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Evidence for a $Na^+/K^+/Cl^-$ Cotransport System in Basolateral Membrane Vesicles from the Rabbit Parotid

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Summary. Sodium (^{22}Na) transport was studied in a basolateral membrane vesicle preparation from rabbit parotid. Sodium uptake was markedly dependent on the presence of both K^+ and Cl^- in the extravesicular medium, being reduced 5 times when K^+ was replaced by a nonphysiologic cation and 10 times when Cl^- was replaced by a nonphysiologic anion. Sodium uptake was stimulated by gradients of either K^+ or Cl^- (relative to nongradient conditions) and could be driven against a sodium concentration gradient by a KCl gradient. No effect of membrane potentials on KCl-dependent sodium flux could be detected, indicating that this is an electroneutral process. A KCl-dependent component of sodium flux could also be demonstrated under equilibrium exchange conditions, indicating a direct effect of K^+ and Cl^- on the sodium transport pathway. KCl-dependent sodium uptake exhibited a hyperbolic dependence on sodium concentration consistent with the existence of a single-transport system with K_m = 3.2 mM at 80 mM KCl and 23°C. Furosemide inhibited this transporter with $K_{0.5} = 2 \times 10^{-4}$ M (23°C). When sodium uptake was measured as a function of potassium and chloride concentrations a hyperbolic dependence on [K] (Hill coefficient = 0.87 ± 0.09) and a sigmoidal dependence on [Cl] (Hill coefficient = 1.31 ± 0.07) were observed, consistent with a Na/K/Cl stoichiometry of 1:1:2. Taken together these data provide strong evidence for the electroneutral coupling of sodium and KCl movements in this preparation and strongly support the hypothesis that a $Na^+/K^+/Cl^-$ cotransport system thought to be associated with transepithelial chloride and water movements in many exocrine glands is present in the parotid acinar basolateral membrane.

Key Words fluid secretion · exocrine gland · chloride transport

Introduction

Salivary gland acini secrete an isotonic plasma-like fluid into the lumen of the secretory endpiece [39]. Although a number of early studies indicated that Na^+, K^+ and Cl^- play important roles in the formation of this primary saliva (e.g., ref. 19), until recently there has been little understanding of this process. Over the past few years, however, several groups have provided evidence that the mechanism underlying primary fluid secretion by the salivary acinar epithelium is similar to the one originally proposed for the shark rectal gland [4, 33] and subsequently suggested for a number of other secretory epithelia [8, 27] including lacrimal glands [7, 32], tracheal epithelium [38] and colon [12]. The main functional components of the proposed system are: (i) a loop-diuretic-sensitive $Na^+/K^+/Cl^-$ cotransporter located in the basolateral membrane of the acinar cells (in some tissues a K^+-independent Na^+/Cl^- cotransporter may instead be involved), (ii) an apical conductive pathway for Cl^-, and (iii) the Na^+,K^+-ATPase. According to this model (*see* Fig. 1) the electrochemical gradient for Na^+ generated by Na^+,K^+-ATPase causes Cl^- to be driven into the acinar cell against its electrochemical gradient via the basolateral cotransporter. Cl^- then diffuses down its electrochemical gradient into the acinar lumen via the apical chloride conductance, and Na^+ follows, presumably by leaking through the tight junctions between the cells, in order to preserve electrical neutrality. The resulting osmotic gradient for NaCl causes a net transepithelial movement of water from blood to lumen. A similar scheme is thought to apply in reverse in some absorptive epithelia where the cotransporter and the chloride channel are localized to the apical and basolateral membranes, respectively [10, 23].

To date the evidence supporting the above model in salivary glands, indeed in most exocrine glands, is somewhat indirect. Several studies have demonstrated inhibition of salivary secretion in perfused submandibular glands when the perfusion solution was modified by Cl^- replacement or by furosemide addition [2, 13, 20, 21, 29]. Poulsen and collaborators [24, 28] have demonstrated that furosemide inhibits Cl^- and Na^+ fluxes in isolated rat parotid acini. Martinez and Cassity [22] have shown that ^{36}Cl uptake into dispersed submandibular acini was reduced 40 to 50% when furosemide was present or when K was replaced in the incubation medium. More recently we have demonstrated

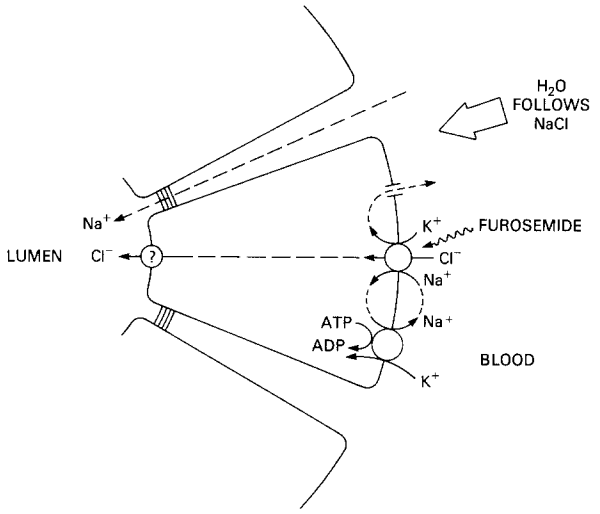

Fig. 1. Model for exocrine gland fluid secretion. *See* text for details

more dramatic reductions (80 to 95%) in ^{36}Cl fluxes in dispersed parotid acini [14] under similar experimental conditions to those employed by Martinez and Cassity.

In the present study we provide direct evidence for the existence of the proposed basolateral $Na^+/K^+/Cl^-$ cotransporter by examining the sodium transport properties of isolated basolateral membrane vesicles from the rabbit parotid.

Materials and Methods

Vesicle Preparation

Basolateral membrane vesicles (BLMV)[1] were prepared from rabbit parotid by the Percoll® gradient method described below. The procedure for one animal (approximately 1 g of parotid tissue) is given. We frequently processed the tissue from 6 to 12 rabbits simultaneously. One mM EDTA was present in all buffers to inhibit a potent phospholipase A_2 known to be present in the rabbit parotid [3].

White New Zealand female rabbits were sacrificed by decapitation (all subsequent steps were carried out at 4°C). The parotid glands were removed, dissected free of lymph nodes, superficial fat and connective tissue and placed in 200 to 300 μl of Homogenization Medium (10 mM Tris/HEPES containing 10% sucrose, 1 mM EDTA and 0.1 mM PMSF). The glands were then minced into approximately 1 mm^3 pieces using fine scissors and diluted in Homogenization Medium to a total volume of 10 ml. This parotid mince was left on ice for several minutes to allow any remaining fatty tissue to float to the top. Floating tissue fragments were removed by aspiration and the volume was again made up to 10 ml with Homogenization Medium. The parotid mince was then homogenized by two 10-sec bursts at power level 5 in a Polytron (Brinkman Instruments, Westbury, N.Y.). The resulting homogenate was spun at 2500 × *g* for 15 min in a Beckman J2-21 centrifuge fitted with a JA-20 rotor. The supernatant from this spin was saved on ice while the pellet was resuspended in 10 ml of Homogenization Medium and rehomogenized and spun as before. The combined supernatants from these two spins were filtered through a fine nylon mesh (Nitex 155μ from Tetko Inc., Elmsford, N.Y.) and spun at 22,000 × *g* for 20 min.

The supernatant from the above high-speed spin was discarded and the pellet was suspended in a few ml of Buffer A (10 mM Tris/HEPES with 100 mM mannitol) containing 1 mM EDTA, passed once through a 25-gauge needle and once through a 30-gauge needle, then diluted to 10.4 ml with the same buffer. Following the addition of 2.6 ml Percoll (20% by volume) this material was spun at 41,500 × *g* for 30 min in a Beckman JA-20.1 rotor. The upper 1.5 to 2.0 ml of the resulting Percoll gradient typically contained no measurable protein. Below this there was a dense white band of membranous material 2.5 to 3.0 ml in volume. The upper 1.25 ml of this band (the "BLMV fraction") was harvested, diluted into 20 ml of Buffer A containing 100 mM KCl and 1 mM EDTA, and spun at 48,000 × *g* for 20 min. This spin resulted in a soft membranous pellet covering a firm Percoll pellet. This membranous pellet was resuspended in 10 ml of Buffer A containing 100 mM KCl and 1 mM EDTA, repelleted and taken up at a protein concentration of 3.75 mg/ml in the same buffer. Aliquots (200 μl) of this material were fast frozen in liquid nitrogen and stored over liquid nitrogen until use.

Uptake Measurements (Rapid Filtration Technique)

On the day of the uptake experiment frozen aliquots of rabbit parotid BLMV were thawed at room temperature for 20 min, diluted 100 times with appropriate media for the uptake experiment, and spun at 48,000 × *g* for 20 min. The resulting pellets were taken up in the same media at a protein concentration of approximately 2 mg/ml.

The procedure for uptake measurements was as follows. A 10-μl aliquot of vesicles was placed in a 12 × 75 test tube and at time zero a 40-μl aliquot of incubation medium containing radioactively labeled ligands and other constituents as required was added. After an appropriate time the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (*see below*). After addition of the stop solution the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter, which retained the BLMV, was then washed with a further 6.0 ml of stop solution, placed in a scintillation vial with 1 ml of water and 10 ml of Filtron X (National Diagnostics) and counted for radioactivity along with samples of the incubation medium and appropriate standards.

The stop solution was Buffer A containing 350 mM KNO_3. The entire stopping and washing procedure took less than 30 sec, during which the vesicles were in contact with the stop solution for less than 15 sec. From control experiments in which the time

[1] *Abbreviations:* 10 mM Tris/HEPES, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffered with Tris to pH 7.4; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsufonyl fluoride; BLMV, basolateral membrane vesicles; NMDG, N-methyl-D-glucamine.

the vesicles were left in the stop solution was prolonged [35], we have established that no significant loss of ^{22}Na occurs during the stopping and washing procedure.

All data were corrected for nonspecific trapping of ^{22}Na by the membranes and filter by subtracting the "uptake" observed at "zero time." "Zero time uptake" (typically 0.07 ± 0.02 nmol/mg protein at 1 mM Na) was determined by dispensing the stop solution onto the vesicles before the addition of the incubation medium, then filtering and washing as usual (*see above*).

The detailed composition of the various media used in each experiment is given in the Figure and Table legends. Tracer ^{22}Na was typically used at a concentration of 20 μCi/ml. All experimental points were carried out in triplicate at 23°C. The errors shown in the Tables and Figures (provided they are large enough to illustrate) are standard deviations. In control experiments (*not shown*) it was established that ^{22}Na uptake was linear with time for at least 15 sec for the entire range of experimental conditions considered in the paper. Unless otherwise noted, all flux measurements were made at 15 sec and therefore represent initial uptake rates. Except where indicated the results of a single representative experiment are shown.

Calculations

In least-squares fits to the data, points were weighted according to their relative experimental errors. The errors quoted in the text on least-squares parameters are standard deviations. Least-squares fits to the Hill equation (*see text*) were carried out as described in ref. [9].

Enzyme Assays

The BLMV preparation procedure was monitored by assaying for the activities of enzymes characteristic of basolateral membranes (K^+-stimulated *p*-nitrophenyl phosphatase), luminal membranes (dipeptidyl peptidase IV—the localization of this enzyme to the luminal membrane of parotid acinar cells has recently been demonstrated by Sahara and Suzuki, ref. [30], mitochondria (succinic dehydrogenase) and lysosomes (acid phosphatase). K^+-stimulated *p*-nitophenyl phosphatase (KpNPPase) was assayed according to reference [37], dipeptidyl peptidase IV was assayed according to reference [17] using Gly-Pro *p*-nitroanilide as substrate, and succinic dehydrogenase was assayed according to reference [26]. Acid phosphatase was measured at pH 4.8 in a 90-mM sodium citrate buffer containing 0.15% Triton X-100 and 5.4 mM *p*-nitrophenyl phosphate as substrate. Protein was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as the standard.

Materials

Radioisotopes were from Amersham (Arlington Heights, Ill.). Furosemide was purchased from Sigma Chemical Co. (St. Louis, Mo.). N-methyl-D-glucamine was from Aldrich (Milwaukee, Wis.) and the methyl sulfate salts of sodium and potassium were from Kodak (Rochester, N.Y.). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

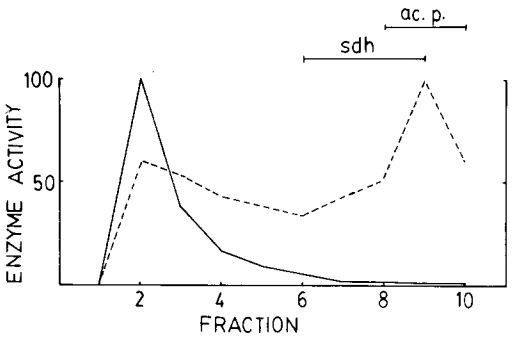

Fig. 2. Distribution of marker enzymes K-stimulated *p*-nitrophenyl phosphatase (solid line) and dipeptidyl peptidase IV (broken line) after centrifugation of 22,000 × *g* crude membrane pellet in a self-orienting Percoll gradient (*see* Materials and Methods). Enzyme activities are plotted as percentage of the maximum activity observed. The locations of the peaks of succinic dehydrogenase (sdh) and acid phosphatase (ac. p.) are also indicated. The upper 1.75 ml of the gradient typically contained no measurable protein and was taken as Fraction 1. The remainder of the gradient was divided into nine 1.25-ml aliquots, the first of which (Fraction 2) was the BLMV preparation discussed in the paper. Results of three independent experiments have been averaged. Error bars have been omitted from the figure for clarity; the standard deviations on the points are typically ≤10% for K-stimulated *p*-nitrophenyl phosphatase and ≤15% for dipeptidyl peptidase IV

Results

Enzymatic Characterization of Vesicle Preparation

Figure 2 shows the distribution of the marker enzymes KpNPPase (basolateral membranes) and dipeptidyl peptidase IV (apical membranes) following isopycnic centrifugation of the "crude membrane fraction" in 20% Percoll (*see* Materials and Methods). The locations of the peaks of the activities of succinic dehydrogenase (mitochondria) and acid phosphatase (lysosomes) are also indicated. KpNPPase activity is clearly localized to a single band on the gradient (Fraction 2) far from the peaks of succinic dehydrogenase and acid phosphatase activities. Dipeptidyl peptidase IV activity on the other hand is more diffusely distributed showing two peaks, one in Fraction 2 and the other in Fraction 9. Based on its high enrichment in KpNPPase activity we have chosen Fraction 2 in Fig. 2 as our BLMV preparation (*see* Materials and Methods). The average activities of KpNPPase, dipeptidyl peptidase IV, succinic dehydrogenase and acid phosphatase in the initial parotid homogenate as well as the recoveries and enrichments of these en-

Table 1. Enzymatic characterization of rabbit parotid basolateral membrane vesicles[a]

Enzyme	Activity in homogenate (μmol/mg) protein/hr)	Enrichment (vesicles/ homogenate)	% Recovery (vesicles/ homogenate)
K^+-stimulated *p*-nitrophenyl phosphatase	0.91 ± 0.26	10.4 ± 1.6	22 ± 1
Dipeptidyl peptidase IV	0.41 ± 0.08	2.0 ± 0.3	4.1 ± 0.2
Succinic dehydrogenase	0.17 ± 0.02	0.51 ± 0.05	1.1 ± 0.2
Acid phosphatase	0.97 ± 0.24	1.67 ± 0.08	3.6 ± 0.6

[a] Values are means ± SD for three independent determinations. Enzyme activities are given as micromoles subtrate consumed per milligram protein per hour.

zymes in the BLMV are given in Table 1. The purity of this BLMV preparation is quite similar to that obtained for a rat parotid BLMV preparation reported previously from our laboratory [34]; however, the present preparation represents a significant improvement in yield, viz., a 22% recovery of basolateral marker in this preparation *vs.* a 10% recovery with our previous method. We typically obtain approximately 2 mg vesicle protein/g rabbit parotid tissue with this procedure.

Initial Characterization of KCL-Dependent Sodium Uptake into Rabbit Parotid BLMV

Our initial studies of sodium uptake into the parotid BLMV preparation described above indicated a dramatic dependence on the presence of both K^+ and Cl^- in the extravesicular medium. These results are illustrated in Table 2. Here we compare the initial uptake rate of 1 mM sodium measured in the presence of an extravesicular to intravesicular KCl gradient, to uptakes measured under similar conditions with various cations and anions replacing K^+ or Cl^-. When chloride is replaced by nitrate or methyl sulfate, sodium flux is reduced by 90 or 95%, respectively. Likewise when potassium is replaced by N-methyl-D-glucamine or tetramethyl ammonium, sodium flux is reduced by 80%. The effects of the loop diuretic furosemide on these fluxes are also shown in Table 2. Furosemide, at a concentration of 3 mM, inhibits KCl-dependent sodium uptake by almost 85%, but has no effect on sodium flux in the absence of K^+ or Cl^-. In control experiments (*not shown*) we have verified that the uptake of [^{3}H]-D-mannitol into the BLMV preparation is not affected by any of the experimental conditions considered in Table 2, indicating that the above effects are not due to nonspecific alterations in vesicle integrity.

Table 2. Effects of K^+, Cl^- and furosemide on the initial rate of ^{22}Na uptake into rabbit parotid BLMV[a]

Incubation medium	Relative uptake	
	− furosemide	+ furosemide
KCl	1.00	0.14 ± 0.01
KNO_3	0.10 ± 0.01	0.09 ± 0.01
K methyl sulfate	0.04 ± 0.01	0.05 ± 0.01
N-methyl D-glucamine Cl	0.20 ± 0.02	0.23 ± 0.02
Tetramethyl ammonium Cl	0.17 ± 0.01	0.17 ± 0.01

[a] BLMV were prepared in Buffer A containing 1 mM EDTA. The incubation media were Buffer A containing (final concentrations) 80 mM of the salt indicated plus 1 mM ^{22}Na methyl sulfate with or without 3 mM furosemide. All results have been normalized to the uptake observed in the presence of KCl.

The results shown in Table 3 demonstrate that sodium uptake into parotid BLMV is not only dependent on the presence of K^+ and Cl^- but also on the presence of gradients of these ions. Sodium uptake is reduced 15% by the elimination of the Cl^- gradient, 55% by elimination of the K^+ gradient and 70% by elimination of both gradients.

The time course of 1 mM sodium uptake into the BLMV preparation in the presence of an initial extravesicular to intravesicular KCl gradient is illustrated in Fig. 3. This sodium uptake curve exhibits a transient "overshoot" of the intravesicular ^{22}Na concentration above its equilibrium value. This well-known overshoot phenomenon [35] indicates that the vesicle preparation is capable of catalyzing the concentrative uptake of sodium in the presence of an inwardly directed KCl gradient.

The above experiments demonstrate that virtually all of the sodium flux into our rabbit parotid BLMV preparation is mediated by a loop diuretic-sensitive, K^+ and Cl^--dependent transport pathway. Maximal sodium uptake via this pathway requires gradients of both of these ions. Moreover, in the presence of a KCl gradient (out > in) this transport system is capable of driving sodium into the vesicles against a concentration gradient. These results are consistent with the existence of the proposed $Na^+/K^+/Cl^-$-coupled transport system in the parotid acinar basolateral membrane.

Effects of Membrane Potentials

It is possible that at least some of the stimulation of sodium flux by K^+ and Cl^- observed in Table 2

Table 3. Effects of K^+ and Cl^- gradients on the initial rate of ^{22}Na uptake into rabbit parotid BLMV[a]

Experimental condition	Relative uptake
KCl gradient	1.00
K gradient, Cl equilibrium	0.84 ± 0.04
K equilibrium, Cl gradient	0.42 ± 0.02
KCl equilibrium	0.33 ± 0.02

[a] BLMV were prepared in Buffer A containing 1 mM EDTA and 100 mM of either NMDG-NO_3, NMDG-Cl, KNO_3 or KCl. The incubation media were such that the final extravesicular concentration of KCl, NMDG-NO_3, and ^{22}Na methyl sulfate were 100, 20 and 1 mM, in every case. All results have been normalized to the uptake observed with a KCl gradient.

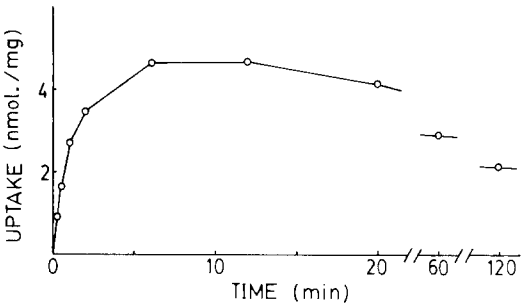

Fig. 3. Timed uptake of 1 mM ^{22}Na in the presence of an initial 80 mM extravesicular to intravesicular KCl gradient. BLMV were prepared in Buffer A containing 1 mM EDTA. The incubation medium was Buffer A containing (final concentrations) 80 mM KCl and 1 mM $^{22}NaCl$

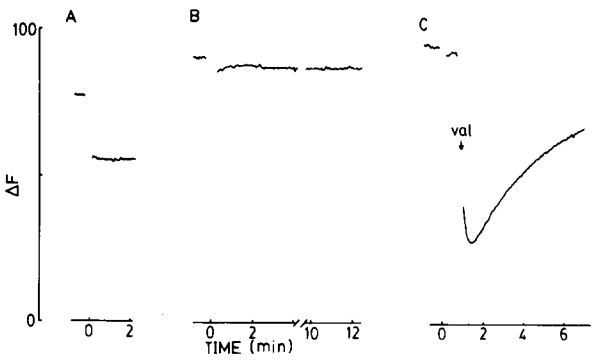

Fig. 4. Effectiveness of NO_3 equilibrium in clamping the BLMV membrane potential at zero. (*A*) Vesicles were prepared in Buffer A containing 1 mM EDTA and 200 mM KCl. At time zero 10 μl of vesicles (approx. 20 μg protein) were added to a cuvette containing 2 ml of Buffer A with 1 mM EDTA, 400 mM mannitol and approximately 0.75 μM diS-C2-(5). Fluorescence was measured in a Perkin Elmer Fluorimeter (Model MPF-44B) with excitation wavelength set at 590 nm and the emission wavelength at 670 nm. Fluorescence is plotted in arbitrary units. (*B*) The protocol was identical in all respects to *A* except that an additional 100 mM NMDG-NO_3 was present in both the intravesicular and extravesicular solutions. (*C*) The protocol was the same as *B* except that 10 μl of a valinomycin stock solution (0.1 mg/ml in EtOH) was added to the cuvette at the time indicated

could arise as the result of diffusion potentials (induced by the various ion gradients employed) acting on a Na^+-conductive pathway. In order to examine this and other questions we have devised a procedure for "clamping" BLMV membrane potentials. The basis of this method is to equilibrate the BLMV with a 100-mM solution of the permeant anion NO_3 and to add 100 mM NO_3 to all extravesicular solutions. In this way one hopes to clamp the *trans*-BLMV membrane potential at the NO_3 diffusion potential (zero). The effectiveness of this maneuver was tested using the membrane potential-sensitive cyanine dye diS-C2-(5) (3,3'-diethylthiadicarbocyanine iodide). Figure 4*A* shows that when BLMV loaded with KCl are diluted into isotonic mannitol containing diS-C2-(5) a significant decrease in fluorescence is observed indicating the induction of an inside-negative KCl diffusion potential. Figure 4*B* shows the results of a similar experiment carried out at 100 mM NMDG-NO_3 equilibrium. Under these conditions a much smaller decrease in fluorescence is seen. A similar decrease is also observed when KCl-loaded vesicles are diluted into isotonic KCl (*data not shown*). Thus this small change in fluorescence is probably due to binding of the (lipophilic) dye to the BLMV. The fluorescence signal in Fig. 4*B* is stable for over 10 min, indicating that there is no significant variation of the potential from zero or near zero levels over this time interval. Figure 4*C* verifies that diS-C2-(5) is still able to respond to membrane potentials under the experimental conditions of Fig. 4*B*. Here a dramatic decrease in fluorescence corresponding to the development of a large negative inside membrane potential is observed when the permeability of the vesicles to potassium is increased by the addition of the ionophore valinomycin.

Table 4 shows the results of an experiment where the effects of chloride and potassium removal on sodium uptake are studied under voltage-clamped conditions. The stimulation of sodium uptake by KCl observed in Table 2 is still present in the results shown in Table 4; thus, this effect could not be electrical in nature. This experiment also demonstrates that the small component of Cl^--dependent, K^+-independent, sodium uptake observed in Table 2 persists under voltage-clamped conditions.

Since sodium uptake into the parotid BLMV preparation obviously involves the participation of several other ions, presumably via a single coupled transport process, it is interesting to ask whether

Table 4. Effects of K^+ and Cl^- substitution on the initial rate of ^{22}Na uptake into rabbit parotid BLMV under voltage-clamped conditions[a]

Incubation media	Relative uptake
KCl	1.00
K methyl sulfate	0.13 ± 0.02
N-methyl-D-glucamine Cl	0.20 ± 0.01
Tetramethyl ammonium Cl	0.20 ± 0.02

[a] BLMV were prepared in Buffer A containing 1 mM EDTA and 100 mM NMDG-NO_3. The incubation media were Buffer A containing (final concentrations) 100 mM NMDG-NO_3, 1 mM ^{22}Na methyl sulfate and 80 mM of the salt indicated. All results have been normalized to the uptake observed in the presence of KCl.

Table 5. Effect of membrane potential on SCN^- uptake and KCl-dependent sodium uptake into rabbit parotid BLMV[a]

Substrate	Uptake (nmol/mg/min)	
	− val	+ val
^{22}Na	1.64 ± 0.12	1.63 ± 0.04
[^{14}C]SCN	2.55 ± 0.23	3.82 ± 0.21

[a] BLMV were prepared in Buffer A containing 1mM EDTA, 100 mM NMDG-NO_3 and 300 mM mannitol with or without valinomycin (5 μg/mg protein). The incubation media were Buffer A containing 100 mM NMDG-NO_3, 150 mM KCl and 1 mM ^{22}Na methyl sulfate or 1 mM K [^{14}C]SCN.

this transport event itself is electrogenic. This question is addressed in the experiment shown in Table 5 where we investigate the effects of membrane potential on the KCl-dependent component of sodium uptake into the BLMV preparation. Here we take advantage of the result shown in Fig. 4*C*, namely that under NO_3 equilibrium conditions a substantial KCl diffusion potential is produced by the addition of valinomycin. Table 5 illustrates that the effect of this diffusion potential can be clearly seen on the uptake of the permeant anion thiocyanate into the BLMV; however, no effect on KCl-dependent sodium uptake is detectable. This experiment suggests that the KCl-dependent component of sodium flux into this preparation occurs via an electroneutral transport process.

Direct Involvement of K^+ and Cl^+ with the Na^+ Transport Pathway

In the results presented above we have specifically ruled out the possibility that KCl-dependent sodium flux in the parotid BLMV preparation occurs via a Na^+-conductive pathway coupled electrically to

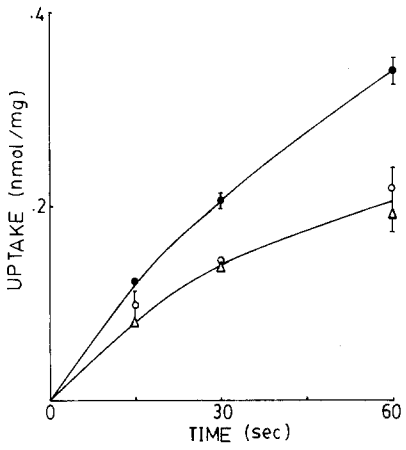

Fig. 5. KCl-dependence of the equilibrium exchange flux of sodium in BLMV. Vesicles were prepared in Buffer *A* containing 1 mM EDTA, 1 mM Na methyl sulfate and 100 mM of either NMDG-Cl (△), K methyl sulfate (○) or KCl (●). The incubation media contained tracer ^{22}Na but were otherwise identical to the above loading buffers

KCl movements (diffusion potentials). It is, however, possible that other types of indirect coupling between Na^+ and Cl^- and/or K^+ fluxes might account for our observations.[2] In the experiment shown in Fig. 5 we examine the roles of K^+ and Cl^- in sodium transport more closely. Here we have measured the equilibrium exchange flux of Na^+ in the presence and absence of these ions. This Figure demonstrates that there is a KCl-dependent component of sodium uptake under equilibrium exchange conditions. Since there are no gradients present in this experiment, this stimulation could not be due to indirect coupling effects and must arise as a result of a direct interaction of both K^+ and Cl^- with the sodium transport pathway. Thus this experiment argues strongly in favor of the existence of a KCl-dependent sodium transport pathway in the parotid BLMV preparation.

Kinetics of KCL-Dependent Sodium Uptake

Figure 6 shows the results of an experiment in which KCl-dependent sodium flux was measured as

[2] For example, Cl^--dependent Na^+ movement across the brush-border membrane of the small intestine has been shown to be due to two independent transport systems, a Na^+/H^+ exchanger and a Cl^-/OH^- exchanger [18]. Thus, in this membrane sodium and chloride fluxes are coupled indirectly via $\Delta\mu_{H^+}$. This type of indirect coupling can also be ruled out here since we have been unable to detect any significant component of either Na^+/H^+ exchange or Cl^-/OH^- exchange in our preparation under the experimental conditions employed in our studies (R.J. Turner, J.N. George and B.J. Baum, *unpublished observations*).

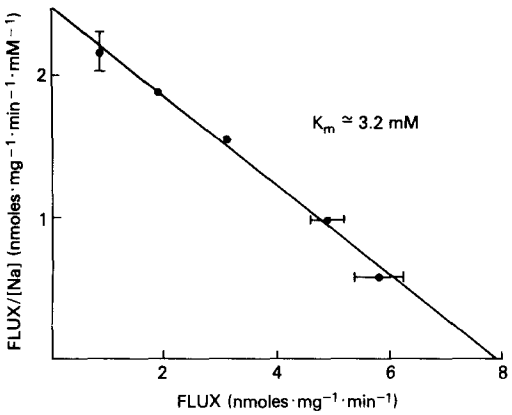

Fig. 6. KCl-dependent sodium flux measured as a function of sodium concentration. Vesicles were prepared in Buffer A containing 1 mM EDTA, 225 mM mannitol and 100 mM NMDG-NO_3. The incubation media were Buffer A containing 100 mM NMDG-NO_3, 100 mM KCl and 0.5 to 12.5 mM NaCl (final concentrations 0.4 to 10 mM). NaCl was replaced isosmotically with NMDG-Cl to obtain the various sodium concentrations tested. Flux observed when chloride was replaced with methyl sulfate was subtracted from all measurements

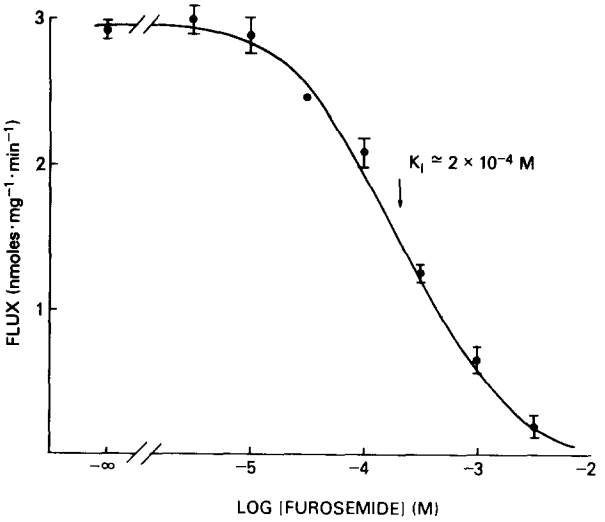

Fig. 7. Inhibition of KCl-dependent sodium flux by various concentrations of furosemide. BLMV were prepared in Buffer A containing 1 mM EDTA, 300 mM mannitol and 100 mM NMDG-NO_3. The incubation media were Buffer A plus 100 mM NMDG-NO_3, 150 mM KCl, 1.25 mM $^{22}NaCl$ and sufficient furosemide to yield the concentrations indicated. Sodium uptake observed when Cl was replaced by methyl sulfate was subtracted from all points

a function of sodium concentration. The data are illustrated as an Eadie-Hofstee plot. The linearity of this plot indicates that the data are fit well by the Michaelis-Menten equation and thus are consistent with the existence of a single KCl-dependent sodium transport system in this preparation. Least-squares analysis of this plot yielded $K_m = 3.2 \pm 0.2$ and $V_{max} = 7.9 \pm 0.3$ with $r = 0.996$. As a routine precaution, in this and the following experiments we have clamped membrane potentials using the NO_3 equilibrium conditions discussed above.

Furosemide Inhibition

The inhibition of KCl-dependent sodium flux by various concentrations of furosemide is illustrated in Fig. 7. The $K_{0.5}$ for furosemide inhibition under these experimental conditions is 2×10^{-4} M.

Dependence on $[K^+]$ and $[Cl^-]$

The dependence of sodium uptake on $[K^+]$ and $[Cl^-]$ is illustrated in Fig. 8. The plot of sodium flux *vs.* $[K^+]$ appears hyperbolic (lower panel) while that of sodium flux *vs.* $[Cl^-]$ is clearly sigmoidal (upper panel). This interpretation is confirmed quantitatively when the data are fit to the Hill equation,

$$\text{flux} = V_m[A^n]/(K_a^n + [A^n]).$$

Least-squares fits yield $K_a = 29.7 \pm 1.4$ mM and $n = 0.87 \pm 0.09$ for K^+ and $K_a = 90.2 \pm 4.0$ mM and $n = 1.31 \pm 0.07$ for Cl^-. As discussed in more detail below, these results indicate the involvement of one potassium ion and multiple chloride ions in the sodium transport event.

Discussion

In this paper we present the results of a series of experiments which characterize the sodium transport properties of rabbit parotid basolateral membrane vesicles. We show that sodium uptake into this preparation is markedly dependent on the presence of both K^+ and Cl^- in the extravesicular medium, being reduced 80% when K^+ is replaced by a nonphysiologic cation and 90% when Cl^- is replaced by a nonphysiologic anion (Table 2). We also show that, relative to KCl equilibrium conditions, sodium uptake is enhanced by both a K^+ and a Cl^- gradient (Table 3) and that sodium transport can be driven against a concentration gradient by a KCl gradient (Fig. 3). The stimulation of sodium flux by KCl is not electrical in nature and thus cannot be due to KCl diffusion potentials acting on a sodium-conductive pathway (Tables 4 and 5). Furthermore, we demonstrate the existence of a KCl-dependent component of sodium flux under equilibrium exchange conditions (Fig. 5). This effect can only be explained by the direct effect of K^+ and Cl^- on the sodium transport pathway. Taken together these

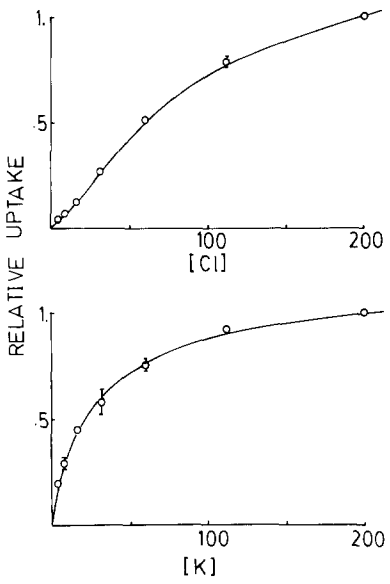

Fig. 8. Dependence of sodium uptake into parotid BLMV on [K] and [Cl]. Vesicles were prepared in Buffer A containing 1 mM EDTA, 100 mM NMDG-NO_3 and 500 mM mannitol. Upper panel: The incubation media were Buffer A plus 100 mM NMDG-NO_3, 1.25 mM $_{22}Na$ methyl sulfate and sufficient concentrations of KCl and K methyl sulfate to yield the final extravesicular chloride concentrations indicated and 200 mM potassium. Lower panel: The incubation media were as for the upper panel except that NMDG replaced potassium to give the concentrations indicated at 200 mM chloride. Data from two independent experiments were normalized to the uptake at 200 mM K (lower panel) or 200 mM Cl (upper panel) and averaged to produce the figure. The lines drawn through the experimental points were calculated from the least-squares fits given in the text

results provide strong evidence for the electroneutral coupling of sodium and KCl movements in this preparation and strongly support the hypothesis that a $Na^+/K^+/Cl^-$ cotransporter, thought to be associated with transepithelial chloride and water movements, is present in the parotid acinar basolateral membrane.

In Fig. 6 we demonstrate that the KCl-dependent component of sodium flux in our parotid BLMV preparation exhibits a simple Michaelis-Menten-type dependence on sodium concentration consistent with the existence of a single transport system with K_m = 3.2 mM at 80 mM KCl and 23°C. The sensitivity of this system to furosemide ($K_{0.5}$ = 2×10^{-4} in Fig. 7) is somewhat lower than that reported for other mammalian $Na^+/K^+/Cl^-$ cotransporters [25]; however, this may be due to the fact that our measurements were not carried out at physiological ionic concentrations or temperatures.

The nearly hyperbolic (n = 0.87 ± 0.09) dependence of sodium flux on potassium concentration shown in Fig. 8 (lower panel) provides good evidence for a Na^+/K^+ stoichiometry of 1 : 1 while the sigmoidal dependence of flux on chloride concentration (Fig. 8, upper panel) indicates that more than one chloride ion is involved in the sodium transport event. A fit of these data to the Hill equation yielded n = 1.31 ± 0.07 for the Cl^-/Na^+ stoichiometry. The derivation of the Hill equation is based on the assumption of strongly cooperative binding sites. In the case of reduced cooperativity the value of n resulting from a Hill-type analysis is expected to be an underestimate of the true stoichiometry, the magnitude of this underestimation increasing with decreasing cooperativity. Thus these results are in good agreement with the proposed 1 : 1 : 2 stoichiometries of the $Na^+/K^+/Cl^-$ cotransporters identified in several other tissues [25, 31]. Note, however, that although the above results provide good evidence for the involvement of one K^+ and two Cl^- in the KCl-dependent sodium transport event, we have not demonstrated directly in this paper that these ions are actually cotransported in this ratio since only sodium fluxes have been measured here (*see* refs. 35 and 36 for a discussion of this point).

The rabbit basolateral membrane vesicle preparation used in our studies was enriched 10 times over the initial gland homogenate in the basolateral membrane marker KpNPPase (Table 1). There was also a modest enrichment (2 times) in the activity of the apical membrane marker dipeptidyl peptidase IV. However, owing to the pyramidal shape of the acinar cells [39], the total area of the basolateral membrane is expected to be at least an order of magnitude greater than that of the apical membrane. Thus the absolute contamination of the BLMV preparation by apical membranes is expected to be very small. The small relative area of the apical membrane may also account for the two peaks of dipeptidyl peptidase IV activity seen in the fractions from the Percoll gradient (Fig. 2). It is expected that at least some plasma membrane fragments will contain regions from both the apical and basolateral surface. Fragments which are predominantly basolateral will copurify with the basolateral membranes and "contaminate" them with their attached apical components. Owing to the large size of the basolateral surface relative to the apical surface, these heterogeneous fragments may account for a significant portion of the apical membranes of the acinar cells and thus a significant portion of the dipeptidyl peptidase IV activity.

$Na^+/K^+/Cl^-$ cotransporters have been studied in vesicle preparations from three other epithelia, the shark rectal gland [6, 11], the renal thick ascending limb of the loop of Henle [5, 15, 16], and the renal cultured cell line LLC-PK1 [1]. The thick ascending limb and the LLC-PK1 cells are absorptive epithelia with the cotransporter localized to the api-

cal membrane, while the shark rectal gland, with the cotransporter localized to the basolateral membrane, is the only other secretory epithelium studied to date. Qualitatively the results from these other vesicle preparations are similar to those presented here in that sodium transport was dependent on the presence of both potassium and chloride and inhibited by loop diuretics. In general, however, the magnitude of the KCl-dependent component of sodium transport is considerably larger in the parotid BLMV preparation described here than in these earlier vesicle systems, particularly when measured relative to the KCl-independent background flux. These observations indicate that the parotid basolateral membrane is relatively free from other KCl-independent sodium transport pathways and may be a richer source of the cotransporter.

In both the thick ascending limb and LLC-PK1 preparations stoichiometric determinations in good agreement with our results were found (i.e., results consistent with a $Na^+/K^+/Cl^-$ stoichiometry of 1 : 1 : 2). The half-saturation constants for sodium and potassium (*cf.* Figs. 6 and 8) in the thick ascending limb preparation (1.3 ± 0.4 and 22.3 ± 9.7 mM, respectively, ref. 16) are close to our values (3.2 ± 0.2 and 29.7 ± 1.4 mM, respectively); however, the half-saturation constant for chloride (15.3 ± 0.7 mM, [16]) is quite different from ours (90.2 ± 4.0 mM). Since these experiments were carried out under very similar conditions with tissue from the same species (rabbit) it is interesting to speculate that this difference in chloride affinity may be related to the tissue of origin, viz., absorptive *vs.* a secretory epithelium.

The results of the present paper provide direct evidence for the existence of a basolateral $Na^+/K^+/Cl^-$ cotransport system in the rabbit parotid and thus strongly support the model for exocrine fluid secretion illustrated in Fig. 1.

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