Regulation of the Sodium Permeability of the Luminal Border of Toad Bladder by Intracellular Sodium and Calcium

Role of Sodium-Calcium Exchange in the Basolateral Membrane

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ABSTRACT Sodium movement across the luminal membrane of the toad bladder is the rate-limiting step for active transepithelial transport. Recent studies suggest that changes in intracellular sodium regulate the Na permeability of the luminal border, either directly or indirectly via increases in cell calcium induced by the high intracellular sodium. To test these proposals, we measured Na movement across the luminal membrane (the Na influx) and found that it is reduced when intracellular Na is increased by ouabain or by removal of external potassium. Removal of serosal sodium also reduced the influx, suggesting that the Na gradient across the serosal border rather than the cell Na concentration is the critical factor. Because in tissues such as muscle and nerve a steep transmembrane sodium gradient is necessary to maintain low cytosolic calcium, it is possible that a reduction in the sodium gradient in the toad bladder reduces luminal permeability by increasing the cell calcium activity. We found that the inhibition of the influx by ouabain or low serosal Na was prevented, in part, by removal of serosal calcium. To test for the existence of a sodium-calcium exchanger, we studied calcium transport in isolated basolateral membrane vesicles and found that calcium uptake was proportional to the outward directed sodium gradient. Uptake was not the result of a sodium diffusion potential. Calcium efflux from preloaded vesicles was accelerated by an inward directed sodium gradient. Preliminary kinetic analysis showed that the sodium gradient changes the V_{max} but not the K_m of calcium transport. These results suggest that the effect of intracellular sodium on the luminal sodium permeability is due to changes in intracellular calcium.

INTRODUCTION

Sodium transport by epithelia such as the toad bladder occurs in two steps; entry into the cell is down an electrochemical gradient and exit is mediated

by sodium, potassium ATPase. When the sodium concentration in the lumen is increased, the net rate of transport saturates, not because of saturation of the ATPase (as might be expected) but rather because of the saturation of sodium entry (12, 43). This behavior of sodium entry makes it the rate-limiting step in transepithelial transport, and hence the sodium channel is a logical site for regulatory influences.

A number of mechanisms, such as a decrease in the transluminal gradient or a reduction in the permeability, could produce saturation. Lindemann et al. (13, 30) found that sudden increases in luminal sodium concentration caused an instantaneous increase in the sodium current that decayed within seconds to a steady level. The peak instantaneous current did not appear to be saturable, whereas the steady-state current was. Because the calculated cell sodium activity did not change, they concluded that external sodium reduces the sodium conductance of the luminal membrane by an allosteric self-inhibition mechanism (31). Recent measurements of sodium activity in some epithelia suggest that intracellular sodium changes upon removal or addition of luminal sodium (15). It is equally plausible, then, that the reduction in luminal permeability on exposure to increasing concentration of external sodium is due also to an increase to cell sodium. Numerous recent studies have implicated increased cell sodium as the signal for a "negative feedback" loop between basolateral and luminal cell membranes (4, 8, 10, 28, 34, 44–46). Changes in the pump rate were found to be accompanied by reciprocal changes in the sodium conductance of the luminal membrane. How increased cell sodium would reduce the luminal sodium conductance remains unknown. The interaction of sodium ions with their channels could lead to saturable behavior, as has been demonstrated for gramicidin channels (11). This interaction is, of course, dependent on channel characteristics such as selectivity, binding constants, and size.

Another possible mechanism involving an indirect interaction has recently been postulated by Grinstein and Erlij (16) and Taylor and Windhager (44). In these proposals, increases in cell sodium increase the calcium influx into the cell, which in turn reduces the sodium conductance much the way that external calcium reduces luminal permeability (6). The mechanism depends on the presence of a sodium-calcium exchanger in the basolateral membrane. The driving force for this exchanger is, therefore, the balance of the electro-chemical gradients for sodium and calcium across this membrane. Changes in cell sodium, therefore, will affect the gradient across the basolateral membrane.

The experiments reported in this paper were designed to examine this mechanism. We demonstrate that reducing the transserosal sodium gradient reduces the luminal sodium permeability. Further, we show that this effect is dependent, in part, on the presence of serosal calcium. Finally, we demonstrate the presence of a sodium-calcium exchanger in purified basolateral membrane vesicles from the toad bladder. These data suggest that the cell calcium activity, which changes in response to the transserosal sodium gradient, may regulate the permeability of the luminal membrane.

METHODS

All studies were performed on the urinary bladder of the Dominican toad *Bufo marinus* (National Reagents, Bridgeport, Conn.). In the intact bladder studies, hemibladders were mounted over nylon mesh onto standard Ussing chambers. The chambers were filled with buffer and bubbled with air. The open-circuit potential difference was recorded via 3 M KCl bridges and calomel cells with an electrometer (Keithley Instruments, Cleveland, Ohio), and those with an open-circuit potential difference of <20 mV were discarded. The bladders were then short-circuited automatically, and the current was recorded.

^{22}Na influx studies were performed on small pieces of toad bladder, using Thompson and Dawson's (45) modification of the method of Biber and Curran (2). Bladders were mounted and glued with Eastman 910 glue (Eastman Kodak Co., Rochester, N.
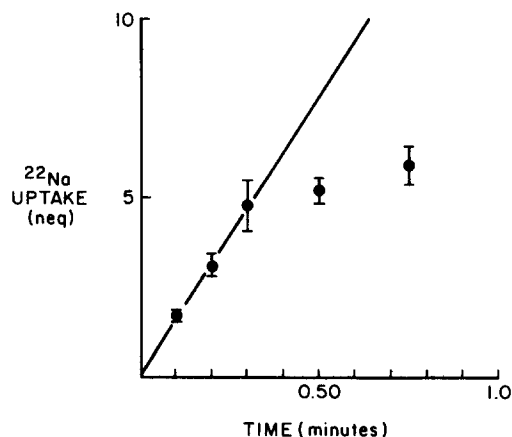

FIGURE 1.   Sodium uptake in toad bladder as a function of time of exposure to mucosal ^{22}Na. The average I_{sc} was 14.4 ± 0.10 neq/min. Sodium uptake was corrected for extracellular contamination, using [^{3}H]mannitol as an extracellular marker. At least three experiments are represented by each point. Mucosal [Na] = 12.5 and serosal [Na] = 112.5.

Y.) onto 2-mm-wide washers cut from siliconized rubber. Each piece, on its washer, was mounted on a small modified Ussing chamber, capped, and placed mucosa down into a 5-ml reservoir containing buffer. 1 ml of buffer was added to the serosal side, and the bladder was short-circuited. Both mucosal and serosal sides were bubbled continuously with air. Bladders whose initial open-circuit potential difference (OCPD) did not exceed 10 mV were discarded. Most bladders, though, had an initial OCPD of >20 mV.

Uptake was measured by briefly (12–18 s) exposing the bladders to ^{22}Na (25 μCi) and [^{3}H]mannitol (50 μCi), inasmuch as preliminary studies showed that uptake was linear with respect to time only for up to 24 s (Fig. 1). After exposure to the isotope, the bladder was washed, blotted on filter paper, cut out with a punch, and dried to a constant weight in a hot-air oven. After the tissue dry weight had been measured, ^{22}Na and ^{3}H were extracted overnight in distilled water, and aliquots were counted in

Bray's solution in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The standard solution used in these experiments, unless otherwise stated, was composed of (millimolar concentrations) NaCl, 110; KCl, 3.5; $CaCl_2$, 1.0; $NaHCO_3$, 2.5. When potassium was omitted, it was without substitution. When mucosal sodium was reduced, choline chloride was added to replace the NaCl. ^{22}Na and [^3H]mannitol were obtained from New England Nuclear, Boston, Mass. and Amersham Corp., Arlington Heights, Ill. A23187 was the generous gift of Dr. R. Hamill, Eli Lilly Co., Indianapolis, Ind.

Calcium transport studies were performed with basolateral membranes obtained from scraped epithelial cells of the toad bladder. The technique used was that of Palmer and Edelman (personal communication). Scraped cells were homogenized in a medium containing 5.7% sucrose, 5 mM Tris, 1 mM EDTA, and 1.0 mM $NaHCO_3$
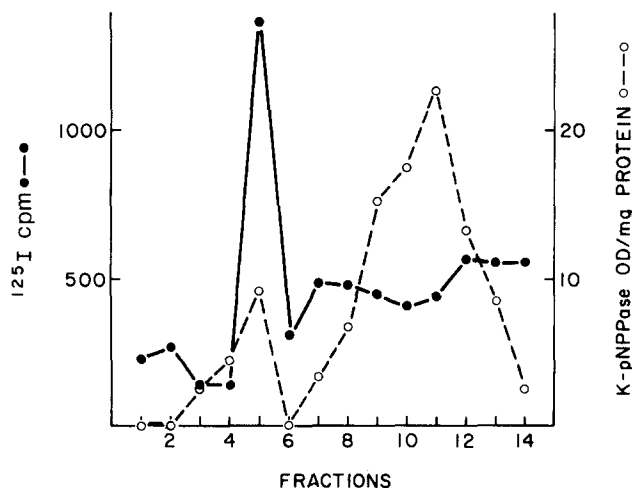

FIGURE 2. Continuous sucrose density gradient of the plasma membrane fraction of toad urinary bladder. Bladders were iodinated from the mucosal side, using glucose oxidase, lactoperoxidose, and ^{125}I (41). The gradient spanned the range 60% (tube 1) to 20% (tube 13).

with a glass Dounce homogenizer, using 11 strokes. Whole cells and nuclei were removed by a single 5-min, 900-g centrifugation. After the mitochondria were removed by two 10,000-g (5-min) spins in a Sorvall RC2B centrifuge (DuPont Instrument-Sorval, Newtown, Conn.), the plasma membranes were pelleted by centrifugation at 22,600 g for 1 h, placed on a continuous sucrose density gradient (ranging from 20 to 60%), and centrifuged overnight at 65,000 g in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.), using an SW-40 rotor. To follow the separation of the membranes, four hemibladders were iodinated on the mucosal side by the method of Rodriguez and Edelman (41) before scraping. The basolateral membrane was followed for enrichment by measuring Na,K ATPase or p-nitrophenyl phosphatase (pNPPase). Fig. 2 shows the separation of two peaks, ^{125}I and K-activated pNPPase, which indicate that this method in fact does separate the

apical and basolateral membranes. In fraction 5, where there was an ^{125}I peak, the K-activated pNPPase was identical to the initial homogenate (not shown). In fraction 11 there was a ninefold enrichment of basolateral membranes. Cytochrome oxidase was measured at all steps of purification, and only in the two pellets obtained after the 10,000-g spins was there significant activity.

For the transport studies, a sucrose step gradient was used to separate the membranes (20, 40, and 60% sucrose). Basolateral membranes were taken from the band between 20 and 40, which corresponded to the peak of pNPPase observed from the continuous gradient. Membranes were centrifuged at 65,000 g for 2 h. These membranes were repelleted at 200,000 g for 1 h. in the buffer that was to be present inside the vesicles.

[45]Ca uptake was measured either by adding isotope to the vesicles and taking aliquots of ~15–30 μg protein or by adding the same quantity of vesicles to isotope-containing buffer. The uptake was terminated by squirting the vesicles onto a prewashed filter and immediately washing with ice-cold buffer (without isotope). This procedure was found to wash away all the extravesicular contamination, as judged by experiments performed with [3H]mannitol as a medium marker. Filters were placed in scintillation vials and counted in Bray's solution or Aquashure (New England Nuclear) in a Beckman scintillation counter. All results are expressed as nanomoles of calcium per milligram of protein. Protein was measured by the method of Lowry as modified by Hartree (18).

RESULTS

Sodium Uptake as a Measure of Influx across the Luminal Membrane

To examine the effects of changing the intracellular ionic milieu on the permeability of the luminal membrane, we measured sodium uptake into the tissue. Under the conditions described in this paper, influx across the luminal membrane is determined as the isotopic rate constant for [22]Na uptake (Fig. 1). Sodium influx is thus the product of the permeability of the luminal membrane and the isotopic electrochemical potential. In the absence of any significant change in cell potential, a change in sodium influx reflects a change in the luminal permeability. Studies with amiloride and ouabain demonstrate this.

In control bladders, sodium influx was identical to the simultaneously measured short-circuit current (I_{sc}) at various luminal sodium concentrations (Fig. 3, Table I). To compare the influx before and after an experimental maneuver in the same bladder, we compared the influx measured after the maneuver to the initial I_{sc}, which is equal to the initial (control) influx. Amiloride, when added to the luminal, side is thought to reduce transport by reducing the permeability of the luminal membrane (17). In our studies, amiloride reduced I_{sc} and influx from 4.6 ($\pm$0.5) to 0.1 ($\pm$0.1) neq/min·mg. Ouabain, which would be expected to inhibit net transport (I_{sc}) but to have no effect on the sodium influx, did just that. I_{sc} fell from 4.4 $\pm$ 0.4 to 1.3 $\pm$ 0.1, whereas there was no change in influx (Table II). As we show below, ouabain does have an effect on influx, depending upon the length of exposure and the luminal sodium concentration.

Reduction in the Transserosal Sodium Gradient

We used two methods to change the sodium gradient across the serosal membrane: (a) we increased the cell sodium, and (b) we reduced serosal sodium.

To increase intracellular sodium, we treated the bladder with ouabain at various mucosal sodium concentrations. Macknight et al. (33) found that the
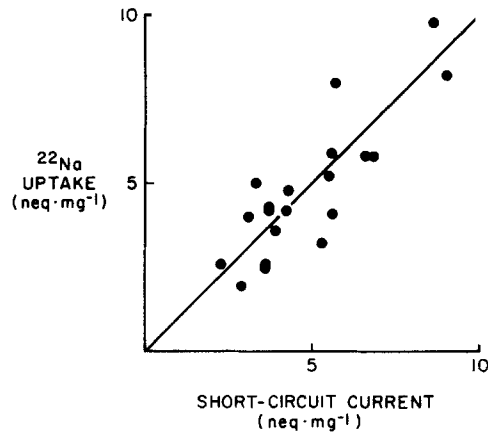

FIGURE 3.　Relationship between sodium uptake and the simultaneously measured I_{sc}. The mucosal sodium concentration was 52.5 mM.

TABLE I

EFFECT OF CHANGES IN THE LUMINAL SODIUM
CONCENTRATION ON THE SODIUM INFLUX AND THE
SIMULTANEOUSLY MEASURED SHORT-CIRCUIT CURRENT

Mucosal sodium concentration	Short-circuit current	Sodium influx
mM	*neq/mg·min*	*neq/mg·min*
2.5 (*n*=3)	3.9±0.5	3.7±0.3
12.5 (*n*=11)	4.9±0.4	4.6±0.5
52.5 (*n*=23)	4.0±0.4	3.8±0.3
70.5 (*n*=3)	9.5±0.8	9.2±0.7

Serosal [Na] was 110 mM. Experiments at each mucosal sodium concentration were performed on different batches of toads.

increase in cell sodium produced by ouabain was due largely to sodium entering from the mucosal side. Hence, the cell sodium concentration would be expected to reflect the mucosal concentration. If increased cell sodium inhibits the luminal permeability, one would expect that the effect of ouabain on the influx would depend on the mucosal sodium concentration. Table II shows that at low mucosal Na (2.5 mM) there was no inhibition of the influx, whereas at higher concentrations there was progressively greater inhibition.

Fig. 4 shows that even at 12.5 mM mucosal Na the inhibition is progressive, such that within 90 min the influx is almost abolished. The gradual reduction in influx is probably the result of the gradual increase in cell sodium (27). Increasing cell sodium by removal of serosal potassium (40) also reduced the influx (4).

Removal of serosal sodium has been shown to reduce I_{sc} (16, 38). To examine the site at which this maneuver inhibited sodium transport, we measured the influx and I_{sc} in toad bladder. We found that removal of serosal sodium reduced both I_{sc} and influx to a similar extent, from 12.4 ± 1.2 to 5.9 ± 0.6 and 5.1 ± 0.5 neq/min·mg, respectively. The decline in current was reversed by mucosal addition of amphotericin B, an agent that increases sodium entry into the cell (Table III*B*) (29). These results demonstrate that the inhibition of net transport was due to a reduction in sodium entry into the cell across the luminal membrane.

TABLE II
EFFECT OF OUABAIN ON SHORT-CIRCUIT CURRENT AND J_i

$[Na]_m$	$[Ca]_s$	Initial I_{sc}	I_{sc}	J_i	J_i/I_{sc} initial
mM	mM		neq/mg·min		%
A. Effect of mucosal Na concentration					
2.5 (n=6)	1.0	4.4±0.4	1.3±0.1	4.4±0.5	100
12.5 (n=7)	1.0	4.8±0.6	0.9±0.2	3.1±0.2	65
52.5 (n=6)	1.0	7.7±0.7	2.0±0.4	4.4±0.8	57
B. Effect of serosal calcium					
52.5 (n=11)	1.0	5.8±0.5	1.6±0.1	1.5±0.1	26
52.5 (n=9)	0.03	5.5±0.8	0.5±0.1	2.4±0.4	44

(*A*) Bladders were exposed to 1.0 mM ouabain on the serosal side for 5 min, after which the short-circuit current and influx were measured simultaneously. [Ca] was 1.0 mM on both serosal and mucosal sides. Values represent means ± SE. I_{sc} is short-circuit current; J_i is sodium influx.

(*B*) The effect of removing serosal calcium on ouabain inhibition (20 min) was studied in bladders exposed to high mucosal sodium (52.5 mM). Because EDTA could not be used in experiments with toad bladder as the transmembrane resistance falls (19), we added verapamil, a drug known to inhibit calcium influx in toad bladder (21). In similarly treated control experiments verapamil had no inhibitory effect on short-circuit current. Sodium influx was lower in the group B than in the group A bladders because of longer exposure to ouabain. (See Fig. 4).

Role of Calcium in Mediating the Effect of a Reduced Transserosal Sodium Gradient

We measured the effect of changing the serosal calcium concentration on the decline in I_{sc} induced by removal of serosal sodium. The inhibition was attenuated by reducing the calcium concentration from 5 to 0.03 mM (Table III*A*). These results are similar to those of Grinstein and Erlij (16). Since the decline in current was shown to be due to a decrease in the influx, these results

demonstrate that the reduced influx is probably related to the change in serosal calcium. We also found that the inhibition of influx caused by ouabain was ameliorated by reduction of serosal calcium from 1 to 0.03 mM (Table IIB). Both of these studies suggest that the effect of the transserosal sodium gradient on the sodium influx is mediated by serosal calcium.[1]

We examined the effect of directly increasing cell calcium on the sodium influx. The calcium ionophore A23187 inhibits I_{sc} in the toad bladder (32, 47), an effect dependent on the presence of calcium, and we found that it reduced both influx and I_{sc} from 6.4 (±0.5) neq/min·mg to 2.6 (±0.3) and 2.7 (±0.4), respectively. This suggests that the reduction in net transport by the ionophore was due to a reduced permeability of the luminal membrane. A23187 is, however, a potent metabolic inhibitor (39), and because amphotericin did not restore the current in our experiments it is not certain that the fall in influx is due only to an increase in cell calcium, nor that the effect of the ionophore is only at the luminal membrane.
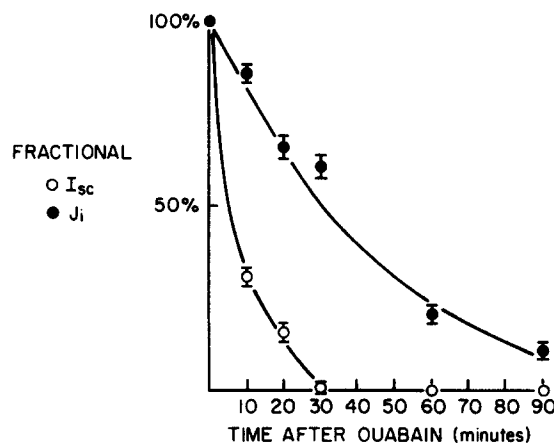

FIGURE 4.   Effect of prolonged incubation in 1 mM ouabain on the sodium influx of toad bladder. The sodium influx was measured as the ^{22}Na uptake in 12 s. The mucosal sodium concentration was 12.5 mM. Six experiments are represented by each point.

Sodium-Calcium Exchange in Basolateral Membrane Vesicles

The fact that lowering the transserosal sodium gradient reduced the sodium influx and that this effect was dependent on serosal calcium suggested the presence of a sodium-calcium exchanger in the basolateral membrane. Calcium uptake was studied in basolateral membranes that were vesicular upon electron microscopy and that behaved like vesicles in that they contained an osmotically active space.

[1] Unlike frog skins, toad bladders rapidly deteriorate upon complete removal of serosal calcium (19). This might explain why we only observed partial effects while Grinstein and Erlij (16) found that chelation of serosal calcium completely prevented the fall in I_{sc} induced by removal of serosal sodium.

The effect of transmembrane ion gradients on calcium uptake was studied by loading the vesicles with 160 mM salt and diluting them in a medium of the same anion, with either a different or a similar cation concentration. When an outward directed sodium gradient was present,^{45}Ca uptake was greatly enhanced (Fig. 5).[2] A reversed sodium gradient (choline inside, sodium outside) resulted in a ^{45}Ca uptake that was lower than the uptake in the absence of a gradient (Fig. 5). Further, ^{45}Ca efflux from preloaded vesicles was greatest when there was an inward directed sodium gradient (Table IV), a finding similar to that of Gmaj et al. (14).

TABLE III

EFFECT OF REMOVING SEROSAL SODIUM ON SHORT-CIRCUIT CURRENT

A. Effect of calcium

Serosal [Ca]	Initial I_{sc}	Final I_{sc}	Fall
mM	μA	μA	%
5.0 (n=15)	205±38	109±20	46±5
1.0 (n=7)	202±58	128±36	36±5
0.03 (n=15)	212±39	150±28	29±9

B. Reversal with amphotericin

Initial I_{sc}	I_{sc} after Na$_s$ removal	I_{sc} after amphotericin	I_{sc} after ouabain
μA	μA	μA	μA
111±10 (n=6)	52±11	87±10	1±1

(A) I_{sc} was measured before and after removal of serosal sodium in the presence of various concentrations of serosal calcium. Mucosal sodium concentration was 12.5 mM. The percent fall at 5.0 mM Ca was significantly greater than the percent fall at 0.03 mM (P < 0.05). Final I_{sc} was measured 5 min after replacement of serosal sodium with choline chloride.

(B) After inhibition of I_{sc} by removal of serosal sodium, amphotericin was added to the mucosal side. After 20 min the I_{sc} was recorded, and ouabain (1.0 mM) was added to the serosal side.

We also studied the effect of lanthanum on calcium transport and found that its effect depends on the side to which it is added. Lanthanum inhibited sodium-calcium exchange when it was added to the same side as calcium (Table V), and the magnitude of this inhibition was greater at a higher lanthanum concentration. When lanthanum was present on the opposite side

[2] We did similar experiments at 110 mM salt, a more physiological solution for amphibia, and obtained identical results. Sodium-loaded vesicles took up 1.53 ± 0.11 nmol of calcium at 0.20 min when diluted into sodium, compared to 3.95 ± 0.48 nmol when diluted into choline chloride. Results are expressed per milligram of protein.
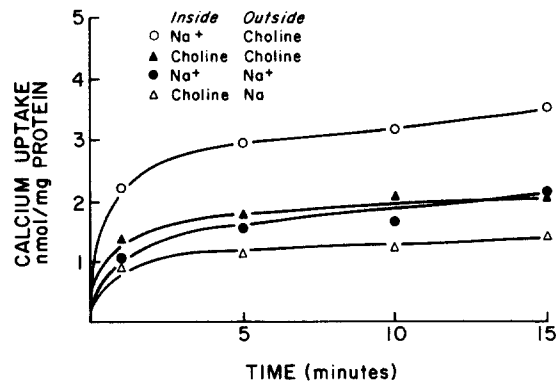
FIGURE 5. ^{45}Ca uptake in basolateral vesicles obtained from toad urinary bladder. Vesicles were loaded with 160 mM NaCl or 160 mM choline chloride and diluted into medium containing either 160 mM NaCl or 160 mM choline chloride. Final outside [Na] in choline outside experiment (O) was 6 mM. All points in this experiment came from the same batch of vesicles prepared from twelve toads.

TABLE IV

^{45}Ca EFFLUX IN BASOLATERAL VESICLES FROM THE TOAD BLADDER

	Inside	Outside	Calcium content	Sodium-dependent calcium efflux
			nmol/mg	
A. Without lanthanum				
a. Initial calcium content			13.62±0.89	
b. Choline		Choline	10.01±0.29	
c. Choline		Sodium	8.29±0.23	Δ = 1.72±0.10
B. With lanthanum (0.1 mM)				
a. Initial calcium content			15.16±1.21	
b. Choline		Choline	10.13±0.26	
c. Choline		Sodium	8.31±0.12	Δ = 1.82±0.08

Vesicles were preincubated in 160 mM choline chloride, 20 mM MOPS, 0.1 mM CaCl$_2$ buffer with ^{45}Ca for 1 h. Aliquots of vesicles were diluted 1/30 into buffer without isotope to which 0.2 mM EGTA had been added. After precisely 18 s of efflux, the vesicles were squirted onto a Millipore filter and filtration apparatus and washed twice with cold buffer. The starting calcium content of the vesicles was measured by taking aliquots of vesicles and placing them directly onto a filter, without previous dilution. $n = 3$ for initial points; $n = 5$ for others.

(i.e., the high sodium side) there was no inhibition of sodium-dependent calcium transport (Table IV). These results are similar to those of Katzung et al. (23), whose studies on sodium-calcium exchange in muscle and nerve demonstrated no effect of lanthanum when it was present on the sodium side and those of Baker et al. (1), who found that lanthanum inhibited sodium-calcium exchange from the calcium side.

To demonstrate that the ^{45}Ca uptake induced by the gradient represented actual calcium movement into the vesicle space rather than binding of ^{45}Ca to the surface, we performed the experiments presented in Fig. 6. In both the

TABLE V

EFFECTS OF LANTHANUM ON SODIUM-DEPENDENT
CALCIUM UPTAKE

Outside solution:	Calcium uptake		Sodium-dependent calcium uptake
	Sodium	Choline	
	nmol/mg protein		
I. Without La^{+++}	1.07±0.04	4.13±0.53	3.06±0.13
With La^{+++} (0.1 mM)	0.50±0.01	1.43±0.21	0.93±0.05
II. Without La^{+++}	1.35±0.10	6.13±0.73	4.78±0.18
With La^{+++} (1.0 mM)	0.04±0.01	0.08±0.03	0.04±0.01

Aliquots of vesicles loaded with 160 mM NaCl, 20 mM MOPS, 0.1 mM CaCl$_2$, pH 7.5, were diluted into either NaCl or choline chloride buffer containing ^{45}Ca. LaCl$_3$ was added in the concentrations noted above. In these experiments the vesicles were preincubated with LaCl$_3$ for 0.5 h before the experiment. n = 6 for each experiment.
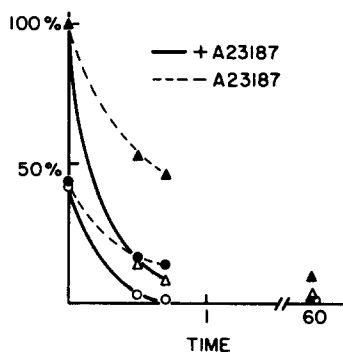

FIGURE 6.   Release of calcium from vesicles with A23187. Vesicles containing 160 mM NaCl MOPS were diluted 1/35 in either NaCl (O, ●) or choline chloride (△, ▲) MOPS buffer containing ^{45}Ca. Calcium uptake was measured after 5 min and expressed as percent of the maximum uptake when choline was the external cation (cf. Fig. 5). Immediately after the 5-min uptake was measured, EGTA was added, to yield a final concentration of 1 mM at T = 0. 10 s later either A23187 (1 μM), carried in dimethyl sulfoxide (DMSO), or DMSO alone was added to the vesicles, and aliquots of the vesicles were taken 15 and 30 s later for the measurement of content. A final aliquot was taken at 1 h. Each point represents the average of two experiments.

presence and the absence of an outward directed sodium gradient, the calcium ionophore A23187 accelerated the efflux of calcium from these vesicles, indicating that the ^{45}Ca taken up was indeed inside the vesicles.

It was possible that the increase in ^{45}Ca uptake in the presence of an outward directed sodium gradient was simply the result of a sodium diffusion potential. To estimate the membrane potential, we measured the uptake of a

TABLE VI

SIMULTANEOUS MEASUREMENT OF CALCIUM AND MTPP$^+$ UPTAKE

Inside	Outside	MTPP$^+$ uptake	Ca uptake
		pmol/mg	nmol/mg
Na	Na	209±15	1.3±0.1
Na	K	177±23	2.7±0.3
K	Na	834±48	1.2±0.1
K	K	227±21	1.6±0.2

Aliquots of vesicles were diluted 1/30 in buffer containing ^{45}Ca and MTPP (^3H). Uptake was measured over 6 s. The buffers were either Na or K (160 mM) gluconate buffered with 20 mM MOPS and Tris to which 200 μM calcium gluconate was added. Cl was present only as $^{45}CaCl_2$ (25 μM). Calcium uptake was calculated using 200 μM as the "cold" calcium activity, although in fact the activity was probably lower due to the high affinity of gluconate for calcium (5). It is, therefore, not possible to compare absolute calcium transport rates between the chloride and gluconate experiments without direct measurements of calcium activity. $n = 5$ for each experiment.

TABLE VII

EFFECT OF EXTERNAL SODIUM ON CALCIUM UPTAKE

Na$_o$	V_{max}	K_m
	nmol/mg · min	μM
155.0	47±17	272±262
5.0	140±15	260±61
3.0	220±19	250±48
2.2	260±17	255±37
1.7	305±14	251±25

Saturation curves measuring initial rates of calcium uptake as a function of external calcium concentration were obtained in the presence of various concentrations of extravesicular Na. V_{max} and K_m (±SE) were obtained from Eadie-Hofstee plots. Experiments were performed by diluting sodium-loaded vesicles into choline buffer containing ^{45}Ca. The extravesicular sodium concentration was adjusted by varying the dilution of the vesicles. For instance, diluting the vesicles 1:50 would yield a [Na] of 155/50 or 3.0. The K_m's were not different. We were able to obtain a value for V_{max} in the absence of extravesicular sodium by plotting V_{max} vs. [Na]$_o$ This yielded a value of 373 nmol/mg·min. For each line drawn, $n = 20$.

lipophilic cation [^3H]methyltriphenyl phosphonium bromide (MTPP$^+$) (42). MTPP$^+$ uptake was maximal in the presence of outward directed potassium gradients (Table VI) and minimal in the presence of an outward directed

sodium gradient, indicating that these vesicles are more permeable to potassium than to sodium, as can be predicted from the Koefoed-Johnsen–Ussing model (24). It is also apparent in Table VI that there was maximal calcium uptake only in the presence of an outward directed sodium gradient, suggesting that calcium uptake was not driven by an inside negative membrane potential.

We studied the details of the interaction between sodium and calcium by measuring apparent initial rates (6 s) of ^{45}Ca uptake at various sodium and calcium concentrations. Since we are not certain that these are true initial rates, the following results should be considered as preliminary estimates. The presence of increasing extravesicular sodium had no effect on K_m, although there was a progressive reduction in V_{max} (Figs. 7 and 8 and Table VII).
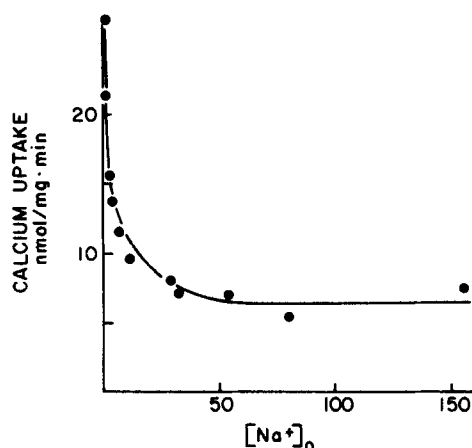

FIGURE 7. Inhibition of ^{45}Ca influx by sodium. The effects of the transvesicular sodium gradient on ^{45}Ca influx were studied in basolateral membrane vesicles prepared from 24 toads. Aliquots of 160 mM NaCl MOPS-loaded vesicles were diluted into ^{45}Ca-containing choline chloride MOPS buffer. The dilution of the NaCl by the choline was adjusted to give different extravesicular sodium concentrations. Uptake was measured by stopping the reaction after 6 s. The final [Ca] was 85 μM. Each point represents the mean of at least five experiments.

The effect of other alkali metal cations on calcium uptake was studied, and the data in Table VIIIA indicate that Cs and Li both were more inhibitory than Na. K was clearly inhibitory to Ca uptake, although not to the same extent as Na, Li, and Cs. Because cell potassium is so plentiful, it was important to examine whether potassium could replace sodium in the exchange with calcium. In the absence of extra-vesicular sodium (Table VIIIB), potassium-loaded vesicles took up as much calcium as did sodium-loaded vesicles. In an experiment under what might be considered in vivo conditions, in which sodium and potassium gradients were in opposite directions, calcium
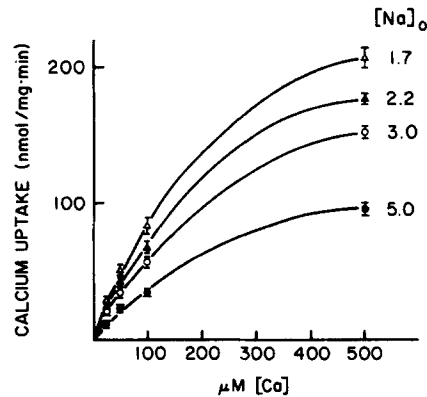
FIGURE 8.   Calcium saturation curves at various extravesicular sodium concentrations. NaCl-loaded vesicles were diluted into choline chloride, and calcium uptake was measured after 6 s, as a function of external calcium concentration, by varying the extravesicular cold calcium. A family of curves at various $[Na]_o$ was generated by varying the dilution of the vesicles (which contained 160 mM NaCl) into choline chloride (see Fig. 7). Each point represents the mean of at least six experiments. Eadie-Hofstee calculations of maximal rates of uptake are given in Table VII.

TABLE VIII
EFFECT OF ALKALI METAL CATIONS ON CALCIUM UPTAKE

Inside	Outside	Calcium uptake
		nmol/mg protein
A. Inhibition of calcium uptake (0.15 min)		
Na	Cs	0.77±0.02
Na	Li	0.91±0.04
Na	Na	0.96±0.02
Na	K	1.06±0.04
Na	Choline	2.75±0.01
B. Calcium uptake in presence of K^+ or Na^+ gradients (0.15 min)		
Na	Na	2.80±0.32
Na	Choline	4.99±0.37
K	K	3.18±0.34
K	Choline	4.95±0.30
C. Calcium uptake in presence of K^+ and Na^+ gradients (15 min)		
Na	Na	1.54±0.05
Na	K	2.27±0.07
K	K	1.51±0.04
K	Na	1.18±0.05

(A) Vesicles loaded with Na were diluted 1/30 in medium containing 160 mM of chloride salts of the alkali metal cations listed. Reaction was measured after 0.15 min. Final [Na] of the external medium was 6 mM in A and B and 7 mM in C when sodium was not the outside buffer. $n = 6$ for each experiment.

(B and C) Vesicles loaded with either Na or K were diluted 1/30 into the buffers on the right, and uptake was measured over time indicated. $n = 8$ for each experiment.

was taken up over control only in sodium-loaded vesicles diluted in potassium (Tables VI and VIIIC).

DISCUSSION

In these studies we have demonstrated that a decrease in the transserosal sodium gradient reduces sodium influx across the luminal membrane, and that this probably represents a reduction in luminal permeability. Because sodium influx across the luminal border is the product of the permeability of the luminal membrane and the transluminal electrical potential, a reduction in ^{22}Na uptake could be due either to a reduction in permeability or to a change in the transluminal potential (cell becoming more positive).

Three lines of evidence suggest that the decline in influx is due to reduced permeability. First, under certain conditions (Fig. 4 and reference 4) the influx is abolished. If the change in cell potential accounted for this near-zero influx, the potential across the luminal border would have to be positive inside under short-circuit conditions, clearly an unlikely event. Second, measurement of cell potential in toad bladder showed that ouabain has little or no effect in the short-circuited state (A. Finn, personal communication). Nor does removal of serosal potassium affect the cell potential in the short-circuited state (9). Finally, using a variety of direct and indirect methods, numerous investigators have found that the resistance of the luminal membrane in tight epithelia increases when intracellular sodium is increased by ouabain (20, 22, 36, 45, 46).

These results show that increasing intracellular sodium reduces the sodium permeability of the luminal membrane. Since the effect of the transserosal gradient was dependent on the presence of serosal calcium and because of the presence of a Na-Ca exchanger in the serosal membrane, we suggest that the effect on luminal sodium permeability was exerted through a change in cytosolic calcium activity. Many of the experiments used maneuvers that are rather harsh, e.g., removal of serosal sodium, calcium, or potassium and the addition of ouabain. Although under such conditions calcium may play a dominant role in "gating" the luminal sodium channels, using these results to explain physiological regulation of sodium transport should be approached with caution. Can calcium play a role in the saturation of transport through the luminal membrane? We estimate below the driving force for the sodium-calcium exchanger and suggest that changes in luminal sodium concentration result in a change in the driving force for this exchanger that is in the correct direction for this mechanism to play such a role under the prevailing physiological conditions.

To estimate the driving force, we assumed that only sodium and calcium are transported by this exchanger and that potassium can be ignored, even though the data in Table VIIIB show that potassium gradients in the absence of sodium can drive calcium uptake. The basis for this assumption is given in Tables VI and VIIIC, which show the effect on calcium uptake of potassium gradients in the presence of opposing sodium gradients. These experiments attempt to reproduce the gradients known to occur across cell membranes, i.e., opposing sodium and potassium gradients. Gluconate or chloride salts

were also used to test for the effect of permeable anions. Calcium was transported only in the presence of an oppositely directed sodium gradient. These results suggest that the binding constant at the sodium "site" has a high Na-K selectivity ratio. The results demonstrating that potassium gradients (in the presence of choline) cause an uptake of calcium indicate that the binding constant for potassium is finite and much greater than that for choline. The quantitative relations among these three ions must await more formal kinetic analysis. However, qualitatively at least, it appears that for conditions similar to the physiological state the operation of the sodium-calcium exchanger is not affected by the presence of potassium to any significant degree.

To calculate the direction of the basolateral exchange, one needs to know electrochemical gradients for sodium and calcium and the stoichiometry (n) of the process. In muscle and nerve most investigators have obtained a value of 3 Na:1 Ca (3, 37). Our studies suggest that the stoichiometry is also 3 sodium:calcium.[3] Using the value of 3 as the stoichiometry, the open-circuit electrical measurements of Narvarte and Finn (35), the cell calcium activity obtained by Lee (25, 26) and an estimate of cell sodium activity (Table IX), one can calculate the direction of the sodium-calcium exchange by calculating the electrochemical potentials for each ion $(\Delta\tilde{\mu})$

$$\Delta\tilde{\mu} = RT \ln \frac{\gamma_o}{\gamma_i} + zF \Delta\psi,$$

where γ is the activity, z is the valence and R, T, and F have their usual meanings. At zero net transport

$$n \, \Delta\tilde{\mu}_{Na} = \Delta\tilde{\mu}_{Ca}.$$

Hence, the driving force for the exchange is $3 \, \Delta\tilde{\mu}_{Na} - \Delta\tilde{\mu}_{Ca}$. Table IX shows that, when mucosal sodium is low, the exchanger operates in a calcium out, sodium in mode; when mucosal sodium is high, calcium moves in and sodium out.

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[3] We calculated the stoichiometry (n) using a Michaelis-Menten treatment of calcium uptake (J_{Ca}) inhibited by external sodium (Na_o)

$$\frac{1}{J_{Ca}} = \left[\frac{1}{J_{Ca}^{max}} \left(1 + \frac{K_{Ca}}{[Ca]} \right) \right] \left(1 + \frac{[Na]_o^n}{K_{Na}} \right). \tag{1}$$

If the values of Table VII are used, this relation is simplified, because they were obtained at "infinite" [Ca]. By rearrangement, the logarithmic form of Eq. 1 evaluated at infinite [Ca] gives the well-known Hill relationship:

$$\ln \left(\frac{J_{Ca}^{max} - J_{Ca}}{J_{Ca}} \right) = n \ln [Na]_o - \ln K_{Na}.$$

J_{Ca}^{max} was obtained by extrapolation of the values in Table VII and thus is probably an overestimate. The calculated value of n varied between 2.3 and 3.3. Because the J_{Ca}^{max} could not be uniquely determined and because of uncertainty regarding J_{Ca} values as initial rates, this estimate is tentative but similar to published values for muscle and nerve (3, 37).

TABLE IX

ESTIMATES OF THE DRIVING FORCES FOR Na AND Ca
ACROSS THE SEROSAL BORDER AT VARIOUS LUMINAL
SODIUM CONCENTRATIONS

	$Na_m = 110$ mM	$Na_m = 2.4$ mM
V_{cs}, mV	26.1	7.1
V_{mc}, mV	23.6	4.8
Na_s, mV	110	110
Na_c, mV	11	4
$\dfrac{\Delta\bar\mu_{Na}}{F}(cs)$, mV	86	94
Ca_s, mM	1.0	1.0
Ca_c, μM	0.1	0.1
$\dfrac{\Delta\bar\mu_{Ca}}{F}(cs)$, mV	286	256
$\left(3\dfrac{\Delta\bar\mu_{Na}}{F} - \dfrac{\Delta\bar\mu_{Ca}}{F}\right)$, mV	−28	+28

V (membrane potential) were obtained from Narvarte and Finn (32). Subscripts "c" refers to cell, "s" to the serosal medium, and "m" to the mucosal medium. Na_c and Ca_c are intracellular activities. $\dfrac{\Delta\bar\mu}{F}$ is the electrochemical gradient (expressed in millivolts). $3\dfrac{\Delta\bar\mu_{Na}}{F} - \dfrac{\Delta\bar\mu_{Ca}}{F}$ is the driving force for an exchanger that transports 3 Na per calcium. When $3\dfrac{\Delta\bar\mu_{Na}}{F} - \dfrac{\Delta\bar\mu_{Ca}}{F}$ is negative, the exchanger operates in a sodium out, calcium in mode; when positive, in the reverse direction.

More direct measurement of cytosolic calcium activity under physiological conditions, as well as measurements of the effects of these calcium activities on the sodium conductance of the luminal membrane, are needed. The latter studies will have to be performed in isolated luminal membrane vesicles, a system that will also allow the study of the direct effect of sodium on its own conductance. Until such studies are completed it will not be possible to quantify the role of each of these mechanisms in the saturable behavior of the luminal membrane.

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