ion transport in
cortical collecting tubule; effect of amiloride

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STONER, L. C., M. B. BURG, AND J. ORLOFF. Ion transport in cortical collecting tubule; effect of amiloride. Am. J. Physiol. 227(2): 453-459, 1974.-Tubules were dissected from rabbit kidneys and perfused in vitro. In cortical collecting tubules the transepithelial electrical potential difference (PD) was -35 mV (lumen negative). There was net active sodium reabsorption from the lumen and net active potassium secretion into the lumen. There was net chloride reabsorption that was probably passive, but the analysis of the mechanism of chloride transport was complicated by the presence of chloride exchange diffusion. Amiloride (10^-5 M) in the lumen inhibited sodium and potassium transport and caused a reversal of the polarity of the PD; 10^-4 M amiloride in the bath had no effect. The positive PD was most likely caused by urinary acidification and decreased toward zero when acetazolamide was added to the bath, or when CO₂ was eliminated from the bathing solutions. In the thick ascending limb of Henle's loop the PD was +5.8 mV, which is attributed to active chloride reabsorption. Amiloride had no effect on either the PD or the net chloride transport. We conclude that amiloride inhibits active sodium and potassium transport in the cortical collecting tubule, but does not inhibit active chloride transport in the thick ascending limb, and that these effects account for the action of the drug previously observed in the intact kidney.

METHODS

Since the techniques of tubule perfusion have been described elsewhere (6-8), only a brief summary that includes pertinent modifications is presented here. Segments of cortical collecting tubules and thick ascending limbs, 0.6-3.9 mm long, were dissected from kidneys of New Zealand white rabbits. The isolated tubules were transferred to a temperature controlled chamber (37°C) and, unless otherwise specified, were perfused with a solution containing, in millimoles per liter: NaCl, 150; K₂HPO₄, 2.5; CaCl₂, 1.0; and MgSO₄, 1.2; titrated to pH 7.4 with HCl. The bathing solution contained NaCl, 115; KCl, 5; NaHCO₃, 25; Na acetate, 10; NaH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 1.0; dextrose, 5.5, and 5% vol/vol calf or rabbit serum. The bathing solution was bubbled with 95% O₂-5% CO₂ gas (pH = 7.4). In some experiments the sodium in the bath and perfusate was replaced with choline, or the chloride was replaced with sulfate (Table 1).

The tubules were perfused using concentric glass pipets and the ends of the tubule were insulated with Sylgard 184 (Dow Corning Corporation, Midland, Mich.), a viscous dielectric liquid. Either a Sage perfusion pump model 255-2 (Sage Instruments, Inc., White Plains, N.Y.) or gravity was used for perfusion. Tubule-fluid samples were collected for analysis under mineral oil by aspiration into a calibrated constriction pipet and the time for each collection was measured.

Sodium and potassium concentrations in bulk solutions were measured with an Instrumentation Laboratory model 143 flame photometer (Instrumentation Laboratory, Inc., Boston). Microsamples of perfused and collected fluid were analyzed for sodium and potassium content with an Aminco helium-glow photometer (33). Chloride was measured in bulk solutions with use of the Cotlove-Aminco chlorideometer (9), and in microsamples of perfused and collected fluid with a modification (6) of the Ramsey method (25). The final osmolality of the bath and perfusion fluids was measured.

administration of the pyrazine diuretic, amiloride, to dog, rat, or man increases the urinary excretion of sodium and decreases both urinary acidification and the excretion of potassium (1, 5, 13, 18, 26, 30). The inhibition of potassium excretion and the relatively small magnitude of the natriuresis led to the conclusion that site of action of the drug is the distal nephron (5, 18). This has been confirmed by result of stop-flow (1) and micropuncture studies (13).

In the present studies the technique of perfusion of isolated tubule segments in vitro has been used to determine directly the effect of the drug on two distal-nephron segments, the cortical collecting tubule and the cortical thick ascending limb of Henle's loop. The two segments have strikingly different electrolyte transport mechanisms. In the cortical collecting tubule the electrical PD is negative in the lumen (8, 17) and there is active reabsorption of sodium from and active secretion of potassium into the lumen. In contrast, the cortical thick ascending limb of Henle's loop exhibits a PD positive in the lumen and there is active transport of chloride out of the lumen (7). In the present studies low concentrations of amiloride in the lumen of cortical collecting tubules caused a reversal of the polarity of the PD and inhibited the active transport of sodium and potassium. Indirect evidence is presented that the positive PD observed under these circumstances is a consequence of continued urinary acidification, i.e., H⁺ secretion or bicarbonate reabsorption. The drug had no effect in the thick ascending limb of Henle's loop.

transepithelial PD; acetazolamide; thick ascending limb; urinary acidification
Fluid absorption was measured with use of $^{[32]}$iothalamate (Abbott) in the perfusate as a volume marker. The leak of $^{[32]}$I to the bath was negligibly small (mean clearance $0.02 \text{ nl mm}^{-1} \text{ tubule length min}^{-1}$).

The transtubular electrical PD was measured between calomel cells connected to the bath and perfusate with 0.16 M NaCl agar bridges. The PD was recorded with a Hewlett-Packard model 320 dual-channel recorder (Hewlett-Packard Co., Pasadena, Calif.) connected via an MPA6 differential preamplifier (Transidyne General Corp., Ann Arbor, Mich.). The electrical resistance of perfused collecting tubules was measured by injecting a known current ($2 \times 10^{-10} \text{ A}$) into the lumen via the perfusion pipet and recording the resulting voltage deflection at both ends of the tubule. The voltage deflection due to the resistance of the perfusion pipet itself was nulled with a bridge circuit (19). The equations used to calculate the electrical resistance have been previously reported (19).

Unidirectional fluxes were measured using $^{42}$K, $^{36}$Cl, $^{22}$Na, and $^{24}$Na (International Chemical & Nuclear Corp.) and calculated as previously (7). Two different experimental protocols were utilized:

1) In one the fluxes of two different ions were measured simultaneously. Lumen-to-bath fluxes were measured by perfusing with a solution containing both radioisotopes and collecting the bath (initially free of radioisotope) for analysis every 5–15 min as described previously (16). Subsequently, the tubule was perfused with a radioisotope free solution and the bath-to-lumen flux was measured by placing the radioisotopes in the bath and measuring the radioactivity of the collected tubule fluid. Lumen-to-bath fluxes were always measured first since it was difficult to decontaminate the bath sufficiently with the tubule in place once a high concentration of radioisotope had been placed in the bath. Either $^{22}$Na and $^{42}$K or $^{24}$Na and $^{36}$Cl were studied simultaneously and the radioactivity of the $^{22}$Na or $^{36}$Cl was determined after that of the $^{42}$K or $^{24}$Na had decayed. Samples for measurement of the fluxes were collected commencing 10–15 min after the radioisotopes were added to the lumen or bath. Flux rates were identical when calculated from consecutive collections in individual tubules, indicating that a radioisotope steady state was achieved by that time.

2) In the second experimental protocol tubules were perfused with a solution containing $^{24}$Na and suspended in a bath containing $^{22}$Na. The lumen-to-bath flux of $^{24}$Na was calculated from the decrease in concentration of $^{22}$Na in the collected perfusion fluid, with the assumption of no significant net fluid reabsorption (see RESULTS). The $^{22}$Na bath-to-lumen flux was calculated from the appearance of the isotope in the collected tubule fluid.

The radioactivity of the bath, perfusate, and collected tubule fluid was measured by liquid scintillation using Multisol liquid scintillation fluid (Isolab, Inc., Akron, Ohio) and a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.)

Net fluxes ($J_i$) of sodium, potassium, and chloride were calculated 1) as the difference between the unidirectional fluxes, or 2) from measurements of the chemical concentration in perfused and collected fluid. If one assumes no net fluid movement (see RESULTS), then (7):

$$J_i = \frac{V_L}{L} (C_o - C_L)$$

where $V_L$ is rate of collection of fluid in nanoliters per second, $L$ is the tubule length in centimeters, and $C_o$ and $C_L$ are the concentrations of sodium or potassium in the perfusion and collected fluids. When measured in the same experiment, the net flux of potassium calculated from unidirectional fluxes of $^{42}$K was not significantly different from that calculated from chemical measurements.

Permeability ($P_i$) to sodium, potassium, or chloride was calculated by dividing the unidirectional flux in the direction opposite to the net flux by the ion concentration ($C_i$).

Ideally, these fluxes should have been measured with zero transepithelial PD. Since it was not possible to short-circuit the tubules, the flux measurements were corrected to zero PD using the constant-field assumption (20). This correction assumes that the voltage gradient through the membrane is constant, that the activities of the ion at the membrane are proportional to those in the bathing solutions, and that the ion does not interact with a membrane carrier (20). Further, these assumptions apply only to a single limiting barrier. Since it is uncertain whether these assumptions are met in the present studies, we recognize that the results of the correction may not be strictly accurate. It is not clear, however, that any other correction would be more accurate and, in any event, corrections of this magnitude do not seriously affect the conclusions which are drawn.

Partial ion conductance ($g_i$) was calculated from the permeability as follows (modified from Ussing (34)):

$$g_i = P_i C_i \frac{F^2}{RT}$$

where $C_i$ is the concentration of the $i^{th}$ ion in the bathing medium, and $R$, $T$, and $F$ are constants. Results are expressed as means $\pm$ standard errors of the means (number of tubules). Statistical significance was estimated with use of the Student $t$ test for paired observations in the individual tubules.

RESULTS

Fluid absorption by cortical collecting tubules. When the bathing solution and the perfusate were equiosmolar the rate of
fluid absorption of collecting tubules was \(0.02 \pm 0.01\) (five tubules) \(\text{nl mm}^{-1} \text{ min}^{-1}\), which is not significantly different from zero. Previously, in comparable experiments at \(25^\circ\text{C}\) there also was no significant net fluid absorption (17).

Transepithelial PD of cortical collecting tubules. Under control conditions the mean transepithelial PD across the cortical collecting tubule was \(-35.4 \pm 3.3\) (16 tubules) \(\text{mV}\), lumen negative (range \(-15\) to \(-85\) mV). Lower mean values noted previously \((\text{-}10\text{ mV} (19) \text{ and } -25\text{ mV} (6)\) were obtained in studies at room temperature \((23^\circ\text{C})\). The difference is presumably due to the higher temperature \((37^\circ\text{C})\) of the solutions in the present studies.

The mean specific resistance \((R_T)\) of five collecting tubules was \(266 \pm 44 \Omega \text{ cm}^2\) (Table 2) and is comparable to the specific resistance of distal convoluted tubule \((4, 23)\). The electrical conductance, the reciprocal of \(R_T\), was \(3.78 \times 10^{-4} \Omega^{-1} \text{ cm}^2\) (Table 2).

**Sodium absorption from cortical collecting tubules.** The mean net sodium flux from lumen to bath was \(8.7 \pm 2.9\) (15 tubules) \(\text{pmol cm}^{-2} \text{ s}^{-1}\). In the previous studies at \(25^\circ\text{C}\) net sodium absorption was \(2.7\) \(\text{pmol cm}^{-2} \text{ s}^{-1}\) (16). The difference is presumably due to the higher temperature \((37^\circ\text{C})\) in the present studies. The net sodium transport must be active since it opposes the electrochemical gradient for sodium (17).

The permeability coefficient and partial ion conductance for sodium (Table 2) were calculated from the backflux of sodium (bath to lumen, Table 3). The sodium permeability was low, \(52 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}\) or \(0.03 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}\) (assuming a lumen diameter of \(20 \mu\text{m}\)). The sodium conductance calculated from the permeability is \(3.4 \times 10^{-9} \Omega^{-1} \text{ cm}^{-2}\), which is equal to approximately 15% of the total conductance, measured electrically (Table 2).

**Potassium secretion in cortical collecting tubules.** The net flux of potassium (from bath to lumen) was \(2.2 \pm 0.7\) (15 tubules) \(\text{pmol cm}^{-2} \text{ s}^{-1}\). The potassium flux ratio was low (Table 3). The PD necessary for such a flux ratio to be explained by passive diffusion was calculated from Ussing’s flux-ratio equation (34), which assumes passive transport and no interaction with the membrane or other ions:

\[
\frac{J_{\text{lb}}}{J_{\text{bl}}} = \frac{C_b}{C_l} \cdot \exp \left( -\frac{zFE_i}{RT} \right)
\]

where \(E_i\) is the required transepithelial PD, \(J_{\text{lb}}\) and \(J_{\text{bl}}\) are fluxes of ion from lumen to bath and bath to lumen, respectively, \(C_b\) and \(C_l\) are the concentrations of ion in bath and lumen, and \(R, T,\) and \(F\) are constants. \(E_i\) was \(-59\text{ mV}\) (Table 3), which is significantly greater than the measured PD of \(-35\text{ mV}\). Therefore, by this criterion potassium transport is not strictly passive. The discrepancy can be accounted for by active potassium transport which is in agreement with the previous finding (17), that potassium transport resulted in concentration gradients at slow flow rates which exceeded the gradients that might theoretically be achieved by passive transport according to the Nernst equation. Although the present finding with respect to the flux ratio is consistent with active potassium transport, it could also be explained by single-file diffusion (21).

The permeability of the tubule to potassium (calculated from the lumen-to-bath flux, Table 3) is an order of magnitude greater than that to sodium (Table 2). The potassium partial ion conductance, however, is lower because of the much lower concentration of potassium in the bathing media. The partial ion conductance of potassium is equal to approximately 5% of the total electrical conductance.

From measurements of the concentration of sodium and potassium in collected fluid, Grantham et al. (17) concluded that there was nearly equal exchange of sodium for potassium in collecting tubules perfused at slow rates. The ratio of the net flux of sodium to potassium was 4 to 1 in the present experiments. Among other differences, the perfusion rate used in the present study was faster (range 5–33 \(\text{nl min}^{-1}\)) to test the effect of perfusion rate, in five additional studies tubules were perfused at a slower rate (1 \(\text{nl min}^{-1}\)). The ratio of net sodium to potassium transport (determined chemically) was \(1.43 \pm 0.15\), which is similar to the earlier result and suggests an as yet undefined effect of perfusion rate on the relative rates of sodium and potassium transport.

**Chloride absorption from cortical collecting tubules.** The mean net chloride flux was \(6.7 \pm 2.7\) (four tubules) \(\text{pmol cm}^{-2} \text{ s}^{-1}\), which is equal to the difference between the mean net sodium absorption \((0.7\text{ peq cm}^{-2} \text{ s}^{-1})\) and potassium secretion \((2.2\text{ peq cm}^{-2} \text{ s}^{-1})\).

The net chloride flux could be passive since it is in the direction of its electrochemical gradient. The chloride flux ratio, however, is near 1 (Table 3) and \(E_{\text{Cl}}\) \((-5\text{ mV}\), Table 3) is significantly lower than the observed PD \((-35\text{ mV}\)). The discrepancy is consistent with chloride exchange diffusion. If a chloride flux by exchange diffusion of 13.5 pmol...
cm⁻¹ s⁻¹ (equal to 85% of the backflux) were subtracted from the flux in each direction, the ratio of the remaining fluxes would fit the measured PD according to the flux-ratio equation.

Chloride permeability and conductance (calculated from the bath-to-lumen flux, Table 3) were high (Table 2). In fact the calculated partial chloride conductance (20.2 × 10⁻² Ω⁻¹ cm⁻²) is greater than the total electrical conductance (3.78 × 10⁻³ Ω⁻¹ cm⁻²). Therefore, most of the measured flux of chloride is electrically silent, presumably by exchange diffusion. From the difference between the total electrical conductance (3.78 × 10⁻³ Ω⁻¹ cm⁻²) and the sum of the partial sodium and potassium conductances (.54 + .17 Ω⁻¹ cm⁻²), the maximum electrical conductance attributable to Cl is 3.07 × 10⁻³ Ω⁻¹ cm⁻², which is 15% of the partial chloride conductance calculated from the radioisotope flux. Therefore, 85% of the observed ³⁶Cl backflux of 15.8 pmol cm⁻¹ s⁻¹ may be exchange diffusion, a finding consistent with the result of the calculation from the Cl flux ratio (Table 2) and PD, given earlier.

The net passive chloride (J_Cl) transport is given by:

\[ J_{Cl} = \frac{g_{Cl} E \left[ (C_{Cl_b} - C_{Cl_l}) - \frac{E_{R}}{RT} \right]}{F \left[ 1 - \frac{E_{R}}{RT} \right]} \]

(modified from ref. 20) where \( C_{Cl_l} \) and \( C_{Cl_b} \) are the chloride concentrations in the perfusate (152 meq liter⁻¹) and bath (122 meq liter⁻¹), respectively, and \( E \) is the observed PD (−35 mV). Assuming that the actual electrical conductance attributable to chloride \( (g_{Cl}) \) is 3.07 × 10⁻³ Ω⁻¹ cm⁻² (or 1.97 × 10⁻⁶ Ω⁻¹ cm⁻¹, assuming a lumen diameter of 20 μm):

\[ J_{Cl} = 8.9 \text{ peq cm}^{-1} \text{ s}^{-1} \]

which is approximately equal to the measured mean net chloride flux (6.7 peq cm⁻¹ s⁻¹). Thus, the chloride conductance, excluding exchange diffusion, is probably great enough so that the observed net chloride flux could be passive.

**Effect of amiloride on cortical collecting tubule.** When tubules were perfused with as little as 10⁻⁵ amiloride, the PD reversed polarity and became positive in the lumen as illustrated in Fig. 1. The change occurred within a few seconds and was immediately reversible when the drug was removed. A decrease of the PD in the presence of amiloride has been observed in anuran membranes (10, 11, 14) and the submaxillary salivary gland duct (28). The mean trans-epithelial PD was unaffected by the presence of 10⁻⁴ M amiloride (mean control PD = −35 mV). Assuming that the actual electrical conductance attributable to chloride \( (g_{Cl}) \) is 3.07 × 10⁻³ Ω⁻¹ cm⁻² (or 1.97 × 10⁻⁶ Ω⁻¹ cm⁻¹, assuming a lumen diameter of 20 μm):

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**Origin of positive PD in cortical collecting tubules.** The positive PD observed when cortical collecting tubules were exposed to 10⁻⁵ M amiloride is not readily explained by the movements of Na or K ions since the net movements of both ions are greatly reduced. A similar result in turtle bladder (29, 31) and toad bladder (22) was attributed to unmasking of the positive PD caused by hy-
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hydrogen ion secretion (or bicarbonate reabsorption) that became evident only when the opposing negative PD caused by sodium transport was eliminated. To test whether the change in PD in the cortical collecting tubule can be similarly explained, sodium transport was inhibited either by replacement of all the sodium in both the bath and perfusion solution by choline, or by exposure of tubules to a bath containing $10^{-5}$ M ouabain. Both manipulations resulted in a positive PD (+9 mV (five tubules) when sodium was replaced with choline and +8 mV (five tubules) when $10^{-5}$ M ouabain was added to the bath). As noted elsewhere, removal of potassium from the bath also inhibits sodium transport and causes the PD to become lumen positive in collecting tubules (17). It is apparent that reversal of the polarity of the PD is not a specific effect of amiloride but results from inhibition of sodium transport per se. To exclude the possibility that chloride transport might account for the positive PD, as it did in some (15) but not other (22) toad bladder studies, cortical collecting tubules were perfused in the absence of chloride (sulfate substitution). Under these conditions, with $10^{-5}$ M amiloride in the lumen, the PD was +18 mV (Fig. 2) even in the absence of chloride. This result excludes chloride transport as a possible source of the lumen-positive PD.

In turtle (29) and toad bladder (22) acidification is believed to be the cause of the positive PD since acetazolamide, a known carbonic anhydrase inhibitor (24), simultaneously abolishes both the positive PD and the acidification process. To test this possibility in the collecting tubule, we added $2 \times 10^{-4}$ M acetazolamide to the bath while amiloride was present in the lumen. As can be seen in Fig. 2, the positive PD under these conditions (Cl free, amiloride in the lumen) was reduced by acetazolamide, suggesting that the PD results from urinary acidification. The result was similar in the presence of chloride (Table 5). Furthermore, the negative PD (in the absence of amiloride) was increased by acetazolamide in the bath (Table 5). This result is consistent with the view that a positive component of the PD due to acidification is present even when the measured PD is negative, as in the control state. Under these conditions the positive component of the PD is obscured by the larger negative PD that results from Na transport.

The urinary acidification system (and the reversal of the PD) of toad bladder (22) and turtle bladder (27, 29) depend in part on the presence of CO$_2$ in the bathing medium. To test this relationship in the cortical collecting tubule, a positive PD was produced by 1) Na$^+$ replacement with choline, or 2) $10^{-4}$ M amiloride in the lumen, or 3) $10^{-5}$ M ouabain in the bath (Fig. 3). In addition, the perfusate (which is free of bicarbonate) was also placed in the bath. As can be seen in Fig. 3, when the solutions were gassed with CO$_2$ there was a positive PD (average +8 mV). When the solutions were gassed without CO$_2$ (100% O$_2$), however, there was a reversible decrease in the PD to a mean value of +3 mV. The pH of the bicarbonate-free solution was 7.4 when gassed with O$_2$ and 6.4 when gassed with 5% CO$_2$. Thus, the exogenous bicarbonate normal-
TABLE 6. Effect of amiloride (10^-4 M) on thick ascending limb of Henle's loop

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Amiloride</th>
<th>Difference</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD, mV</td>
<td>5.8</td>
<td>5.4</td>
<td>0.4 ± 0.2</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Net chloride flux,peq cm^-1 s^-1</td>
<td>9.3</td>
<td>9.0</td>
<td>0.3 ± 0.5</td>
<td>&gt;1</td>
</tr>
</tbody>
</table>

Amiloride was placed in both the lumen and the bath, in each of four tubules. Triplicate control or experimental measurements were made over a period of 9 to 15 min. The experimental period began 10 min after the amiloride was added and the after-control period, 10 min after the drug was removed. Control values represent the means of the pre- and postcontrol periods.

DISCUSSION

The transepithelial PD across the cortical collecting tubule, which is ordinarily oriented negatively in the lumen, apparently results from the balance of several ion transport processes. The negative PD is caused by active Na absorption out of the lumen, and may be shunted by the accompanying passive transport of Cl from the lumen or by the active and passive transport of K into the lumen. In addition, urinary acidification (hydrogen ion secretion or bicarbonate reabsorption) may generate a small opposing positive PD.

The "K-sparing effect" of amiloride (1, 5, 13, 18, 26, 30) is readily explained by inhibition of the potassium secretion previously found in the distal convoluted tubule (13) and the nearly complete inhibition of K secretion demonstrated in the present studies in the cortical collecting tubule. It is interesting that although it greatly inhibits sodium transport in the cortical collecting tubule, the drug causes only a limited natriuresis or diuresis under control conditions (1, 5, 13, 26). The drug had no effect on NaCl reabsorption in the thick ascending limb of Henle's loop (present studies) or in the more proximal segments (1, 13), and had an equivocal effect on Na transport in the distal convoluted tubule (13). Normally as a result of transport of NaCl out of earlier segments including the thick ascending limb of Henle's loop and the distal convoluted tubule, the amount of Na which reaches the cortical collecting tubule is relatively small and limits the net sodium reabsorption from this segment. Thus, inhibition of Na transport in the cortical collecting tubule could normally result in only a relatively small increase in Na excretion. Amiloride causes a larger increase in sodium excretion when Na excretion has already been elevated by other diuretics (1, 26). Under these conditions a larger than normal fraction of the filtered Na reaches the collecting tubule and is reabsorbed, so that inhibition of the Na transport by amiloride has a larger effect.

There is also a marked decrease in urinary acidification in (1, 18, 26) dog, rat, and man in the presence of amiloride, which is in apparent contradiction to the result of the present study in which the positive PD in the presence of the drug was attributed to persistent urinary acidification. Guignard and Peters (18), however, have suggested that the decreased acidification in vivo may result from a decrease in the transepithelial PD which they assumed to occur in the distal nephron. They pointed out that a high, negative PD favors the acidification of urine by passive mechanisms and that acidification might be reduced along with the PD as a result of the action of amiloride. Although the persistence of an acetazolamide-sensitive, positive PD in the presence of amiloride in the collecting tubules suggests that there is an active urinary acidification process which is not inhibited by the drug, there could be a decrease in the total rate of acidification because of inhibition of the passive component. Direct studies of acidification in the collecting tubule are required to confirm this hypothesis.

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