Contribution of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter NKCC1 to Cl\(^{-}\) secretion in rat OMCD

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Received 5 October 2000; accepted in final form 20 December 2000

The cellular composition of rat OMCD is heterogeneous: 60–64% of cells are principal cells whereas 36–40% are α-intercalated cells (16). Although principal cells mediate robust rates of Na\(^{+}\) transport (33), they mediate little Cl\(^{-}\) transport (34). Therefore, it remains to be determined whether significant transepithelial movement of Cl\(^{-}\) occurs in rat OMCD. If Cl\(^{-}\) secretion is observed in rat OMCD, it might occur through anion exchange- and/or NKCC1-mediated Cl\(^{-}\) uptake across the basolateral membrane in series with Cl\(^{-}\) efflux across the apical membrane.

In rabbit OMCD, Cl\(^{-}\) is secreted in parallel with H\(^{+}\) secretion (HCO\(_3\)\(^{-}\) absorption) (39). In this segment, Cl\(^{-}\) secretion and HCO\(_3\)\(^{-}\) absorption are eliminated with addition of SITS, an inhibitor of anion exchange (39). Therefore, secretion of Cl\(^{-}\) and absorption of HCO\(_3\)\(^{-}\) (secretion of H\(^{+}\) equivalents) are mediated by Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchange on the basolateral membrane in series with efflux of H\(^{+}\) and Cl\(^{-}\) across the apical membrane.

Like rabbit OMCD, rat OMCD absorbs HCO\(_3\)\(^{-}\) and secretes NH\(_4\)\(^{+}\) (9) However, Cl\(^{-}\) transport pathways in this segment and their possible contribution to transepithelial Cl\(^{-}\) transport are not understood. The purpose of the present study was to determine whether rat OMCD secretes Cl\(^{-}\} and whether trans-

Am J Physiol Renal Physiol

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epithelial transport of Cl\(^-\) is mediated, at least in part, by NKCC1.

**METHODS**

Tubules from the inner stripe of the OMCD were dissected from pathogen-free male Sprague-Dawley rats weighing 65–120 g (Harlan, Indianapolis, IN). Animals were housed in microisolator cages and fed a low-Na\(^+\), 0.8% K\(^+\) diet (Zeigler Bros., Garners, PA) (41). Rats received 5 mg deoxycorticosterone pivalate (DOCP; CIBA-Geigy Animal Health, Greensboro, NC) by intramuscular injection 5–7 days before death. To induce a rapid diuresis, animals were injected with furosemide (5 mg/100 g body wt ip) 45 min before death by decapitation. This furosemide-induced diuresis reduces the inner medullary axial solute concentration gradient (41) and attenuates changes in the extracellular osmolality of the tubule.

Coronal slices were cut from the kidneys and placed into a dissection dish containing the chilled experimental solution (11°C). Solution compositions are given in Table 1. The dissection solution was either solution 1 or solution 2 as appropriate, to match the NH\(_4\)Cl concentration of the perfusate and bath solution used when measurements were performed. To dissect OMCD tubules from the inner stripe of the outer medulla, a cut was made between the inner and outer stripe of the outer medulla using a razor blade, and OMCD tubules were dissected as reported previously (9). Tubules were mounted on concentric glass pipettes and perfused in vitro at 37°C.

Experiments were performed with symmetrical solutions in the bath and perfusate. Osmolality was measured in all experiments (41). To maintain the desired CO\(_2\) concentration in HCO\(_3\)/CO\(_2\)-buffered solutions, the perfusate was passed through jacketed concentric tubing, through which 95% air-5% CO\(_2\) was blown in a countercurrent direction around the perfusate line (41, 42). To maintain pH in HCO\(_3\) containing solutions, the bath fluid was constantly bubbled with furosemide (5 mg/100 g body wt ip) 45 min before death by decapitation. This furosemide-induced diuresis reduces the inner medullary axial solute concentration gradient (41) and attenuates changes in the extracellular osmolality of the tubule.

Table 1. **Solution composition**

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<th>Solution No.</th>
<th>1</th>
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<tr>
<td>Choline-Cl</td>
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<td></td>
</tr>
<tr>
<td>Choline-HCO(_3)</td>
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</tr>
</tbody>
</table>

Composition is in mM.
was varied between 7.0 and 7.8. The other details of pH measurement in tubules perfused in vitro were performed as described previously by our laboratory (41). Fluid flux. To measure fluid flux ($J_v$), changes in raffinose concentration of the luminal fluid were measured, using the assay described by Garvin and Knepper (12). Raffinose concentration in collected fluid samples was measured by using an enzymatic assay in which raffinose is converted to galactonolactone and NADH. This assay was purchased as a kit (Boehringer Mannheim, Mannheim, Germany). Fluorescence of NADH was measured using a continuous-flow ultramicrofluorometer. Samples were collected into the lower chamber (17 nl) of a double-constriction pipette. An enzyme solution was employed that contained 3.5 mg/ml NADH$^+$ in citrate buffer (pH 4.5) and 4.5 U/ml $\alpha$-galactosidase. This enzyme solution was drawn into the pipette until the second chamber (237 nl) was filled. Because the enzymatic conversion of raffinose to NADH and galactonolactone is complete after 6 min at 37°C (data not shown), all samples were incubated for 7–9 min at 37°C and then injected into a flowing stream. The flowing stream was drawn by a constant-withdrawal pump at 166 nl/s and contained 0.3 U/ml $\beta$-galactosidase in potassium diphosphate buffer (pH 8.6). By using this assay, raffinose concentration is linear from at least 0 to 203 pmol (not shown).

Fluid absorption was calculated by using the equation

$$J_v = (C_L / C_o - 1)Q/L$$

where $C_L$ is the concentration of raffinose in the collected fluid, $C_o$ is the concentration of raffinose in the perfused fluid, $Q$ is the collection rate in nanoliters per minute, and $L$ is the tubule length.

Measurement of $J_v$ requires use of a volume marker that has a low permeability in rat OMCD. Raffinose permeability ($P_{raf}$) was therefore measured in rat OMCD tubules. To measure $P_{raf}$, tubules were bathed in solution 8, which contained 10 mM raffinose. The perfusate solution was the same solution, except that raffinose was replaced with NaCl to match the osmolality of the bath solution. The concentration of raffinose was measured in the collected fluid samples. Samples for raffinose concentration were taken at the same time points as were the Cl$^-$ samples. $P_{raf}$ was calculated by using the following equation

$$P_{raf} = J_{raf}(C_o / L)$$

Raffinose flux, $J_{raf}$, was calculated as collected raffinose concentration (in mM) times the luminal flow rate ($Q$; in nl/min) divided by tubule length ($L$). $A_s$ is the surface area per unit length of tubule, and $C_o$ is the mean concentration gradient across the epithelium. $P_{raf}$ in three OMCD tubules averaged 1.0 ± 0.5 × 10$^{-6}$ cm/s. This compares with $P_{raf}$ of 3.0 × 10$^{-6}$ cm/s reported in rabbit proximal tubule (12). Thus OMCD is relatively impermeable to raffinose, making it a suitable volume marker in this segment.

To measure $V_T$, the solution in the perfusion pipette was connected to an electrometer (model KS-700, World Precision Instruments, New Haven, CT) through an agar bridge saturated with 0.16 M NaCl and a calomel cell as described previously (41). The reference was an agar bridge from the bath to a calomel cell.

Statistics. In all experiments wherein either Cl$^-$ or raffinose concentration was assayed, two to three replicate measurements were made in a single tubule. The mean of all measurements made in a single tubule was used in the statistical analysis, where $n$ represents the number of tubules studied. Statistical significance was determined by using a paired or unpaired two-tailed Student’s t-test as appropriate. For multiple comparisons, ANOVA was used with specific contrasts by the Bonferroni method. Statistical significance was achieved with $P < 0.05$. Data are displayed as means ± SE.

RESULTS

Role of anion exchange in $J_{CI}$. Ion transport pathways along the collecting duct have been studied extensively in DOCP-treated rats (9, 27, 41, 42). Therefore, to explore Cl$^-$ transport pathways in rat OMCD, DOCP-treated rats were employed both to facilitate comparison with these previous studies and to stimulate Cl$^-$ transport pathways such as anion exchange (17).

The role of anion exchange in Cl$^-$ secretion in rat OMCD was explored by testing the effect of 0.5 mM H$_2$DIDS on pH$_i$ and $J_{CI}$ in rat OMCD. The effect of H$_2$DIDS on pH$_i$ is shown in Fig. 1. pH$_i$ increased nearly 0.4 pH units ($n = 3, P < 0.05, solution 1$) 4 min after the addition of 0.5 mM H$_2$DIDS to the bath solution. However, no change in pH$_i$ was noted after the addition of the NKCC1 transport inhibitor bumetanide (100 μM). The effect of H$_2$DIDS on $J_{CI}$ is shown in Fig. 2 (Table 2). (Table 2). Rat OMCD tubules secreted Cl$^-$ with a $J_{CI}$ of −11.9 ± 1.7 pmol·mm$^{-1}$·min$^{-1}$ ($n = 5, solution 2$). In the presence of H$_2$DIDS, $J_{CI}$ was −7.9 ± 1.9 pmol·mm$^{-1}$·min$^{-1}$ [$n = 5, P = not significant (NS)$]. Although no change in Cl$^-$ secretion was detected with the application of H$_2$DIDS, an effect of stilbene inhibitors on $J_{CI}$ cannot be excluded from these data. Nevertheless, these data raise the possibility that along the basolateral membrane, other Cl$^-$ transport pathways might participate in the transepithelial transport of Cl$^-$.

Effect of bumetanide on $J_{CI}$. To determine the role of NKCC1-mediated Cl$^-$ uptake in the process of transepithelial transport of Cl$^-$, the effect of bumetanide on $J_{CI}$ was tested. Results are shown in Fig. 2 (Table 2). Cl$^-$ secretion ($−13.5 ± 0.8$ pmol·mm$^{-1}$·min$^{-1}$, $n = 5, solution 2$) was attenuated in a dose-dependent fashion with bumetanide, an inhibitor of NKCC1, when added to the peritubular bath. $J_{CI}$ was inhibited by 55% with 10 μM bumetanide ($P < 0.05$) and 78% by 100 μM bumetanide ($P < 0.05$).

The effect of bumetanide on $V_T$ was tested in separate tubules when they were perfused and bathed in the presence of solution 2. As shown in Fig. 3, although bumetanide inhibited $J_{CI}$, an effect of bumetanide on $V_T$ could not be demonstrated. Because of the variability in measured $V_T$, the effect of ethoxzolamide on $V_T$ was tested as a positive control (29) to determine our ability to detect changes in $V_T$. Addition to the bath of

\footnote{1An effect of DOCP on $J_{CI}$ could not be detected in rat OMCD. $J_{CI}$ was $−2.9 ± 1.1$ pmol·mm$^{-1}$·min$^{-1}$ ($n = 5$) in untreated controls and $−7.1 ± 1.6$ pmol·mm$^{-1}$·min$^{-1}$ in tubules from DOCP-treated rats ($solution 2$, $n = 7, P = 0.075$, unpaired Student’s t-test).

\footnote{2At an H$_2$DIDS concentration of 0.5 mM, 66% of Na$^+$-independent anion exchange is inhibited along the basolateral membrane of intercalated cells in rat CCD (6).}
Strated, however, either under baseline conditions anide on membrane potential could not be demon-
strate for NKCC1, the effect of Na
+ with bumetanide. With 10
51
V
+ tenuated in a dose-dependent fashion
But, an effect of bumetanide-sensitive Cl
uptake occurs when operating as a Na
+ cotransporter.
In many cell types, NH
+ substitues for K
+ on NKCC1 (8, 43). Therefore, we asked whether bumetanide-sensitive Cl
secretion persists in rat OMCD when NH
+ is substituted for K
+ . At a K
+ concentration of 2 mM (NH
+ absent), J
+ was low in either the presence or the absence of bumetanide (Fig. 4, Table 2).
Moreover, with substitution of NH
+ for choline (2 mM K
+ , 6 mM NH
+ , solution 6), secretion of Cl
was significant (−9.9 ± 1.1 pmol·mm
−1·min
−1, n = 5). Thus both total and bumetanide-sensitive Cl
secretion are very low. However, at a K
+ concentration of 20 mM (solution 5, Fig. 4, Table 2), significant Cl
secretion was observed, which was reduced with the application of bumetanide to the bath. Therefore, total and bumetanide-sensitive Cl
secretion are dependent on K
+ in the bath and perfusate.

In other cell types, such as in salivary glands, NKCC1 mediates Cl
secretion,
which gives rise to secretion of fluid (28). We therefore tested whether fluid secretion is observed in parallel with Cl− secretion in rat OMCD. Tubules were perfused and bathed in the presence of 5 mM raffinose (solution 7), which was employed as a volume marker. Results are displayed in Table 3. In symmetrical solutions, low levels of fluid secretion were observed in this segment ($J_V = -0.042 \pm 0.015 \text{ nmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$, $n = 5$), which was unchanged with the application of bumetanide to the bath. However, because of the variability in the measurements, we cannot exclude a small effect of bumetanide on $J_V$.

It is possible that $J_V$ is low because of raffinose-induced changes in $J_{Cl}$. Therefore, as a control, $J_{Cl}$ was measured in the presence and absence of raffinose. Cl− secretion was similar in the presence ($J_{Cl} = -10.4 \pm 1.4 \text{ pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$, $n = 3$, solution 7) and in the absence ($J_{Cl} = -10.6 \pm 2.3 \text{ pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$, $n = 3$, solution 2) of 5 mM raffinose in the perfusate and bath ($P = \text{NS}$). Therefore, our inability to detect significant rates of total and bumetanide-sensitive $J_V$ did not result from a raffinose-induced change in Cl− secretion.

**DISCUSSION**

This study demonstrates that in OMCD tubules perfused in vitro from DOCP-treated rats, Cl− is secreted into the luminal fluid through Cl− uptake across the basolateral membrane, mediated by both NKCC1 and anion exchange, in series with Cl− efflux across the apical membrane. This contribution of NKCC1 to Cl− secretion represents a novel mechanism of transepithelial Cl− transport in rat OMCD.

Significant Cl− secretion was observed in the OMCD in the present in vitro study. In vivo studies suggest that Cl− secretion might occur in rat OMCD. After NaCl stress, Cl− delivery to the base of the collecting duct is greater than Cl− delivery at the level of the superficial distal tubule (18). One explanation for these results is that Cl− secretion occurs along the collecting duct in vivo in one or more nephron segments, which

![Figure 3. Effect of bumetanide on transepithelial potential difference ($V_T$). $V_T$ was measured in OMCD tubules perfused in the presence of solution 2. Under baseline conditions, a lumen positive potential difference was observed (4.9 ± 1.9 mV, $n = 4$). Addition of 100 μM bumetanide did not alter $V_T$ (4.6 ± 1.5 mV, $n = 4$, $P = \text{NS}$, unpaired Students t-test). The lumen positive $V_T$ was abolished with the addition of the carbonic anhydrase inhibitor ethoxzolamide (100 μM) to the bath solution (0.2 ± 0.1 mV, $n = 3$). In the presence of ethoxzolamide, $V_T$ was unchanged with the addition of bumetanide (100 μM) to the bath ($-0.1 ± 0.1$ mV, $n = 4$, $P = \text{NS}$).](http://ajprenal.physiology.org/)

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**Table 2. Chloride flux in OMCD from DOCP-treated rats**

<table>
<thead>
<tr>
<th>Tubule Length, mm</th>
<th>Bath pH</th>
<th>Collection Rate/ Tubule Length, nM/mm/min</th>
<th>Perfusate Cl− Concentration, mM</th>
<th>Collected Cl− Concentration, mM</th>
<th>$J_{Cl}$, pmol/mm/min</th>
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</thead>
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<tr>
<td><strong>Effect of H2DIDS</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control ($n = 5$)</td>
<td>0.56 ± 0.04</td>
<td>7.34 ± 0.02</td>
<td>2.30 ± 0.24</td>
<td>137.8 ± 1.7</td>
<td>140.3 ± 1.6</td>
</tr>
<tr>
<td>H2DIDS ($n = 5$)</td>
<td>0.57 ± 0.05</td>
<td>7.40 ± 0.01</td>
<td>2.15 ± 0.13</td>
<td>136.6 ± 1.4</td>
<td>140.4 ± 2.1</td>
</tr>
<tr>
<td><strong>Effect of bumetanide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ($n = 5$)</td>
<td>0.61 ± 0.09</td>
<td>7.32 ± 0.03</td>
<td>2.69 ± 0.28</td>
<td>128.1 ± 0.8</td>
<td>143.1 ± 1.3</td>
</tr>
<tr>
<td>10 μM bumetanide ($n = 5$)</td>
<td>0.50 ± 0.02</td>
<td>7.34 ± 0.01</td>
<td>2.72 ± 0.13</td>
<td>137.4 ± 0.6</td>
<td>139.7 ± 0.5</td>
</tr>
<tr>
<td>100 μM bumetanide ($n = 5$)</td>
<td>0.65 ± 0.06</td>
<td>7.33 ± 0.02</td>
<td>2.63 ± 0.14</td>
<td>141.5 ± 1.6</td>
<td>142.4 ± 1.3</td>
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<td><strong>Effect of Na− removal</strong></td>
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<tr>
<td>Na-free ($n = 5$)</td>
<td>0.57 ± 0.08</td>
<td>7.43 ± 0.01</td>
<td>2.31 ± 0.22</td>
<td>132.6 ± 1.5</td>
<td>133.1 ± 1.3</td>
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<td>Na-free + bumetanide ($n = 5$)</td>
<td>0.65 ± 0.08</td>
<td>7.41 ± 0.01</td>
<td>2.23 ± 0.13</td>
<td>132.0 ± 0.5</td>
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<td><strong>Effect of extracellular K+</strong></td>
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<tr>
<td>K+ = 2 mM ($n = 5$)</td>
<td>0.55 ± 0.04</td>
<td>7.39 ± 0.03</td>
<td>2.30 ± 0.21</td>
<td>137.7 ± 1.4</td>
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<tr>
<td>K+ = 2 mM + bumetanide ($n = 6$)</td>
<td>0.63 ± 0.08</td>
<td>7.40 ± 0.03</td>
<td>2.04 ± 0.11</td>
<td>137.1 ± 1.0</td>
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<td>K+ = 20 mM ($n = 5$)</td>
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<td>7.35 ± 0.02</td>
<td>1.67 ± 0.05</td>
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<tr>
<td>K+ = 20 mM + bumetanide ($n = 6$)</td>
<td>0.55 ± 0.04</td>
<td>7.34 ± 0.01</td>
<td>2.03 ± 0.11</td>
<td>137.7 ± 0.9</td>
<td>139.8 ± 1.0</td>
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<td><strong>Effect of extracellular NH4+ at a K+ concentration of 2 mM</strong></td>
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<td>NH4+ = 6 mM ($n = 5$)</td>
<td>0.67 ± 0.06</td>
<td>7.36 ± 0.03</td>
<td>2.21 ± 0.21</td>
<td>137.9 ± 1.2</td>
<td>142.4 ± 1.1</td>
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<tr>
<td>NH4+ = 6 mM + bumetanide ($n = 6$)</td>
<td>0.50 ± 0.04</td>
<td>7.32 ± 0.01</td>
<td>2.24 ± 0.18</td>
<td>140.1 ± 0.5</td>
<td>141.6 ± 0.8</td>
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Values are means ± SE, n, No. of tubules; $J_{Cl}$, transepithelial Cl− flux; OMCD, outer medullary collecting duct; DOCP, deoxycorticosterone pivalate; $^aP < 0.05$. $^\dagger P = $ not significant.
lie between the superficial distal tubule and the base of the collecting duct. The contribution of each segment that falls between these two micropuncture sites to the increment in Cl⁻ delivered to the base of the collecting duct is not known. The present study demonstrates that the OMCD is one segment in this region of the nephron that secretes Cl⁻ in vitro. The pathway(s) that mediates Cl⁻ uptake across the basolateral membrane of the α-intercalated cell in rat OMCD were therefore explored in more detail.

Anion exchange mediates Cl⁻ uptake and HCO₃⁻ exit across the basolateral membrane. Application of H₂DIDS led to a marked increase in pHi. Although pHi changes might be due to nonspecific effects of H₂DIDS, the most likely explanation for these observations is that H₂DIDS prevents Cl⁻/HCO₃⁻ exchange from mediating net HCO₃⁻ exit. The anion exchanger responsible for the increase in pHi after the addition of H₂DIDS to the bath solution is probably AE1, a Na⁺-independent, Cl⁻/HCO₃⁻ exchanger, which is stillbene sensitive and highly expressed along the basolateral membrane of the α-intercalated cell in rat OMCD (7). Because Cl⁻ secretion was not eliminated with 0.5 mM H₂DIDS, other Cl⁻ uptake pathways along the basolateral membrane of rat OMCD might also be important in the process of transepithelial Cl⁻ transport.

We demonstrated that JCl⁻ is reduced in a dose-dependent fashion with bumetanide. These data are consistent with previous reports of the dose response of bumetanide to rat NKCC1 (14, 28). At a concentration of <10 μM, bumetanide is a relatively specific inhibitor of NKCC1 (15). Therefore, significant Cl⁻ uptake across the basolateral membrane is mediated by NKCC1 because >50% of JCl⁻ is inhibited by low concentrations of bumetanide (10 μM) when added to the bath.

In rat, NKCC1 is fully inhibited at a bumetanide concentration of 100 μM (14, 28). However, at this bumetanide concentration, at least partial inhibition of other Cl⁻ transporters, such as Cl⁻/HCO₃⁻ exchange (AE1) (46), Cl⁻ channels (19), and KCl cotransport (19), has been observed. Cl⁻ secretion sensitive to 100 μM bumetanide may therefore overestimate the contribution of NKCC1 to total transepithelial Cl⁻ transport in rat OMCD. However, ion substitution experiments demonstrated that the bumetanide-sensitive component of Cl⁻ secretion is dependent on extracellular Na⁺ and either K⁺ or NH₄⁺. This ion dependency is consistent with Cl⁻ uptake mediated by NKCC1 instead of through these other transporters. Although Cl⁻ uptake across the basolateral membrane can be attributed to both anion exchange and NKCC1, the mechanism of Cl⁻ transport across the apical membrane is unknown.

Whether Cl⁻ uptake across the basolateral membrane of the α-intercalated cell in rat OMCD is mediated by NKCC1 or NKCC2 cannot be determined directly. In the rat outer medulla, the absorptive isoform

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Table 3. Fluid flux in OMCD tubules from DOCP-treated rats

<table>
<thead>
<tr>
<th>Tubule Length, mm</th>
<th>Bath pH</th>
<th>Collection Rate/Tubule, nl·mm⁻¹·min⁻¹</th>
<th>J₀, transepithelial fluid flux, nl·mm⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>0.57 ± 0.05</td>
<td>7.39 ± 0.01</td>
<td>2.22 ± 0.10</td>
</tr>
<tr>
<td>Bumetanide (n = 4)</td>
<td>0.48 ± 0.03</td>
<td>7.40 ± 0.02</td>
<td>2.73 ± 0.24</td>
</tr>
</tbody>
</table>

Values are means ± SE, n, No. of tubules; J₀, transepithelial fluid flux. *P = not significant.

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Fig. 4. Effect of extracellular K⁺ and NH₄⁺ on JCl⁻. A: tubules were perfused and bathed in HCO₃⁻/CO₂-buffered solutions containing 2 mM KCl, but in the absence of NH₄Cl (solution 4). JCl⁻ was low and not reduced further with the application of bumetanide (100 μM) to the bath (P > NS, unpaired Student’s t-test). B: tubules were perfused and bathed in the same solution as described in A, but one in which the K⁺ concentration was increased to 20 mM (solution 5). Under these conditions, Cl⁻ secretion was observed, which was attenuated with the application of bumetanide (100 μM) to the bath (P < 0.05, unpaired Student’s t-test). C: tubules were perfused and bathed in HCO₃⁻/CO₂-buffered solutions containing 2 mM KCl, and 6 mM NH₄Cl (solution 6). Significant Cl⁻ secretion was observed, which was attenuated with the application of bumetanide (100 μM) to the bath (P < 0.05, unpaired Student’s t-test).

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Anion exchange mediates Cl⁻ uptake and HCO₃⁻ exit across the basolateral membrane. Application of H₂DIDS led to a marked increase in pHi. Although pHi changes might be due to nonspecific effects of H₂DIDS, the most likely explanation for these observations is that H₂DIDS prevents Cl⁻/HCO₃⁻ exchange from mediating net HCO₃⁻ exit. The anion exchanger responsible for the increase in pHi after the addition of H₂DIDS to the bath solution is probably AE1, a Na⁺-independent, Cl⁻/HCO₃⁻ exchanger, which is stillbene sensitive and highly expressed along the basolateral membrane of the α-intercalated cell in rat OMCD (7). Because Cl⁻ secretion was not eliminated with 0.5 mM H₂DIDS, other Cl⁻ uptake pathways along the basolateral membrane of rat OMCD might also be important in the process of transepithelial Cl⁻ transport.

We demonstrated that JCl⁻ is reduced in a dose-dependent fashion with bumetanide. These data are consistent with previous reports of the dose response of bumetanide to rat NKCC1 (14, 28). At a concentration of <10 μM, bumetanide is a relatively specific inhibitor of NKCC1 (15). Therefore, significant Cl⁻ uptake across the basolateral membrane is mediated by NKCC1 because >50% of JCl⁻ is inhibited by low concentrations of bumetanide (10 μM) when added to the bath.

In rat, NKCC1 is fully inhibited at a bumetanide concentration of 100 μM (14, 28). However, at this bumetanide concentration, at least partial inhibition of other Cl⁻ transporters, such as Cl⁻/HCO₃⁻ exchange (AE1) (46), Cl⁻ channels (19), and KCl cotransport (19), has been observed. Cl⁻ secretion sensitive to 100 μM bumetanide may therefore overestimate the contribution of NKCC1 to total transepithelial Cl⁻ transport in rat OMCD. However, ion substitution experiments demonstrated that the bumetanide-sensitive component of Cl⁻ secretion is dependent on extracellular Na⁺ and either K⁺ or NH₄⁺. This ion dependency is consistent with Cl⁻ uptake mediated by NKCC1 instead of through these other transporters. Although Cl⁻ uptake across the basolateral membrane can be attributed to both anion exchange and NKCC1, the mechanism of Cl⁻ transport across the apical membrane is unknown.

Whether Cl⁻ uptake across the basolateral membrane of the α-intercalated cell in rat OMCD is mediated by NKCC1 or NKCC2 cannot be determined directly. In the rat outer medulla, the absorptive isoform
of the Na-K-2Cl cotransporter, NKCC2, has been localized to the thick ascending limb by in situ hybridization and immunolocalization studies (5, 25). Although NKCC2 message has also been detected in rat OMCD (45), it is unlikely that the isoform of the cotransporter detected in the present study is NKCC2. First, NKCC2 protein expression has not been detected in rat OMCD (5), although NKCC1 protein has been clearly demonstrated in this segment (13). Therefore, NKCC2 is most probably not as abundant in this segment as NKCC1. Second, bumetanide inhibits Cl\(^-\) secretion when applied to the bath, consistent with localization of NKCC1 to the basolateral membrane (13).

In the present study it was observed that total and bumetanide-sensitive \(J_{\text{Cl}}\) vary greatly over an extracellular K\(^+\) concentration range of 2–20 mM. Because the interstitium of the rat outer medulla is not accessible to micropuncture, interstitial K\(^+\) concentration in vivo is not known. However with medullary recycling of K\(^+\), interstitial K\(^+\) concentration in the outer medulla is expected to be greater than serum values (23), which vary in rat\(^3\) between 2 and 7 mM (3, 4), but less than interstitial values in the inner medulla, which range from 6 to 54 mM (2). Thus interstitial K\(^+\) concentration in the interstitium of the outer medulla is probably \(>2\) but \(<50\) mM. For NKCC1 the Michaelis-Menten constant \((K_m)\)\(^4\) for Rb\(^+\) (a K\(^+\) congener) is 2-15 mM (22, 30, 43). Thus NKCC1-mediated Cl\(^-\) uptake should vary greatly over a K\(^+\) concentration range of 2–50 mM\(^5\). If so, changes in K\(^+\) concentration in the interstitium of the rat outer medulla in vivo should markedly alter Cl\(^-\) secretion in the OMCD through changes in NKCC1-mediated Cl\(^-\) transport.

Although total and bumetanide-sensitive \(J_{\text{Cl}}\) in rat OMCD vary greatly with changes in extracellular Na\(^+\) concentration, it is less likely that changes in interstitial Na\(^+\) concentration in vivo significantly regulate NKCC1-mediated Cl\(^-\) uptake. In rat, serum Na\(^+\) concentration ranges from 95 to 200 mM (20). Through countercurrent multiplication, interstitial Na\(^+\) concentration in rat outer medulla is therefore probably \(>95\) mM, although it has not been measured directly. Because the \(K_m\) for Na\(^+\) reported for mammalian NKCC1 is generally less than 50 mM (22, 30), the Na\(^+\) concentration of the rat outer medullary interstitium probably always approaches maximal transport rate conditions for NKCC1. Thus changes in interstitial Na\(^+\) concentration over the physiological range expected in vivo probably do not significantly alter NKCC1-mediated Cl\(^-\) uptake.

Although Cl\(^-\) transporters such as AE1 clearly participate in transepithelial secretion of net H\(^+\) equivalents, the role of NKCC1 in renal physiology is not known. The Na-K-2Cl cotransporter has been implicated in a number of cell functions, including the secretion of HCl (38) and KCl (32). However, its primary physiological function is in volume regulation and the vectorial transport of water and NaCl (25). Therefore, rat \(\alpha\)-intercalated cells, which express high levels of NKCC1 relative to other cells in rat kidney, may serve physiological functions other than secretion of H\(^+\) equivalents.

In vivo studies have demonstrated secretion of NaCl and fluid along rat IMCD (36, 40). However, more recent studies of Wallace and co-workers (44) have reported secretion of fluid in vitro in rat initial IMCD (44), a segment that contains \(\alpha\)-intercalated cells and expresses NKCC1 (13). Fluid secretion, determined by measuring luminal diameter over a 5- to 12-h period in tubules with sealed ends, was attenuated with the addition of bumetanide to the bath (44). In the present study, very low levels of fluid secretion were observed in rat OMCD, with a \(J_w\) similar to that reported previously in rat CCD (1) and rat tIMCD (42). Low levels of fluid secretion observed in rat OMCD in the present study are compatible with observations of Wallace et al. (44) in initial IMCD. However, in rat OMCD no change in \(J_w\) was detected with inhibition of NKCC1. Therefore, under the conditions of the present study a role of NKCC1 in fluid secretion or absorption in OMCD tubules from DOCP-treated rats could not be demonstrated.

Previous studies have suggested a role of the Na-K-2Cl cotransporter in NaCl secretion in the rat IMCD (31). The natriuresis and chloruresis observed in rats given a NaCl load occurs in part through atrial natriuretic factor (ANF) (10, 37, 40). In rat initial IMCD, Rocha and Kudo (31) observed that with the application of ANF to the bath fluid, bath-to-lumen flux of Na\(^+\) and Cl\(^-\) is increased, whereas the lumen-to-bath flux of these ions is reduced. This ANF-induced change in Na\(^+\) and Cl\(^-\) secretion was fully inhibited with low concentrations of furosemide when added to the bath solution. The authors concluded that ANF reduced Na\(^+\) and Cl\(^-\) absorption through inhibition of apical Na\(^+\) channels while stimulating Na\(^+\) and Cl\(^-\) secretion mediated by the Na-K-2Cl cotransporter. The possible role of NKCC1 in mediating NaCl excretion after NaCl stress will require further study.

In conclusion, rat OMCD secretes Cl\(^-\). Cl\(^-\) secretion in this segment occurs in part through Cl\(^-\) uptake across the basolateral membrane, mediated by NKCC1, in series with Cl\(^-\) efflux across the apical membrane. The physiological role of this transport process remains to be determined. However, under the conditions of the present in vitro study, a role of NKCC1 in fluid secretion or absorption was not demonstrated in this segment. The cosecreted cation or

\(^3\)Interstitial ion concentration in cortex is taken to be equivalent to serum levels. In inner medulla, interstitial ion concentrations are assumed to reflect values measured in vasa recta plasma at the same level along the corticomedullary axis (24).

\(^4\)The \(K_m\), or apparent affinity, of a transport protein reflects the substrate concentration needed to achieve half the maximal transport rate \((V_{\text{max}})\).

\(^5\)For mouse NKCC1, our laboratory has reported a \(K_m\) for K\(^+\) of 4.6 mM (45). Assuming that NKCC1 follows Michaelis-Menten kinetics, at an extracellular K\(^+\) concentration of 2 mM, NKCC1-mediated Cl\(^-\) uptake should operate at \(-28\%\) of \(V_{\text{max}}\). If a perfusate flow rate of 2 nl-mm\(^{-1}\)-min\(^{-1}\) is employed, the predicted change in perfusate Cl\(^-\) concentration with the application of bumetanide should be 0.8 mM, which is beyond the limit of detection of this assay (11).
counterion, which accompanies bumetanide-sensitive Cl− secretion, remains to be established.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-46493 (to S. M. Wall).

REFERENCES


