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Modulation of Apical Na Permeability of the Toad Urinary Bladder by Intracellular Na, Ca, and H

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Summary. The Na conductance of the apical membrane of the toad urinary bladder was measured at different concentrations of Na both in the external medium and in the cell. Bladders were bathed in high K-sucrose medium to reduce basal-lateral resistance and voltage, and the transepithelial currents measured under voltage-clamp conditions. Amiloride was used as a specific blocker of the apical Na channel. At constant external Na, the internal Na concentration was increased by blocking the basal-lateral Na pump with ouabain. With high Na activity in the mucosal medium (86 mM), increases in intracellular Na activity from 10 to over 40 mM increased the amiloride-sensitive slope conductance at zero voltage while apical Na permeability, estimated from current-voltage plots using the constant field equation, decreased by less than 20%. Lowering the serosal Ca concentration from 1 to 0.1 mM had no effect on the change in P_{Na} with increasing Na_c, but increasing serosal Ca to 5 mM enhanced the reduction in P_{Na} with increasing Na_c, presumably by increasing Ca influx into the cell. P_{Na} was also reduced by serosal vanadate (0.5 mM), a putative blocker of ATP-dependent Ca extrusion from the cell, and by acute exposure to CO_2, which presumably acidifies the cytoplasm. Current-voltage relationships of the amiloride-sensitive transport pathway were also measured in the absence of a Na gradient across the apical membrane. These plots show that outward current passes through the channels somewhat less easily than does inward current. The shape of the *I-V* relationships was not significantly altered by changes in cellular Na, Ca or H, indicating that the effects of these ions on P_{Na} are voltage independent.

Key Words tight epithelium · apical Na permeability · amiloride-sensitive Na channels · intracellular Na activity · intracellular Ca activity · intracellular pH

Introduction

Changes in the intracellular ionic environment have been implicated in the control of Na transport of tight epithelia. Increases in cellular Na (Lewis & Diamond, 1976; Turnheim, Frizzell & Schultz, 1978) or Ca (Grinstein & Erlij, 1978; Taylor & Windhager, 1979; Chase & Al-Awqati, 1981, 1983) are thought to mediate down-regulation or feedback inhibition of Na permeability of the apical membrane of these tissues. I have used the K-depolarized toad urinary bladder, in which the electrical properties of the apical membrane can be conveniently studied (Palmer, Edelman & Lindemann, 1980; Palmer, 1984*a*) to examine apical Na permeability as a function of both internal and external Na, and during presumed changes in cell Ca and pH. The goals of this study were first, to assess the effect of internal Na itself, as opposed to actions mediated through changes in Ca or H, on Na permeability, and second, to investigate the possibility that these internal cations might interact directly with the Na channels by plugging the pore through which the ions move. Such a mechanism should lead to a voltage-dependent block of Na permeability.

Materials and Methods

Toads (*Bufo marinus*, female, Dominican origin) were obtained from National Reagents (Bridgeport, Conn.). They were kept in tanks with access to fresh water prior to use. Urinary bladders were excised from double-pithed toads and mounted in Lucite® chambers. Mounting and electrical connections were as described previously (Palmer, 1982*b*). Current-voltage relationships were obtained by applying ramps to the command port of the voltage clamp, as described previously (Palmer, 1984*b*). Data were corrected for series solution resistance.

Solutions

Control serosal solutions contained KCl (85 mM), sucrose (50 mM), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM), glucose (5 mM) and K-phosphate (3.5 mM, buffered to pH 7.5).

Control mucosal solutions contained NaCl (115 mM), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM) and K phosphate (3.5 mM, buffered to pH 6.0). The activity of Na was calculated to be 86 mM, assuming an activity coefficient of 0.75. Solutions containing reduced Na activities were obtained with equimolar substitution for NaCl by either choline Cl, in the experiment of Fig. 1, or N-methyl-D-glucamine Cl (NMDG Cl) in all other cases. The activity coefficient for Na was assumed to remain constant. The electrical

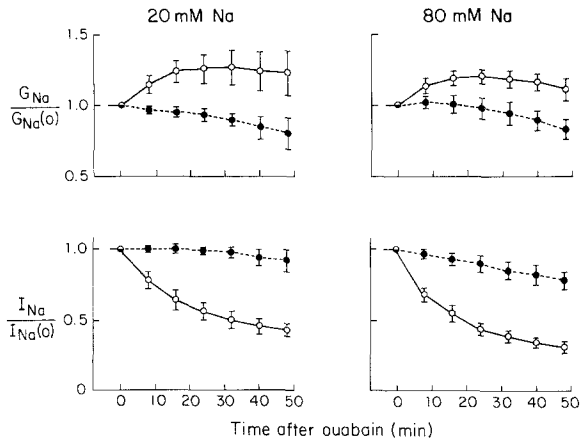

Fig. 1. Effect of ouabain on I_{Na} and G_{Na}. Paired hemibladders were preincubated with either 20 or 80 mM NaCl on the mucosal side. At time zero, ouabain (5 mM) was added to the serosal side of the experimental hemibladders. I_{Na} and G_{Na} were measured at 10-min intervals for 50 min, and values were normalized to those at $t = 0$. Initial values were, in $\mu A/cm^2$ and mS/cm^2, 20 mM Na: $I_{Na} = 6.4 \pm 1.4$ (control), 8.3 ± 2.5 (experimental); $G_{Na} = 1.3 \pm 0.3$ (control), 1.7 ± 0.6 (experimental). 80 mM Na: $I_{Na} = 19 \pm 4$ (control), 20 ± 3 (experimental); $G_{Na} = 3.6 \pm 0.8$ (control), 4.1 ± 0.6 (experimental). ○ = ouabain-treated. ● = control. Data are given as means ± SEM for five hemibladder pairs at each Na activity

properties of the tissue were not measurably different in choline and NMDG-containing solutions (Palmer, 1984*b*).

CO_2 tension of the mucosal medium was increased by bubbling the solutions with 6% CO_2 and adding 1.3 mM $KHCO_3$ to maintain a constant pH. Amiloride (a gift of Merck, Sharp and Dohme) was dissolved in deionized water at 10^{-2} M and added to the mucosal solutions at concentrations of 0.1 μM (sub-maximal dose) or 10 μM (maximal dose). Ouabain (Sigma) was dissolved directly in the serosal solution at a concentration of 5 mM. Na_3VO_4 (Sigma) was dissolved in deionized water at a concentration of 0.5 M and added to the serosol solution to a final concentration of 0.5 mM.

I-V Relationships with no Na Gradient

In several series of experiments current-voltage relationships were obtained with equal Na activities in the mucosal solution and in the cytoplasm. Tissues were Na-loaded under short-circuit conditions by incubation with 5 mM serosol ouabain for various times (10 to 40 min) in the presence of 86 mM mucosal Na. The mucosal Na activity was then rapidly reduced, resulting in an outward or reversed Na current which decayed as the cell Na activity approached that of the mucosal solution (Morel & LeBlanc, 1975; Van Driessche & Erlij, 1983). When the current was zero the *I-V* relationship was measured. Although I_{Na} was not strictly in a steady state under these conditions, the rate of change was slow (worst case about 2 $\mu A/cm^2 \cdot$ min) relative to the time interval required to obtain the *I-V* curves (about 1 sec). Amiloride (10 μM) was then added to the mucosal solution and the *I-V* relationship remeasured. The latter relationship was used to correct the former for currents not mediated by Na channels. Since the short-circuit current was zero both before and after addition of amiloride, the drug had no effect on short-circuit current but reduced the currents for $V \neq 0$.

Analysis of Data

As in previous studies, the transepithelial potential was assumed to reflect that of the apical membrane, and the amiloride-sensitive transepithelial resistance was assumed to reflect that of the apical membrane Na channels (Palmer et al., 1980; Palmer, 1984*a*). The transepithelial resistance in the presence of amiloride was 5 to 20 kohm · cm^2. Typically, the conductance in the presence of amiloride was 10 to 30% that in its absence. The short-circuit current in the presence of amiloride was close to zero (*see* Palmer et al., 1980; Palmer, 1984*b*).

In some experiments, the amiloride-sensitive *I-V* or I_{Na}-*V* relationship was fitted with the constant field equation over a voltage range including $V = 0$ and the reversal potential of $I_{Na}(E_{Na})$ as done previously (Palmer et al., 1980). In this study, a somewhat simplified fitting method was employed. First, the intracellular Na activity (Na_c) was calculated from E_{Na} using the relationship:

$$E_{Na} = \frac{RT}{F} \ln (Na_c/Na_o) \quad (1)$$

where Na_o is the Na activity in the mucosal solution. The apical Na permeability (P_{Na}) was then computed from the relationship:

$$I_{Na}(O) = FP_{Na}(Na_o - Na_c) \quad (2)$$

where $I_{Na}(O)$ is the amiloride-sensitive short-circuit current. The parameters P_{Na} and Na_c defined in this way are relatively model-independent, and do not depend on the *a priori* assumption that the constant-field equation applies. The reversal potential measurement of Na_c depends only on the assumption that the channels are perfectly Na selective. This assumption has been justified (Palmer, 1982*b*). The calculation of P_{Na} can be generalized to Eyring rate-theory models, in which diffusion through the channel is discontinuous, as long as the channel is of low occupancy, i.e. far from saturation with respect to Na (*see* Appendix).

In other experiments, in which paired hemibladders were studied, only the amiloride-sensitive currents at $V = 0$ and $V = -10$ mV were measured. To compare these results with those in which the whole *I-V* relationship was measured, P_{Na} and Na_c were estimated from Eq. (2) and from the additional relationship:

$$G_{Na}(O) = \frac{F^2}{RT} P_{Na} \left[\frac{Na_o + Na_c}{2}\right] \quad (3)$$

where $G_{Na}(O)$ is the amiloride-sensitive slope conductance at $V = 0$. These two equations can be readily solved for the two unknowns P_{Na} and Na_c.

Results

The effect of ouabain on the directly measured parameters I_{Na} and G_{Na} at 20 and 80 mM mucosal Na activities is shown in Fig. 1. Paired hemibladders which did not receive ouabain served as controls. The tissues were short-circuited except for brief 10-

Table 1. Effect of ouabain on electrical properties of the apical membrane[a]

	Fractional change			Na_c^i (mM)	Na_c^f (mM)
	I_{Na}	G_{Na}	P_{Na}		
Na 20					
Control	0.85 ± 0.08	0.85 ± 0.09	0.82 ± 0.07	1.3 ± 0.2	0.6 ± 0.3
Ouabain	0.39 ± 0.03*	1.21 ± 0.17*	0.89 ± 0.13	1.4 ± 1.0	10.5 ± 1.2*
$n = 5$					
Na 80					
Control	0.78 ± 0.06	0.80 ± 0.07	0.79 ± 0.08	6.2 ± 3.9	8.8 ± 5.9
Ouabain	0.32 ± 0.03*	1.11 ± 0.08*	0.76 ± 0.05	6.3 ± 2.7	49 ± 4*
$n = 5$					
Na 80					
Normal Ca + ouabain	0.24 ± 0.06	0.67 ± 0.18	0.52 ± 0.14	2.8 ± 2.8	34 ± 8
Low Ca + ouabain	0.30 ± 0.06	0.80 ± 0.14	0.57 ± 0.13	1.2 ± 0.7	34 ± 5
$n = 5$					
Na 80					
Low Ca + ouabain	0.39 ± 0.04	1.21 ± 0.08	0.83 ± 0.05	5.1 ± 1.5	45 ± 3
High Ca + ouabain	0.33 ± 0.02	0.80 ± 0.12*	0.57 ± 0.07*	6.8 ± 3.6	37 ± 4
$n = 9$					
Na 80					
Low Ca	0.94 ± 0.05	1.03 ± 0.07	0.99 ± 0.06	7.8 ± 2.8	10.7 ± 3.3
High Ca	1.04 ± 0.06	1.04 ± 0.04	1.02 ± 0.06	5.8 ± 1.9	4.4 ± 2.7
$n = 8$					

[a] I_{Na} and G_{Na} were measured directly at the beginning and end of the experimental time period. P_{Na} and Na_c were calculated from the constant field equation. Number of experiments is given by n.
* Indicates statistically significant difference between values in the upper and lower rows ($P < 0.05$ by paired Student's t-test).

mV displacements of the clamping voltage to measure conductance. The short-circuit current and conductance of Na-independent pathways were measured at the beginning and end of the experiments by removing Na from the mucosal medium. Values before and after the experiment were not significantly different and were averaged for use in correcting the currents and conductances measured in the presence of Na. With both high and low mucosal Na, I_{Na} fell continuously after addition of ouabain to the serosal medium. G_{Na} initially rose and then remained fairly constant compared with controls. The fall in I_{Na} is attributable to the blocking of the basal-lateral Na pump resulting in an increase in internal Na which reduces the driving force for Na entry across the apical membrane (Palmer et al., 1980). The increase in G_{Na} can be attributed to the rise in internal Na as predicted from the constant-field equation (Eq. 3).

To see if Na permeability as well as the Na driving force across the apical membrane was affected by ouabain, P_{Na} and Na_c were estimated according to Eqs. (2) and (3). As can be seen in Table 1, increases in Na_c to 10.5 and 49 mM were accompanied by decreases in P_{Na} of 11 and 24%, respectively. However, in paired tissues serving as time controls, P_{Na} fell by roughly the same amount without a significant change in Na_c. Thus the effect on P_{Na} was largely a time-dependent one and could not be attributed to the increase in Na_c.

Chase and Al-Awqati (1981) suggested that decreases in Na permeability with increased cellular Na in nondepolarized ouabain-treated toad bladders may result from inhibition of Na-Ca exchange at the basal-lateral membrane leading to an increased cellular Ca activity (Chase & Al-Awqati, 1981). To evaluate this possibility, the experiments described above were repeated using different serosal Ca concentrations. In these protocols the paired hemibladders were preincubated with different concentrations of serosal Ca, and both hemibladders received ouabain. As shown in Table 1, lowering Ca from 1 to 0.1 mM had no apparent effect on P_{Na}, which fell by equivalent amounts in both Ca concentrations. On the other hand, raising Ca to 5 mM led to a significantly greater fall in P_{Na} after ouabain addition. Similar results have been obtained by Garty and Lindemann (1984). In the absence of ouabain, increasing serosal Ca had no effect on I_{Na} or G_{Na}. One interpretation of the results is that at increased cell Na and serosal Ca, a Na-Ca exchanger in the basal-lateral membrane mediates the exchange of cytoplasmic Na for serosal Ca. The increase in cell Ca could then inhibit Na permeability.

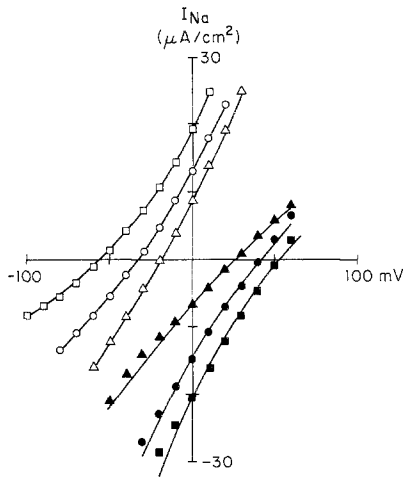

Fig. 2. Current-voltage relationships for forward and reversed Na currents. The first *I-V* relationship (□) was obtained after the hemibladder had reached a steady state in 86 mM mucosal Na. Ouabain was then added to the serosal medium and additional *I-V* relationships were obtained 2 min (○) and 5 min (△) later. After 20 min of exposure to ouabain, the mucosal Na activity was rapidly reduced to 3.75 mM, causing a reversal of the short-circuit current. *I-V* relationships were obtained 15 sec (■), 1.5 min (●) and 7 min (▲) after reduction of mucosal Na activity. The *I-V* relationships were corrected for amiloride-insensitive currents obtained in the presence of 10 μM amiloride at the beginning (86 mM Na) and at the end (3.75 mM Na) of the experiment. The solid lines represent theoretical *I-V* relationships derived from the constant field equation using the following parameters:

	P_{Na}(10^{-6} cm/sec)	Na_c(mM)
□	0.86	10.1
○	0.71	23
△	0.65	40
■	2.71	37
●	3.38	18.5
▲	3.43	10.3

The effect of cell Na on Na permeability was further evaluated in a second series of experiments shown in Figs. 2–4. Here unpaired hemibladders were incubated in 86 mM mucosal Na and short-circuited except for periods in which *I-V* relationships were obtained. After a steady state was reached, ouabain was added to the serosal medium. *I-V* curves were obtained at intervals of 1 to 5 min for about 30 min, after which Na_c, measured from the reversal potential of I_{Na}, had reached 35 to 45 mM. At this time, the mucosal Na activity was abruptly dropped to 3.75 mM, eliciting a reversal of the short-circuit current due to the reversal of the activity gradient for Na across the apical membrane (Morel & Leblanc, 1975; Van Driessche & Erlij, 1983). The reversed current gradually decayed with net Na efflux from the cell. Additional *I-V* relationships were obtained during the decay of current.

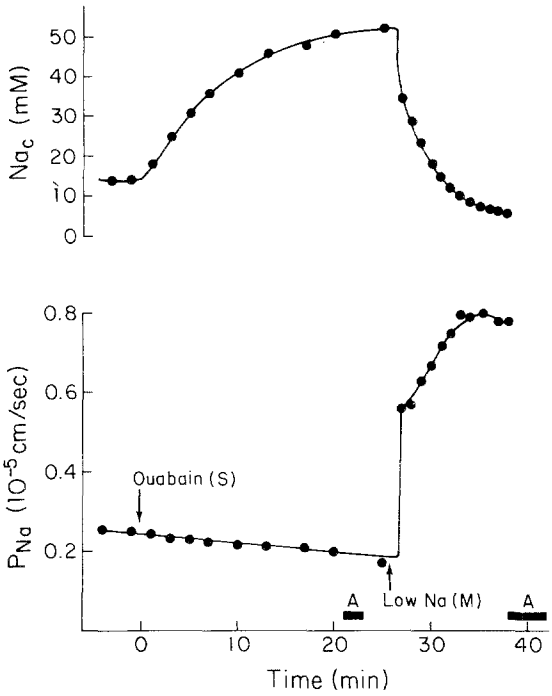

Fig. 3. Time course of changes in P_{Na} and Na_c after ouabain addition and lowering of mucosal Na activity. The protocol was that described in Fig. 2. Values of P_{Na} and Na_c were calculated from the constant field equation fits to the I_{Na}-*V* relationships. The bars marked *A* indicate application of amiloride to the mucosal solution. The arrows indicate addition of ouabain to the serosal solution (t = 0) and the reduction of mucosal Na from 86 to 3.75 mM (t = 26 min)

The *I-V* curves were corrected using curves measured in the same solutions but with the addition of amiloride before the experiment (in 86 mM Na) and at the end of the experiment (in 3.75 mM Na). In some experiments the *I-V* relationship was checked by briefly adding amiloride to the mucosal solution before reducing the Na activity. No significant change in the amiloride-insensitive *I-V* relationship was observed at the different times throughout the experiment. The I_{Na}-*V* curves obtained in this way were fitted with the constant field equation as shown in Fig. 2.

During Na loading, P_{Na}, estimated from the I_{Na}-*V* relationship as described in the Materials and Methods section, decreased by about 20% as shown in Fig. 3 for a typical bladder. This may have been due in part to a time-dependent, rather than a Na-dependent decrease as in the experiment in Fig. 1. When mucosal Na was lowered, P_{Na} rose rapidly, more than doubling within the time required to change the solution and obtain the new *I-V* relationship. An additional, smaller increase was observed as Na_c diminished. This increase in P_{Na} is similar in terms of percentage change, to the initial decrease

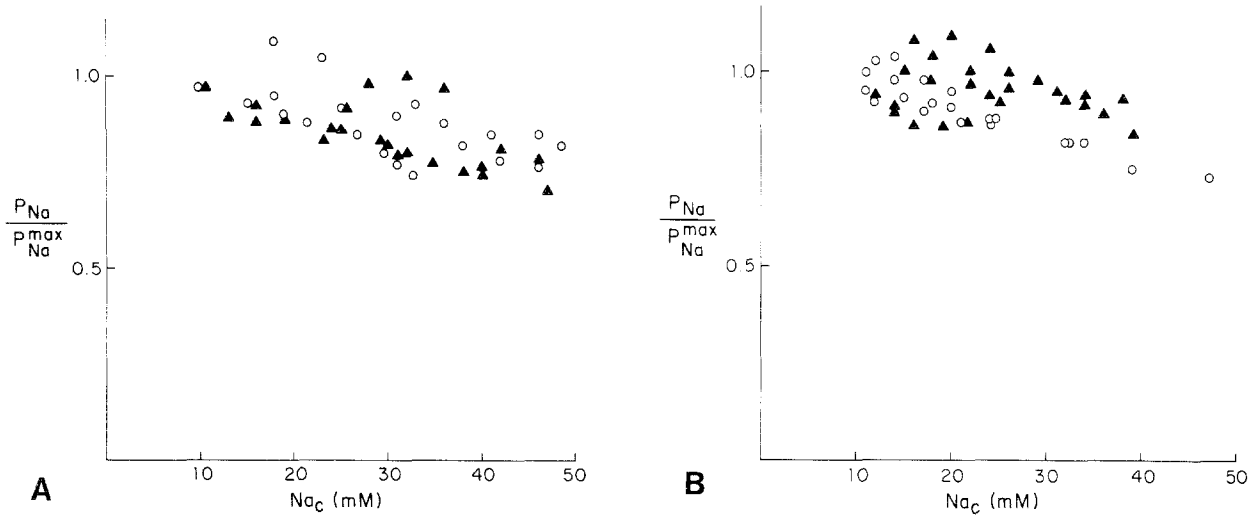

Fig. 4. Correlation of P_{Na} with Na_c. Values of P_{Na} are plotted as a function of Na_c for seven experiments similar to that shown in Fig. 3. Filled triangles indicate four experiments with 1 mM Ca and open circles three experiments with 0.1 mM Ca. *A*. Increasing Na_c. P_{Na} and Na_c were measured after ouabain addition with 86 mM mucosal Na. P_{Na} values were normalized to the value before ouabain addition (mean Na_c = 7.6 ± 1.5 mM). *B*. Decreasing Na_c. P_{Na} and Na_c were measured after reduction of mucosal Na to 3.75 mM. P_{Na} values were normalized to the value after Na_c had fallen below 10 mM (mean Na_c = 8.3 ± 0.4 mM)

with increasing Na_c, and is presumably not time dependent. Note that the value of Na_c dropped abruptly after lowering the mucosal Na activity. This fall was too rapid to be accounted for by a net loss of Na from the cell, and may reflect solute polarization in the vicinity of the apical membrane.

Values of P_{Na} were plotted as a function of Na_c in Fig. 4*A* for Na loading in high mucosal Na and in Fig. 4*B* for Na unloading in low mucosal Na. These Figures show data from seven experiments. To facilitate comparison, P_{Na} was normalized to its maximum value, obtained with the lowest value of Na_c recorded. The maximum P_{Na} was 3.4 ± 0.4 times higher with low mucosal Na than with high mucosal Na (mean ± SEM). The fall in P_{Na} with increasing Na_c was similar in both cases, and roughly linear. Linear regression analysis revealed correlation coefficients of 0.60 (loading) and 0.59 (unloading) with slopes of 0.48 and 0.52%/mM, respectively.

I_{Na}-V Relationships with Equal Na Activities on Both Sides of the Membrane

A possible mechanism for decreases in Na permeability associated with increased cellular Na is binding of Na to sites within the lumen of the pore, thus mechanically and/or electrostatically preventing Na transit from mucosa to cell (*see* Edmonds, 1982). Such a mechanism could imply a change in the shape of the I_{Na}-V curve as intracellular Na is increased. In order to test this possibility over as wide a voltage range as possible, the I_{Na}-V relationship was measured between −200 and +200 mV under conditions of equal Na activities on both sides of the apical membrane. Cells were loaded with Na in the presence of 86 mM mucosal Na and serosal ouabain for 40 to 50 min. The mucosal Na activity was then reduced to 43 mM and, after the short-circuit current reached zero following the decay of the outward current transient the *I-V* relationship was measured first in the absence and then in the presence of amiloride. The amiloride was washed off, and the procedure repeated by further reducing the mucosal Na activity to 21.5 and 10.75 mM. This protocol gave I_{Na}-V curves which passed through the origin, indicating that there was no net movement of Na through the channels when the voltage was zero.

I_{Na}-V curves obtained in this way are shown in Fig. 5. Each curve was normalized by dividing by the slope conductance at $V = 0$ (G^0_{Na}) to eliminate differences in the total channel density from tissue to tissue. Normalized currents at each voltage were averaged for seven hemibladders. The normalized I_{Na}-V curves were similar at different Na concentrations, although at lower Na activities the currents showed a slightly greater tendency to level off at both positive and negative voltages.

As a positive control to show that voltage-dependent block could be demonstrated under these conditions, I_{Na}-V plots in the absence and presence of a submaximal concentration of amiloride (0.1 μM) were obtained with equal Na activities (20 mM)

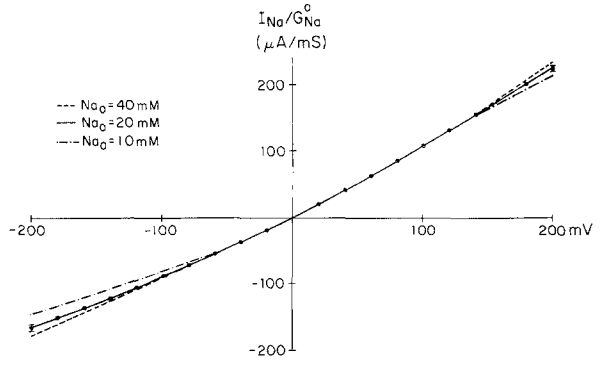

Fig. 5. I_{Na}-V relationships in the absence of a Na gradient. Hemibladders were incubated with serosal ouabain and 86 mM mucosal Na. The mucosal solution was abruptly changed to one containing 43 mM Na and the I-V relationship obtained when the short-circuit current was zero. The mucosal Na activity was then further lowered to 21.5 mM and 10.75 mM and I-V relationship was remeasured when I_{sc} was zero. The I-V curves were corrected for amiloride-insensitive currents measured in the presence of 10 μM amiloride with each mucosal Na activity at the end of the experiment. Values of I_{Na} at each voltage were normalized by dividing by the slope conductance at $V = 0$ (G^o_{Na}). Mean values of normalized currents for seven experiments are plotted. Standard errors are shown at $V = \pm 200$ mV and were comparable or smaller at other voltages. Mean values of G^o_{Na} were 1.2 ± 0.2 mS (10.75 mM Na), 2.1 ± 0.2 mS (21.5 mM Na), and 2.4 ± 0.2 mS (43 mM Na)

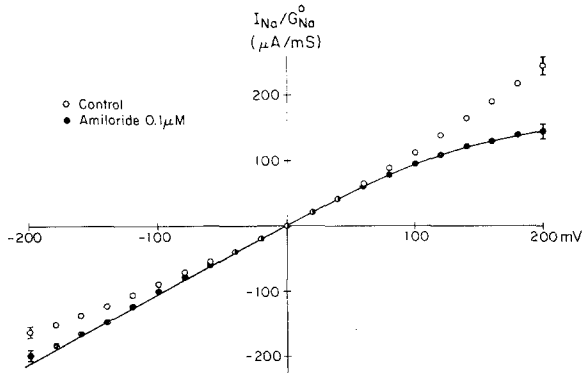

Fig. 6. Voltage-dependence of amiloride block. I_{Na}-V relationships were obtained with 21.5 mM Na on both sides of the apical membrane in the presence (●) and absence (○) of 0.1 μM amiloride as described in the text. Data represent means ± SEM of I_{Na} values normalized to G^o_{Na} for six experiments. The solid line is a theoretical curve describing the predicted I_{Na}-V relationship in the presence of 0.1 μM amiloride assuming the amiloride inhibition constant is voltage dependent according to the relationship $K_I(V) = K_I(O)e^{-\delta FV/RT}$ with $K_I(O) = 0.18$ μM and $\delta = 0.13$

on both sides of the apical membrane. Cells were Na loaded by incubation with 86 mM mucosal Na and 5 mM serosal ouabain as described above. The mucosal Na activity was dropped to 21.5 mM and, when the short-circuit current reached zero, I-V relationships were obtained with no amiloride, 0.1 μM amiloride and 10 μM amiloride in the mucosal solution. The latter curve was used to correct the former two curves for amiloride-insensitive currents. I_{Na}-V relationships from six hemibladders were normalized by dividing by G^o_{Na} and averaged. The normalized, averaged curves with and without 0.1 μM amiloride are shown in Fig. 6. The normalized currents were lower in the presence of amiloride at positive voltages, confirming that the blocker becomes more potent in the presence of an electric field which tends to drive the molecule into the membrane. At negative voltages, the normalized currents are higher in the presence of amiloride, indicating that the fractional block of I_{Na} by the drug is reduced in this voltage range relative to $V = 0$. The absolute current was always lower in the presence of amiloride at all voltages.

Palmer (1984*b*) showed that under non-Na-loaded conditions amiloride block was increased at positive voltages. The increase was consistent with the hypothesis that the amiloride binding site senses about 12% of the electric field across the apical membrane. The solid line in Fig. 6 shows the predicted normalized I_{Na}-V curve for equal Na activities assuming

$$K_I(V) = K_I(O) \exp(-\delta FV/RT)$$

where $K_I(V)$ is the voltage-dependent inhibition constant for amiloride and δ is the fraction of the field sensed by the amiloride binding site. Values of $K_I(O)$ and δ were 0.18 μM and 0.13, respectively. The theoretical curve fits the data reasonably well except at the most negative potentials ($V = -200$ mV). This extends previous results obtained for amiloride block in the voltage range 0 to +200 mV and confirms that voltage-dependent block can be observed in both the positive and negative voltage domains in the absence of a transmembrane Na gradient.

Effect of Increased Intracellular Ca on the I_{Na}-V Relationship

To test whether cytoplasmic Ca might interact with the Na channel in a voltage-dependent manner, I_{Na}-V relationships were determined with Na-loaded tissues in the absence and presence of Ca loading. Internal Na was first increased by incubation for 5 to 10 min with ouabain in the presence of low (0.1 mM) serosal Ca. I_{Na}-V curves were generated as before with 20 mM Na in the mucosal medium and in the cell. Then the tissues were incubated for a

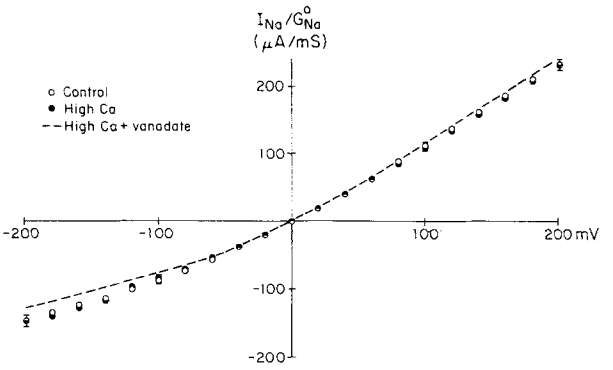

Fig. 7. Effect of Ca loading on I_{Na}-V relationship. I_{Na}-V relationships were obtained as in Fig. 5 with 21.5 mM Na in the mucosal solution and 0.1 mM serosal Ca (○), 5 mM serosal Ca (●) and 5 mM serosal Ca plus 0.5 mM vanadate (---) as described in the text. Currents were normalized as in Fig. 5 and plotted as means ± SEM for six experiments. Values of G^{o}_{Na} were 0.53 ± 0.10 mS (0.1 mM Ca), 0.28 ± 0.04 mS (5 mM Ca) and 0.13 ± 0.02 mS (5 mM Ca plus vanadate)

Table 2. Effects of CO_2 and vanadate on electrical properties of the apical membrane[a]

	I_{Na} (μA/cm^2)	P_{Na} (10^{-5} cm/sec)	Na_c (mM)
Control	10.7 ± 2.9	0.143 ± 0.041	6.7 ± 1.5
6% CO_2	5.1 ± 1.4	0.069 ± 0.020	7.9 ± 1.2
Fractional change	0.48 ± 0.04	0.48 ± 0.05	1.2 ± 0.1
Control	17.8 ± 2.2	0.239 ± 0.031	8.7 ± 1.1
0.5 mM vanadate	8.4 ± 1.0	0.118 ± 0.015	12.3 ± 1.0
Fractional change	0.47 ± 0.02	0.50 ± 0.02	1.47 ± 0.20

[a] P_{Na}, Na_c and $I_{Na}(O)$, the amiloride-sensitive short-circuit current, were estimated before and after addition of 6% CO_2 + 1.3 mM $KHCO_3$ to the mucosal solution, and before and after addition of 0.5 mM Na_3VO_4 to the serosal solution. Data represent means ± SEM for five experiments for CO_2 and four experiments for VO_4.

further 30 to 40 min in the presence of 5 mM serosal Ca. As shown in Table 1, this procedure results in a Ca-dependent decrease in P_{Na}, presumably due to increased Ca influx across the basal-lateral membranes.

G^{o}_{Na} was decreased in Na + Ca-loaded conditions by 47% over that in Na-loaded controls, confirming the finding presented in Table 1 for high *vs.* low Ca with ouabain. There was, however, no significant change in the shape of the I_{Na}-V relationship, as shown in Fig. 7. Thus the Ca-dependent block of P_{Na} is voltage independent.

In an attempt to produce further increases in Ca in the cell, the same tissues were incubated for an additional 10 to 15 min with Na_3VO_4 in the serosal medium. VO_4^{-3} is known to block Ca-ATPase in the plasma membranes of red cells and in squid axons (DiPolo & Beaugé, 1981, Rossi, Garrahan & Rega, 1981), and causes a rapid reduction in I_{Na} and P_{Na} in non-Na-loaded K-depolarized bladders (Table 2). I_{Na}-V relationships in the absence of a Na gradient were obtained after incubation with vanadate. Within 20 min, G^{o}_{Na} fell to 46% of the value before vanadate. Once again, the normalized I_{Na}-V relationship was not significantly changed, indicating that the inhibition of P_{Na} by vanadate was not voltage-dependent.

Effect of CO_2 on P_{Na}

In addition to Na-Ca exchange processes, a Na-H exchanger has been reported in the basal-lateral membrane of at least one renal epithelium, the proximal tubule of *Necturus* (Boron & Boulpaep, 1983). Thus cytoplasmic H activity could be affected by changes in cytoplasmic Na, and might also be involved in negative-feedback control of P_{Na}. To see if cytoplasmic pH affected P_{Na}, CO_2 mixture was added to the mucosal medium together with sufficient HCO_3^- to maintain constant external pH. This manuever is known to acidify the cytoplasm in squid axons (Boron & De Weer, 1976). Increases in CO_2 from 0 to 6% resulted in a rapid, reversible decrease in G_{Na} and P_{Na} as shown in Fig. 8 and Table 2. Note that although P_{Na} decreased with CO_2, the intracellular Na activity increased slightly. This could signify an inhibition of the basal-lateral Na pump by cellular acidification, as proposed by Eaton, Hamilton and Johnson (1984) for the rabbit urinary bladder, in addition to the effect on Na permeability.

Since the inhibition of I_{Na} by mucosal protons is voltage dependent (Palmer, 1984*b*) the voltage dependence of the CO_2-induced inhibition was also studied. I_{Na}-V relationships were determined with 20 mM Na in the cell and in the mucosal medium as described above. Then 6% CO_2 was added to the mucosal medium and the measurement repeated approximately 5 min later. As shown in Fig. 9, the normalized I_{Na}-V curves from control and CO_2-treated tissues were nearly superimposable. Thus, like the effects of increased Na, high Ca and vanadate, inhibition of P_{Na} by internal protons is voltage independent.

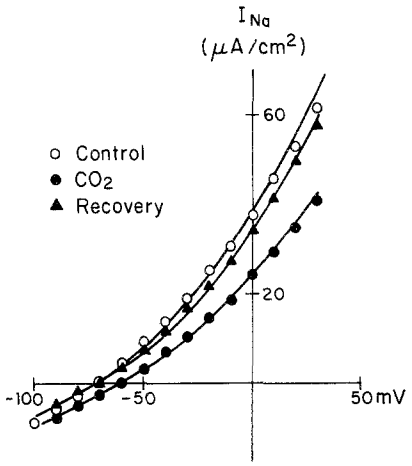

Fig. 8. Effect of CO_2 on the I_{Na}-V relationship. A hemibladder was equilibrated with 86 mM mucosal Na in the absence and presence of 10 μM amiloride. After the amiloride was washed off, CO_2 (6%) was added to the mucosal medium, along with $KHCO_3$ to maintain constant pH. After 4 min, I-V curves were remeasured in the absence and presence of amiloride. After amiloride was washed off, the mucosal solution was replaced with the original CO_2 and HCO_3-free medium. After 5 min the I-V relationships were obtained again. The I_{Na}-V relationships for control (○), CO_2 (●) and recovery (▲) are plotted. The solid lines are theoretical I-V curves obtained from the constant field equation with the following parameters:

	$P_{Na}(10^{-5}$ cm/sec)	Na_c
control	0.16	5.2 mM
CO_2	0.10	8.1 mM
recovery	0.15	5.2 mM

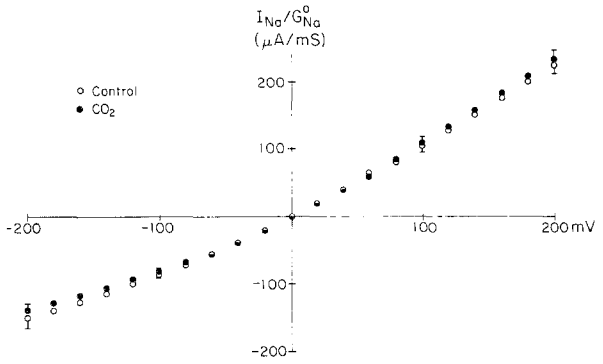

Fig. 9. Effect of CO_2 on the I_{Na}-V relationship. I_{Na}-V curves were obtained as in Fig. 5 with 21.5 mM mucosal Na. The mucosal solution was then replaced with one containing 6% CO_2 as in Fig. 8. After 5 to 10 min the I_{Na}-V relationship was remeasured. Currents were normalized as in Fig. 5, and are plotted as means ± SEM for seven experiments. Mean values of G^o_{Na} were 1.4 ± 0.3 mS (control) and 0.5 ± 0.1 mS (CO_2)

Discussion

Shape of the I_{Na}-V Relationship with Equal Na Activities on Both Sides of the Apical Membrane

In a number of tight epithelia the I-V characteristic of the amiloride-sensitive Na transport pathway in the apical membrane can be described by the constant field equation over a substantial voltage range (Fuchs, Hviid Larsen & Lindemann, 1977; Palmer et al., 1980; Thompson, Suzuki & Schultz, 1982; Thomas et al., 1983). When the Na activity is equal on the two sides of the membrane the constant field equation predicts a linear I-V relationship passing through the origin.

One major conclusion from the work presented here is that the I_{Na}-V relationship does become substantially linearized when the Na gradient is abolished as evidenced by an amiloride-sensitive short-circuit current of zero (Figs. 5, 6, 7 and 9). This implies that most of the rectification seen under more physiological conditions is a consequence of asymmetric Na activities on the two sides of the membrane.

There is, however, a relatively small but consistent curvature in the I_{Na}-V relationship even in the absence of a Na gradient. The curve was slightly concave upward, and the outward current at $V = -200$ mV was on the average about 30% lower than the inward current at $V = +200$ mV. There are several possible explanations. First, the assumption that the basal-lateral potential is zero could be wrong. A cell potential of +5 to +10 mV under short-circuit conditions would explain the results according to the constant field equation since the cell Na activity would be lower than that of the mucosal medium. Second, the assumption that the basal-lateral membrane resistance is negligible compared to that of the apical membrane could break down at negative potentials, making the true transapical potential smaller than the transepithelial clamping potential. Third, outward current could be diminished more than inward current by depletion of Na in the solution near the apical membrane. This could be due to a more restricted ionic diffusion in the cytoplasm, or to a difference in the surface charge density at the inner and outer sides of the membrane. This could also explain the rapid reduction in Na_c measured during reversal of the Na current in Fig. 3. Finally, the channel itself might be asymmetrical. Helman (1981) has previously proposed that the flow of Na across the apical membrane of the frog skin is rectified, based on the I-V properties of that membrane.

Inhibition of P_{Na} by Cell Na

The second major conclusion from this work is that intracellular Na ion activity *per se* has little or no effect on P_{Na} between 0 and 40 mM, a range that encompasses that likely to be encountered under physiological conditions. Thus the present results confirm and extend findings of weak dependence of P_{Na} on Na_c over a smaller range in the toad bladder and the rabbit urinary bladder (Palmer et al., 1980; Eaton, 1981).

The results presented in Figs. 1 and 4 need reconciliation. Although both show a decrease in Na permeability of about 20% as Na_c increases from less than 10 to over 40 mM, in Fig. 1 this decrease seems to be time dependent rather than Na dependent, as P_{Na} in controls fell by a similar amount. In Fig. 3, however, a subsequent increase in P_{Na} was observed as Na_c is lowered, apparently reversing the effects of increased Na_c. One explanation is that the controls in Fig. 1 are not true controls, since the control hemibladders have a larger short-circuit current, and hence a larger metabolic demand, throughout the experiment. This could lead to a metabolism-dependent decrease in P_{Na} (Palmer et al., 1980) which by coincidence is similar to the Na_c-dependent decrease in the experimental tissues. Another possibility is that changes in P_{Na} during Na loading are Na independent, but changes during Na unloading are Na dependent. This could be the case if internal Na and external Na were competitive inhibitors of P_{Na}.

It is not clear how much of this effect can be attributed to direct interactions of intracellular Na with the Na channel, and the 20% diminution of P_{Na} is in any case of minimal importance in the physiological regulation of Na reabsorption. The data do indicate quite strongly, however, that the inhibition of P_{Na} seen with increasing mucosal Na concentration in the K-depolarized toad bladder (Li et al., 1982) is a direct effect of external Na and is not secondary to an increase in Na_c. This conclusion conflicts with a recent report of Chase and Al-Awqati (1983) who found no effect of Na concentration on tracer Na fluxes in apical membrane vesicles from the toad bladder.

Inhibition of P_{Na} by Cell Ca

A number of investigators have proposed that intracellular Ca might inhibit apical Na permeability in tight epithelia (Grinstein & Erlij, 1978; Taylor & Windhager, 1979; Chase & Al-Awqati, 1981, 1983). This proposal is based on a variety of evidence, including the observation that Na transport is reduced by lowering serosal Na, a maneuver expected to raise cytoplasmic Ca by reducing extrusion of Ca from the cell in exchange for serosal Na. In the K-depolarized bladder, in which the apical conductive pathway can be studied more directly, serosal Na is absent under control conditions, eliminating this pathway for Ca efflux. Recently Garty and Lindemann (1984), using K-depolarized toad bladders in which intracellular Na was increased by serosal ouabain, found that P_{Na} was reversibly inhibited by increasing serosal Ca. Their interpretation was that the basal-lateral Na-Ca exchange process runs backwards under these conditions, exchanging cell Na for serosal Ca, thus increasing the cytoplasmic Ca activity. These observations were confirmed in the present study (Table 1). It should be stressed that the Ca-dependent decrease in Na conductance required high levels of intracellular Na. In tissues not treated with ouabain increased serosal Ca did not affect Na transport (Table 1). This provides further evidence that Ca influx through the Na-Ca exchanger is responsible for the decrease in P_{Na}.

In the absence of Na-dependent Ca extrusion from the cell, it is likely that an ATP-dependent, Na-independent Ca pump is largely responsible for maintaining low cytoplasmic Ca activity. This transport mechanism has been found in the plasma membranes of other tissues (Schatzmann, 1966; DiPolo, 1978; Gmaj, Murer & Kinne, 1978). Since this Ca pump is inhibited by vanadate in other preparations (DiPolo et al., 1979; Rossi et al., 1981), it seemed possible that vanadate could lead to increased cytoplasmic Ca and decreased P_{Na}. The second prediction was verified: serosal vanadate decreased P_{Na} by 50% under conditions where Na_c was raised by less than 4 mM (Table 2). The small increase in Na_c was presumably a consequence of inhibition of the Na pump (Cantley et al., 1978). In addition, vanadate decreased G_{Na} in bladders where the Na pump had previously been blocked with ouabain (Fig. 7). These results are consistent with those of Beauwens, Crabbé and Rentmeesters (1981), who reported that serosal vanadate decreased the short-circuit current and the net Na flux in the toad bladder, with a loss of intracellular K that was much less than that observed in the presence of ouabain. Thus increased cytoplasmic Ca could account for these effects of vanadate on the isolated toad bladder and might also contribute to the high potency of vanadate as a natriuretic agent in the rat (Balfour, Grantham & Glynn, 1978). This interpretation should, however, be made with caution, as vanadate inhibits other enzymes involved in phosphate metabolism (Van Etten, Waymack & Rehkop, 1974; Lo-

pez, Stevens & Lindquist, 1976) and may not affect P_{Na} exclusively through changes in cell Ca.

Effect of CO_2 on P_{Na}

Increased CO_2 has been used to acidify the cytoplasm of squid axons, presumably acting by diffusing more rapidly into the cell than does HCO_3, becoming hydrated and releasing a proton (Boron & DeWeer, 1976). This maneuver caused a rapid, reversible fall in I_{Na} and P_{Na}, consistent with the idea that intracellular protons block Na channels. A similar effect of CO_2 on Na transport by the frog skin was reported by Ussing and Zerahn (1951). However, changes in cell pH and pCa are difficult to separate, and decreases in cell pH have been associated with increases in cytoplasmic Ca in some tissues (Meech & Thomas, 1977; DiPolo & Beaugé, 1982). Thus changes in P_{Na} with acidification of the cytoplasm may be mediated by increased Ca activity.

Thus the third conclusion of this work is that putative increases in cytoplasmic Ca and/or pH can decrease P_{Na}. In the absence of direct measurements of the Ca and H ion activities in the cytoplasm in these experiments and in different physiological conditions, it is yet to be established if these ions are important physiological regulators of this system.

It should also be stressed that these results were all obtained using K-depolarized epithelia. Clearly, the regulation of intracellular ionic composition in this preparation will differ significantly from that of nondepolarized bladders. Intracellular Ca homeostasis, for example, is probably affected by both the absence of serosal Na ions and the reduction in the basal-lateral membrane electrical potential. In addition, Thomas et al. (1983) have cited differences in the regulation of intracellular Na activity in depolarized *vs.* nondepolarized *Necturus* urinary bladders. I have assumed that the behavior of the channels themselves in response to changes in the intracellular milieu is similar in the depolarized and more physiological states. Although I know of no evidence to the contrary, this assumption should be made with caution.

Mechanism of Block by Cytoplasmic Cations

A final major conclusion of this study is that inhibition of Na permeability by intracellular Na, H and Ca is voltage independent. This has implications for the mechanism of inhibition by these ions. In many instances, voltage-dependent block of channels by impermeant or slowly premeant ions has been interpreted as indicating that the binding site for the blocking ion is within the lumen of the channel. Examples include block of K channels by Ba, quaternary ammonium ions and Cs (Benzanilla & Armstrong, 1972; Eaton & Brodwick, 1980; Coronado & Miller, 1982), block of excitable Na channels by H (Woodhull, 1973; Begenisich & Danko, 1983) and block of epithelial Na channels by amiloride and alkali metal cations (Palmer, 1984*b*). Thus inhibition of Na channels by internal Ca is not analogous to block of K channels by internal Ba. If a divalent blocking cation, present at a concentration giving half-maximal inhibition at $V = 0$, senses 1% of the electric field at its binding site, an applied voltage of 200 mV would alter the degree of block by 17%, which would have been easily detected in the experiments shown in Fig. 7. Thus Ca could act directly on the Na channels at a site which senses less than 1% of the membrane field or, alternatively, could act indirectly, as through a Ca-dependent protein kinase.

In the case of block by H, the interpretation is complicated by the fact that the channel is permeable to H (Palmer, 1982*b*). This can reduce the voltage dependence of block (Woodhull, 1973; Begenisich & Danko, 1983). Nevertheless, block of the toad bladder Na channel by external protons was found to be voltage dependent (Palmer, 1984*b*) as was block of excitable Na channels in squid axon by both external and internal protons (Woodhull, 1973; Bengenisich & Danko, 1983). Therefore, the voltage independence of the effect of cellular acidification is evidence that internal protons are not titrating an acidic group within the lumen of the pore under these conditions. As in the case of Ca, the effects of decreased cell pH could be to titrate a charge near the mouth of the channel, or could be mediated by parallel changes in cell Ca or by inhibition of metabolism, which is known to decrease Na permeability (Palmer et al., 1980).

In the case of Na, reduction in P_{Na} by internal Na could reflect saturation of a binding site for Na within the pore. According to the data shown in Fig. 4, such a site might be about 20% saturated at 40 mM interval Na activity, assuming that the entire fall in P_{Na} in that experiment is due to direct effects of internal Na. This would be consistent with a binding site with a K_m for Na of 160 mM. As this site could be within the electric field of the channel, a saturation of the site, and hence the effect of Na on P_{Na}, might be voltage dependent. In this case, however, the voltage dependence may well be quite weak. In the Appendix, theoretical I-V curves for a symmetrical 3-barrier Eyring rate-theory model of a channel are derived for various degrees of channel

saturation. Interaction of internal Na with a binding site which senses 30% of the electric field of the membrane is still consistent with the weak voltage dependence of the effects of internal Na.

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Appendix

In this paper, as in previous reports (Fuchs et al., 1977; Palmer et al., 1980; Thompson et al., 1982; Thomas et al., 1983) the constant field equation (CFE) has been used to describe the *I-V* relationship of the amiloride sensitive transport pathway in tight epithelia and to derive voltage-independent permeability coefficients in various conditions. This equation is based on the Nernst-Planck equation which describes electrodiffusion of ions through a homogeneous medium. There is no theoretical justification for its application to single-file ion migration through narrow, inhomogeneous biological membrane pores. The purpose of this Appendix is first to demonstrate that *I-V* relationships very similar to those predicted from the CFE can be accounted for by discontinuous diffusion or Eyring rate-theory models of ion translocation (Eyring, Lumry & Woodbury, 1949; Läuger, 1973; Hille, 1975). Second, it will be demonstrated that two features of Na channel *I-V* relationships reported here—asymmetry of current in the absence of a Na gradient and the relative independence of the shape of the *I-V* curve with changing Na concentrations—can be readily accounted for by such models. The purpose is not to present a unique or even a best-fit rate theory model of the presented data. Rather, somewhat general schemes

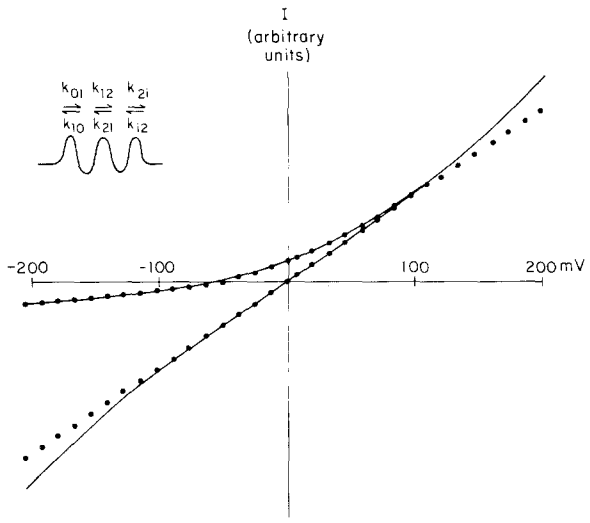

Fig. A1. *I-V* relationships predicted from the constant field equation (dotted lines) and from the 3-barrier Eyring model (solid lines) illustrated with $k_{10} = k_{12} = k_{21} = k_{2i}$ and $\delta_1 = \delta_2 = 0.3$ and $\delta_m = 0.4$ in the limit of $P_o = 1$ (low channel occupancy). The models are compared for the cases $S_i = S_o$ and $S_i = 0.1\ S_o$. In both models the currents in the two cases converge at high positive voltages. The parameters of the models were adjusted so that they predicted the same currents and conductances at $V = 0$

which qualitatively describe the observed features and are consistent with the experimental data will be described.

The basic model to be examined has three energy barriers for ion translocation and two binding sites or energy wells as illustrated in the inset to Fig. A1. Translocation of ions through the channel is described kinetically by the scheme:

$$S_o + C_o \underset{k'_{10}}{\overset{k'_{01}}{\rightleftharpoons}} SC_1 \underset{k'_{21}}{\overset{k'_{12}}{\rightleftharpoons}} SC_2 \underset{k'_{i2}}{\overset{k'_{2i}}{\rightleftharpoons}} S_i + C_o$$

where S_o and S_i are the concentrations of permeant ions on the two sides of the membrane, C_o is the number of unoccupied channels and SC_1 and SC_2 the channels occupied at the two sites, respectively. For simplicity it is assumed that the two sites cannot both be occupied. The k' parameters represent voltage-dependent rate constants and are given by:

$$k'_{01} = k_{01} \exp(\delta_1 U/2) \qquad k'_{10} = k_{10} \exp(-\delta_1 U/2)$$
$$k'_{12} = k_{12} \exp(\delta_m U/2) \qquad k'_{21} = k_{21} \exp(-\delta_m U/2)$$
$$k'_{21} = k_{2i} \exp(\delta_2 U/2) \qquad k'_{i2} = k_{i2} \exp(-\delta_2 U/2)$$

where the k parameters represent rate constants in the absence of a transmembrane electric field, $U = FV/RT$, δ_1, δ_m and δ_2 are the fractions of the transmembrane voltage expressed across the first, middle and third barrier, respectively. Current flow through this system is given by:

$$I(U) = BC_o \left[\frac{S_o \exp(U/2) - S_i \exp(-U/2)}{k_{2i}k_{10} \exp((\delta_2 - \delta_1)U/2) + k_{2i}k_{12} \exp(\delta_2 + \delta_m)U/2)} \right.$$
$$\left. + k_{21}k_{10} \exp(-(\delta_1 + \delta_m)U/2) \right]$$

where $B = k_{o1}k_{12}k_{2i} = k_{i2}k_{21}k_{1o}$.

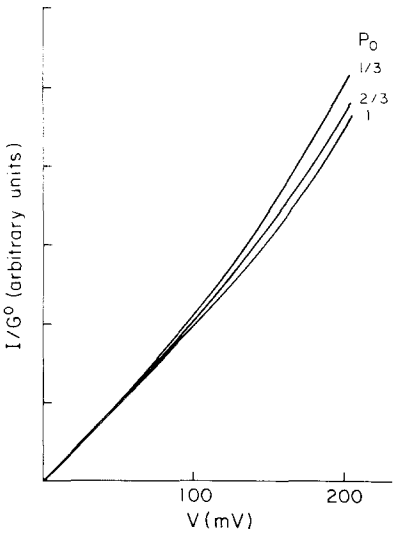

Fig. A2. Normalized *I-V* relationships from the model of Fig. A1 for $S_o = S_i$ and open channel probabilities (P_o) of 1, $\frac{2}{3}$ and $\frac{1}{3}$. Since the channel is symmetric the points at negative voltages are omitted

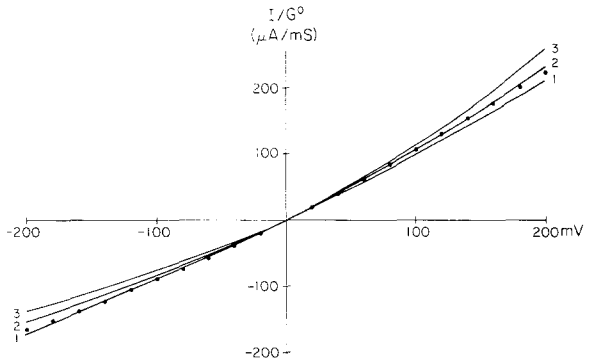

Fig. A3. Normalized *I-V* relationships from the model of Fig. A1 with $S_o = S_i$ and $P_o = 1$ but with $\delta_1 = 0.3$, $\delta_2 = 0.2$ and $\delta_m = 0.5$ (curve 1), $\delta_1 = 0.35$, $\delta_2 = 0.15$ and $\delta_m = 0.5$ (curve 2), and $\delta_1 = 0.28$, $\delta_2 = 0.22$ and $\delta_m = 0.5$ (curve 3). Data points from Fig. 5 (Na_o = 20 mM) are also plotted on the same scale

If the concentrations S_o and S_i are sufficiently small, $C_o \approx C_T$, the total number of channels. This is likely to be a good approximation for the Na channel under physiological conditions, as the Na channel current is a linear function of mucosal Na in this range (Van Driessche & Lindemann, 1979). The *I-V* relationship predicted by Eq. (2) for small S_o and S_i is similar to that of the CFE when the values of the different rate constants and δ's are similar. An example is illustrated in Fig. A1, for $k_{2i} = k_{10} = k_{12} = k_{21}$ and $\delta_1 = \delta_2 = 0.3$ and $\delta_m = 0.4$ *I-V* relationships with equal Na activities and a 10 : 1 Na gradient across the membrane are shown together with the comparable *I-V* relationships derived from the CFE. Significant divergence is seen only at high voltages. The particular values of *k*'s and δ's chosen are not critical, and the divergence can be reduced by increasing the number of barriers and sites. In the limit of an infinite number of barriers with the same *k* and δ values, the CFE will be reproduced exactly (Woodbury et al., 1970). Lindemann (1982) also found that *I-V* curves for 2-barrier, 1-site channels at low occupancy were very similar to those predicted by the constant-field equation.

Note that at $V = 0$, $I = BC_o(S_o - S_i)/(k_{2i}k_{10} + k_{2i}k_{12} + k_{21}k_{10})$. Thus the parameter $P \equiv I(O)/(S_o - S_i)$ depends only on the total number of channels and the voltage-independent rate constants, as long as S_o and S_i are sufficiently small. Thus this parameter is in effect a permeability coefficient. A decrease in *P* with increasing ion activities can be explained by saturation of the channel, in which case $C_o < C_T$. For example, a 20% reduction in the number of unoccupied channels will reduce *P* by 20% and would account for the data shown in Fig. 4. In general, channel occupancy will be voltage dependent. The voltage dependence predicted on the basis of the model under consideration will, however, be weak. Figure A2 shows the predicted normalized *I-V* curves for such a model with the $S_o = S_i$ adjusted to give 0, $\frac{1}{3}$ and $\frac{2}{3}$ occupancy. The essential point is that the shapes of the *I-V* relationships are rather independent of the degree of occupancy, and hence of ion concentration, particularly at relatively low occupancy levels.

Finally, asymmetric *I-V* curves are predicted even in the presence of symmetrical solutions, providing the channel itself is asymmetric. Figure A3 shows the *I-V* relationship expected from the same channel discussed above but with $\delta_1 \neq \delta_2$. For comparison the data from Fig. 5 is also shown. Asymmetries as shown in Figs. 5, 6, 7 and 9 can be accounted for by this sort of channel asymmetry. As discussed in the text, however, other explanations are also feasible.

In summary, the electrical properties of the epithelial Na channel can be accounted for by an Eyring rate-theory model with three (or more) barriers which have similar potential drops and rate constants across them, and with two (or more) energy wells which are mostly unoccupied at physiological concentrations of Na. A more precise description will require measurements made under conditions which are better defined with respect to series resistance barriers and intracellular blocking ions, and which can be varied over a larger range, particularly with respect to the concentrations of permeant ions.