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Current-Voltage Analysis of Apical Sodium Transport in Toad Urinary Bladder: Effects of Inhibitors of Transport and Metabolism

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Summary. The basal-lateral surface of the epithelium of the urinary bladder of the toad (*Bufo marinus*) was depolarized by exposure of the serosal surface to 85 mM KCL and 50 mM sucrose. The extent of depolarization appeared to be virtually complete, as evaluated by the invariance in the transepithelial electrical potential difference and conductance on addition of nystatin (a monovalent cation ionophore) to the serosal medium. The Na-specific current (I_{Na}) was defined as the current sensitive to the removal of Na from the mucosal medium or inhibitable by addition of amiloride to this medium. In the presence of the high K-sucrose serosal medium, rapid, serial, stepwise clamping of the transepithelial voltage (V) yielded a curvilinear dependence of I_{Na} on V; which is taken to represent the I–V curve of the apical Na channels. The constant field equation (Goldman, D.E. 1943; *J. Gen. Physiol.* **27**:37) fits the I–V data points closely, allowing estimates to be made of the permeability to Na of the apical membrane (P_{Na}) and of the intracellular Na activity (Na_c). Exposure of the apical surface to amiloride (5×10^{-7} M) decreased P_{Na} in proportion to the decrease in I_{Na} (i.e., ~70%) but decreased Na_c only 25%. In contrast, an equivalent reduction in I_{Na} elicited by exposure of the basal-lateral surface to ouabain was accompanied by only a 20% decrease in P_{Na} and a sixfold increase in Na_c. The effects of amiloride on P_{Na} and ouabain on Na_c are consistent with the primary pharmacological actions of these drugs. In addition, P_{Na} appears to be under metabolic control, in that 2-deoxyglucose, a specific inhibitor of glycolysis, decreased I_{Na} and P_{Na} proportionately, and lowered Na_c marginally, effects indistinguishable from those obtained with amiloride.

Transepithelial transport of Na by the toad urinary bladder has been considered to be a two-step process, as proposed for the frog skin by Koefoed-Johnsen and Ussing (1958). According to this scheme Na diffuses from the urine across the apical membrane into the epithelial cells, driven by the electrochemical activity gradient, and is subsequently extruded across the basal-lateral membrane by the energy-dependent Na pump. At the present time, however, little is known about the electrical properties of the apical and basal-lateral plasma membranes. One approach to the problem is the use of intracellular microelectrodes to record electrical events across each surface separately. This technique, however, is difficult to apply in tissues where cells are small and is subject to errors due to impalement artifacts (Lindemann, 1975; Higgins, Gebler & Frömter, 1977; Nelson, Ehrenfeld & Lindemann, 1978).

In the present study, we used a noninvasive technique to evaluate the electrical properties of the apical membrane of the toad bladder (Fuchs, Hviid Larsen & Lindemann, 1977). The basal-lateral membrane was depolarized by raising the concentration of K in, and adding sucrose to, the serosal medium. Depolarization was assessed with the monovalent cation ionophore, nystatin. The results indicate that under these conditions, the electrical potential and resistance properties of the epithelium are dominated by those of the apical membrane which are then conveniently studied with electrodes in the external solutions. The sodium current-voltage (I_{Na}–V) relationships of the depolarized toad bladder were used to measure the permeability of the apical membrane to Na (P_{Na}) under a variety of conditions.

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Materials and Methods

Animals

Bufo marinus (obtained from Lemberger, Germantown, Wisconsin) were kept in tanks with *ad lib* access to tap water. Animals were pithed, and the urinary bladders were quickly excised and mounted either as sacs or as flat sheets in chambers.

For sac preparations, hemibladders were mounted either mucosal side out or serosal out on plastic syringes, filled with 7 ml of solution and immersed in 70 ml of solution in plastic containers (Rossier, Wilce & Edelman, 1974). Electrical connections were made through agar bridges to Ag/AgCl (current) and calomel (voltage) electrodes. Open-circuit potential differences and short-circuit currents (I_{sc}) were measured as described previously (Rossier et al., 1974; Spooner & Edelman, 1975).

For flat sheet preparations, hemibladders were stretched slightly over a plastic ring (inside diameter 2 cm) with the mucosal side up and tied with thread to the outside groove. The serosal side of the tissue was supported by a piece of Melitta Kaffee filter No. 1 paper cut to fit the plastic ring. The entire assembly was mounted in a plastic chamber with a serosal volume of 18 ml open to the atmosphere and stirred by aeration. The mucosal solution (1 ml) was replaced continuously, at variable rates, by gravity feed from a 1-liter reservoir. A constant positive hydrostatic pressure difference of 10 cm of H_2O was maintained across the tissue. A ring-shaped gasket was made by cutting the same filter paper to match the plastic ring, dipping the filter in Sylgard which was partially cured with 1% (wt/wt) catalyst, draining the excess Sylgard and storing overnight at room temperature. This gasket was placed over the mucosal side of the bladder to improve sealing in the chamber and minimize edge damage.

Solutions

Serosal solutions consisted either of a conventional Na-Ringer's solution: NaCl, 110; $CaCl_2$, 1; $MgSO_4$, 0.5; K-phosphate, 3.5 (adjusted to pH 7.5); glucose 5 (all in mM) or high K-sucrose Ringer's solution in which NaCl was replaced by 85 mM KCl and 50 mM sucrose. Some experiments used Na-free, choline Ringer's solution in which NaCl was replaced with choline Cl. Nystatin (Sigma) was dissolved in methanol at 5 mg/ml, sonicated just before use, and added to the serosal medium to a final concentration of 35 μg/ml. When nystatin was used, the filter paper support on the serosal side of the tissue had to be eliminated as nystatin apparently did not penetrate the filter paper. Ouabain (Sigma) was dissolved in high K-sucrose solution and added to a final concentration of 10^{-3} to 10^{-2} M. Isotonic (236 mM) 2-deoxy-glucose (Sigma) was added to the serosal media to a final concentration of 5 mM.

Mucosal solutions usually contained Na_2SO_4 plus K_2SO_4 at a constant combined activity of 60 mM, Ca gluconate, 1 mM, and tris-H_2SO_4, 5 mM, pH 7.5 (Fuchs et al., 1977). Amiloride was dissolved in deionized water and added to a final concentration of 5×10^{-7} M for partial inhibition or $2.4\text{–}10\times10^{-5}$ M for complete inhibition of Na transport.

Electrical

Bladders in sac preparations were continuously open-circuited except for brief periods ($\sim$10 sec) for measurement of I_{sc}. In sheet preparations, bladders were voltage clamped. Electrical conductance was measured by displacing the clamping voltage from 0 to 10 mV and measuring the current difference. Capacitance was estimated by application of repetitive constant current pulses across the epithelium and recording the voltage change on an oscilloscope. In the absence of mucosal Na, with high K-sucrose on the serosal side the time dependence of the voltage rise was approximated by a single exponential curve. The capacitance was computed from the time required to reach half the final voltage value, according to the equation $C=t_{1/2}/(1n2\times R)$ where C is the capacitance in μF, $t_{1/2}$ the half-rise time in msec and R the resistance in kilohms. When the resistance of the basal-lateral membrane is small compared to the of the apical membrane, the transepithelial capacitance, measured by the half-rise time technique, should approximate that of the apical membrane. Basal-lateral membrane resistance was reduced by use of the high K-sucrose solution.

Current-voltage relationships were generated by stepwise voltage clamping and measuring the corresponding currents. The voltages were changed in steps of -5 mV, from $+20$ to -100 mV (mucosal relative to serosal). Step duration was 5 msec which was sufficient for the current to reach a constant value. The total sweep time was 120 msec. Apical Na permeability and intracellular Na activity were estimated by curve fitting of the I–V data points with the constant field equation (Goldman, 1943). The I–V parameters were measured in the presence of Na (20 mM activity) in the mucosal medium, and either in Na-free mucosal medium or in the presence of maximally effective concentrations of amiloride ($2.4\text{–}10\times10^{-5}$ M) or both. The Na-specific current (I_{Na}) was estimated at each voltage by subtraction of the measured current in the absence of Na transport from that obtained with 20 mM Na in the mucosal medium. Elimination of transepithelial Na transport by all three methods (Na-free, amiloride, or both) gave similar reference I–V curves (Na-independent pathway). Equivalent estimates of P_{Na} and Na_c were obtained with all three methods. The sequence of measurements (plus then minus Na transport or vice versa) had no effect on the results.

Control of the clamping voltage, storage of measured current values, subtraction of reference I–V relationships, and curve fitting with the constant field equation were done with the aid of a computer (Data General, Nova 1230). The details of the curve fitting technique are described in Fuchs et al. (1977).

Cell Volume

Cell water content was measured by incubating hemibladders in beakers in either conventional Na-Ringer's solution or depolarizing solutions (made up with various concentrations of KCl and sucrose) that contained ^{3}H-insulin (0.14 mCi/mg, New England Nuclear) at a final activity of 1 μCi/ml. Bladders were removed from the solutions, blotted briefly with Whatman #1 paper, and the epithelial cells were quickly scraped from the supporting tissue with a glass microscope slide, onto a piece of tared aluminum foil. The wet weight was measured immediately and the dry weight was measured after drying overnight at 98 °C. The dried epithelial scrapings were extracted in 1.0 ml of 0.1 N HNO_3 at room temperature for 24 to 48 hr. Aliquots of the extract (0.5 ml) were added to 10 ml of Aquasol (New England Nuclear) and assayed by liquid scintillation spectroscopy (Analytic 92, Searle). All solutions were water-clear and at the same pH, thus no corrections were made for self-absorption. The extracellular space was calculated as wet wt-dry wt-inulin space and normalized to the dry wt.

Results

Depolarization of the Basal-Lateral Membrane

Conductance Changes: Replacement of the Na-Ringer's with the KCl-sucrose solution resulted in

an immediate rise in total transepithelial conductance (g) and a lesser fall in I_{sc}; the new steady-state was reached in 30 min. The leak conductance (g_l) was measured, after stabilization, by replacement of the mucosal solution with the Na-free medium, and the Na-specific conductance (g_{Na}) was calculated from $g_{Na}=g-g_l$. Similarly I_{Na} was calculated from: $I_{Na}=I_{sc}-I_l$, where I_l is the short-circuit current measured in the absence of mucosal Na. Substitution of the KCl-sucrose solution for the Na-Ringer's solution doubled g_{Na}, had no effect on g_l and reduced I_{Na} by 24% (Table 1). Inasmuch as I_{Na} of the control hemibladders fell 14% in the same interval, the differential fall in I_{Na} (i.e., that attributable to the presence of the depolarizing solution) averaged 12%, and did not achieve statistical significance.

Nystatin Experiments: The extent of depolarization was evaluated by addition of nystatin to the serosal media: Paired hemibladders were mounted as sacs with Na-Ringer's on the serosal side and Na-free, choline Ringer's on the mucosal side. Despite the large serosal to mucosal Na activity gradient, I_{sc} was very small, as illustrated in Fig. 1. The failure to obtain a reversed I_{sc} presumably was due to the low Na permeability of the basal-lateral membrane and the tight junctions. Within 10 min after the addition of nystatin to the serosal medium, a large outward current (reversed I_{sc}) developed, presumably because the ionophore enabled Na to flow freely across the basal-lateral membrane. When amiloride was then added to the mucosal medium, the outward current returned to near base-line levels, indicating that the reversed current was passing through the Na-selective, amiloride-sensitive, apical membrane channels. These results confirm the monovalent cation ionophore action of nystatin on the basal-lateral plasma membrane since any effects on the parallel intercellular pathway should be insensitive to amiloride (Hong & Essig, 1976).

To evaluate the depolarizing action of the high K-sucrose solution, nystatin was added to the serosal side of open-circuited hemibladders, and the effects on transepithelial potential differences (V) were recorded. With Na-Ringer's solution on both sides (no transepithelial Na gradient) nystatin caused a rapid and monotonic decline in both V and I_{sc}, as expected for an induced increase in Na permeability of the basal-lateral membrane (Figs. 2 and 3). Substitution of choline for Na in the serosal medium modified

Table 1. Effects of the high K-sucrose serosal solution on transepithelial Na transport and conductance[a]

	I_{Na} (μA)	g_{Na} (mhos)	g_l (mhos)
Control			
Initial value	37 ± 3	350 ± 60	430 ± 160
Fractional change[b]	0.86 ± 0.06	0.88 ± 0.08	1.05 ± 0.08
High K-sucrose			
Initial value	40 ± 10	370 ± 90	420 ± 110
Fractional change[b]	0.76 ± 0.05	2.00 ± 0.27[c]	1.00 ± 0.04

[a] Paired hemibladders were mounted as flat sheets and preincubated in Na-Ringer's solution for 1–2 hr. Total transepithelial conductance (g) and I_{sc} were then measured. Leak conductance (g_l) and leak currents (I_l) were measured by substitution with Na-free mucosal solution. The Na specific values were computed from:

$I_{Na}=I_{sc}-I_l$

$g_{Na}=g-g_l$.

[b] The fractional changes were estimated as the ratio of the values recorded 60 min after replacement of the serosal solutions (high K-sucrose, or fresh Na-Ringer's) to the values recorded just before replacement (i.e., each hemibladder served as its own control) and given as means ± SEM, $n=5$ experiments.

[c] Denotes statistical significance $P<0.01$.

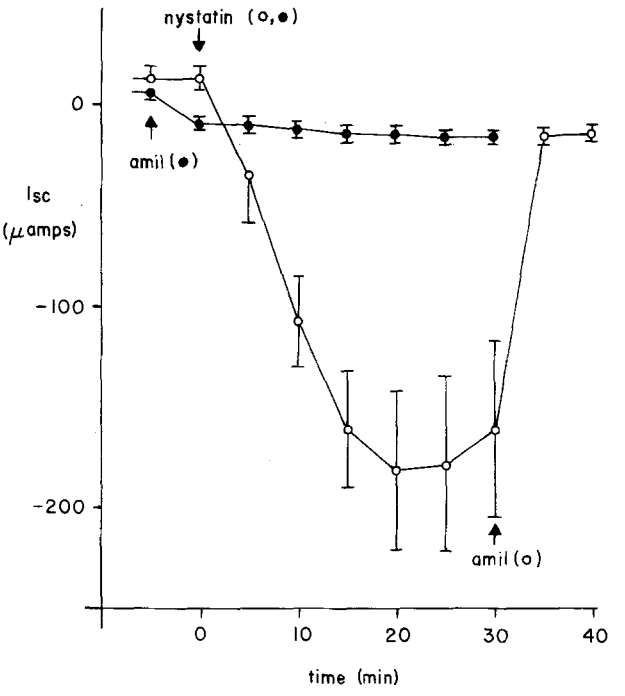

Fig. 1. Reverse short-circuit current (I_{sc}) induced by nystatin. Paired hemibladders were mounted as sacs with the conventional Na-Ringer's solution (Na = 100 mM) on the serosal side and choline-Ringer's (Na-free) solution on the mucosal side. Control hemibladders were preincubated for 5 min with amiloride on the mucosal side (final concentration 10^{-4} M). At time zero nystatin (35 μg/ml) was added to the serosal side of both the experimental and control bladder. After 30 sec, amiloride (final concentration 10^{-4} M) was added to the mucosal side of the experimental hemibladders. The bladders were in the open circuited state except for brief measurements of I_{sc}. The points and vertical lines represent the mean ± SEM of pairs of hemibladders

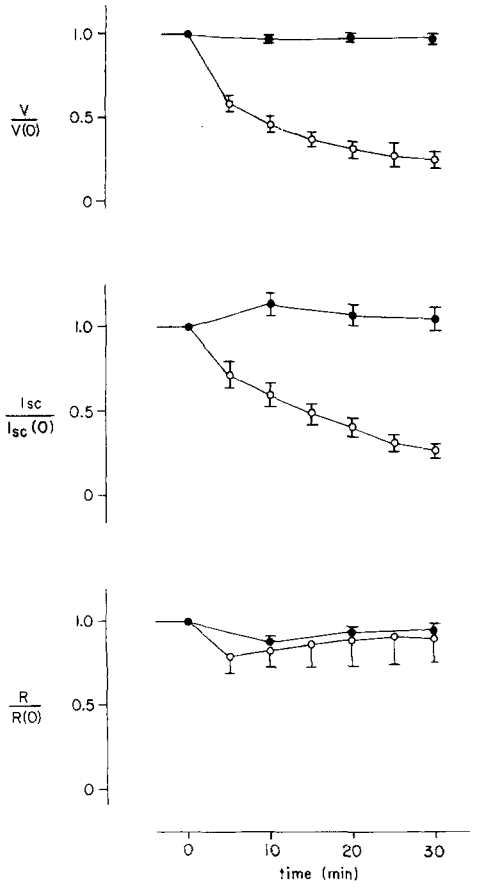

Fig. 2. Effect of nystatin on V, I_{sc} and R (Na-Ringer's solution). Paired hemibladders were mounted as sacs with Na-Ringer's solution (110 mM) on both sides. At time zero nystatin (35 μg/ml) was added to the serosal side of the experimental hemibladders, and diluent to that of the control. The bladders were in the open circuited state except for brief measurements of I_{sc}. Resistance was calculated as the ratio of the open-circuit voltage to the short-circuit current. Fractional changes were recorded as the ratio of a value at a given time after addition of nystatin or diluent to that recorded at time zero, just before the addition. The points and vertical lines represent the mean $\pm$SEM of 7 pairs of hemibladders. Initial values were for controls: $V=112\pm6$ mV; $I_{sc}=335\pm61$ μA; $R=385\pm45$ ohms; for experimentals: $V=107\pm7$ mV; $I_{sc}=325\pm75$ μA; $R=395\pm49$ ohms

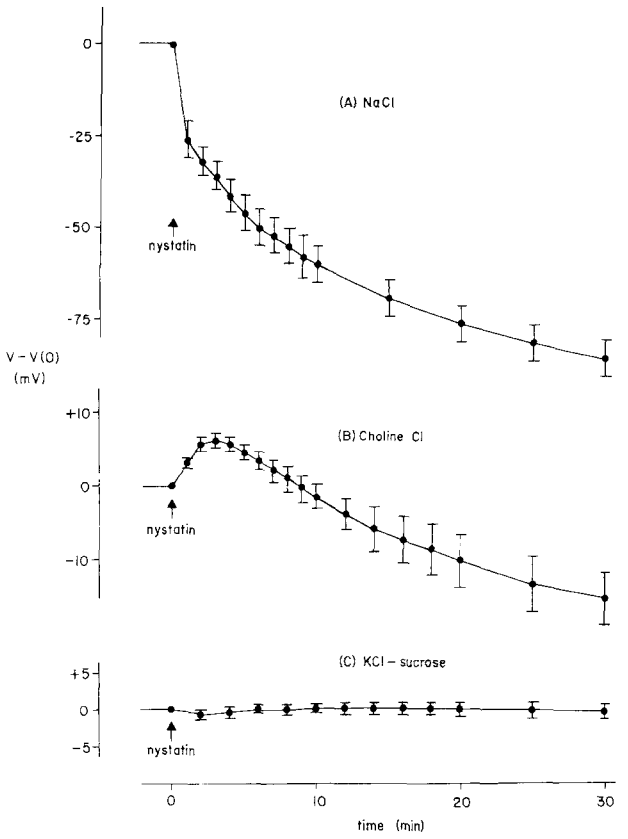

Fig. 3. Effect of nystatin on open-circuit potential. Hemibladders were mounted as sacs with Na-Ringer's solution on the mucosal side and either Na-Ringer's solution (*A*), choline-Ringer's solution (*B*) or high K-sucrose Ringer's solution (*C*) on the serosal side. At time zero, nystatin (35 μg/ml) was added to the serosal medium and open-circuit transepithelial potential monitored as a function of time. Means $\pm$SEM of 7 hemibladders. Initial values were: 112 ±6 mV (*A*); 43 ± 9 mV (*B*); 37 ± 8 mV (*C*). Paired hemibladders were used in (*B*) and (*C*). Hemibladders in (*A*) were from a different batch of toads

the response to nystatin, which now evoked a transient hyperpolarization, followed by a rapid depolarization (Fig. 3). In the high K-sucrose medium, however, addition of nystatin did not induce any of the changes seen in Na-Ringer's or choline-Ringer's: V, I_{sc} and R remained invariant for at least 30 min. The invariance in R and V on addition of nystatin implies that the basal-lateral membrane resistance to K was already low and that the potential difference (PD) across this membrane was already close to zero before addition of the ionophore.

I–V Relationship in High K-sucrose: The existence of a distinct nonlinearity in the reference *I–V* relationship (i.e., independent of transepithelial Na transport) provided an additional means of evaluating the inference that the high K-sucrose serosal medium completely depolarized the basal-lateral membrane. Figure 4 shows the *I–V* plot in the nontransporting state, with no Na and 80 μM amiloride in the mucosal medium. Under these conditions, the resistance of the apical membrane should be large compared to that of the basal-lateral membrane. With Na-Ringer's on the serosal side, the slope of the curve increases at large negative potentials, at about -100 mV. After depolarization (i.e., replacement with high K-sucrose on the serosal side) rectification of the outward current was more distinct and the break-point voltage (V') occurred at less negative potentials. Subsequent application of nystatin did not shift V' further (Fig. 4). At large negative potential (i.e., greater than V') the increased slope conductance obtained by substitution of the high K-sucrose solution presumably reflects

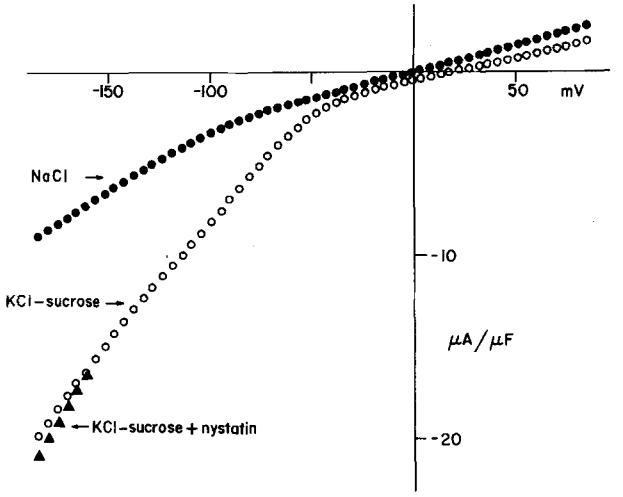

Fig. 4. *I–V* relationship with Na-free mucosal solutions. A single hemibladder was mounted as a flat sheet with choline-Ringer's solution plus 80 μM amiloride on the mucosal side and Na-Ringer's solution on the serosal side (filled circles). After the *I–V* curve was measured, the serosal medium was replaced with high K-sucrose and allowed to equilibrate for 60 min. The *I–V* curve was then remeasured (open circles). Nystatin (35 μg/ml) was then added to the serosal medium and the *I–V* curve measured again after 20 min. The curve is super-imposed on the high-K-sucrose data except at large negative voltages (triangles). Voltage staircase was +100 to −200 mV in 5-mV steps, and the step duration was 5 msec

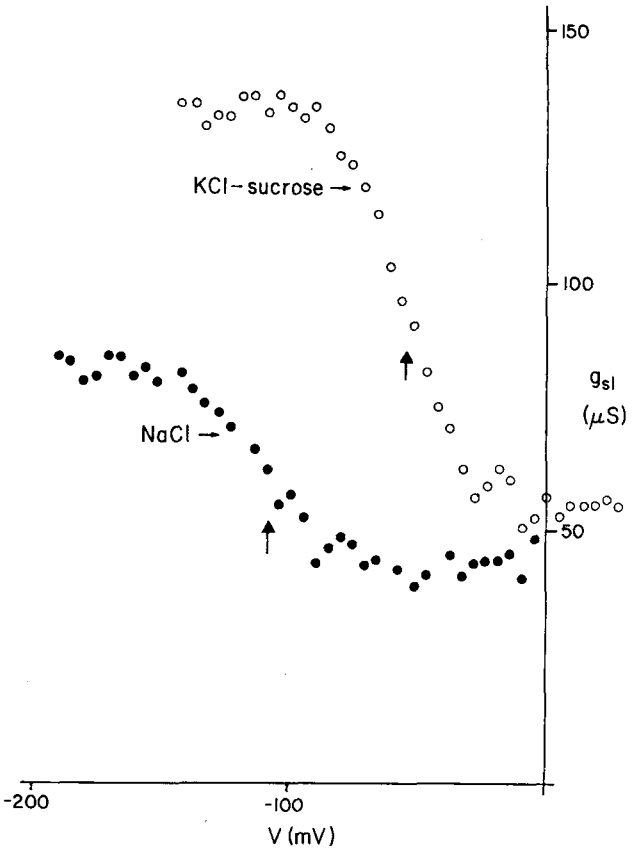

Fig. 5. Slope conductance as a function of transepithelial PD. The filled circles represent measurements with Na-Ringer's solution on the serosal side and the open circles the measurements with high K-sucrose on the serosal side. Data were calculated from the experiment shown in Fig. 4. Arrows represent the voltages at which the conductance was the mean of the two plateau values. These voltages were: −55 mV (Na-Ringer's solution); −110 mV (high K-sucrose)

a marked increase in conductance of the basal-lateral membrane. This implies a substantial contribution of the basal-lateral membrane resistance to the total transcellular resistance in the nondepolarized tissue over this voltage range. Furthermore, the augmentation in rectification of the outward current by depolarization of the basal-lateral membrane implies that the apical membrane leak conductance rather than the paracellular shunt conductance has rectifier properties. Thus, if the nonlinearity is a property of the apical membrane, the shift in V provides a rough estimate for the change in potential across the basal-lateral membrane elicited by the high K-sucrose medium. The shift in V can be evaluated more precisely from *g–V* plots. Figure 5 shows the conductance computed from the slopes in the *I–V* curve, as a function of voltage, before and after depolarization with high K-sucrose. The conductance is constant at low PD's, then increases sharply at the break point to a new constant level at hyperpolarizing potentials. The voltage at which the conductance was the mean of the two plateau values (i.e., half-point in the sigmoid curve) was −105 mV in Na-Ringers's and −55 mV in high K-sucrose medium. Thus, the K-dependent change in the series basal-lateral membrane voltage was estimated to be about 50 mV (*see* Discussion). This voltage change was not increased further by nystatin, implying complete depolarization before addition of the ionophore. These data are consistent with a "well-type" potential profile under these conditions with an intracellular potential of about −50 mV.

Cell Volume: As substitution of KCl for NaCl was expected to produce cell swelling, a range of concentrations of sucrose and KCl were prepared at a constant osmolality of 220 mOsm/liter, and the effects on cell volume were estimated by measurement of the noninulin water volume of the scraped epithelium. All solutions contained 3.5 mM K-phosphate, 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$. Hemibladders were incubated either in conventional Na-Ringer's or in a K-sucrose mixture for 60 min at room temperature in beakers. The results are expressed, in units of mg H_2O/mg dry wt, as absolute differences from the control. Substitution of sucrose of KCl resulted in a curvilinear, monotonic decrease in epithelial cell volume

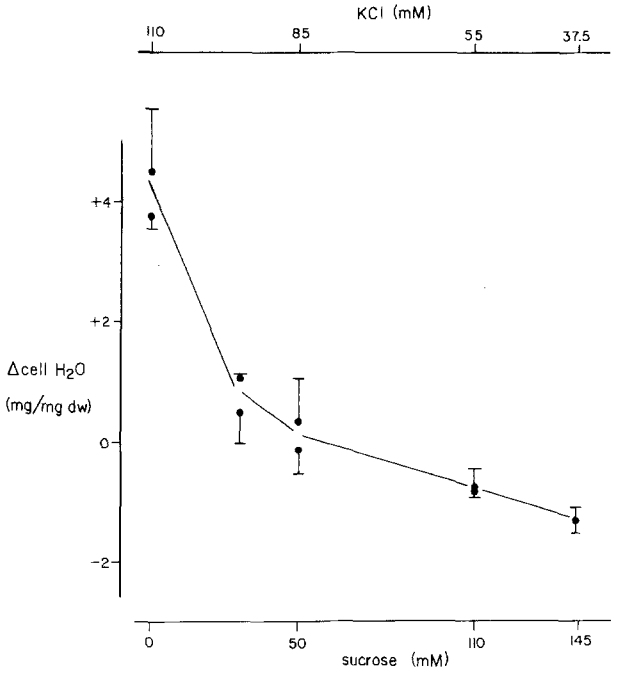

Fig. 6. Epithelial cell water as a function of serosal K and sucrose. Hemibladders were incubated for 1 hr in either Na-Ringer's solution or solutions in which NaCl was replaced by various iso-osmotic combinations of KCl+sucrose. The scraped epithelium was then assayed. Data are plotted as the absolute difference in water content of the hemibladders in Na-Ringer's solution from those in high K-sucrose solution, after correction for inulin space and expressed in mg/mg dry wt. Each point represents the mean ± SEM for 4 hemibladders. Water content in Na-Ringer's solution was 3.76 ± 0.16 mg/mg dry wt (13 hemibladders)

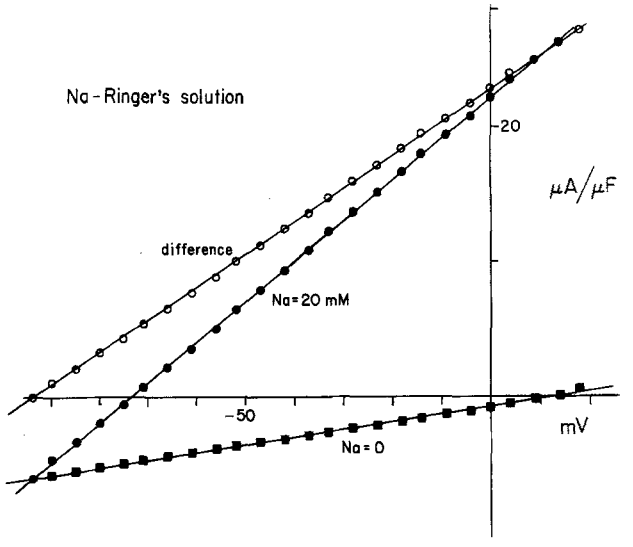

Fig. 7. *I–V* relationships with and without mucosal Na. A hemibladder was mounted as a flat sheet with Na-Ringer's on the serosal side. The mucosal solution was Na-free (filled squares) during measurement of the first *I–V* curve. The mucosal solution was then replaced with 20 mM Na (filled circles) and the *I–V* curve was remeasured. The difference curve is represented by the open circles

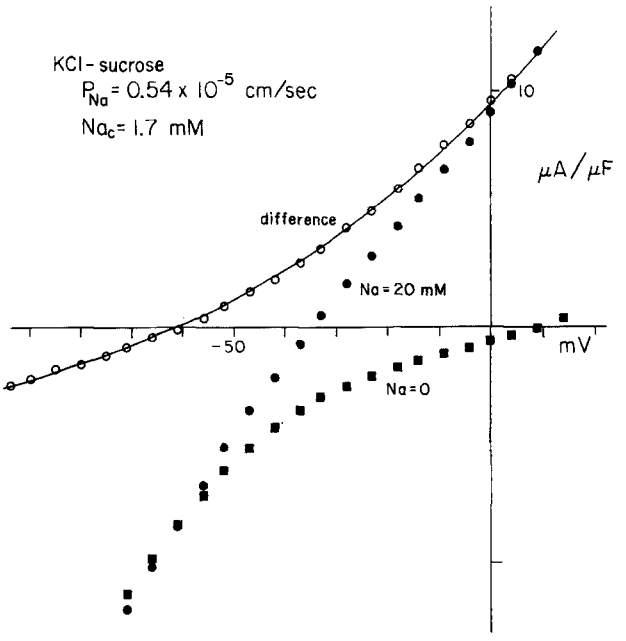

Fig. 8. *I–V* relationships with high K-sucrose serosal medium. The hemibladder analyzed in Fig. 7 was incubated in high K-sucrose for 60 min and the *I–V* curves were measured sequentially with Na-free (filled squares) and 20 mM Na (filled circles) mucosal solutions. The difference curve (open circles) is fitted with the constant field equation (Goldman, 1943) and yielded values of $P_{Na} = 0.54 \times 10^{-5}$ cm/sec, and $Na_c = 1.7$ mM

(Fig. 6). No significant change in volume was obtained at 85 mM KCl −50 mM sucrose. Thus, this combination was used in all of the standard depolarizing experiments.

I–V Relationship of the Apical Membrane

Application of the Constant Field Equation: With Na-Ringer's solution on the serosal side, the *I–V* curves both in the presence of 20 mM Na and in Na-free mucosal media are linear over the full range of applied voltages (+20 to −100 mV; serosal side as the reference) (Fig. 7). Sixty min after replacement of the serosal medium with high K-sucrose, the *I–V* curves in the presence of either 20 mM Na or 0 mM Na in the mucosal media now show outward current rectification at −50 mV, the slope conductances increased at large negative potentials. The difference curve, representing the *I–V* relationship of the Na transport pathway was nonlinear but in contrast, the slope conductance decreased at large negative voltages (Fig. 8). The *I–V* curves under depolarizing conditions, therefore, cannot be analyzed in terms of a linear equiva-

lent circuit but, as in frog skin (Fuchs et al., 1977), may be quantified by use of the constant field equation for a single permeant ion:

$$I = P_{Na} \frac{F^2 \cdot V/RT}{1-\exp(-FV/RT)} \cdot [Na_m - Na_c \exp(-FV/RT)] \quad (1)$$

where F is the Faraday constant, T the absolute temperature in degrees Kelvin, R the gas constant, and Na_m the activity of Na in the mucosal solution. The variables used to fit the equation to the data were P_{Na} (the apical membrane permeability coefficient) and the Na_c (Na ion activity in the epithelial cell near the apical membrane). The solid line in Fig. 8 represents the curve drawn from the constant field equation for $P_{Na} = 0.54 \times 10^{-5}$ cm/sec and $Na_c =$ 1.7 mM, and fits the data points closely.

The constant field equation fit the I–V curves well over the voltage domain of +10 to −60 mV in virtually every bladder tested, provided that the two I–V curves to be subtracted from each other were measured within 5 min. Rarely (less than 1 in 20) I–V curves could not be satisfactorily fitted with the constant field equation, in which case the data were rejected.

Two protocols, both of which gave satisfactory fits to the constant field equation, were used interchangeably according to the needs of a particular experiment: (1) The reference curve was first obtained with the Na-free mucosal solution (no amiloride) and the $I_{Na} - V$ curve was remeasured after replacement of the mucosal medium with one of the Na-containing Ringer's solutions. The second I–V curve was recorded as soon as I_{sc} stabilized. (2) The I–V curve in the presence of mucosal Na was recorded first, followed by addition of amiloride ($2.4–10 \times 10^{-5}$ M) and the reference I–V curve was recorded as soon as I_{sc} was stabilized.

In control hemibladders exposed to the high K-sucrose serosal medium and the 20 mM Na mucosal medium for at least 1 hr and with I_{sc} at steady state, the constant field equation applied to the I–V curves yielded values of $P_{Na} = 0.52 \pm 0.40 \times 10^{-5}$ cm/sec, and $Na_c = 1.9 \pm 0.8$ mM (mean ± SD; $n = 51$ hemibladders). These bladders were assayed 1 to 3 hr after mounting and were maintained in 10 mM serosal glucose throughout.

For the calculation of P_{Na} the surface area of the exposed epithelium was assumed to be proportional to the capacitance of the tissue measured in the absence of mucosal Na, with a proportionality constant of 1 cm^2/μF (Lewis, Eaton & Diamond, 1976).

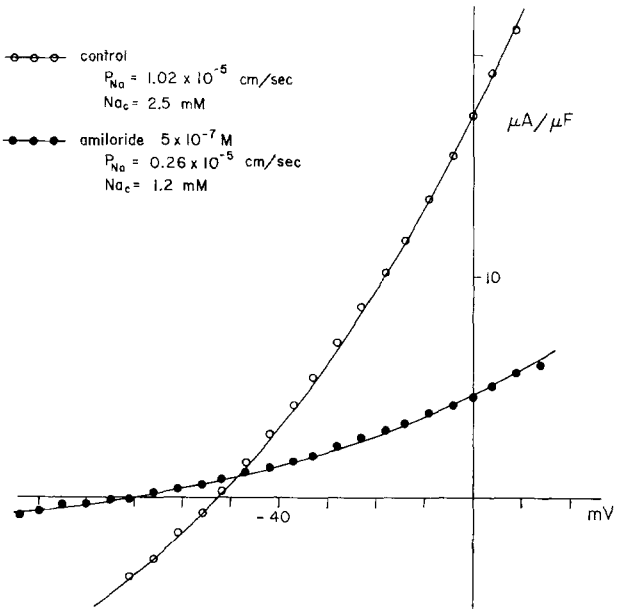

Fig. 9. Effect of amiloride on I–V relationships. A hemibladder was counted as a flat sheet with high K-sucrose serosal medium and the I–V difference curves were measured with 20 mM Na and Na-free mucosal solutions (*see* Fig. 8) before (open circles) and after (closed circles) the addition of a submaximal inhibitory dose of amiloride (final concentration = 5×10^{-7} M) to the mucosal medium. P_{Na} and Na_c were computed from the best fit of the constant field equation to the data

Effect of Amiloride: When amiloride was applied to the mucosal surface of the bladders at submaximal inhibitory concentrations I_{sc} fell immediately and stabilized within 1 min. I–V curves in the control and inhibited states were obtained with mucosal solutions that contained 20 mM Na, or 20 mM Na + 0.5 μM amiloride. To correct for the I–V characteristics of the passive, parallel (shunt) pathway, measurements were also made with 0 Na + 24 μM amiloride on the mucosal side. A representative experiment is shown in Fig. 9. In 6 experiments, a 70% reduction in I_{Na} was accompanied by a 71% fall in P_{Na} and a 26% fall in Na_c (Table 2). According to Eq. (1), the fall in Na_c should produce a small increase in the driving force for movement of Na across the apical membrane. Thus, the amiloride-dependent decrease in I_{Na} is accounted for by the decrease in P_{Na}.

Effect of Ouabain: I–V curves were measured before and at various times after the addition of ouabain (10^{-3} M) to the serosal medium (Fig. 10). At 10^{-3} M, ouabain elicited a slow continuous fall in I_{sc}. After 60 min, I_{Na} fell to 34% of its initial value (Table 2). I–V analysis at the point of 66% inhibition of I_{Na}, showed a 20% fall in P_{Na} and a sixfold rise in Na_c. The rise in Na_c presumably reflects blockade of the

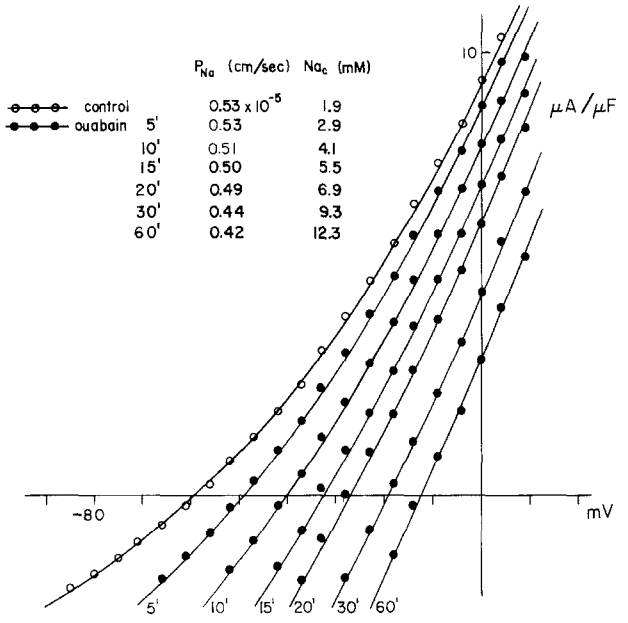

Fig. 10. Effect of ouabain on *I–V* relationships. A hemibladder was mounted as a flat sheet with high K-sucrose serosal medium and the *I–V* difference curves were measured with 20 mM Na and Na-free mucosal solution (*see* Fig. 8) before (open circles) and after (filled circles) the addition of ouabain (10^{-3} M) to the serosal medium at the indicated times. The values from P_{Na} and Na_c were computed from the best fit of the constant field equation to the data

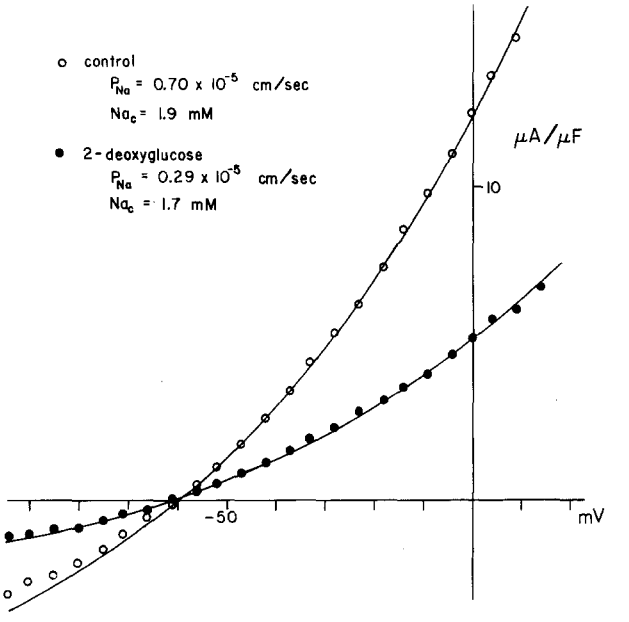

Fig. 11. Effect of 2-DG on *I–V* relationships. A hemibladder was mounted as a flat sheet with high K-sucrose serosal medium and the *I–V* difference curves were measured with 20 mM Na and Na-free mucosal solutions (*see* Fig. 8) before (open circles) and after (filled circles) the addition of 2-DG to the serosal medium. P_{Na} and Na_c were computed from the best fit of the constant field equation to the data

Table 2. Effects of amiloride, ouabain and 2-deoxyglucose on *I–V* parameters[a]

No. of Expts.	Addition	I_{Na} (μA/μF)	P_{Na} (10^{-5} cm/sec)	Na_c (mM)
6	Amiloride (5×10^{-7} M)			
	Initial value	11.5 ±4.3	0.67±0.26	1.89±0.21
	Fractional change[b]	0.30±0.01[c]	0.29±0.01[c]	0.74±0.14
7	Ouabain (10^{-3} or 10^{-2} M)			
	Initial value	15.0 ±3.3	0.89±0.20	2.27±0.29
	Fractional change[b]	0.34±0.01[c]	0.80±0.02[c]	6.1 ±0.9[c]
5	2-Deoxyglucose (5 mM)			
	Initial value	8.25±1.96	0.48±0.11	2.10±0.15
	Fractional change[b]	0.37±0.02[c]	0.37±0.03[c]	0.84±0.13

[a] Hemibladders were mounted as flat sheets and were preincubated in KCl-sucrose Ringer's solution for 1 or more hr. Just before the addition of one of the agents, the *I–V* relationships were measured with and without transcellular Na transport. Amiloride was added to the mucosal medium, and ouabain or 2-DG was added to the serosal media. The *I–V* curves were repeated 5 min after addition of amiloride, 30–60 min after ouabain and 50 min after 2-DG.

[b] The fractional changes were estimated as the ratio of the values recorded after addition of one of the agents to the values recorded at zero time (i.e., each hemibladder served as its own control). All results are given as means ±SEM.

[c] $P<0.001$.

basal-lateral Na pump in the presence of little hindrance to Na entry.

Effect of 2-Deoxyglucose: Although the transport of Na across the apical membrane has been characterized as a passive process, the metabolic state of the epithelium may have a significant influence on apical P_{Na}. This possibility was tested with 2-deoxyglucose (2-DG), a well-characterized inhibitor of glycolysis and secondarily of oxidative phosphorylation (Webb, 1966). Addition of 2-DG (5 mM) to the serosal medium inhibited I_{sc} progressively over a period of ~1 hr. Accordingly, the *I–V* curves were measured before and 50 min after addition of 2-DG (Fig. 11). To correct for the contribution of the shunt pathway at "zero time", Na-free mucosal medium was used just before addition of the 2-DG, while amiloride (24 μM) was used to make this correction after exposure to 2-DG, at the end of the experiment. Both methods (i.e., Na-free or addition of amiloride) yielded similar *I–V* curves and similar corrections for the shunt pathway. The concentration of 2-deoxyglucose was chosen to produce ~65% inhibition of I_{sc}. As a basis for

interpreting the findings with 2-DG, two model compounds were used for comparison: amiloride, a selective inhibitor of apical permeability to Na, and ouabain, a selective inhibitor of basal-lateral Na pump. The concentration of amiloride and the time of exposure to ouabain were also chosen to produce ~65% inhibition of I_{Na}. As noted above, amiloride elicited proportionate falls in I_{Na} and P_{Na} (~70%), and a 26% fall in Na_c (not statistically significant) (Table 2). The entire effect on I_{Na} is attributable to the fall in P_{Na}. In contrast, ouabain inhibited P_{Na} minimally compared to the fall in I_{Na} (20 and 66%, respectively), and evoked a sixfold increase in Na_c. In response to 2-DG, I_{Na} and P_{Na} fell proportionately, decreasing to 37% of their respective initial values and Na_c fell slightly (16%, statistically insignificant) (Table 2). The differences in the electrophysiological actions of ouabain and 2-DG are clearly shown in Figs. 9 and 10. At 0 mV, ouabain elicited a small increase in the slope conductance and a ~20% decrease in P_{Na} when I_{sc} was reduced by 65%, whereas 2-DG decreased the slope conductance and reduced P_{Na} and I_{sc} proportionately. In contrast to these differences, the effects of 2-DG and amiloride on P_{Na} and Na_c, at equivalent degrees of inhibition of I_{Na}, were indistinguishable. These results imply metabolic regulation of apical Na conductance at least to the same extent as the metabolic dependence of the basal-lateral Na pump.

Discussion

In nondepolarized toad bladders, the transepithelial *I–V* relationship is approximately linear over a voltage range of ±100 mV, under a variety of experimental conditions (Civan, 1970; Saito, Lief & Essig, 1974; Spooner & Edelman, 1975). We confirmed this behavior in our experimental system. The increase in slope conductance with large hyperpolarizing voltages has also been observed previously. In toad bladder, Civan (1970) found an increase in slope conductance at about −130 mV and a decrease in slope conductance upon further hyperpolarization to −180 mV. Pronounced rectification was also noted by Helman and Fisher (1977) who found a break point in the *I–V* relationship in frog skin at about −130 mV, where the slope conductance either increased or decreased, depending on the skin and the experimental conditions. In our studies, as in the earlier experiments on frog skin by Fuchs et al. (1977), the inflection was evident both in the presence of Na transport and when Na transport was blocked by serosal ouabain. Thus, the inflection does not appear to be a property of the Na^+ pump (or E_{Na}) as proposed by Helman and Fisher (1977). In Na-free, amiloride-containing mucosal media, the cellular resistance pathway should be dominated by the apical membrane. The strongly rectifying element therefore is most probably resident in the apical membrane, in parallel with the amiloride-sensitive pathway, either in the apical membrane or in the paracellular pathway. Augmentation of the inflection by depolarization with high serosal K^+ implies an apical membrane location. The physical nature, ion selectivity, and physiological significance of the rectifier are as yet unknown.

Substitution of the high K-sucrose solution on the serosal side results in a shift in the inflection toward a less negative voltage, implying that the basal-lateral membrane provides a voltage in series with the nonlinear element. The extent of depolarization of the basal-lateral plasma membrane can be estimated from the shift in the inflection on the substitution of the high K-sucrose medium. The shift in the inflection point should be influenced by changes in both the resistance divider ratio (apical/basal-lateral) and the voltage profiles. When transepithelial Na transport is nil (i.e., Na-free mucosal medium or mucosal amiloride) the apical resistance should be dominant (Lewis et al., 1976) and contributions of changes in the resistance divider ratio to the shift should be minimal. It is reasonable, therefore, to assign the shift of about 50 mV to depolarization of the basal-lateral PD on substitution of high K-sucrose, under the conditions of our experiments.

Most microelectrode studies on the toad bladder indicate that in the open circuit the profiles are two-stepped with approximately equal voltage differences (20–40 mV) and resistances, determined by the apical and basal-lateral membranes (Frazier, 1962; Frazier & Leaf, 1963; Civan & Frazier, 1968; Reuss & Finn, 1974, 1975). In the short-circuited toad bladder, intracellular negative potentials of only −4 to −8 mV have been reported (Frazier, 1962; Reuss & Finn, 1974; DeLong & Civan, 1978). However, in the rabbit urinary bladder, Lewis et al. (1976) found a basal-lateral PD of −40 mV, independent of the transport state of the tissue, and Higgins et al. (1977) recorded basal-lateral PD's of 80–90 mV in *Necturus* urinary bladder. In both of these structures, most of the transcellular resistance was located at the apical membrane. In these tissues, the epithelial cells are larger and more easily impaled by microelectrodes than in toad bladder. Moreover, considerable care was taken to avoid impalement and tip artifacts in the latter studies.

The estimation of the magnitude of depolarization from the shift in the inflection is dependent on the inference that rectification is a property of the apical plasma membrane. Therefore, nystatin was also used

to probe the depolarizing action of the high K-sucrose solution. This polyene antibiotic forms monovalent cation conducting channels in cell membranes and lipid bilayers (Cass, Finkelstein & Krespi, 1970; Marty & Finkelstein, 1975; Russell, Eaton & Brodwick, 1977; Lewis, Wills & Eaton, 1978). Addition of nystatin to the serosal medium of the toad bladder, in the presence of a serosal-to-mucosal Na gradient elicited an amiloride-sensitive reversed I_{sc} (Fig. 1). This result is the expected consequence of formation of Na permeable channels in the basal-lateral membrane. To evaluate the effects of nystatin on K conductance (without Na loading of the cells) serosal Na was replaced by choline which presumably does not penetrate the nystatin channels (Fig. 3). Under these conditions, nystatin caused a transient hyperpolarization followed by a monotonic decline in the open-circuit PD and I_{sc}. The transient hyperpolarization is most probably a K diffusion potential via the nystatin-induced pores owing to the orientation of the electrochemical potential gradient for K, higher in the cell than the medium (Delong & Civan, 1979). The subsequent decline in PD presumably reflects the loss of K from the cell to the medium, either with Cl or in exchange for choline. The invariance of the PD on addition of nystatin to the high K-sucrose serosal medium implies that K is close to electrochemical and chemical equilibrium under these conditions. The finding that, in the presence of high K-sucrose, nystatin did not shift further the inflection in the I–V curve also supports the inference that the basal-lateral membrane is almost completely depolarized by this medium. Moreover, in toad bladder epithelium, recent measurements with K-sensitive microelectrodes yielded estimates of intracellular K activity of 81 ± 2 mM (DeLong & Civan, 1979). Thus, the use of 85 mM K to depolarize the basal-lateral membrane is quite suitable.

Although depolarization of the basal-lateral membrane with the high K-sucrose medium (i.e., replacement of serosal Na with K) may have effects on a variety of membrane processes, the net effect on I_{Na} was small (Table 1). This small effect could be a consequence of a complex set of events, some of which augment and others diminish Na transport. Reduction in the energy barrier across the basal-lateral membrane could augment "uphill" movement of Na through the pump. Contrary effects, however, could result from a decrease in the transapical PD (under short-circuited conditions) which might reduce net Na influx from the lumen, or from impaired Na:Ca exchange at the basal-lateral surface as postulated by Taylor and Windhager (1979). The latter effect could raise intracellular Ca and thereby inhibit either apical entry of Na or extrusion across the basal-lateral membrane by the Na pump (Grinstein & Erlij, 1978). Evaluation of these possibilities awaits further study.

The I–V curves of the amiloride-sensitive current (I_{Na}) of the toad bladder were linear under nondepolarizing conditions and curvilinear with depolarization. The curvilinear $I_{Na}-V$ plots conformed to the constant field equation, as previously described in the frog skin (Fuchs et al., 1977). These results are in accord with the predictions of a model consisting of an apical membrane with constant field $I_{Na}-V$ properties in series with a basal-lateral membrane with a significant resistance and open-circuit voltage. This model yields an approximately linear overall I–V relationship (Lindemann & Van Driessche, 1978).

In epithelia, under normal Na/K concentrations in the media (i.e., not depolarized), elimination of the apical Na^+ current should shift the apical PD and thereby modify net flux of Cl^-. This will result in discrepancies between the actual I_{Na} and that measured as the amiloride-sensitive current, if the conductance of Cl^- is of significant magnitude. Removal of Na from the mucosal solution reduced the short-circuit current to small, usually negative values (*see* Figs. 4, 7, and 8) despite the presence of a large transepithelial Cl gradient. This implies that the permeability to Cl of the apical membrane is probably low. This finding is consistent with those of Macknight (1977), who reported that mucosal Cl did not exchange with intracellular Cl at a measurable rate in the toad bladder. Moreover, $I_{Na}-V$ analysis was done with the serosal surface depolarized by high K. Under these conditions, the apical PD will depend primarily on the magnitude of the voltage clamp rather than the Na current.

The closeness of fit obtained with the constant field equation implies that, in contrast to the more complex parameter of slope conductance, P_{Na} of the apical membrane is independent of voltage (within the range of the intercepts on the I, V axes) and can be assigned a single value. The fit also yields an estimate of Na activity on the cytoplasmic side of the apical Na channels. In toad bladder epithelium, exposed to Na-Ringers on both sides, intracellular Na concentration has been estimated to be ~ 40 mM by chemical analysis (Macknight et al. 1971). Only 25% of the total intracellular Na, or ~ 10 mM, was found to be exchangeable with Na in the mucosal solution (Macknight, Civan & Leaf, 1975). Recently, estimates of 13 mM have been obtained by electron probe X-ray microanalysis (Rick et al., 1978), in close agreement with the figure of 10 mM for exchangeable Na. Our mean value of 1.9 mM for Na_c need not agree closely with the microprobe analysis for the following reasons: (1) The microprobe measures the concentration of Na averaged over a significant part of the cytoplas-

mic compartment. In contrast, the I–V curves estimate Na activity at the cytoplasmic boundary of the channel. (2) The Na activity coefficient in cytoplasm may be significantly less than in Cl-Ringer's (0.75) or in SO_4-Ringer's (0.55). (3) The microprobe does not distinguish between free cytoplasmic Na and bound or sequestered (in organelles) Na. (4) The measurements reported herein were made with mucosal Na concentrations of 20 mM and with Na-free (high K-sucrose) serosal solutions. It is of interest, however, that both methods yielded estimates of cytoplasmic to apical solution Na concentration ratios of 1:10, when active transport was not impaired.

Amiloride and ouabain were used to distinguish the electrophysiological effects of perturbing either the apical Na channels or the basal-lateral Na pump. Abundant evidence indicates that amiloride selectively blocks Na transit across the apical membrane and ouabain specifically inhibits the Na pump or its enzymatic equivalent, the $(Na^+ + K^+)$-dependent ATPase (Bentley, 1968; Ehrlich & Crabbé, 1968; Dörge & Nagel, 1970; Nagel & Dörge, 1970; Bonting & Canaday, 1964; Herrera, 1966; Erlij & Smith, 1973). The $I_{Na} - V$ responses obtained with these drugs revealed a predominant decrease of P_{Na} effected by amiloride and a predominant increase of Na_c elicited by ouabain. Both responses are in agreement with the known primary effects of these inhibitors and support the validity of $I_{Na} - V$ analysis of depolarized epithelia. The effect of ouabain will be analyzed further in a subsequent paper (L.G. Palmer and B. Lindemann, *in preparation*).

Previous reports noted a reduced uptake of radiosodium from the mucosal solution into the epithelium of toad bladder, after removal of K from or addition of ouabain to the serosal medium (Essig & Leaf, 1963; Finn, 1975). A proposed mechanism for this effect is down-regulation of apical Na conductance secondary to the increased Na_c (MacRobbie & Ussing, 1961; Erlij & Smith, 1973; Larsen, 1973; Lewis et al., 1976; Turnheim, Frizzell & Schultz, 1978). A secondary increase of Ca_c may mediate this phenomenon (Grinstein & Erlij, 1978; Taylor & Windhager, 1979). In agreement with such results Hong and Essig (1976) observed a ouabain-dependent decrease in the amiloride-sensitive transcellular conductance of nondepolarized toad bladder epithelia. More recently, Helman, Nagel and Fisher (1979) found that ouabain decreased both basal-lateral and apical conductance of nondepolarized frog skin epithelium considerably. It appears that in nondepolarized epithelia inhibition of the Na-pump causes a secondary inhibition of apical Na uptake. In contrast, in our preparations, which were depolarized by high serosal K, inhibition of the Na-pump had only a marginal effect on apical Na-permeability (−20%). Despite the minor decrease in P_{Na} and in the face of a 65% decrease in I_{Na}, the slope conductance at 0 mV (g_{Na}) actually increased slightly (Fig. 10). This effect is explained by the overriding increase in Na_c, since g_{Na} is a function of both P_{Na} and Na_c. The relevant equation, derived from Eq. (1) (*see* Lindemann, 1977) based on

$$g_{Na} = \left(\frac{dI}{dV}\right)_{V=0},$$

is:

$$g_{Na} = (F^2 P_{Na}/RT) \cdot (Na_m + Na_c)/2. \quad (2)$$

Lipton and Edelman (1971) proposed that energy metabolism simultaneously regulates apical Na conductance and the output of the basal-lateral Na pump based on two sets of findings: (1) Augmentation of metabolism by addition of substrate to substrate-depleted bladders or inhibition of metabolism with rotenone had no effect on intraepithelial Na^+ concentrations despite striking changes in I_{sc} (both stimulatory and inhibitory). (2) Biber (1971) found that the initial rate (30 sec) of uptake of radiosodium by frog skin from the outside solution was reduced by 40% after 30 min of anaerobiosis. In the present study, 2-DG, a well-characterized inhibitor of glucose metabolism was used to evaluate the role of energy metabolism in the regulation of I_{Na} (Webb, 1966). A predominant action of 2-DG on the apical boundary should mimic the response to amiloride, whereas a predominant action on the basal-lateral Na pump should resemble the response to ouabain. In fact, the response to 2-DG was similar to that of amiloride, indicative of a significant effect on the apical membrane (*cf.* Figs. 9 and 11, Table 2). Hong and Essig (1976) reported an inhibition of the conductance of the cellular Na^+ transport pathway by 2-DG, a finding consistent with our observed fall in apical permeability. In conclusion, although apical Na transit is considered to be passive, this process, nevertheless, is subject to metabolic control. This mechanism may serve to coordinate apical entry of Na with the energy available for active extrusion into the interstitium.

A variety of pathways could couple P_{Na} and energy metabolism: Grinstein and Erlij (1978) proposed that metabolic inhibition, e.g., with CN, could release mitochondrial Ca. Mitochondrial release of Ca may also eventuate on addition of 2-DG owing to a decrease in both availability of oxidative substrates (pyruvate and acetyl CoA derived from glycolysis) and depletion of cytoplasmic ATP, ADP and P_i secondary to sequestration of phosphate as 2-DG (Webb, 1966). A decrease in ATP concentration could also inhibit Ca-ATPase-mediated extrusion of Ca from the cell.

The subsequent rise in cytoplasmic Ca could, in turn, decrease P_{Na}.

Na channels could also be opened or closed by a variety of other energy-dependent processes, including: (1) the local binding of adenine nucleotides, which may control the activities of some enzymes, (Lehninger, 1971) and some membrane transport processes, such as Na-Ca exchange in squid axons (Blaustein, 1977) or Na/K exchange ratio of the Na-K-ATPase (DeWeer, 1973); (2) the phosphorylation or dephosphorylation of membrane proteins (DeLorenzo et al., 1973; Liu & Greengard, 1974); (3) recruitment of precursor proteins from underlying granules (Masur, Holtzman & Walter, 1972); or (4) changes in the phospholipid composition of the membrane (Lien, Goodman & Rasmussen, 1975; Scott, Reich & Goodman, 1979). Obviously elucidation of the dependence of apical Na translocation on metabolism awaits further experimental analysis.

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