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# Interactions of Amiloride and Other Blocking Cations with the Apical Na Channel in the Toad Urinary Bladder

Lawrence G. Palmer
Department of Physiology, Cornell University Medical College, New York, New York 10021

**Summary.** A simple model of the action of amiloride to block apical Na channels in the toad urinary bladder was tested. According to the model, the positively charged form of the drug binds to a site in the lumen of the channel within the electric field of the membrane. In agreement with the predictions of the model: (1) The voltage dependence of amiloride block was consistent with the assumption of a single amiloride binding site, at which about 15% of the transmembrane voltage is sensed, over a voltage range of ±160 mV. (2) The time course of the development of voltage dependence was consistent with that predicted from the rate constants for amiloride binding previously determined. (3) The ability of organic cations to mimic the action of amiloride showed a size dependence implying a restriction of access to the binding site, with an effective diameter of about 5 angstroms. In a fourth test, divalent cations (Ca, Mg, Ba and Sr) were found to block Na channels with a complex voltage dependence, suggesting that these ions interact with two or more sites, at least one of which may be within the lumen of the pore.

**Key Words** amiloride · Na channels · epithelial Na transport · voltage-dependent block

## Introduction

Amiloride is a K-sparing diuretic that acts by blocking Na reabsorption through apical membrane channels in tight epithelia (Benos, 1982). In a previous study, I presented evidence that amiloride block of Na transport by the toad urinary bladder is voltage dependent (Palmer, 1984). One interpretation of this finding is that amiloride binds to a site within the transport pathway for Na through the apical channels, which senses 10 to 15% of the transmembrane electric field. The experiments reported in this paper were designed to test this simple model, which is able to account satisfactorily for most of the results. In addition, the question of which ions could block Na channels in a voltage-dependent manner was addressed, and the implications for the possible geometry of the outer mouth of the channel is discussed.

## Materials and Methods

Toads (*Bufo marinus*, female, Dominican origin) were obtained from National Reagents (Bridgeport, Connecticut). 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 as described previously (Palmer, 1982*a*). Current-voltage relationships were obtained by applying ramps to the command port of the voltage clamp, as described previously (Palmer, 1984).

### 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).

To examine the effects of monovalent blocking cations, the Na concentration in the mucosal bath was reduced to $\frac{1}{4}$ that of the standard solution by replacing 86 mM NaCl with N-methyl-D-glucamine (NMDG) Cl. NMDG was assumed not to block the Na channels. NMDG Cl was then replaced with the Cl salts of various cations, and the fractional decrease in the amiloride-blockable current measured as a function of voltage. In some cases (amino guanidinium, hydroxyl guanidinium, methyl guanidinium) the sulfate salt was used. Here the reference solution contained $(NMDG)_2SO_4$ instead of NMDG Cl so that the anion composition of the solution remained constant. When the effects of divalent cations were tested, 86 mM NMDG Cl was replaced with 29 mM $CaCl_2$, $MgCl_2$, $BaCl_2$ or $SrCl_2$ to maintain constant ionic strength and thus equal Na activities. Since we have not found a "control" divalent cation which does not appear to affect the channel the monovalent cation NMDG has been used as a control in this series of experiments. Thus to preserve constant ionic strength, both the osmolarity and the Cl activity of the test solutions had to be reduced. We have assumed that these reductions do not affect the channels. If this assumption is incorrect, the results obtained in Fig. 6 and Table 2 would be altered.

In some experiments, epithelial cell Na was increased by incubation with 5 mM ouabain in the serosal solution to abolish the electrochemical activity gradient across the apical membrane, as previously described (Palmer, 1982*b*; Palmer, 1985).

## Current Transients

To measure current transients during step changes in transepithelial voltage, fixed voltage pulses were applied to the command of the voltage clamp using a stimulator driven by a laboratory computer. The clamp current was digitized and sampled at 50 to 100 Hz with the computer.

## Analysis of Data

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

To measure the currents passing through the apical Na channels, the transepithelial current in the presence of 10 $\mu$M amiloride was subtracted from the total transepithelial current for each condition. The amiloride-sensitive current was assumed to equal the Na channel-mediated current.

The voltage dependence of block by amiloride and other organic cations was analyzed according to a one-site binding model in which a fraction of the transmembrane voltage is sensed at the binding site (Woodhull, 1973; Latorre & Miller, 1983; Palmer, 1984). Thus:

$$I^B_{Na}(V) = \frac{I_{Na}(V)}{1 + \dfrac{B}{K^B_I \exp\left(-\dfrac{ZF}{RT}\delta V\right)}} \tag{1}$$

where $I_{Na}$ and $I^B_{Na}$ are the currents in the absence and presence of blocker $B$, and $K^B_I$ is the inhibition constant at zero voltage. Rearrangement gives:

$$\ln\left[\frac{I_{Na}(V)}{I^B_{Na}(V)} - 1\right] = \ln\left[\frac{(B)}{K^B_I}\right] + \frac{ZF}{RT}\cdot\delta\cdot V. \tag{2}$$

Thus $K^B_I$ and $\delta$ can be estimated from the intercept and slope of a plot of the expression on the left *vs.* $V$.

The voltage dependence of block by divalent cations was analyzed according to a more complex model with two binding sites in series having different dissociation constants and which sense different fractions of the membrane voltage (*see* Palmer, 1984).

The basic equation describing the voltage dependence of block in this model system is:

$$I^B_{Na}(V) = \frac{I_{Na}}{1 + \dfrac{(B)}{K^{eff,B}_I}} \tag{3}$$

where

$$K^{eff,B}_I = \frac{K^B_I \exp\left(-\dfrac{ZF}{RT}\delta_1 V\right)\cdot K^B_{12}\exp\left(-\dfrac{ZF}{RT}\delta_{12}V\right)}{1 + K^B_{12}\exp\left(-\dfrac{ZF}{RT}\delta_{12}V\right)}. \tag{4}$$

$K^B_I$ and $K^B_{12}$ are the dissociation constants for sites $S_1$ and $S_2$, and $\delta_{12} = \delta_2 - \delta_1$ where $\delta_1$ and $\delta_2$ are the fractions of $V$ sensed at $S_1$ and $S_2$, respectively. It is assumed that occupancy of the two sites by the blocking ion is mutually exclusive.

The analysis of current transients in the presence of submaximal doses of amiloride is based on the assumption that amiloride binding is described by the two-state model:

$$A + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AR$$

where $R$ and $AR$ represent open and amiloride-blocked channels, respectively. Changing the voltage will perturb the equilibrium since the binding constant, and therefore the individual rate constants, are voltage dependent. The net change in the number of open channels with time is given by:

$$d(R)/dt = k'_{off}(AR) - k'_{on}(A)(R)$$

where $k'_{off}$ and $k'_{on}$ represent the rate constants at the new voltage. Substituting $(AR) = (R_T) - (R)$, where $(R_T)$ is the total number of blocked and unblocked channels gives:

$$d(R)/dt = k'_{off}(R_T) - k'_{off}(R) - k'_{on}(A)(R)$$
$$= k'_{off}(R_T) - [k'_{off} + k'_{on}(A)](R).$$

The solution of this simple differential equation is:

$$R(t) = R(\infty) + [R(0) - R(\infty)]\exp(-rt)$$

where $R(0)$ and $R(\infty)$ are the number of open channels just after the voltage pulse and in the steady state, respectively, and $r$ is the chemical relaxation rate and is given by:

$$r = k'_{off} + k'_{on}(A). \tag{5}$$

Since the current is assumed to be proportional to the number of open channels, $I^A_{Na}$ can be substituted for $R$ in Eq. (5):

$$I^A_{Na}(t) = I^A_{Na}(\infty) + [I^A_{Na}(0) - I^A_{Na}(\infty)]\exp(-rt). \tag{6}$$

Thus the current in response to a voltage step in the presence of a submaximal dose of amiloride should follow an exponential time course with a time constant of $1/r$.

The amplitude of the current transient, which is the difference between the initial and steady-state currents, will be given by (*see* Eq. 1):

$$I^A_{Na}(0) - I^A_{Na}(\infty) = I_{Na}(V')/(1 + A/K^A_I(V))$$
$$- I_{Na}(V')/(1 + A/K^A_I(V')) \tag{7}$$

where $V$ and $V'$ refer to the voltages before and after the voltage change. The term $1/(1 + A/K^A_I(V))$ represents the fraction of channels blocked before and, by inference, immediately after the voltage change, while $1/(1 + A/K^A_I(V'))$ represents the fraction blocked in the steady state at the new voltage $V'$.

Since all the other parameters in Eq. (7) are directly measurable, this equation can be used to estimate $K^A_I(V') = K_I(0)\exp(-FV'/RT)$ for voltage jumps from zero to any voltage $V'$, and therefore to obtain an independent estimate of $\delta$.

This analysis assumes that the time-dependent changes in the current in the presence of amiloride are due to changes in amiloride binding only, and that the leak current and the total number of channels do not change over the course of the current transient. In fact, the current in the presence of a maximal dose of amiloride was constant after the decay of the capacitance

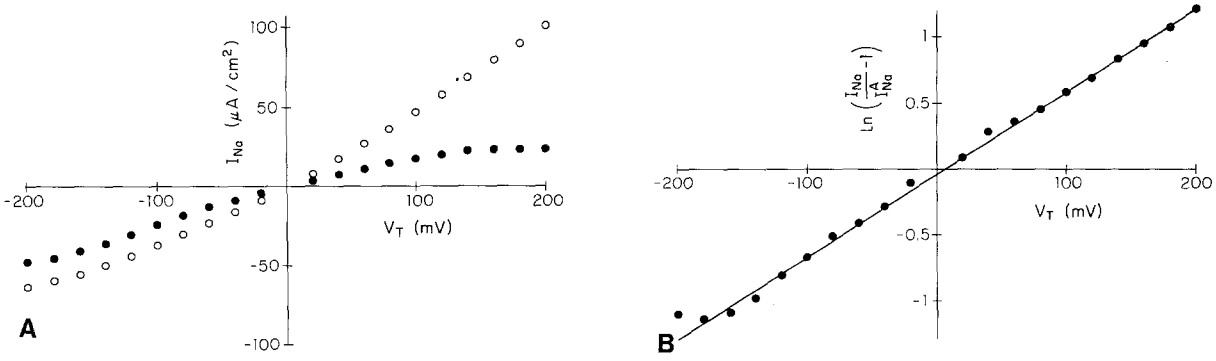


**Fig. 1.** Voltage dependence of amiloride block. A hemibladder was preincubated in the presence of ouabain (5 mM) in the serosal solution, and high Na (115 mM) in the mucosal solution. The mucosal Na concentration was then rapidly reduced to 20 mM, causing the short-circuit current to fall to approximately zero. Current-voltage relationships were then measured with 0, 0.1 and 10 $\mu$M amiloride in the mucosal solution. Currents measured with 10 $\mu$M amiloride were subtracted from the other sets of current values at each voltage. The corrected currents, representing the amiloride-sensitive *I-V* relationship, are shown for no amiloride (open circles) and 0.1 $\mu$M amiloride (filled circles). In *B*, the fractional inhibition of current by 0.1 $\mu$M amiloride is plotted as a function of voltage as in Eq. (2). The straight line represents a least-squares fit using Eq. (2) with parameters $K_I^A = 0.104$ $\mu$M and $\delta = 0.162$

transient. The total current in the absence of amiloride, which presumably reflects the total number of channels, tended to increase somewhat with time at the highest voltages (*see* Fig. 2). In some experiments a correction factor was applied to the amiloride transients to account for this change. Since the corrected values of the rate constants were not significantly different from the uncorrected ones, this procedure was not used in the analyses presented here.

## Results

### Voltage Dependence at Negative and Positive Voltages

The voltage dependence of amiloride block was previously examined at voltages between zero and 200 mV mucosa positive. Over this range the apparent binding constant decreases as the voltage increases. The simple model proposed to account for the voltage dependence predicts that amiloride block should become weaker at negative voltages. This could not be tested, as small changes in the reversal potential complicated the analysis.

Using a protocol previously designed to increase epithelial cell Na and abolish the transapical driving force for Na (Palmer, 1982*b*), amiloride block of outward Na currents at negative voltages could also be tested. $I_{Na}$-*V* curves under these conditions were described previously (Palmer, 1985).

Figure 1 shows *I-V* relationships for a single Na-loaded hemibladder under control conditions and with 0.1 $\mu$M amiloride in the mucosal solution. When analyzed according to Eq. (1), the apparent $K_I$ for amiloride is a monotonic function of voltage over the entire voltage range. The slope of the plot in Fig. 1*B* is proportional to $\delta$, the fraction of the transmembrane voltage sensed at the amiloride binding site, and in constant from +200 to −160 mV. At more negative voltages small systemic deviations from linearity were seen in plots like Fig. 1*B*, which may reflect a more complex set of events at these large, hyperpolarizing potentials.

In a series of six hemibladders, the mean value of $\delta$ determined between 0 and +200 mV was 0.15 ± 0.01, and that determined between 0 and −160 mV was also 0.15 ± 0.01. This value is close to that of 0.12 to 0.13 determined previously in non-Na-loaded tissues (Palmer, 1984).

### Relaxation Curves for Amiloride Block

Another prediction of the hypothesis that amiloride binds within the lumen of the channel is that the increase in blocking efficiency at high voltages should be expressed with a time course determined by the chemical rate for amiloride block (*see* Analysis of Data).

Figure 2*A* shows the results of an experiment in which the voltage was changed abruptly from −40 to +200 mV in the presence of several concentrations of amiloride. The capacitative transients, which do not show up in these traces, are very brief in this time frame. This is because the time constants for these transients are given approximately by the product RC of the series solution resistance (<40 ohms) and the effective capacitance of the tissue (3 to 6 $\mu$F) and are consequently shorter than 1 msec. In the absence of amiloride, the currents were either constant, or increased slightly over a period of 1 sec. In the presence of 10 $\mu$M amiloride, a maximal dose, currents are much smaller, and are

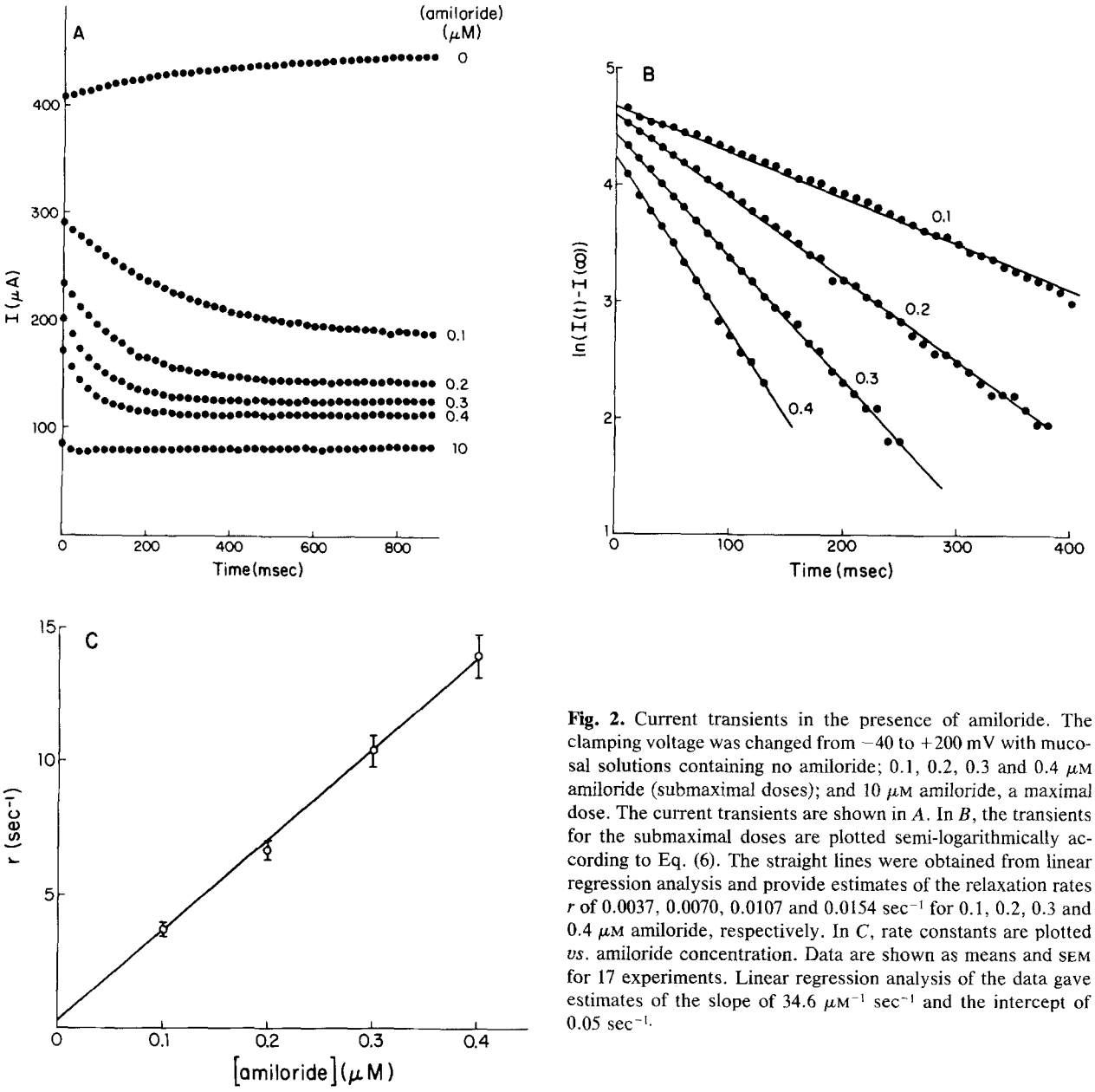


**Fig. 2.** Current transients in the presence of amiloride. The clamping voltage was changed from −40 to +200 mV with mucosal solutions containing no amiloride; 0.1, 0.2, 0.3 and 0.4 μM amiloride (submaximal doses); and 10 μM amiloride, a maximal dose. The current transients are shown in *A*. In *B*, the transients for the submaximal doses are plotted semi-logarithmically according to Eq. (6). The straight lines were obtained from linear regression analysis and provide estimates of the relaxation rates *r* of 0.0037, 0.0070, 0.0107 and 0.0154 $sec^{-1}$ for 0.1, 0.2, 0.3 and 0.4 μM amiloride, respectively. In *C*, rate constants are plotted *vs.* amiloride concentration. Data are shown as means and SEM for 17 experiments. Linear regression analysis of the data gave estimates of the slope of 34.6 $\mu M^{-1}$ $sec^{-1}$ and the intercept of 0.05 $sec^{-1}$.

also constant with time. With a submaximal concentration of amiloride, the current reaches a peak just after the increase in voltage and then declines exponentially to a new steady-state level. This relaxation apparently results from the increased proportion of amiloride-blocked channels at positive voltages.

Relaxation rates were estimated with semi-log plots of current *vs.* time (*see* Eq. 6). The chemical rates (*r*) for amiloride binding are given by the slopes of these plots (Eq. 5). Figure 2*B* shows the fits of the relaxation data for four submaximal doses of amiloride. Most of the relaxation curve could be described by a single exponential whose time constant decreased with increasing amiloride concentration.

In Fig. 2*C*, the rate of the exponential relaxation is plotted *vs.* the amiloride concentration. The slope of this plot gives the on-rate constant $k_{on}$ and the intercept the off-rate constant $k_{off}$ (*see* Eq. 5). Qualitatively, these data are consistent with the hypothesis that the voltage-dependent block by amiloride is associated with a voltage-dependent binding of the drug to the channel.

To assess the hypothesis more quantitatively, the amplitudes of the current transients were used

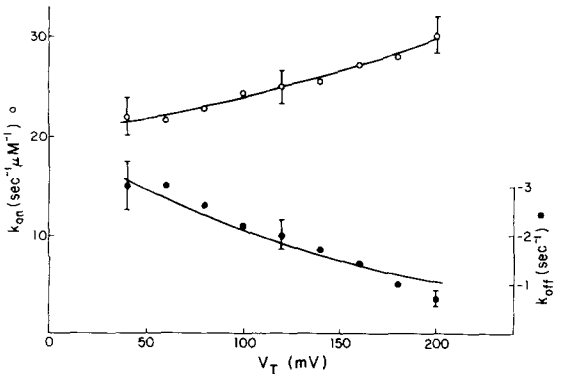


**Fig. 3.** Voltage dependence of the amiloride rate constants. The on-rate constant $k_{on}$ and the off-rate constant $k_{off}$ were determined from the slope and intercept of the rate-concentration plots at each voltage. The data represent the mean and SEM for 12 experiments

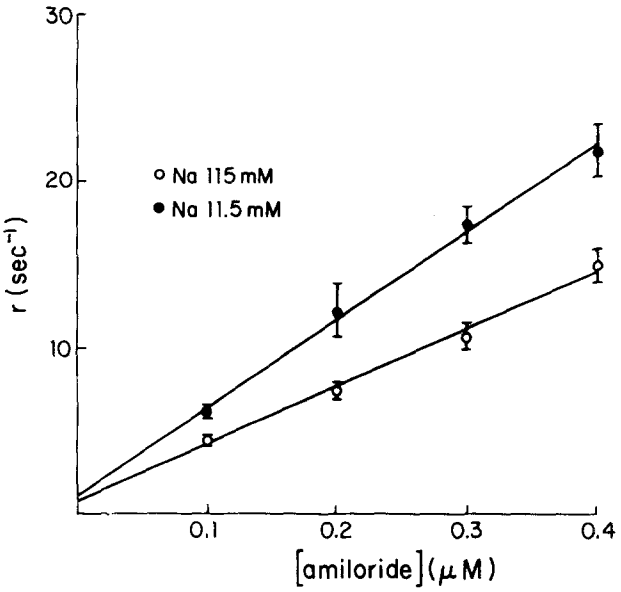


**Fig. 4.** Dependence of amiloride rate constants on mucosal Na. The relaxation rates of the current transients in the presence of different amiloride concentrations were determined with 115 and 11.5 mM Na in the mucosal solution. The data represent means and SEM for six experiments. Linear regression analysis gave slopes of 35.1 $\mu$M$^{-1}$ sec$^{-1}$ (high Na) and 52.7 $\mu$M$^{-1}$ sec$^{-1}$ (low Na) and intercepts of 0.6 sec$^{-1}$ (high Na) and 1.3 sec$^{-1}$ (low Na). The voltage was 120 mV

to compute the change in the fraction of amiloride-blocked channels. This was then used to compute a value of $\delta$ using Eq. (7). In a series of 10 experiments, the mean value was $\delta = 0.15 \pm 0.01$ for steps of 100 to 200 mV. There was no obvious dependence of $\delta$ on voltage. For the same tissues, $\delta$ was also computed from the steady-state current values at each voltage using Eq. (2). This value was $\delta = 0.15 \pm 0.01$. The close agreement in the two estimates of $\delta$ implies that the analysis is self-consistent and that the entire voltage dependence of amiloride block is accounted for by the current transients as shown in Fig. 2.

The voltage dependence of the amiloride rate constants, computed from rate *vs.* concentration plots, is shown in Fig. 3. As $V$ increases, $k_{on}$ increases while $k_{off}$ decreases. At the lowest voltage (40 mV) at which the rate constants could be measured, $k_{on}$ was 22 $\mu$M$^{-1}$ sec$^{-1}$ and $k_{off}$ was 3 sec$^{-1}$. These values are in good agreement with those obtained under short-circuit conditions using fluctuation analysis: $k_{on} = 17$ $\mu$M$^{-1}$ sec$^{-1}$ and $k_{off} = 2$ to 3 sec$^{-1}$ (Li et al., 1982).

The data in Fig. 3 were fitted by equations of the form:

$$k_{on} = k_{on}(0) \exp(\delta_{on} FV/RT).$$

The values obtained from this fit were $k_{on}(0) = 19.7$ M$^{-1}$ sec$^{-1}$ and $\delta_{on} = 0.05$. Analysis of $k_{off}$ using an analogous equation:

$$k_{off} = k_{off}(0) \exp(-\delta_{off} FV/RT)$$

gave values of $k_{off}(0) = 4$ sec$^{-1}$ and $\delta_{off} = 0.17$. Thus the off-rate constant is somewhat more voltage dependent than the on-rate constant. A similar conclusion was reached by Warncke and Lindemann (1985) who have analyzed admittance spectra in the presence of amiloride.

Since at each voltage $K_I^A = k_{off}/k_{on}$, the sum of $\delta_{on} + \delta_{off}$ should equal $\delta$, which averaged 0.15. The source of this discrepancy may be in the determination of $\delta_{off}$ since the values of $k_{off}$ are not very reliable at the highest voltages where the intercepts of the rate-concentration plots are close to zero (Fig. 2*C*).

The dependence of the rate constants on mucosal Na was assessed by measuring transients at 115 and 11.5 mM mucosal Na. As shown in Fig. 4, lowering mucosal Na increased $k_{on}$ by about 50%, consistent with a competitive interaction between Na and amiloride. The values of $k_{off}$ were not significantly different.

## Voltage Dependence of Block by Organic Cations

A third prediction of the working model is that the binding site within the pore should discriminate among cations on the basis of their size. To test this idea, a number of "organic" or N-based cations were screened for the ability to block Na channels in a voltage-dependent manner.

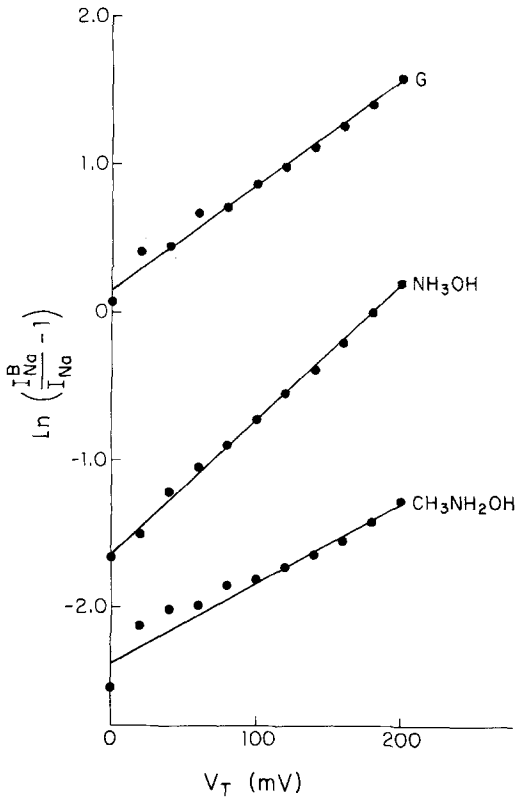


**Fig. 5.** Voltage dependence of block by guanidinium (G), hydroxylammonium ($NH_3OH$) and methylhydroxylammonium ($CH_3NH_2OH$). $I_{Na}$-$V$ curves were obtained with mucosal solutions containing 29 mM Na and 86 mM NMDG Cl, G Cl, $NH_3OH$ Cl or $CH_3NH_2OH$ Cl. The fractional inhibition of $I_{Na}$ by the three blocking cations is plotted *vs.* voltage according to Eq. (3). The straight lines are least-squares fits to the data points obtained by linear regression. The parameters derived from the fits are:

| | $K_I(0)$ (mM) | δ |
|---|---|---|
| G | 74 | 0.168 |
| $NH_3OH$ | 443 | 0.236 |
| $CH_3NH_2OH$ | 929 | 0.138 |

Data for three such ions—guanidinium, hydroxylammonium and methylhydroxylammonium—are shown in Fig. 5. As in Fig. 1, the slopes of the plots are proportional to the δ value for each ion, while the intercepts represent the inhibition constants at $V = 0$ ($K_I(0)$). While values of $K_I(0)$ were quite different for the three ions, δ values were similar, although not identical.

Mean values for those parameters are given for a number of cations in Table 1. Several points deserve mention. First, with one exception (methylammonium), all the δ values fell between 0.125 and 0.235. This implies that all of the blockers penetrate the electric field of the membrane and, by inference, the channels themselves, to a similar degree. Second, there is no obvious correlation between δ and $K_I(0)$. Third, all ions with a diameter less than 5 Å showed voltage-dependent block, whereas those larger than 5 Å did not. Thus, the cation blocking site acts as a molecular sieve.

**Table 1.** Voltage-dependent block by monovalent cations[a]

| Blocking ion | $K_I$ (mM) | δ | $n$ |
|---|---|---|---|
| $NH_4$ | 220 ± 40 | 0.14 ± 0.1 | 5 |
| $CH_3NH_3$ | 660 ± 110 | 0.085 ± 0.017 | 5 |
| $NH_3OH$ | 760 ± 180 | 0.235 ± 0.020 | 6 |
| $CH_3NH_2OH$ | 1800 ± 400 | 0.18 ± 0.01 | 5 |
| formamidinium | 530 ± 120 | 0.140 ± 0.004 | 6 |
| guanidinium | 88 ± 13 | 0.179 ± 0.006 | 10 |
| methyl guanidinium | 100 ± 16 | 0.163 ± 0.006 | 6 |
| dimethyl guanidinium | 456 ± 35 | 0.125 ± 0.005 | 4 |
| amino guanidinium | 145 ± 33 | 0.195 ± 0.007 | 6 |
| hydroxyl guanidinium | 148 | 0.205 | 2 |
| $(CH_3)_2NH_2$, $(CH_3)_3NH$, $(CH_3)_4N$, $(CH_3CH_2)_4N$, Choline, Tris, NMDG | Did not block. | | |

[a] Values of $K_I$ and δ were estimated from linear regression analysis as in Fig. 5. The concentration of the blocking ion was 86 mM, and the concentration of Na was 29 mM. Data represent mean ± SEM. $n$ = number of determinations.

## Voltage Dependence of Block by Divalent Cations

The working model predicts that divalent cation blockers should exhibit twice the voltage dependence of analogous monovalent blockers. To assess this prediction, the block of Na channels by the divalent cations Ca, Mg, Ba and Sr was studied. NMDG Cl was replaced by the Cl salts of divalent cations to maintain constant ionic strength.

All four divalent cations exhibited voltage-dependent block of Na currents. The situation was more complex, however, than for amiloride. There was little or no voltage dependence of block between 0 and +60 mV. At higher voltages dependence was quite steep.

Blocking affinity is plotted as a function of voltage for a representative experiment in Fig. 5. The solid lines represent fit to the series two-site model developed previously to account for block by K, Rb and Cs (Palmer, 1984) and described by Eqs. (3) and

(4). For simplicity, the outer binding site was assumed to be outside the membrane electric field. The best fits were obtained assuming the second site has a $\delta$ value of 0.25 to 0.30. Thus the divalent cations can apparently penetrate further into the field of the channel than can amiloride. A similar conclusion was reached for K, Rb and Cs (Palmer, 1984).

## Discussion

The voltage dependence of amiloride block at both positive and negative potentials, and the relaxation behavior of amiloride block during changes in voltage, are consistent with a simple model of the interaction of amiloride with the epithelial Na channel. In this model the positively charged amiloride molecule binds to a fixed negatively charged site within the lumen of the pore, acting as a plug to prevent ion conduction through the channel. The voltage dependence at both positive and negative voltages follows the prediction for interaction at a single site within the electric field of the membrane over a substantial voltage range. In addition, the kinetics of the current transients after step changes in voltage are the same as those found for amiloride block itself at steady state (Li et al., 1982). This is consistent with the idea that the voltage-dependent step is that of the binding of amiloride to the channel. A similar conclusion was drawn by Warncke and Lindemann (1985) who used admittance measurements to study amiloride-dependent relaxation effects.

The experiments with organic or N-based cations indicate that these molecules block Na transport with a voltage dependence that is similar to that of amiloride block, although with much lower affinities. The inference is that these ions bind at or near the amiloride binding site. Although this has not been proven, it seems reasonable that guanidinium, for example, would interact with the channel at the same site as the guanidinium moiety of amiloride.

If this inference is valid, the data in Table 1 indicate the specificity of this site. The major conclusion is that all molecules smaller than, or the same size as, guanidinium block the channel to some extent, while ions larger than guanidinium do not. This is consistent with the notion that the ions must enter a cylindrical pore of diameter about 6 Å, the largest diameter of guanidinium. Since guanidinium is planar, the cross-section of the pore could be smaller in one dimension. In addition, H bonding by the amino groups could reduce the effective size of the molecule, allowing for an even narrower pore size (Hille, 1975). This would explain the finding that dimethyl ammonium, whose size and shape are similar to that of guanidinium, but which cannot form H bonds, does not block the Na channel to a measurable extent.

The one notable exception to the size is amiloride itself, which is of course much larger than guanidinium. It is possible, however, that only the guanidinium moiety of amiloride penetrates the channel, with the pyrazine ring interacting at sites outside the narrow pore. Such a corking mechanism for block by amiloride, benzamil and triamterene was suggested on the basis of their molecular structures by Cuthbert (1976).

If the pyrazine ring of amiloride is excluded from the pore entirely, the guanidinium portion could penetrate the channel by only about 5 Å. This would imply that 15% of the voltage drop across the channel occurs over a 5-Å distance. This is not unreasonable; Miller (1982), studying the K channel from sarcoplasmic reticulum, concluded that 65% of the voltage drop occurred over a distance of 6 to 7 Å.

In general, guanidinium derivatives were more potent blockers of the Na channel than were the ammonium derivatives, although the voltage dependence of block was similar in the two groups of blockers. A single methyl group seems to reduce voltage dependence of block somewhat ($CH_3NH_3$ *vs.* $NH_4$; $CH_3NH_2OH$ *vs.* $NH_3OH$, $CH_3$-guanidinium *vs.* guanidinium) without substantial changes in binding affinity.

The divalent alkaline earth cations Ca, Mg, Ba and Sr also blocked the Na channel in a voltage-dependent manner, and the voltage dependence was steeper, at least in the high-voltage range, than that of the monovalent cations. This could be expected if both monovalent and divalent blockers interact with the same site within the electric field of the membrane. The situation is complicated, however, because of the complex nature of the voltage dependence of divalent cation block, as shown in Fig. 6. Qualitatively similar results were found previously for block by monovalent alkali metal cations, K, Rb and Cs (Palmer, 1984), suggesting that both classes of blocking ions interact with the channel at more than one site. If both sets of results are interpreted according to the two-site model of Eq. (3) and the simplifying assumption is made that the first site does not sense the electric field, then the second site must sense about 30% of the field both for the monovalent (Palmer, 1984) and the divalent (Table 2) blockers. This result rests on several rather arbitrary assumptions. It suggests, however, that monovalent and divalent blockers *could* be interacting with the channel at the same or similar sites.

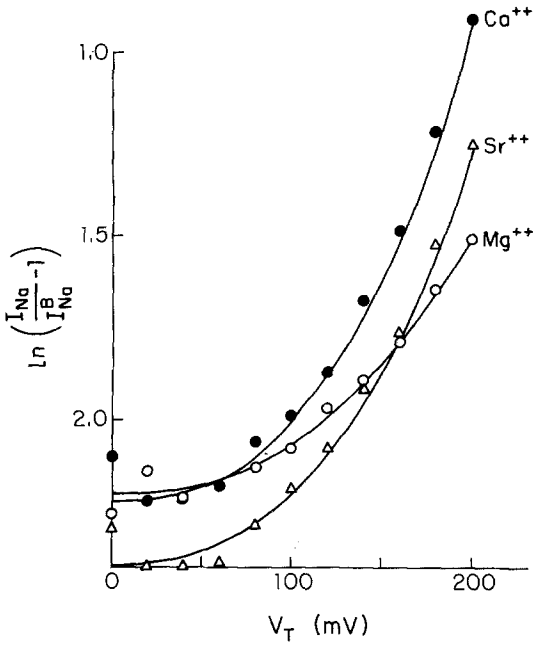


**Fig. 6.** Voltage dependence of block by divalent cations. $I_{Na}$-$V$ curves were obtained with mucosal solutions containing 29 mM Na and 86 mM NMDG Cl or 29 mM $CaCl_2$, $MgCl_2$, $BaCl_2$ or $SrCl_2$. Data are plotted as in Fig. 4. Data for $BaCl_2$ were omitted for clarity. The solid lines are theoretical plots derived using a two-site model as described in Materials and Methods. The parameters used for the theoretical lines are:

| | $K_1$ (mM) | $K_{12}$ | $\delta_1$ | $\delta_2$ |
|---|---|---|---|---|
| Ca | 273 | 40 | 0 | 0.60 |
| Sr | 326 | 40 | 0 | 0.57 |
| Mg | 267 | 40 | 0 | 0.48 |
| Ba | 305 | 70 | 0 | 0.57 |

Although the pore-plugging model of amilorde block is consistent with the data obtained so far, other schemes are also compatible. For example, amiloride and other blockers could bind to a site on the outside of a channel. Binding could bring about a conformational change closing the channel, in which a charged moiety within the channel moves across 15% of the electric field. The binding site could be recessed within a pocket on the surface of the protein, giving rise to the size requirement for blocking ions. In this scheme, the voltage dependence does not depend on the charge of amiloride itself. If could be tested by studying an amiloride analogue with a charge of other than +1, if such a blocker were found.

A third possible interpretation is that amiloride can be displaced from the channel by intracellular Na or another cation whose binding within the channel is alleviated at large positive voltages. Such a mechanism, in which the voltage dependence lies in the entry of the permeant ion, would also predict that the voltage dependence of block should also be independent of the charge of the blocking molecule.

**Table 2.** Voltage-dependence of block by divalent cations[a]

| | $K_1(0)$ (mM) | $K_{12}$ | $\delta_1$ | $\delta_2$ |
|---|---|---|---|---|
| Mg | 360(270–590) | 70(40–100) | 0 | 0.27(0.24–0.29) |
| Ca | 370(270–610) | 40(20–50) | 0 | 0.29(0.26–0.30) |
| Sr | 490(320–890) | 70(40–100) | 0 | 0.32(0.28–0.34) |
| Ba | 940(300–2300) | 50(10–70) | 0 | 0.28(0.27–0.30) |

[a] Parameters were obtained by fitting the blocking affinity *vs.* voltage curves with Eq. (4) as in Fig. 6. In each case, $\delta_1$ was assumed to be zero. Values for the other parameters are given as means and ranges for four experiments.

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