ influx. (B) Dose–response curves for the effects of amiloride (□; N=4; $I_{50}\approx$150 μmol l^{-1}) and EIPA (■; N=2; $I_{50}\approx$3.3 μmol l^{-1}) on ouabain-resistant $^{86}Rb^+$ influx. The samples were incubated at 11 °C for 1 h prior to the addition of $^{86}Rb^+$ to allow the reagents to take full effect. In both graphs $^{86}Rb^+$ influx is expressed as a percentage of the ouabain-resistant $^{86}Rb^+$ influx in the absence of any other inhibitor. Where not visible, the error bars fall within the symbols. Values are means±S.E.M.

Table 1. *Effects of transport inhibitors and of external ion replacement on ouabain-resistant $^{86}Rb^+$ influx*

Treatment	Inhibitor concentration (mmol l^{-1})	$^{86}Rb^+$ influx (% of control)	N	P
Bumetanide	0.1	92±3	10	(*)
H74	0.3	86±4	7	*
Ba^{2+}	2	11±2	24	**
Amiloride	2	14±3	8	**
EIPA	0.10–0.15	15±3	6	**
Quinine	0.5	68±4	6	**
TEA^+	20	37±2	5	**
SITS	0.1	97±2	4	NS
Na^+ replacement	–	97±4	6	NS
Cl^- replacement	–	86±4	7	*

The inhibitors were added to the suspension at least 1 h prior to the addition of $^{86}Rb^+$.

In the ion replacement experiments the cells were washed five times in a solution from which either Na^+ or Cl^- was omitted. Na^+ was replaced iso-osmotically with $NMDG^+$. Cl^- was replaced iso-osmotically with NO_3^-.

Ouabain-resistant $^{86}Rb^+$ influx is expressed as a percentage of that for cells suspended in a normal lamprey Ringer's solution (with $[K^+]_e$=5 mmol l^{-1}) in the absence of any of the inhibitors tested.

P indicates the significance of the difference between the $^{86}Rb^+$ influx in the treated and control cells; ** $P<0.01$; * $0.01<P<0.05$; (*) $0.05<P<0.1$; NS, not significant.

EIPA, ethylisopropylamiloride; TEA^+, tetraethylammonium; $NMDG^+$, *N*-methyl-D-glucamine.

Fig. 3 shows the time-dependence for the onset of inhibition of ouabain-resistant $^{86}Rb^+$ uptake by Ba^{2+} and amiloride. Ba^{2+} exerted its full inhibitory effect within the time required to obtain the first time point (i.e. within approximately 3 min). The inhibitory effect of amiloride developed (in a biphasic manner) over a period of more than 1 h; in data averaged from four similar experiments the mean ouabain-resistant $^{86}Rb^+$ uptake remaining approximately 3 min after the addition of amiloride (38±3 % of the control value) was significantly different both from that in the control sample ($P<0.01$) and from that remaining 1 h after the addition of amiloride (16±4 % of the control value, $P<0.05$).

The effects of different inhibitors, as well as the effect of external Na^+ or Cl^- replacement, on the Ba^{2+}-sensitive component of the ouabain-resistant $^{86}Rb^+$ influx were tested and the results are given in Table 2. Quinine (0.5 mmol l^{-1}) and TEA^+ (20 mmol l^{-1}) both caused a significant decrease in the Ba^{2+}-sensitive $^{86}Rb^+$ influx. The other treatments had little effect.

Effect of $[K^+]_e$ on Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ influx

The effects of $[K^+]_e$ on the Ba^{2+}-sensitive component of $^{43}K^+$ and $^{86}Rb^+$ uptake

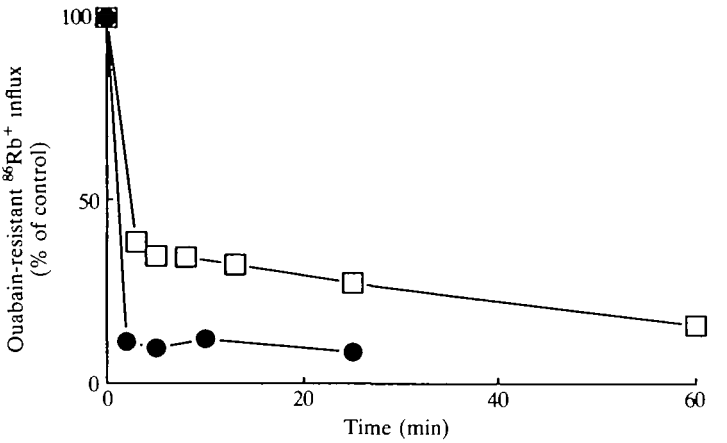

Fig. 3. Time-dependence of the inhibitory effect of Ba^{2+} (●, 2 mmol l^{-1}) and of amiloride (□, 1.6 mmol l^{-1}) on ouabain-resistant $^{86}Rb^+$ influx. The experiments were commenced by adding either Ba^{2+} or amiloride to cells suspended in lamprey Ringer (at a haematocrit of approximately 3 %), pretreated with ouabain and preincubated for 10 min at 11 °C. After a predetermined time (ranging from 1 to 58 min), $^{86}Rb^+$ was added (in a 125 mmol l^{-1} KCl solution) to give an activity of approximately 6 μCi ml^{-1}, a $[K^+]_e$ of 5 mmol l^{-1} and a final sample volume of 700 μl. The samples were incubated for a further 4 min, after which $^{86}Rb^+$ uptake was terminated by transferring two 300 μl portions of the suspension to separate Eppendorf tubes containing 800 μl of ice-cold lamprey Ringer (to which had been added 1 mmol l^{-1} $BaCl_2$) layered over 250 μl of dibutylphthalate. The tubes were immediately centrifuged and processed as described in Materials and methods. The abscissa indicates the time elapsed since the addition of inhibitor and the time points correspond to the middle of each 4-min flux incubation. $^{86}Rb^+$ uptake is expressed as a percentage of that measured in the absence of both Ba^{2+} and amiloride. The data shown are from a single experiment. The Ba^{2+} data are representative of that from two such experiments and the amiloride data of that from four such experiments.

by lamprey erythrocytes are shown in Fig. 4. As $[K^+]_e$ was increased, the Ba^{2+}-sensitive components of the influx rate constants decreased. When the corresponding K^+ fluxes (calculated from the Ba^{2+}-sensitive components of k^K and k^{Rb} using equation 2) are plotted as a function of $[K^+]_e$ the resultant curves resemble those for a transport system displaying saturation kinetics (Fig. 4A). Fig. 4B shows the corresponding double-reciprocal plot.

Effect of cell volume on Ba^{2+}-sensitive $^{86}Rb^+$ influx

In erythrocytes from many of the higher vertebrates, ouabain-resistant K^+ transport pathways are activated by cell swelling and they thereby play a primary role in mediating a regulatory volume decrease (Hoffman and Simonsen, 1989). The effects of cell volume on the Ba^{2+}-sensitive pathway of the lamprey erythrocyte were therefore investigated. Fig. 5 shows the response of Ba^{2+}-sensitive $^{86}Rb^+$ influx to both a decrease and an increase in osmotic pressure. The

Table 2. *Effects of transport inhibitors and of external ion replacement on Ba^{2+}-sensitive $^{86}Rb^{+}$ influx*

Treatment	Inhibitor concentration ($mmol\,l^{-1}$)	Ba^{2+}-sensitive $^{86}Rb^{+}$ influx (% of control)	N	P
Bumetanide	0.1	103±3	4	NS
H74	0.3	94±3	3	NS
Quinine	0.5	72±3	4	*
TEA^{+}	20	32±3	4	**
Na^{+} replacement	–	90±5	4	NS
Cl^{-} replacement	–	101±7	5	NS

The experiments were carried out as described in Table 1.

The Ba^{2+}-sensitive $^{86}Rb^{+}$ influx (obtained by subtracting k^{Rb} measured in the presence of $2\,mmol\,l^{-1}$ Ba^{2+} from k^{Rb} measured in its absence) is expressed as a percentage of that for cells suspended in a normal lamprey Ringer's solution in the absence of any of the inhibitors tested.

P indicates the significance of the difference between the $^{86}Rb^{+}$ influx in the treated and control cells; ** $P<0.01$; * $0.01<P<0.05$; NS, not significant.

TEA^{+}, tetraethylammonium.

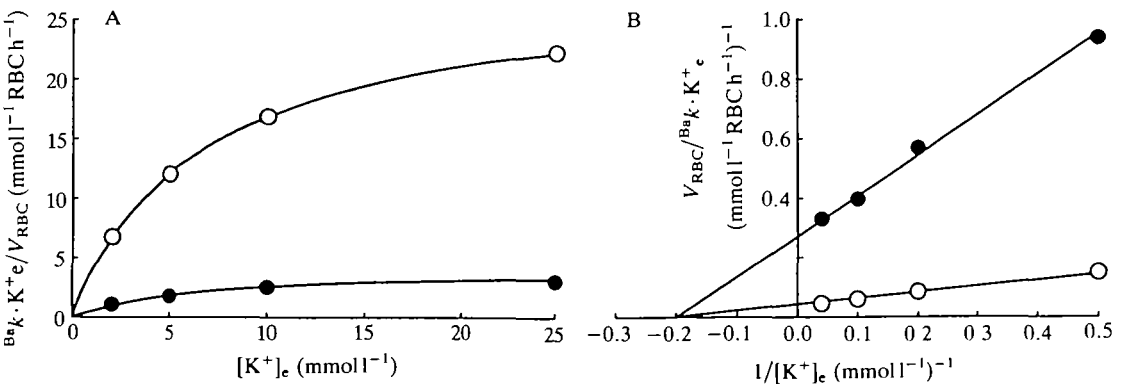

Fig. 4. (A) Variation of the Ba^{2+}-sensitive component of $^{43}K^{+}$ (○) and $^{86}Rb^{+}$ (●) influx with $[K^{+}]_e$. The ordinate indicates the unidirectional K^{+} influx values calculated from the Ba^{2+}-sensitive components of k (denoted ^{Ba}k) for $^{43}K^{+}$ or $^{86}Rb^{+}$ using equation 2. $[K^{+}]_e$ was varied by replacing NaCl in the suspending solution with an equimolar amount of KCl. (B) Double reciprocal plot for the variation of Ba^{2+}-sensitive K^{+} influx [calculated from either the Ba^{2+}-sensitive components of k^{K} (○) or k^{Rb} (●)] with $[K^{+}]_e$ concentration.

addition of H_2O (16.7% v/v) to erythrocyte suspensions caused the RCV to increase to 1.13±0.01 ($N=3$, $P<0.01$) and reduced the Ba^{2+}-sensitive $^{86}Rb^{+}$ uptake by 13.4±4% ($N=3$, $P<0.10$). The addition of a hypertonic solution ($120\,mmol\,l^{-1}$ NaCl, $1\,mol\,l^{-1}$ sucrose; 16.7% v/v) caused the RCV to decrease to

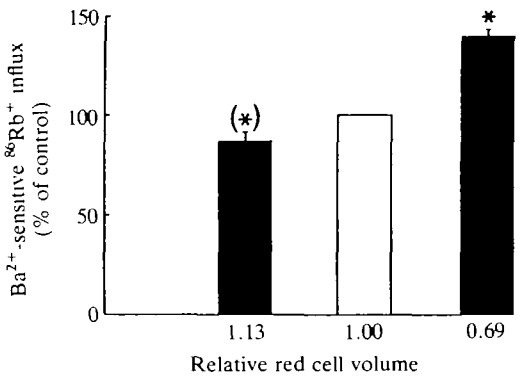

Fig. 5. Ba^{2+}-sensitive $^{86}Rb^+$ influx in cells swollen or shrunk osmotically. Cells were swollen by the addition of H_2O (16.7% v/v) to the suspension and shrunk by the addition of a hypertonic solution (16.7% v/v) containing 120 mmol l^{-1} NaCl and 1 mol l^{-1} sucrose to the suspension, immediately prior to commencing the flux. Relative cell volumes (RCV) were measured (in separate experiments) immediately following the addition of the hypo- or hypertonic solutions. Ba^{2+}-sensitive $^{86}Rb^+$ influx is expressed as a percentage of that measured in samples to which an equivalent volume of isotonic (120 mmol l^{-1}) NaCl solution had been added immediately prior to commencing the flux (open bar). The normalised influx rates and RCV estimates were each averaged from three separate experiments. Values are means+S.E.M. * $0.01<P<0.05$; (*) $0.05<P<0.1$.

0.69±0.02 ($N=3$, $P<0.05$) and increased the Ba^{2+}-sensitive $^{86}Rb^+$ uptake by 40±3% ($N=3$, $P<0.05$).

Effect of pHi and pHe on Ba^{2+}-sensitive $^{86}Rb^+$ influx

The effect of pHe on Ba^{2+}-sensitive $^{86}Rb^+$ uptake is shown in Fig. 6. A decrease in the pHe from its physiological value of 7.6 to 6.6 caused a very slight decrease in the Ba^{2+}-sensitive $^{86}Rb^+$ influx. An increase in pHe from 7.6 to 8.4 resulted in a modest (10±3%, $N=4$) increase in the Ba^{2+}-sensitive $^{86}Rb^+$ influx.

Table 3 shows the effects of pHe as well as a number of different treatments on both pHi and RCV. As has been shown previously (Nikinmaa, 1986), variations in pHe were reflected in corresponding (albeit smaller) variations in pHi. Variations in pHi at a constant pHe were brought about by the addition to the suspension of a weak acid or base. The addition of NH_4Cl (20 mmol l^{-1}) to cells suspended in a Hepes buffer caused a significant increase in pHi while leaving pHe unchanged. Preincubation of the cells with the proton ionophore DNP (20 μmol l^{-1}) caused a significant decrease in pHi without affecting pHe and a similar intracellular acidification followed the addition of HCO_3^-/CO_2 to cells suspended in a nominally HCO_3^--free Hepes buffer. Amiloride was found to have no significant effect on pHi ($P>0.10$). None of the treatments had any significant effect on RCV ($P>0.10$).

Fig. 7 shows the effect of NH_4^+, DNP and HCO_3^-/CO_2 on $^{86}Rb^+$ uptake *via* the Ba^{2+}-sensitive pathway. Intracellular alkalisation (following an NH_4^+ pre-

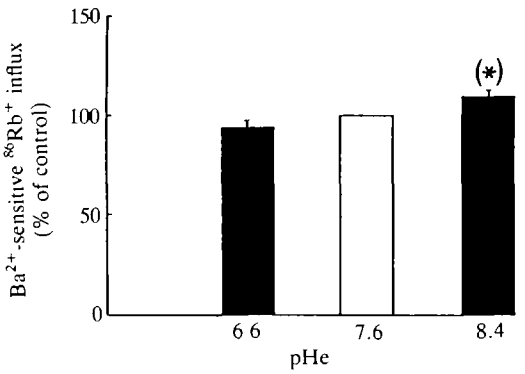

Fig. 6. Effect of pHe on Ba^{2+}-sensitive $^{86}Rb^{+}$ uptake. Cells were collected and washed as described in Materials and methods. The suspension was then subdivided into three portions each of which was washed five times in lamprey Ringer's solution containing 20 mmol l^{-1} Hepes and having pH values of 6.6, 7.6 or 8.4. The cells were incubated in the appropriate Ringer for 1 h at 20 °C to allow pHi to reach a steady value (Nikinmaa, 1986) before commencing the flux measurements. Ba^{2+}-sensitive $^{86}Rb^{+}$ influx is expressed as a percentage of that measured for cells suspended at pHe=7.6 (open bar). The normalised influx rates were averaged from at least three separate experiments. Values are means+S.E.M. (*) $0.05<P<0.1$.

Table 3. *Effect of pHe, HCO_3^- (20 mmol l^{-1})/CO_2, DNP (20 μmol l^{-1}), NH_4^+ (20 mmol l^{-1}) and amiloride (2 mmol l^{-1}) on pHi and relative cell volume (RCV)*

Treatment	pHi	*P*	RCV	*N*
pHe 6.6	7.33±0.03	*	1.03±0.04	3
pHe 7.6	7.48±0.06	–	1	3
pHe 8.4	7.59±0.04	*	1.02±0.06	3
HCO_3^-/CO_2	7.32±0.04	*	1.04±0.01	3
DNP	7.37±0.05	*	0.96±0.03	3
NH_4^+	7.61±0.02	**	1.00±0.03	3
Amiloride	7.49±0.05	NS	0.99±0.01	3

Samples were prepared as described in the legends to Figs 6 and 7.

The NH_4^+ and HCO_3^-/CO_2 solutions were added immediately before making the measurements, whereas in the case of the amiloride- and DNP-treated samples the cells were incubated with the reagents for 1 h at 20 °C prior to making the measurements so as to allow them to take full effect (Fig. 3 and Nikinmaa, 1986).

RCV values are quoted relative to that measured for cells suspended in lamprey Ringer's solution at pHe 7.6 in the absence of any other reagents.

P indicates the significance of the difference between pHi measured in the control cells (pHe 7.6) and the treated cells. None of the treatments tested gave rise to a significant change in RCV ($P>0.10$). ** $P<0.01$; * $0.01<P<0.05$; NS, not significant.

DNP, dinitrophenol chloride.

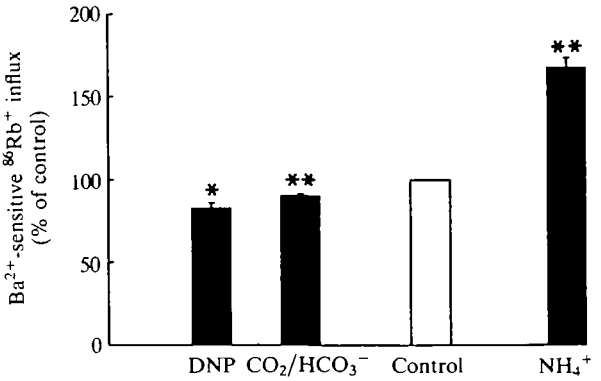

Fig. 7. Effect of NH_4^+, dinitrophenyl chloride (DNP) and HCO_3^-/CO_2 on Ba^{2+}-sensitive $^{86}Rb^+$ influx. Cells were suspended in a lamprey Ringer's solution containing 20 mmol l^{-1} Hepes (pH 7.6). NH_4^+ was added as an iso-osmotic NH_4Cl solution to give a final concentration of 20 mmol l^{-1}. HCO_3^-/CO_2 was added as an iso-osmotic $NaHCO_3$ solution (saturated with CO_2) to give a final HCO_3^- concentration of 20 mmol l^{-1}. Both the NH_4^+ and HCO_3^-/CO_2 solutions were added immediately before commencing the flux measurements. DNP was added as a dilute solution in lamprey Ringer (to give a final concentration of 20 μmol l^{-1}) at least 1 h before commencing the flux measurements so as to allow protons to equilibrate across the cell membrane (Nikinmaa, 1986). Ba^{2+}-sensitive $^{86}Rb^+$ influx is expressed as a percentage of that measured in controls in which the NH_4^+, HCO_3^-/CO_2 or DNP solutions were substituted with an equivalent volume of lamprey Ringer's solution (open bar). The normalised influx values were averaged from four separate experiments. Values are means+S.E.M. ** $P<0.01$; * $0.01<P<0.05$.

pulse) caused a 67±6 % ($N=4$, $P<0.01$) increase in influx *via* this pathway. Acidification of the cell interior by a HCO_3^-/CO_2 prepulse caused a 10±1 % ($N=4$, $P<0.01$) decrease in Ba^{2+}-sensitive $^{86}Rb^+$ uptake. In cells preincubated for 1 h with DNP (20 μmol l^{-1}), Ba^{2+}-sensitive $^{86}Rb^+$ uptake was reduced by 17±3 % ($N=4$, $P<0.05$).

Effect of intra- and extracellular Ca^{2+} on Ba^{2+}-sensitive $^{86}Rb^+$ influx

Fig. 8 shows the Ba^{2+}-sensitive $^{86}Rb^+$ uptake for cells collected, washed and resuspended in a Ca^{2+}-free solution containing EGTA. In four such experiments the Ba^{2+}-sensitive influx measured in cells suspended in a Ca^{2+}-free solution (in the absence of ionomycin) was 101±2 % of that measured in cells suspended in a solution containing 1 mmol l^{-1} Ca^{2+}. Addition of the Ca^{2+} ionophore ionomycin to cells suspended in a Ca^{2+}-free solution containing EGTA caused a substantial diminution ($P<0.10$) of the Ba^{2+}-sensitive $^{86}Rb^+$ uptake (Fig. 8).

Distribution of [^{14}C]TPP

On addition of the membrane potential probe [^{14}C]TPP to a suspension of lamprey erythrocytes pretreated with TPB ($[K^+]_e=5$ mmol l^{-1}), the extracellular

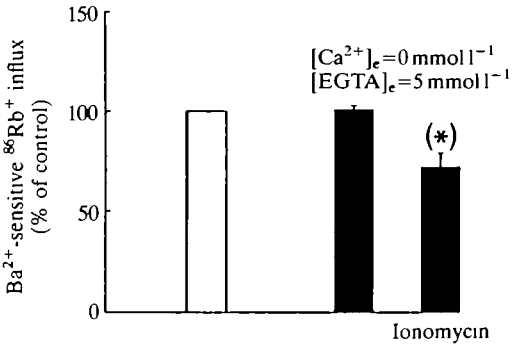

Fig. 8. Effect of EGTA and ionomycin on Ba^{2+}-sensitive $^{86}Rb^+$ influx. Cells were collected into heparinised saline ($[Ca^{2+}]$=0 mmol l^{-1}, [EGTA]=0.1 mmol l^{-1}), then washed five times in a lamprey Ringer's solution from which Ca^{2+} had been omitted. Samples of the suspension were dispensed into Eppendorf tubes to which either Ca^{2+} or EGTA (pH adjusted to 7.6) was then added to give final extracellular concentrations of 1 and 5 mmol l^{-1}, respectively. Where appropriate, ionomycin was added (to samples containing EGTA) to give a final concentration of 8 μmol l^{-1}. The samples were incubated for 10 min at 11 °C prior to commencing the flux measurements. Ba^{2+}-sensitive $^{86}Rb^+$ influx is expressed as a percentage of that measured for cells suspended in normal lamprey Ringer's solution ($[Ca^{2+}]$=1 mmol l^{-1}, open bar). The normalised influx values were averaged from at least four separate experiments. Values are mean+S.E.M. (*) $0.05<P<0.1$.

concentration of the radiolabel reached a steady state (indicating equilibration of the cation between the intra- and extracellular compartments) within 6 min (results not shown). Fig. 9A shows the effects of $[K^+]_e$ on [^{14}C]TPP distribution. As $[K^+]_e$ was increased (from 0.5 to 50 mmol l^{-1}) extracellular [^{14}C]TPP concentration (measured after a 6 min preincubation) increased dramatically. A similar increase in extracellular [^{14}C]TPP concentration followed the addition of Ba^{2+} to cells suspended in a low-K^+, high-Na^+ solution (Fig. 9B).

Discussion

Ouabain-sensitive $^{43}K^+$ and $^{86}Rb^+$ transport

The observation that ouabain caused a significant (and virtually identical) decrease in k^K and k^{Rb} (Fig. 1) is indicative of the presence in the lamprey erythrocyte membrane of the Na^+/K^+ pump known to be inhibited by ouabain and to carry $^{43}K^+$ and $^{86}Rb^+$ at similar rates (Bourne and Cossins, 1984). This finding accounts for the high $[K^+]_i$ and low $[Na^+]_i$ of these cells (Nikinmaa and Weber, 1984) but contrasts with the finding of Asai *et al.* (1976) that erythrocytes from a different lamprey species (*Entosphenus japonicus*) showed a complete absence of Na^+/K^+-ATPase activity. The magnitude of the ouabain-sensitive K^+ influx in the lamprey erythrocyte at 11 °C (2.2 mmol l^{-1} RBC h^{-1} at $[K^+]_e$= 5 mmol l^{-1}) is somewhat less than that in trout erythrocytes at 20 °C (8.6 mmol l^{-1} RBC h^{-1} at $[K^+]_e$=6 mmol l^{-1}; Bourne and Cossins, 1984) but is

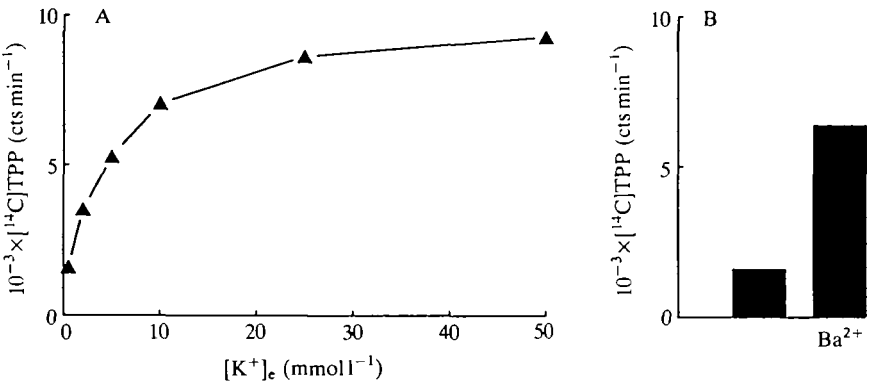

Fig. 9. (A) Effect of $[K^+]_e$ on $[^{14}C]TPP$ distribution across the lamprey erythrocyte membrane. $[K^+]_e$ was varied by replacing NaCl in the suspending solution with an equimolar concentration of KCl. (B) Effect of Ba^{2+} (2 mmol l^{-1}) on $[^{14}C]TPP$ distribution in cells suspended in a low-K^+ solution ($[K^+]_e$=0.5 mmol l^{-1}). In both graphs the ordinate indicates the amount of the radiolabel in the extracellular solution following a 6 min preincubation. The haematocrit was 2.7 %. The results shown are from a single experiment and are representative of those obtained in three similar experiments.

similar to that in guinea pig and human erythrocytes at 37°C (2.4 and 1.5 mmol l^{-1} RBC h^{-1}, respectively, at $[K^+]_e$=5 mmol l^{-1}; Hall and Willis, 1984; Ellory *et al.* 1987).

Cl^--dependent $^{86}Rb^+$ transport

In human erythrocytes there are thought to be two distinct Cl^--dependent K^+ transport pathways, the so-called $Na^+/K^+/Cl^-$ and K^+/Cl^- cotransport systems (Stewart and Ellory, 1989). $^{86}Rb^+$ transport through both systems is inhibited completely by replacement of Cl^- with NO_3^- in the suspending solution. However, the two systems may be distinguished from one another on the basis of their relative sensitivities to a number of different inhibitors (Stewart and Ellory, 1989). $^{86}Rb^+$ transport *via* the $Na^+/K^+/Cl^-$ cotransport system is inhibited almost completely by 0.1 mmol l^{-1} bumetanide (Stewart and Ellory, 1989) but is unaffected by 0.3 mmol l^{-1} H74 (Ellory *et al.* 1990). $^{86}Rb^+$ transport *via* the K^+/Cl^- cotransport system is reduced only slightly (approximately 20 %; Stewart and Ellory, 1989) by 0.1 mmol l^{-1} bumetanide but is reduced by approximately 80 % by 0.3 mmol l^{-1} H74 (Ellory *et al.* 1990). The two systems may be similarly differentiated on the basis of their Na^+-dependence; replacement of extracellular Na^+ with $NMDG^+$ causes a significant reduction in $^{86}Rb^+$ transport *via* the $Na^+/K^+/Cl^-$ cotransport system but has no effect on K^+/Cl^- cotransport (Stewart and Ellory, 1989).

In lamprey erythrocytes, replacement of extracellular Cl^- with NO_3^- reduced the ouabain-resistant $^{86}Rb^+$ influx by an average of 14 % (Table 1), as did H74 (0.3 mmol l^{-1}). Bumetanide (0.1 mmol l^{-1}) caused a much smaller (8 %) decrease

in the ouabain-resistant $^{86}Rb^+$ influx and the replacement of extracellular Na^+ with $NMDG^+$ had no significant effect. These observations are consistent with the hypothesis that a small fraction of the ouabain-resistant $^{86}Rb^+$ influx was *via* a K^+/Cl^- cotransport system (rather than a $Na^+/K^+/Cl^-$ cotransport system) similar to that shown to be present in the erythrocytes of a number of mammalian species.

Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ transport

Although a small fraction of $^{86}Rb^+$ influx into the lamprey erythrocytes may have been *via* a K^+/Cl^- cotransport system, it is clear from Fig. 1 and Tables 1 and 2 that most of the $^{43}K^+$ and $^{86}Rb^+$ influx was *via* a pathway with quite different characteristics. The pathway operated in the presence of the cotransport inhibitors bumetanide and H74, and in the absence of extracellular Na^+ or Cl^-. However, it could be inhibited *fully* by Ba^{2+}, amiloride or EIPA (as is evident from the observation in Fig. 1 that the flux of $^{43}K^+$ measured in the presence of Ba^{2+} or amiloride was no higher than that of $^{86}Rb^+$) and it was inhibited also (though less effectively) by quinine and TEA^+. The pathway showed a large (seven- to eightfold) preference for $^{43}K^+$ over $^{86}Rb^+$. $^{86}Rb^+$ is clearly not a valid congener for K^+ in this system and it is not possible to estimate the K^+ influx directly from k^{Rb} (using equation 2). Nevertheless, $^{86}Rb^+$ (which has a much longer half-life than $^{43}K^+$) did serve as a convenient tool with which to investigate the characteristics of the pathway.

Evidence for a Ba^{2+}-sensitive K^+ conductance pathway

$[^{14}C]TPP$ is a lipophilic cation and its (equilibrium) distribution between the intra- and extracellular solutions of a cell suspension should therefore reflect E_m. Hyperpolarisation of the membrane should lead to an increase in the intracellular concentration and a decrease in the extracellular concentration of the ion; depolarisation of the membrane should have the opposite effect.

The dramatic increase in extracellular $[^{14}C]TPP$ concentration that accompanied an increase in $[K^+]_e$ (Fig. 9A) implies that the lamprey erythrocyte membrane depolarised as $[K^+]_e$ was increased from 0.5 to 50 mmol l^{-1}. This suggests that the K^+ conductance of the membrane was sufficiently high for the transmembrane K^+ distribution to have contributed significantly to E_m. Furthermore, it suggests that the K^+ conductance of the membrane was higher than the Na^+ conductance; if the opposite were true then increasing $[K^+]_e$ at the expense of $[Na^+]_e$ should have hyperpolarised the membrane and led to a decrease in extracellular $[^{14}C]TPP$ concentration.

The increase in extracellular $[^{14}C]TPP$ concentration that followed the addition of Ba^{2+} to cells in a low-K^+ solution (Fig. 9B) indicates that, under these conditions, Ba^{2+} caused a depolarisation of the membrane. This is consistent with Ba^{2+} having inhibited the K^+ conductance pathway responsible for the dependence of E_m on $[K^+]_e$.

Dependence of Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ influx on $[K^+]_e$

The observed dependence on $[K^+]_e$ of Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ influx (Fig. 4) conformed to that expected for a simple, saturable carrier transporting $^{43}K^+$ and $^{86}Rb^+$ into the cell with similar K_m values but with a V_{max} for $^{43}K^+$ uptake some 8 times higher than that for $^{86}Rb^+$ uptake. However, the same result might have arisen if Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ transport were *via* a non-saturable conductive pathway. The depolarisation of the cell membrane that accompanied the increase in $[K^+]_e$ represents a decrease in the thermodynamic driving force for the conductive movement of cations into the cell. For univalent cations (such as K^+ and Rb^+) moving into the cell *via* a conductive pathway, a decrease in the driving force will cause the influx rate constants to decrease in direct proportion to one another (providing that the fluxes are under thermodynamic rather than kinetic control). Any such decrease in k^K and k^{Rb} will result in a proportional variation in the calculated K^+ influx rates (equation 2), thereby giving the *appearance* of a saturable system that carried the two cations with similar K_m but different V_{max} values. In view of the results of the [^{14}C]TPP experiments (which provide evidence for the presence in the membrane of a Ba^{2+}-sensitive K^+ conductance pathway), this latter explanation seems the more likely.

Dependence of Ba^{2+}-sensitive $^{86}Rb^+$ influx on cell volume

Osmotic shrinkage of lamprey erythrocytes caused a significant (40 %) increase in Ba^{2+}-sensitive $^{86}Rb^+$ influx; swelling of the cells caused a modest (13 %) decrease in the influx (Fig. 5). This effect may have been due either to a genuine activation of the Ba^{2+}-sensitive pathway with decreasing cell volume or to an increase in the driving force for $^{86}Rb^+$ influx. The concentrations of intracellular ions vary inversely with the cell volume and the increase in $[K^+]_i$ with cell shrinkage (at constant $[K^+]_e$) may have given rise to a significant hyperpolarisation with a consequent increase in the driving force for the conductive transport of $^{86}Rb^+$ into the cells. However, such an explanation assumes that the K^+ conductance of the membrane was greater than the Cl^- conductance, which may not have been the case.

Irrespective of the origin of the cell volume-dependence, the main significance of this result is that it shows that the Ba^{2+}-sensitive pathway was *not* activated by cell swelling. The pathway is, therefore, unlikely to be a primary regulatory volume decrease mechanism.

Dependence of Ba^{2+}-sensitive $^{86}Rb^+$ influx on pHi and pHe

Decreasing pHe from its physiological value of 7.6 to 6.6 caused pHi to decrease by 0.15 units (Table 3). Addition of HCO_3^-/CO_2 to the cells or preincubation of the cells with DNP (at pHe 7.6) caused a similar decrease in pHi. All of these treatments brought about a small (6–17 %) decrease in the Ba^{2+}-sensitive $^{86}Rb^+$ influx.

Increasing pHe from 7.6 to 8.4 caused pHi to increase by 0.11 units and resulted

in a 10% increase in the Ba^{2+}-sensitive $^{86}Rb^+$ influx. Addition of NH_4^+ caused pHi to increase by a similar amount (0.13 units) but resulted in a much greater (67%) increase in $^{86}Rb^+$ influx *via* the Ba^{2+}-sensitive pathway. As with the cell volume effects, it is uncertain whether the increase in Rb^+ influx brought about by the NH_4^+ prepulse reflects an increase in the activity of the $^{86}Rb^+$ pathway or an increase in the driving force (perhaps due to the activation or inhibition of another pathway). The results do suggest, however, that the response of the cells to an increase in pHi depends upon pHe. Neither treatment had a significant effect on the RCV (Table 3) and volume effects (of the sort depicted in Fig. 5) may, therefore, be discounted. One possibility is that the activity of the Ba^{2+}-sensitive pathway is regulated by the pH gradient across the cell membrane – specifically, that the activity is increased in response to an inwardly directed proton gradient. It has recently been shown in human placental brush-border membrane vesicles that there is a (Ba^{2+}-sensitive) K^+ conductance that is markedly stimulated in the presence of an inwardly directed proton gradient (Vatish and Boyd, 1991). In the context of lamprey erythrocytes it is interesting to note that erythrocytes from lampreys exposed to hypoxic conditions show a large inward pH gradient (of 0.15 units; Nikinmaa and Weber, 1984). This may act to increase the activity of the Ba^{2+}-sensitive leak pathway and this may, in turn, be responsible for the pronounced decrease in the intracellular K^+ concentration following hypoxic exposure (Nikinmaa and Weber, 1984).

Dependence of Ba^{2+}-sensitive $^{86}Rb^+$ influx on Ca^{2+}

The Ba^{2+}-sensitive pathway operated normally in the complete absence of extracellular Ca^{2+}. However, $^{86}Rb^+$ influx *via* the pathway decreased when cells were pretreated with ionomycin and EGTA (Fig. 8). The combination of ionomycin and EGTA might be expected to have decreased the intracellular Ca^{2+} content. The observed decrease in $^{86}Rb^+$ uptake might, therefore, suggest a role for intracellular Ca^{2+} in the operation of the pathway.

The mode of action of amiloride

The inhibition of $^{86}Rb^+$ influx by Ba^{2+} occurred within the time required to make a single flux measurement (i.e. within a few minutes). In contrast, the inhibitory effect of amiloride developed (in a biphasic manner) over a period of more than 1 h (Fig. 3). If the amiloride acted at an intracellular site it is possible that the time-dependence of the inhibition reflected the time-dependence of the accumulation of the drug inside the cell. Alternatively (or additionally), the effect might be explained by amiloride having acted by an indirect mechanism rather than on the transport pathway itself. Amiloride is known to inhibit Na^+/H^+ exchange, Na^+ channels, Na^+/Ca^{2+} exchange and the Na^+/K^+ pump (Frelin *et al.* 1987) as well as adenylate cyclase (Mahé *et al.* 1985) and various protein kinases (Holland *et al.* 1983; Besterman *et al.* 1985). In frog kidney, amiloride has been shown to decrease K^+ conductance indirectly by inhibiting Na^+/H^+ exchange and thereby inducing a decrease in pHi (Oberleithner *et al.* 1986). The

lamprey erythrocyte is known to have an active Na^+/H^+ exchange system (Nikinmaa, 1986; Nikinmaa *et al.* 1986); however, as is evident from Table 3 (and as has been shown previously by Nikinmaa *et al.* 1986), amiloride had no significant effect on the pHi of lamprey erythrocytes suspended in a nominally HCO_3^--free solution. In contrast, the replacement of extracellular Na^+ (Nikinmaa *et al.* 1986), preincubation of the cells with DNP (Table 3 and Nikinmaa, 1986) or the addition of HCO_3^-/CO_2 (Table 3 and Nikinmaa *et al.* 1986) all caused a significant decrease in pHi yet produced only a slight reduction in $^{86}Rb^+$ transport *via* the Ba^{2+}- and amiloride-sensitive pathway (Table 2 and Fig. 7). It therefore seems unlikely that the inhibitory effect of amiloride was mediated by an effect on pHi.

An alternative explanation is that the maintenance of the Ba^{2+}- and amiloride-sensitive pathway in its operational state is dependent upon the activity of adenylate cyclase and/or a protein kinase. In trout erythrocytes, amiloride inhibits adenylate cyclase with an I_{50} of 6 μmol l^{-1} (Mahé *et al.* 1985), less than the I_{50} for the inhibition of $^{86}Rb^+$ uptake obtained here (approximately 150 μmol l^{-1}, Fig. 2B). Amiloride is a less potent inhibitor of various protein kinases, including cyclic AMP-dependent protein kinase (which is 60% inhibited by 100 μmol l^{-1} amiloride; Holland *et al.* 1983) and protein kinase C (which is inhibited with an I_{50} of approximately 1 mmol l^{-1}; Besterman *et al.* 1985). The I_{50} for the inhibition of protein kinase C by amiloride is somewhat higher than that obtained for the uptake of $^{86}Rb^+$ into lamprey erythrocytes, though it should be noted that the negative E_m is likely to have led to the accumulation of amiloride (a cation) within the cell, with the result that the *intracellular* concentrations of the drug (and therefore the I_{50}) may have been substantially higher than those indicated in Fig. 2B. A role for protein kinase C in maintaining the pathway in its active form might also account for the apparent dependence of Ba^{2+}-sensitive $^{86}Rb^+$ influx on intracellular Ca^{2+} (Fig. 8).

The possible identity of the Ba^{2+}-sensitive K^+ pathway

As has been discussed, the experiments with the membrane potential probe [^{14}C]TPP provided evidence for the presence in the lamprey erythrocyte membrane of a Ba^{2+}-sensitive K^+ conductance pathway. The observed $[K^+]_e$-dependence of Ba^{2+}-sensitive $^{43}K^+$ and $^{86}Rb^+$ influx was consistent with the Ba^{2+}-sensitive transport having been mediated by such a pathway. Ba^{2+}, quinine and TEA^+ are all known to block Ca^{2+}-activated K^+ channels and it therefore seems likely that a channel of this sort was responsible for the Ba^{2+}-sensitive K^+ transport in these cells. Erythrocytes from a wide range of species (mammal, bird, amphibian and fish) have previously been shown to have present in their membrane a Ca^{2+}-activated K^+ channel (the so-called Gardos channel) (Lew and Ferreira, 1978). However, the Ba^{2+}-sensitive pathway in the lamprey erythrocyte membrane showed a number of fundamental differences from the Ca^{2+}-activated K^+ channels of the erythrocytes from higher vertebrates. First, the Ca^{2+}-activated K^+ channels of the higher vertebrate erythrocytes are generally quiescent under

normal conditions and are only activated by an increase in intracellular [Ca^{2+}], usually in response to an influx of Ca^{2+} from the extracellular solution. The Ba^{2+}-sensitive pathway in the lamprey erythrocyte was apparently active under normal conditions and its activity was not dependent upon the presence of Ca^{2+} in the extracellular solution (Fig. 8). Second, the Gardos channel of human cells is thought to have a slight (1.5-fold) preference for Rb^+ over K^+ (Simons, 1976). The lamprey pathway showed a marked preference for K^+ over Rb^+ (Fig. 1). Third, there is a marked difference in the sensitivities of the pathways to the different inhibitors studied. In experiments with human erythrocytes, $^{86}Rb^+$ influx *via* the Gardos channel (in cells pretreated with a Ca^{2+} ionophore) was almost completely (>99 %) inhibited by 0.3 mmol l^{-1} quinine; Ba^{2+} (1.8 mmol l^{-1}) was rather less effective and brought about only a 90 % decrease in $^{86}Rb^+$ influx, while amiloride (2 mmol l^{-1}) had no significant effect. The pathway of interest in the lamprey erythrocyte membrane was only partially (28 %) inhibited by 0.5 mmol l^{-1} quinine (Table 2) but was inhibited completely by Ba^{2+} at less than 1 mmol l^{-1} and by 2 mmol l^{-1} amiloride (Figs 1 and 2).

It is clear that if Ba^{2+}-sensitive K^+ transport across the lamprey erythrocyte membrane is *via* a Ca^{2+}-activated K^+ channel, the pathway is quite dissimilar from the Gardos channel. It bears a closer resemblance (particularly in terms of its selectivity for K^+ over Rb^+) to the high-conductance ('maxi-K^+') channels that have been described previously in many cell types in higher vertebrate species (Latorre *et al.* 1989). However, such channels have not previously been found in erythrocytes, and they have not been shown to be susceptible to inhibition by amiloride (or its derivative, EIPA).

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