Na^+-H^+ Exchange and Na^+ Entry across the Apical Membrane of *Necturus* Gallbladder

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ABSTRACT The role of Na^+-H^+ exchange in Na^+ transport across the apical membrane was evaluated in *Necturus* gallbladder epithelium by means of intracellular Na^+ activity (aNa_i) and $^{22}Na^+$ uptake measurements. Under control conditions, complete replacement of Na^+ in the mucosal solution with tetramethylammonium reduced aNa_i from 14.0 to 6.9 mM in 2 min ($P < 0.001$). Mucosal addition of the Na^+-H^+ exchange inhibitor amiloride (10^{-3} M) reduced aNa_i from 15.0 to 13.3 mM ($P < 0.001$), whereas bumetanide (10^{-5} and 10^{-4} M) had no effect. Na^+ influx across the apical membrane was studied by treating the tissues with ouabain, bathing them in Na-free solutions, and suddenly replacing the mucosal solution with an Na-containing solution. When the mucosal solution was replaced with Na-Ringer's, aNa_i increased at ~11 mM/min. This increase was inhibited by 54% by amiloride (10^{-3} M, $P < 0.001$) and was unaffected by bumetanide (10^{-5} M). Amiloride-inhibitable Na^+ fluxes across the apical membrane were also induced by the imposition of pH gradients. Na^+ influx was also examined in tissues that had not been treated with ouabain. Under control conditions, $^{22}Na^+$ influx from the mucosal solution into the epithelium was linear over the first 60 s and was inhibited by 40% by amiloride (10^{-3} M, $P < 0.001$) and by 19% by bumetanide (10^{-5} M, $P < 0.025$). We conclude that Na^+-H^+ exchange is a major pathway for Na^+ entry in *Necturus* gallbladder, which accounts for at least half of apical Na^+ influx both under transporting conditions and during exposure to ouabain. Bumetanide-inhibitable Na^+ entry mechanisms may account for only a smaller fraction of Na^+ influx under transporting conditions, and cannot explain influx in ouabain-treated tissues. These results support the hypothesis that NaCl entry results primarily from the operation of parallel Na^+-H^+ and Cl^--HCO_3^- exchangers, and not from a bumetanide-inhibitable NaCl cotransporter.

INTRODUCTION

Leaky epithelia such as gallbladder, renal proximal tubule, and intestine accomplish transepithelial transport of NaCl by a neutral process. (For reviews see Diamond, 1968; Frizzell et al., 1979*a*; Warnock and Eveloff, 1982.) Numerous studies have demonstrated an interdependence of net transepithelial Na^+ and

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/84/01/0057/18 $1.00 57
Volume 83 January 1984 57–74

Cl^- fluxes (Quay and Armstrong, 1969; Nellans et al., 1973; Frizzell et al., 1975; Cremaschi and Hénin, 1975), and an Na^+ requirement for the maintenance of intracellular chloride activity (aCl_i) at a level greater than that predicted from equilibrium distribution (Duffey et al., 1978; Spring and Kimura, 1978; Reuss and Grady, 1979; Garcia-Diaz and Armstrong, 1980; Oberleithner et al., 1982). These results have been interpreted to imply the existence of direct coupling of Na^+ and Cl^- fluxes through a ternary-complex NaCl cotransporter at the apical membrane (Frizzell et al., 1979*a*). However, the possibility of neutral, coupled NaCl transport resulting from the simultaneous operation of Na^+-H^+ and Cl^--OH^- exchangers has been suggested (Turnberg et al., 1970; Liedtke and Hopfer, 1977) and has recently been the subject of considerable investigation (Petersen et al., 1981; Warnock and Yee, 1981*a*; Liedtke and Hopfer, 1982*a*, *b*). Distinguishing between these two mechanisms is difficult because many of their predictions are the same.

The conclusion that coupled NaCl entry results from a single cotransporter is based in part on the observation that the process is inhibited by furosemide or bumetanide (Eveloff et al., 1978; Ericson and Spring, 1982*a*). Although these drugs have been demonstrated to inhibit many coupled ion transport processes, their specificity has not been established.

The case for parallel ion exchangers is also inconclusive and is based on the demonstration of both Na^+-H^+ and Cl^--OH^- exchange processes in apical membrane vesicles from leaky epithelia (Murer et al., 1976; Kinsella and Aronson, 1980; Warnock and Yee, 1981*a*; Liedtke and Hopfer, 1982*a*, *b*). In addition, attempts to demonstrate the existence of a ternary-complex NaCl cotransporter have been unsuccessful (Liedtke and Hopfer, 1982*a*).

Our previous studies (Weinman and Reuss, 1982*a*) used extracellular and intracellular pH measurements to demonstrate the existence of Na^+-H^+ exchange at the apical membrane of *Necturus* gallbladder. This Na^+-H^+ exchange process occurs continuously under control conditions, but its magnitude could only be estimated crudely from the extracellular pH measurements. In the present study, we evaluate the role of Na^+-H^+ exchange in transapical Na^+ transport by measurements of intracellular sodium activity (aNa_i) and unidirectional $^{22}Na^+$ uptake in *Necturus* gallbladder. The results demonstrate that in ouabain-treated tissues, at least 50% of apical Na^+ entry results from amiloride-inhibitable Na^+-H^+ exchange, and that under control conditions, 40% of the unidirectional $^{22}Na^+$ uptake is amiloride inhibitable. Apical membrane Na^+-H^+ exchange is therefore a major pathway for transepithelial Na^+ transport. Preliminary results of these studies have been reported (Weinman and Reuss, 1982*b*, 1983).

MATERIALS AND METHODS

Mud puppies (*Necturus maculosus*) were purchased from Nasco Biologicals (Ft. Atkinson, WI), kept in aquaria at ~10°C, and fed live fish. Gallbladders were removed, mounted mucosal side up, and continuously perfused on both sides in a modified Ussing chamber as previously described (Reuss and Finn, 1975, 1977). Na-Ringer's solution had the following composition (mM): 109.2 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.0 Hepes. TMA-Ringer's contained 109.2 mM tetramethylammonium (TMA) Cl instead of NaCl. These solutions

were titrated with KOH and equilibrated with room air to have a final pH of 7.7. HCO_3^--Ringer's was made by replacing 10 mM NaCl with 10 mM $NaHCO_3$ and omitting the Hepes. This solution was equilibrated with 1% CO_2-99% air and had a final pH of 7.6. Amiloride was a generous gift of Merck, Sharp & Dohme, West Point, PA; bumetanide was a generous gift of Hoffman-La Roche, Somerville, NJ. A stock solution (10^{-3} M) was prepared in Na-Ringer's titrated to pH 9. ^{22}NaCl and [^{3}H]mannitol were purchased from New England Nuclear, Boston, MA.

Electrical Potential Measurements

Transepithelial (V_{ms}), apical membrane (V_{mc}), and basolateral membrane (V_{cs}) potentials were measured as described previously (Reuss and Finn, 1975, 1977). The serosal reference was an Ag-AgCl electrode connected to the solution via an Na-Ringer's agar bridge. The mucosal solution potential was measured with a calomel electrode connected to the mucosal solution by a flowing, saturated KCl bridge. V_{ms} was referred to the serosal side; V_{mc} and V_{cs} were referred to the respective bathing solutions. Transepithelial current pulses were passed via two Ag-AgCl electrodes connected to the respective solutions by agar bridges.

Micropipettes were pulled from 1-mm-OD inner fiberglass capillaries (Hilgenberg, Malsfeld, Federal Republic of Germany) on a horizontal electrode puller (Narishige, Japan). The pipettes were filled with either 3 M or 0.5 M KCl and had resistances of 15–40 MΩ when filled with 3 M KCl and immersed in Na-Ringer's. Cells were observed with an MS inverted microscope (Nikon, Inc., Garden City, NY) and impaled with either a motorized remote control micromanipulator (Stoelting, Chicago, IL) or a 3-D hydraulic microdrive (Narishige). Impalements with conventional microelectrodes were accepted when (*a*) the potential change upon impalement was abrupt and monotonic, (*b*) the cell potential was stable for at least 2 min, and (*c*) simultaneous impalement with a second, usually Na-selective, microelectrode revealed that both impaled cells had the same apparent ratio of membrane resistances (see Results).

Intracellular Na^+ Activity Measurements

Intracellular Na-selective microelectrodes were constructed as described previously (Reuss et al., 1983). Micropipettes were pulled similarly to those used for conventional microelectrodes; when filled with 3 M KCl and immersed in Na-Ringer's, they had resistances of 10–30 MΩ. Pipettes were dried at 160°C and exposed to vapor of hexamethyldisilazane (Sigma Chemical Co., St. Louis, MO) for ~1 h. After cooling, the tips were filled with Na^+ resin (0.2 μl). An inner pipette filled with Na-Ringer's was then inserted into the back of the electrode to make contact with the resin within 100 μm of the tip. The electrode was backfilled with Na-Ringer's and an Ag-AgCl wire was inserted and sealed in place with wax.

The Na^+ resin used was that described by Steiner et al. (1979), and consisted of a 10% wt/wt solution of Na-ligand I (Fluka Chemical Co., Hauppauge, NY) in *o-n*-octyl oxynitrobenzene (Alfa Products, Danvers, MA) with 0.5% Na-tetraphenylborate (Fluka Chemical Co.) added. Potential measurements with these electrodes were made with an FD223 ultra-high impedance electrometer (W-P Instruments, Inc., New Haven, CT).

Slope and selectivity (Na^+ over K^+) for these electrodes were ~52–55 and 30–100 mV/decade, respectively. It was noted that the slopes of the electrodes in pure KCl and NaCl solutions were frequently different. Electrodes were therefore calibrated in six solutions containing 120 mM KCl and various concentrations of NaCl ranging from 2 to 50 mM, in a method similar to that described by Armstrong and Garcia-Diaz (1980). Intracellular

Na^+ activity was measured by impaling two cells simultaneously with a KCl-filled microelectrode and an Na-selective microelectrode, respectively. The potential measured by the conventional electrode (V_{ce}), that measured by the Na-selective electrode (V_{Na}), and the difference ($V_{Na} - V_{ce}$) were displayed on digital panel meters and a stripchart recorder (Gould, Inc., Cleveland, OH). Intracellular Na^+ activity was determined from $V_{Na} - V_{ce}$ by interpolation onto the calibration curve for that particular electrode.

The use of two simultaneous impalements rather than separate single impalements at different times allowed us to have a continuous record of aNa_i. In addition, it allowed the use of more stringent criteria to validate the impalements. These criteria have been described previously (Reuss and Weinman, 1979; Weinman and Reuss, 1982*a*) and involve a comparison of the voltage deflections measured by each electrode when the cell potential was changed by either transepithelial current pulses or mucosal solution ionic composition changes. An example of these criteria is presented in Fig. 1 (see Results).

Intracellular Na^+ Activity Measurements in Ouabain-treated Tissues

In some experiments, gallbladders were treated with ouabain (10^{-4} M) on the serosal side for at least 45 min and aNa_i was measured during exposure to TMA-Ringer's on either the mucosal side only or on both sides. The influx of Na^+ across the apical membrane was assessed by suddenly replacing the mucosal TMA-Ringer's with Na-Ringer's and recording the resulting changes in aNa_i. In some tissues the Na^+ influx rate, when the tissue was re-exposed to control Na-Ringer's, either declined or increased as a function of time. For this reason, influx rates in the presence of amiloride or bumetanide were always compared with control ones measured both before and after the exposure to the drug. In two out of nine tissues, the control influx rate changed by a factor of >4 from beginning to end of ouabain exposure. Results from these tissues were discarded.

Unidirectional $^{22}Na^+$ Influx

The flux of $^{22}Na^+$ from the mucosal solution into the epithelium (J_{me}) was measured in an apparatus similar to that described by others (Schultz et al., 1967; Frizzell et al., 1975). Four gallbladders were mounted on a plexiglass base, serosal side down, resting on filter paper wetted with Na-Ringer's. The mucosal surface was then isolated by placement of an upper chamber with cylindrical wells on top of the tissues. The seals were made by rubber O-rings and the exposed mucosal surfaces each had an area of 0.42 cm^2. The mucosal solution (200 μl) was gently stirred by bubbling with air. The bathing solution could be removed by suction and added by pipetting directly into the top of the chamber. Gallbladders mounted in this chamber were exposed on the mucosal side to Na-Ringer's for 20–30 min. After this preincubation, the mucosal solution was removed by suction and replaced by Na-Ringer's containing $^{22}NaCl$ (~15 μCi/ml) and [^{3}H]mannitol (~10 μCi/ml). After a timed interval (15–90 s), the tracer solution was removed by suction and the tissue was rinsed by washing for 2 s with 5 ml of isotonic sucrose solution at ~5°C. The gallbladder was then punched out, blotted gently, and transferred to a scintillation vial. Gallbladders were digested by incubation at 65°C for 1 h with 200 μl $HClO_3$ (60%) and 20 μl H_2O_2 (30%) (Mahin and Lofberg, 1966). After digestion and cooling, 10 ml Budget solve (Research Products International, Mount Prospect, IL) was added, and samples were counted in a Tri Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) for ^{3}H and ^{22}Na. ^{3}H counts were corrected for the fraction of the ^{22}Na spectrum that counted in the ^{3}H window (2.10% of the total ^{22}Na cpm). The ^{3}H counting efficiency in the ^{22}Na window was zero. $^{22}Na^+$ influx was corrected for the $^{22}Na^+$ present in extracellular fluid contamination as estimated by the distribution of [^{3}H]mannitol. This was generally <5% of the total ^{22}Na counts. Uptake was shown to be linear for 60 s (see Results), and

uptake rates were determined from 45-s exposures. When the effects of amiloride or bumetanide on J_{me} were tested, the tissues were preincubated as described and exposed to drug-containing solution for 1 min prior to exposure to the drug-containing radioactive tracer solution.

Statistics

Results are presented as means ± SE. When specified, comparisons were made by conventional paired data analysis. Otherwise, comparisons were made by Student's *t* test; a value of $P < 0.05$ was considered significant.

RESULTS

Validation of Impalements

Intracellular Na^+ activity (aNa_i) was determined by simultaneous impalement with two electrodes, a KCl-filled microelectrode and a liquid-membrane Na-selective microelectrode. The criteria used to validate impalements were those described previously (Reuss and Weinman, 1979; Weinman and Reuss, 1982*a*). The most rigorous tests consisted of demonstrating equal changes of membrane potentials of the two impaled cells both during transepithelial current pulses and during ionic substitutions of the mucosal solution. Since mucosal Na^+ removal leads to an immediate change in aNa_i, however, the depolarization produced by K^+ for Na^+ substitution could not be used for validation. As demonstrated in Fig. 1, mucosal Na^+ replacement with TMA^+ leads to a fall of aNa_i and a new steady state is reached in ~2 min. If at this time TMA^+ is replaced with K^+, very large changes in apical and basolateral membrane potentials occur that are identical in both impaled cells. This observation makes it very unlikely that the recorded values of aNa_i are in error because of nonspecific impalement damage of the apical membrane. All impalements in this study were validated by passage of transepithelial current pulses (Weinman and Reuss, 1982*a*) and only occasionally was the selectivity criterion applied.

Approximately one-third of the simultaneous conventional and Na-selective impalements that satisfied the criteria of abruptness and stability produced different basolateral membrane voltage deflections across the two impaled cells. These impalements were discarded.

Intracellular Na^+ Activity: Effects of Na^+ Removal, Amiloride, and Bumetanide

When *Necturus* gallbladder was bathed on both sides with Na-Ringer's (1 mM Hepes), aNa_i was 14.1 ± 1.9 mM ($n = 52$ tissues). When tissues were bathed on both sides with 10 mM HCO_3-Ringer's, aNa_i was 15.6 ± 1.2 mM ($n = 10$ tissues).

In order to evaluate the mechanisms responsible for the maintenance of steady state aNa_i, the effects of mucosal Na^+ removal or mucosal addition of amiloride (10^{-3} M) or bumetanide (10^{-5} M) were studied. As illustrated in Fig. 2 and summarized in Table I, Na^+ replacement with TMA^+ reduced aNa_i by ~50% in 2 min. Amiloride, after 2 min, caused a 12% reduction, which was statistically significant, and bumetanide caused no measurable change. Similar results were obtained with amiloride and bumetanide in 10 mM HCO_3-Ringer's (Table I). The initial rates of change of aNa_i in Na-Ringer's, calculated over the first 20 s,

were 10.8 ± 1.6 mM/min after Na^+ removal (n = 20) and 2.2 ± 0.4 mM/min after amiloride addition (n = 20).

Changes in aNa_i do not provide quantitative information about the inhibition of the Na^+ influx step by these experimental perturbations. In the case of complete Na^+ substitution with TMA^+, all apical Na^+ entry has certainly stopped, but the decline of aNa_i may be partly due to Na^+ exit across the apical membrane. In the case of amiloride, the small magnitude of the change may in part reflect a changing basolateral Na^+ extrusion rate or cell shrinkage, which would tend to blunt the change of aNa_i (Spring and Ericson, 1982). In the case of bumetanide,

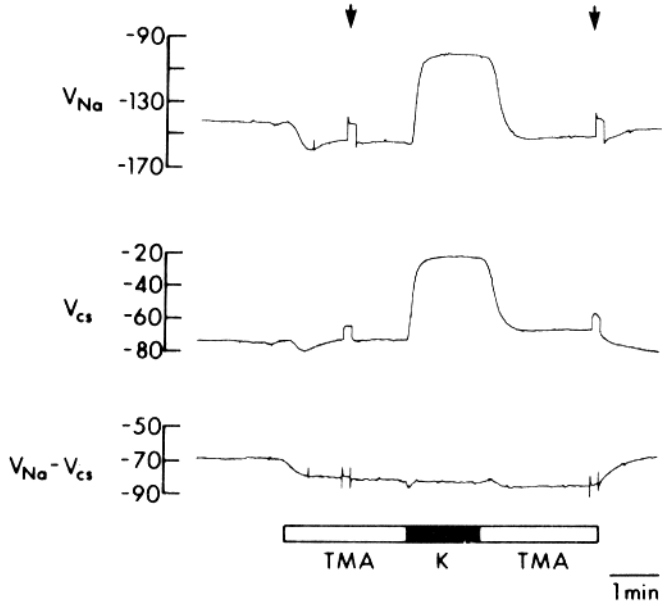

FIGURE 1. Validation of aNa_i measurements. The records begin with both KCl-filled and Na-selective microelectrodes in cells. Potentials are in millivolts as defined in Materials and Methods. At the two arrows, transepithelial current pulses (100 $\mu A/cm^2$, 10 s duration) were passed. At the beginning and end of the record, the mucosal solution was Na-Ringer's. For the periods indicated by the bar, it was replaced with TMA-Ringer's, K-Ringer's, and TMA-Ringer's. In the lower trace, a downward change represents a decline in aNa_i.

however, the absence of any effect on aNa_i would suggest that there has been no inhibition of Na^+ influx. Even if cell volume decreased in parallel with a reduction of Na^+ content, aNa_i would be expected to decrease, since Na^+ constitutes a small fraction of the intracellular cation pool.

Since Larson and Spring (1983) have reported that bumetanide causes a large, rapid reduction of aNa_i at a higher concentration (10^{-4} M), we also tested this concentration in four experiments. No significant changes in aNa_i were observed (aNa_i values were 11.3 ± 1.2, 12.3 ± 1.1, and 11.7 ± 1.0 mM before, during [3 min], and after exposure to bumetanide, respectively).

Effect of Ouabain on aNa_i

Since changes in the rate of Na^+ extrusion by the basolateral Na^+-K^+ ATPase might tend to minimize changes in aNa_i produced by inhibition of Na^+ entry, we chose to study the Na^+ entry step after inhibiting the basolateral pump with ouabain. After exposure to ouabain, the cells became somewhat more difficult to impale, but valid simultaneous impalements could be obtained.

The gallbladder was bathed in Na-Ringer's on both sides, and ouabain (10^{-4} M) was added to the serosal bathing solution. After a 5–10-min lag, aNa_i increased roughly linearly at a rate of 0.8 mM/min (Fig. 3). This increase in aNa_i reduces the driving force for net Na^+ entry across the apical membrane and therefore makes it difficult to study this process. Hence, we reduced aNa_i in ouabain-treated tissues by complete replacement of Na^+ with TMA^+, either bilaterally or on the mucosal side only. When tissues were first exposed to

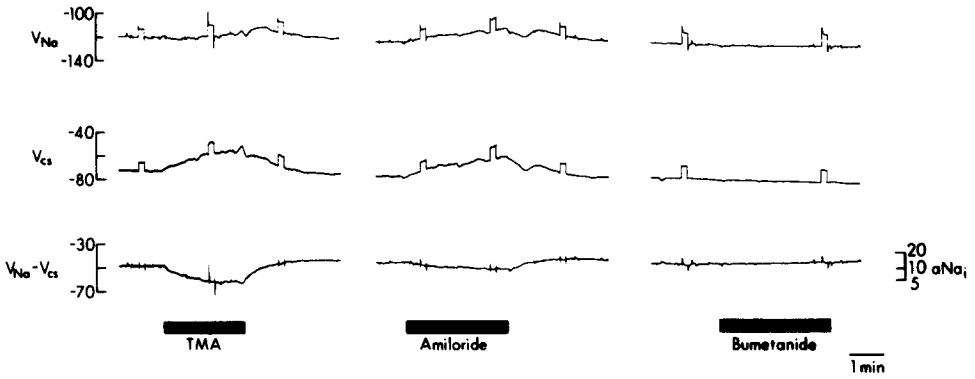

FIGURE 2. Effects of Na removal, amiloride, and bumetanide on aNa_i. Potentials were measured and transepithelial current pulses were passed as in Fig. 1. All records start with both microelectrodes in cells. At the times indicated by the bars, mucosal Na-Ringer's was replaced with TMA-Ringer's, Na-Ringer's plus 10^{-3} M amiloride, or Na-Ringer's plus 10^{-5} M bumetanide.

ouabain in Na-containing solutions and then Na^+ was replaced on the mucosal side only, aNa_i could be lowered to ~20 mM. If, however, Na^+ was replaced by TMA^+ on both sides of the epithelium, aNa_i could be consistently lowered to ~5 mM. The need to remove Na^+ from both sides in order to lower aNa_i could reflect either a component of Na^+ entry across the basolateral membrane or a serosa-to-mucosa paracellular Na^+ leak followed by apical membrane Na^+ entry.

Na^+ Influx in Ouabain-treated Tissues: Effects of Amiloride and Bumetanide

Tissues were treated with ouabain for at least 45 min while aNa_i was kept low by exposure to TMA-Ringer's on either the mucosal side only or on both sides of the tissue. After a steady value of aNa_i was measured by simultaneous impalements, the mucosal solution was suddenly changed from TMA-Ringer's to Na-Ringer's containing either no drug (control), amiloride (10^{-3} M), or bumetanide (10^{-5} M). The increases in aNa_i after 1 min were compared for these

three conditions. As illustrated in Fig. 4 and summarized in Table II, upon re-exposure of the mucosal side of the tissue to Na-Ringer's, Na^+ enters the cells and causes a rise in aNa_i of ~11 mM in the first minute. This increase is inhibited by ~50% by amiloride; it is unaffected by bumetanide.

Transepithelial and cell membrane potentials before and after re-exposure to Na^+ under these conditions are presented in Tables III and IV. It is interesting to note that there is no significant difference between control, amiloride, or bumetanide. This suggests that the mechanism of Na^+ entry across the apical membrane is electrically silent (see Discussion).

Effect of External pH on aNa_i

The observation that ~50% of Na^+ entry in ouabain-treated gallbladders can be inhibited by 1 mM amiloride suggests that Na^+-H^+ exchange accounts for a substantial fraction of entry. In principle, however, inhibition of Na^+ entry by amiloride does not prove that it results from Na^+-H^+ exchange. As a result of

TABLE I

Effect of Mucosal Solution Replacement on aNa_i

	Control solution (before)	Experimental solution	Control solution (after)	n	P
Hepes-buffered					
TMA-Ringer's	14.0±1.2	6.9±0.6	13.8±1.2	21	<0.001
Amiloride (10^{-5} M)	15.0±1.8	13.3±1.6	15.9±1.8	21	<0.001
Bumetanide (10^{-5} M)	14.4±2.7	14.6±2.7	14.7±3.1	13	NS
HCO_3-buffered					
Amiloride (10^{-5} M)	16.1±1.4	15.0±1.2	16.1±1.2	9	<0.025
Bumetanide (10^{-5} M)	13.3±1.3	13.6±1.2	14.2±1.5	8	NS

Values are intracellular Na^+ activities (in millimolar) measured in Na- or HCO_3-Ringer's before, 2 min after solution change, and after returning to the control solution. Drugs were added to the appropriate Ringer's solution at the indicated concentrations.

inhibiting Na^+-H^+ exchange, amiloride decreases intracellular pH in *Necturus* gallbladder (Weinman and Reuss, 1982*a*). Conceivably, the lower rate of Na^+ entry in amiloride-treated tissues could result from a difference in intracellular pH after re-exposure to Na^+. Even if the only effect of amiloride were to block Na^+-H^+ exchange, the intracellular acidification thus caused might inhibit another pathway for Na^+ entry, such as NaCl cotransport. This possibility was ruled out by the experiments shown in Fig. 5 and Table V. In these experiments, the external Na^+ concentration was adjusted to keep the Na^+ activity ratio across the apical membrane near unity. If Na^+-H^+ exchange were the mechanism by which Na^+ crosses the membrane, sizable net fluxes could then be induced by mucosal solution pH changes.

Tissues were exposed to TMA-Ringer's with ouabain on the serosal side and TMA-Ringer's containing 11 mM Na^+ on the mucosal side. As shown in Fig. 5, changing the pH of the mucosal solution from 7.7 to 6.1 (HCl titration), at constant mucosal Na^+ concentration, caused a fall in aNa_i, consistent with a net Na^+ flux mediated by Na^+-H^+ exchange. When the experiment was repeated

with amiloride added to the pH 6.1 solution, $a\mathrm{Na}_i$ changes were abolished. A subsequent mucosal acidification without amiloride demonstrated that the tissue retained the ability to respond to mucosal solution pH changes. The results of similar experiments in five tissues are presented in Table V. They demonstrate that pH gradients across the apical membrane induce large changes in $a\mathrm{Na}_i$ in ouabain-treated *Necturus* gallbladder. These changes are abolished by amiloride.

Unidirectional $^{22}Na^+$ Influx

The studies on ouabain-treated *Necturus* gallbladder have shown bumetanide-insensitive, amiloride-inhibitable Na^+-H^+ exchange to be a major mechanism of Na^+ flux across the apical membrane. However, it is possible that the magnitude of Na^+-H^+ exchange in tissues exposed to ouabain is different from that under control conditions. It was thus necessary to establish whether Na^+-H^+ exchange is also a sizable component of Na^+ entry in tissues in which the Na^+ pump is

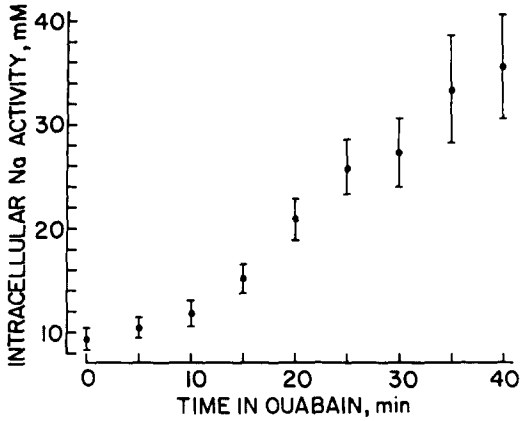

FIGURE 3. Intracellular Na^+ activity as a function of time of exposure to ouabain. $a\mathrm{Na}_i$ was determined from continuous intracellular recordings after ouabain (10^{-4} M) was added to the serosal solution. For each point n ranges from 8 to 13 tissues.

operative. This was accomplished by measurements of $^{22}Na^+$ influx from mucosal solution into the epithelium with methods similar to those used by others (Schultz et al., 1967; Frizzell et al., 1975).

The time course of $^{22}Na^+$ entry into the epithelium is shown in Fig. 6. During the first 60 s, influx is linear and can therefore be presumed to represent the entry process without any significant contribution of $^{22}Na^+$ backflux (Schultz et al., 1967).

The effects amiloride and bumetanide on J_{me} are summarized in Table VI. J_{me} was reduced by 40% by the presence of amiloride (10^{-3} M) in the mucosal solution. This result is statistically significant ($P < 0.001$) and is in good quantitative agreement with the 54% reduction of Na^+ entry estimated from $a\mathrm{Na}_i$ measurements in ouabain-treated tissues (Table II). Bumetanide (10^{-5} M) reduced J_{me} by 19%. This result is also statistically significant ($P < 0.05$) and may reflect a smaller component of Na^+ influx that is bumetanide inhibitable.

DISCUSSION

The importance of the results presented in this paper lies in how they relate to the mechanism of neutral NaCl entry in leaky epithelia. Na^+ and Cl^- entry are linked in many systems and this observation has been interpreted to result from either a direct NaCl cotransporter (Frizzell et al., 1979*a*), or parallel Na^+-H^+, Cl^--OH^- exchangers (Turnberg et al., 1970; Liedtke and Hopfer, 1977).

The proposal of an apical membrane NaCl cotransporter is based on demon-

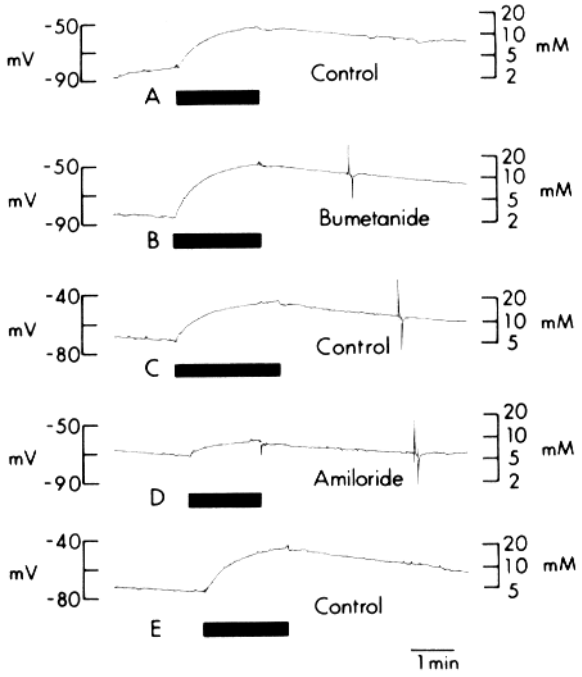

FIGURE 4. Changes in $a\mathrm{Na}_i$ upon sudden exposure to mucosal Na^+ in a ouabain-treated gallbladder. These are five consecutive records obtained in a single tissue. The interval between traces is 1–4 min. The left-hand scale is $V_{Na} - V_{cs}$, the right-hand one is $a\mathrm{Na}_i$. The tissue was perfused on both sides with TMA-Ringer's and, at the times indicated by the bars, the mucosal solution was replaced with either Na-Ringer's without drugs (*A*, *C*, *E*), or with bumetanide (*B*), or amiloride (*D*). The deflections in the latter half of traces *B*, *C*, and *D* were produced by transepithelial current pulses.

strations of interdependence of Na^+ and Cl^- fuxes and the effects of furosemide or bumetanide on cell volume changes and Na-dependent Cl^- fluxes or intracellular Cl^- activity (Quay and Armstrong, 1969; Frizzell et al., 1975, 1979*b*; Cremaschi and Hénin, 1975; Eveloff et al., 1978; Oberleithner et al., 1982; Ericson and Spring, 1982*a*). However, these arguments are inconclusive because flux interdependence can also be explained by parallel exchangers, and because the implied specificity of the loop diuretics is uncertain, particularly since

TABLE II

Intracellular Na^+ Activity Changes in Ouabain-treated Gallbladders

	Control before	Drug	Control after	n	P
Amiloride (10^{-5} M)	11.3±1.6	5.2±0.8	11.4±2.7	7	<0.001
Bumetanide (10^{-5} M)	10.6±1.8	10.1±1.5	10.5±1.6	7	NS

Values are the change in $a\mathrm{Na}_i$ (in millimolar) measured 1 min after a rapid change of the mucosal solution from TMA-Ringer's to Na-Ringer's. Each line represents the magnitude of this change measured in each tissue when the Na-Ringer's contained either no drug (controls), amiloride, or bumetanide. n = number of tissues. P compares columns 1 and 2 and was determined by the paired t test.

furosemide and bumetanide inhibit purely anionic exchange processes (Brazy and Gunn, 1976; Cousin and Motais, 1976; Aronson and Seifter, 1983). Furthermore, a kinetic analysis of Cl^--Cl^- exchange at equilibrium, in intestinal luminal membrane vesicles, revealed no dependence on Na^+, which suggests the absence of a ternary-complex NaCl cotransporter (Liedtke and Hopfer, 1982*a*). In addition, in two preliminary studies in renal brush-border vesicles, the existence of NaCl cotransport could not be demonstrated (Seifter et al., 1980; Warnock and Yee, 1981*b*).

Both Na^+-H^+ and Cl^--OH^- exchangers have been identified in apical membrane vesicles (Murer et al., 1976; Kinsella and Aronson, 1980; Warnock and Yee, 1981*a*; Liedtke and Hopfer, 1982*a*, *b*), but their existence does not prove that they are present in the apical membrane of intact cells or permit a quantitative assessment of their role in fluid transport.

Previous studies (Weinman and Reuss, 1982*a*) have demonstrated conclusively the existence of Na^+-H^+ exchange at the apical membrane of *Necturus* gallbladder epithelial cells under control in vitro conditions. This process was also shown to participate in the regulation of intracellular pH, but its contribution to transepithelial Na^+ transport could not be established quantitatively. The results of this study demonstrate that Na^+-H^+ exchange accounts for a substantial fraction of apical Na^+ entry, and that NaCl cotransport (estimated from the bumetanide-sensitive Na^+ entry) is quantitatively less important. In ouabain-treated tissues, $a\mathrm{Na}_i$ increases resulting from imposed transapical membrane Na^+ gradients are inhibited by ~50% by amiloride, and large amiloride-inhibitable $a\mathrm{Na}_i$ changes are generated by imposition of pH gradients across the apical membrane. In control tissues not treated with ouabain, 40% of unidirectional apical ^{22}Na uptake is inhibited by amiloride. In contrast, bumetanide had no effect on $a\mathrm{Na}_i$ changes

TABLE III

Transepithelial and Cell Membrane Potentials in Ouabain-treated Tissues

	V_{ms}	V_{mc}	V_{cs}	n
TMA/Na	+33.3±2.9	−70.6±4.7	−37.4±3.5	4
TMA/TMA	+3.0±1.6	−54.2±8.8	−51.3±7.8	5

Values are in millivolts; polarities are as defined in Materials and Methods. The first row is for tissues bathed by TMA-Ringer's on the mucosal side, and by Na-Ringer's on the serosal side. The second row is for tissues bathed by TMA-Ringer's on both sides. n = number of tissues.

TABLE IV

Changes in Transepithelial and Cell Membrane Potentials Accompanying Na^+ Entry in Ouabain-treated Tissues

	ΔV_{ms}	ΔV_{mc}	ΔV_{cs}	n
Control	−35.2±2.0	+18.3±3.1	−17.3±2.3	8
Amiloride (10^{-5} M)	−34.7±2.4	+23.3±2.6	−11.4±2.3	8
Bumetanide (10^{-5} M)	−35.2±2.2	+17.7±2.7	−17.6±2.2	7

Values are the changes in potential (in millivolts) produced by replacement of mucosal TMA-Ringer's by Na-Ringer's with or without the appropriate drug. They were measured 1 min after the solution change. n = number of tissues. Neither drug caused significant changes in ΔV_{ms}, ΔV_{mc}, or ΔV_{cs}.

in ouabain-treated tissues and caused a smaller inhibition (19%) of unidirectional ^{22}Na uptake.

Na^+ Entry Results Predominantly from Na^+-H^+ Exchange

Measurements of aNa$_i$ changes produced by both transapical Na^+ and pH gradients have demonstrated that Na^+ entry in ouabain-treated tissues results largely from Na^+-H^+ exchange. If the cells swell upon addition of Na^+ to the mucosal solution, then the magnitude of the observed aNa$_i$ changes would underestimate the actual Na^+ influx. Although only 54% of the aNa$_i$ change was inhibited by amiloride, the fraction of entry resulting from Na^+-H^+ exchange could be even greater if the kinetic constants of amiloride inhibition of Na^+-H^+ exchange in *Necturus* gallbladder are similar to those determined in renal brush-border vesicles (Kinsella and Aronson, 1981). In addition, the lack of effect of bumetanide at 10^{-5} M, i.e., a concentration much higher than that required to block cotransport in other systems (see Schlatter et al., 1983), indicates that

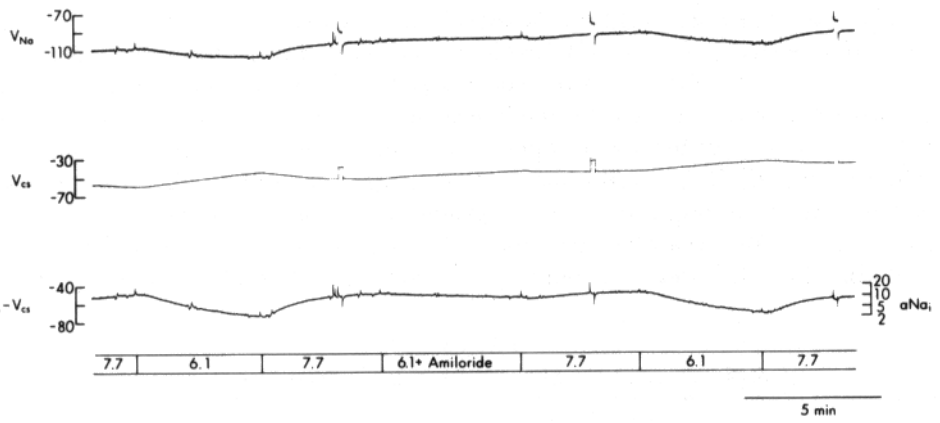

FIGURE 5. Effect of mucosal solution pH changes on aNa$_i$ in a ouabain-treated gallbladder. Simultaneous intracellular recordings with Na-selective and KCl-filled microelectrodes are shown as in Figs. 1, 2, and 4. The mucosal solution Na^+ concentration was kept constant at 11 mM throughout. The serosal solution was TMA-Ringer's. At the times indicated, the mucosal solution pH was changed from 7.7 to 6.1 in the presence or absence of amiloride (10^{-3} M).

TABLE V

Effect of Mucosal Solution pH on aNa_i at Constant Mucosal Na^+ Concentration

	Mucosal solution pH				
	7.7	6.1	7.7	*n*	*P*
Control	16.3±2.7	8.5±1.7	17.7±3.5	5	<0.01
Amiloride (10^{-3} M)	16.0±2.0	15.4±2.0	18.0±3.5	5	NS

Values are intracellular Na^+ activities (in millimolar) measured in tissues bathed by TMA-Ringer's on the serosal side and 11 mM Na^+, 98 mM TMA^+-Ringer's on the mucosal side. The first column is aNa_i at mucosal solution pH 7.7. The second column is aNa_i 4–5 min after mucosal solution pH change to 6.1. The third column is aNa_i 4–5 min after a return to mucosal pH 7.7. In the experiments summarized in the second row, amiloride was added only to the pH 6.1 solution. *n* = number of tissues. *P* compares columns 1 and 2 and was determined by the paired *t* test.

under the conditions of this study, bumetanide-inhibitable ion transport processes do not play a major role in apical membrane Na^+ entry.

The Na^+ entry process observed in this study is also not measurably electrogenic, and therefore not electrodiffusional. aNa_i increased at a rate of 11 mM/min (Table II) when Na^+ was suddenly added to the mucosal bathing solution. Assuming no cell volume changes, a Na^+ transport mechanism carrying one positive charge per Na^+ would result in a current of ~73 $\mu A/cm^2$ across the apical membrane. If the cells swell during Na^+ entry, the hypothetical Na^+ current would be even greater. Estimates of apical membrane resistance in this tissue range from 1,220 (Suzuki et al., 1982) to 4,470 Ωcm^2 (Frömter, 1972). The expected change in apical membrane potential resulting from this Na^+ current would therefore be from 90 to 325 mV, but the observed change is only 18 mV (Table IV). Two considerations reveal that this depolarization does not

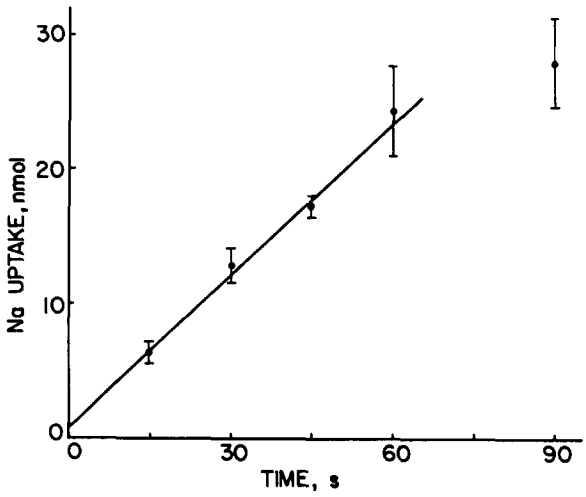

FIGURE 6. Time course of $^{22}Na^+$ uptake. The points represent uptake into single gallbladders (0.42 cm^2). *n* ranges from 5 to 15. The line is the linear least-squares fit to the first four points.

result from an inward Na^+ current across the apical membrane. First, mucosal substitution of TMA-Ringer's with Na-Ringer's results in a large paracellular biionic potential, as reflected by a mucosa-negative V_{ms} change of ~35 mV. This by itself results in a depolarization of V_{mc} that could account for most of the observed changes (Table IV). Second, although amiloride reduced Na^+ entry by ~50%, it had no significant effect on the membrane potential changes produced by the substitution of TMA-Ringer's with Na-Ringer's. We therefore conclude that the Na^+ entry process illustrated in Fig. 4 is electrically silent.

Apical Na^+ Entry in the Absence of Ouabain

Although Na^+-H^+ exchange is a major component of Na^+ entry in ouabain-treated tissues, it is possible that the conditions of the experiment enhanced the rate of Na^+-H^+ exchange above that in normal transporting tissues. To test whether Na^+-H^+ exchange accounts for significant Na^+ entry under control conditions, we measured unidirectional $^{22}Na^+$ uptake from the mucosal solution into the epithelium.

As summarized in Table VI, amiloride and bumetanide reduced uptake by 40

TABLE VI

Effects of Amiloride and Bumetanide on $^{22}Na^+$ Influx

	J_{mc}	n	P
	$\mu eq\ cm^{-2}h^{-1}$		
Na-Ringer's	3.35±0.16	15	—
Na-Ringer's + amiloride (10^{-3} M)	2.00±0.23	9	<0.001
Na-Ringer's + bumetanide (10^{-5} M)	2.72±0.19	10	<0.025

Values were determined from 45-s uptakes. n = number of tissues. P compares the influx rate in drug-containing solution with that in Na-Ringer's and was determined by the unpaired t test.

and 19%, respectively. The effect of amiloride cannot be explained by an indirect effect on pH_i because we have previously shown that intracellular acidification does not become apparent until ~2 min after exposure to the drug (see Fig. 9 of Weinman and Reuss, 1982*a*). We therefore conclude that in transporting gallbladders not treated with ouabain, Na^+-H^+ exchange still accounts for a substantial fraction of apical Na entry.

Unidirectional uptakes measured in this way are only estimates of net transapical Na^+ uptake. The control rate of uptake, 3.3 μeq cm^{-2} h^{-1}, is at least three times the rate of net transepithelial transport and therefore could include Na^+-Na^+ exchange and Na^+ entry into the lateral intercellular spaces in excess of the extracellular volume marker, but the linearity of uptake (Fig. 6) argues that the latter fraction must be small. These problems make quantitative interpretation of these results difficult, but the large effect of amiloride in our study suggests that Na^+-H^+ exchange is a significant mechanism of Na^+ entry even when the tissue has not been perturbed by exposure to Na-free solutions and/or ouabain. The smaller effect of bumetanide may indicate that there is a smaller component of Na^+ influx mediated by a bumetanide-inhibitable process such as NaCl

cotransport. However, the quantitative uncertainties of the $^{22}Na^+$ uptake experiments, the lack of any effect of bumetanide on Na^+ uptake as estimated from aNa_i measurements, and the possibility of inhibition of other transport processes by bumetanide (*vide supra*), make this conclusion less certain.

Intracellular Activity Changes in Tissues Not Treated with Ouabain

The above results clearly implicate Na^+-H^+ exchange in the apical Na entry process. It therefore, at first, seems paradoxical that amiloride addition produces only a small change in aNa_i (Table I). The magnitude of the aNa_i fall upon inhibition of entry is difficult to predict because it depends upon factors including changes of the rate of active basolateral Na^+ extrusion, the magnitude of Na^+ backleak through the basolateral membrane, and cell volume changes. After complete mucosal Na^+ removal, the initial rate of fall, 10.8 mM/min, may reflect apical membrane Na^+ exit as well as cessation of entry and persistent basolateral exit. For a cell height of 35 μm (Spring and Hope, 1979), ignoring cell volume changes, the calculated Na^+ flux would correspond to an isotonic fluid transport rate of ~22 $\mu l\ cm^{-2}\ h^{-1}$, i.e., a value much higher than that measured under isotonic conditions (Reuss et al., 1979; Persson and Spring, 1982).

Since pharmacological inhibition of Na^+ entry would not induce a backflux across the apical membrane, the rate of decline of aNa_i would certainly be smaller. The initial rate of fall of aNa_i after amiloride addition (2.2 mM/min) would correspond to a volume flux of ~5 $\mu l\ cm^{-2}\ h^{-1}$, a value in good agreement with that measured recently in this tissue (Larson and Spring, 1983; Peterson and Reuss, 1983). The small magnitude of the aNa_i change caused by amiloride could therefore be compatible with the blockage of a significant fraction of apical Na^+ entry. This result is similar to the observation of Eaton (1981) that although amiloride abolished the short-circuit current in rabbit urinary bladder, it only had a small effect on intracellular sodium activity.

Comparison with Other Studies in Necturus Gallbladder

The results obtained in this study do not agree with the conclusions drawn by Spring and colleagues (Ericson and Spring, 1982*a*, *b*; Larson and Spring, 1983), who measured cell volume in *Necturus* gallbladder epithelial cells. They concluded that volume regulation is the result of the operation of parallel Na^+-H^+ and Cl^--HCO_3^- exchangers, and that NaCl entry under control conditions is mediated by a NaCl cotransporter. In addition, recent results from the same laboratory indicate large falls of both aNa_i and aCl_i after addition of bumetanide to the mucosal medium (Larson and Spring, 1983).

The results reported in this paper disagree with the observation that aNa_i falls after application of mucosal bumetanide. This difference cannot be explained by differences in Ringer's solution, since we obtained similar results using 10 mM HCO_3-Ringer's (Table I). In addition, the activity of our bumetanide was verified in isolated bullfrog cornea (reduction of short-circuit current) and in vivo rat (diuretic effect). Finally, artifactual impalements with our Na^+ electrodes are unlikely, given the strict adherence to our validation criteria.

An explanation of the difference between our results and those of Spring and co-workers is not apparent. One possibility is that NaCl cotransport and double

exchange (Na^+-H^+, Cl^--HCO_3^-) represent different transport modes of the same carriers. Perhaps as yet unidentified experimental conditions have selected one or another of these modes.

In conclusion, the results reported in this paper demonstrate that at least 50% of Na^+ entry across the apical membrane of *Necturus* gallbladder is mediated by neutral, amiloride-inhibitable Na^+-H^+ exchange. A single NaCl cotransporter at the apical membrane cannot explain our observations. Whether or not Na^+-H^+ exchange in parallel with Cl^--HCO_3 exchange explains the coupling of Na^+ and Cl^-fluxes observed in gallbladder, and whether ternary-complex cotransport and double exchange may be different modes of operation of the same transporter, remain to be determined.

We are grateful for the help of Drs. E. Bello-Reuss, K.-U. Petersen, and P. Reinach in this work. We also wish to thank V. Creasy for excellent chamber construction and L. Larkin and S. Eads for secretarial help.

This work was supported by grant AM-19580 from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, and National Research Service Award GM 07200 from the National Institute of General Medical Sciences.

Received for publication 11 April 1983 and in revised form 12 July 1983.

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