Calcium Reduces the Sodium Permeability of Luminal Membrane Vesicles from Toad Bladder

Studies Using a Fast-Reaction Apparatus

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ABSTRACT Regulation of the sodium permeability of the luminal membrane is the major mechanism by which the net rate of sodium transport across tight epithelia is varied. Previous evidence has suggested that the permeability of the luminal membrane might be regulated by changes in intracellular sodium or calcium activities. To test this directly, we isolated a fraction of the plasma membrane from the toad urinary bladder, which contains a fast, amiloride-sensitive sodium flux with characteristics similar to those of the native luminal membrane. Using a flow-quench apparatus to measure the initial rate of sodium efflux from these vesicles in the millisecond time range, we have demonstrated that the isotope exchange permeability of these vesicles is very sensitive to calcium. Calcium reduces the sodium permeability, and the half-maximal inhibitory concentration is 0.5 μM, well within the range of calcium activity found in cells. Also, the permeability of the luminal membrane vesicles is little affected by the ambient sodium concentration. These results, when taken together with studies on whole tissue, suggest that cell calcium may be an important regulator of transepithelial sodium transport by its effect on luminal sodium permeability. The effect of cell sodium on permeability may be mediated by calcium rather than by sodium itself.

INTRODUCTION

Sodium crosses tight epithelia such as the toad bladder in two steps; it enters the cell passively down its electrochemical gradient and is then actively transported across the basolateral membrane by the sodium-potassium ATPase (26). Sodium entry across the luminal membrane is the rate-limiting step in transepithelial transport, and regulation of transport is accomplished primarily by changes in the sodium permeability of the luminal membrane (11, 14, 47, 49, 53).

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Although the luminal and basolateral membranes have different ion permeabilities, they are not independent of each other (13, 38, 46). Among the conditions in which membrane-to-membrane communication occurs is the so-called negative feedback effect, first noted by MacRobbie and Ussing (38), in which inhibition of the basolateral pump reduces the sodium permeability of the luminal membrane (5, 7, 12, 22, 23, 33, 44, 52, 54). Another example is the effect of changing the mucosal sodium concentration on luminal and basolateral ion permeability. An increase in the mucosal sodium concentration decreases the luminal sodium permeability and increases the basolateral potassium conductance (9, 15, 20, 35, 42). This inverse relationship between mucosal sodium concentration and luminal membrane sodium permeability allows the cells to maintain a high rate of transport when the luminal sodium concentration is low and prevents the cell from excessive sodium gain when luminal sodium is high.

A number of possible mechanisms could mediate the effect of mucosal sodium on the luminal sodium permeability. Lindemann (34–36) suggested that sodium, acting on the external surface, exerts a self-inhibitory effect on the membrane sodium permeability, causing saturation of the rate of sodium transport as the mucosal sodium concentration increases. In these studies, the calculated cell sodium did not change appreciably upon increasing mucosal sodium and the authors concluded that the effect of increasing mucosal sodium on luminal sodium permeability was mediated at the external face of the sodium channel. In epithelia where cell sodium activity has been measured with ion-selective electrodes, however, it has been demonstrated that the cell sodium activity increases as the luminal sodium concentration is raised (11, 18). Thus, the reduction in luminal sodium permeability caused by an increase in luminal sodium concentration could be due to the effect of an increase in cell sodium rather than the increase in extracellular sodium. Intracellular sodium could regulate luminal sodium permeability either directly or indirectly through an intermediate such as calcium. Grinstein and Erlij (19) and Taylor and Windhager (50) have suggested that changes in cell sodium were coupled to changes in cell calcium by the action of a sodium-calcium exchange mechanism in the basolateral membrane and that cell calcium regulated the permeability of the luminal membrane.

To test these proposals, we measured the permeability of the luminal membrane of intact toad bladder and found that an increase in cell sodium, under a variety of conditions, decreased the sodium permeability of the luminal membrane (4, 5). We also found that this change depended on the presence of serosal calcium, a result that further strengthened the argument that the effects of an increase in cell sodium on luminal sodium permeability were indirect and mediated by cell calcium.

The notion that calcium regulates the permeability of the luminal membrane sodium permeability has much appeal, not only because of the numerous examples of calcium-regulated cellular events, but also because calcium could mediate other changes known to occur in concert with changes in the luminal sodium permeability. It is becoming evident that variations in the rate of sodium entry into the cell across the luminal membrane are associated
with reciprocal changes in the basolateral potassium conductance (9, 20, 47). Because sodium and potassium are exchanged by the basolateral pump, any increase in sodium entry and net sodium flux through the pump requires a commensurate increase in passive potassium efflux across the basolateral membrane, lest the cell gain potassium. Calcium could be responsible for both the reduction in luminal sodium permeability and the increase in basolateral potassium permeability (47). There is evidence from many tissues that calcium can increase the potassium conductance of membranes (30, 40, 41, 43), including data from Germann and Dawson (17) and Bello-Reuss et al. (3) that this occurs in epithelia.

Much of the evidence implying a role for cell calcium in the regulation of the luminal sodium permeability, however, has been indirect. To provide more compelling results, it is necessary to demonstrate the existence of a sodium-calcium exchanger in the basolateral membrane, to show that cytosolic calcium changes under varying physiological conditions and to provide evidence that calcium, in these concentrations, directly affects the sodium permeability of the luminal membrane. Some of these questions have already been answered. We recently demonstrated the existence of a sodium-calcium exchanger in the basolateral membrane of toad bladder (5). Also, Lorenzen et al. (37) and Lee et al. (32) showed that changes in cell calcium activity in Necturus tubule were coupled to the sodium gradient across the serosal membrane. As yet, unfortunately, no measurement of cell calcium as a function of luminal sodium is available.

The present study was undertaken to test directly the effects of calcium on the sodium permeability of luminal membrane vesicles of the toad bladder. To measure sodium permeability in vesicles and study these effects, it is necessary to obtain initial rates of isotope flux. Because the sodium permeability of the luminal membrane is $\sim 2.5 \times 10^{-5}$ cm/s in whole bladders at a low luminal sodium concentration (5, 35, 53), a vesicle of 0.3 μm diam will exchange half of its contents within $\sim 140$ ms. To perform these studies, therefore, we had to use a fast-reaction method. We report here results of experiments using a flow-quench method that demonstrate the presence of a fast, amiloride-sensitive sodium flux in membrane fractions of the toad bladder enriched with a luminal marker. The sodium permeability of these membranes, as measured by this flux, is exquisitely sensitive to calcium in concentrations known to be present in the cytosol. Also, there is little effect, if any, of sodium on its own permeability.

**METHODS**

**Preparation of the Membranes**

The procedure for isolation of luminal membranes has been described in detail in a previous publication (5). However, because there have been important modifications in the procedure, we will describe it briefly, highlighting the changes. Toads (*Bufo marinus*) obtained from National Reagents, Bridgeport, CT, were doubly pithed. The hearts were perfused with Ringer’s buffer containing, in millimoles/liter: 110 NaCl, 3.5 KCl, 2.5 NaHCO₃, and 1.0 CaCl₂. When the bladders became bloodless they were removed and placed in a beaker of ice-cold Ringer’s buffer, cut open, and blotted
with filter paper. The cells were scraped with a glass slide and placed in homogenizing buffer made of 5.7% sucrose, 1.0 mM NaHCO₃, 1.0 mM EDTA, and 5.0 mM Tris base and brought to pH 8.2 with HCl. After a single wash, the scraped cells were homogenized using 11 strokes in a tight-fitting Dounce homogenizer in a volume five times the wet weight of the cells. The homogenate was centrifuged in a Sorvall RC2B (Dupont Instruments-Sorvall Biomedical Div., Wilmington, DE) centrifuge at 900 g for 5 min to separate whole cells and nuclei from the membranes contained within the supernatant. Mitochondria were next removed by two 5-min spins at 10,000 g.

The plasma membranes were then pelleted by centrifugation at 22,600 g for 30 min and placed on a discontinuous sucrose gradient made of three steps, 20, 40, and 60% sucrose (wt/wt) in homogenizing buffer. The membranes were centrifuged at 65,000 g for 1 h in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) using an SW-40 rotor. Membranes lighter than 40% sucrose formed a band at the 20–40% interface and were predominantly basolateral, based on our studies of sodium-potassium ATPase. Membranes heavier than 40% sucrose formed a band at the 40–60% interface and, based on our previous and present studies, were predominantly luminal membranes. These membranes were washed and re-repelled twice in the buffer to be used during the experiment. The membrane pellet was resuspended into vesicles by adding ~500–1,000 μl of buffer to 400–600 μg of membrane protein. With this method of membrane preparation each toad bladder yielded ~75 μg of protein (see below). The membranes were drawn in and out of a syringe, first with a 22-gauge needle, next with a 26-gauge needle, and were sonicated with a Branson bath sonicator (Branson Sonic Power Co., Danbury, CT) for 60 s.

After the completion of most of the studies in this paper, we noticed that the luminal membrane vesicles, when left to stand at room temperature, became surrounded by a stringy fibrous material. This material was loosely attached to the membranes, and the fact that mild treatments such as cooling the membranes or incubating them in a high-salt buffer (see below) could remove it suggests that they are not integral membrane proteins. Also, when present in a high concentration, they formed a gel, which strongly suggests that they were composed of cytoskeletal proteins. Indeed, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the material showed several proteins, the most prominent being a 43,700-dalton protein that is probably actin, whose molecular weight is 41,900 (27). Other proteins were also noted. Of course, more definitive immunochemical methods need to be performed in order to unequivocally identify these proteins.

We developed two techniques for removing these proteins so that the results of our studies on the effects of ions and inhibitors would not be confounded by the presence of the cytoskeleton. After the luminal vesicles were harvested from the 40–60% sucrose band, they were diluted in homogenizing buffer and spun at 22,000 g for 15 min. The pellet was resuspended in a high-salt buffer containing 500 mM NaCl, 1.0 mM MgCl₂, 1 mM EGTA, and 5 mM Tris, brought to a pH of 7.5 with H₂SO₄. The membranes were placed on ice for 20 min and then were repelled at 22,000 g for 15 min. The supernatant, containing about one-third of the original protein, was poured off and the pellet was resuspended in the buffer in which the experiment was going to be performed. As the pellet was broken up by drawing it in and out of a 18-gauge needle attached to a 1.0-ml tuberculin syringe, it was clear that there were stringy fibrous strands which were easily pelleted at 1,230 g for 10 min, leaving membranes in the supernatant. The membranes were then pelleted at 22,000 g for 15 min, and the final membrane pellet represented ~20% of the original protein obtained from the 40–60% band on the sucrose gradient.

A second, simpler method of removing cytoskeletal proteins was to place the
resuspended luminal vesicles on ice for 1 h. During this time, the easily recognizable stringy material (see above) settled to the bottom and could be pelleted by centrifuging at 400 g for 2 min. The supernatant containing the luminal vesicles could easily be removed. Removal of the cytoskeletal proteins had no effect on the efflux rate coefficient, but it did increase the sodium space per milligram of protein (see Table II).

Efflux Studies

The efflux of $^{22}\text{Na}$ from luminal membrane vesicles was measured using a rapid-reaction apparatus of the flow-quench type modeled after that of Thayer and Hinkle (51). Fig. 1 shows a schematic diagram of the apparatus. Vesicles, preloaded with isotope, were drawn up into a Hamilton gas-tight, 100-µl syringe (Hamilton Co., Reno, NV) (syringe marked 3 in Fig. 1). 1,000 µl of the dilution buffer was drawn into syringe 2 and was the same as that of the vesicles except that it was isotope free. During the experiment, the contents of syringes 2 and 3 were mixed in mixing chamber 1, after which they passed through Teflon tubing of variable length. (The volume of 1 cm was 10 µl.) Into another syringe (number 1 in the diagram) was
drawn ice-cold "stop" solution, which quenched the reaction by stopping the efflux of isotope from the vesicles when the effluent from mixer 1 reached mixer 2. The syringes in the apparatus were emptied by the force of a moving piston powered by compressed air released suddenly into the top of the piston. We timed the descent of the syringes using an oscilloscope (Tektronix, Inc., Beaverton, OR) to measure the velocity of the fluid moving from mixer 1 to mixer 2. Because we knew both the volume of fluid from the syringes that passed through the Teflon mixing tube over the measured time and the volume of the mixing tube (1 cm = 10 μl), we could calculate the time of the flux by the equation (volume of mixing tube)/(syringe volume/descent time) = flux time.

After the vesicles, diluent, and stop solution passed through mixer 2, the solution was sucked down an ion-exchange column that adsorbed the 22Na free in solution, after which the column was washed twice with 1 ml of 250 mM sucrose-Tris. The effluent was collected in a scintillation vial, counted in a Beckman gamma-counter (Beckman Instruments, Inc., Fullerton, CA), and the protein was measured using the Bio-Rad microassay (Bio-Rad Laboratories, Richmond, CA) with albumin as the standard.

The ion-exchange column was modified from that described by Gasko et al. (16) and was made by placing a 12-in. glass column into a 1-ml blue pipette tip (Sarstedt, Princeton, NJ). The point of the tip was pushed into a rubber stopper which in turn was placed into a 250-ml Erlenmeyer flask connected to a vacuum pump (see Fig. 1) that helps pull the fluid down the column quickly. The column was packed with AG 50W-X8, 20-50 mesh and 50-100 mesh, obtained from Bio-Rad Laboratories, and the beads were pretreated with 3 M Tris base until their pH in water was >7.5. The column was packed first by filling the blue pipette tip with the larger beads and then by packing the rest of the column with the small beads. The column was washed first with bovine serum albumin, 2-5 mg, and then with 20 ml of water.

Calculation of the Results

Results are expressed as an apparent sodium space, in microliters per milligram of protein and the isotopic rate coefficient is calculated from the change in this space over time. The sodium space was calculated as:

\[
\text{apparent sodium space} = \frac{[\text{cpm}_{\text{vesicles}} - \text{cpm}_{\text{background}}][\text{cpm}_{\text{medium}}/\mu l]^{-1}[\text{mg prot}]^{-1}}{\mu l/mg},
\]

where cpm_{vesicles} is the counts per minute of the total effluent collected in the scintillation vial, cpm_{background} is the counts per minute that are present in the buffer and have escaped the ion-exchange column, and cpm_{medium} is the number of counts per minute per microliter of buffer. The column did not adsorb all the isotope present in the buffer free in solution and not associated with the vesicles. Although this represents a small fraction of the total counts per minute of the experiments with vesicles, it was necessary to subtract from the vesicle-associated counts per minute those counts per minute that escaped the column. To estimate the background counts in each experiment, we performed an initial experiment identical to an efflux experiment except for the absence of vesicles. This was followed by three fluxes with vesicles in syringe 1 and a final flux without vesicles in syringe 1. Using the background counts per minute at the beginning and end of each experiment, it was possible to estimate the background counts per minute for experiments with vesicles.

By changing the length of Teflon tubing from mixer 1 to mixer 2, the efflux time could be varied from 10 to 100 ms and with five or more time points an efflux rate constant was obtained. The rate coefficient for isotope loss can be described by

\[
\frac{dN_{\text{a}}}{dt} = -kN_{\text{a}},
\]
where Na* is the isotope tracer (in moles) at time $t$, and $k$ is the efflux rate coefficient. It is convenient to express the vesicle isotope as a sodium space, the advantage being that the space per milligram of protein changes little from day to day, whereas the actual tracer concentration may vary greatly. By integrating and dividing Na* by the specific activity $[Na^*]$ and expressing it per milligram of protein, we get

$$\ln Na_t = -kt + \ln Na_{t=0},$$

where $Na_t$ is the sodium space (microliters per milligram of protein) at time $t$, and $Na_{t=0}$ is the space at time zero.

The efflux experiment was performed by diluting the vesicles into a finite, rather than an infinite, volume of buffer and it was necessary to take the dilution into account in calculating the true rate constant. Eq. 2 was modified to

$$\ln[Na_t - Na_t\to\infty/Na_{t=0} - Na_t\to\infty] = -kt,$$

where $Na_{t=\infty}$ is at $t = \infty$. For the $Na_{t=0}$ value we used the $t = 0$ intercept from a plot of $\ln Na_t = -kt + \ln Na_{t=0}$. For $Na_{t=\infty}$ we used $Na_{t=\infty}/11$ (100 µl of vesicles were diluted into 1,000 µl buffer and at equilibrium $Na_{t=\infty} = Na_{t=0}/11$). Because the vesicles are not uniform in size and do not represent a homogeneous population, we would not expect a single rate coefficient to define the isotopic efflux in these vesicles. To reduce the day-to-day variation with respect to vesicle size and contamination, we divided each batch of vesicles into two groups, control and experimental. The efflux reaction was performed between 0 and 100 ms in each, and the results were calculated by linear regression. Because the control and experimental groups were treated identically, the rate coefficients can be used to compare the effects of an experimental maneuver on the sodium exchange permeability.

**Operation of the Flow-Quench Apparatus**

Proper function of this kind of apparatus requires that the velocity of flow be uniform, mixing be instantaneous, and the reaction be stopped completely. We used an oscilloscope to measure the descent time of the syringes to test for uniformity of flow. After an initial inertial phase not lasting more than 10 ms, the rate of descent was constant over the rest of the time, which was on the order of 0.5 s in all experiments.

To test for the occurrence of instantaneous mixing, we measured the alkaline hydrolysis of dinitrophenylacetate. In this reaction dinitrophenylacetate (colorless) is hydrolyzed with base (NaOH), which generates the yellow compound dinitrophenol (DNP). The reaction is then quenched with acid. Fig. 2 shows an experiment that demonstrates that the reaction is linear over the first 100 ms, which indicates that the overall mixing in the apparatus is uniform. The intercept of $\sim0$ suggests that mixing is instantaneous. Were it not, one would expect an $x$ intercept of a few milliseconds. There may be some delay in mixing, but because the reaction is both initiated and quenched by mixing, the delays, if consistent, would offset each other and yield a zero intercept.

**Stopping the Efflux Reaction**

To measure sodium fluxes in the millisecond time range, we needed to stop the efflux reaction so that as the vesicles were being harvested they would not lose additional isotope. For the stop solution we tested calcium, which we had reason to believe might block sodium channels, and amiloride, which we knew closed sodium channels in intact tissue (2, 21). In Fig. 3 are the results of three experiments in which we tested the use of calcium or amiloride as inhibitors that could stop the efflux reaction. In these experiments, luminal vesicles were preincubated in media containing $^{22}Na$ in...
buffer with no added calcium. The vesicles were diluted during the efflux reaction into isotope-free buffer containing either amiloride (0.1 mM), as seen in the middle panel, or calcium (1 mM), as seen in the panel to the right. In the far left panel is a control experiment in which the vesicles were diluted into the original buffer without either amiloride or calcium added. In all three experiments, syringe number 1 contained both amiloride and calcium. The experiment was performed without preincubation in the inhibitors in order to test specifically whether these agents could quench the efflux on contact with the vesicles. From the figure it is apparent that only in the control flux is there a significant loss of isotope.

![Graph](https://example.com/graph.png)

**Figure 2.** Hydrolysis of dinitrophenylacetate. The alkaline hydrolysis of dinitrophenylacetate (colorless) to dinitrophenol (yellow) was measured in the flow-quench apparatus between 10 and 100 ms. In syringe 3 was dinitrophenylacetate (0.62 mM), in 2 was sodium hydroxide (0.35 M), and in syringe 1 was the stop solution, HCl (0.4 M).

Since it seemed likely that the membranes might contain both inside-out and right-side-out vesicles, it was surprising that amiloride and calcium were each able to abolish the efflux. (We assume that amiloride blocks only from the outside, whereas calcium, at least in concentrations below 1 mM, blocks only from the cytosolic face; see below.) On close examination of the results in Fig. 3 it appears that there is some flux in the presence of 1 mM calcium. In experiments presented later in the paper (Table II), it appears that the vesicles are largely right-side out. One possible explanation of the effect of 1 mM calcium is that these mostly right-side-out vesicles have some finite calcium permeability, and because the inside surface is exquisitely sensitive to calcium (see below), enough calcium could have diffused into the vesicles to cause inhibition. If some of the vesicles were inside out, it is likely that amiloride was able to penetrate sufficiently rapidly to reach blocking concentrations on the inside given the high surface-to-volume ratio of these vesicles and a reasonable amiloride permeability (10).
RESULTS

The purpose of the present study was to measure the effect of calcium and sodium on the sodium permeability of the luminal membrane in the absence of electrochemical gradients using a rapid-reaction apparatus. Before performing these experiments, however, we needed to show that the plasma membrane fractions used were indeed derived from the luminal membrane and that sodium fluxes in that fraction resembled those in the native state.

**Tracer Exchange Flux in Luminal Membranes**

The results of a typical $^{22}\text{Na}$ efflux experiment are shown in Fig. 4. In the left panel, luminal (apical) membrane vesicles, as defined biochemically from our previous studies (5), were diluted into isotope-free buffer. The fractional loss of isotope is plotted as a semilogarithmic function of time and the slope of the curve is the efflux rate coefficient. The half-time of isotope loss in this experiment was $\sim 175$ ms. For comparison, similar experiments were performed with basolateral and mitochondrial membranes from the toad bladder. In these vesicles there is no loss of isotope over 100 ms. Thus, only in the fraction that we had previously determined to be luminal membranes is there a fast sodium flux.

Luminal membranes are undoubtedly contaminated with basolateral and mitochondrial membranes. Because these contaminating membranes are tight to sodium over the 100-ms time range during which the flux is measured and have larger apparent volumes per milligram of protein (see legend to Fig. 4), contamination would reduce the calculated rate coefficient of efflux. Since there was no way to actually measure the degree of contamination, to reduce the effect of contamination on the measurement of the luminal membrane...
permeability we simultaneously ran control and experimental fluxes prepared from the same batch of membranes. Any difference could thus be attributed to the effect of the experimental maneuver rather than to the day-to-day variability in the purity of the luminal fraction. It is remarkable, however, that in spite of the presumed day-to-day variability the isotope fluxes over a variety of conditions were similar (see Tables I and III and Figs. 6 and 7).

The isotope efflux rate coefficient is a measure of membrane sodium permeability only if the loss of isotope represents movement of sodium through the membrane rather than debinding of sodium from the surface of the vesicle. To measure the extent of binding and its influence on the efflux rate coefficient, we tested the effect of increasing the ambient osmotic pressure. Fig. 5 shows the sodium isotope content of vesicles as a function of the reciprocal of the external osmolality and hence vesicle volume. At an infinite osmolarity (1/osmol = 0), the predicted vesicle volume becomes vanishingly small, and such vesicles, in the absence of binding, should contain no isotope. The lower curve is an experiment at 110 mM sodium chloride and demonstrates that the vesicles contain no isotope when the internal volume is reduced to 0 by increasing the extravesicular osmolarity. At 1 mM NaCl, however, there is some isotope left associated with the vesicle even when the predicted volume is 0. This presumably represents binding and is 31% of the isotope content at isoosmolarity. This result was confirmed by measuring the isoosmotic intravesicular space with glucose (14C) simultaneously with 22Na in vesicles at 1 mM sodium using the filtration method (5). The glucose space was 5.71 ± 0.17 µl/mg, whereas the sodium space was 8.69 ± 0.09. As in the previous experiment, ~30% of the apparent space in the low sodium experiments was due to binding.

![Graph showing isotope efflux rate coefficient in different membrane fractions.](image-url)
When we compared the rate coefficient of vesicles at 110 mM sodium with that measured in 1 mM sodium, we found that they were similar (Table I). Because the rate of loss of isotope from the vesicles in the presence and absence of binding is approximately the same, the loss of sodium isotope from the vesicles under either condition represents transmembrane translocation rather than rebinding. The $^{22}$Na efflux rate coefficient measured over 100 ms in the presence or absence of binding is thus a measure of luminal membrane isotope exchange sodium permeability.

**Figure 5.** Estimation of $^{22}$Na binding in luminal membrane vesicles at high and low sodium concentrations. From the same preparation, vesicles were divided in half and incubated in either low-sodium chloride (1 mM) or high-sodium chloride (110 mM) buffer containing 1 mM EGTA, 20 mM HEPES, and Tris, pH 7.5. The ionic strength was kept constant in the low-sodium buffer by the addition of 110 mM choline chloride. Each batch was then subdivided into three small aliquots into which a very concentrated aliquot of 60% sucrose (in buffer) was added to bring the final osmolarity up to ~375 (n = 2) and 600 mosmol (n = 2). The isotope was added and the vesicles were incubated for 1 h. The membranes were sent through the apparatus to be diluted into an identical buffer without isotope in a short mixing tube whose length was calculated to allow efflux for 10 ms before the reaction was stopped. The values on the curve thus do not represent an initial sodium space. During this short efflux time the vesicles lost some isotope (~0.16 μl/mg), which explains why the y intercept for the high sodium experiment is <0. The slopes of the lines drawn are not significantly different, P > 0.05.

**Effect of Amiloride**

Exquisite amiloride sensitivity of sodium transport is one of the distinguishing features of the sodium channel present in tight epithelia such as the toad bladder (2, 8). The drug abolishes transport at extremely low concentrations ($K_1 \sim 10^{-7}$ M). The composite results of 12 experiments with amiloride are displayed in Fig. 6, in which the calculated rate constant for different experiments at different amiloride concentrations is plotted as a function of...
TABLE I

EFFECT OF BINDING ON THE ISOTOPE EFFLUX RATE COEFFICIENT

| [Na] (mM) | Na<sub>i</sub> = 0 (µl/mg s<sup>-1</sup>) | k (s<sup>-1</sup>) |
|-----------|---------------------------------|-----------------|
| 1 (n = 4) | 7.66±0.53                        | -4.46±0.41      |
| 110 (n = 3)| 4.27±0.30                        | -3.89±0.37      |

Luminal membrane vesicles were preincubated in either a high- or low-sodium-containing buffer, and the efflux rate coefficient was measured over 100 ms. The low-sodium buffer contained (in mM): 1 NaCl, 110 choline-Cl, 20 HEPES, 1 EGTA, and Tris buffered to a pH of 7.5. The high-sodium buffer contained 110 NaCl, 20 HEPES, 1 EGTA, and Tris buffered to a pH of 7.5. The results under these two conditions were pooled from many different experiments performed on different days. Because ~30% of the sodium space in these vesicles is bound sodium (see Fig. 5), it was necessary to calculate the true rate coefficient from the observed rate coefficient by subtracting the bound space from the total space. The apparent rate coefficient for the vesicles at 1 mM sodium was -2.84 ± 0.78 as compared with -4.46 ± 0.41 for the true rate coefficient, which is not significantly different from that at 110 mM sodium, 0.05 < P < 0.10.

![Figure 6](image-url)

**Figure 6.** Effect of amiloride on the sodium permeability of luminal membranes. <sup>22</sup>Na efflux was measured in luminal membrane vesicles and each point on the curve represents the rate coefficient ± the standard error of the slope of a single experiment at a given amiloride concentration in which there were at least six different time points. Both vesicles (syringe 3) and diluent (syringe 2) contained amiloride in the concentration indicated. The control point represents the rate coefficient calculated from a single curve with at least 20 time points from the six experiments run in tandem with the amiloride experiments. The buffer for all experiments was: 110 mM choline-Cl, 1 mM NaCl, 20 mM HEPES, 1 mM EGTA, and Tris base, pH 7.5. Amiloride was added to the diluent and vesicles as a concentrate from a stock solution of 5 mM. k = -2.82 ± 0.35 s<sup>-1</sup> for control; -2.54 ± 0.37, 1 nM amiloride; -1.91 ± 0.29, 10 nM; -1.13 ± 1.27, 100 mM; -0.40 ± 1.01, 1 µM; and -0.003 ± 0.90, 10 µM. The flux, calculated as J*/[Na*] was: 0.28 µl/mg·100 ms at 10 µM amiloride and 2.12 in control. J* is the isotope flux in cpm/100 ms·mg; and [Na*] is the isotope concentration in cpm/µl at t = 0.
the amiloride concentration. The vesicles were preincubated in amiloride for 1 h to allow the drug to exert an effect on the outside face of a sodium channel in case some or all of the vesicles were inside out. The experiment demonstrates that amiloride has a potent effect on the rate constant and therefore on sodium isotope exchange permeability. The $K_i$ of the amiloride inhibition is 50 nM, almost an order of magnitude lower than the $10^{-7}$ M found in previous experiments on whole toad bladder by us (unpublished observations) and by others (8). The likely explanation for this difference is that these experiments were performed in 1 mM sodium, a situation well known to increase the affinity of amiloride for the sodium channel (8).

Our conclusion regarding the action of amiloride assumes that amiloride specifically inhibits the transmembrane translocation of sodium rather than displaces a vesicle-bound component of sodium ions. These studies were performed at 1 mM sodium concentration, where $\sim$30% of the initial sodium space represents bound sodium. As presented above, however, even in the presence of binding, the rate coefficient is a measure of transmembrane transport rather than debinding. Any inhibition of the isotopic efflux rate coefficient, therefore, represents inhibition of transport. Furthermore, because amiloride did not change the apparent internal sodium space, the drug did not inhibit initial binding; $\text{Na}_i-0$ was $8.31 \pm 0.81 \mu l/mg$ for control ($n = 5$) and $8.00 \pm 0.95$ ($n = 5$) for the amiloride-treated vesicles. Moreover, the amiloride-sensitive sodium flux is $\sim$85% of the total flux (see the legend of Fig. 6) and is much greater than the total amount bound ($\sim$30%). Finally, amiloride gave the same inhibition in the presence of 110 mM sodium (results are presented in the next section), a situation in which there is no binding present. Thus, the effect of amiloride on the efflux rate coefficient was the result of inhibition of transmembrane transport rather than the result an effect on the bound sodium pool.

Effect of Calcium on the Permeability of the Luminal Membrane

We measured the rate coefficient of $^{22}\text{Na}$ efflux from the luminal membrane vesicles at different calcium concentrations and the results appear in Fig. 7. The vesicles were exposed to the calcium-EGTA-buffered solutions on both sides of the membrane for at least 1 h before the measurement of the efflux. At the time of the experiments we were not sure whether the vesicles were right-side or inside out and we wanted to allow sufficient time for equilibration of the internal vesicular calcium concentration. Calcium clearly reduced the permeability of the luminal membrane vesicles, and the half-maximal inhibition calculated from a Hill plot is 0.5 $\mu$M, very much within the range of cytosolic calcium activity as measured by ion-selective microelectrodes (31, 32, 37). We assume that because the permeability of the vesicles is sensitive to calcium in the submicromolar range, and in whole tissue there seems to be little effect of external millimolar calcium on luminal sodium permeability, the effect of calcium in these experiments was exerted on the cytosolic face of the membrane (6).

If we assume that the inhibition of sodium transport by calcium in the submillimolar range is due to an effect solely on the cytosolic face of the
channel, then it is possible to test for the orientation of the vesicles by examining the sidedness of the effect of calcium. Table II illustrates this point. The sodium flux was measured in luminal membrane vesicles (at 110 mM sodium) in the presence or absence of calcium. When vesicles were preincubated with EGTA but no calcium and diluted into a buffer where the final concentration of calcium was 100 μM, there was no inhibition of sodium efflux. On the other hand, when the vesicles were preincubated with calcium so that the inside of the vesicle contained 100 μM calcium, there was inhibition of sodium efflux even when the vesicles were diluted into EGTA. The results thus indicate that only when calcium is on the inside of the vesicles is there inhibition of sodium transport, which suggests that the vesicles are predominantly right-side out. (This conclusion assumes that there is no day-to-day variation in the sidedness of these vesicles.)

The effect of calcium on reducing the sodium isotope efflux rate coefficient could have been due to calcium-induced aggregation of vesicles. We tested for

\[ -k_{2} \]

\[ \text{CALCIUM (M)} \]

**Figure 7.** Effect of calcium on the permeability of the luminal membrane. \(^{23}\text{Na} \text{ efflux was measured in luminal membrane vesicles in the presence of various amounts of calcium. The calcium scale refers to the calculated free calcium based on the total [calcium] and [EGTA]. The association constant used to calculate this number was } 6.76 \times 10^{7} \text{ obtained from Sillen and Martell (48) as calculated for different pHs by Pershadsingh and MacDonald (45). To make the preparation of the buffers uniform and quick, } CaCl_{2} (1 \text{ mM}) \text{ was added to the standard buffer containing (in mM): 1 NaCl, 110 choline chloride, 20 HEPES, and Tris buffer, pH 7.5. To this was added various amounts of EGTA. Each point on the curve represents the rate coefficient calculated from at least six points obtained at that particular free-calcium concentration. The control point represents experiments pooled from a number of experiments all performed in } 1 \text{ mM EGTA with no added calcium. The Hill coefficient from these data is } 0.82 \pm 0.14. \]

1 Dilution of calcium-loaded vesicles into EGTA will result in elution of calcium from its binding site only if the \( \text{OFF} \) rate constant of the calcium:channel complex and the \( \text{ON} \) rate constant of the calcium:EGTA complex are sufficiently fast to allow instantaneous removal of the bound calcium. Until these parameters are known, the conclusions from this particular experiment remain ambiguous. However, the result is consistent with those of the more conclusive experiments in Table II, which in the aggregate indicate that the majority of those vesicles are right-side out.
aggregation by measuring light scattering of a highly concentrated vesicle preparation (1 mg/ml) in a fluorometer. For these experiments we had to remove much of the associated cytoskeletal proteins (see Methods) because actin polymerization increases light scattering and would therefore interfere with the use of this technique to estimate vesicle aggregation (27). We found that ambient calcium concentration of >1 mM had no effect on light scattering (data not shown). These results, taken together with the fact that only when calcium was present on the inside of the vesicles was there inhibition of transport (Table II), suggest that the effect of calcium on sodium transport is not due to an effect on vesicle aggregation.

**Effect of Sodium on the Permeability of the Luminal Membrane**

To test directly the effect of sodium on its own permeability, we measured the rate coefficients of luminal membrane vesicles at different ambient sodium concentrations (Tables I and III). In these and in all the experiments presented in this paper, there were no ionic gradients across the membranes, with the exception of the $^{22}\text{Na}$. The observed rate constant for vesicles in 110 mM sodium was higher than that of vesicles in 1 mM sodium (see the legend to Table I). It is also apparent that the initial sodium spaces ($N_{a_{t=0}}$) were

| Vesicle [Ca] | Inside | Outside | $J^*/[\text{Na}^*]$ | $N_{a_{t=0}}$ |
|-------------|--------|---------|---------------------|--------------|
|             | µl/mg·100 ms | µl/mg |
| 0           | 0      | 0       | 3.25                | 11.49        |
| 0           | 100 µM | 100 µM  | 3.75                | 9.82         |
| 100 µM      | 100 µM | 0       | 0                   | 9.95         |
| 100 µM      | 3 nM   | 0       | 0                   | 9.67         |

In these experiments vesicles were resuspended in 110 mM NaCl, 20 mM HEPES, and Tris, pH 7.5, to which the above concentrations of calcium were added. Efflux was measured from 10 to 50 ms and the efflux rate coefficients were calculated as described in the Methods. Each condition represents a single experiment in which there were at least four individual time points that went into the calculation of the rate coefficient and flux. Most of the actin in the preparation was removed prior to the experiment (see Methods), which explains why the sodium space per milligram of protein is higher than in the other experiments. $J^*$ is the isotope flux in counts per minute per 100 ms per milligram; and $[\text{Na}^*]$ is the isotope concentration in counts per minute per microliter at $t = 0$.

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2 We had to repeat the standard experiments with amiloride and calcium to ensure that the treatment procedure used to remove cytoskeletal proteins did not affect the sodium flux through the luminal membrane. We found that after treatment, in 110 mM sodium, the flux ($J^*/[\text{Na}^*]$) was 2.5 µl/mg·100 ms in the control, 0 in the presence of 200 µM amiloride, and 0.5 in the presence of 100 µM calcium.
different because of greater binding of $^{22}$Na isotope to the vesicle membranes at 1 mM sodium. Because the rate coefficient is calculated from the fractional change of isotope content of the vesicles, constant binding of isotope to vesicles throughout the 100-ms flux would have the effect of reducing the observed rate coefficient. Thus, to compare the sodium permeabilities under the two conditions, it was necessary to compare either the true rate coefficient (after the effect of binding is eliminated) or the actual isotopic fluxes rather than the apparent isotopic rate coefficients (the effect of binding not being eliminated). From Table III it is apparent that the isotopic flux in the presence of 110 mM sodium was reduced by 28% compared with the flux at 1 mM sodium. Thus, sodium itself may exert a small but significant self-inhibitory effect on its permeability in the luminal membrane.

DISCUSSION

The Sodium Flux

A constant problem in the study of transport phenomena in isolated membranes is the search for a specific and sensitive identifying label for the membrane fraction of interest, not to mention similar labels for possible contaminating membranes. For the luminal membrane of the toad urinary bladder, we propose the exquisite amiloride sensitivity of sodium transport ($K_I \sim 100$ nM) as the minimum identifying label. The necessity for this stringent criterion is that it is evident that other cell membranes contain sodium-sodium, sodium-lithium, and sodium-hydrogen exchangers, transport processes that are amiloride sensitive as well but for which the $K_I$ is on the order of 10–100 μM (24, 25). The use of qualitative amiloride sensitivity can therefore be misleading. For instance, LaBelle and co-workers (28, 29) isolated a microsomal fraction from the toad bladder and kidney in which sodium transport was inhibited by amiloride in the millimolar range. The membrane fraction we used had a low $K_I$ for amiloride, essentially identical to that found in intact bladder.

An additional identifying label for sodium channel-containing vesicles should be the rapidity of the isotope exchange efflux reaction. An average-sized vesicle (0.2 μm diam) in 100 mM sodium, containing a single sodium channel through which pass $10^6$ ions/s, would lose half of its isotope in <1 s. The rate coefficients we observed in our preparation are thus consistent with a channel transport mechanism. On the other hand, LaBelle and co-workers (28, 29) isolated an amiloride-sensitive sodium flux, the $t_{1/2}$ of which was on

3 That is, if $N_{a_0}$ represented both a true sodium space, $N_{a_0}$, and an apparent space due to binding, $N_{a_b}$, the slope of

$$\ln\left(\frac{N_{a_t} + N_{a_b}}{N_{a_0} + N_{a_b}}\right) = -kt$$

would become less steep as the binding component increased. Binding, therefore, reduces the observed rate constant.

4 The volume of a vesicle 0.2 μm in diameter is $4.2 \times 10^{-15}$ cm$^3$. At 110 mM sodium, there are $\sim 2.5 \times 10^5$ ions per vesicle. If $\sim 10^6$ ions pass through a single channel per second, as a minimum estimate, then the vesicle will lose approximately half of its sodium in 100 ms.
the order of minutes rather than seconds. This result, in addition to the high $K_1$ for amiloride, makes it unlikely that the flux they observed is through the sodium channel.

Lastly, the membranes we isolated were enriched with a covalent label of the luminal membrane that we had inserted biochemically (5). This fact, in conjunction with the low $K_1$ for amiloride and the time course of sodium efflux in these vesicles, indicates that this fraction contains the luminal membrane and its sodium permeability.

### Table III

**EFFECT OF SODIUM ON THE SODIUM PERMEABILITY OF LUMINAL MEMBRANE**

| [Na] (mM) | $j^*/[Na^*]$ (µl/mg·100 ms) | Percent fall compared with [Na] = 1 mM |
|-----------|-----------------------------|--------------------------------------|
| 1 (n = 4) | 1.86±0.21                   |                                      |
| 55 (n = 1) | 1.44                        | 23%                                  |
| 110 (n = 3) | 1.35±0.01                   | 27%                                  |

In this experiment luminal membrane vesicles were preincubated in buffers with differing amounts of sodium to test the effect of sodium on the membrane sodium permeability. For convenience, an isotopic flux was calculated as the change in the measured sodium space per milligram of protein per 100 ms. This was done to eliminate the effect of binding and day-to-day variability of the sodium spaces on the calculated rate coefficients. Also, we made the assumption that the ambient sodium concentration does not affect significantly the surface to volume ratio of the vesicles. Choline-Cl replaced NaCl to maintain osmolarity and ionic strength. The data at 1 and 110 mM sodium are from the same experiments as those in Table I. $j^*$ is the isotopic flux in counts per minute per 100 ms per milligram; and [Na$^*$] is the isotopic concentration in counts per minute per microliter at $t = 0$. The flux at 1 mM [Na] is significantly greater than at 110 mM, $P < 0.05$.

**Effect of Calcium and Sodium on the Exchange Permeability of the Luminal Membrane**

We have demonstrated that the amiloride-sensitive sodium efflux from luminal vesicles is inhibited by very low concentrations of calcium. There are a number of ways in which calcium could reduce the sodium efflux in the luminal membrane vesicles. Calcium could specifically interact with the sodium channel and inhibit transport. Alternatively, calcium could alter the surface charge of the membrane and indirectly affect the permeation of sodium through the channels. It is unlikely that calcium acts primarily by affecting the surface charge of the lipid bilayer because in artificial membranes, divalent cations change the surface potential only when the concentration is in the millimolar range. The association constant for the effect of divalent cations such as calcium and the bilayer is many orders of magnitude greater than the $K_1$ of the effect of calcium on sodium permeability (39). The fact that the
concentration of monovalent cations is high also makes this explanation unlikely.

Lindemann (34) has suggested an interesting model of a “floating receptor interaction” for the effect of external sodium whereby an increase in luminal sodium concentration increases the frequency of collision of sodium channels and this collision causes inactivation and hence reduced permeability. This kind of translational diffusion of membrane proteins is now known to be controlled by the cytoskeleton, which in turn is regulated by cytosolic calcium. It is possible that cytosolic calcium could produce effects on sodium transport by influencing the cytoskeleton and the insertion and maintenance of open sodium channels. Studies in living cells whose sodium channels are labeled by some ligand will be needed before any final resolution of this issue can be achieved.

One interesting point that emerged from our attempt to remove contaminating proteins from the luminal membrane vesicles was that the luminal membranes, as compared with the basolateral membranes, were loaded with a 43,700-dalton protein that had the same migration on sodium dodecyl sulfate gel electrophoresis as actin. The co-purification of actin is of note because it suggests that the actin may in some way be bound to the surface of the luminal membranes, either directly or indirectly. Many recent studies have demonstrated that cytoskeletal elements are attached to important integral membrane proteins, including channels such as band 3 of the red cell (1). Although calcium does inhibit sodium transport in the luminal membrane vesicles in which much of the actin has been removed, we in no way removed all the actin. It is thus possible that calcium inhibits transport by interacting with the cytoskeleton, even in this in vitro membrane preparation, and does not interact directly with the sodium channel. This important issue will not be resolved until reconstitution of the sodium channel is accomplished, allowing for direct study of the role of the cytoskeletal proteins in maintaining open sodium channels.

The data thus suggest that calcium acts directly on the luminal membrane and perhaps the sodium channel. The Hill coefficient of 0.8, calculated from the experiments in Fig. 7, does not necessarily mean a one calcium:one channel relationship. Because the Hill coefficient was obtained from a plot of the sodium efflux rate coefficient rather than from single-channel measurements, it would have a value near 1 even if calcium acted as a purse string and closed a number of channels simultaneously. Clearly, more experiments directed specifically at these issues will be needed to examine the mechanism by which calcium inhibits sodium transport through the sodium channels.

The permeability of the luminal membrane vesicles is only minimally reduced in the presence of a high sodium ion concentration. In fact, it is not clear that the 27% reduction of the sodium flux seen in the presence of a high sodium concentration actually represents a reduction in the permeability of the membranes. The fall may be simply due to competition by nonisotopic sodium for a binding site within the channel, thereby reducing the observed rate coefficient. In any case, the effect is small by comparison with that of
calcium. It is unlikely that the effect of an increase in cell sodium in intact tissue is due to the effect of sodium itself. Rather, the effect on luminal permeability is probably due to the direct actions of calcium. As for the effect of mucosal sodium on the luminal membrane sodium permeability, we cannot eliminate the possibility that mucosal sodium might have some effect on the insertion and deletion of channel-containing membrane. Noise analysis suggests that an increase in luminal sodium does decrease the number of open channels, which could be due either to a decrease in channel number present in the membrane or to a closing of channels already present within the membrane. Thus, although we have shown that sodium has little direct effect on the isolated luminal membrane sodium permeability, it is certainly possible that in the intact cell, extracellular (mucosal) sodium causes a decrease in either the number of channels or in the fraction of open channels in the luminal membrane. Until a covalent ligand for the channel is developed, this question will remain unanswered.

Correlation between the In Vivo and In Vitro Responses

Only recently has cytosolic calcium activity been measured in epithelia with ion-selective electrodes. Lee and associates (32, 37) have measured cell calcium in Necturus proximal tubule under a variety of conditions, including removal of serosal sodium and the addition of ouabain. We had previously performed identical experiments in intact toad bladder and found that the permeability of the luminal membrane was reduced under these conditions. Table IV displays data from Lee and co-workers from Necturus tubule which show that there is an increase in calcium activity after either adding ouabain or removing serosal sodium. Using the results in Fig. 7 and assuming that similar changes in calcium activities occur in the toad bladder, we calculated the extent of reduction in luminal membrane sodium permeability. The decrease in net sodium transport observed in the intact bladder can be accounted for by the

| Maneuver                     | [Ca] Before | [Ca] After | k Before | k After | Vesicles Fall | Bladder Fall |
|------------------------------|-------------|------------|----------|---------|---------------|--------------|
| Removal of serosal sodium    | 100 nM      | 380 nM     | -2.1     | -1.4    | 33%           | 36%          |
| Ouabain                      | 50 nM       | 630 nM     | -2.6     | -1.2    | 54%           | 74%          |

Lee and co-workers (31, 32, 37) have measured the change in cytosolic calcium activity that follows ouabain and removal of serosal sodium. We also performed these experiments in intact tissue (5), in which we measured the sodium permeability of the luminal membrane. In this table we have listed both the results of Lee and co-workers and of our previous experiments, in which we measured the fall in the permeability of the luminal membrane after both of the maneuvers. Using the measured values of the change in calcium activity from Lee and co-workers, we estimated from Fig. 7 the fall in the efflux rate coefficient in the vesicles after the particular maneuver. In the column to the right we have compared the decline in luminal permeability in whole tissues with the measured fall in permeability of the vesicles.
effects on luminal permeability induced by a change in cell calcium. However, in order to conclude that calcium is an important physiological regulator of luminal sodium permeability, one must obtain measurements of cytosolic calcium in the toad urinary bladder. The importance of calcium in regulating transepithelial sodium transport will be proved only by demonstrations that changes in the cell calcium level correlate in the appropriate manner to changes in the rate of sodium transport.

Conclusions

To define the properties of a channel it is necessary to have both electrophysiological data and tracer kinetics. The method of rapid tracer studies should provide insight into the molecular mechanism of ion translocation through the channel. There are limitations, however, the most serious being that the vesicles may not be oriented symmetrically; that is, some are inside out and others are right-side out. Preliminary evidence (Table II) suggests that most of the vesicles are right-side out. Because it is not likely that the cytosolic and extracellular faces of the sodium channel are identical, it will not be possible to study important issues such as voltage dependence and topography until the issue of sidedness is definitively resolved.

We were greatly saddened by the death of James V. Bone, who worked in the early phase of these experiments. This paper is dedicated to the memory of this most engaging and energetic youth.

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