J. Membrane Biol. 50, 365–387 (1979)

Effect of Amiloride on the Apical Cell Membrane Cation Channels of a Sodium-Absorbing, Potassium-Secreting Renal Epithelium

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Received 1 May 1979

Summary. The effect of the K-sparing diuretic amiloride was assessed electrophysiologically in the isolated cortical collecting tubule of the rabbit, a segment which absorbs Na and secretes K. Low concentrations of amiloride in the perfusate caused a rapid, reversible, decrease in the magnitude of the lumen negative transepithelial potential difference, V_{te}, transepithelial conductance G_{te}, and equivalent short-circuit current, I_{sc}, with an apparent $K_{1/2}$ of approximately 7×10^{-8} M. The effects of a maximum inhibitory concentration of amiloride (10^{-5} M) were identical to those observed upon Na removal from lumen and bath (Na removal from the bath alone has no effect). Removal of Na in the presence of 10^{-5} M amiloride had no affect on V_{te}, G_{te}, or I_{sc}, and is consistent with the view that amiloride blocks the Na conductive pathways of the apical cell membrane. Further, in the absence of Na, the subsequent addition of amiloride had no influence. In tubules where active Na absorption was either spontaneously low, or abolished by removal of Na from lumen and bath, the elevation of K from 5 to 155 meq/liter in the perfusate caused a marked change of the V_{te} in the negative direction and an increase in the G_{te}. These effects could be attributed to a high K permeability of the apical cell membrane and not of the tight junctions. Amiloride (10^{-5} M) had no effect on these responses to K. It is concluded that amiloride selectively blocks the apical cell membrane Na channels but has no effect on the K conductive pathway(s). This selective nature of amiloride may indicate that Na and K are transported across the apical cell membrane via separate conductive pathways.

The diuretic amiloride is known to have both a natriuretic and antikaliuretic influence on the kidney (Glitzer & Steelmann, 1966; Baer *et al.*, 1967; Bull & Laragh, 1968). This effect of amiloride has been shown to result from a reduction in Na absorption from the lumen and K secretion into the lumen of the distal nephron (Duarte, Chomety & Giebisch, 1971). While the site of action has been identified, the mechanism by which amiloride exerts its influence on Na and K transport

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0022-2631/79/0050-0365 $04.60

in renal epithelia has not been rigorously evaluated. Indeed, our understanding of the mechanism of action of amiloride is primarily based upon studies of its action on Na transport in nonrenal epithelia, such as the urinary bladder (Bentley, 1968; Ehrlich & Crabbé, 1968; Cuthbert & Shum, 1975; Lewis, Eaton & Diamond, 1976; Frömter & Gebler, 1977; Sudou & Hoshi, 1977), colon (Schultz, Frizzell & Nellans, 1977; Frizzell & Turnheim, 1978; Thompson & Dawson, 1978; Wills, Lewis & Eaton, 1979), and amphibian skin (Dörge & Nagel, 1970; Salako & Smith, 1970; Biber, 1971; Erlij & Smith, 1973; Cuthbert & Shum, 1974; Fuchs, Larsen & Lindemann, 1977; Helman & Fisher, 1977; Lindemann & Van Driessche, 1977). These studies have established that amiloride binds to a receptor at the outer or apical cell membrane decreasing the Na permeability at that cell border, thus blocking Na uptake into the cell. However, unlike the epithelia of the distal nephron, these nonrenal epithelia are not avid secretors of K, with the possible exception of the mammalian colon. Thus, they may provide little or no information about the mechanism of action of amiloride with respect to inhibition of transcellular K secretion.

Studies on distal nephron segments indicate that amiloride reduces the magnitude of the lumen negative values of the transepithelial potential difference (Stoner, Burg & Orloff, 1974; Gross, Imai & Kokko, 1975; Barratt, 1976; Boudry, Stoner & Burg, 1976; O'Neil & Helman, 1977; Stoner, 1977; Shareghi & Stoner, 1978) and causes a decrease in the transepithelial conductance (Stoner *et al.*, 1974), effects consistent with inhibition of active Na absorption (Duarte *et al.*, 1971; Stoner *et al.*, 1974; O'Neil & Helman, 1977; Shareghi & Stoner, 1978). In a more recent study utilizing equivalent circuit analysis, Helman and O'Neil (1977) determined that the reduction in the net fluxes of Na and K of the cortical collecting tubule during exposure of several hours to amiloride is due to a fall in the equivalent transepithelial conductances for these two ions, although the effect on the K conductance was comparatively small relative to the effect on the Na conductance. Whether secondary effects of this long-term exposure to amiloride, such as intracellular ion concentration changes, may have in part influenced these results, especially as regards the effects on K, could not be assessed. In addition, these studies did not allow localization of the intraepithelial site of action.

The purpose of the present study was to assess the acute influence and mechanism of action of amiloride on the electrophysiological properties of the isolated cortical collecting tubule of the rabbit. In particular, the study was designed to determine whether amiloride may exert its

effect by reducing the Na and/or K conductance of the apical cell membrane. The results of the study show that amiloride selectively blocks the Na conductive channels of the apical cell membrane, but does not appear to affect the K conductive pathway(s). These results have been presented, in part, in abstract form (O'Neil & Boulpaep, 1978, 1979).

Materials and Methods

Tubule Preparation and Perfusion

New Zealand white female rabbits (1.5–3 kg) were used which had been maintained on standard Purina Laboratory Rabbit Chow (Na content = 170 meq/kg, K content = 360 meq/kg) and tap water *ad libitum*. On the day of experimentation, a rabbit was killed (by a blow to the base of the skull), the right kidney was quickly removed, and several sagittal slices of kidney (~1 mm thick) obtained and placed in control bathing solution at room temperature. Segments of cortical collecting tubule (CCT), 0.3 to 2.5 mm long, were dissected from the kidney slices and perfused *in vitro* at room temperature (~23 °C) according to the methods of Burg *et al.* (1966).

Briefly, tubular segments were perfused via a glass perfusion pipette (~20 μm OD) which was inserted into one end of the tubular lumen. The other end of the tubule was held in a glass collection pipette by means of a small amount of the liquid dielectric, Sylgard 184 (Dow Corning). The Sylgard formed a liquid seal between the outer border of the tubule and the glass pipette, providing electrical insulation between the tubular lumen and bath at this end of the tubule. At the perfusion end of the tubule, electrical insulation between tubule lumen and bath was achieved by a relatively tight mechanical seal between the glass perfusion pipette and the luminal border of the tubule. In early experiments, the perfusion end of the tubule was insulated around the outer border of the tubule by means of liquid Sylgard held between the tubule and an additional outer concentric pipette (Lutz, Cardinal & Burg, 1973). This procedure proved to be unnecessary in the cortical collecting tubules as the Sylgard seal at the perfusion end did not alter either the absolute magnitude of the resting transepithelial electrical potential, or the cable properties of the tubule.

The perfusion pipette arrangement permitted rapid exchange of the control perfusate with a test solution within 10–20 sec. This was accomplished by perfusing either control perfusate or test solution through an exchange pipette inserted inside of the perfusion pipette as far as possible. The bath solution also could be rapidly exchanged within 10–20 sec, by flowing test solution into one end of the bathing chamber, via an entrance port, while simultaneously aspirating the fluid from the opposite end of the chamber, keeping the bath volume constant at approximately one ml.

Control Solutions

In all experiments, the tubules were initially perfused with a control NaCl perfusate containing (in mM): 115 NaCl, 35 Na isethionate, 2.25 K_2HPO_4, 0.5 KH_2PO_4, 1.0 $MgSO_4$, 1.0 Ca lactate, and adjusted to pH 7.4. The tubules were initially bathed in a control NaCl bathing solution containing (in mM): 115 NaCl, 25 $NaHCO_3$, 10 Na acetate, 2.25 K_2HPO_4, 0.5 KH_2PO_4, 1.0 $MgSO_4$, 1.0 Ca lactate, 5.5 glucose, to which was added

5% (vol/vol) newborn calf serum (Flow Laboratories). The bathing medium was gassed with 5% CO_2–95% $/_2$, maintaining the pH at 7.4. Thus control perfusate and bathing solutions were identical in ionic composition except that the 35 mM isethionate of the perfusate isomotically replaced the HCO_3 and acetate of the bathing solution. Substitution of the HCO_3 and acetate in the perfusate for isethionate had no influence upon the values of transepithelial potential difference and conductance when the tubules were perfused at fast flow rates as in the present study (*manuscript in preparation*). Under the conditions of our experiments the NaCl perfusate and bathing solutions can therefore be considered as functionally symmetrical solutions. In addition, the removal of the 5% calf serum and 5.5 mM glucose from the bathing solution, for periods of up to 15–20 min, had no noticeable influence upon the values of transepithelial potential difference and conductance.

Measurement of V_{te} and G_{te}

The transepithelial potential difference, V_{te}, was monitored continuously through the perfusion pipette via an agar bridge (150 mM NaCl in 3% agar) placed in direct contact with the perfusion solution. A Ag-AgCl wire connected the bridge to a high input impedance amplifier ($10^{10}\,\Omega$, model M-4 WPI) whose output was recorded on one channel of a strip chart recorder (Grass Instruments). The bathing solution was grounded through a second identical agar bridge and Ag-AgCl wire. The small asymmetry between the bridge connections to the control perfusate and bath solution was nulled with a voltage reference source. Under experimental conditions were ion composition was unilaterally altered in the perfusate, the values of V_{te} were corrected for the asymmetry in bridge diffusion potentials. The magnitude of this asymmetry was estimated in the absence of a tubule as the change in potential difference of the perfusion pipette, relative to a flowing 3-M KCl reference electrode, when the ionic composition was varied.

The transepithelial conductance, G_{te}, was measured using cable analysis as described by Helman, Grantham and Burg (1971) by passing pulses of constant current (10–50 nA amplitude; 500 msec duration) into the perfusion end of the tubule lumen and recording the resulting voltage deflections at both ends of the tubule. An electrical bridge arrangement was used at the perfusion end so that a single electrode could be used to pass the constant current pulse, ΔI_O, into the tubule lumen while simultaneously recording the resulting voltage deflection at that end, ΔV_O. The voltage deflection due to the resistance of the perfusion pipette could readily be nulled with the bridge arrangement of the electrometer before the tubule was mounted so that during an experiment, only that voltage deflection due to the presence of the tubule was recorded. However, since the nulling of the voltage drop across the pipette resistance could not be continuously adjusted during an experiment, an additional correction had to be made for those test solutions whose volume resistivities differed from the control perfusate. This correction term was estimated in the absence of a tubule both before and after an experiment by measuring the observed changes in ΔV_O when the control perfusate was exchanged for a test solution. At the collecting end of the tubule, the voltage deflection, ΔV_L, was monitored via a NaCl agar bridge connected to a second high input impedance amplifier ($10^{10}\,\Omega$, model M-4 WPI) whose output was recorded on a second channel of the strip chart recorder.

The G_{te} of the tubule was calculated according to one dimensional cable theory for a terminated cable of length L:

$$L/\lambda = \cosh^{-1}(\Delta V_o/\Delta V_L) \tag{1}$$

$$G_{te}(\text{mS cm}^{-2}) = \frac{\Delta I_o}{2\pi a \Delta V_o \lambda} \coth(L/\lambda) \quad (2)$$

where L is the optically measured length of tubule between perfusion and collection pipets, λ is the space constant of the tubule and a is the observed luminal radius, all in cm. Both ΔV_O and ΔV_L were read within the first 100 msec of the current pulse. The transepithelial resistance, R_{te}, was calculated as

$$R_{te}(\Omega \text{ cm}^2) = 1/G_{te}.$$

The resistance per unit length of the luminal core of fluid could also be estimated from the cable equations (*see* Helman *et al.*, 1971), whereupon knowing the volume resistivity of the perfusate, the apparent radius of the electrical cable, a_e, could be estimated. As a check on the applicability of the cable theory, a_e was compared with a in each tubule. Only those tubules were used where the two estimates of radius agreed to within 1.0–1.5 μm.

Influence of Amiloride on V_{te} and G_{te}

After the tubules were allowed to equilibrate for 2–3 hr in the control bath and perfusion solutions, achieving steady values of V_{te} and G_{te}, the influence of amiloride (Merck, Sharp and Dohme) was assessed by changing the perfusion solution to one containing amiloride at the concentrations specified in the *Results*. Upon reaching a new steady-state, the values of V_{te} and G_{te} were noted over a period of several minutes. Subsequently, the amiloride-containing perfusate was replaced with the control perfusate. The observed changes in V_{te} and G_{te} with amiloride were recorded as the steady-state changes relative to the average steady-state control values before and after amiloride. Thus, in dose-response studies, amiloride was flushed from the tubular lumen after testing each concentration.

Influence of Amiloride on the Na and K Conductances

To assess the Na conductive properties of the epithelium, the Na ion was completely replaced with Tetramethylammonium ion (TMA) in both perfusate and bath for periods of 2 to 10 min, and the steady-state changes in V_{te} and G_{te} were recorded. In some experiments, 10^{-5} M amiloride was added to the Na-free perfusate to determine whether amiloride had an effect on ion transport that was independent from Na transport.

To evaluate the K conductive properties of the apical membrane (*see Results*), the K was elevated from 5 to 155 meq/liter in the luminal fluid and the effect on V_{te} and G_{te} noted. Potassium was either substituted for Na, or alternatively, to obviate the effect of Na, for TMA. In the latter case, Na was first replaced with TMA in both lumen and bath, and then K substituted for TMA. The influence of amiloride was assessed by adding 10^{-5} M amiloride to the high K perfusate.

Statistical Analysis

All average values are presented as mean values ± SEM. Differences between means were analyzed by paired or unpaired t tests as appropriate. Correlations between parameters were determined by linear regression analysis.

Results

Kinetics of Amiloride Action

The addition of amiloride to the luminal perfusate of the isolated cortical collecting tubule of the rabbit caused a very rapid, but reversible reduction in the magnitude of the lumen negative transepithelial potential difference, V_{te}, and transepithelial conductance, G_{te}. As shown by the example in Fig. 1, the addition of 10^{-5} M amiloride completely abolished the lumen negative value of V_{te} while simultaneously reducing the value of G_{te}. The onset of the effect was very rapid and, in the example of Fig. 1, was essentially complete within 3–4 sec. In tubules where a supramaximal dose of amiloride (10^{-4} M) was used, the inhibitory effect was 90% complete within one to two sec.

The reversal of amiloride inhibition during "wash-out" of the lumen, occurs at a much slower rate than the initial inhibition. In general, when the amiloride (10^{-5} or 10^{-4} M) containing perfusate was exchanged for the control perfusate in the tubular lumen, the return of the V_{te} and G_{te} to control values required 1 to 2 min. As discussed below, several factors may be responsible for this slow recovery.

To facilitate the analysis of the inhibitory influence of amiloride on the ionic currents, the instantaneous equivalent short-circuit current, I_{sc}, was estimated for individual tubules. Since the current-voltage relationship of the epithelium is linear between open-circuit and short-circuit conditions (Helman & O'Neil, 1977), I_{sc} was simply calculated as: $I_{sc} = -V_{te}G_{te}$. The dose-response curve for amiloride inhibition given as % of maximum change, $\%\Delta$, with 10^{-4} M amiloride, was estimated for both V_{te} and I_{sc}. Since the changes in G_{te} were small and variable at the lower doses of amiloride, it was practical to describe a reliable dose-response relationship for G_{te}, The data for V_{te} and I_{sc} are summarized in Fig. 2. The solid curve gives the expected theoretical relationship for simple Michaelis-Menten type kinetics for amiloride inhibition assuming a $K_{1/2}$ of 7×10^{-8} M. While the data appear to fit the theoretical curve reasonably well, the "goodness of fit" may better be assessed using linearization procedures. When the data were thus transformed into Eadie plots, ($\%\Delta$ *vs.* $\%\Delta$/[amil]) the data for most tubules could be adequately described by a linear relationship. However, to fully evaluate whether the data conform to simple first-order rate kinetics, more studies are required utilizing a greater number of amiloride concentrations, especially in the lower concentration range. The inhibitory influence of amiloride

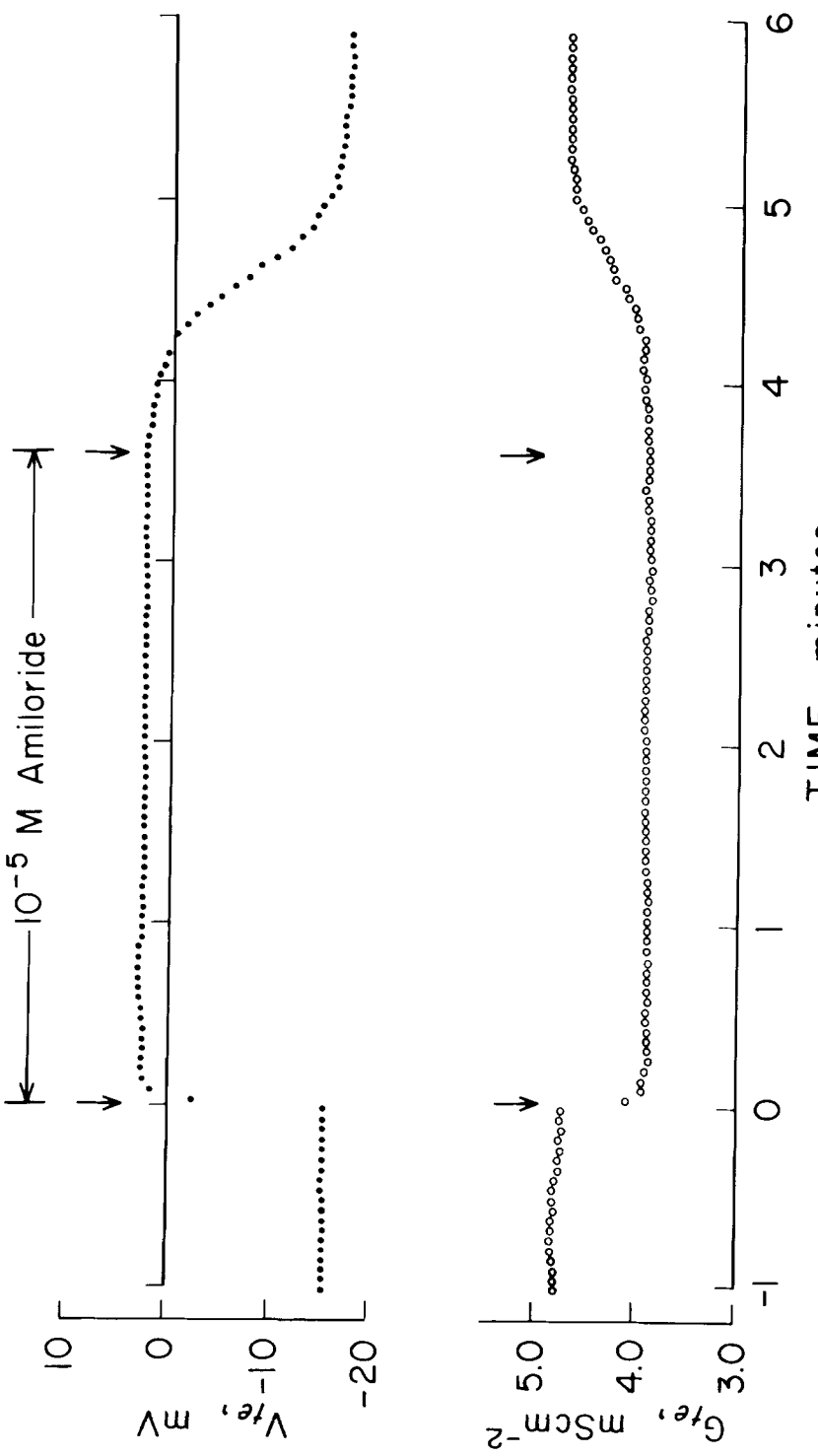

Fig. 1. Time course showing the effect of 10^{-5} M amiloride on the values of transepithelial potential difference, V_{te}, and transepithelial conductance, G_{te}. The data points were obtained at intervals of 3.3 sec

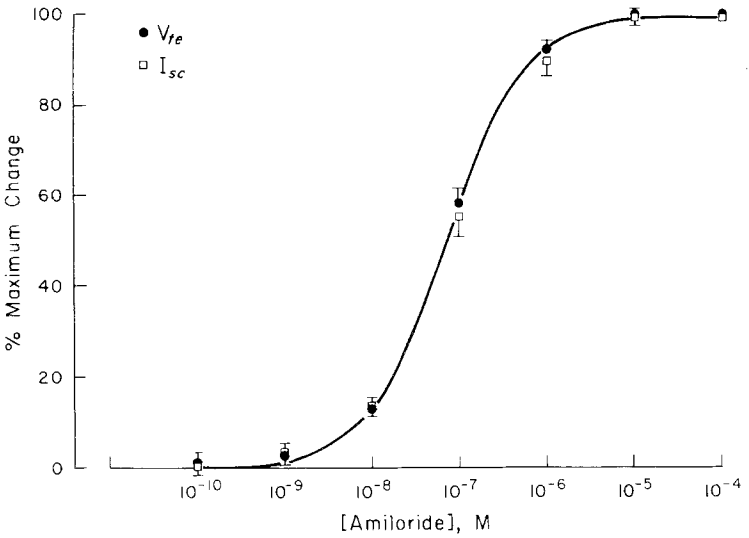

Fig. 2. Amiloride dose-response relationship for V_{te} ($n=6$) and I_{sc} ($n=4$). The data are plotted as the percent change at each amiloride concentration relative to the maximum change observed with 10^{-4} M amiloride. The vertical bars represent one SEM for a given mean value. The solid curve gives the theoretical relationship for Michaelis-Menten type kinetics assuming a $K_{1/2}$ of 7×10^{-8} M

at a concentration of 10^{-5} M is indistinguishable from the maximum effect at 10^{-4} M. 10^{-5} M amiloride has previously been shown to completely inhibit Na absorption (O'Neil & Helman, 1977).

Nature of Maximum Response

Since 10^{-5} M amiloride has a maximum affect on V_{te} and I_{sc}, we have used this concentration to assess the transport properties of the epithelium in greater detail. As shown in Fig. 3, the steady-state control values of V_{te} varied considerably among tubules, including both positive and negative values. When 10^{-5} M amiloride was added to the perfusate, not only were the negative values of V_{te} abolished, but as first noted by Stoner *et al.* (1974), the V_{te} actually became positive in value, thus suggestive of the persistence of an amiloride-insensitive current, or reverse current. The mean values are summarized in Table 1. As shown in Fig. 4, the amiloride-sensitive component of the V_{te}, defined as $\Delta V_{te}^{amil} = V_{te}^{amil} - V_{te}^{Control}$ was highly dependent upon the initial control value of V_{te}; the slope of the best-fit straight line, -1.02 ± 0.05, does not differ from unity. The difference between ΔV_{te}^{amil} and $V_{te}^{control}$ is equal to V_{te}^{amil}, the

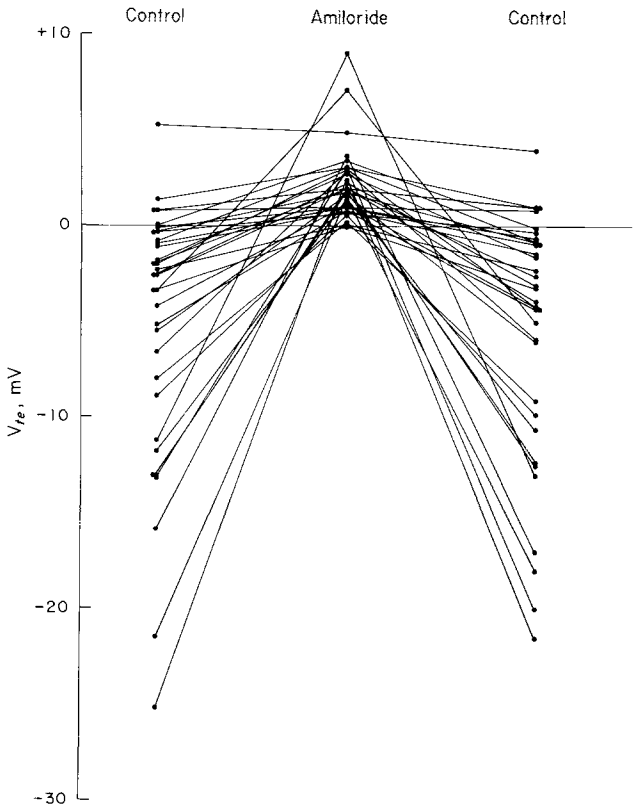

Fig. 3. The effect of 10^{-5} M amiloride on the values of V_{te}, showing the reversibility. Solid lines between points connect the values from individual tubules ($n=33$)

Table 1. Influence of 10^{-5} M amiloride on selected electrical parameters ($n=33$)

	Control	Amiloride	Δ
V_{te} (mV)	-5.5 ± 1.2	$+2.2 \pm 0.3$	$+7.8 \pm 1.2$[a]
G_{te} (mS cm^{-2})	6.31 ± 0.55	5.96 ± 0.55	-0.36 ± 0.07[a]
R_{te} (Ω cm^{-2})	197 ± 17	218 ± 22	$+21 \pm 5$[a]
I_{sc} (μA cm^{-2})	26.7 ± 5.2	-12.5 ± 2.5	-39.2 ± 5.7[a]

Transepithelial potential difference, V_{te}; transepithelial conductance, G_{te}; transepithelial resistance, R_{te}; and equivalent short-circuit current, I_{sc}.

[a] Significant pair difference ($P<0.001$).

amiloride-insensitive component of V_{te}, and is represented in Fig. 4, as the displacement of the solid regression line from the broken line whose slope is also equal to one but passes through the origin. As can be seen from Fig. 4, this displacement of the regression line is constant

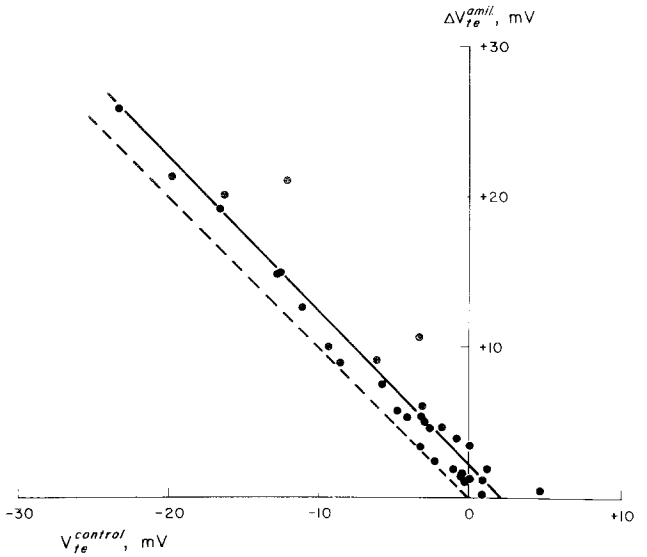

Fig. 4. The relationship between the control values of V_{te}, $V_{te}^{control}$, and the amiloride-sensitive component of V_{te}, ΔV_{te}^{amil}. The broken line represents the line of identity between the two values, where $\Delta V_{te}^{amil} = -V_{te}^{control}$. The solid line is the linear regression best-fit line of the data given as: $\Delta V_{te}^{amil} = +2.2 - 1.02\ V_{te}^{control}$

at approximately +2.2 mV along the ordinate for all values of $V_{te}^{control}$ along the abscissa. This implies that the amiloride insensitive component of V_{te} is independent of the initial control values. It also follows that amiloride should have little or no effect in those tubules which spontaneously exhibit a control V_{te} of near +2 mV as indeed is demonstrated in Fig. 4.

A similar relationship also exists for the equivalent short-circuit current of the epithelium. As shown in Fig. 5, the amiloride-sensitive component, $\Delta I_{sc}^{amil} = I_{sc}^{amil} - I_{sc}^{control}$, is highly dependent upon the magnitude of the control I_{sc}. The slope of the regression line is -0.99 ± 0.08 and again does not differ from unity. The amiloride-insensitive component of the I_{sc}, i.e., the reverse I_{sc}, represented by the displacement of the regression line from the broken line in Fig. 5, remains relatively constant near a mean negative value of $-12.5\ \mu A\ cm^{-2}$ for all control values of I_{sc}. Again, it follows that amiloride fails to affect tubules which spontaneously exhibited a reverse I_{sc} of near $-12\ \mu A\ cm^{-2}$. Thus, the partial ionic currents responsible for the reverse I_{sc} may not be influenced by amiloride. Conversely, the normal positive component of I_{sc} which is thought to reflect active Na absorption, appears to be the only amiloride-sensitive component of the I_{sc}. Indeed, the mean amiloride-sensitive com-

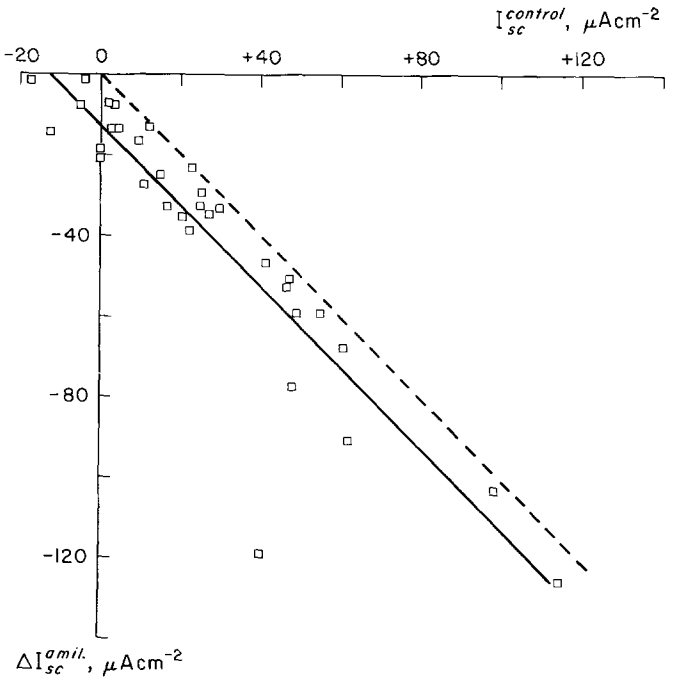

Fig. 5. The relationship between the control values of I_{sc}, $I_{sc}^{control}$, and the amiloride-sensitive component of I_{sc}, ΔI_{sc}^{amil}. The broken line represents the line of identity between the two values, where $\Delta I_{sc}^{amil} = -I_{sc}^{control}$. The solid line is the linear regression best-fit line of the data given as: $\Delta I_{sc}^{amil} = -12.9 - 0.99\ I_{sc}^{control}$. Note that amiloride has no effect on tubules when the $I_{sc}^{control}$ is spontaneously near -12 μA cm^{-2}

ponent of the I_{sc} was 39.2 ± 5.7 μA cm^{-2} (*see* Table 1) and does not differ from the Na short-circuit current, I_{sc}^{Na}, of 45.3 μA estimated from previous direct measurements of net Na flux obtained near short-circuit conditions (*see Discussion*). Consequently, in those tubules where amiloride has little or no effect on V_{te} or I_{sc}, i.e., in tubules which spontaneously exhibit a reverse I_{sc}, the rate of active Na transport may be spontaneously low if not zero.

Addition of 10^{-5} M amiloride to the luminal perfusate caused the G_{te} to decrease by 0.36 ± 0.07 mS cm^{-2} from a control value of 6.31 mS cm^{-2} (*see* Table 1). In tubules where the control I_{sc} was initially zero or negative in value, amiloride had an insignificant effect on the G_{te}, consistent with the lack of effect of amiloride on the V_{te} and I_{sc} in these tubules. Only in tubules where significant active Na transport was thought to occur, i.e., in tubules with negative values of V_{te} and positive values of I_{sc}, did amiloride usually cause a sizeable reduction in the G_{te}, likely due to a fall in the Na conductance of the epithelium.

Cellular and Ionic Basis of Amiloride Action

As noted in the *Introduction,* amiloride is thought to exert its influence on Na transport by blocking Na entry at the apical border of the cell. This notion has not been previously assessed for amiloride-sensitive renal epithelia. To evaluate the mechanism and site of action in the cortical collecting tubule, we took advantage of the fact that the basolateral membrane and tight-junctions of the cortical collecting tubule appear to be relatively impermeable to the passive flow of Na as evidenced by the finding that removal of Na from the bathing medium has little or no influence on the V_{te} and G_{te} of the tissue (*manuscript in preparation*; also, *see* O'Neil & Helman, 1977). Consequently, removal of Na from both lumen and bath (tetramethylammonium ion, TMA, replacement) should mainly act on the apical cell border and thus block active Na transport and essentially abolish the Na conductance of the epithelium. If the action of amiloride is to block the Na conductance of the apical membrane, the observed effect of either complete Na removal or amiloride addition, should be identical. This identity was examined in experiments where the influence of Na removal and amiloride (10^{-5} M) addition were assessed in paired comparison on the same tubule. In other experiments, the additive effect of Na removal and amiloride was evaluated.

As shown in Fig. 6*A* and Table 2*A* and *B*, the change in V_{te} upon

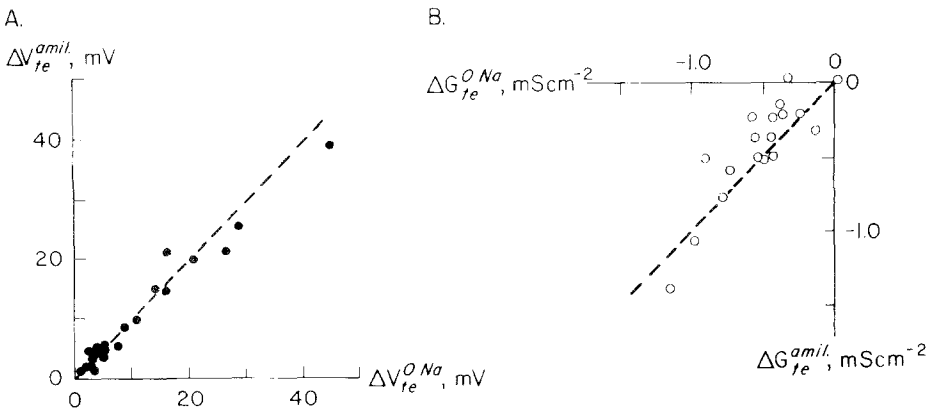

Fig. 6. Comparison between the influence of Na removal from lumen and bath *vs.* addition of 10^{-5} M amiloride. The broken line represents the line of identity between the two values. (*A*): Change in V_{te} upon Na removal, $\Delta V_{te}^{0\,Na}$, compared to the change upon amiloride addition, ΔV_{te}^{amil}. Values are in mV. (*B*): Change in G_{te} upon Na removal, $\Delta G_{te}^{0\,Na}$, compared to the change upon amiloride addition, ΔG_{te}^{amil}. Values are in mS cm^{-2}

Table 2.

A. Influence of Na removal in the absence of amiloride ($n=18$)

	−Amiloride		
	150 Na	0 Na	Δ
V_{te} (mV)	−7.6 ±1.8	+2.2 ±0.7	+9.8 ±2.0[a]
G_{te} (mS cm^{-2})	5.73±0.67	5.25±0.67	−0.48±0.08[a]
I_{sc} (μA cm^{-2})	34.2 ±7.4	−8.4 ±1.9	−42.6 ±7.7[a]

B. Influence of 10^{-5} M amiloride addition in the presence of Na ($n=18$)

	150 Na		
	−Amiloride	+Amiloride	Δ
V_{te} (mV)	−7.6 ±1.7	+1.9 ±0.5	+9.5 ±1.9[a]
G_{te} (mS cm^{-2})	5.96±0.72	5.52±0.72	−0.44±0.08[a]
I_{sc} (μA cm^{-2})	+34.3 ±7.0	−8.3 ±1.6	−42.8 ±7.1[a]

C. Influence of Na removal in the presence of 10^{-5} M amiloride ($n=8$)

	+Amiloride		
	150 Na	0 Na	Δ
V_{te} (mV)	+2.4 ±0.6	+2.9 ±0.8	+0.5 ±0.4
G_{te} (mS cm^{-2})	4.72±0.55	4.75±0.54	+0.04±0.04
I_{sc} (μA cm^{-2})	−11.2 ±2.7	−13.4 ±4.0	−2.2 ±1.9

D. Influence of 10^{-5} M amiloride addition in the absence of Na ($n=3$)

	0 Na		
	−Amiloride	+Amiloride	Δ
V_{te} (mV)	+2.5 ±0.8	+2.8 ±0.8	+0.3 ±0.1
G_{te} (mS cm^{-2})	2.97±0.32	2.95±0.34	−0.02±0.02
I_{sc} (μA cm^{-2})	−7.2 ±2.8	−8.3 ±2.6	−1.1 ±0.3

Values in panels *A* and *B* are paired observations on the same tubules.

[a] Significant pair difference ($P<0.001$).

Na removal from lumen and bath, $\Delta V_{te}^{0\,Na}$, was identical to the change in V_{te} observed upon amiloride addition, ΔV_{te}^{amil}. The slope of the linear regression of ΔV_{te}^{amil} on $\Delta V_{te}^{0\,Na}$ is 1.10 ± 0.05 ($n=21$) and does not differ from unity. Similarly, as shown in Fig. 6*B* and Table 2*A* and *B*, the observed changes in G_{te}, $\Delta G_{te}^{0\,Na}$ and ΔG_{te}^{amil}, were identical, the slope of the linear regression of ΔG_{te}^{amil} on $\Delta G_{te}^{0\,Na}$ is 0.99 ± 0.18 ($n=18$).

In other experiments, amiloride was first added to the perfusate, and Na subsequently removed in the continued presence of amiloride. As shown in Fig. 7*A*, 7*B*, and in Table 2*C*, in the presence of a maximum inhibitory dose of amiloride (10^{-5} M) the removal of Na (or subsequent reversal) had no effect on either the V_{te} or G_{te} of the tissue. Clearly V_{te} and G_{te} would have been altered upon removal of Na if "open" Na conductive channels still existed in the apical (luminal) membrane after amiloride. Thus, amiloride apparently blocks the Na conductive channels of the apical cell membrane in this renal epithelium.

In these same studies, the influence of amiloride addition in the absence of Na was also assessed. The results are given in Fig. 7*C* and *D* and in Table 2*D*. Under these conditions, amiloride had no influence on the V_{te} and G_{te}. If in the absence of extracellular Na, the Na current is abolished, it would appear that amiloride does not influence any other ionic currents, at least not in the absence of Na.

The K permeability properties of the apical cell membrane were evaluated from the changes in V_{te} and G_{te} observed upon elevation of K from 5 meq/liter to 155 meq/liter in the luminal perfusate. Since the tight junctions of the epithelium appear to be relatively impermeable to K, while the apical cell membrane is highly permeable to K (Grantham *et al.*, 1970; Stoner *et al.*, 1974; O'Neil & Boulpaep, 1979), the observed responses of V_{te} and G_{te} were taken to reflect properties of the apical cell membrane (*see Discussion*). To obviate the influence of Na transport, the experiments were performed either on tubules which spontaneously exhibited a minimal rate of Na transport as evidenced by zero or positive values of V_{te}, or on tubules where Na transport was abolished by replacement of Na with TMA. Potassium was then elevated in the perfusate in exchange for the predominant cation, either Na or TMA. The influence of amiloride was assessed by adding 10^{-5} M amiloride to the high K perfusate and repeating the experiments in the same tubules.

Elevation of K in the luminal perfusate caused a rapid decrease in the V_{te} to more lumen negative values and a rapid increase in the G_{te}, both parameters approaching maximum changes within 10–20 sec. The maximum changes in the values of V_{te}, $\Delta V_{te}^{155\,K}$, and G_{te}, $\Delta G_{te}^{155\,K}$,

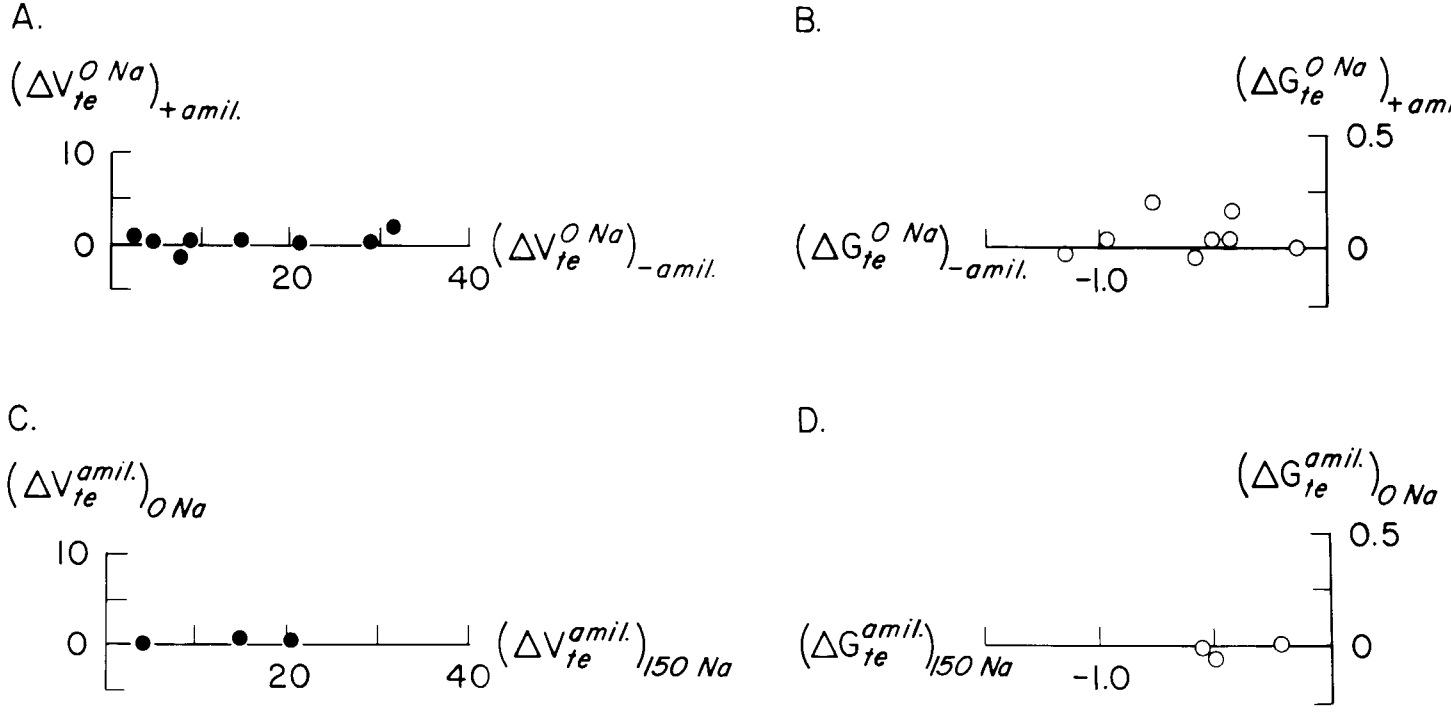

Fig. 7. *Upper panel:* Effect of 10^{-5} M amiloride upon the tubular response to Na removal from lumen and bath. (*A*): Change in V_{te} upon Na removal in the absence of amiloride, $(\Delta V_{te}^{0Na})_{-amil}$, compared to the change in the presence of amiloride, $(\Delta V_{te}^{0Na})_{+amil}$. Values are in mV. (*B*): Change in G_{te} upon Na removal in the absence of amiloride, $(\Delta G_{te}^{0Na})_{-amil}$, compared to the change in the presence of amiloride, $(\Delta G_{te}^{0Na})_{+amil}$. Values are in mS cm^{-2}. *Lower panel:* Effect of Na removal upon the tubular response to addition of 10^{-5} M amiloride. (*C*): Change in V_{te} upon amiloride addition in the presence of Na, $(\Delta V_{te}^{amil})_{150\,Na}$, compared to the change in the absence of Na, $(\Delta V_{te}^{amil})_{0\,Na}$. Values are in mV. (*D*): Change in G_{te} upon amiloride addition in the presence of Na, $(\Delta G_{te}^{amil})_{150\,Na}$, compared to the change in the absence of Na, $(\Delta G_{te}^{amil})_{0\,Na}$. Values are in mS cm^{-2}.

Table 3. Influence of 10^{-5} M amiloride on the tubular response to elevation of K in the luminal fluid from 5 to 155 meq/liter ($n=8$)

	5 K	155 K	Δ	5 K	155 K +amiloride	Δ
V_{te} (mV)	+1.6	−13.9	−15.5	+2.0	−13.7	−15.7
	±0.5	± 3.3	± 3.3	±0.4	± 3.3	± 3.4
G_{te} (mS cm $^{-2}$)	6.18	9.00	+ 2.82	6.01	8.67	+ 2.66
	±1.37	±2.40	± 1.28	±1.21	±2.08	± 1.13

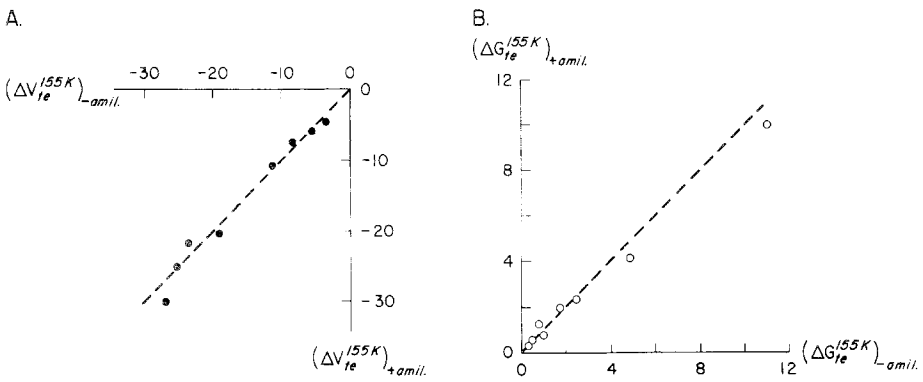

Fig. 8. Effect of 10^{-5} M amiloride on the tubular response to luminal K elevation. The broken lines represent the lines of identity between the values compared. (*A*): Change in V_{te} upon K elevation in the absence of amiloride, $(\Delta V_{te}^{155K})_{-amil}$, compared to the change in the presence of amiloride, $(\Delta V_{te}^{155K})_{+amil}$. Values are in mV. (*B*): Change in G_{te} upon K elevation in the absence of amiloride, $(\Delta G_{te}^{155K})_{-amil}$, compared to the change in the presence of amiloride, $(\Delta G_{te}^{155K})_{+amil}$. Values are in mS cm^{-2}

are summarized in the left panel of Table 3. The large changes in V_{te}, −15.5 mV, and G_{te}, +2.82 mS cm^{-2}, indicate a relatively high K permeability of the apical cell membrane. The influence of amiloride on this K permeability was assessed by repeating these experiments in the same tubules, but now with the addition of 10^{-5} M amiloride to the high K perfusate. As can be seen in Fig. 8 and Table 3, the changes in both V_{te} and G_{te} were not influenced by the addition of amiloride to the perfusate. These results indicate that amiloride does not affect the K permeability or conductance of the apical cell membrane.

Transepithelial Partial Conductance and Equivalent Driving Force for Na

If it is considered that the only effect of amiloride on the ionic conductance was to block the Na conductance of the epithelium, the change in G_{te} observed upon the addition of 10^{-5} M amiloride to the perfusate should reflect a reduction in the transepithelial Na conductance, G_{te}^{Na}, to near zero, so that: $G_{te}^{Na} \simeq -\Delta G_{te}^{amil}$. Defined in this manner, the mean transepithelial G_{te}^{Na} was 0.36 mS cm^{-2} (*see* Table 1) or alternatively the mean Na resistance, $R_{te}^{Na}(\equiv 1/G_{te}^{Na})$, was 2800 Ω cm^2.

The equivalent transepithelial driving force for Na transport, E_{te}^{Na}, could also be estimated in these studies according to the relationship $I_{sc}^{Na} = G_{te}^{Na} E_{te}^{Na}$. Since I_{sc}^{Na} could be estimated as $-\Delta I_{sc}^{amil}$ and G_{te}^{Na} as $-\Delta G_{te}^{amil}$, E_{te}^{Na} could be evaluated readily as $\Delta I_{sc}^{amil}/\Delta G_{te}^{amil}$ with the aid of amiloride. Limiting the analysis to those tubules where ΔG_{te}^{amil} was of sufficient magnitude so that it could be measured reliably, i.e., $\Delta G_{te}^{amil} \geqq 0.1$ mS cm^{-2}, E_{te}^{Na} was estimated to be -117 ± 11 mV ($n=29$). This is very similar to our previous estimates of E_{te}^{Na} for the cortical collecting tubule of -119 mV using different methodology (Helman & O'Neil, 1977).

Discussion

The K-sparing diuretic amiloride is known to exert its influence on the kidney by reducing Na absorption and K secretion in the distal nephron. In the isolated cortical collecting tubule (CCT) of the rabbit, a segment which not only actively absorbs Na, but also actively secretes K (Grantham *et al.*, 1970; Stoner *et al.*, 1974; Helman & O'Neil, 1977) amiloride has been shown to markedly reduce the net flux of both of these ions (Stoner *et al.*, 1974; O'Neil & Helman, 1977; Shareghi & Stoner, 1978). In the present study, amiloride had a rapid but reversible effect on the electrophysiological properties of the cortical collecting tubule. The acute addition of amiloride to the luminal perfusate caused a reduction in the magnitude of the lumen negative V_{te}, the positive I_{sc} and the G_{te}, effects consistent with inhibition of Na uptake across the apical cell border.

The onset of the effect of amiloride in the cortical collecting tubule was very rapid and for the V_{te} was 90% complete within 1–2 sec for a supramaximal dose of amiloride. The actual rate of amiloride inhibition of ion transport is likely to be much faster than that observed for V_{te} as the response may be limited by the rate of exchange of the tubular fluid. Furthermore, the rate of binding of amiloride to a membrane

receptor may itself exceed the rate of transport inhibition. In contrast, the reversal of the amiloride effect during "wash out" occurs at a slow rate, requiring 1–2 min for a complete recovery. Several factors may be responsible for this slow return to control conditions. Firstly, dissociation of amiloride from the specific receptor may be slow relative to the rate of binding. Secondly, a large pool of nonspecifically-bound amiloride may be released slowly into the tubular lumen during *wash-out*. During the early phase of *wash-out*, a finite tubular volume and limited flow rate may result in a significant luminal amiloride concentration thus delaying the recovery. Finally, because drug-receptor interactions and the biological responses of such interactions are not linearly related to drug concentration, and moreover, because drug concentration does not change linearly with time during *wash-in* and *wash-out* of the drug, the time course of the tubular response to either increasing or decreasing the amiloride concentration may differ considerably.

The experimental observations of the present study are all consistent with a primary effect of amiloride on the Na conductance of the apical cell membrane. Indeed, with 10^{-5} M amiloride in the luminal perfusate, a dose sufficient to completely inhibit active Na absorption under conditions similar to those of the present study (O'Neil & Helman, 1977), amiloride appeared to completely block the Na conductive pathways of the apical cell membrane. The total abolition of the apical membrane Na conductance or permeability is evidenced by the finding that varying the perfusate Na concentration from 0 to 150 meq/liter in the presence of 10^{-5} M amiloride had no effect on either the V_{te} or G_{te}. Such a mechanism of action of amiloride on Na transport is supported by previous observations of the CCT where it was determined indirectly by equivalent circuit analysis that 10^{-5} M amiloride reduced the equivalent transepithelial Na conductance to zero (Helman & O'Neil, 1977). Further, this is consistent with the effects of amiloride in Na absorbing, nonrenal epithelia, where amiloride has been shown to reduce the apical cell membrane conductance (Reuss & Finn, 1975; Lewis *et al.*, 1976; Frömter & Febler, 1977; Helman & Fisher, 1977; Schultz *et al.*, 1977; Wills *et al.*, 1979) to reduce isotopic Na uptake across the apical cell membrane (Dörge & Nagel, 1970; Salako & Smith, 1970; Biber, 1971; Erlij & Smith, 1973; Frizzell & Turnheim, 1978; Thompson & Dawson, 1978) and to reduce the passive Na permeability of the apical cell membrane (Fuchs *et al.*, 1977; Lindemann & Van Driessche, 1977; Sudou & Hoshi, 1977).

Unlike the effect of amiloride on Na transport, inhibition of K secre-

tion by amiloride does not appear to involve a similar mechanism of action. The acute addition of 10^{-5} M amiloride to the luminal perfusate did not detectably influence the K conductive pathways of the apical cell membrane. In these studies, however, it should be noted that the K conductive properties of the apical cell membrane were evaluated from the changes in V_{te} and G_{te} that occurred when K was elevated in the luminal perfusate either in the presence or absence of amiloride (10^{-5} M). It is imperative, therefore, that the observed responses are predominantly due to a significant K permeability of the apical cell membrane and not of the tight junction. Several lines of evidence indicate that the tight junction K permeability is relatively low. Firstly, Grantham *et al.* (1970) demonstrated that the CCT could generate and maintain luminal K concentrations of up to 130–140 meq/liter corresponding to a K electrochemical gradient of near 40 mV in excess of what would be predicted from the V_{te} for a passively distributed ion. Secondly, Stoner *et al.* (1974) demonstrated that the bidirectional isotopic flux ratio for K likewise deviated considerably from that predicted from the V_{te} for a passively distributed ion. Thirdly, in recent studies in our own laboratory we have observed that the magnitude of the changes in the V_{te} and G_{te} upon unilateral elevation of K in either the lumen or bath, were highly asymmetric. On the average, the magnitude of the changes for both V_{te} and G_{te} were 3–5 times greater when K was elevated in the luminal perfusate as opposed to when K was elevated in the bath (O'Neil & Boulpaep, 1979), thus implying that the K permeability is not determined by a dominant tight junctional permeability to K. Further, the epithelial cells were often observed to swell when K was elevated in the luminal perfusate, supposedly due to KCl influx into the cell, thus implying a significant K permeability of the apical cell membrane. These data provide collective evidence in support of a relatively high K permeability of the apical cell membrane compared to that of the tight junction.

In view of the above evidence, it was considered that the changes in V_{te} and G_{te} observed upon elevation of the K in the luminal perfusate were primarily the result of a significant K permeability of the apical cell membrane. Since 10^{-5} M amiloride did not affect the observed responses of V_{te} and G_{te}, we concluded that amiloride does not alter the K conductive pathways of this membrane.

Since the acute influence of amiloride on K secretion does not appear to be a direct effect of the drug on the K conductance of the apical cell membrane, indirect effects may be responsible for the inhibition

of K transport, such as a reduction in the magnitude of a favorable lumen negative V_{te} subsequent to inhibition of active Na transport. During long-term exposure to amiloride, other secondary factors may become important. For example, inhibition of Na absorption may be accompanied by a reduction in the Na-K exchange at the basolateral membrane pump sites which could depress intracellular K activity and hence the rate of K secretion across the apical membrane. In turn, secondary changes in intracellular ion concentrations could conceivably alter membrane permeabilities and conductances. In a recent study Helman and O'Neil (1977) noted that during prolonged exposure to amiloride, the equivalent transepithelial K conductance of the cortical collecting tubule was decreased relative to control tubules. Since the acute addition of amiloride in the present study does not appear to influence the K conductance of the apical cell membrane, the effect on the equivalent K conductance during long-term exposure to amiloride may be the result of secondary effects such as alterations in intracellular ion concentrations.

Recently, McKinney and Burg (1978) demonstrated that amiloride may reduce the rate of bicarbonate absorption (or H secretion) in the cortical collecting tubule. Interestingly, removal of Na from lumen and bath had the opposite effect, stimulating bicarbonate absorption. The mechanism(s) of these effects is not known. In any event, under the conditions of the present study, no evidence was obtained that amiloride may alter ionic currents and conductances other than for Na. As shown, in the absence of Na in the lumen and bath, the subsequent addition of amiloride to the perfusate had no effect on either V_{te}, G_{te}, or I_{sc} of the CCT. Furthermore, in those tubules which under control conditions spontaneously exhibited a lumen-positive value of V_{te} and negative I_{sc} (reverse I_{sc}), indicating an absence or reduced rate of active Na absorption, amiloride had little or no effect on V_{te}, G_{te}, or I_{sc}. In addition, the amiloride-sensitive value of $I_{sc}(\equiv -\Delta I_{sc}^{amil})$ of 39.2 ± 5.7 μA cm^{-2} does not significantly differ from the Na short-circuit current (I_{sc}^{Na}) of 45 μA cm^{-2} which was estimated from actual measurements of the net Na flux near short-circuited conditions in a previous study[1] (Helman

[1] We have previously measured the net rate of Na absorption in the cortical collecting tubule under conditions similar to those of the present study (Helman & O'Neil, 1977; O'Neil & Helman, 1977). The V_{te} averaged -8.5 mV and the open-circuit Na current, I_{oc}^{Na}, estimated from the net Na flux, averaged 42.1 μA cm^{-2} (assuming a 20 μm luminal diameter). Since the net driving force for Na transport, E_{te}^{Na}, also was estimated in these studies, averaging -119 mV, the I_{sc}^{Na} could be estimated as:

$$I_{cc}^{Na} = E_{te}^{Na} G_{te}^{Na} = E_{te}^{Na} I_{oc}^{Na} / (V_{te} - E_{te}^{Na}).$$

The I_{sc}^{Na} thus estimated is 45.3 μA cm^{-2}.

& O'Neil, 1977). Likewise, the amiloride-sensitive conductance ($\equiv -\Delta G_{te}^{amil}$) of 0.36 ± 0.07 mS cm^{-2} obtained in the present study does not differ from previous estimates of the equivalent Na conductance of 0.31 mS cm^{-2} (assuming a 20 μm luminal diameter). Even the present estimates of E_{te}^{Na} of -117 ± 11 mV, which depend upon estimating G_{te}^{Na} and I_{sc}^{Na} from the amiloride-sensitive components of G_{te} and I_{sc}, are similar to our previous estimates of E_{te}^{Na} of -119 ± 4 mV (Helman & O'Neil, 1977). Furthermore, these values are well within the range of values usually reported for E_{te}^{Na} of near -100 to -130 mV for numerous other nonrenal *tight* epithelia (*see* Helman, O'Neil & Fisher, 1975; Schultz *et al.*, 1977; Lewis, Wills & Eaton, 1978). These results clearly demonstrate that even in this Na-absorbing, K-secreting renal epithelium, the K-sparing diuretic amiloride is a selective blocker of the apical cell membrane Na current, the effect on other transepithelial ionic currents apparently being secondary to the inhibition of the Na current. In addition, it follows from this analysis that the relatively small effect of amiloride on the G_{te} may be explained by the low partial Na conductance of the CCT. Indeed, as previously shown, the Cl conductance predominates in this epithelium (Helman & O'Neil, 1977).

It may be of some significance that amiloride is particularly selective for Na at a membrane which is thought to have a high passive permeability for both Na and K. In view of the selective nature of amiloride it is unlikely that Na and K are traversing the apical membrane via a common channel since amiloride can selectively and completely block the permeability of one cation without altering the permeability of the second cation. This is supported further by the observation that a decrease in the luminal pH of the cortical collecting tubule can reduce K secretion by nearly 50% while having little or no effect on Na absorption (Boudry *et al.*, 1976). Also, a separation of the Na and K pathways would be consistent with the nonlinear properties of the current-voltage relationship of the epithelium, as previously suggested by Helman and O'Neil (1977). Based upon these earlier investigations and the present findings that amiloride can selectively and completely block the apical membrane Na conductance, we propose that Na and K are transported via separate channels across the apical cell membrane. Thus, the amiloride receptor of the apical cell membrane may be intimately associated with the Na channel and hence the selective nature of this diuretic.

This work was supported by U.S. Public Health Service Research Grant 5-R01-AM-13844 and Program Project AM-17433 from the National Institute of Arthritis, Metabolic

and Digestive Diseases. During the course of these studies R.G. O'Neil held a Postdoctoral Fellowship from the National Institute of Arthritis, Metabolic and Digestive Diseases, F32-AM-05161. The technical assistance of Ms. Lynne DesJardins was greatly appreciated.

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