Chloride Transport by the Rabbit Cortical Collecting Duct:
Dependence on H,K-ATPase

XIAOMING ZHOU, SHEN-LING XIA, and CHARLES S. WINGO
Laboratory of Epithelial Transport, Division of Nephrology, Hypertension, and Transplantation, University of Florida; and Nephrology Section, Department of Veterans Affairs Medical Center, Gainesville, Florida.

Abstract. The rabbit cortical collecting duct (CCD) exhibits the capacity for active chloride absorption when basolateral Na-K-ATPase is inhibited by ouabain. The present studies examine the contribution of H,K-ATPase to this ouabain-insensitive Cl absorption and to related ion fluxes. Rabbits were fed a KCl-rich diet with no measurable Na for 4 to 13 d before isolation of the CCD for microperfusion. Application of peritubular ouabain (0.1 mM) significantly increased (P < 0.001) net luminal absorptive chloride flux (J^N_{cl}) without an effect on lumen-to-bath isotopic ^36Cl flux (J^P_{cl}). The H,K-ATPase inhibitor Sch 28080 (1 to 10 μM) abolished ouabain-insensitive J^N_{cl}, but transepithelial voltage (V_T) was not significantly affected. The contribution of H,K-ATPase activity on active Cl flux (J^A_{cl}) and passive Cl flux (J^P_{cl}) was also assessed. Ouabain significantly increased J^A_{cl} and Sch 28080 inhibited J^A_{cl}, but J^P_{cl} was not affected by Sch 28080. To assess the contribution of changes in net bicarbonate flux (J^CO2_{bic}) to changes in J^N_{cl}, J^CO2_{bic} was measured under identical conditions as for J^N_{cl}. Ouabain significantly increased J^CO2_{bic}, and this ouabain-insensitive bicarbonate flux was inhibited by Sch 28080 without significantly affecting V_T. To assess the possibility that the CCD may possess mechanisms for neutral salt absorption, lumen-to-bath ^86Rb efflux (K_{Rb}), and ^22Na efflux (K_{Na}) were also measured. Ouabain significantly increased K_{Rb}, and Sch 28080 inhibited this ouabain-insensitive K_{Rb}. Furthermore, Sch 28080 and A80915a (a structurally distinct H,K-ATPase inhibitor) significantly inhibited K_{Na} in the presence of 1 mM lumenal amiloride. These observations suggest that, in addition to potassium, sodium can be transported via the H,K-ATPase. Although the CCD contains more than one cell population, the data could be fitted very well to the function of the B-type intercalated cell. A cell model is proposed for the hypothesis that ouabain-insensitive chloride absorption is mediated by the parallel operation of an apical H,K-ATPase with an apical Cl-HCO_3 exchanger and that the H,K-ATPase can function, under certain conditions, as a mechanism of Na absorption.

The pathways of chloride (Cl) transport by the cortical collecting duct (CCD) are incompletely understood (1,2). Luminal Cl absorption is widely accepted to occur via a voltage-driven paracellular pathway (3–6), apical Cl-bicarbonate (HCO_3) exchange (7–9), and sodium chloride (NaCl) absorption (10,11). However, despite a lumen-negative voltage, we have observed net luminal Cl secretion in the CCD (12–14), which indicates that this segment also has the capacity for active Cl secretion. These studies further demonstrated that Cl secretion was dependent on K secretion in the CCD, which could be attributed to luminal KCl secretion (13). Basolateral application of ouabain inhibited active Cl secretion, which suggests that Cl secretion requires the presence of a functional basolateral Na,K-ATPase (13). However, when ouabain inhibited basolateral Na,K-ATPase, the CCD exhibited significant rates of active Cl absorption (13). These results suggest that active Cl absorption is mediated by the action of a ouabain-insensitive pump. Because a luminal Cl-HCO_3 exchanger is present at the apical membrane of B-type intercalated cells of the CCD (7), we postulated that active Cl absorption may be coupled to HCO_3 secretion. Having demonstrated the presence of H,K-ATPase in the inner stripe of the outer medullary collecting duct (15–17) and in the CCD (17,18), we speculated that active Cl absorption may be dependent on the activity of H,K-ATPase in the collecting duct. Thus, we measured net and active Cl flux in the K-replete CCD in the presence of basolateral ouabain, and assessed the contribution of H,K-ATPase to net and active Cl flux as determined by Sch 28080 (19), a potent K-site H,K-ATPase inhibitor (20–22). To investigate the possibility that the CCD

Received February 20, 1998. Accepted July 6, 1998.
Correspondence to Dr. Charles S. Wingo, Nephrology and Hypertension (111G), Department of Veterans Affairs Medical Center, Gainesville, FL 32608-1197
1046-6673/9912-2194$03.00/0
Journal of the American Society of Nephrology
Copyright © 1998 by the American Society of Nephrology

\[\text{Ouabain, 3-[(6-deoxy-\alpha-L-mannopyranosyl)oxy]-1,5,11α,14,19-pentahydropyrano[20-(22)-enolide; Sch 28080, 2-methyl-8-(phenylimethoxy)imidazo[1,2-a]pyridine-3-acetonitrile. A80915a, 3,4-dichloro-3,4,4a,10α-tetrahydro-6,8-dihydropyrazole-2,7-trimethyl-10a-[(2,2-dimethyl-3-chloro-6-methylencyclohexyl) methyl]-2H-naphtho[2,3-b]pyran-5,10-dione.}\]

\[\text{The term "active Cl absorption" is used to denote Cl transport that cannot be attributed to passive driving forces. This term includes both primary and secondary active transport, as well as facilitated and exchange diffusion. Strictly speaking, it should be referred to as "nonpassive" Cl transport.}\]
may possess mechanisms for ouabain-insensitive neutral salt absorption, in addition to its role in acid-base regulation, we examined the effect of ouabain on net HCO₃ flux and rubidium efflux in the absence or presence of Sch 28080. Finally, to examine whether the H,K-ATPase may transport sodium (Na) ions, as previously suggested (23), the effect of H,K-ATPase inhibition on the ⁸⁶⁹Na rate coefficient (K₉⁹Na) was measured in the absence or presence of either Sch 28080 or A80915a when conductive Na efflux was fully blocked by 1 mM luminal amiloride.

Materials and Methods

Animal Preparations

New Zealand White female rabbits (1.5 to 2.0 kg) receiving ad libitum tap water were maintained on a semisynthetic KCl-rich diet for 4 to 13 d before experimental use. The diet was identical to a "basic" diet used in previous studies (12,24), replete in all nutrients necessary for normal animal growth but containing an additional 241 mmol KCl/kg diet (K = 1.23%, Cl = 0.91%, Na = 0.00005%; TD 85004, Teklad, Madison, WI). Thus, the K and Cl content of the diet used in the present studies was comparable with normal rabbit chow (Purina Laboratory Rabbit Diet, Richmond, IN) and, unlike the normal chow, the diet contained nearly measurable Na. The experiments were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Florida and the Sub-Committee on Animal Studies of the Gainesville Department of Veteran Affairs Medical Center.

Solutions

The composition of the dissection, luminal perfusion, and bath solutions for each protocol is listed in the Table 1 (in mM). The flow rate of the perfusate (Vₑ) was maintained between 4 and 6 ml/min. The bath solution was exchanged at a constant flow rate of 0.64 ml/min. The luminal perfusion solutions for all experiments contained 50 μCi of [methoxy-³H]inulin exhaustively dialyzed by the method of Schafer et al. (25). Bath collections before and after each set of experiments were analyzed for [methoxy-³H]inulin to ensure that there were no mechanical leaks. In most tubules, the leak rate was <2% and those tubules with leak rates of >5% were discarded. Radioisotopes were obtained from New England Nuclear (Boston, MA). Fetal calf serum (5% vol/vol) was added to the bath and dissection solutions. All solutions were gas-gassed with 95% O₂ and 5% CO₂. Sch 28080 was graciously supplied by Dr. James Kaminsky of Schering (Bloomfield, NJ), and A80915a was graciously supplied by Margaret Niedenthal of Eli Lilly (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO).

CCD Isolation and Microperfusion

Standard techniques were used, as described previously (12,13,24). In brief, the rabbits were decapitated, and the left kidney was quickly removed and sliced into 1- to 2-mm sections that were placed into a chilled artificial ultrafiltrate of plasma. Dissection proceeded superficially from the corticomedullary junction. Tubules were transferred to a thermostatically controlled chamber (37°C), and the two ends of the tubule were aspirated into holding pipettes. The perfusing pipette was advanced into the lumen approximately 100 μm beyond the holding pipette. After a minimum of 30 min of perfusion or stabilization of transepithelial voltage (Vₜ), samples of effluent were collected into a constant volume pipette, and Vₜ was recorded throughout the experiment with a KS-700 high impedance electrometer (World Precision Instruments, Sarasota, FL). The reported voltages represent the time-averaged voltage over the period of the tracer or chemical flux collection. In all experiments the collection of samples for analysis was delayed until Vₜ was stabilized.

Ion Transport Measurements

Four protocols (I through IV) were used in this study. To avoid time-dependent effects, the order of the periods in these protocols was rotated, according to a balanced Latin-squares design (26). No statistically significant order-dependent effect on any of the fluxes was observed. In protocols I through III, there was one collection period in which the perfusate contained 0.1% DMSO (vehicle for Sch 28080) (control period), one collection period in which ouabain was present basolaterally and vehicle was present in the perfusate (ouabain period), and one collection period in which 0.1 mM basolateral ouabain and luminal Sch 28080 were present (Sch 28080 period). In protocol IV, a two-period protocol was used: Sch 28080 or A80915a was present luminally in one period (Sch 28080 or A80915a period) and 0.1% DMSO was present in the perfusate during the other period (control period).

Protocol I examined the effect of ouabain and Sch 28080 on Vₜ and net Cl flux (J⁺Cl, in pmol · mm⁻¹ · min⁻¹). We defined J⁺Cl as the total of both paracellular and transcellular Cl fluxes from lumen to bath. Cl concentration in the perfused and collected fluid was measured by electrotitrator (model ETI; World Precision Instruments) following the identical procedure used previously (12). The instrument displayed a linear response over the entire sample range. Three collections were obtained for each period, and each collection was analyzed at least in triplicate. J⁺Cl was calculated as follows:

\[ J⁺Cl = (Vₑ/L)[(C₁)ₑ - (C₁)₀] \]

Table 1. Composition of solutions I through IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaGlucodate</td>
<td>97.4</td>
<td>97.4</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>7.6</td>
<td>7.6</td>
<td>25.5</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Na₃HPO₄</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>NaCH₃CO₂</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>8.3</td>
<td>8.3</td>
<td>8.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>CaGlucodate</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td></td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(perfusion only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>⁸⁶⁹Rb (μCl)</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>³⁸Cl (μCl)</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>⁹⁹Na (μCl)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The terms HCO₃ and total CO₂ content (tCO₂) are used interchangeably throughout the text because the majority of total CO₂ content reflects HCO₃ ion concentration at physiologic pH (7.4).
where \([\text{Cl}]_p\) and \([\text{Cl}]_b\) are the initial concentration of Cl ion in the perfusate and the concentration of Cl ion in the collected (perfused) samples (in mmol/L), respectively; \(V_o\) is the flow rate of the perfusate (in nl/min); and \(L\) is the length of the tube (in mm).

Protocol II examined the effect of ouabain and Sch luminal 28080 on \(V_T\), total HCO\(_3\) flux, and the rate of \(^{86}\text{Rb}\) tracer efflux (\(K_{\text{Rb}}\)). Samples of effluent were analyzed for total CO\(_2\) content (tCO\(_2\)) using a picamethometer (GVH-I, World Precision Instruments) that displayed a linear response throughout the concentration range used. The average sensitivity of the instrument was 100 U/(mEq/L) and could reliably detect tCO\(_2\) differences of approximately 1.0 mEq/L. This value is approximately one-quarter of the mean difference in perfused and collected fluid tCO\(_2\) under basal conditions. Net tCO\(_2\) flux \((J_{\text{CO}_2}\), in pmol · mm\(^{-1}\) · min\(^{-1}\)) was calculated by the following formula:

\[
J_{\text{CO}_2} = (V_o/L)(([\text{CO}_2])_p - [\text{CO}_2])_b
\]

where \(V_o\) is the collected fluid rate (in nl/min), \(L\) is the length of the tubule (in mm), and \([\text{CO}_2]\) and \([\text{CO}_2]_b\) are the perfused and collected tCO\(_2\) concentrations (in mmol/L), respectively. Positive values of \(J_{\text{CO}_2}\) denote net absorption.

Collected perfusate samples for \(^{86}\text{Rb}\) tracer efflux (\(K_{\text{Rb}}\)) were analyzed with an LS-7800 liquid scintillation counter (Beckman Instruments, Irvine, CA), using standard quenching and overlap procedures as described previously (24). \(K_{\text{Rb}}\) was used as a tracer marker for measurement of \(K_{\text{Cl}}\). The rate coefficient of the disappearance of \(^{86}\text{Rb}\) (in nm/s) from the perfusate was evaluated by the following equation:

\[
K_{\text{Rb}} = 2(V_o/L)(Rb^+_b - Rb^-_b)/(Rb^+_b + Rb^-_b)
\]

where \(Rb^+_b\) and \(Rb^-_b\) are the \(^{86}\text{Rb}\) counts per minute (cpm) per nanoliter in the perfused and collected fluid. At least three collections in each period were obtained for measurement of \(K_{\text{Rb}}\).

Protocol III examined the effect of ouabain and Sch 28080 on active and passive Cl flux, and unidirectional (lumen-to-bath) \(^3\text{Cl}\) flux. \(J^p_{\text{Cl}}\) was assessed in an identical manner as described in protocol I. Passive paracellular Cl flux \((J^p_{\text{Cl}}, \text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1})\) was calculated according to the Goldman Constant Field Equation:

\[
J^p_{\text{Cl}} = P_{\text{Cl}} \cdot V_T \cdot (zF/RT) \cdot (1 - \text{exp}(zFV_T/(RT)))
\]

where \([\text{Cl}]_b\) is the luminal Cl concentration, \([\text{Cl}]_b\) is the Cl concentration in the bath, and \(z, F, R, T\) have their usual meaning. A value of \(6.9 \times 10^{-6}\) cm/s was used for the Cl permeability \((P_{\text{Cl}})\) (12,27). Active transcellular Cl flux \((J^a_{\text{Cl}}, \text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1})\) was then assessed according to the following equation:

\[
J^a_{\text{Cl}} = J^p_{\text{Cl}} - J^p_{\text{Cl}}
\]

Unidirectional Cl flux was assessed by measurement of isotopic \(^3\text{Cl}\) flux \((J^b_{\text{Cl}}, \text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1})\) and measured as follows:

\[
J^b_{\text{Cl}} = (V_o/L)(\text{Cl}^+_b - \text{Cl}^-_b)/g
\]

where \(V_o\) is the flow rate of the collected fluid (nl/min), \(L\) is the length of the tubule (mm), \(\text{Cl}^+_b\) and \(\text{Cl}^-_b\) are the \(^3\text{Cl}\) cpm per nanoliter in the perfused and collected fluid, respectively, and \(g\) is the specific activity of \(^3\text{Cl}\) in cpm per pmol.

Protocol IV was conducted to determine whether the H,K-ATPase transports Na ion. We examined the effects of Sch 28080 as well as A80915a on the \(^2\text{Na}\) lumen-to-bath rate coefficient \((K_{\text{Na}2}\), nm/s, assessed in an identical manner to \(K_{\text{Rb}}\) with adjustment of scintillation counter parameters). Conductive Na entry and any hypothetical Na-H antipporter function was inhibited by the luminal application of 1 mM amiloride.

**Statistical Analyses**

Statistical analyses were performed by ANOVA or \(t\) test, as appropriate, and post hoc comparisons were performed by the Ryan-Einot-Gabriel-Welch \(F\)-test, using Crunch Statistical Package (version 4, Crunch Software Co., Oakland, CA). The null hypothesis was rejected at the 0.05 level. Values are reported as the mean ± SEM.

**Results**

Net Cl Flux (Protocol I)

Net Cl flux was measured on six tubules of average length of 1.3 ± 0.1 mm. Figure 1 shows the summary of the measurements from these individual tubules in three periods. \(V_T\) increased significantly \((P < 0.05)\) in the presence of 0.1 mM ouabain (13.7 ± 3.7 mV) relative to the control period (1.4 ± 10.4 mV) and was not significantly affected by the further application of luminal 10 μM Sch 28080 (Sch 28080 period, 14.2 ± 3.0 mV). Compared with the control period (4.3 ± 1.8 pmol · mm\(^{-1}\) · min\(^{-1}\)), ouabain significantly increased net Cl flux (11.6 ± 2.1 pmol · mm\(^{-1}\) · min\(^{-1}\) · P < 0.001) in all six tubules (Figure 1B). In contrast, addition of Sch 28080 abolished \(J^p_{\text{Cl}}\) (−1.5 ± 1.7 pmol · mm\(^{-1}\) · min\(^{-1}\)) in the continued presence of basolateral ouabain. Net Cl flux was significantly decreased during the Sch 28080 period (when compared with either the control period \(P < 0.01\)) or ouabain period \(P < 0.001\). There was no significant difference in \(V_o\) of the perfusate during the three periods of the protocol (control period, 4.35 ± 0.1 nl/min; ouabain period, 4.45 ± 0.1 nl/min; Sch 28080 period, 4.36 ± 0.1 nl/min).

Total HCO\(_3\) Flux (Protocol II)

The measurement of \(J_{\text{CO}_2}\) was performed on nine tubules of average length of 1.5 ± 0.1 mm. As shown in Figure 2A, \(V_T\) was significantly more positive during the ouabain period (14.2 ± 3.6 mV; \(P < 0.001\)) compared with the control period (−17.4 ± 8.6 mV). When compared with the control period, \(V_T\) during the Sch 28080 period was also significantly more positive (15.7 ± 2.7 mV; \(P < 0.001\)), but there was no significant change of \(V_T\) between the Sch 28080 period and the ouabain period. As indicated in Figure 2B, \(J_{\text{CO}_2}\) increased in eight of nine tubules when 0.1 mM ouabain was present in the bath solution (control period, −1.6 ± 1.1 pmol · mm\(^{-1}\) · min\(^{-1}\) versus ouabain period, 2.1 ± 1.1 pmol · mm\(^{-1}\) · min\(^{-1}\); \(P < 0.01\)). Luminal application of 10 μM Sch 28080 reduced \(J_{\text{CO}_2}\) in six tubules (Figure 2), and the mean value of \(J_{\text{CO}_2}\) was significantly less (−1.3 ± 1.5 pmol · mm\(^{-1}\) · min\(^{-1}\)) during the Sch 28080 period, when compared with the ouabain period \((P < 0.01)\). There was no significant difference in the perfusate \(V_o\) during the experiments (4.8 ± 0.1 nl/min in control period, 4.6 ± 0.2 nl/min in ouabain period, and 4.7 ± 0.1 nl/min in Sch 28080 period).

\(^{86}\text{Rb}\) Tracer Efflux (Protocol II)

\(^{86}\text{Rb}\) tracer efflux \((K_{\text{Rb}})\) was measured as a qualitative marker of Cl efflux in the same experiments as \(J_{\text{CO}_2}\), and the
results are summarized in Figure 3. \(K_{\text{Rb}}\) was increased significantly during the ouabain period (53.1 ± 8.3 nm/s; \(P < 0.01\)) when compared with the control period (24.2 ± 6.1 nm/s) (Figure 3A). Ten micromolar Sch 28080 inhibited \(^{86}\text{Rb}\) efflux in six of nine tubules, and the mean value for the Sch 28080 period was significantly less (31.4 ± 7.5 nm/s; \(P < 0.01\)) than during the ouabain period (Figure 3B).

**Active and Passive \(^{36}\text{Cl}\) Fluxes (Protocol III)**

Measurements were performed on nine tubules of average length of 1.5 ± 0.1 mm. In Figure 4A, similar to protocols I and II, the basal \(V'_T\) became significantly more lumen-positive during application of 0.1 mM ouabain (control period, −8.3 ± 8.8 mV; ouabain period, 11.4 ± 4.1 mV; \(P < 0.01\)), and this lumen-positive voltage was not significantly affected by the presence of 1 \(\mu\)M Sch 28080 (13.9 ± 5.4 mV). As shown in Figure 4A, and similar to the findings observed in protocol I, \(J^N_{\text{Cl}}\) significantly increased from −1.4 ± 0.6 pmol \(\cdot\) mm\(^{-1}\) \(\cdot\) min\(^{-1}\) during the control period to 7.6 ± 1.9 pmol \(\cdot\) mm\(^{-1}\) \(\cdot\) min\(^{-1}\) (\(P < 0.05\)) in the presence of 0.1 mM ouabain. This increase in \(J^N_{\text{Cl}}\) was reversed in six of nine tubules (figure not shown) by the addition of 1 \(\mu\)M Sch 28080 (−0.2 ± 4.0 pmol \(\cdot\) mm\(^{-1}\) \(\cdot\) min\(^{-1}\); \(P < 0.05\)). The effect of Sch 28080 on net \(\text{Cl}\) flux is qualitatively similar at 1 \(\mu\)M (in protocol III) and 10 \(\mu\)M (in protocol I). The \(V_o\) of the perfusate was very constant throughout the experiments (4.8 ± 0.1 nl/min in control period, 4.6 ± 0.2 nl/min in ouabain period, and 4.7 ± 0.1 nl/min in Sch 28080 period).

**Figure 1.** Effect of ouabain and Sch 28080 on net \text{Cl} flux (\(J^N_{\text{Cl}}\)) in the K-replete rabbit cortical collecting duct (CCD). (A) Transepithelial voltage \((V'_T)\) and \(J^N_{\text{Cl}}\) were compared. Solution I was used for the bath and the lumenal fluid (see Table 1). *, statistically different from control period; **, statistically different from ouabain period. (B) \(J^N_{\text{Cl}}\) changes in the six individual tubules. Basolateral 0.1 mM ouabain and luminal 10 \(\mu\)M Sch 28080 (in the presence of basolateral ouabain) were used in the microperfusion measurements.

**Figure 2.** Effect of ouabain and Sch 28080 on net \(\text{HCO}_3\) flux (\(J_{\text{HCO}_3}\)). (A) Transepithelial voltage \((V'_T)\) and \(J_{\text{HCO}_3}\) were compared. Solution II was used in the experiments. *, statistically different from control period; **, statistically different from ouabain period. (B) \(J_{\text{HCO}_3}\) changes in response to basolateral application of 0.1 mM ouabain and luminal application of 10 \(\mu\)M Sch 28080 (in the presence of basolateral ouabain). A total of nine tubules was examined.
Figure 3. Effect of ouabain and Sch 28080 on $^{86}$Rb tracer efflux ($K_{Rb}$). (A) $K_{Rb}$ as a qualitative marker for K ion flux, was measured in the presence of basolateral ouabain (0.1 mM) and in the presence of luminal Sch 28080 (10 μM) and basolateral ouabain. The measurements were made on the same tubes as those in Figure 2 and used solution II. *, statistically different from control period; **, statistically different from ouabain period. (B) The distribution of $K_{Rb}$ changes in response to ouabain and Sch 28080 in the nine tubules.

As shown in Figure 4B, there was no significant change in $J_{b}^{b}$ upon addition of 0.1 mM ouabain, and there was a trend (0.05 < $P$ < 0.1) toward decreased $J_{b}^{b}$ in the presence of 1 μM Sch 28080 when compared with either the control period or ouabain period. Ouabain significantly reduced passive paracellular Cl flux ($J_{cl}^{p}$) from 3.1 ± 2.9 pmol · mm$^{-1}$ · min$^{-1}$ during control period to −3.4 ± 1.5 pmol · mm$^{-1}$ · min$^{-1}$ during ouabain period ($P$ < 0.01). Addition of 1 μM luminal Sch 28080 did not significantly affect $J_{cl}^{p}$ (−4.0 ± 1.9 pmol · mm$^{-1}$ · min$^{-1}$). However, ouabain produced a very significant increase in active transcellular Cl flux ($J_{cl}^{a}$, 11.0 ± 1.7 pmol · mm$^{-1}$ · min$^{-1}$) compared with the control period (−4.5 ± 3.0 pmol · mm$^{-1}$ · min$^{-1}$; $P$ < 0.01). One micromolar Sch 28080 significantly inhibited ouabain-insensitive $J_{cl}^{a}$ (3.8 ± 3.6 pmol · mm$^{-1}$ · min$^{-1}$; $P$ < 0.05) (Figure 4B) in six of nine tubules (Figure 4C).

$^{22}$Na Efflux (Protocol IV)

Measurement of $V_{T}$ and $^{22}$Na lumen-to-bath efflux coefficient ($K_{Na}$, nm/s) was performed on six tubules of average length of 1.7 ± 0.2 mm. For both the Sch 28080 and the A80915a protocols, three tubules were perfused with vehicle and then with the active drug, and in three tubules the order was reversed. Conductive Na entry and any hypothetical apical Na-H exchange was fully inhibited by the luminal application of 1 mM amiloride. The $V_{o}$ of the perfusate was not affected by
the addition or removal of 10 μM Sch 28080 (5.6 ± 0.1 nl/min in the control period; 5.5 ± 0.2 nl/min in the Sch 28080 period). $V_T$ was not affected by applying Sch 28080 (8.7 ± 1.2 mV during the control period versus 9.0 ± 1.2 mV during the Sch 28080 period). However, addition of 10 μM Sch 28080 to the perfusate significantly decreased $^{22}\text{Na}$ efflux ($K_{Na}$) from 61.3 ± 8.1 nm/s (control period) to 44.7 ± 7.7 nm/s (Sch 28080 period; $P < 0.02$). The data collected from each individual tubule are shown in Figure 5B.

We also tested under the same experimental conditions the effect of luminal A80915a in six tubules of average length of 1.3 ± 0.2 mm. As shown in Figure 5C, $V_T$ were not affected by the addition or removal of 10 μM A80915a in the experiments (5.8 ± 0.9 mV during the control period; 5.2 ± 0.8 mV during the A80915a period). However, addition of 10 μM A80915a to the perfusate decreased $K_{Na}$ in five of six tubules (Figure 5D). The mean value of $K_{Na}$ decreased by approximately 18%, from 51.3 ± 11.1 nm/s (control period) to 42.3 ± 12.3 nm/s (A80915a period, $P < 0.03$). There was no difference in $V_o$ of the perfusate during the two periods of the protocol (4.3 ± 0.2 nl/min in control period; 4.3 ± 0.2 nl/min in A80915a period).

### Discussion

The present study provides evidence for the contribution of H,K-ATPase to Cl transport in the CCD under K-replete and Na-restricted conditions. The results of this study are consistent with the hypothesis that the component of Cl absorption that is ouabain-insensitive is mediated by the action of parallel transporters, wherein a primary active H,K-ATPase provides the driving force for proton secretion and K absorption and, as a consequence, secondary active apical Cl-HCO$_3^-$ exchange and Cl absorption take place. This parallel operation may serve as a mechanism for neutral salt absorption rather than acid-base regulation.

Previous observations suggested that ouabain unmasked the presence of a Cl absorptive process that is not observed by measurement of $J^\text{P}_{Cl}$ because of the presence of active Cl secretion (12,13). In the present study, we observed that ouabain significantly enhanced $J^N_{Cl}$ (Figures 1 and 4A) with no change in the lumen-to-bath Cl flux (Figure 4B) and, therefore, bath-to-lumen secretory Cl flux decreased. Because $V_T$ became significantly more lumen-positive in the presence of ouabain, which would alter passive driving forces, we calculated the components of net Cl flux that could be attributed to passive paracellular movement and active transcellular movement. Ouabain significantly increased active transcellular Cl flux from lumen to bath (Figure 4, B and C), but this may be equally described as a decrease in active transcellular transport of Cl (Cl secretion) from bath to lumen. These data are consistent with apical Cl secretion that is dependent on basolateral Na,K-ATPase. Importantly, in the presence of basolateral ouabain, a substantial ouabain-insensitive Cl absorptive flux persisted.

Ouabain-insensitive $J^N_{Cl}$ was abolished by addition of the H,K-ATPase inhibitor Sch 28080 (Figure 1), which suggests that ouabain-insensitive net Cl absorptive flux occurs in parallel with H,K-ATPase. The H,K-ATPase activity reported herein may reflect the activity of two H,K-ATPase isoforms known to be present in the kidney: the “gastric” isoform (HK$_{a_1}$) and the “colonic” isoform (HK$_{a_2}$). However, the available data indicate that the former is Sch 28080-sensitive (20–22), whereas the latter is relatively Sch 288,080-insensitive (28). The apical application of ouabain significantly inhibits colonic H,K-ATPase activity in the rat and guinea pig colon (28,29), but in previous studies we have observed that the majority of H,K-ATPase flux in the rabbit CCD is sensitive to luminal Sch 28080 and not to luminal ouabain (18). Such observations suggest that the major component of H,K-ATPase activity in the present study is most consistent with HK$_{a_1}$ activity. Previous studies have also demonstrated that Sch 28080 is a selective H,K-ATPase inhibitor of luminal acidification in the inner stripe of the outer medullary collecting duct (30,31).

We further examined the nature of this Cl absorptive flux by measuring changes in $J_{CO_2}$ at the same conditions as we

![Figure 5](image-url)

**Figure 5.** Effect of luminal Sch 28080 or A80915a on $^{22}\text{Na}$ lumen-to-bath rate coefficient ($K_{Na}$). (A and B) $K_{Na}$ was reduced by luminal application of Sch 28080 (10 μM), as measured in six tubules. (C and D) $K_{Na}$ was reduced by luminal application of A80915a (10 μM) in five of six tubules. Experiments in Panel D used different tubules from Panel B. Luminal amiloride (1 mM) was added to solution IV, which was used in all experiments. *$K_{Na}$ in Sch 28080 or A80915a period was statistically different from the control period.
measured changes in $J_{\text{Cl}}^N$. Ouabain significantly increased $J_{\text{CO}_2}$ and shifted $V_T$ to more lumen-positive values (Figure 2). The ouabain-insensitive bicarbonate flux was inhibited by additional Sch 28080 without significantly affecting $V_T$. These findings suggest that ouabain-insensitive Cl absorptive flux could couple a ouabain-insensitive bicarbonate exchange in the apical membrane of rabbit CCD.

However, the magnitude of ouabain-insensitive $J_{\text{Cl}}^N$ (Figure 1A) significantly exceeded the magnitude of ouabain-insensitive $J_{\text{CO}_2}$ (Figure 2A). This could be because ouabain-sensitive Cl secretion (from bath to lumen) significantly decreased, or other ouabain-insensitive Cl absorptive fluxes may also exist. The magnitude of Sch 28080-inhibited $J_{\text{Cl}}^N$ (Figure 1A) also significantly exceeded the magnitude of Sch 28080-inhibited $J_{\text{CO}_2}$ (Figure 2A). This observation suggests that a substantial amount of apical $\text{HCO}_3^-$ secretion accompanies apical proton secretion via H,K-ATPase. This nonvectorial apical proton and $\text{HCO}_3^-$ secretion results in no net acid-base transport but allows for a mechanism of apical Cl absorption. We cannot exclude a direct effect of Sch 28080 on Cl transport. However, we speculate that, if any, the effect would be very minimal.

Nevertheless, these observations suggest that a component of ouabain-insensitive Cl absorption may be mechanistically described by Cl uptake via an apical Cl-$\text{HCO}_3^-$ exchanger, which is driven by the action of the apically oriented H,K-ATPase proton pump. Recent intracellular pH studies support the presence of both a Cl-$\text{HCO}_3^-$ exchanger and an H,K-ATPase at the apical membrane of the CCD for B-type intercalated cells (32–34). The current study establishes a relationship between an apical Cl-$\text{HCO}_3^-$ exchanger and an apical H,K-ATPase of the CCD (although we do not know the intermediate steps of the coupling at the present time). Because our observations suggest the possible involvement of the B-type intercalated cell in this ouabain-insensitive Cl flux, we have included a proposed cell model as shown in Figure 6. Because the CCD possesses more than one cell population, the cell model proposed here, although most consistent with characteristics for the B-type intercalated cell and supported by the present data, is not exclusive of other cell types in the CCD.

We also carried out additional experiments to assess the possibility that the CCD possesses a mechanism for neutral salt absorption. Previously, we have demonstrated that peritubular application of 0.1 mM ouabain significantly increased $^{86}$Rb efflux by the CCD of rabbits conditioned to a K-restricted diet and that this effect of ouabain was abolished when 10 $\mu$M Sch 28080 was present in the perfusate (18). These observations led to the proposal that the effect of ouabain on K/Rb absorption requires the presence of a functional H,K-ATPase (18). Consistent with this hypothesis, Laski (35) has observed that ouabain significantly enhances total CO$_2$ flux in the rabbit CCD. Similar results were observed in the present studies in which ouabain enhanced both Sch 28080-sensitive total CO$_2$ flux (Figure 2), as well as Sch 28080-sensitive $^{86}$Rb efflux (Figure 3). These observations are most consistent with the predominant apical location of an H,K-ATPase, and suggest that inhibition of Na,K-ATPase may enhance the turnover of the apical H,K-ATPase, resulting in enhanced Rb efflux and net bicarbonate absorption. A mechanism accounting for this has not been investigated but may be a function of intracellular potassium or ATP/ADP concentrations.

The regulation of Na excretion in the CCD has been ascribed to the apical amiloride-sensitive Na conductance that is stimulated by aldosterone (36). However, evidence suggests that an amiloride-insensitive mechanism of Na transport may also be present in the apical membrane of this segment of the kidney. Specifically, aldosterone stimulates an amiloride-resistant Na absorptive flux in the in vitro perfused CCD (37) and there is evidence that aldosterone stimulates renal H,K-ATPase (38,39). Of note, the animal model in our current studies has been shown to increase plasma aldosterone, since the animals were on a Na-restricted diet for up to 13 d (24). Our previous studies of the CCD from K-restricted rabbits demonstrated competition of Na with K for transport on H,K-ATPase (23), and enzymatic studies of Buffin-Meyer et al. (40) in the rat CCD are consistent with this observation. Additionally, it has been demonstrated that Na can, under certain conditions, activate the dephosphorylation step of hog gastric vesicle H,K-ATPase, thus serving as a K analog (41). The combined evidence indicates that H,K-ATPase is not entirely selective for K.

![Figure 6](image-url)
and may allow Na transport via this enzyme. However, the question that remains to be answered is whether this Na transport also occurs in a normal diet rabbit model.

On the basis of the preceding observations, we examined the effect of demonstrated inhibitors of the gastric H,K-ATPase on Na efflux under conditions in which Na absorption via conductive pathways was fully inhibited by amiloride, but active basolateral Na extrusion was allowed via the Na,K-ATPase. We found significant inhibition of Na efflux by Sch 28080. Although the Sch 28080-sensitive contribution to Na absorption (approximately 27%; Figure 5B) is smaller than that to ouabain-sensitive K/Rb absorption (approximately 41%; Figure 3B), these observations indicate that the H,K-ATPase contributes to Na absorption. Further evidence supporting this comes from the experiments with A80915a, a functionally and structurally dissimilar gastric H,K-ATPase inhibitor (42). The inhibition of Na absorption by A80915a was approximately 18% (Figure 5D). The proposed cell model notes these observations (Figure 6).

Active Cl absorption was H,K-ATPase-dependent and Na efflux was significantly reduced by H,K-ATPase inhibitors. These findings suggest that a mechanism(s) for neutral salt absorption is present in the CCD, which incorporates active K and Na transport by H,K-ATPase. These consistent findings warrant further consideration because they not only may have significant bearing on the presently accepted cell models for the CCD, but also may help to explain the Na retention and hypertension that is observed with ingestion of low K diets (43).

Acknowledgments
This work was supported by grants from the Medical Research Service of the Department of Veterans Affairs and National Institutes of Health (DK49750). We thank Jeanette Lynch and Scott Straub for technical support.

References