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Voltage-Dependent Block by Amiloride and Other Monovalent Cations of Apical Na Channels in the Toad Urinary Bladder

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Summary. Inhibition of the Na conductance of the apical membrane of the toad urinary bladder by amiloride, alkali cations and protons was voltage dependent. Bladders were bathed with a high K-sucrose serosal medium to reduce series basal-lateral resistance and potential difference. Transepithelial current-voltage relationships were measured over a voltage range of ±200 mV with a voltage ramp of frequency 0.5 to 1 Hz. Na channel *I-V* relationships were obtained by subtraction of currents measured in the presence of maximal doses of amiloride (10 to 20 μM). With submaximal doses of amiloride (0.05 to 0.5 μM), the degree of inhibition of the Na channel current (I_{Na}) increased as the mucosal potential was made more positive. The data can be reasonably well explained by assuming that amiloride blocks Na transport by binding to a site which senses ~12% of the transmembrane voltage difference. I_{Na} was reduced in a qualitatively similar voltage-dependent manner by mucosal K, Rb, Cs and Tl (~100 mM) and by mucosal H (~1 mM). Block by these cations cannot be explained in terms of interactions with a single membrane-voltage-sensing site; a model in which there are two or more blocking sites in series provides a better description of the data. On the other hand, amiloride block was reduced competitively by mucosal Na and K, suggesting that occupation of the channel by one cation excludes occupancy by the others. ADH and ouabain also reduce the apparent affinity of amiloride for its blocking site. Thus, intracellular Na may also compete with amiloride for occupancy of the channel.

Key Words toad urinary bladder · epithelial Na channels · amiloride · voltage-dependent inhibition

Introduction

Permeability to Na of the apical membranes of the toad urinary bladder and frog skin is thought to be mediated by ion channels (Lindemann & Van Driessche, 1977; Li et al., 1982; Palmer, 1982*a*). A pharmacological hallmark of these channels is their sensitivity to the diuretic drug amiloride, which acts on these tissues from the mucosal side (Bentley, 1968). The active form of amiloride is a monovalent cation (Benos et al., 1976; Cuthbert, 1976). Other positively charged ions reduce Na permeability when present in the mucosal solution, including protons, K and other large alkali cations, and Na itself (Leaf, Keller & Dempsey, 1964; Fuchs, Hviid Larsen & Lindemann, 1977; Benos, Mandel & Simon, 1980).

In this paper, I show that amiloride, K, Rb, Cs and H block the Na channels in a voltage-dependent manner. The data suggest that these blockers occupy sites near the luminal surface which sense 10 to 35% of the total voltage drop across the apical membrane. I propose that these sites are within the lumen of the channel, and that occupancy by a blocking ion obstructs the passage of Na through the pore.

Materials and Methods

Animals

Female toads (*Bufo marinus*) were obtained from National Reagents (Bridgeport, Conn.) and kept in tanks with access to tap water prior to use.

Solutions

Serosal solutions contained (in mM): KCl 85, sucrose 50, $CaCl_2$ 1, $MgCl_2$ 0.5, glucose 5, and K phosphate 3.5, buffered to pH 7.6. The standard mucosal solution contained (in mM): NaCl 115, $CaCl_2$ 1, $MgCl_2$ 0.5 and K phosphate 3.5, buffered to pH 6.0. In some experiments NaCl was reduced, being replaced isotonically with choline Cl, KCl, RbCl, CsCl, or tetraethylammonium Cl. In other experiments, the pH of the mucosal solution was varied by changing the ratio of monobasic to dibasic K phosphate at constant K concentration. In experiments where the effects of Tl were measured, the NO_3 salt of Na and Tl and the SO_4 salts of Ca and Mg were used. N-methyl-*d*-glucamine (NMDG) base neutralized with HNO_3 and was used to replace $TlNO_3$.

Electrical

Tissues were mounted as flat sheets and voltage-clamped as described previously (Palmer, 1982*b*) except that the current-passing electrodes were made of platinum and were coated with platinum black. Voltage electrodes were placed close to the tissue to minimize series solution resistance (<40 ohms). These electrodes were in direct contact with the bathing solutions.

To measure current-voltage relationships, tissues were maintained at a transepithelial voltage (V_T) of zero until a steady state was attained. V_T, assumed to approximate the apical membrane voltage, was then changed to −200 mV and swept to +200 mV using a voltage ramp applied to the voltage command input of the voltage clamp with a function generator (Tektronix FG 501A). All voltages are given as the mucosal voltage relative to that of the serosal solution. The voltage range varied slightly in different experiments. The transepithelial voltage and the resulting transepithelial current were measured using two channels of a Nicolet (Model 2090-III) digital storage oscilloscope. The traces were stored on diskettes for later analysis. The duration of the voltage ramp was usually one second. This speed was chosen to be slow relative to the rate of amiloride block, estimated from the corner frequency measured with noise analysis (20 sec^{-1} at an amiloride concentration of 1 μM (Li et al., 1982)), but to be sufficiently fast to minimize redistribution of ions. As a check on whether ion accumulation or depletion was affecting the results, ramps with durations of 0.5 to 2 sec were used; the results were independent of ramp speed, except for the capacitative currents. Furthermore, several ramps could be applied consecutively without affecting the *I-V* relationship. These findings suggest that the voltage displacement was not causing major redistributions of ions across the membrane.

In all cases there was a small offset current due to the membrane capacitance. This current will be given by $I_c = C(dV/dt)$ and will be constant for a given ramp speed, adding to the ionic currents flowing across the membrane. All data are corrected for currents measured in the presence of a maximal dose of amiloride (*see below*). The capacitative currents, which will be the same with and without amiloride, will also be subtracted out by this procedure.

To compare *I-V* relationships with different mucosal solutions, tissues were allowed to reach steady state with one solution, at $V_T = 0$. An *I-V* curve was obtained, and immediately afterward the mucosal medium was replaced by a second solution. When the solution change was complete and a new steady-state short-circuit current was achieved (generally within a few seconds) a second *I-V* curve was generated. This process was repeated to produce up to five *I-V* relationships. All changes in these relationships were fully reversible. Finally, a second series of mucosal solutions was used, identical to the first except for the addition of a maximal dose of amiloride to each solution. The same protocol was used to generate a second set of *I-V* curves. To obtain the amiloride-sensitive current-voltage (I_{Na}–V) relationship for any given condition, the *I-V* curve obtained with maximal amiloride was subtracted from that measured without maximal amiloride. The difference curve was computed by subtracting currents obtained at each voltage (Palmer, Edelman & Lindemann, 1980).

Chemicals

Antidiuretic hormone (ADH) was added as aqueous pitressin (Park Davis, Detroit, Mich.) to the serosal bath. Amiloride was a gift of Merck, Sharp and Dohme (West Point, Pa.). Stock solutions of 10^{-2} or 5×10^{-4} M were prepared in deionized water and added to the mucosal bath as required.

Data Analysis

All data given as clamp current are corrected for amiloride-insensitive current. When short-circuit current was continuously monitored, current values obtained for any given experimental condition were corrected by subtracting the short-circuit current measured under the same conditions except for the addition of 10^{-5} M amiloride to the mucosal solution. In current-voltage plots, all *I-V* curves were corrected using a second *I-V* relationship in the presence of 10^{-5} M amiloride (*see above*). Corrected current values are denoted by I_{Na} or, when H ion currents were measured, by I_H.

The effects of a blocking ion B were analyzed assuming a first-order interaction between the blocker and its site of interaction(s) with the channel:

$$B + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} BS.$$

When the BS complex B is formed, the channel is assumed to be in a nonconducting state. Thus:

$$I_{Na}^B = \frac{I_{Na}}{1 + \frac{(B)}{K_I^B}} \tag{1}$$

where I_{Na} and I_{Na}^B are Na currents in the absence and presence of B, (B) the concentration of B in the mucosal solution and $K_I^B = k_{-1}/k_1$ the apparent inhibition constant. If the site S is within the electric field of the membrane, K_I^B will be voltage-dependent.

To assess voltage dependence, I_{Na}-V relationships were measured in the presence and the absence of the blocker. The effect of an impermeant blocker B was analyzed according to a treatment used previously (Woodhull, 1973; Latorre & Miller, 1983) using the relationship:

$$I_{Na}^B(V) = \frac{I_{Na}(V)}{1 + \frac{B}{K_I^B \exp\left(-\frac{ZF}{RT}\delta V\right)}} \tag{2}$$

where I_{Na} and I_{Na}^B are now functions of voltage V, K_I^B is the inhibition constant at zero voltage and δ is the fraction of the membrane voltage sensed at the site of interaction of B with the channel. A positive V will drive mucosal blocking cations into the membrane, decreasing the apparent inhibition constant. Equation (2) can be arranged to give:

$$\ln\left\{\left[\frac{I_{Na}(V)}{I_{Na}^B(V)}\right] - 1\right\} = \ln\left[\frac{(B)}{K_I^B}\right] + \frac{ZF}{RT} \cdot \delta \cdot V. \tag{3}$$

The expression on the left is therefore expected to be a linear function of the membrane voltage. This simplified treatment ignores effects of competition between permeant and blocking ions (*see* Discussion).

A second method of analysis involved a more complex interaction with two blocking sites in series:

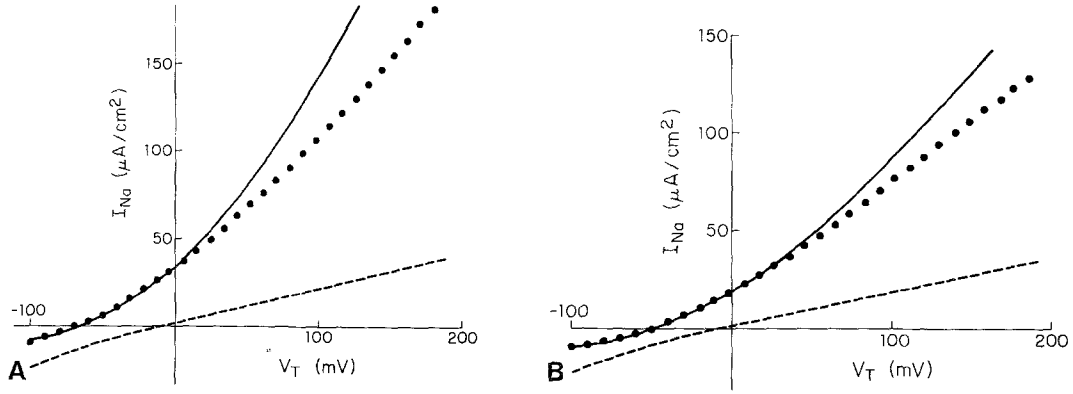

Fig. 1. I_{Na}-V relationships with high and low mucosal Na. Transepithelial current-voltage relationships were obtained in the presence and absence of amiloride (10 μM). I_{Na}-V relationships were computed by subtracting the currents measured in the presence of amiloride from those measured in its absence at the same voltage. The solid lines represent the I_{Na}-V relationship predicted from the constant field equation, forced to pass through the data points at $V = 0$ and $I_{Na} = 0$ as described in the text. The dashed line represents the I-V relationship in the presence of amiloride. *A*. Mucosal Na concentration 115 mM. Parameters used to generate the solid curve were $P_{Na} = 0.41 \times 10^{-5}$ cm/sec and $Na_c = 6$ mM. *B*. Same hemibladder as in *A*, with mucosal Na concentration 29 mM. Parameters used to generate the solid curve were $P_{Na} = 1.00 \times 10^{-5}$ cm/sec and $Na_c = 3.1$ mM

$$B + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} BS_1 \underset{K_{21}}{\overset{k_{12}}{\rightleftharpoons}} BS_2.$$

Thus the channel can be unblocked, blocked at site S_1, or blocked at site S_2. Assuming that I_{Na} is proportional to the number of channels in which neither S_1 nor S_2 is occupied by B,

$$I^B_{Na}(V) = \frac{I_{Na}}{1 + \dfrac{(B)}{K_I^{eff,B}}} \tag{4}$$

where

$$K_I^{eff,B} = \frac{K_1^B \exp\left(-\dfrac{ZF}{RT}\delta_1 V\right) \cdot K_{12}^B \exp\left(-\dfrac{ZF}{RT}\delta_{12} V\right)}{1 + K_{12}^B \exp\left(-\dfrac{ZF}{RT}\delta_{12} V\right)}. \tag{5}$$

$K_I^B = \dfrac{k_{-1}}{k_1}$ and $K_{12}^B = \dfrac{k_{21}}{k_{12}}$ are the dissociation constants for sites S_1 and S_2, and $\delta_{12} = \delta_2 - \delta_1$ where δ_1 and δ_2 are the fractions of V sensed at S_1 and S_2, respectively.

As discussed previously, the transepithelial voltage was assumed to be a good approximation to the transepithelial membrane voltage in the presence of the KCl-sucrose serosal solutions (Palmer et al., 1980; Warncke & Lindemann, 1981; Palmer, 1982*b*; Palmer & Lorenzen, 1983).

Data are expressed as means ± standard errors (SEM). Statistical inferences were made using Student's *t*-test.

Results

Shape of the I_{Na}-V Relationship

In previous studies, the I_{Na}-V relationship of the toad bladder was well described by the constant field equation (CFE) over the voltage range between $V = 0$ and the reversal potential (Palmer et al., 1980; Li et al., 1982; Palmer et al., 1980). I_{Na}-V relationships over a more extended voltage range, obtained with 115 and 29 mM Na in the mucosal solution, are shown in Fig. 1. The solid lines represent I-V curves calculated from the CFE with the free parameters for the Na permeability (P_{Na}) and the cellular Na activity (Na_c) chosen to fit the data in the upper left-hand quadrant. To obtain these parameters, $I_{Na}(0)$, the current at $V = 0$, and E_{Na}, the reversal potential or voltage at $I_{Na} = 0$, were obtained by linear interpolation of the data points near the intersections with the V and I axes. Na_c was computed directly from $E_{Na} = -(RT/F) \ln (Na_o/Na_c)$, where Na_o is the Na activity in the mucosal solution. P_{Na} was computed from the relationship $I_{Na}(0) = FP_{Na}(Na_o - Na_c)$. With these parameters, the CFE describes the data reasonably well at negative voltages. At positive voltages, currents are smaller than those predicted from the CFE.

Mean values of P_{Na} and Na_c obtained in this way in the absence of mucosal blocking ions were $P_{Na} = 0.22 \pm 0.13 \times 10^{-5}$ cm/sec and $Na_c = 13 \pm 7$ mM (means ± SD, $n = 18$) for 115 mM mucosal Na, and $P_{Na} = 0.57 \pm 0.32 \times 10^{-5}$ cm/sec and $Na_c = 2.8 \pm 0.9$ mM ($n = 13$) at 29 mM mucosal Na. The latter values are in good agreement with those reported previously for similar conditions (Palmer et al., 1980).

The I-V relationship in the presence of a maximal dose of amiloride is also shown. This relationship is approximately linear at positive voltages,

and becomes steeper at voltages more negative than −50 mV, as reported previously (Palmer et al., 1980).

Effects of Amiloride on I_{Na}-V

I_{Na}-V relationships with and without a submaximal dose of amiloride (0.4 μM) are shown in Fig. 2. Compared with the control curve, the amiloride-blocked I_{Na}-V relationship shows a reduced short-circuit current (intersection with the I axis), a more negative reversal potential (intersection with the V axis), reflecting a reduced intracellular Na activity, and, most strikingly, at high positive potentials, a greatly reduced slope, becoming nearly flat at potentials greater than 150 mV.

These data, and those for three other I_{Na}-V curves obtained in the same experiment using different amiloride concentrations, are plotted according to Eq. (3) in Fig. 3. The linearized fractional inhibition relationships were fitted with straight lines using least-squares linear regression on the data points at voltages between 100 and 200 mV. In this experiment, and in general, correlation coefficients were >0.99. Below 100 mV the data fell near the regression line but showed considerably more scatter.

In principle, Eq. (3) should be applicable to data in the negative voltage range as well. Currents in this range, however, are quite sensitive to changes in intracellular Na activity; at $V = -200$ mV inward Na flux should be small, implying that I_{Na} is carried mainly by an outward Na flux. This flux should increase as intracellular Na increases. Since intracellular Na generally decreases with amiloride, as evidenced by the shift in the reversal potential to the left in Fig. 2, outward currents cannot be reliably used in this protocol to test for voltage dependence of ionic blockers.

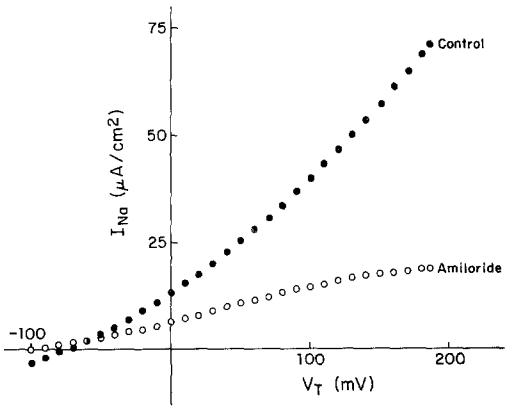

Fig. 2. I_{Na}-V relationships of a hemibladder under control conditions and with 0.4 μM amiloride in the mucosal solution. Mucosal Na concentration was 115 mM. Transepithelial I-V relationships were measured sequentially with 0, 0.1, 0.2, 0.3 and 0.4 μM amiloride. Currents in the presence of 10 μM amiloride were subtracted from each of the other I-V relationships to generate the I_{Na}-V plots. Only data from control (●) and 0.4 μM amiloride (○) traces are shown

Calculated values of K_I^{amil} and δ were essentially independent of amiloride concentration. Mean values of these parameters from six experiments with 115 mM Na were 0.36 ± 0.12 μM and 0.122 ± 0.003, respectively. The value for K_I^{amil} obtained from the linear regression analysis is in good agreement with the value calculated from the decrease in amiloride-sensitive short-circuit current according to Eq. (1) (0.38 ± 0.07 μM; Table 1).

Effects of K, Rb and Cs on I_{Na}-V

Figure 4 shows I_{Na}-V curves from the same hemibladder with three different mucosal solutions: (1) (control) Na 29 mM, choline 86 mM; (2) (amiloride-blocked) Na 29 mM, choline 86 mM and amiloride 0.08 μM; (3) (K-blocked) Na 29 mM, K 86

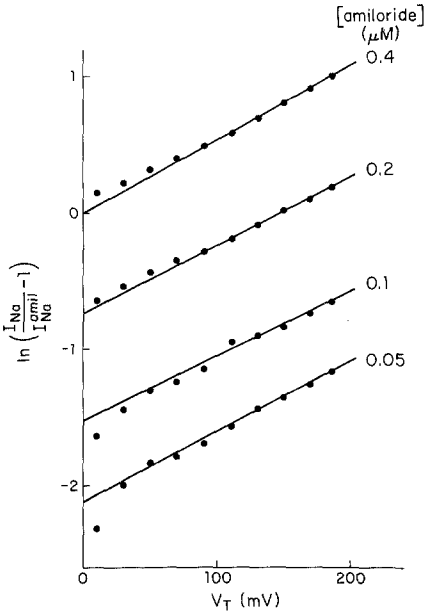

Fig. 3. Voltage dependence of amiloride block. Data are from the experiment shown in Fig. 1. The mucosal concentration was 115 mM. At each amiloride concentration, the fractional inhibition of current was calculated and plotted as a function of voltage as in Eq. (3). The straight lines show linear least-squares fit of the data between 100 and 200 mV. Values of K_I and δ for amiloride computed from the fitted lines are:

(amiloride) (μM)	K_I (μM)	(δ)
0.05	0.42	0.13
0.10	0.46	0.12
0.20	0.42	0.13
0.4	0.40	0.14

mM. The K and amiloride solutions fortuitously produced very similar reductions in I_{Na}. From the general shape of the I_{Na}-V curves it is evident that amiloride and K blocks had similar voltage-dependencies. When analyzed according to Eq. (3), however, (Fig. 5), and K block showed somewhat steeper voltage dependence at high voltages.

This analysis assumes that choline ions, which are present in the mucosal solutions when the control I-V relationships in these experiments were measured, do not themselves block the Na channel. This cannot be shown rigorously. However, replacement of choline with tetramethylammonium, tetraethylammonium or NMDG at constant Na concentration had no effect on the I-V relationship. This implies that all four of these ions block the channel to the same extent. The simplest interpretation is that they do not block at all, possibly due to their large ionic radii.

Similar voltage dependencies were observed for blocks by K, Rb and Cs. Figure 6 shows a typical experiment plotted as blocking affinity *vs.* V_T as in Fig. 3. The Figure indicates the relative apparent affinities of the inhibitory site are in the sequence K > Rb > Cs, the order being independent of voltage. Fitting these curves with Eq. (3) was not satisfactory, as the data did not fall on a straight line. Nevertheless, the slopes of these curves between 100 and 200 mV, denoted by δ_+, give a rough indication of the voltage dependence of the block (*see* Discussion), and by this index, the voltage dependence was similar for the three ions. Mean values of δ_+, along with values of the apparent inhibition constants measured at $V_T = 0$, are given in Table 1.

Table 1. Values of K_I and δ_+ for blocking ions[a]

		δ_+	K_I
amiloride	(6)	0.122 ± 0.003	0.38 ± 0.07 μM
K	(7)	0.155 ± 0.007	200 ± 40 mM
H	(9)	0.059 ± 0.007	3.2 ± 0.8 mM
K	(5)	0.168 ± 0.005	170 ± 20 mM
Rb	(5)	0.152 ± 0.018	250 ± 20 mM
Cs	(5)	0.130 ± 0.007	500 ± 50 mM
Tl	(5)	0.154 ± 0.006	74 ± 9 mM

[a] Values of K_I were obtained from the fractional inhibition of I_{Na} at $V_T = 0$, according to Eq. (1). Values of δ_+ were obtained from the slope of the blocking *vs.* voltage curves as plotted in Figs. 3, 5, 6 and 12 using linear regression analysis of the data between 100 and 200 mV. Mucosal Na concentrations were 115 mM in the case of amiloride and H, 29 mM for K, Rb and Cs and 86 mM for Tl. Blocker concentrations were 0.05 to 0.5 μM for amiloride, 0.63 mM for H, 86 mM for K, Rb and Cs and 29 mM for Tl. Data are expressed as mean ± SEM. The number of observations is given in parentheses.

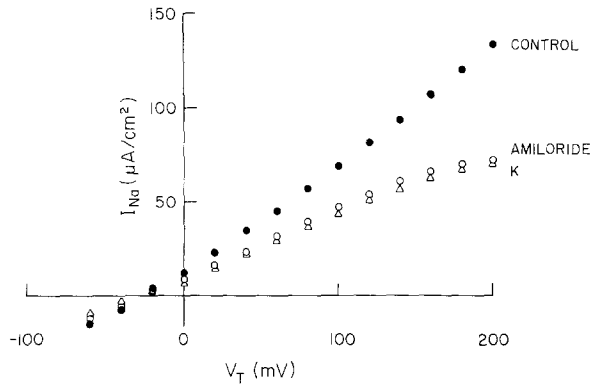

Fig. 4. The effects of amiloride and K on I_{Na}-V relationships. Mucosal Na concentration was 29 mM throughout. I-V relationships were measured sequentially with 86 mM choline Cl and 0, 0.08, 0.16 and 10 μM amiloride, as in Fig. 1. The amiloride was then washed off, and the I-V relationships measured with choline Cl, KCl replacing choline Cl, and KCl and 10 μM amiloride. I_{Na} curves for control (i.e., choline Cl, no amiloride), (●), 0.8 μM amiloride (○) and K (△) are shown

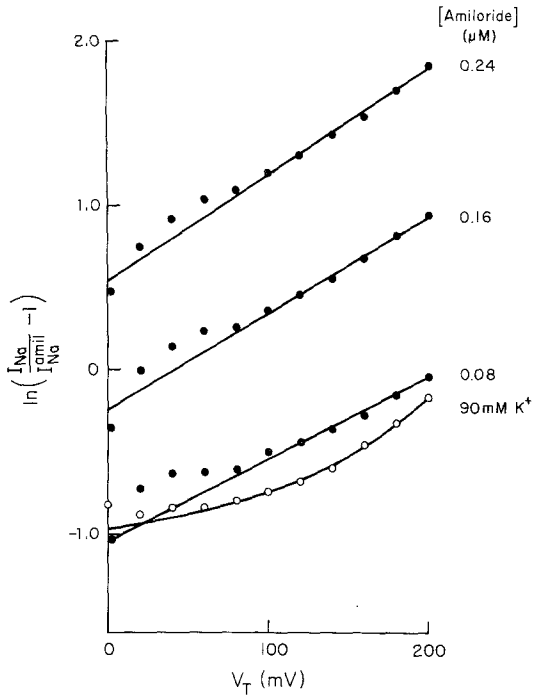

Fig. 5. Voltage-dependence of amiloride and K inhibition. Data are from the experiment shown in Fig. 4. The Na concentration was 29 mM throughout. Data are plotted as in Fig. 2. Values of K_I and δ for amiloride obtained from the regression lines are:

(amiloride) (μM)	K_I^{amil} (μM)	(δ)
0.08	0.23	0.13
0.16	0.20	0.15
0.24	0.14	0.16

The line drawn through the K data was obtained from Eq. (5) with K_1 = 260 mM, K_{12} = 7, δ_1 = 0 and δ_2 = 0.3

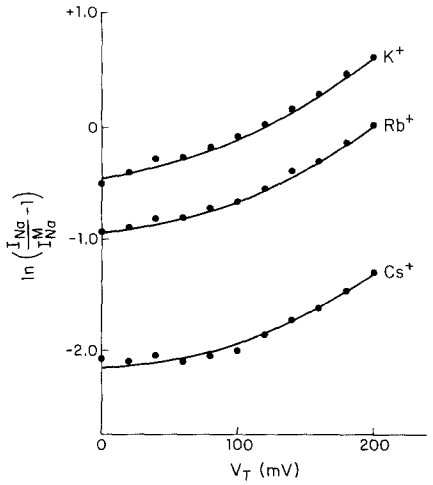

Fig. 6. Voltage dependence of K, Rb, and Cs block. *I-V* relationships were obtained with 29 mM Na and 86 mM choline, K, Rb and Cs in the mucosal solution. I_{Na}-V relationships were obtained by subtracting currents obtained with the same solutions but with 10 μM amiloride. Data plotted as in Figs. 2 and 4. The solid lines were drawn according to Eq. (5) with the following parameters:

	δ_1	δ_2	K_I (mM)	K_{12}
K	0	0.35	155	6.5
Rb	0	0.35	246	8.5
Cs	0	0.35	551	10.0

A more satisfactory fit to the data could be made with Eq. (5), assuming that the cations interacted with two sites in series within the electric field of the membrane. This involves four free parameters, the binding constants and δ values for binding to the first and second sites. In order to reduce the number of free parameters, the first site was assumed to be near the outer end of the electric field, and δ_1 was arbitrarily assigned a value of zero. The curve for K was fitted by trial and error, and the other curves were fitted using the same values for $\delta_2 = \delta_{12}$, the fraction of the electric field sensed at the second site. In general, all three curves could be described using values of δ_2 between 0.30 and 0.35. In Fig. 6, the three curves are fitted using $\delta_2 = 0.35$ and values of K_{12} of 6.5 for K, 8.5 for Rb and 10 Cs. Because of the simplifying assumptions made, these are not unique fits to the data, but are merely meant to show that the two-site model, from which Eq. (5) was derived, offers a reasonable explanation of the data.

In a separate series of experiments, the voltage dependence of block by $TlNO_3$ (replacing NMDG NO_3) was measured. The results were similar to those obtained with K, Rb and Cs, except that K_I at $V_T = 0$ averaged 74 ± 9 mM, implying that Tl is a more potent blocker than is K. Values of the slope (δ_+) at $V_T > 100$ mV were similar to those for the other cations. NMDG itself apparently had no effect on the Na channels, as replacement of choline Cl with NMDG did not detectably alter the I_{Na}-V relationship.

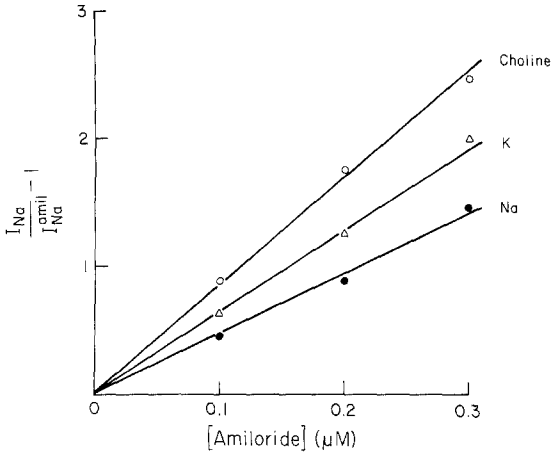

Fig. 7. Effects of Na and K on amiloride dose-response relationships. Short-circuit current was measured with amiloride concentrations of 0, 0.1, 0.2, 0.3, and 10 μM in the mucosal solutions under three different conditions in the same hemibladder. (○) 29 mM NaCl, 86 mM choline Cl; (●) 115 mM NaCl; and (△) 29 mM NaCl, 86 mM KCl. Each current value was corrected by subtracting the current at 10 μM amiloride. The data were analyzed according to Eq. (1) by linear regression of $\frac{I_{Na}}{I_{Na}^{amil}} - 1$ *vs.* amiloride concentration. Values of I_{Na}, the Na current in the absence of amiloride, were 7.8, 14.8, and 6.0 μA/cm^2, respectively. Values of K_I^{amil} obtained from the linear regression analysis were 0.118, 0.213 and 0.157 μM

Competition between Amiloride and K

The similar voltage-dependence of K block and amiloride block suggests that the two ions may interact with the same site, or closely related sites, within the channel. If this were the case, the two blockers should interact competitively. This point was tested in the experiment shown in Fig. 7. I_{Na} was measured under short-circuit conditions with 29 mM Na in the mucosal medium and 86 mM choline or K. K_I^{amil} was assessed according to Eq. (1) by plotting $\frac{I_{Na}}{I_{Na}^{amil}} - 1$ against amiloride concentration. The apparent inhibition constant for amiloride was increased in the presence of K, as indicated by the decreased slope of this plot. Assuming that this increase is due to a competitive interaction between K and amiloride, an estimate of K_I^K can be obtained from the relationship:

$$K_I^{amil}(K) = K_I^{amil}(0)\,[1 + [K]/K_I^K].$$

Table 2. Effects of Na, K, ADH and ouabain on K_I for amiloride[a]

		Control	Experimental	Exp/Control	K_I^M (mM)
1. K *vs.* choline	(5)	0.097 ± 0.009	0.135 ± 0.010	1.40 ± 0.03	166 ± 14
2. Na *vs.* choline	(7)	0.100 ± 0.008	0.169 ± 0.012	1.70 ± 0.05	104 ± 10
3. ADH (20 mU/ml)	(5)	0.18 ± 0.02	0.26 ± 0.02	1.46 ± 0.10	
4. Ouabain (0.5 mM)	(5)	0.26 ± 0.07	0.38 ± 0.10	1.44 ± 0.09	

[a] Values of K_I^{amil} were obtained from amiloride dose-response relationships as shown in Fig. 6. Conditions for K *vs.* choline and Na *vs.* choline were as in Fig. 7. In row 3, K_I^{amil} was measured before and 30 to 45 min after addition of ADH. In row 4, K_I^{amil} was measured before and 20 to 30 min after addition of ouabain to the serosal solution. The increase in I_{Na} after addition of ADH was 2.0 ± 0.3-fold. The decrease in I_{Na} after addition of ouabain was 0.57 ± 0.05-fold. The column on the right gives the calculated values for K_I for K and Na based on the reduction of K_I^{amil} assuming competitive interactions (*see text*).

From the measured values of K_I^{amil} at two different K concentrations, K_I^{amil}, the apparent inhibition constant in the absence of K, and K_I^K can be calculated. In 5 experiments, the value of K_I^K determined in this way was 166 ± 14 mM. In the same set of experiments, K_I^K values obtained more directly from the reduction in I_{Na} when K replaced choline using Eq. (1) was 180 ± 13 mM (Table 2).

If the site occupied by amiloride is within the lumen of the channel, in the pathway normally taken by Na ions as they traverse the membrane, then the block by amiloride should also be competitive with mucosal Na. As shown in Fig. 7, increasing the mucosal Na concentration from 29 t 115 mM increased the apparent inhibition constant for amiloride. From this increase, an apparent dissociation constant (K_I^{Na}) for Na binding to the amiloride site (or to a site where Na binding displaces amiloride binding) was calculated to be 104 ± 10 mM. Thus Na was somewhat more effective than K at displacing amiloride.

In a separate series of experiments, amiloride dose-response relationships were obtained at four different Na concentrations. The apparent K_I^{amil} increased with increasing Na as shown in Fig. 8. The data are reasonably well described by a straight line with a positive slope, consistent with competitive interaction between Na and amiloride. Linear regression analysis of the data gave values of K_I^{amil} = 0.059 μM and K_I^{Na} = 59 mM. Averaging values of K_I^{Na} obtained from individual experiments gave a mean value of 68 ± 15 mM. Averaging all individual values of K_I^{Na} from both sets of experiments gives 86 ± 10 mM.

It is also of interest to calculate the apparent K_m for Na transport, based on the saturation of I_{Na} with increasing mucosal Na, using the equation:

$$I_{Na} = \frac{I_{max} \cdot (Na)}{(Na) + K_m}$$

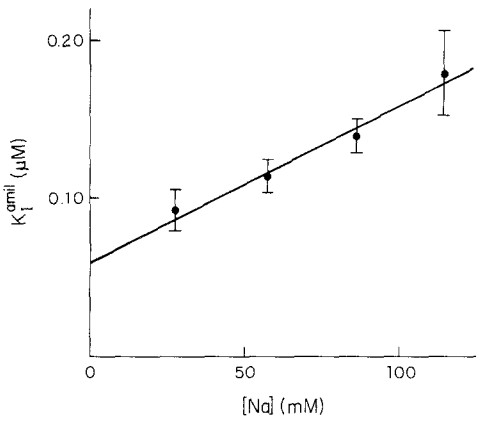

Fig. 8. Effects of Na on amiloride dose-response relationship. Short-circuit current was measured with amiloride concentrations of 0, 0.1, 0.2, 0.3, and 10 μM in the mucosal solution with Na concentration of 115, 86, 57.5 and 29 mM. Ionic strength was maintained constant with choline Cl. Values of K_I^{amil} were obtained as in Fig. 6, and plotted *vs.* the mucosal Na concentration. The straight line was obtained by linear regression, and gave values of 0.059 μM for the apparent K_I^{amil} (in the absence of Na) and 59 mM for the apparent K_I^{Na}. Data are presented as mean ± SEM for six experiments

(Frazier, Dempsey & Leaf, 1962). Assuming that this relationship holds, a value of K_m can be estimated from measurements of I_{Na} at 115 and 29 mM Na. In 7 experiments, the mean value was 32 ± 3 mM. This is somewhat higher than that reported previously (17 mM activity, Li et al., 1982) using a lower concentration range. It is nonetheless significantly lower than that obtained in the same tissues from the decrease in the apparent affinity of amiloride.

Inhibition of I_{Na} by Low pH

Figure 9 shows the inhibition of I_{Na} as mucosal pH was reduced. Figure 9*A* is a retracing of a record of short-circuit current as the mucosal pH was changed rapidly in steps from 6.0 to 3.2 and back. The effects of these brief exposures to low pH were usually reversible. The fractional inhibition of I_{Na} relative to the value at pH 6.0 is shown in Fig. 9*B*. These data could be fitted only roughly with a simple titration curve. The two curves drawn in Fig. 7*B* correspond to titrations of sites with pK_a's of 3.2 and 3.0. The latter was obtained by linear regression using all of the data points. The point at pH 3.2 was heavily weighted in this regression. The other curve was obtained by omitting the point at pH 3.2 from the regression analysis.

Unlike K and amiloride, protons appear to be quite permeant through the amiloride-sensitive Na

channel (Palmer, 1982*b*). It was therefore of interest to compare the pH dependence of H transport to that of H block of Na transport. Bladders were equilibrated with mucosal solutions in which all the Na had been replaced by K, and which were buffered to different pH values. Short-circuit current was continuously monitored. The mucosal solution was then exchanged for one with identical composition except for the addition of 10^{-5} M amiloride. Figure 10*A* shows the response to amiloride at various values of pH in a representative experiment. At pH 5 to 6, the responses were usually small and often positive, i.e., in a direction opposite to the normal effect of amiloride on Na current. These positive responses, whose origins are unclear, were also observed previously (Palmer, 1982*b*). Under the assumption that these current changes are unrelated to proton movements, they are subtracted from those at lower pH to calculate the amiloride-inhibitable proton current. At lower pH, the response was always negative, consistent with the inhibition of amiloride of a proton current from mucosa to cell. Figure 10*B* summarizes the mean changes in current after amiloride application as a function of mucosal pH. The data can be reasonably well described by a hyperbolic saturation curve of the form described in Eq. (6) with a K_m for protons of 0.13 mM.

Further evidence that protons and Na may be using the same amiloride-blockable channel is presented in Fig. 10*C*, in which the value of I_H, obtained as described above, is plotted against a value of I_{Na} from the same bladder. The solid line was obtained with linear regression, and was forced to pass through the origin. The regression coefficient is 0.93, indicating a significant correlation between I_H and I_{Na}. The slope of the regression line is 0.012. Assuming intracellular Na activity of 10 mM, intracellular pH 7.0, and estimating the mucosal Na activity to be 86 mM, the permeability ratio for H and Na can be calculated from:

$$\frac{I_H}{I_{Na}} = \frac{P_H}{P_{Na}} \cdot \frac{(H)_m - (H)_{cell}}{(Na)_m - (Na)_{cell}}$$

to be about 5. This is in reasonable agreement with the value of 6 reported previously (Palmer, 1982*b*).

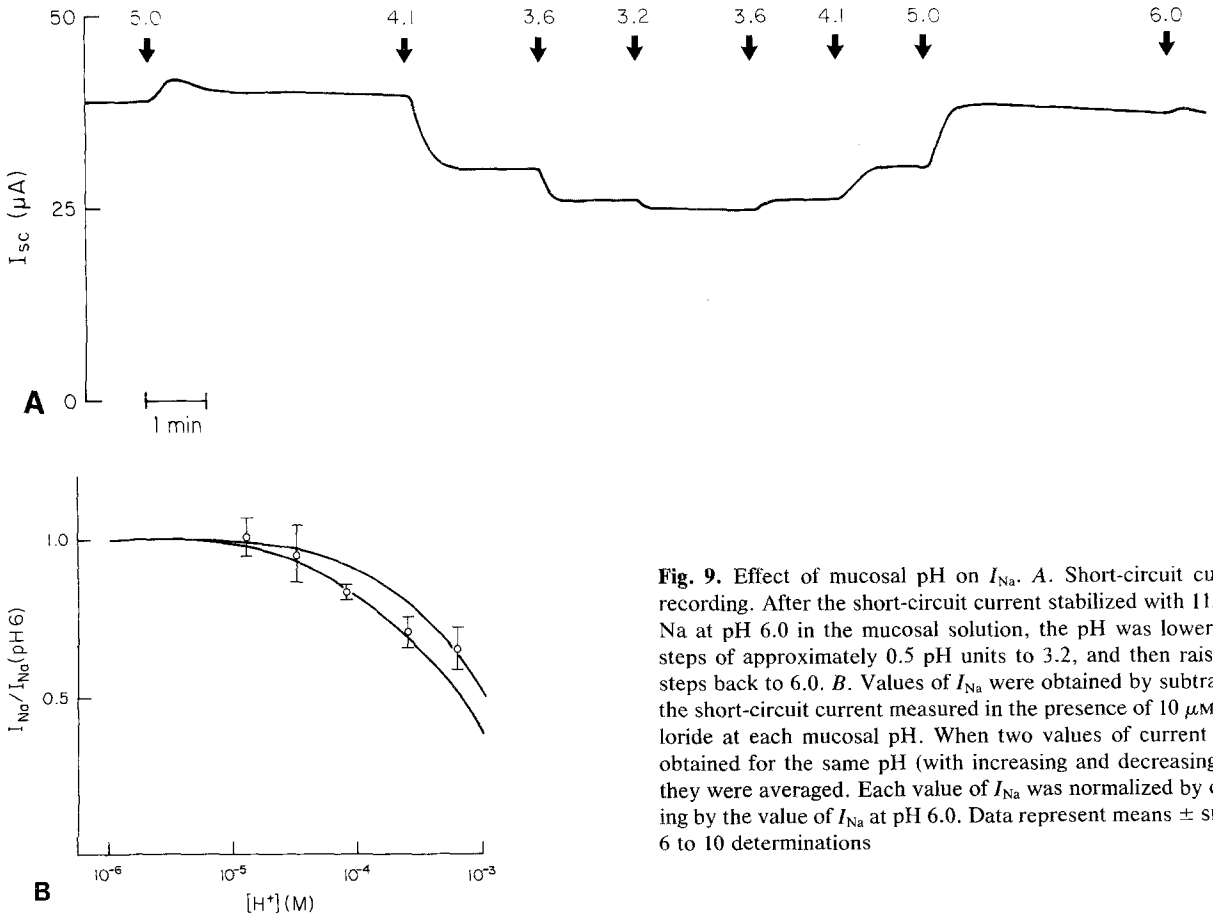

Fig. 9. Effect of mucosal pH on I_{Na}. *A*. Short-circuit current recording. After the short-circuit current stabilized with 115 mM Na at pH 6.0 in the mucosal solution, the pH was lowered in steps of approximately 0.5 pH units to 3.2, and then raised in steps back to 6.0. *B*. Values of I_{Na} were obtained by subtracting the short-circuit current measured in the presence of 10 μM amiloride at each mucosal pH. When two values of current were obtained for the same pH (with increasing and decreasing pH) they were averaged. Each value of I_{Na} was normalized by dividing by the value of I_{Na} at pH 6.0. Data represent means ± SEM of 6 to 10 determinations

The voltage dependence of the proton block of I_{Na} is shown in Figs. 11 and 12. As was the case for K, the linearized fractional inhibition plot is nonlinear, and showed a significant positive slope only at large positive voltages (Fig. 12). The mean value of δ_+ obtained by fitting the data between +100 and +200 mV to a straight line is 0.059, substantially lower than that for the other blockers (Table 2). A fit using the two-site model with $\delta_1 = 0$ and $\delta_2 = 0.20$ is shown in Fig. 12.

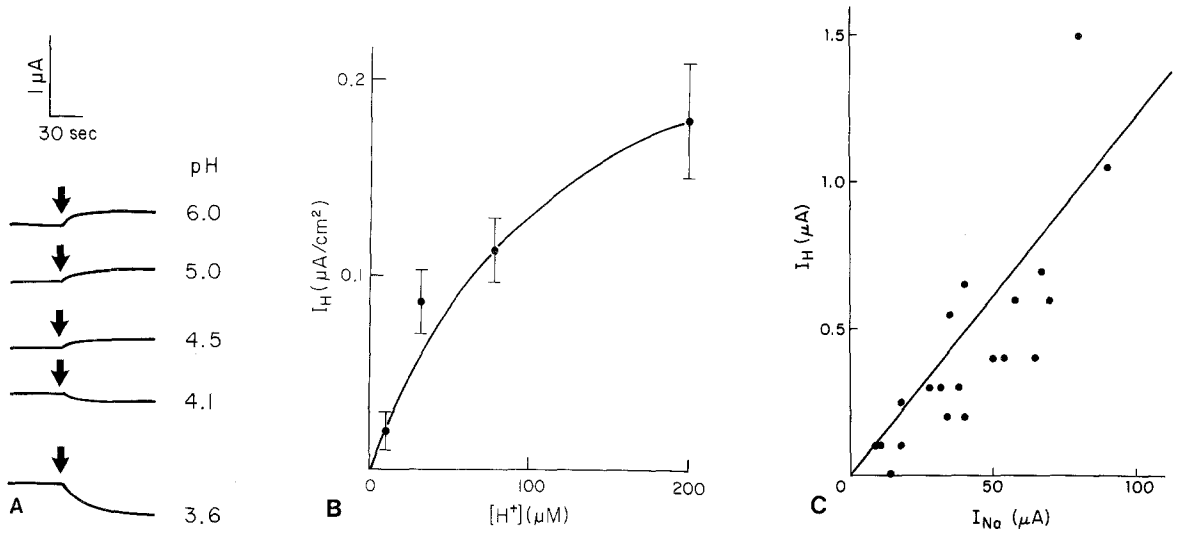

Fig. 10. Amiloride-sensitive H currents. *A*. Changes in short-circuit current after addition of 10 μM amiloride at various mucosal pH values in the absence of Na. At each pH, short-circuit current was allowed to reach a steady level. The mucosal solution was then exchanged for one containing amiloride. The absolute values of short-circuit current before the addition of amiloride were: pH 6.0: −0.8 μA; pH 5.0: −0.1 μA; pH 4.5; +0.4 μA; pH 4.1: +2.4 μA; pH 3.6: + 2.8 μA. *B*. Values of amiloride-sensitive H current (I_H) plotted as a function of mucosal pH. I_H was calculated as the difference observed after addition of amiloride, minus the current difference observed at pH 6.0. Data are given as means ± SEM for 14 experiments. The solid line is a linear least-squares fit to the data, assuming a relationship of the form in Eq. (6). Parameters derived from this analysis were $I_{max} = 0.29$ μA/cm² and $K_M = 124$ μM. *C*. Correlation of I_H and I_{Na}. I_H values were obtained as in part *B* at pH 3.6. Value of I_{Na} were obtained as the amiloride sensitive short-circuit current in the presence of 115 mM mucosal Na. The solid line represents a linear regression analysis of the data, constrained to pass through the origin. The slope of the regression line $I_H/I_{Na} = 0.012$. The correlation coefficient is 0.93

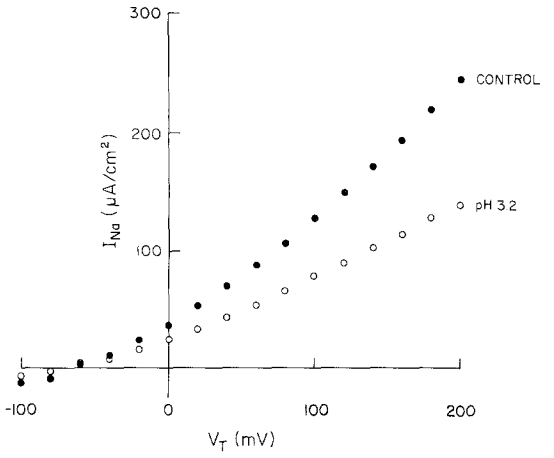

Fig. 11. Effect of reduced mucosal pH on I_{Na}-V relationship. Mucosal Na concentration was 115 mM. *I-V* relationships were measured at pH 6.0 and pH 3.2, and then at the same pH values in the presence of 10 μM amiloride. I_{Na}-V curves were constructed for pH 6.0 (●) and 3.2 (○)

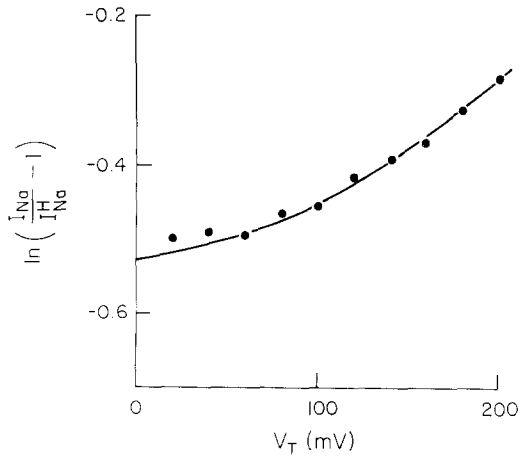

Fig. 12. Voltage dependence of proton block. Data from Fig. 9 are plotted as in Figs. 2, 4 and 5. The line drawn through the data points was obtained from Eq. (5) with $K_1 = 1.16$ μM, $K_{12} = 13$, $\delta_1 = 0$ and $\delta_2 = 0.2$

Variation in Blocking Ion Affinities

One puzzling aspect of these studies was the variation from tissue to tissue in the apparent K_I for amiloride, which, measured under identical conditions, ranged from 0.21 to 0.42 μM, while the apparent K_I for K ranged from 116 to 369 mM. This variability cannot be accounted for by experimental errors.

One source of variation might be differences in the intracellular milieux. In support of this idea, both ADH, which increases intracellular cAMP, and ouabain, which increases intracellular Na, altered K_I^{amil} when added to the serosal bath. Amiloride dose-response curves, similar to those shown in Fig. 6, were obtained before and during stimulation with 20 mU/ml ADH, and before and during inhibition by 0.5 mM ouabain. As shown in Table 2, both of these maneuvers significantly increased K_I^{amil}. While these tests are obviously far from exhaustive, they do indicate that interactions of blocking ions with the channel can be affected by intracellular events.

Discussion

Shape of the I_{Na}-V Relationship

The CFE has been used to describe the I_{Na}-V relationship of the apical membranes of a number of tight epithelia including frog skin, toad urinary bladder, rabbit descending colon and *Necturus* urinary bladder (Fuchs et al., 1977; Palmer et al., 1980; Thompson, Suzuki & Schultz, 1982; Thomas et al., 1983). In the present study, a systematic deviation from the CFE was found at positive voltages, the measured values of I_{Na} being smaller than those predicted from the CFE.

It should be stressed that there is no *a priori* reason why the I_{Na}-V relationship should follow the CFE. Ion diffusion through narrow biological channels is likely to involve significant interactions among ions and between ions and the pore itself, violating the assumptions of independent ion diffusion used to derive the CFE. On the other hand, Henrich and Lindemann (1983) have recently found a decrease in the apparent number of conducting channels at positive voltages using fluctuation analysis. This phenomenon would reduce I_{Na} at positive voltages, even if the single-channel I-V relationship were to obey the CFE. Finally, the existence of a finite series resistance imposed by the basal-lateral membrane would tend to reduce the slope of the measured I_{Na}-V curve.

The main conclusion of this work is that a number of univalent cations block Na conduction through the apical Na channel in a voltage-dependent manner. This voltage dependence is similar for amiloride, which blocks Na transport at micromolar concentrations, and for alkali cations K, Rb and Cs which block at concentrations of hundreds of millimolar. The direction of the voltage dependence is consistent with the idea that these ions bind to sites within the channel itself, and that these sites sense a portion of the transmembrane electrical potential.

The estimate of 12% for the fraction of the electrical potential sensed by the amiloride binding site refers, strictly speaking, to the transepithelial potential difference. As this potential is increased, some part of the voltage change must occur across the basal-lateral membrane in series with the apical Na channels. Thus 12% may be an underestimate of the fraction of the apical membrane voltage sensed at the site. Under the conditions used, the apical/basal-lateral resistance ratio at short circuit is probably large (Palmer et al., 1980; Warncke & Lindemann, 1981; Palmer & Lorenzen, 1983). Whether this ratio is different at large positive voltages such as those used in this study is not known. A decrease in the resistance ratio in this range would also cause δ to be underestimated.

The voltage-dependence of the amiloride block could also be affected by the competitive interaction between Na and amiloride, providing that Na binding is also voltage-dependent. Assuming that Na and amiloride bind to the same site within the lumen of the pore, at which a fraction of the membrane voltage is sensed, and that Na can subsequently be translocated through the pore, model calculations show that values of δ calculated from Eq. (3) can either underestimate or overestimate the true value of δ. The magnitude and direction of the discrepancy depends upon the rate constants for Na entering and leaving the site, and on their voltage-dependence. Available information is insufficient to predict these effects. However, they are probably fairly minor under the conditions used, as changing the mucosal Na concentration from 29 to 115 mM had little influence on the voltage-dependence of the amiloride block (*compare* Figs. 2 and 5).

In a number of studies of channels from excitable tissues, voltage-dependent ionic block has been interpreted as indicating that the blocking ions interact with the channel at sites within the lumen of the pore (Woodhull, 1973; Hille, 1975; Eaton & Brodwick, 1980; French & Shoukimas, 1981; Latorre & Miller, 1983). This is an attractive explanation for the data presented in this paper. It is consistent with the conclusion of Cuthbert (1976) that the guanidinium moiety of the amiloride molecule inter-

acts with negatively charged sites within the membrane, perhaps acting as a molecular plug. Thus the positive charge of the guanidinium may penetrate the channel far enough to cross a portion of the transmembrane electric field.

A number of questions arise from this interpretation of the data, which cannot be fully answered at this time, and are discussed below:

1. Is a one-site model adequate to describe block by various cations?

The obvious nonlinearity of the plots in Figs. 5, 6 and 12 indicates that the simple model of one blocking site within the membrane electric field, and the predicted voltage-dependence of block described in Eq. (3), do not adequately describe the data obtained with K, Rb, Cs and H. A more complex model which does account for these findings is one in which the blocking ions can bind to two or more sites within the channel. The second site is assumed to be in series with the first, and binding to one site is assumed to exclude binding to the other. The predicted voltage-dependence of block in such a system is given in Eq. (5). The concave upward shapes of the curves in Figs. 5, 6 and 12 can be explained if one site senses a relatively small fraction of the field and has a relatively high affinity for the ion. The curve in Fig. 5 drawn through the data for K block was generated by assuming that the two sites sense 0 and 30% of the electric field, and that the affinity of the first site for the ion is seven times that of the second. Other fits are shown in Figs. 6 and 12.

At very high voltages, only the inner site will be occupied, and the voltage-dependence will approach that of the simple one-site model. Thus the slope of the plots in the high voltage range (100 to 200 mV) will estimate the voltage-dependence, and the location within the electric field of inner site. For K, Rb, Cs and Tl this is about 15% of the way through the field (Table 1). This will in general be an underestimate as the voltage range may be insufficient to achieve the limiting case. Other estimates of the farthest point of penetration of the ions can be made from the values of δ_2 obtained from applying Eq. (5) to the data. For K, Rb and Cs fits were achieved for $\delta_1 = 0$ and $\delta_2 = 0.30$ to 0.35. Thus a more precise, albeit more model dependent, estimate for the maximum point of penetration of these ions is 30 to 35% of the electric field.

The fits generated with the two-site model are not unique, but merely illustrate the plausibility of this type of explanation. For example, somewhat different parameters could be obtained by assuming that the first site sensed a small fraction of the electric field. Furthermore, a model with three sites, or a continuum of sites, seems equally likely. Until direct evidence for a two-site or other model is obtained, more elaborate treatments of the data will be inappropriate.

In the case of amiloride, the voltage-dependence of block is closer to that predicted from a one-site model. This might be expected from the much stronger and presumably more specific binding of amiloride to its blocking site. Nevertheless, small deviations from the one-site model, which are seen for example at low voltages in Fig. 5, could also be accounted for by a multiple site interaction with the channel.

2. Does Na interact with the blocking ion sites?

If cations such as amiloride and K bind to sites within the lumen of the channel, excluding, by steric and/or electrostatic interactions, the occupation of the channel by Na, it is likely that Na ions themselves interact with these sites on their way through the channel.

This interpretation leads to the prediction that the rate of Na transport through the channels should reach a limiting value as the mucosal Na concentration is increased, reflecting saturation of the cation binding site within the pore. Indeed, saturation of this transport system has been observed, with half-maximal rates of transport at around 20 mM mucosal Na (Frazier et al., 1962; Li et al., 1982). Using fluctuation analysis of Na transport in frog skin, however, Van Driessche and Lindemann (1978) observed that the transport rate through single open channels did not show saturation behavior at mucosal Na activities of up to 60 mM. Thus the saturation could be explained by a down regulation of the number of conducting channels as mucosal Na is increased.

An independent estimate of the affinity of Na for the external cation blocking sites can be made from measurements of Na-amiloride competition. The apparent dissociation constant for Na binding which is competitive with amiloride inhibition was about 100 mM, considerably higher than the Na concentration required for half-maximal Na transport. This implies that the amiloride binding site and the high affinity Na self-inhibition site are distinct. Benos, Mandel and Balaban (1979) reached a similar conclusion in studies of amphibian skin preparations.

In my current working hypothesis, amiloride binds relatively tightly to a specific site within the lumen of the Na channel, within the transmembrane electrical field but to the outside of the region of high Na selectivity. K also binds, although with much lower affinity, to sites near that for amiloride. Occupation of the lumen of the pore by K and amiloride are mutually exclusive. Na interacts with this part of the channel in much the same way that K

does, but the affinity for Na is about twice as high. Thus most of the very high selectivity for Na over K in the overall transport process occurs downstream from this site.

The selectivity sequence for binding to the outer site appears to be Na > Tl > K > Rb > Cs. The same sequence was reported by Benos et al. (1980) for reduction of tracer Na permeability across the short-circuited frog skin. The sequence corresponds to that expected of a high field strength anionic site (Eisenman, 1962).

3. Why is the block by protons less voltage-dependent than that made by amiloride or K?

The voltage dependence of proton block illustrated in Figs. 11 and 12 can be explained by the same sort of two-site model used to describe K block. The apparent value of δ, measured as the slope in Fig. 12 between 100 and 200 mV, is less for H block than for K block, implying either that the innermost binding site is not as far into the channel as that for K, or that the affinity of the outer site relative to the inner site is larger for K than for H.

The analysis of proton block is complicated, however, by at least two additional factors. First, protons appear to be conducted through the Na channel (Fig. 9). The voltage dependence of a permeable blocker can be reduced, as the membrane potential difference can promote both ion entry into and exit from the channel (Woodhull, 1973). Second, changes in mucosal pH could lead to changes in the charge density at the outer surface of the apical membrane, which can in turn alter the instantaneous current-voltage relationship (Frankenhauser, 1960; Lindemann, 1982).

If a single fixed negative charge within the pore controlled both Na and H movement through the channel, then the apparent pK_a for proton block of Na transport should be lower than that for proton translocation itself due to ion competition for the fixed site. The difference between these pK_a values (about 1 pH unit, Figs. 8 and 9) was larger than expected. The evaluation of the discrepancy is difficult, however, due to the uncertainties involved in measuring the proton currents quantitatively and assigning a K_m value for proton transport from the data in Fig. 9.

4. What accounts for the variation in blocking ion affinity?

Variations in the apparent K_I for the blocking ions were observed both from tissue to tissue, and in a given tissue upon perturbation with ADH or with ouabain (Table 2). An increase in the apparent K_I for amiloride during ADH stimulation was also reported by Cuthbert and Shum (1975). Li et al. (1982), using much larger amiloride concentrations, found a smaller and statistically insignificant increase in K_I.

The change in amiloride affinity with ADH could reflect the recruitment of additional Na channels with different binding properties. Alternatively, it might result from any number of possible changes in the intracellular milieu. One interesting possibility is that the effect might be mediated by an increase in intracellular Na activity which was demonstrated previously under similar conditions (Li et al., 1982). This might explain why no significant changes in the microscopic rate constants were observed after ADH stimulation (Li et al., 1982), as those measurements were made using high amiloride concentrations. The resulting reduction in Na permeability might minimize the increase in intracellular Na. The increase in K_I^{amil} observed after ouabain treatment is also consistent with an effect of intracellular Na on amiloride binding.

Such an effect could be easily incorporated into a model of the epithelial Na channel proposed recently by Edmonds (1982). Here a binding site for Na, or other cations, near the inner mouth of the pore, is invoked to account for decreased Na permeability when intracellular Na is increased (Erlij & Smith, 1973; Cuthbert & Shum, 1977; Turnheim, Frizzell & Schultz, 1978). When such a site is occupied, external ions (Na itself or a blocking ion) would be excluded electrostatically from the channel. As pointed out by Edmonds (1982), such a multisite pore which can be occupied by at most one ion is consistent with measurements of flux ratio exponents near 1 for this transport system (Palmer, 1982*a*).

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