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Role of Cl^- in Electrogenic H^+ Secretion by Cortical Distal Tubule

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Abstract. The presence of an electrogenic H^+-ATPase has been described in the late distal tubule, a segment which contains intercalated cells. The present paper studies the electrogenicity of this transport mechanism, which has been demonstrated in turtle bladder and in cortical collecting duct. Transepithelial PD (V_t) was measured by means of Ling-Gerard microelectrodes in late distal tubule of rat renal cortex during in vivo microperfusion. The tubules were perfused with electrolyte solutions to which 2×10^{-7} M bafilomycin or 4.6×10^{-8} M concanamycin were added. No significant increase in lumen-negative V_t upon perfusion with these inhibitors as compared to control, was observed as well as when 10^{-3} M amiloride, 10^{-5} M benzamil or 3 mM Ba^{2+} were perfused alone or in combination. The effect of an inhibition of electrogenic H^+ secretion, i.e., increase in lumen-negative V_t by 2–4 mV, was observed only when Cl^- channels were blocked by 10^{-5} M 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB). This blocker also reduced the rate of bicarbonate reabsorption in this segment from 1.21 ± 0.14 ($n = 8$) to 0.62 ± 0.03 (8) nmol.cm^{-2}.sec^{-1} as determined by stationary microperfusion and pH measurement by ion-exchange resin microelectrodes. These results indicate that: (i) the participation of the vacuolar H^+ ATPase in the establishment of cortical late distal tubule V_t is minor in physiological conditions, but can be demonstrated after blocking Cl^- channels, thus suggesting a shunting effect of this anion; and, (ii) the rate of H^+ secretion in this segment is reduced by a Cl^- channel blocker, supporting coupling of H^+-ATPase with Cl^- transport.

Key words: H^+-ATPase — Transepithelial PD — Distal tubule — Bicarbonate reabsorption

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Introduction

The mammalian cortical distal tubule has been shown to be an important site of urine acidification. It possesses the ability to reabsorb or to secrete bicarbonate, according to the prevailing acid-base conditions (Capasso et al., 1987; Chan, Malnic & Giebisch, 1989; Levine, Vandorpe & Iacovitti, 1990; Wesson, Dolson & Babino, 1991). The nature of the processes underlying H^+ secretion in this segment is now partly defined. Na^+-H^+ exchange is the major mechanism in the early distal tubule and an H^+-adenosine triphosphatase (H^+-ATPase) predominates in the late distal tubule (Wang et al., 1993; Fernández et al., 1994). An H^+-K^+ ATPase similar to that of gastric mucosa was found in the more distal portions of this segment (Wingo et al., 1990; Gifford, Rome & Galla, 1992).

We have defined the distal tubule as the part of the nephron between the macula densa region and the first confluence of that nephron with another. The early distal tubule includes the distal convoluted tubule (DCT), whereas the late distal tubule includes the connecting tubule (CNT) and the initial collecting tubule (ICT). These segments contain several structural components similar to those of the adjacent segments. There are intercalated cells in the connecting segment and in the initial collecting duct, which are portions of the late distal tubule (Kriz & Kaissling, 1985). It is, therefore, probable that the mechanism of acidification of the late distal tubule may possess similarities with that of the cortical collecting duct. In 1982, Koeppen and Helman (1982) have shown that the rabbit cortical collecting duct studied in vitro when perfused and bathed in 0 Na^+/0 K^+ Ringer developed a lumen positive transepithelial electrical potential difference (V_t). This lumen positive V_t was reduced by acetazolamide, was increased by mineralocorticoids, was sensitive to the PCO_2 of the bathing solutions but was not dependent on Cl^- in either the

luminal or bath solutions. They concluded that the secretion of H^+ by the cortical collecting tubule is electrogenic, similar to H^+ secretion in the turtle bladder, mediated by a vacuolar H^+-ATPase.

If an electrogenic H^+-ATPase were to participate in late distal acidification, the inhibition of this pump is expected to increase the lumen negative V_t due to abolition of a positive pump current into the lumen (Al-Awqati, Mueller & Steinmetz, 1977). However, in previous experiments we have observed that the perfusion with 2×10^{-7} M bafilomycin does not affect V_t significantly. V_t was -37.8 ± 3.52 mV in controls *vs.* -34.0 ± 2.41 mV with bafilomycin (Fernández et al., 1994). These experiments suggest that the participation of H^+ secretion in the establishment of distal tubule V_t is minor and that the tranfer of H^+ is possibly being electrically neutralized by the flow of other co- or counterions.

The aim of the present work is to study the role of an H^+-ATPase in the establishment of late distal cortical tubule V_t, and to determine why its effect on V_t is low, investigating the nature of the ionic shunt current that might be responsible for the neutralization of the electrogenicity of this pump. For this purpose we used the in vivo microperfusion technique, applying specific inhibitors of the H^+-ATPase: bafilomycin A1 and concanamycin, and inhibitors of the other ionic currents: K^+ (Ba^{2+}), Na^+ (amiloride), and Cl^- (NPPB).

Materials and Methods

Two groups of male Wistar rats weighing 180–240 g were used in the experiments. Control rats received tap water and a standard pellet diet containing 10% protein up to the time of the experiment, and received isotonic saline plus 3% mannitol at a rate of 0.07 ml/min during the experiments. In a second group, chronic metabolic acidosis was induced by adding 75 mM NH_4Cl to distilled drinking water for 72 hr. These rats received 75 mM NH_4Cl and 75 mM NaCl plus 3% mannitol at 0.07 ml/min during the experiment.

After anesthesia by 100 mg/kg Inactin (Byk-Gulden, Konstanz, Germany) given peritoneally, the rats were prepared for micropuncture as described previously (Gil & Malnic, 1989). In brief, the left jugular vein and carotid artery were cannulated for infusions and blood withdrawal, respectively. A tracheostomy was performed. The kidney was isolated by a lumbar approach and immobilized *in situ* by Ringer-agar in a lucite cup. It was superfused with mammalian Ringer solution at 37°C.

Transepithelial PD Measurement

The microperfusion procedure involved impalement of a proximal loop with a double barreled micropipette made from theta glass tubing (R & D Optical Systems, Spencerville, MD). One barrel was filled with the control perfusion solution colored with 0.05% FD & C green and the other with the experimental perfusion solution. The control perfusion solution was used to detect early and late distal tubule loops. When two surface loops of a distal tubule were detected, the double-barreled micropipette was tranferred to the first loop in order to perfuse the late distal segment. Perfusions were performed by applying pressure to the double barreled micropipettes via hand-held oil filled syringes. This method allowed for perfusions at a rate sufficient to minimize alterations in the composition of the solutions by the transport mechanisms of the segment between site of perfusion and measurement. After control perfusions, solutions of the same electrolyte composition but containing bafilomycin A1 or concanamycin were used, followed again by the control solution.

A Ling-Gerard microelectrode made from 1.2 mm o.d. borosilicate glass with an internal filament (Hilgenberg, Malsfeld, Germany) was inserted into a late distal loop. It contained 1 M KCl colored by FD & C green and the transepithelial electrical potential difference (V_t) was the difference between this microelectrode and ground. Input resistance of these electrodes was between 3 and 10 MΩ. The rat was grounded by connecting its tail to a Ag/AgCl electrode. Voltages were read by a WPI (Sarasota, FL) model FD 225 differential electrometer in single-ended configuration, the output of which was recorded on a Beckman (Model R511A) Dynograph. Junction potentials between the electrode tip and the perfusion solutions were determined in vitro by superfusion of the kidney with the used solutions after impalements, the measured V_t being corrected for this value.

Stopped-Flow Microperfusion

The last group of experiments was designed to analyze the effect of NPPB on late distal tubule acidification. The microperfusion procedure, which has been previously described (Fernández et al., 1994), involved impalement of a proximal loop with a double-barreled micropipette. One barrel was filled with Sudan black colored castor oil used to block fluid columns to keep them stationary in the tubule lumen, and the other with the control perfusion solution (solution 1) colored with 0.05% FDC-green, which was used to detect early and late distal loops. Further downstream in the proximal tubule or in the early distal loop, a single micropipette containing the perfusion solution with 10^{-5} M NPPB was inserted. After the control perfusion a column of oil was injected into the late proximal tubule lumen, blocking the flow of fluid. By this procedure, initial bicarbonate concentrations, after the lumen was blocked with oil, were near the 25 mM of the perfusion solution. An asymmetric double—barreled microelectrode made from Hilgenberg (Malsfeld, Germany) filament containing glass capillaries was then inserted into a late distal loop. pH was measured as the voltage difference between the two barrels of the microelectrode. The larger barrel contained a H^+-sensitive ion exchange resin (Fluka, Buchs, Switzerland) as described by Ammann (1981), and the smaller one contained 1 M KCl colored by FD & C green (reference barrel). Transepithelial electrical potential difference (V_t) was the difference between the reference barrel and ground. The microelectrode slope in bicarbonate buffered solutions was 58.4 ± 1.85 mV/pH unit ($n = 9$) (Lopes & Malnic, 1993).

Voltages were read by means of a differential electrometer (WPI mod. FD 223), the output of which was recorded on a Beckman mod. R511A Dynograph and digitized in 1-sec intervals by means of an AD conversion board (Data Translation model DT 2801, Marlborough, MS) mounted on a 386 DX (DELL 333D) IBM-PC compatible microcomputer.

From the time course of the pH change, the concentration of HCO_3^- was calculated by the Henderson-Hasselbalch equation, assuming a pCO_2 equivalent to that in systemic blood (Mello Aires, Lopes & Malnic, 1990). The log of $(HCO_3^-)t - (HCO_3^-)s$ was plotted against time in seconds; the subscript t refers to time t, and s to the stationary situation. This plot generated a straight line, indicating that the buffer concentrations approach their steady state value in an exponential manner. The half-time ($t½$) of the approach of bicarbonate concentrations to their steady-state value was calculated from the slope of these lines. $JHCO_3^-$ per square centimeter of tubule epithelium was obtained according to the following relation (Gil & Malnic, 1989):

Table 1. Composition of solutions

	Solution 1 10 mM K^+	Solution 2 10 mM K^+ + Benzamil	Solution 3 0 mM Cl^- + Ba + Amil.	Solution 4 0 mM Cl^- + NPPB	Solution 5 0 mM Cl^- + Ba Amil. + NPPB	Solution 6 Peritubular perfusion
NaCl	80	80				
KCl	10	10				
$CaCl_2$	1	1				
$MgSO_4$	1.2	1.2	1.2	1.2	1.2	1.2
$NaHCO_3$	25	25	25	25	25	
Na^+ Gluconate			80	80	80	80
K^+ Gluconate			5	5	5	5
Ca^{++} Gluconate			1	1	3	3
Hepes						17.8
Amiloride			1		1	
Ba^{2+}			3		3	
Benzamil		10^{-5} M				
NPPB				10^{-5} M	10^{-5} M	

Values are mM except for benzamil and NPPB. All the solutions have raffinose to reach isotonicity.
All the solutions have a pH of 7.8 except solution 6 which has a pH of 7.4.
NPPB: 5′-Nitro-2-(3-Phenylpropyl-amino)-benzoic acid
Ba: Ba^{2+}
Amil.: Amiloride

$$JHCO_3^- = (ln\ 2/t½) \cdot (HCO_3^- o - HCO_3^- s) \cdot (r/2)$$

where r is the tubular radius.

The pH microelectrodes were calibrated before and after every impalement on the kidney's surface by superfusion with 20 mM phosphate Ringer buffer solutions containing 120 mM NaCl at 37°C. Their pH was adjusted to 6.5, 7.0 and 7.5.

The composition of perfusion solutions is described in Table 1. They contain raffinose to reach isotonicity to avoid volume changes during the measurements. The experimental perfusion solution in each case had the same composition as the control solution plus 2×10^{-7} M bafilomycin A or 4.6×10^{-8} M concanamycin. Amiloride was obtained from Merck, Sharp & Dohme (Rahway, NJ). Benzamil was obtained from Prof. E.J. Cragoe, Jr. Bafilomycin A1 was obtained from Prof. K. Altendorf, University of Osnabrück, Germany. Barium chloride was obtained from Sigma, St. Louis, MO. Concanamycin A was obtained from Mikrobielle Chemie, Ciba, Basel, Switzerland, and NPPB (5-Nitro-2-(3-phenylpropylamino)-benzoic acid) from Prof. R. Greger, Freiburg University, Germany.

Blood pH and pCO_2 were measured by means of an Instrumentation Laboratory model 11/329 blood gas system. Statistical comparisons were made by the paired *t*-test.

Results

A total of 40 control rats had a mean arterial blood pH of 7.37 ± 0.007 (mean ± SE), blood PCO_2 of 33.4 ± 0.90 mm Hg and plasma bicarbonate of 18.7 ± 0.46 mM. Acidotic rats had arterial blood pH of 7.20 ± 0.02, PCO_2 of 38.1 ± 3.35 mm Hg and bicarbonate of 14.4 ± 1.25 mM ($n = 5$).

In a preliminary series of experiments, the V_t of the late distal tubule was −34 ± 3.16 mV ($n = 10$) when perfused by solution 1 and the perfusion with the same solution plus 3 mM $BaCl_2$ (a blocker of K^+ channels) results in a hyperpolarization of V_t: −52 ± 3.46 mV ($n = 10$), as expected in an epithelium whose cells express a significant K^+ conductance in the apical membrane.

Table 2 shows experiments in which paired comparisons between control and bafilomycin- (or concanamycin-) containing solutions were performed. In these experiments, surface late distal tubules (connecting segment and initial collecting tubule) were localized by perfusion of proximal tubules with FDC-green colored perfusion fluid. They were then perfused with two solutions of the same ionic composition (*see* Table 1), one containing bafilomycin or concanamycin (experimental) and the other not (control). The late distal segment was impaled by a microelectrode and V_t measured during perfusion with the two solutions. Every tubule was perfused with only one pair of solutions. In the first group of tubules the lumen was perfused with 10 mM K^+ plus other ionic components made up to approximately resemble physiological late distal solution (solution 1), or with solutions containing blockers of Na^+ channels (benzamil or amiloride) or of K^+ channels (Ba^{2+}) (solutions 2 and 3). In most experiments there were no significant alterations of V_t of late distal tubule when 2×10^{-7} M bafilomycin was added to the perfusion fluid. In rats submitted to chronic (3–5 days) metabolic acidosis induced by drinking 75 mM NH_4Cl in order to stimulate H^+ secretion, a lumen-positive V_t was seen in control perfusions, which is compatible with electrogenic secretion of H^+ by a H^+-ATPase. However, the perfusion with bafilomycin resulted in an increase in the luminal positivity, contrary to what was expected by inhibiting the H^+-ATPase.

Table 2. Transepithelial potential difference (V_t) in mV of late distal cortical tubules perfused with solutions which contain 2×10^{-7} M bafilomycin (experimental) or not (control).

	n	Control	Experimental	*P*
10 mM K^+ (Solution 1)	7	−46.52 ± 6.23	−42.29 ± 5.85	NS
10 mM K^+ + Benzamil (Solution 2)	9	−11.78 ± 2.63	−11.21 ± 2.13	NS
0 mM Cl^- + Ba + Amil. (Solution 3)	8	−2.02 ± 1.58	0.83 ± 1.31	NS
0 mM Cl^- + Ba + Amil. (Acidotic rats)	18	2.09 ± 1.57	3.94 ± 1.38	0.014
0 mM Cl^- + NPPB (Solution 4)	7	−17.8 ± 1.95	−22.2 ± 2.68	0.02
0 mM Cl^- + Ba + Amil. + NPPB (Solution 5)	12	−0.07 ± 1.32	−2.31 ± 1.42	0.01
0 mM Cl^- + Ba + Amil. + NPPB (Solution 5—Acidotic rats)	12	−0.78 ± 1.53	−3.61 ± 1.64*	0.001
0 mM Cl^- + Ba + Amil. + NPPB (Luminal perfusion) 0 mM Cl^- + HEPES (Peritubular perfusion)	25	−3.33 ± 1.69	−4.66 ± 1.72	0.01

n = number of tubules. Values are means ± SE; compared by the paired *t*-test.
Ba: Ba^{2+}. Amil.: Amiloride
* This experimental perfusion contains 4.6×10^{-8} M concanamycin.

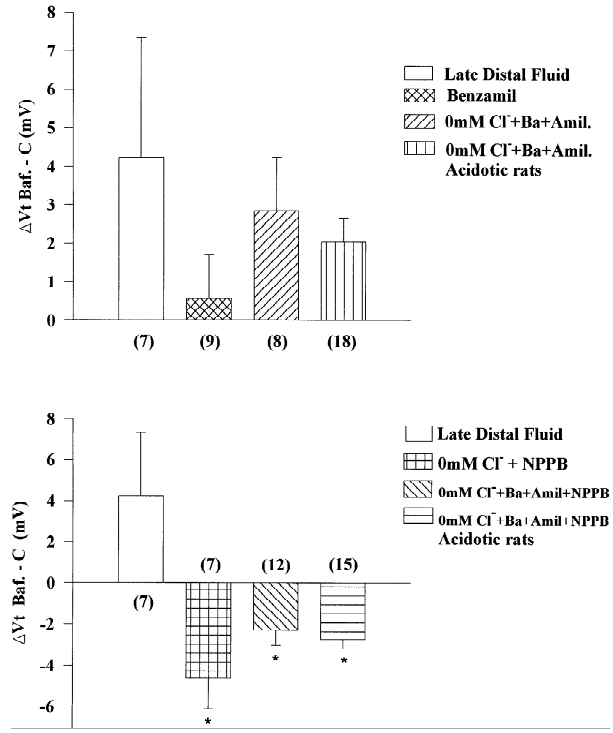

Fig. 1. Mean paired differences of V_t (bafilomycin—control) in the absence (*top*) and presence of 10^{-5} M NPPB (*bottom*). Values are means ± SE; $*P < 0.05$ compared with 0 mV by paired *t* test; ( ), number of tubules.

If the H^+-pump current were small, its contribution to the V_t could be concealed by other ionic currents in the apical membranes of tubule cells. This effect or incomplete blocking of channels of the other cations could be responsible for the lumen-negative V_t as well as for the absence of the expected effect of inhibition of the vacuolar H^+-ATPase. A summary of these results is given in the upper portion of Fig. 1.

The fact that during blockade of apical Na^+ and K^+ channels no significant alterations of V_t during perfusion with bafilomycin was observed led us to block chloride channels, which might carry a shunt for the current carried by the H^+-ATPase. For this purpose, we utilized 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), a specific blocker of Cl^- channels in the basolateral membrane of thick ascending limb of Henle's loop (Wangemann et al., 1986). Table 2 shows that during the perfusion with 10^{-5} M NPPB and zero chloride (solution 4) V_t was −17.8 ± 1.95 mV and turned significantly more negative by the perfusion with bafilomycin: −22.2 ± 2.68 mV. Also when Na^+, K^+ and Cl^- channels were blocked (solution 5), bafilomycin or concanamycin caused an increase in lumen-negative V_t (*see* Table 2 and Fig. 1). Thus, the reduction of chloride transport by the apical membrane allows to detect the electrogenic component of H^+ secretion.

To rule out Cl^- recycling between the peritubular space and tubular lumen via the paracellular path, the capillaries next to the studied tubule were perfused with a zero chloride solution (solution 6). When the tubular lumen was perfused with solution 5 (with similar ionic composition as solution 6) the transepithelial PD depolarized but did not become positive (Table 2). As in the previous group, this depolarization was partially reverted when the tubule was perfused with bafilomycin. Thus, only when the apical chloride current was eliminated a small electrogenic effect of the H^+-ATPase became evident.

In the last series of experiments, bicarbonate reabsorption was measured during perfusion with solution 1 in the presence and absence of NPPB 10^{-5} M. The results are given in Fig. 2 and Table 3. There was no significant difference between stationary pH in controls (6.30 ± 0.14) and with NPPB (6.42 ± 0.09, $n = 8$) (Table 3). The half-time of bicarbonate disappearance from the lumen ($t½$) was significantly and consistently increased in all the tubules perfused with NPPB (7.44 ± 0.25 *s* in control *vs.* 13.57 ± 1.11 *s* with NPPB, $n = 8$), and as a consequence the rate of bicarbonate reabsorption was

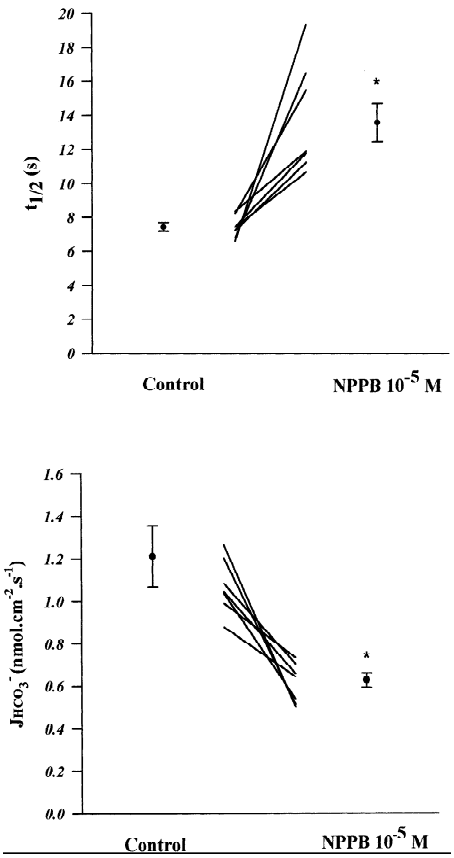

Fig. 2. Acidification half-times ($t_{1/2}$) and bicarbonate reabsorption (J_{HCO3}) in late distal tubule during luminal perfusion with 10^{-5} M NPPB compared to controls. Lines connect means of perfusion data obtained in the same segment. *$P < 0.05$, paired t test.

Table 3. Effects of 10^{-5} M NPPB on stationary pH (pHs) and V_t of late distal tubules

	n	pHs	V_t (mV)
Control	8	6.30 ± 0.14	−38.4 ± 4.87
NPPB	8	6.42 ± 0.09	−32.8 ± 4.82
Recovery	7	6.56 ± 0.12	−31.7 ± 3.63

Values are means ± SE; n, of no. of tubules. pHs, stationary pH; V_t, transepithelial potential difference.

significantly decreased (1.21 ± 0.14 nmol · cm^{-2} · sec^{-1} in control *vs.* 0.62 ± 0.03 nmol · cm^{-2} · sec^{-1} with NPPB, $n = 8$) (Fig. 2). These data show that Cl^- conductance does not only shunt the electrogenicity of the H^+ pump, but is also important to maintain its normal transport activity. The effect of NPPB was not completely reversible, as noted by the fact that the J_{HCO3}- during the recovery period is decreased relative to the control period (0.80 ± 0.08 *vs.* 1.21 ± 0.14 nmol · cm^{-2} · sec^{-1}), although higher than during NPPB perfusion (0.62 ± 0.03 nmol · cm^{-2} · sec^{-1}).

Discussion

Role of H^+-ATPase in Transepithelial V_t

The last surface loop of the cortical distal tubule is constituted of either connecting segments or initial collecting ducts; both segments contain intercalated cells besides connecting tubule cells and principal cells, respectively (Kriz & Kaissling, 1985). The apical membrane of α intercalated cells is the site of two major acid secreting mechanisms: a vacuolar H^+ ATPase and a gastric-type H^+-K^+ ATPase. The presence of these mechanisms is supported by several lines of evidence, including microperfusion studies with inhibitors, biochemical determinations of carrier molecules and immunocytochemical localization (Brown, Hirsch & Gluck, 1988; Planelles et al., 1991; Wang et al., 1993; Fernández et al., 1994). The β intercalated cells secrete HCO_3^- and absorb Cl^- via apical Cl^-/HCO_3^- exchange.

The transepithelial PD across the late distal cortical tubule, which is ordinarily oriented in a lumen-negative way (−20 to −50 mV), results from the balance of the several ion transport processes present in the apical and basolateral membranes. The lumen negative PD is caused mostly by Na^+ transport from lumen to cell via Na^+ channels in the apical membrane along a favorable electrochemical potential gradient created by the basolateral sodium pump, and may be shunted by the accompanying passive transport of Cl^- from the lumen or by the transport of K^+ into the lumen. The secretion of H^+ into the lumen by the apical vacuolar H^+ ATPase may generate a small opposing positive PD. From the data of previous work (Fernández et al., 1994) and applying Ohm's law ($V = I \times R$), where V is the transepithelial voltage, I is the H^+ current into the lumen, obtained from a mean rate of distal HCO_3^- reabsorption of 1 nmol/cm^2.sec and Faraday's constant, and taking a resistance of 100 Ω.cm^2 as the resistance, R, of distal tubule (Bermudez & Windhager, 1975), the voltage generated by the proton currents in the late distal tubule may be of up to 10 mV. Blocking all conductive pathways R should be higher, and also the pump contribution to late distal voltage is expected to be higher. However, this reasoning is an oversimplification, since the distal epithelium is a heterogeneous structure. This aspect will be considered below (*see* Appendix).

The perfusion of rabbit cortical collecting duct with Na^+-free solutions and/or 10^{-5} M amiloride has been shown to revert its polarity, causing a lumen-positive PD (Stoner, Burg & Orloff, 1974; Koeppen & Helman,

1982). The effect caused in the late cortical distal tubule by the blockade of the apical pathway for Na^+ was not as extensive (Table 2). The simultaneous blockade of apical pathways for Na^+ and K^+ determined an abrupt reduction in V_t (Table 2) but did not lead to a reversion like that found in rabbit cortical collecting duct. Only when H^+ secretion was stimulated by chronic metabolic acidosis a PD reversion, (2 mV lumen positive), was observed. This result is compatible with a small positive current generated by the H^+ ATPase. It is not clear why the perfusion with bafilomycin resulted only in this group in an increase in the luminal positivity.

What could be the reason for the difficulty in recognizing an electrogenic effect of the distal tubule H^+-ATPase? The tranfer of H^+ might be electrically shunted by cofluxes of anions or by counterfluxes of cations. Among the ions that may be responsible for this shunting the most important are Na^+, K^+ and Cl^-. The model described in the appendix tries to answer this question. Figure 3 (upper) shows a schematic drawing of the equivalent electrical circuits of principal and intercalated cells. The latter include an apical Cl^- channel which may shunt the H^+ current of the H^+-ATPase. The paracellular pathway includes an electromotive force (EMF) depending on transepithelial ion gradients and selectivities. Since a major part of the current flowing across the epithelium moves across principal cells and the paracellular pathway, it is not surprising that the H^+ current normally does not cause significant modifications of V_t. This model allows a relationship to be obtained between V_t and the ratio of H^+ and Cl^- conductances of the apical membrane of the α intercalated cell in the absence of Na^+ and K^+ currents across the principal cell. Figure 3 (lower) shows that a reduction of Cl^- conductance may induce a lumen-positive V_t due to H^+ current, but only when there is a low chloride electromotive force across the shunt path. Thus, the model shows that under physiological conditions (significant shunt path EMF) distal transepithelial PD may be lumen negative even after blocking Na^+ channels.

In our experiments, we have blocked the participation of Na^+ by using solutions containing the sodium channel blockers benzamil or amiloride. In addition, the participation of K^+ ions was minimized by the use of Ba^{2+}. The possibility of shunting by apical Cl^- is also of relevance. The importance of shunting of H^+ fluxes by maxi Cl^- channels has been demonstrated in several intracellular organelles (Al-Awqati, 1995). Chloride channels have been described in both α and β intercalated cells. Patch-clamp studies on a rabbit collecting duct cell line that exhibits several α properties indicate the presence of a basolateral Cl^- channel with a single channel conductance of 35–75 pS. The apical membrane of these cells has a Cl^- channel with a large conductance of 305pS, inhibited by DPC and NPPB. The channel is

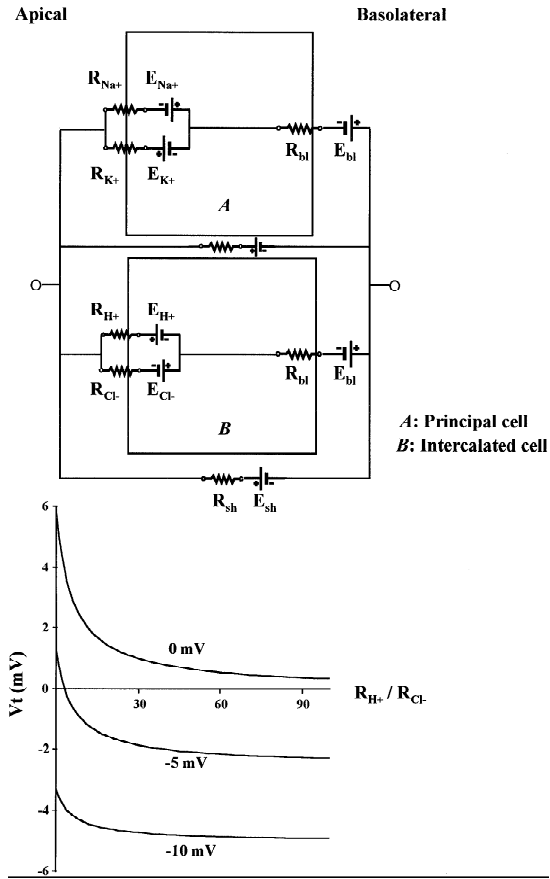

Fig. 3. *Top.* Electrical equivalent circuit model of the late distal tubule of rat. The apical membrane of the α intercalated cell contains the H^+-ATPase and a parallel shunt current carried by Cl^-. R_{K+}, R_{Na+}, R_{Cl-}, R_{H+}: Equivalent resistance of K^+, Na^+, Cl^- and H^+ respectively. E_{K+}, E_{Na+}, E_{Cl-}, E_{H+}: Electromotive force of K^+, Na^+, Cl^- and H^+ respectively. R_{sh}: Paracellular shunt resistance. E_{sh}: Paracellular electromotive force, E_{bl}: Electromotive force of the basolateral membrane, R_{bl}: Basolateral resistance. *Bottom.* Simulated V_t for some values of the shunt electromotive force (0, −5 and −10 mV) as a function of the ratio R_{H+}/R_{Cl-}. The values utilized for the calculation of V_t by Eq. 4 are: E_+ 100 mV, E_{bl} −30 mV (Muto et al., 1990), R_{sh} 100 Ω.cm^2 (Bermudez & Windhager, 1975), R_{H+} 1000 Ω.cm^2, R_{bl} 100 Ω.cm^2 (Froemter, 1982).

inactive in the basal, unstimulated state, but the formation of inside-out patches and large depolarizing voltage steps activate it (Light et al., 1990; Schwiebert et al., 1992; Dietl & Stanton, 1992). Flux studies of perfused rabbit cortical collecting ducts suggest that β intercalated cells also have a basolateral Cl^- conductance (Schuster & Stokes, 1987). In addition, at the apical membrane of rabbit β cells there is evidence for a stilbene-inhibitable Cl^- conductance (Furuya, Breyer & Jacobson, 1991).

An extensive search for effective Cl^- channel blockers in the thick ascending limb of the loop of Henle (TAL) resulted in the identification of diphenyl-amine-2-carboxylate (DPC), which showed an IC50 value of 30 μM for the basolateral TAL Cl^- channel. Screening of

the effect of 198 different compounds structurally related to DPC in *in vitro* perfused TAL segments has led to the identification of NPPB, the most potent member of the group (Cabantchick & Greger, 1992).

The results of the present work show that NPPB may also affect an apical Cl^- channel in the late distal tubule. However, we have no means of distinguishing the cell type that may be the site of the described action. When the apical pathway for Cl^- is inhibited the positive current generated by the H^+ ATPase becomes evident (Fig. 1, bottom). This finding suggests that the late cortical distal tubule may preferentially utilize Cl^- to shunt electrogenic acidification, as was suggested for the rabbit medullary collecting duct (Stone et al., 1983).

An additional path that might be responsible for shunting the H^+ current is the paracellular path. Considering that the distal tubule is a tight epithelium of relatively high resistance (Bermudez & Windhager, 1975), the role of this path is expected to be minor. The small effect of the paracellular shunt in this segment is demonstrated by the fact that peritubular perfusion with a solution in which Cl^- was substituted by gluconate, did not enhance the change in V_t obtained by the luminal perfusion with NPPB (Table 2). However, the model described above shows that the paracellular path is able to maintain a significant lumen-negative V_t in the presence of Cl^-, even after blocking Na^+ channels, which explains findings such as the V_t of −11 mV in benzamil-perfused tubules (*see* Table 2). There has not been reported any evidence that NPPB might block paracellular Cl^- conductance. However, if such action should occur, V_t would be less lumen-negative and the rheogenic effect of H^+ transport would also be more apparent (*see* Fig. 3).

Dependence of Distal H^+ Transport on Cl^-

It has been reported that not only the electrogenicity of H^+-ATPase is dependent on Cl^-, but also that H^+ transport by this transporter depends on the presence of Cl^- (Gluck, 1993). Hilden, Johns & Madias (1988), working with an endosomal fraction isolated from rabbit renal cortex, showed that the ATP-driven H^+ pump had an absolute requirement for the presence of Cl^-. In addition, when the activity of H^+ ATPase was measured by microfluorometry at the single-cell level in rat proximal tubules loaded with BCECF, Zimolo et al. (1992) observed that H^+ extrusion is reduced by lowering intracellular Cl^-. These findings led us to study the chloride dependence of H^+ secretion in the late cortical distal tubule of the rat, a segment in which the main mechanism of H^+ secretion is an H^+ ATPase (Wang et al., 1993; Fernández et al., 1994). The results showed that the half-time of acidification was increased by 80% and net $JHCO_3^-$ decreased by 52% when this blocker was added to luminal perfusate (Fig. 2). These data demonstrate that H^+ secretion by the late distal tubules depends on an apical chloride current.

The chloride-dependence of H^+ ion secretion in distal tubule may reflect entry of Cl^- into the tubular lumen through a conductance pathway, compensating the transfer of positive charge by the H^+-ATPase. However, it may also be a consequence of stimulation of proton pump activity by Cl^-. Kaunitz, Gunther & Sachs (1985) obtained evidence in rat renal medullary microsomal vesicles, that H^+-ion secretion does not depend on transvesicle PD since shunting by valinomycin/potassium did not restore H^+-ion secretion in the absence of chloride ions; they attributed this *electrogenic symport* mechanism to the interaction of Cl^- with a specific site on the H^+-ATPase molecule.

It may appear surprising that blocking Cl^- channels and thereby causing a change of a few mV in transepithelial PD should cause a large modification in H^+-ion secretion rates. According to the model discussed above and in the appendix, the blockade of Cl^- channels will result in hyperpolarization of the apical membrane of α intercalated cells. The PD across the apical membrane of these cells is larger than V_t calculated by the model, due to shunting by the paracellular path. A sizeable hyperpolarization of the apical membrane of the intercalated cell may limit flow of H^+ across the membrane. This change in PD will be detected only marginally across the whole epithelium because it occurs only in a minority group of cells. Nevertheless, perfusion with NPPB tends to reduce V_t, as may be observed in Tables 2 and 3, which is compatible with the analysis of Fig. 3.

In conclusion, the results of the present study demonstrate that: (i) the participation of the vacuolar H^+-ATPase in the establishment of cortical late distal tubule V_t is minor in physiological conditions, but can be demonstrated after blocking Cl^- channels, thus suggesting a shunting effect of this anion; and, (ii) the rate of H^+ secretion in this segment depends on the presence of an apical chloride current, supporting the view that a coupling of H^+-ATPase with Cl^--transport, whatever its mechanism, is an essential aspect of the function of this ATPase.

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Appendix

Equivalent Electrical Circuit for Distal Tubule Epithelium

The equivalent electrical circuit in Fig. 3 assumes that the epithelium contains two cell types: principal cells and intercalated cells (proton-secreting). Sodium and potassium channels in the apical membrane of principal cells are supposed to be fully blocked respectively by amiloride and by Ba^{2+}. In this condition, circular currents flow through paracellular conductance and intercalated cells, and the circuit is greatly simplified. We assume that in the apical membrane of intercalated cells the predominant conductive pathways are the chloride channels (Dietl & Stanton, 1992) and the rheogenic H-pump. This pump is represented, in the circuit, by a protonmotive force (E_H) in series with a resistance. The basolateral membrane is represented by a lumped conductance (R_{bl}) and electromotive force (E_{bl}). The electromotive force in the paracellular pathway accounts for diffusion potentials which arise from ionic gradients between luminal and peritubular compartments and selectivity of the shunt. R_{sh} represents the resistance of this pathway. The Thevenin equivalent for the intercalated cell apical membrane resistance is:

$$R_a = \frac{R_H R_{Cl}}{R_H + R_{Cl}} \tag{1}$$

and for the apical electromotive force:

$$E_a = \frac{R_H}{R_H + R_{Cl}} E_{Cl} + \frac{R_{Cl}}{R_H + R_{Cl}} E_H \tag{2}$$

The transepithelial electrical potential difference (V_t) is given by:

$$V_t = E_{sh} + R_{sh}\frac{E_H - E_{bl} - E_{sh} + \frac{R_H}{R_{Cl}}(E_{Cl} - E_{bl} - E_{sh})}{R_H + R_{bl} + R_{sh} + \frac{R_H}{R_{Cl}}(R_{bl} + R_{sh})} \quad (3)$$

Assuming an equilibrium distribution for chloride across the basolateral membrane:

$$E_{Cl} = E_{bl}$$

eq. (3) simplifies to:

$$V_t = E_{sh} + R_{sh}\frac{E_H - E_{bl} - E_{sh}\left(1 - \frac{R_H}{R_{Cl}}\right)}{R_H + R_{bl} + R_{sh} + \frac{R_H}{R_{Cl}}(R_{bl} + R_{sh})} \quad (4)$$

According to Eq. (4), V_t is a function of the ratio R_H/R_{Cl} when the remaining parameters of the circuit remain constant. In addition when there is a concentration gradient for Cl^- between the luminal and peritubular compartments, an electromotive force may develop across the shunt pathway, depending on its selectivity. Figure 3 (bottom) shows the simulated V_t for some values of the shunt electromotive force as a function of the ratio of internal H-pump resistance to Cl^- resistance. Reversal of transepitelial potential difference (lumen positive) by the rheogenic proton secretion can be observed only in conditions of very small diffusion potentials across the shunt (equivalent to absence of a Cl^- gradient or low Cl^- selectivity) and a very low ratio of the H-pump resistance to Cl^- resistance in the apical membranes of intercalated cells.