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Characterization of a Na^+-K^+-$2Cl^-$ cotransport system in oocytes from *Xenopus laevis*

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In order to characterize the transport systems mediating K^+ uptake into oocytes, flux studies employing ^{86}Rb were performed on *Xenopus* oocytes stripped of follicular cells by pretreatment with Ca^{2+}-Mg^{2+}-free Barth's medium. Total Rb^+ uptake consisted of an ouabain-sensitive and an ouabain-insensitive flux. In the presence of 100 mmol/l NaCl and 0.1 mmol/l ouabain the ouabain-insensitive flux amounted to 754.7 ± 59.9 pmol/oocyte per h ($n = 30$ cells, i.e., 10 cells each from three different animals). In the absence of Na^+ (Na^+ substituted by *N*-methylglucamine) or when Cl^- was replaced by NO_3^- the ouabain-insensitive flux was reduced to 84.4 ± 42.9 and 79.2 ± 12.1 pmol/oocyte per h, respectively ($n = 50$ cells). Furthermore, this Na^+- and Cl^--dependent flux was completely inhibited by 10^{-4} mol/l bumetanide, a specific inhibitor of the Na^+-K^+-$2Cl^-$ cotransport system. These results suggest that K^+ uptake via a bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport system represents a major K^+ pathway in oocytes.

Introduction

The oocyte of *Xenopus laevis* represents a powerful tool for studying many of the functions of a variety of cells through the micro-injection of foreign mRNAs and subsequent expression of these mRNAs as functional proteins [1–8]. It is, however, also important to look at the oocytes themselves to understand better which endogenous systems the cells have. To this end much work has been done involving transport systems for amino acids [9–11], ions, particularly channels [2,12–16], D-glucose [17] as well as cellular receptors [2]. The oocyte Na^+/K^+-ATPase has been studied in terms of its electrophysiology [15] and in terms of ouabain-sensitive isotopic cation (e.g. $^{86}Rb^+$) fluxes [18]. With regard to the latter experiments it was noted by Richter et al. [18] that a portion of the $^{86}Rb^+$ flux was insensitive to ouabain pointing to mechanisms other than the Na^+/K^+-ATPase responsible for this uptake. Chipperfield and Fry [19] noted an ouabain-insensitive but furosemide-sensitive $^{42}K^+$ flux in the immature oocytes of the toad *Bufo bufo* suggesting the possible participation of a Na^+-K^+-Cl^- cotransport system in the K^+ uptake process. In the present study it was found that the ouabain-insensitive Rb^+ uptake was dependent on Na^+ as well as Cl^-, bumetanide inhibitable and had a stoichiometry of $1Rb^+ : 1Na^+ : 2Cl^-$. These properties of the Rb^+ flux strongly suggest the operation of a Na^+-K^+-$2Cl^-$ cotransport system in addition to the Na^+/K^+-ATPase. The two systems together account for almost all of the $^{86}Rb^+$ uptake in the oocytes used in these experiments.

Materials and Methods

Oocytes, stages V and VI according to Dumont [20], were removed from anesthetized animals (exposed for 20 min to 0.15% MS-222 in fresh water) through an abdominal incision and immediately placed in Barth's medium ((in mmol/l) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 5 Hepes, pH 7.6 with Tris). The cells were washed and separated into single cells in the same medium. After a period of 1–2 h the follicular cells were removed by incubating the oocytes in Ca^{2+}-Mg^{2+}-free Barth's medium for 30–60 min at 18°C [21] followed by removal to a fresh aliquot of normal Barth's medium. The denuded oocytes were stored in Barth's medium at 18°C overnight before use in uptake studies.

Measurement of $^{86}Rb^+$ uptake was carried out in the following manner. Ten oocytes were placed in a vial containing 1 ml of the uptake buffer (see figure legends

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for the exact composition of the buffers) which was then allowed to equilibrate to room temperature (20–22°C). In some experiments Na^+ and Cl^- were replaced isosmotically by *N*-methylglucamine (NMG) and nitrate, respectively. Kinetic experiments were carried out in the same medium with varying ratios of Na^+/NMG for the Na^+-dependent kinetics and varying ratios of Cl^-/NO_3^- for the Cl^--dependent kinetics of Rb^+ (K^+) uptake. Both K^+ and Rb^+ were also tested for the ability to inhibit the uptake of $^{86}Rb^+$, the adjustments to the incubation medium for these experiments are noted in the corresponding figure legends. The inhibition constant K_i for bumetanide was also determined by incubating cells for 1 h in media containing varying concentrations of this drug.

The transport reaction was initiated by adding 10 μCi $^{86}Rb^+$ (Amersham, specific activity 1–8 mCi/mg) to each vial followed by gentle mixing. The cells were incubated in this medium for the required time interval after which they were quickly washed three times with 3 ml ice-cold stop solution containing *N*-methylglucamine chloride or $NaNO_3$ instead of NaCl. Each cell was placed in a scintillation vial and 200 μl 20% SDS were added to each followed by at least a 2-h period to dissolve the cell and release any contained radioactivity. 7 ml of Beckman Ready Protein scintillation fluid were added and the samples were counted in a Beckman liquid scintillation counter. Bumetanide (10^{-4} mol/l, Hoechst), added to the above described media, was tested for the ability to inhibit $^{86}Rb^+$ uptake.

Uptake values were expressed as pmol/oocyte with a 5-μl aliquot of the radioactive uptake buffer plus 200 μl 20% SDS serving as the standard. Blank values were determined by counting 200 μl 20% SDS in 7 ml scintillation fluid (without oocytes) and were subtracted from the data to correct for background. Student's *t*-test was used to measure significance of difference where appropriate, and *P* values of less than or equal to 0.05 were considered significant. The animals used in these experiments were obtained either from Drs. W. De Rover (Turnhout, Belgium) or Kähler (Hamburg, F.R.G.)

Results

First the effect of 0.1 mmol/l ouabain, the inhibitor of the Na^+/K^+-ATPase, and bumetanide (10^{-4} mol/l), an inhibitor of Na^+-K^+-$2Cl^-$ cotransport [22–24], on rubidium uptake after 1 h was investigated (Table I). Ouabain alone inhibited $^{86}Rb^+$ uptake by about 70%, i.e. about 30% of the uptake was insensitive to ouabain thus indicating the participation of an ouabain-insensitive component in addition to Na^+/K^+-ATPase in the overall uptake process. This finding is in agreement with Chipperfield and Fry [19] who worked with $^{42}K^+$ fluxes and Richter et al. [18] who have shown a ouabain-insensitive $^{86}Rb^+$ flux in *Xenopus* oocytes. When bumetanide was included in the uptake medium (without ouabain) $^{86}Rb^+$ uptake was inhibited by about 30% (i.e., about 70% of the control uptake remained). When both ouabain and bumetanide were present the uptake of $^{86}Rb^+$ was inhibited by approximately 98% when compared to the complete absence of inhibitors. Additionally, when one calculates the bumetanide-sensitive portion of uptake both in the presence and absence of ouabain there is no statistically significant difference between the two values (Table I).

TABLE I

Effect of ouabain and bumetanide on 1-h Rb^+ uptake in Xenopus oocytes

$N = 30$ cells per mean value.

	pmol Rb/oocyte per h (mean ± S.E.)	Percent of control uptake (mean ± S.E.)
Control		
no ouabain		
no bumetanide	2602.0 ± 270.3	100
0.1 mmol/l		
bumetanide	1848.2 ± 256.4	70.7 ± 3.2 [a]
0.1 mmol/l		
ouabain	754.7 ± 59.9	29.4 ± 3.7 [a]
Bumetanide		
and ouabain		
(both 0.1 mmol/l)	48.3 ± 6.5	1.9 ± 0.1
Bumetanide-sensitive		
(no ouabain)	753.8 ± 54.5	29.3 ± 3.2 [b]
Bumetanide-sensitive		
(with ouabain)	706.3 ± 56.2	27.6 ± 3.7 [b]

[a] Significant difference as compared to control value.
[b] Bumetanide-sensitive fluxes are not significantly different from one another.

In the presence of 100 mmol/l NaCl and 0.1 mmol/l ouabain the uptake of Rb^+ was found to be linear up to 2 h. Bumetanide (10^{-4} mol/l) inhibited the uptake by about 93% after 30 min, 96% after 60 min and 96% after 120 min again indicating a transport sensitive to this inhibitor. In order to determine whether this bumetanide-sensitive transport represents the Na^+-K^+-$2Cl^-$ cotransporter the effect of replacement of Na^+ and Cl^-, with *N*-methylglucamine and nitrate respectively, was investigated. Removal of Na^+ resulted in a decrease in uptake which was roughly equivalent to the inhibition by bumetanide (Fig. 1). When Cl^- was replaced by nitrate a strong reduction in transport of approximately the same magnitude was observed (Fig. 1).

The kinetics of the Na^+-dependent Rb^+ (K^+) uptake mechanism were investigated by incubating cells for 1 h in media containing varying Na^+ concentrations (isosmotic replacement with *N*-methylglucamine). After the

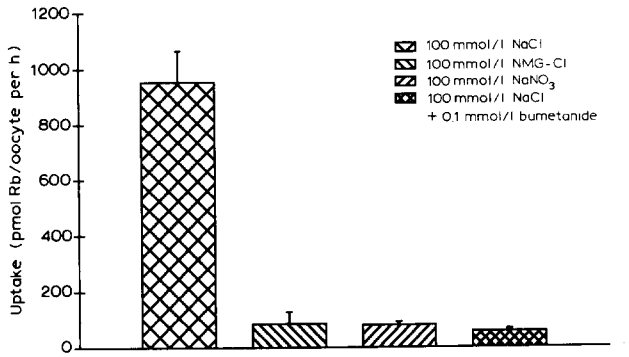

Fig. 1. One hour rubidium uptake from media containing (in mmol/l) 100 NaCl, 2.4 $KHCO_3$, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 5 Hepes, 0.1 ouabain and 10 μCi $^{86}Rb^+$/ml (pH 7.6). For Na^+-free experiments all components remained the same except that NaCl was replaced by 100 mmol/l *N*-methylglucamine chloride (NMG-Cl). For Cl^--free experiments NaCl was replaced by 100 mmol/l $NaNO_3$, $MgCl_2$ and $CaCl_2$ were replaced by $Mg(NO_3)_2$ and $Ca(NO_3)_2$, respectively. For experiments with bumetanide the medium was as for NaCl plus 10^{-4} M bumetanide. Each bar represents the mean ± S.E. of 40 cells, i.e., 10 cells each from four different animals.

incubation time the cells were treated as previously described. Fig. 2 presents the results of experiments with the cells from four animals (10 cells/point). The uptake of Rb^+ appears to follow simple saturation kinetics. When plotted in reciprocal form (not shown) the data indicated an apparent K_m and V_{max} of 39.9 mmol/l Na^+ and 913.8 pmol Rb/oocyte per h, respectively. Fig. 2 also shows analysis of the data by the following Hill-type equation taken from Turner [25]: $V = V_{max} \cdot [A^n]/(K_{0.5}^n + [A^n])$. Using the kinetic parameters derived from the Lineweaver-Burk plot it is possible to calculate the theoretical Na^+ concentration dependence of Rb^+ uptake for different stoichiometries. At all Na^+ concentrations the experimental data correpond best to the theoretical calculation of stoichiometry of $1Na^+ : 1Rb^+(K^+)$. A Hill plot of the data yielded a straight line ($r^2 = 0.971$) with a slope of 0.93 ± 0.12 (not shown) further indicating a minimum stoichiometry of 1 : 1.

The kinetics of Cl^--dependent Rb^+ (K^+) uptake were also examined in the same manner as the Na^+ kinetics (Fig. 3). The Cl^- concentration was altered by partial replacement with NO_3^-. The sigmoid shape of the curve indicates a stoichiometry greater than 1 : 1 and indeed when these data are plotted according to the Eadie-Hofstee plot (not shown) it is apparent that a stoichiometry of 1 : 1 is not sufficient to account for the observed results ($r^2 = 0.497$). Assuming a ratio of 2 : 1, however, yielded a straight line ($r^2 = 0.997$). When the kinetic parameters from the Eadie-Hofstee plot, apparent K_m and V_{max} 47.2 mmol/l Cl^- and 961.6 pmol Rb/oocyte per h, respectively, are used in the Hill-type equation (see above) and theoretical Cl^--dependent Rb^+ uptakes are calculated the experimental data best fit the theoretical calculation described by a $2Cl^- : 1Rb^+(K^+)$

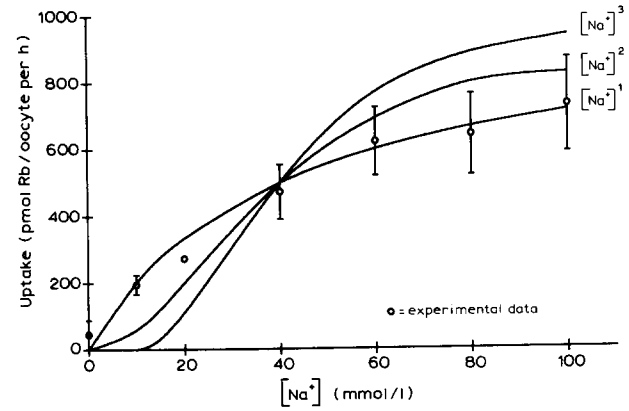

Fig. 2. The Na^+ concentration dependence of bumetanide-sensitive rubidium uptake. The incubation medium (in mmol/l, 2.4 $KHCO_3$, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 5 Hepes, 0.1 ouabain and 10 μCi $^{86}Rb^+$/ml (pH 7.6)) contained varying ratios of NMG-Cl/NaCl to provide 100 mmol/l Cl^- and the concentrations of Na^+ tested. The incubation period was 1 h. Uptake in the presence of bumetanide (10^{-4} M) was subtracted from the data to provide the bumetanide-sensitive uptake. Each point represents the mean ± S.E. of determinations made with 40 cells, i.e., 10 cells each from four different animals. The curves represent the theoretical calculation of cotransport stoichiometry according to a Hill-type equation with kinetic parameters derived from a Lineweaver-Burk plot. The experimental data (open circles) fit best to the theoretical calculation of $1Na^+ : 1Rb^+$. The equation used was: $V = V_{max} \cdot [A^n]/(K_{0.5}^n + [A^n])$ where A is the activator (in this case Na^+) and n represents the stoichiometry.

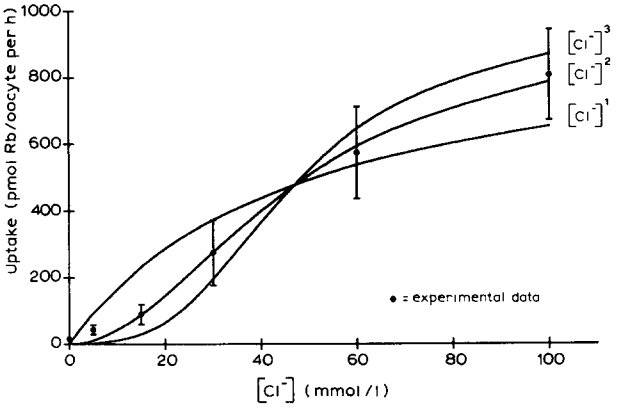

Fig. 3. The Cl^- concentration dependence of bumetanide-sensitive rubidium uptake over 1 h. The incubation medium (in mmol/l, 2.4 $KHCO_3$, 0.82 $Mg(NO_3)_2$, 0.74 $Ca(NO_3)_2$, 5 Hepes, 0.1 ouabain and 10 μCi $^{86}Rb^+$/ml (pH 7.6)) contained varying ratios of $NaNO_3$/NaCl to provide 100 mmol/l Na^+ and the concentrations of Cl^- tested. Uptake in the presence of bumetanide (10^{-4} M) was subtracted from the data to provide the bumetanide-sensitive uptake. Each point represents the mean ± S.E. of determinations made with 50 cells, i.e., 10 cells each from five different animals. The curves represent the theoretical calculations of cotransport stoichiometry according to a Hill-type equation with kinetic parameters derived from an Eadie-Hofstee plot. The experimental data (closed circles) fit best to the theoretical calculation of $2Cl^- : 1Rb^+$. The equation used was: $V = V_{max} \cdot [A^n]/(K_{0.5}^n + [A^n])$ where A is the activator (in this case Cl^-) and n represents the stoichiometry.

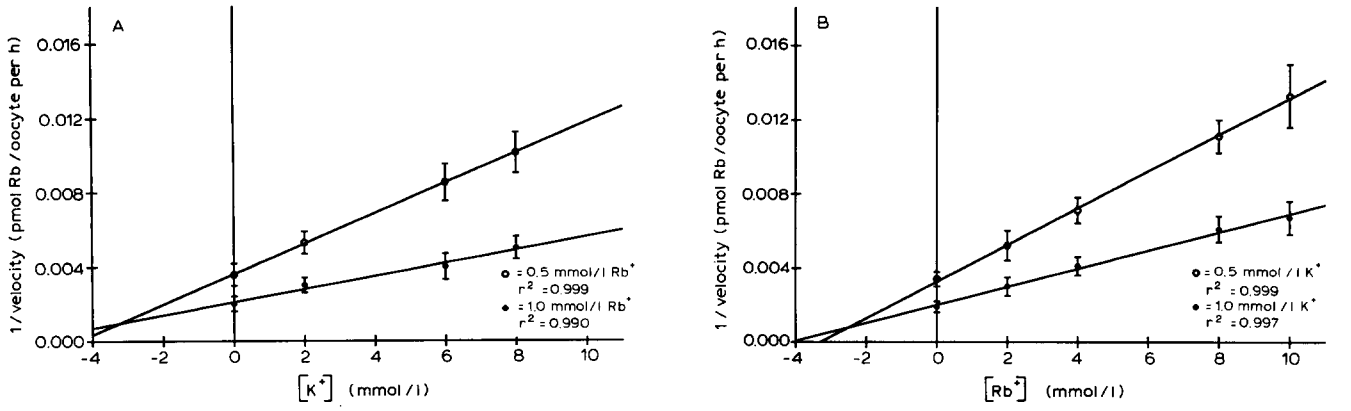

Fig. 4. (A) Dixon plot analysis of K^+ inhibition of rubidium uptake. The incubation medium contained in mmol/l, 100 NaCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 5 Hepes, 0.1 ouabain and 10 μCi $^{86}Rb^+$/ml. Non-isotopic $RbNO_3$ was either 0.5 mmol/l (upper curve) or 1.0 mmol/l (lower curve). $KHCO_3$ (0, 2, 6 and 8 mmol/l) was used to alter the K^+ concentration and the pH of each buffer was adjusted to 7.6. The K_i was found to be 3.1 ± 0.3 mmol/l K^+. (B) Dixon plot analysis of Rb^+ inhibition of rubidium uptake. The incubation medium contained in mmol/l, 100 NaCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 5 Hepes, 0.1 ouabain and 10 μCi $^{86}Rb^+$/ml. [K^+], provided as $KHCO_3$, was either 0.5 mmol/l (upper curve) or 1.0 mmol/l (lower curve) and the pH of each buffer was adjusted to 7.6. $RbNO_3$ (0, 2, 4, 8 and 10 mmol/l) was used to alter the Rb^+ concentration. The K_i was found to be 2.6 ± 0.3 mmol/l Rb^+.

stoichiometry (shown also in Fig. 3). Hill plot analysis of the data (not shown) yielded a straight line ($r^2 = 0.997$) with a slope of 2.17 ± 0.12.

Inhibition of $^{86}Rb^+$ uptake by K^+ and Rb^+ was also investigated and the results are presented in Fig. 4. In both cases the $1/V$ was plotted against the varied inhibitor ion concentration (K^+ or Rb^+) while holding K^+ or Rb^+ concentration constant at either 0.5 mmol/l or 1.0 mmol/l. The calculated regression lines intersect above the X-axis indicating competitive inhibition. Moreover, the $1/V$ values best fit to the inhibitor concentration to the first power demonstrating a minimum 1 : 1 stoichiometry for this interaction. The K_i values for K^+ and Rb^+ taken from the figures are 3.1 ± 0.3 mmol/l K^+ and 2.6 ± 0.3 mmol/l Rb^+ and are not significantly different from each other.

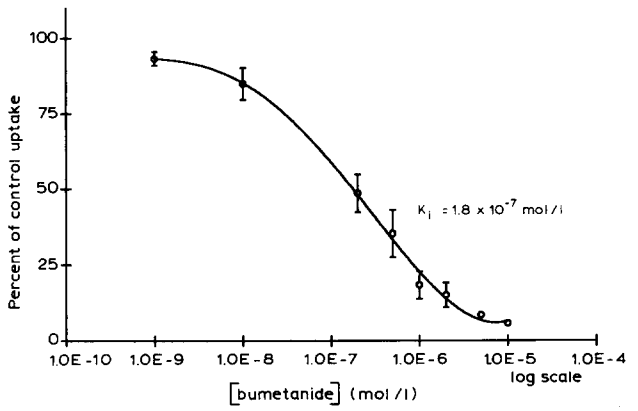

Fig. 5. Bumetanide dose-response curve plotted on a log scale. The incubation medium was as described for uptake in the presence of 100 mmol/l NaCl. Ouabain concentration was 0.1 mmol/l and bumetanide concentration was varied as indicated. The inhibition constant calculated from the data was $1.8 \cdot 10^{-7}$ mol/l bumetanide.

Bumetanide, in a concentration range from 10^{-9} to 10^{-5} mol/l, revealed a dose-dependent inhibition of 1 h $^{86}Rb^+$ uptake (Fig. 5). The concentration of bumetanide producing half-maximal inhibition was calculated to be $1.8 \cdot 10^{-7}$ mol/l. Additional experiments with other known inhibitors of the Na^+-K^+-$2Cl^-$ cotransporter, namely furosemide and piretanide (data not shown), suggested the following order of inhibition: bumetanide > piretanide > furosemide. This order of inhibition has been reported for the Na^+-K^+-$2Cl^-$ cotransporter of flounder intestine [26] and also for avian erythrocytes [27].

Discussion

The inability of ouabain alone to completely block Rb^+ flux in the oocytes leads to the conclusion that other systems participate in this process. Indeed, when bumetanide (an inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter) and ouabain are present together in the incubation medium a further, and almost complete, inhibition of Rb^+ uptake is observed. These observations seem to indicate the participation of two major pathways for Rb^+ uptake in oocytes: (1) a Na^+/K^+-ATPase and; (2) a bumetanide-sensitive pathway. Ion replacement studies aimed at exploring the latter uptake mechanism pointed to a Na^+- as well as Cl^--dependent mechanism as being responsible for the bumetanide-sensitive flux of Rb^+. This evidence, though not directly demonstrating coupling between the ion fluxes, lends support to the notion of the presence of Na^+-K^+-Cl^- cotransporter in the oocytes.

The apparent lack of potassium channels is not surprising in light of previous reports in the literature. Parker and Miledi [28] reported transient outward potassium currents in *Xenopus* oocytes which were ob-

served only when the oocyte membrane was depolarized using microelectrodes. Further, this potassium current was abolished by the removal of the follicle cell layer which surrounds the oocyte. It would thus appear that potassium channels are indeed present in the oocyte but require special treatment to become active.

The Na^+ concentration dependence of Rb^+ uptake demonstrates a clear one-to-one relationship as has been observed for the cotransporter of other cells [29–31]. The apparent K_m for sodium (39 mmol/l in the presence of 100 mmol/l Cl^- and 2.4 mmol/l K^+) observed in the present study is somewhat higher than that observed for other systems. Brown and Murer [31] who investigated the renal epithelial cell line LLC-PK_1, observed an apparent K_m for sodium of 0.42 mmol/l or a factor of 100 lower than observed in the oocytes. Koenig et al. [32] reported an apparent K_m of 1.3 mmol/l Na^+ in TALH membrane vesicles. Aiton et al. [33] demonstrated an apparent K_m for sodium of 25 mmol/l in HeLa cells and a value of 35 mmol/l has been reported for human red blood cells by Chipperfield et al. [34,35]. The last two values may be most comparable to the present data because in both cases $^{86}Rb^+$ uptake was measured as a function of the Na^+ concentration. The differences between the present data and those of Brown and Murer [31] and Koenig et al. [32] most likely represents differences in experimental design specifically concerning the magnitude of the ion gradient employed to stimulate uptake and differences in the cell systems studied. The V_{max} value (914 pmol Rb/oocyte per h) is difficult to compare because of the extreme difference in oocyte size compared to the size of other cells and membrane vesicles which have been shown to possess such a cotransport system. Preparation of membrane vesicles from the oocytes, if possible, might indeed yield a different result than is observed in the intact cell.

The apparent $K_{0.5}$ observed for Cl^- concentration dependent bumetanide-sensitive Rb^+ uptake (47 mmol/l in the presence of 100 mmol/l Na^+ and 2.4 mmol/l K^+) in the present study compares more favorably with the data obtained from other systems. Greger et al. [36] reported an apparent $K_{0.5}$ for Cl^- of 49 mmol/l in isolated perfused tubules from rabbit kidney cortex using electrophysiological methods. The same value was obtained by Saier and Boyden [37] for MDCK cells using $^{86}Rb^+$ uptake stimulated by a varying Cl^- concentration. Other $K_{0.5}$ values reported in the literature range from 15.3 mmol/l in TALH membrane vesicles [31] to 100 mmol/l in HeLa cells [33]. The value reported here for *Xenopus* oocytes fits comfortably within this range. As with the K_m for Na^+ the differences observed in apparent Cl^- affinities are most likely due to experimental design as well as cell system differences. The V_{max} for the Na^+ and the Cl^- concentration dependence of Rb^+ uptake (914 pmol Rb/oocyte per h and 962 pmol Rb/oocyte per h, respectively) are not significantly different from one another. This observation would be expected since in the range of V_{max} the NaCl concentration for both kinetics experiments is 100 mmol/l.

Nitrate has been shown to be accepted and translocated by the rabbit kidney medulla cotransporter in the presence of residual chloride [38] and thus could create problems for the determination of $K_{0.5}$ if nitrate is used to partially replace chloride. Brown and Murer [31], who worked with LLC-PK_1 cells, and Silva et al. [39], who worked with isolated perfused shark rectal gland, demonstrated a shift in coupling ratio from 2 : 1 when gluconate was used to a ratio of 1 : 1 when nitrate was used to replace chloride. Although no data are presented here with regard to gluconate replacement the results clearly show a coupling ratio of 2 : 1 when nitrate is used to replace chloride. Partial anion replacement experiments with the oocytes have thus far indicated no stimulation by nitrate at low chloride (up to 50 mmol/l) concentrations (data not shown). This observation taken together with the evidence showing a sigmoid relationship between rubidium uptake and chloride concentration and the theoretical calculations for different coupling ratios (Fig. 3) seems to justify the use of nitrate as a chloride substitute for measuring the kinetics of the oocyte Na^+-K^+-$2Cl^-$ cotransporter. The observed difference in nitrate interaction with the cotransporter may point to a difference in anion specificity between the oocyte system and the systems of the rabbit renal medulla [38] and the LLC-PK_1 cells [31].

The inhibition of $^{86}Rb^+$ uptake by both K^+ and Rb^+ clearly demonstrates the competitive interaction of 1 Rb^+ or 1 K^+ with the cotransporter and is consistent with the idea of 1 Rb^+ or 1 K^+ being cotransported. The apparent affinity (K_i) for rubidium, 2.6 ± 0.3 mmol/l and that for potassium, 3.1 ± 0.3 mmol/l (both measured at 100 mmol/l NaCl) were not significantly different from each other indicating the ability of Rb^+ to effectively replace K^+ in the cotransport process. Some differences between the present values and those reported in the literature should be noted. Koenig et al. [32] reported a K_m for K^+ of 22.3 mmol/l in TALH membrane vesicles. These experiments were carried out at lower-than-saturating (0.5 mmol/l) concentrations of Na^+. Kinne et al. [40] later reported a K_m of less than 1 mmol/l for the same vesicle system when the Na^+ concentration was raised to 100 mmol/l to match the Cl^- concentration. Aiton et al. [33] reported an apparent affinity of 1.0 mmol/l K^+ in HeLa cells (also determined at high concentrations of NaCl, 137 mmol/l) and Brown and Murer [31] observed an apparent K_m of 11.9 mmol/l K^+ measured at 100 mmol/l NaCl. Although some apparent cell type differences exist the values presented here for the oocytes fall within the range reported for other cell types.

It is at this point perhaps pertinent to compare the affinity constants discussed above to those reported for a $K^+:Cl^-$ cotransport mechanism. The K_m values typically reported for $K^+(Rb^+)$ in a $K^+:Cl^-$ cotransport (in Na^+-free medium) are 4–8-times higher (12.4 mmol/l [41] and an average of 25 mmol/l [42]) than those found in the current study for the Na^+-K^+-$2Cl^-$ cotransport supporting the conclusion that it is in fact the Na^+-K^+-$2Cl^-$ cotransporter we are measuring and not a $K^+:Cl^-$ cotransport. Duhm [41] also reports a K_m for Rb^+ in 100 mmol/l Na^+ medium of 2.9 mmol/l which is not different from the 3 mmol/l found in 100 mmol/l Na^+ medium in the present study. Since uptake of Rb^+ was minimal in the absence of Na^+ (Fig. 1) we conclude that Na^+ is necessary for the major part of the Rb^+ to occur. This finding is in contrast to a $K^+:Cl^-$ cotransport which would not require Na^+ for the transport process.

The action of bumetanide on Rb^+ uptake revealed a dose-dependent inhibition as has been seen in other cells and systems [43–46]. Bumetanide at a concentration of $1.8 \cdot 10^{-7}$ mol/l produced half-maximal inhibition of Rb^+ uptake. The K_i found for bumetanide in the present study (0.18 μM) compares well to that found for other cell types. Amsler and Kinne [43] reported a K_i of 1.13 μM in LLC-PK_1/Cl_4 cells and Owen and Prastein [44] reported a K_i of 0.1 μM for cultured fibroblasts (at 150 mmol/l NaCl). Haas and McManus [45] working with duck red cells demonstrated a K_i at 100 mM Cl^- of 0.176 μM which is perhaps the best comparison to the present study because the measurement was also performed at 100 mM Cl^-. Thus, the oocyte cotransporter seems to be quite similar to the cotransporter of other cells as far as sensitivity to bumetanide is concerned.

The apparent slow rate of transport is not surprising when one considers the size of the oocytes. The cells used in these transport studies range in diameter from 1 to 1.4 mm and calculations of volume yield an average value of about 500 nl. This is a large volume compared to that of membrane vesicles from rabbit TALH cells or shark rectal gland cells both of which have been shown to possess a NaCl-dependent bumetanide-sensitive Rb^+ uptake [47,48]. Costa et al. [16] found the intracellular value for K^+ in *Xenopus* oocytes to be about 109 mmol/l so even at 2 h incubation time only a small fraction of the oocyte K^+ has been exchanged for Rb^+.

The Na^+-K^+-$2Cl^-$ cotransport mechanism has been shown in avian erythrocytes [49–52] to participate in cell volume regulation. Osmotic shrinking in these cells leads to an increase in the activity of the Na^+-K^+-$2Cl^-$ cotransport and thus a restoration of the normal volume followed by inactivation of the cotransporter. A similar activation of the cotransporter following osmotic shrinkage has also been documented in Ehrlich ascites tumor cells [24] although it does not lead to a restoration of volume unless the cells have been previously adjusted to hypotonic medium. No such evidence as yet exists for the cotransport activity in the oocytes, however, one could postulate possible roles for the oocyte cotransporter in any case.

Fertilization of eggs from the frog *Rana pipiens* has been shown to be accompanied by changes in potassium and chloride conductances [53] and fertilization in sea urchin eggs [54] is accompanied by a Na^+-dependent intracellular alkalinization coupled to a transient increase in intracellular sodium activity presumably brought about via the Na^+/H^+ exchanger owing to the amiloride-sensitivity of the changes. The restabilization of intracellular sodium activity following fertilization is inhibited by ouabain indicating the participation of the Na^+/K^+-ATPase in this process. Perhaps fertilization is also accompanied by an increase in volume in which case the Na^+-K^+-$2Cl^-$ cotransporter would be a prime candidate to initiate such an action.

The cotransport may also be important to the embryo by providing a volume regulatory mechanism during early development. Assuming an even distribution of cotransport proteins across the oocyte surface each of the new cells of the developing embryo would receive a portion of the cotransporter molecules. An uneven distribution of cotransporters would mean that only certain cells would retain the cotransporter and could thus provide a basis for further differentiation of the cells.

For studies on the expression of ion transport systems in the oocytes the presence of this basal activity of Na^+-K^+-$2Cl^-$ cotransport has to be taken into account in particular when isotope flux measurements are performed.

In summary evidence has been presented showing the presence of a Na^+- and Cl^--dependent and bumetanide inhibitable Rb^+ (K^+) flux in the *Xenopus* oocytes. The almost total inhibition of uptake by bumetanide (in the presence of ouabain) along with the evidence showing a dependence on the presence of NaCl for transport strongly suggests that there are only two major sources of $^{86}Rb^+(K^+)$ uptake in the *Xenopus* oocytes used in this study, an ouabain-sensitive Na^+/K^+-ATPase and a NaCl-dependent bumetanide-sensitive system, possibly a Na^+-K^+-Cl^- cotransport. Further evidence based on kinetic analysis of $^{86}Rb^+$ transport also suggests that the system is a Na^+-K^+-Cl^- cotransport system which seems to share properties with the cotransport system investigated in other cells: (1) the apparent affinities for the cotransported ions fall within the ranges reported for other systems (with the possible exception of the Na^+ data); (2) Rb^+ and K^+ are transported equally and are competitive inhibitors of each other; (3) the apparent stoichiometry of $1Na^+:1K^+:2Cl^-$ suggests an electroneutral cotransport as reported for other cells; and (4) bumetanide inhibits Rb^+ uptake in a dose-dependent manner and the inhibition constant is within

the range documented for other cell types. These data suggest the presence of a Na^+-K^+-$2Cl^-$ cotransport system in *Xenopus* oocytes which is not unlike the system of other cells.

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