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Stoichiometry and Ion Affinities of the Na-K-Cl Cotransport System in the Intestine of the Winter Flounder (*Pseudopleuronectes americanus*)

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Summary. Na-K-Cl cotransport stoichiometry and affinities for Na, K and Cl were determined in flounder intestine. Measurement of simultaneous NaCl and RbCl influxes resulted in ratios of 2.2 for Cl/Na and 1.8 for Cl/Rb. The effect of Na and Rb on Rb influx showed first order kinetics with $K_{1/2}$ values of 5 and 4.5 mM and Hill coefficients of 0.9 and 1.2, respectively. The effect of Cl on rubidium influx showed a sigmoidal relationship with $K_{1/2}$ of 20 mM and a Hill coefficient of 2.0. The effects of variations in Na and Cl concentration on short-circuit current (I_{sc}) were also determined. The $K_{1/2}$ for Na was 7 mM with a Hill coefficient of 0.9 and the $K_{1/2}$ for Cl was 46 mM with a Hill coefficient of 1.9. Based on the simultaneous influx measurements, a cotransport stoichiometry of 1Na:1K:2Cl is concluded. The Hill coefficients for Cl suggest a high degree of cooperativity between Cl binding sites. Measurements of the ratio of net Na and Cl transepithelial fluxes under short-circuit conditions (using a low Na Ringer solution to minimize the passive Na flux) indicate that the Cl/Na flux ratio is approximately 2:1. Therefore, Na recycling from serosa to mucosa does not significantly contribute to the I_{sc}. Addition of serosal ouabain (100 μM) inhibited Rb influx, indicating that Na-K-Cl cotransport is inhibited by ouabain. This finding suggests that a feedback mechanism exists between the Na-K-ATPase on the basolateral membrane and the apical Na-K-2Cl cotransporter.

Key Words cotransport · ion transport · stoichiometry · intestinal transport · winter flounder

Introduction

Na-K-Cl cotransport is the principle way salt is absorbed across the brush border membrane of the intestine of the winter flounder [9, 26]. This cotransport is inhibited on its luminal side by furosemide [10] and on its cellular side by cyclic GMP [30]. Similar Na-K-Cl cotransport systems have been found in other epithelial tissues such as the luminal (apical) membranes of the thick ascending limb of Henle's loop in rabbit kidney [15], the distal tubule of *Amphiuma* kidney [27], and MDCK cells [25]. There is suggestive evidence for its existence in the basolateral membranes of Cl-secreting cells such as shark rectal gland and teleost Cl cells [8, 16, 19, 22]. Na-K-Cl cotransport has also been demonstrated in several nonepithelial cells, including human and avian erythrocytes [4, 5, 29], squid giant axon [28], cultured human fibroblasts [25], and Ehrlich ascites cells [11].

In the present study we have determined the effects of various Na and Cl concentrations on short-circuit current (I_{sc}) and of various Na, Rb and Cl concentrations on ^{86}Rb influx. The apparent affinity constants ($K_{1/2}$) for each ion have been calculated and compared with values obtained for other epithelial and nonepithelial cells and tissues. We have also established the stoichiometry of Na-K-Cl cotransport by simultaneous dual measurements of Na and Cl influxes and of Rb and Cl influxes.

Materials and Methods

Bumetanide was obtained from Hoffmann-Laroche, Inc. N-methyl-D-glucamine (NMDG), N-2-hydroxyethyl-piperazine propane sulfonic acid (EPPS), triethylammonium (TEA), and Na,K,Mg,Ca gluconate salts were purchased from Sigma. ^{86}Rb (1.7 Ci/mmol), ^{22}Na (2.3Ci/mmol), ^{36}Cl (0.36 Ci/mmol) and [^{3}H]-PEG (900 mol wt) were obtained from New England Nuclear.

Animals

Winter flounder (*Pseudopleuronectes americanus*) were obtained from Mount Desert Island Biological Laboratory, ME (MDIBL) and from Southampton College Marine Station, Long Island, NY. Animals held at MDIBL were kept in large seawater aquaria at a temperature of 15°C. Flounder were starved at least three days before use. Flounder from NY were transported to Chicago and maintained at the John G. Shedd Aquarium. These flounder were held at 15°C ± 2° and initially fed twice a week. After a three-week acclimation period, the flounder were starved for one week and then used for experimentation. At Columbia University, winter flounder from Shinnecock Bay, Long Island, were held in a 200-gal aquarium at 5°C and starved for at least 1 week before use.

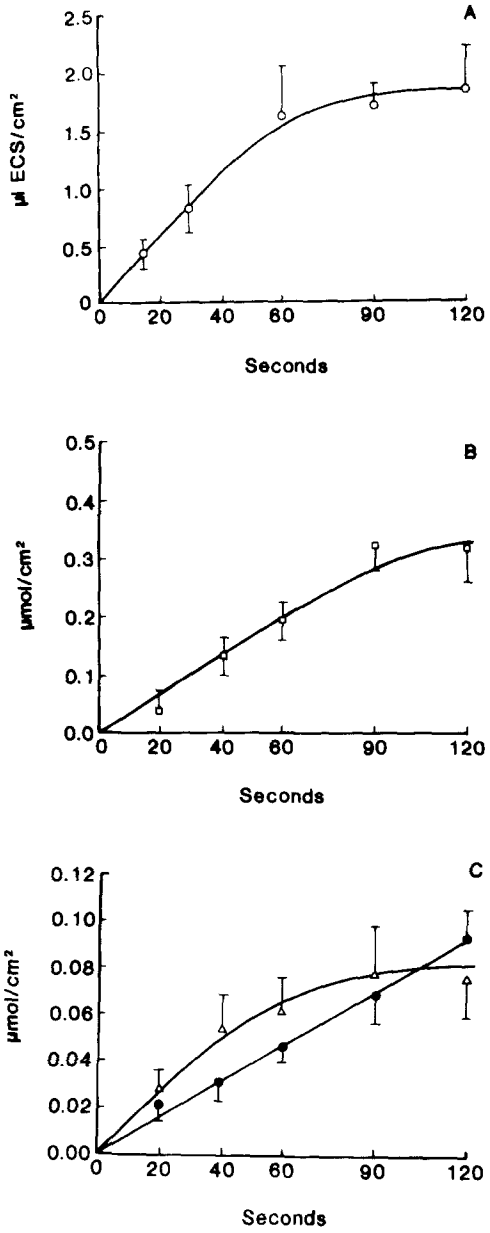


Fig. 1. (*A*) Time course of [^{3}H] PEG influx into the intestinal epithelium of the winter flounder. (*B*) ^{36}Cl influx into flounder intestinal epithelium. Both PEG and ^{36}Cl uptake are linear up to 60 sec. Each point represents the mean ± SE of four determinations for both ^{36}Cl and [^{3}H] PEG. (*C*) Time course of ^{22}Na and ^{86}Rb influx from the mucosal solution into the intestinal epithelium. ^{86}Rb influx is linear for at least 2 min. ^{22}Na influx remains linear for at least 40 sec. Each point represents the mean ± SE of four determinations (Δ, Na; ●, Rb)

Unidirectional Influx Measurements

Simultaneous unidirectional influxes of either ^{22}Na and ^{36}Cl or ^{86}Rb and ^{36}Cl were determined under short-circuit conditions using influx chambers similar to those described by Thompson and Dawson [34]. Intestines were removed and stripped of circular and longitudinal musculature as previously described [7]. Tissues were bathed on both sides with identical Ringer solutions which contained in mmol/liter : 20, NaCl; 53, NMDG-Cl; 5, KCl; 1, $CaCl_2$; 1, $MgSO_4$; 3, $NaHPO_4$; 20, glucose; 110, mannitol; and 5, EPPS (N-2-hydroxyethyl piperazine propane sulfonic acid, pH 8.0). Solutions were bubbled with room air and maintained at 15°C. Portions of stripped mucosa were mounted and placed in the influx chamber and allowed to incubate until the short-circuit current (I_{sc}) became stable (15–30 min). The mucosal surface of the tissue was then exposed to a test solution (5 ml) containing [^{3}H]PEG, mol wt 900 (10 μCi), ^{36}Cl (5 μCi) and either ^{22}Na (2–3 μCi) or ^{86}Rb (2–3 μCi). After 15–120 sec of exposure the inner chamber holding the tissue was rapidly removed and the influx terminated by washing the mucosal surface of the tissue with a large volume (100 ml) of cold 300 mM Mannitol solution for 2–3 sec. The tissue was then punched-out, blotted with absorbent paper, and placed in 1.0 ml of 0.2 N NaOH. After 12 hr, samples were vigorously vortexed and 0.2-ml samples removed, acidified with 0.1 ml 1 N HCl and assayed for radioactivity. The uptake of [^{3}H] PEG served as an estimate of the extracellular volume adherent to the mucosal surface after blotting. The "space" for ^{22}Na, ^{86}Rb or ^{36}Cl (i.e., (^{22}Na/cm² tissue)/(^{22}Na/ml medium)) minus that for PEG represented the influx of Na, Rb or Cl into the epithelium. Bumetanide-inhibitable influx was determined after preincubation with 10^{-4}M bumetanide for 20 min. Bumetanide reduced the I_{sc} to nearly zero within this time period. Bumetanide-inhibitable influx represented the difference between the remaining influx after preincubation with bumetanide and the total influx measured in a paired tissue from the same animal.

The uptakes of [^{3}H]-PEG, ^{22}Na, ^{86}Rb and ^{36}Cl were measured as functions of time over 120 sec (*see* Fig. 1*A–C*). The influxes of Rb and Cl proved to be linear for at least 60 sec. The Na influx was linear for at least 40 sec. Sampling of the serosal bathing solution after 60 sec indicated that virtually no radioisotope had crossed the tissue. Since Rb and Cl uptake appears to be linear for at least 60–90 sec, a 60-sec end point was chosen so that the measurements could be made during the linear phase of uptake and at a time at which extracellular PEG (900 mol wt) uptake had already reached equilibrium. With Na and Cl, influx begins to deviate from linearity before reaching 60 sec. Therefore an end point of 30 sec was selected so that these measurements could also be taken during the linear portion of the influx. However, the extracellular space is probably underestimated since PEG has not reached equilibrium and Na will presumably equilibrate faster in the extracellular space than does PEG. The resulting overestimate of Na influx, however, must be less than 10–15% since the extracellular space correction never exceeded this range. Moreover, since only the bumetanide-inhibitable portion of the Na and Cl influxes are being considered the ratio of Na to Cl entry should not be affected by any error in the determination of extracellular space.

The unidirectional influx of ^{86}Rb across the luminal border was also measured using influx chambers modified from those described by Frizzell et al. [10]. The initial Ringer solution contained in mmol/liter: 160, NaCl; 5, RbCl; 1.25, $CaCl_2$; 1.1, $MgCl_2$; 0.3, NaH_2PO_4; 1.65, Na_2HPO_4; 20, glucose; 5, EPPs (pH 8.0). Na was replaced with either tetraethylammonium (TEA) or N-methyl-D-glucamine (NMDG). Cl was replaced with gluconate (Na, Ca or Mg salts) and NO_3 (Rb salt). Solutions were maintained at 15°C and bubbled with room air. After a 30–60 min preincubation period ^{86}Rb and [^{3}H]-PEG, mol wt 900, were added, and after 45 sec tissues were quickly punched-out and processed for assays of radioactivity as described in the preceding paragraph.

Table 1. Na-Rb-Cl unidirectional influx measurements

	Na	Cl	Rb	Cl
Total	4.0 ± 0.6	8.3 ± 0.6	6.2 ± 0.5	9.3 ± 1.8
+Bumetanide	2.0 ± 0.6	3.9 ± 0.9	3.7 ± 0.6	4.6 ± 0.9
Bumetanide inhibitable	2.0	4.4	2.5	4.7
Ratio	Cl/Na = 2.2		Cl/Rb = 1.8	

Results shown are the initial rates of uptake (influx) of ^{22}Na, ^{86}Rb and ^{36}Cl from the mucosal solution into the mucosa. Rates of Na and Cl uptake were made simultaneously as were Rb and Cl so that a comparison between the ratio of Na/Cl uptake could be made with that of Rb/Cl.

Uptakes of Na and Cl and Rb and Cl were measured after 30 and 60 sec, respectively.

Values shown are μmol/hr/cm^2 ± SEM.

Effects of Na and Cl Concentrations on Short-Circuit Current (I_{sc})

Tissues were mounted in Ussing chambers and initially bathed on both sides with a Ringer solution containing in mmol/liter: 170, NaCl; 5, KCl; 1, $CaCl_2$; 1, $MgSO_4$; 3, Na_2HPO_4; 20, glucose; 5, EPPS (pH 8.0). After the I_{sc} had stabilized, the mucosal and serosal solutions were serially diluted with either Na-free or Cl-free Ringer solutions. After each Na or Cl dilution step the I_{sc} was allowed to stabilize and the new steady-state I_{sc} was recorded. This process could be repeated at least 6–8 times on each tissue. In most cases 85–90% of the original I_{sc} was restored when normal Ringer solution was added at the conclusion of the experiment. Estimates of $K_{1/2}$ were obtained from Eadie-Hofstee plots. Hill coefficients were determined from the slope of the Hill plots. Lines were drawn by the method of least squares.

Transepithelial Flux Measurements

The effects of bumetanide on transepithelial fluxes of Na and Cl were determined on tissues bathed on both sides with a Ringer solution containing in mmol/liter: Na, 50; K, 5; NMDG, 120; Ca, 1; Mg, 1; Cl, 180; PO_4, 3; EPPS, 5 (pH 8.0) and glucose, 20. Solutions were bubbled with room air and cooled to 7°C. Flux measurements were begun 45–60 min after tissues were mounted to allow the PD and I_{sc} to stabilize. Twenty min following addition of radioisotopes, control fluxes were measured over a 30-min period. Bumetanide (0.1 mmol/liter) was then added to the mucosal side. After a 20-min re-equilibration period, the post-bumetanide fluxes were measured over 30 min. In preliminary experiments we established that the I_{sc} and resistance are constant for at least 24 hr.

Results

Simultaneous Influx Experiments for NaCl and RbCl

Double-label influx measurements for ^{22}Na and ^{36}Cl and for ^{86}Rb and ^{36}Cl are shown in Table 1. Ratios of the bumetanide-inhibitable influxes of Cl and Na and of Cl and Rb were 2.2 and 1.8, respectively.

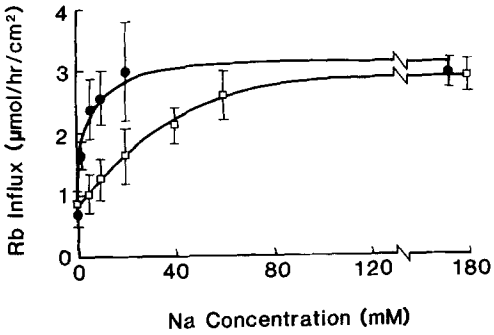


Fig. 2. Effect of Na concentration on ^{86}Rb influx from the mucosal solution into the intestinal epithelium. ^{86}Rb influx was measured over a 45-sec time period at each Na concentration. Each point shows the mean ± SE of four measurements. (●, Rb influx using NMDG as the substituted cation; □, Rb influx using TEA as the substituted cation)

These ratios do not differ significantly from 2.0 or from each other. Comparison of ^{86}Rb influx in RbCl Ringer with ^{86}Rb influx in KCl Ringer indicated no significant difference in total or bumetanide-inhibitable influx (*data not shown*). ^{86}Rb influx in Rb-Ringer and ^{42}K influx in K-Ringer were also not significantly different. Rb appears, therefore, to be a suitable substitute for K. The above results are consistent with a stoichiometry for Na, K and Cl influxes of 1Na : 1K : 2Cl.

Effect of Na,K and Cl on ^{86}Rb Influx

The effects of variations in Na, K and Cl concentration on ^{86}Rb influx are shown in Figs. 2–4. The influxes remaining in the absence of Na or Cl were assumed to be paracellular. When Na was replaced with NMDG the Na-dependence curve obeyed simple Michaelis-Menten kinetics and had a Hill coefficient of 1.09 (Fig. 2). The average $K_{1/2}$ for Na was 7 mM. When Na was replaced with TEA the Na-dependence curve no longer obeyed simple Michaelis-

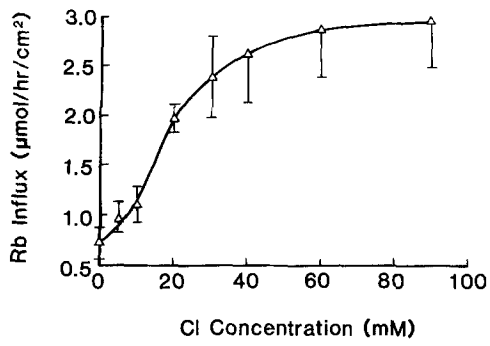


Fig. 3. Effect of Cl concentration on ^{86}Rb influx into the intestinal epithelium. Cl was replaced with gluconate. ^{86}Rb influx was measured over a 45-sec period at each Cl concentration. Each point shows the mean ± SE of five determinations.

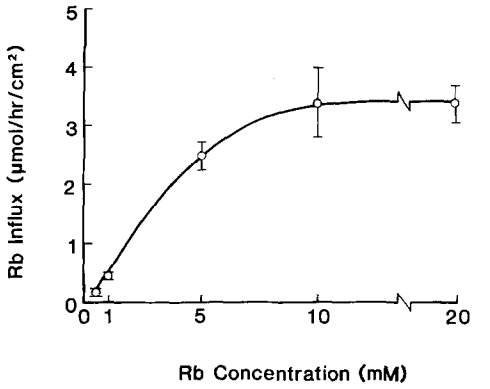
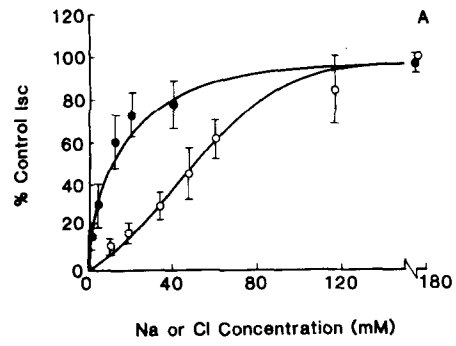


Fig. 4. Effect of Rb concentration on ^{86}Rb influx into the intestinal epithelium. Rb was replaced with Na. ^{86}Rb influx was measured for 45 sec at each Rb concentration. Each point shows the mean ± SE of four determinations



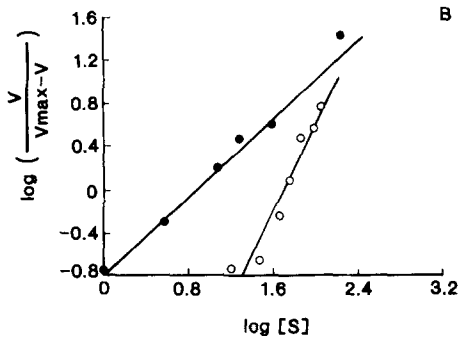


Fig. 5. (*A*) Effect of Na or Cl concentration on short-circuit current (I_{sc}). Na was substituted with NMDG-Cl and Cl concentration was maintained at 177 mM for each measurement. Cl was substituted with gluconate while Na was maintained at 176 mM. The $K_{1/2}$ for Na is 7 mM and the $K_{1/2}$ for Cl is 46 mM. (●, Na; n = 8 ± SE; ○, Cl; n = 7 ± SE). (*B*) Hill plots of Na and Cl concentration effects on flounder I_{sc}. The Hill coefficients (n) for Na and Cl are 0.9 and 1.95, respectively

Menten kinetics and the Hill plot was biphasic. Furthermore, in the presence of TEA, the $K_{1/2}$ for Na shifted from 7 to 38 mM, suggesting some competition between TEA and Na for the Na site. A sigmoidal relationship was obtained for the effect of Cl concentration on ^{86}Rb influx (Cl substituted with gluconate). The hill coefficient for Cl was 2.06 and the $K_{1/2}$ was 20 mM (Fig. 3). Changes in Rb concentration resulted in a curve similar to that for Na with a Hill coefficient of 1.19 (Fig. 4). The $K_{1/2}$ for Rb was 4.5 mM.

Effects of Na and Cl Concentrations on I_{sc}

Figure 5*A* shows a Michaelis plot of the dependence of I_{sc} on Na and Cl concentrations. Their effects on I_{sc} are qualitatively similar to those observed for ^{86}Rb influx. Na was replaced with NMDG; its effect on I_{sc} follows simple saturation kinetics. However, the results of Cl replacement with gluconate show a sigmoidal relationship between I_{sc} and Cl concentration. The $K_{1/2}$ values for Na and Cl were obtained from Eadie-Hofstee plots. The $K_{1/2}$ for Na was 7 mM and that for Cl was 46 mM (the Eadie-Hofstee plot was linear when I_{sc} was plotted against I_{sc_2} [Cl]). This $K_{1/2}$ for Cl differs significantly ($P < 0.05$) from that obtained for Rb influx (20 mM). Hill plots in Fig. 5*B* give Hill coefficients of 0.90 and 1.9 for Na and Cl, respectively.

Effect of Ouabain on ^{86}Rb Influx

The effect of ouabain (10^{-4}M serosal side addition) on ^{86}Rb influx was measured after I_{sc} had decreased to near zero (usually 45–60 min). This concentration of ouabain eliminated the I_{sc} and decreased unidirectional ^{86}Rb influx from 3.1 ± 0.22 μEq/hr/cm² to 0.9 ± 0.17 μEq/hr/cm² (mean of four paired tissues, $P < 0.01$ different from control). Omission of Ca^{2+} from the serosal solution did not prevent this

Table 2. Transepithelial Na and Cl fluxes across short-circuited flounder intestine

	Na			Cl			I_{sc}
	$M \rightarrow S$	$S \rightarrow M$	Net	$M \rightarrow S$	$S \rightarrow M$	Net	
Control	3.96 ± 0.46	1.82 ± 0.24	2.15 ± 0.26	8.11 ± 1.2	3.79 ± 0.82	4.45 ± 0.51	−2.7 ± 0.8
Bumetanide (10^{-4} M)	2.15 ± 0.46	1.67 ± 0.33	0.46 ± 0.25	3.21 ± 0.30	2.99 ± 0.60	0.39 ± 0.11	−0.21 ± 0.1

Tissues were bathed in 50 mM Na Ringer solution as stated in the text. The low Na concentration was used to reduce the paracellular flux of Na so that a more accurate estimate of the cellular component of the unidirectional Na fluxes could be determined. The effect of [Na] on I_{sc} (Fig. 5A) shows that the $K_{1/2}$ for Na is 7 mM. Values shown are μmol/hr/cm² ± SE for nine experiments.

effect (data not shown), suggesting that serosal Na/Ca exchange was not responsible for this effect of ouabain.

Transepithelial Flux Measurements

The effects of bumetanide on Na and Cl fluxes were determined in a modified Ringer solution ([Na] = 50 mM; [Cl] = 177 mM) after the I_{sc} had decreased to nearly zero (Table 2). Bumetanide inhibited 80% of the net Na flux and 90% of the net Cl flux. After adding bumetanide, the net Na flux did not differ significantly from zero, but a small residual net Cl flux of 0.39 ± μEq/hr/cm² remained ($P < 0.05$). This may represent Cl/HCO_3 exchange, since HCO_3 secretion of about 0.4 μEq/hr/cm² appears to be present (Q. Al-Awqati and M. Field, *unpublished*).

It is worth noting that, under short-circuit conditions, the ratio of net Cl to net Na fluxes was 2.07 before the addition of bumetanide. The ratio of the changes in net Na and net Cl fluxes produced by bumetanide was 2.40. These ratios do not differ significantly from each other or from 2.0.

Discussion

The winter flounder, like other marine fishes, compensates for osmotic and urinary water losses by drinking seawater and absorbing salt and water from the gastrointestinal tract [21, 24, 33]. Excess NaCl is then excreted by the gills [2, 6, 8, 22]. A model showing the cellular mechanism of salt and water absorption of flounder intestinal cells is illustrated in Fig. 6. The four major elements of NaCl transport are: (i) an electrically neutral Na-K-Cl cotransport system located on the brush border membrane with a transport stoichiometry of 1 Na, 1 K, and 2 Cl; (ii) a Ba-sensitive K channel, also on the apical membrane; (iii) Na-K-ATPase, in the basolateral membrane; and (iv) Cl conductance in the basolateral membrane. When identical Ringer solutions are placed on both sides of the tissue, a serosa-negative transepithelial PD is observed. The Na-K-ATPase maintains a low intracellular Na concentration, thereby creating a driving force for Na entry into the cell. The presence of a Na-K-Cl cotransporter in the apical membrane couples Cl entry

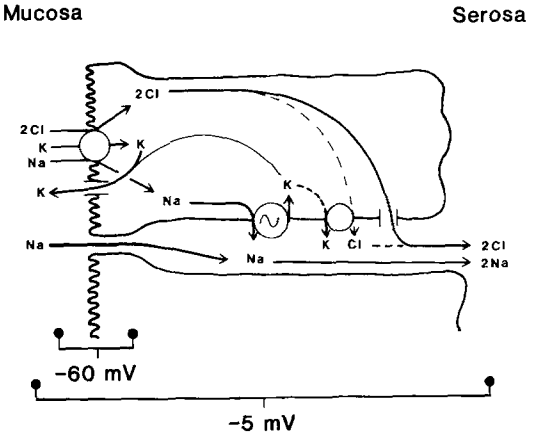


Fig. 6. A model of salt transport across the intestinal epithelium of winter flounder intestine. When the tissue is bathed on both sides with identical Ringer solutions, there is a serosa-negative transepithelial potential difference and a cell potential of about −60 mV. A Na-K-2Cl cotransporter couples Cl entries to Na entry. The entering Na is pumped out across the basolateral membrane by Na, K-ATPase. The entering K recycles to the lumen through apical K channels. The entering Cl accumulates in the cell above electrochemical equilibrium, by virtue of its coupling to the Na gradient. A driving force exists, therefore, for Cl to exit across the basolateral membrane. Chloride appears to do so via an electrogenic pathway (Cl channel) and, to a lesser extent, by an electroneutral pathway, probably KCl cotransport. A major advantage of this system is that for each Na ion pumped across the basolateral membrane, 2 Cl ions are transported. The resulting transepithelial electric potential difference causes movement of an additional Na ion through cation-selective intercellular junctions. Thus Na-K-2Cl cotransport in combination with an apical K channel and a basolateral Na pump, provides an energetically efficient means of transporting large quantities of salt and water across the tissue

to Na entry, permitting Cl to accumulate in the cell above electrochemical equilibrium. This mechanism provides an energetically efficient way of transporting large quantities of NaCl since the electroneutral cotransport system effectively uncouples apical Na and Cl entries from the electric potential of the cell. Although this uncoupling diminishes the driving force for Na entry (medium-to-cell PD is about −60 mV), it generates a driving force for Cl entry since the medium Cl concentration (180 mM) is greater than cell Cl (40 mM) [3]. Furthermore, since 2 Cl are transported for each Na, the driving force for Cl entry (RT [ln Ci/Co]/F) is doubled [20]. Potassium, which enters with Na and Cl against a steep concentration difference for K, recycles back to the lumen through apical K channels (cell K is about 15 mV above electrochemical equilibrium [18]). Since Cl accumulates in the cell above electrochemical equilibrium there is a driving force for the exit of Cl ion across the basolateral membrane. The Cl permeability in this membrane has not been fully characterized, but appears to be a Cl-selective channel. A portion of the serosal efflux of Cl may be due to KCl cotransport [18].

Initial studies on transport in this system suggested that the major permeability in the apical membrane was a Na-Cl cotransport system and that Na recycling occurred across a cation-selective tight junction between the cells [7]. The process was proposed as an explanation of the serosa-negative transepithelial PD. The ratio of net Cl to net Na fluxes in Table 2 (2.07) indicates that the magnitude of Na recycling is near zero. The serosa-negative PD appears to be due predominantly to the conjunction of Na-K-2Cl cotransport with an apical K channel that permits the recycling of the entering K. The short-circuit current is thus a function of both transcellular movements of Na + Cl and active K secretion. If one assumes that all of the incoming K through the cotransporter is recycled, then the source for net secretion of K is the Na-K ATPase in the basolateral membrane. In this sense, recycling of K entering through the cotransporter does not directly contribute to the tissue I_{sc}. The secreted K (resulting from Na-K ATPase activity), however, contributes directly to the short-circuit current, accounting for approximately 20% (0.7 μeq/cm^2/hr K secretion relative to a I_{sc} of 3.58 μeq/cm^2/hr). The majority of the I_{sc} measured in this system can be viewed as the difference between net Na and Cl fluxes across the tissue. (J(net)Cl − J(net)Na) = 2.3 μeq/cm^2/hr relative to a I_{sc} of 2.7 μeq/cm^2/hr from data in Table 2). Therefore the I_{sc} is, for the most part, an indirect measurement of chloride transport. It is dependent on a functional cotransport system since inhibitors of cotransport like bumetanide abolish (95% inhibition) the I_{sc} and net Cl flux. Inhibition of cotransport also results in an inhibition of net K secretion. This is presumably due to the fact that Na-K ATPase turnover, responsible for net K entry across the basolateral membrane, is regulated by the rate of Na entry across the brush border. Na entry can be reduced to near zero with bumetanide or furosemide. When cotransport is inhibited by these diuretics, hyperpolarization of the apical membrane occurs and K approaches electrochemical equilibrium. This reduces the driving force for K secretion. Thus the entire I_{sc}, including the K secretion component, can be viewed as a direct function of the rate of Na-K-2Cl cotransport.

The stoichiometry of Na-K-Cl cotransport has been determined in Ehrlich ascites cells, duck and ferret red cells, and squid giant axon by measurements of isotopic influx. In Ehrlich ascites cells [11] the stoichiometry was determined from experiments in which the extracellular concentrations of two of the three ions were kept constant while the concentration of the third was varied. The influxes of the two constant ion species were then plotted as a function of the third and the stoichiometry derived from the slope ratios. The stoichiometry was 1Na : 1K : 2Cl. The same stoichiometry was reported for catecholamine-stimulated duck red cells as estimated from the ratio of furosemide-inhibitable (Na + K) to Cl influx [20]. However, in ferret red cells the ratios of bumetanide-sensitive influxes into SITS + DIDS treated cells at 21°C gave a stoichiometry of 2 Na : 1K : 3Cl [17]. In squid giant axon a similar 2 : 1 : 3 stoichiometry was measured by comparing simultaneous bumetanide-inhibitable influx ratios of Na/K and Cl/K [32]. Thus in nonepithelial cells Na-K-Cl cotransport processes appear to be electroneutral but display at least two different stoichiometries, depending upon the cell type.

In epithelial tissues the stoichiometry of Na-K-Cl cotransport has not been so well established. In MDCK cells a stoichiometry of 1Na : 1K : 2Cl was proposed by McRoberts et al. [25] based on the following indirect evidence: (i) transport of any one ion occurs only in the presence of the other two ions, (ii) the Hill coefficient for Cl dependence of Na and K uptake was greater than one suggesting more than one Cl site relative to Na or K, and (iii) valinomycin had no effect on transport, suggesting that the cotransport is electroneutral. In the rabbit TALH, the effect of Na, K and Cl concentrations on Na uptake was measured in a membrane vesicle preparation by Koenig et al. [23]. The Hill coefficients for Na, K and Cl were 1.0, 0.98, and 1.8, respectively. In earlier studies on intact tubules, Greger and Schlatter [15] and Greger, [13], mea-

sured I_{sc} as a function of Na concentration and chloride concentration. Their observations led them to suggest that the cotransporter has a stoichiometry of 1Na : 2Cl and is thus electrogenic. This stoichiometry seemed consistent with the lumen-positive transepithelial PD found there. In subsequent studies, however, they found that luminal K was required for Na and Cl cotransport and this suggested to them a stoichiometry of 1Na : 1K : 2Cl [14]. In each case the results from studies in epithelial tissues suggest electroneutral cotransport but whether the stoichiometry is 1 : 1 : 2 or 2 : 1 : 3 or some other combination has not been rigorously established.

In the present study we determined the stoichiometry of Na-K-Cl cotransport in flounder intestine by measuring the bumetanide-inhibitable influxes of NaCl and RbCl into the tissue. The stoichiometry was determined by calculating the ratio of Cl influx relative to Na and Rb influxes. Secondly, we measured ^{86}Rb influx at different Na, Rb and Cl concentrations and estimated the degree of cooperativity of Cl binding sites from a comparison of Hill coefficients for each ion. Finally we examined the effects of Na and Cl concentrations on I_{sc} as did Greger [13] in the TALH in rabbit kidney. Our results are similar to those reported for rabbit kidney TALH where the dependence of I_{sc} on Cl concentration showed a sigmoidal relationship.

In previous studies, such as in MDCK cells and rabbit TALH vesicles, the effects of Na, K and Cl concentrations on ^{86}Rb or ^{22}Na uptake have been measured and Hill coefficients reported. In these studies the sigmoidal relationship observed with changes in Cl concentration (when substituted with gluconate) suggested that more than one binding site exists for Cl. Since the stoichiometry of the cotransport process was not directly measured in these studies, the degree of cooperativity between Cl binding sites could not be determined. In flounder intestine, measurement of ^{86}Rb influx and I_{sc} as a function of Cl concentration both resulted in sigmoidal relationships. Analysis of the Hill coefficients in view of the stoichiometry established by simultaneous tracer uptake measurements indicates that Cl binding to the cotransporter is highly cooperative.

Measurement of I_{sc} as a function of Na concentration in flounder intestine with NMDG as the substituted cation resulted in an apparent affinity ($K_{1/2}$) of 7 mM. This $K_{1/2}$ estimate was determined from dilution experiments where the Na concentration of both mucosal and serosal solutions was diluted using Na-substituted Ringer solution. When these experiments were performed by initially eliminating Na from both solutions and then adding Na back to both sides of the tissue, a higher $K_{1/2}$ value of 19 mM was found (data not shown). Greger [12] found a $K_{1/2}$ of 3.6 mM for the effect of Na concentration on I_{sc} in rabbit kidney. In his experiments Na was replaced by either choline, TEA or Tris; no differences in the $K_{1/2}$ for Na were observed for any of these cation substitutions. In a recent paper by Owen and Prastein [28], Rb influx into fibroblasts was measured as a function of Na concentration using choline and lithium as substitutes for Na. Both showed Michaelis-Menten type saturation kinetics, but Li appeared to be transported to some degree and proved to be less effective than choline in inhibiting Rb influx. The $K_{1/2}$ for Na was estimated to be 15 mM (choline substitution). In flounder intestine, the effect of Na concentration on ^{86}Rb influx yielded a different $K_{1/2}$ for NMDG substitution (5 mM) than for TEA substitution (38 mM). Thus the measured affinity for Na of the Na-K-Cl cotransporter depends in part on the cation used to substitute for Na.

The Cl concentration-dependence of Na-K-Cl cotransport in flounder intestine (gluconate substituted for Cl) showed a sigmoidal relationship with a $K_{1/2}$ of 46 mM with respect to I_{sc} and 20 mM with respect to Rb influx. In rabbit TALH, Greger [13] found a similar $K_{1/2}$ value for Cl (50 mM) when Cl was substituted with NO_3. The reason for the difference we observed between $K_{1/2}$ values for I_{sc} and for ^{86}Rb influx measurements is uncertain. It is worth noting that I_{sc} reflects the *net* fluxes of Na and Cl across the apical membrane whereas ^{86}Rb influx measurements reflect unidirectional fluxes. This suggests that under some circumstances there is a significant back flux of these ions from cell to lumen via the cotransporter. Measurements of intracellular Cl at varying extracellular Cl concentrations are needed to further evaluate this possibility.

Previous measurements in fibroblasts, Hela cells and ferret red cells have shown that the shape and $K_{1/2}$ of Cl titration curves is dependent upon the substituted anion [1, 28]. In fibroblasts, substitution with gluconate gave a sigmoidal relationship with a $K_{1/2}$ of 30 mM. Anions such as Br, acetate, I, and SCN caused an increase in the $K_{1/2}$ value and gave hyperbolic curves, instead of the sigmoidal relationship observed with gluconate. The reason for the differences observed with various substituted anions is presently unclear, but suggests that the substituted anions were transported in place of the second Cl.

Although ouabain, by eliminating ion gradients, would be expected to inhibit net transport, it is somewhat surprising that it also inhibits unidirectional influx through the cotransporter. There therefore appears to be a mechanism for feedback inhibition of cotransport. Preliminary experiments

suggest that the Na/K pump inhibitors ouabain and vanidate increase cGMP concentration in flounder intestine (M.C. Rao and M. Field, *unpublished*). Since cGMP inhibits Na-K-Cl cotransport in flounder intestine [30], this could be the mechanism for feedback inhibition.

In conclusion, we have determined the stoichiometry of Na-K-Cl cotransport in flounder intestine to be 1Na : 1K : 2Cl. Analysis of Cl concentration effects on ^{86}Rb uptake and on I_{sc} indicate a high degree of cooperativity between the two Cl binding sites. Comparison of the ratio of net transepithelial Na and Cl fluxes (1 : 2) indicates that Na recycling from the serosa to mucosa does not significantly contribute to the I_{sc}. It also suggests that in the open-circuit condition the cation selectivity of the tight junction allows for passive Na uptake in response to the transepithelial electric potential. Finally, the effects of serosal ouabain on Na-K-2Cl cotransport suggest that a feedback mechanism between the Na-K ATPase and Na-K-2Cl cotransporter exists and may be mediated by cGMP.

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References

1. Aiton, J.F., Chipperfield, A.R., Lamb, J.F., Ogden, P., Simmons, N.L. 1981. Occurrence of passive furosemide-sensitive transmembrane potassium transport in cultured cells. *Biochim. Biophys. Acta* **646:**389–398
2. Degnan, K.J., Karnaky, K.J., Zadunaisky, J.A. 1977. Active chloride transport in the in vitro opercular skin of a teleost (*Fundulus heteroclitus*), a gill like epithelium rich in chloride cells. *J. Physiol. (London)* **271:**155–191
3. Duffey, M.F., Thompson, S.M., Frizzell, R.A., Schultz, S.G. 1979. Intracellular chloride activities and active chloride absorption in the intestinal epithelium of the winter flounder. *J. Membrane Biol.* **50:**331–341
4. Dunham, P.B., Stewart, G.W., Ellory, J.C. 1980. Chloride activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA* **77:**1711–1715
5. Ellory, J.C., Stewart, G.W. 1982. The human erythrocyte Cl-dependent Na-K cotransport system as a possible model for studying the action of loop diuretics. *Br. J. Pharmacol.* **75:**183–188
6. Evans, D.H., Mallery, C.H., Kravitz, L. 1973. Sodium extrusion by a fish acclimated to sea water; physiological and biochemical description of a Na for K exchange system. *J. Exp. Biol.* **58:**627–636
7. Field, M., Karnaky, K.J., Smith, P.L., Bolton, J.E., Kinter, W.B. 1978. Ion transport across the isolated intestinal mucosa of the winter flounder (*Pseudopleuronectes americanus*). *J. Membrane Biol.* **41:**265–293
8. Foskett, J.K., Scheffey, C. 1982. The chloride cell: Definitive identification as the salt-secretory cell in teleosts. *Science* **215:**164–166
9. Frizzell, R.A., Halm, D.R., Musch, M.W., Stewart, C.P., Field, M. 1984. Potassium transport by flounder intestinal mucosa. *Am. J. Physiol.* **246:***F*946–*F*951
10. Frizzell, R.A., Smith, P.L., Vosburgh, E., Field, M. 1979. Coupled sodium-chloride influx across brush border of flounder intestine. *J. Membrane Biol.* **46:**27–39
11. Geck, P., Pietvgyk, C., BurckHardt, B.C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na,K and Cl in Ehrlich cells. *Biochim. Biophys. Acta* **600:**432–447
12. Greger, R. 1981. Chloride reabsorption in the rabbit cortical thick ascending limb of the hoop of Henle. *Pfluegers Arch.* **390:**38–43
13. Greger, R. 1981. Coupled transport of Na and Cl in the thick ascending limb of Henle's loop of rabbit nephron. *Scand. Audiol. Suppl.* **14:**1–15
14. Gregor, R., Schlatter, E. 1981. Presence of luminal K, a prerequisite for active NaCl transport in cortical thick ascending limb of Henle's loop in rabbit kidney. *Pfluegers Arch.* **392:**92–94
15. Greger, R., Schlatter, E. 1983. Cellular mechanisms of the action of loop diuretics on the thick ascending limb of Henle's loop. *Klin. Wochenschr.* **61:**1019–1027
16. Greger, R., Schlatter, E. 1984. Mechanism of NaCl secretion in the rectal gland of spiny dogfish (*Squalus acanthias*) I. *Pfluegers Arch.* **402:**63–75
17. Hall, A.C., Ellory, J.C. 1985. Measurement and stoichiometry of bumetanide-sensitive (2Na : 1K : 3Cl) cotransport in ferret red cells. *J. Membrane Biol.* **85:**205–213
18. Halm, D.R., Krasny, E.J., Frizzell, R.A. 1985. Electrophysiology of flounder intestinal mucosa: Conductance properties of the cellular and paracellular pathways. *J. Gen. Physiol. (in press)*
19. Hannafin, J., Kinne-Saffran, E., Friedman, D., Kinne, R. 1983. Presence of a sodium-potassium chloride cotransport system in the rectal gland of *Squalus acanthias*. *J. Membrane Biol.* **75:**73–83
20. Hass, M., Schmidt, W.F., McManus, T.J. 1982. Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically neutral [Na + K + 2Cl] cotransport. *J. Gen. Physiol.* **80:**125–147
21. Hickman, C.P. 1968. Ingestion, intestinal absorption and elimination of sea water and salts in the southern flounder, *Paralichthys lethostigma*. *Can. J. Zool.* **46:**457–466
22. Karnaky, K.J. 1980. Ion secreting epithelia: Chloride cells in the head region of *Fundulus heteroclitus*. *Am. J. Physiol.* **238:***R*185–*R*198
23. Koenig, B., Ricapito, S., Kinne, R. 1983. Chloride transport in the thick ascending limb of Henle's loop: Potassium dependence and stoichiometry of the NaCl cotransport system in plasma membrane vesicles. *Pfluegers Arch.* **399:**173–179
24. Krasny, F.J., Frizzell, R.A. 1984. Intestinal ion transport in marine teleosts. *In:* Chloride Transport Coupling in Biological Membranes and Epithelia. G.A. Gerencer, editor. pp. 205–220. Elsevier, New York
25. McRoberts, J.A., Erlinger, S., Rindler, M.J., Saier, M.H. 1982. Furosemide sensitive salt transport in Madin-Darby canine kidney cell line. Evidence for the cotransport of Na,K and Cl. *J. Biol. Chem.* **257:**2260–2266

26. Musch, M.W., Orellana, S.A., Kimberg, L.S., Field, M., Halm, D.R., Krasny, E.J., Frizzell, R.A. 1982. Na-K-Cl cotransport in the intestine of a Marine teleost. *Nature (London)* **300:**351–353
27. Oberleithner, H., Guggino, W., Glekisch, G. 1983. Effect of furosemide on luminal sodium, chloride and potassium transport in the early distal tubule of *Amphuima* kidney. *Pfluegers Arch.* **396:**27–33
28. Owen, N.E., Prastein, M.L. 1975. Na/K/Cl cotransport in cultured human fibroblasts. *J. Biol. Chem.* **260:**1445–1451
29. Palfrey, H.C., Alper, S.L., Greengard, P. 1980. Protein phosphorylation and the regulation of a cation cotransport. *J. Exp. Biol.* **89:**103–115
30. Rao, M.C., Nash, N.T., Field, M. 1984. Differing effects of cGMP and cAMP on ion transport in winter flounder intestine. *Am. J. Physiol.* **246:***C*167–*C*177
31. Russell, J.M. 1979. Chloride and sodium influx: A coupled uptake mechanism in the squid giant axon. *J. Gen. Physiol.* **73:**801–818
32. Russell, J.M. 1983. Cation coupled chloride influx in squid axon: Role of potassium and stoichiometry of the transport process. *J. Gen. Physiol.* **81:**909–925
33. Sleet, R.B., Weber, L.S. 1982. The rate and manner of sea water ingestion by a marine teleost and corresponding sea water modification by the gut. *Comp. Biochem. Physiol.* **72A:**469–475
34. Thompson, S.M., Dawson, D.C. 1978. Sodium uptake across the apical border of the isolated turtle colon: Confirmation of the two-barrier model. *J. Membrane Biol.* **42:**357–374

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