CFTR is required for PKA-regulated ATP sensitivity of Kir1.1 potassium channels in mouse kidney

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The cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel plays vital roles in fluid transport in many epithelia. While CFTR is expressed along the entire nephron, its function in renal tubule epithelial cells remains unclear, as no specific renal phenotype has been identified in cystic fibrosis. CFTR has been proposed as a regulator of the 30 pS, ATP-sensitive renal K channel (Kir1.1, also known as renal outer medullary K [ROMK]) that is critical for K secretion by cells of the thick ascending limb (TAL) and distal nephron segments responsive to aldosterone. We report here that both ATP and glibenclamide sensitivities of the 30 pS K channel in TAL cells were absent in mice lacking CFTR and in mice homozygous for the ΔF508 mutation. Curcumin treatment in ΔF508-CFTR mice partially reversed the defect in ATP sensitivity. We demonstrate that the effect of CFTR on ATP sensitivity was abrogated by increasing PKA activity. We propose that CFTR regulates the renal K secretory channel by providing a PKA-regulated functional switch that determines the distribution of open and ATP-inhibited K channels in apical membranes. We discuss the potential physiological role of this functional switch in renal K handling during water diuresis and the relevance to renal K homeostasis in cystic fibrosis.

Introduction

The regulation of salt and water excretion by the kidney is crucial for volume and osmotic homeostasis and requires the complex coordinated effects of many different ion, water, and solute transport proteins that are differentially distributed along the nephron. Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent, PKA-regulated Cl− channel (1) that is highly expressed in all segments of the mammalian nephron (2–6). While CFTR plays vital roles in fluid transport in many epithelia, such as those from lung, sweat duct, and intestine, its function in renal tubule epithelial cells remains unclear (2, 7). This is underscored by the apparent lack of an overt renal phenotype in cystic fibrosis.

CFTR not only functions as a Cl− channel but has also been proposed as a regulator of other ion channels, including the renal secretory renal outer medullar potassium (ROMK) channel (8–11). ROMK (or Kir1.1; gene Kcnj1) is a 30 pS K channel that is a member of the inward rectifier family of K channels (Kir; ref. 11) which proposed as a regulator of other ion channels, including the renal secretory renal outer medullar potassium (ROMK) channel (8–11). ROMK (or Kir1.1; gene Kcnj1) is a 30 pS K channel that is a member of the inward rectifier family of K channels (Kir; ref. 11) which also includes the ATP-sensitive K channels (KATP, Kir6.x; ref. 12). ROMK mediates apical K recycling in the thick ascending limb (TAL) and net K secretion by aldosterone-sensitive distal nephron cells in the connecting segment and cortical collecting duct (CCD) (11, 13). Loss-of-function mutations in the human ROMK gene (KCNJ1) cause type II Bartter syndrome with renal salt wasting (14, 15), consistent with the crucial role of ROMK in supporting NaCl reabsorption in the TAL. Studies in the ROMK-null mouse (16) have confirmed that ROMK forms the 30 pS K recycling/secretry channel in the TAL and collecting duct (17) and is also required for expression of the 70 pS K recycling channel in the TAL (18).

ROMK channel expression and function is intricately regulated by a wide variety of factors, including intracellular pH and both extracellular and cytosolic ATP (11). Mg-ATP concentrations of ≥1 mM and micromolar concentrations of the sulfonylurea glibenclamide inhibit the 30 pS K channels in the TAL and CCD (19). In vitro studies have suggested that CFTR may be required for inhibition of ROMK by cytosolic ATP (20) and glibenclamide (20, 21). These in vitro effects of CFTR on ROMK function are likely mediated through binding of both proteins via PSD95/DLG/ZO-1 (PDZ) interactions to the scaffolding protein Na+H+ exchange regulatory factor (NHERF; ref. 22) and require the transmembrane domain 1, the first nucleotide-binding fold, and the regulatory domain on CFTR (23, 24). In addition, CFTR-ROMK functional interaction can be regulated by cAMP-dependent protein kinase A (PKA), since increasing cAMP abrogates the effect of CFTR on glibenclamide sensitivity of ROMK in Xenopus laevis oocytes (21). However, other ABC proteins, the sulfonylurea receptors (SUR1/2), have been implicated in the effect of glibenclamide on ROMK (25) as well as ATP and glibenclamide sensitivities of the other ATP-sensitive inward rectifier channels (KATP, Kir6.x; refs. 12, 13, 26). Since SUR mRNA and protein are expressed in the distal nephron (11), the potential role of CFTR on ROMK function in vivo is unclear.

In the present study we examined whether CFTR is required for ATP sensitivity of ROMK in mouse kidney and whether CFTR’s effects are altered by cAMP/PKA. Studies were performed using 2 different mouse models of cystic fibrosis, a CFTR-null strain (cftr−/−, cfrf508B; ref. 27) and a ΔF508-CFTR strain (cftr+/ΔF508; ref. 28). In each model, ATP and glibenclamide sensitivities were drastically reduced or eliminated, consistent with CFTR as a criti-
Cytosolic-side pH provides an important and fundamental gating mechanism for ROMK, with reductions in pH to ≤6.9 reversibly inhibiting channel activity (11). To determine whether the absence of CFTR at the plasma membrane globally affects the gating of ROMK, we examined the effect of pH on ROMK channel activity. Figure 2 shows that NPFo was reversibly inhibited in both wild-type and mCFTR –/– mice by lowering cytosolic pH from 7.4 to 6.9 (Figure 2, A and B). NPFo decreased in wild-type mice from 5.02 ± 0.82 to 0.05 ± 0.02 (n = 4) and in mCFTR –/– mice from 6.72 ± 0.23 to 0.04 ± 0.01 (n = 5). Thus the absence of CFTR did not affect the ability of ROMK to be inhibited by an acidic cytosolic pH.

CFTR is not required for regulation by extracellular ATP or low concentrations of intracellular ATP. ROMK is complexly regulated by both extracellular and intracellular ATP (11), and one potential way in which CFTR could modulate ROMK activity is by affecting one of these regulatory mechanisms. For example, purinergic receptors are expressed along the entire nephron (30), and extracellular ATP has been shown to inhibit the apical 30 pS ROMK channel in mouse CCD principal cells through activation of a P2Y2 receptor in the apical membrane (31). In addition, CFTR has been postulated to mediate efflux of ATP out of the cell (32) or to enhance another physiological characteristic to the mCFTR transgenic mouse, mCFTR–/–; ref. 27) or mouse CFTR trafficking to the plasma membrane with the ΔF508 mutation (ΔF508-CFTR; ref. 28) altered ROMK channel activity (NPfo see “Statistics” in Methods), measured as number of channels (N) × single-channel open probability (Po), or single-channel characteristics in TAL cells. Figure 1, A and B, shows typical K channel current traces from excised, inside-out patches of cortical TAL apical membranes from both CFTR transgenic mouse models. The associated I–V curves showed slight inward rectification typical for ROMK (17). Table 1 shows that the average single-channel conductance and Po was not different from our previous observations in TAL from wild-type mice (17). The dwell-time histograms (Figure 1, A and B) show 1 open time and 1 closed time that were similar to those in patches of TAL from wild-type mice (Table 1; ref. 17). Thus, CFTR is not required for trafficking of ROMK to the plasma membrane of TAL cells, and the absence of CFTR at the plasma membrane does not alter single-channel electrical properties of ROMK.

Table 1
Single-channel characteristics of the apical K channel in distinct CFTR genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>G (pS)</th>
<th>Po</th>
<th>Tmo (ms)</th>
<th>Toc (ms)</th>
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<tbody>
<tr>
<td>Wild-type (n = 4)A</td>
<td>31.5 ± 0.23</td>
<td>0.88 ± 0.02</td>
<td>28.28 ± 0.23</td>
<td>1.21 ± 0.13</td>
</tr>
<tr>
<td>mCFTR–/– (n = 4)</td>
<td>32.1 ± 0.12</td>
<td>0.87 ± 0.16</td>
<td>30.47 ± 1.21</td>
<td>1.69 ± 1.23</td>
</tr>
<tr>
<td>DF508-CFTR (n = 3)</td>
<td>30.46 ± 0.28</td>
<td>0.89 ± 0.13</td>
<td>24.59 ± 2.94</td>
<td>1.51 ± 0.46</td>
</tr>
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Channel conductance (G) was calculated between −40 and −80 mV from inside-out patches. *Data cited from ref. 17. Tmo, open time; Toc, closed time.
extracellular ATP (Figure 3B) in TAL cells. In mCFTR the principal cells (31) from CCDs, but also in TAL cells (Figure 3A).

In addition, the absence of mouse CFTR did not alter the effect of extracellular ATP on ROMK activity in inside-out patches from TAL or principal cells (40). In vitro studies in X. laevis oocytes suggest that these inhibitors of ROMK require coexpression of CFTR (20, 21). The effects of glibenclamide in representative current traces from inside-out patches of apical membranes from wild-type and mCFTR−/− mice are shown in Figure 5, A and B, and the data are summarized in Figure 5C. Addition of 20 μM glibenclamide reversibly inhibited wild-type ROMK channel activity (Figure 5A); NPo increased from 0.03 ± 0.01 to 2.57 ± 0.24 by addition of 500 μM Mg-ATP (Figure 4A; n = 8); this response was similar in TAL from mCFTR−/− mice, where NPo increased from 0.01 ± 0.02 to 2.61 ± 0.31 after addition of 250 μM Mg-ATP (Figure 4B; n = 9).

CFTR is required for inhibition of ROMK by micromolar glibenclamide and millimolar intracellular ATP. Cytosolic concentrations of Mg-ATP ≥1 mM or micromolar concentrations of the sulfonylurea glibenclamide inhibit ROMK channel activity in inside-out patches from TAL or principal cells (40).

In the apical membrane of TAL from wild-type and mCFTR−/− mice, single-channel recordings show pH 6.9 reversibly inhibited ROMK channels in inside-out patches in both wild-type (A) and mCFTR−/− (B) mice. Bottom: Fast time resolution tracings obtained at time points indicated by corresponding numbered arrows at top.

Figure 3
Extracellular ATP inhibits the ROMK channel in the apical membrane of TAL. Low concentration of Mg-ATP (200 μM) reversibly reduced ROMK channel activity in cell-attached patches, likely through stimulation of a P2Y2 receptor, in wild-type (A) and mCFTR−/− mice (B) held at a −V of 0 mV.
reduce PKA-mediated phosphorylation (42). In addition, curcumin is an ER Ca\textsuperscript{2+}-ATP completely inhibited channel activity (31). The representative current trace from a mCFTR\textsuperscript{+} mouse in Figure 6B shows that ATP sensitivity was dramatically altered. Increases in Mg-ATP up to 8 mM had no significant effect on NP\textsubscript{p} (n = 4). A Mg-ATP-free, Ca\textsuperscript{2+}-containing Ringers bath solution was used to allow channel rundown to the baseline current (0 + Ca\textsuperscript{2+} in Figure 6B). Following rundown, addition of 1 mM Mg-ATP showed the typical partial recovery (Figure 6B). Figure 6C shows a representative current trace in a TAL cell from a ΔF508-CFTR mouse illustrating that the Mg-ATP inhibitory effect was also virtually abolished. The Mg-ATP sensitivities for each of the genotypes are compared in Figure 6D. While 2 mM Mg-ATP completely inhibited 30 pS K channel activity in wild-type mice (Table 2; Hill coefficient = 4.4 ± 2.6), 10 mM Mg-ATP only minimally reduced NP\textsubscript{p} from 10.44 ± 0.23 to 7.34 ± 0.31 (~32.3%; n = 3) in mCFTR\textsuperscript{+} mice and from 8.46 ± 0.72 to 6.77 ± 0.63 (~21.4%; n = 3) in ΔF508-CFTR mice. The Mg-ATP IC\textsubscript{50} values were 10.9 mM (Table 2) for mCFTR\textsuperscript{+} mice and 12.6 mM for ΔF508-CFTR mice. Thus, Mg-ATP sensitivity of the 30 pS K channel required expression of CFTR at the plasma membrane.

Curcumin partially rescues ATP sensitivity of ROMK in ΔF508-CFTR mice. Curcumin, a constituent of the spice turmeric, exerts complex actions on CFTR function, including both direct and indirect effects. In cultured cells, acute administration of curcumin can act as a CFTR channel opener (41, 42) and can reduce PKA-mediated phosphorylation (42). In addition, curcumin is an ER Ca\textsuperscript{2+}-ATPase inhibitor that has been reported to increase plasma membrane expression of ΔF508-CFTR in baby hamster kidney cells (43), although this effect on ΔF508-CFTR trafficking in cultured cells is controversial for this mutant channel (44–47) as well as for other ABC transporters (47). In ΔF508-CFTR mice, both increases (43) and no effects (44) in CAMP-stimulated transepithelial potentials in CFTR-expressing epithelia have been observed.

Curcumin has been suggested to partially recover the defect in CFTR function in ΔF508-CFTR mice (43). Thus in a subset of ΔF508-CFTR mice, curcumin was administered orally for 3–7 days, and the ATP sensitivity of the 30 pS K channel was assessed. Either the rectal potential difference (RPD) and/or the nasal potential difference (NPD) was measured prior to curcumin treatment and at the end of the treatment period (n = 4) as described previously (43). In these mice the average baseline NPD after treatment decreased by 12.3 ± 0.09 mV, approaching the baseline values of wild-type mice. In addition, perfusion with the low Cl\textsuperscript{−} solution resulted in a modest hyperpolarization of the NPD similar to that seen in wild-type animals (4.14 ± 1.99 mV). Finally, perfusion with isoproterenol enhanced this effect further, such that the resultant potential difference was similar to that of wild-type mice (3.5 ± 0.91 mV). As described previously (43), these data suggest there was a partial correction in the NPD following treatment with curcumin. Similar alterations were observed in the RPD assay in treated mice. As shown in Figure 7A, increases in cytosolic Mg-ATP 6 mM partially inhibited 30 pS K channel activity in TAL cells from curcumin-treated ΔF508-CFTR mice. The effect of curcumin is summarized in Figure 7B and shows that this compound restored partial sensitivity to Mg-ATP.

Cytosolic acidification enhances ATP sensitivity of ROMK in the presence and absence of CFTR. Cytosolic pH is a major regulator of ROMK, with reductions in pH lowering channel activity (11). In earlier studies, we showed a low intrinsic Mg-ATP sensitivity of ROMK expressed in X. laevis oocytes and that this sensitivity could be 

Figure 5
Glibenclamide sensitivity of ROMK in inside-out patches from TAL apical membrane of wild-type and mCFTR\textsuperscript{+} mice. (A) Low concentration of glibenclamide reversibly reduced ROMK channel activity with an IC\textsubscript{50} of 15 μM in wild-type mice. (B) Glibenclamide had no significant effect on ROMK channel activity at 0.2 mM (IC\textsubscript{50}, 0.68 mM) in mCFTR\textsuperscript{+} mice. (C) Glibenclamide dose response of the normalized NP\textsubscript{p}.
enhanced by reducing cytosolic pH from 7.4 to 7.2 (48). To determine whether lowering cytosolic pH could also partially restore Mg-ATP sensitivity of 30 pS K channels in TAL cells in the absence of CFTR, we assessed the effect of lowering intracellular pH from 7.4 to 7.2 in mCFTR+/− mice (Figure 8 and Table 2). Representative currents traces from wild-type and mCFTR+/− mice are shown in Figure 8, A and B, respectively, and the results are summarized in Figure 8C. In wild-type mice, reduction in cytosolic pH from 7.4 to 7.2 reduced the IC50 for Mg-ATP from 0.88 mM to 0.61 mM (Table 2; n = 8). In mCFTR+/− mice the same change in pH produced a dramatic shift of Mg-ATP sensitivity (IC50 fell from 10.12 mM to 3.22 mM; Table 2; n = 14). Thus, expression of CFTR produced 5-fold and 10-fold increases in the sensitivity of ROMK at cytosolic pH values of 7.4 and 7.2, respectively. This weak intrinsic Mg-ATP sensitivity of the 30 pS K channel observed in TAL cells in the absence of CFTR is consistent with the ability of the cytosolic regions of ROMK to bind nucleotides (49, 50).

Curcumin has recently been reported to alter the channel gating and phosphorylation state of CFTR (41, 42) and might therefore affect the gating of other channels. Since many agents or conditions can alter the pH sensitivity of ROMK and pH can affect Mg-ATP sensitivity (Table 2; ref. 49), we questioned whether curcumin might alter the pH gating of ROMK and thereby affect Mg-ATP sensitivity. Indeed, similar effects on ATP sensitivity of cytosolic pH reduction in mCFTR+/− mice (Figure 8C) and curcumin treatment in ΔF508-CFTR mice (Figure 7B) would be consistent with this possibility. Thus we assessed the acute effects of 10 μM curcumin on the ability of 2 mM Mg-ATP to inhibit 30 pS K channel activity in TAL cells and on the pH sensitivity of ROMK channels expressed in X. laevis oocytes.

Figure 9A shows a representative current trace from an inside-out patch of a TAL cell isolated from a wild-type mouse that demonstrates the effects of curcumin on Mg-ATP–mediated inhibition of 30 pS K channel activity. An approximately 5-minute cytosolic exposure to 10 μM curcumin had no effect on channel activity (Figure 9, A and B; NP0, 7.16 ± 1.80 versus 6.98 ± 1.92 after curcumin; NS; n = 6). The ability of 2 mM Mg-ATP to abolish 30 pS K channel activity in TAL cells was also unaltered (Figure 9, A and B). Figure 9C summarizes the effect of cytosolic pH on ROMK activity in excised inside-out giant patches from X. laevis oocytes (n = 3). The pH sensitivity of ROMK was not altered by extracellular exposure to 10 μM curcumin. Thus direct curcumin effects on 30 pS (ROMK) channels did not account for the increase in Mg-ATP sensitivity observed in ΔF508-CFTR mice following treatment with curcumin (Figure 7).

Enhanced PKA phosphorylation abolishes the inhibitory action of 2 mM cytosolic ATP in TAL and principal cells from wild-type mice. Our previous studies in X. laevis oocytes with coexpression of human CFTR and rat ROMK2 showed that forskolin stimulation abolished the sensitivity of ROMK to glibenclamide (21). PKA phosphorylates both ROMK (35, 36) and the regulatory domain of CFTR (51–53), and these phospholyations activate both channels. Thus, the PKA phosphorylation state of the cell may modulate the functional interactions of CFTR and ROMK.

Since CFTR was required for high sensitivity of ROMK to glibenclamide (Figure 5C) and Mg-ATP (Figure 6D), we examined whether PKA-dependent phosphorylation would also modulate Mg-ATP sensitivity in TAL cells from wild-type mice. Pretreatment of TAL with 2 μM forskolin and 300 μM 3-isobutyl-1-methylxanthine

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>pH</th>
<th>NP0</th>
<th>IC50 ATP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>7.4</td>
<td>2.22 ± 0.49</td>
<td>0.88</td>
</tr>
<tr>
<td>+/+</td>
<td>7.2</td>
<td>1.15 ± 0.18</td>
<td>0.61</td>
</tr>
<tr>
<td>−/−</td>
<td>7.4</td>
<td>5.68 ± 0.68</td>
<td>10.9</td>
</tr>
<tr>
<td>−/−</td>
<td>7.2</td>
<td>4.16 ± 0.51</td>
<td>3.2</td>
</tr>
</tbody>
</table>

+/, wild-type; −/−, CFTR knockout.
(IBMX) prior to patching abolished the sensitivity of 30 pS channels in TAL cells to 1–2 mM Mg-ATP (Figure 10A; n = 3; compare with Figure 6, A and D).

ROMK is also expressed in principal cells from the CCD, and PKA stimulates 30 pS K channel activity in these cells (11). Figure 10C shows a typical current trace from a principal cell, illustrating that 1 mM Mg-ATP reversibly inhibited K channel activity in a manner similar to the TAL (Figure 6A). Pretreatment of principal cells with forskolin and IBMX also abolished the ability of 1–2 mM Mg-ATP to inhibit channel activity (Figure 10, B and D). Thus, unlike the apparent fixed interaction of Kir6.x subunits with their ABC partners (SUR proteins) in KATP channels (12, 13, 26), the state of PKA phosphorylation rapidly modulated the functional interaction of ROMK and CFTR with regard to Mg-ATP sensitivity in both TAL and principal cells.

Discussion

Our results in 2 transgenic mouse models of cystic fibrosis demonstrate that CFTR was necessary for both glibenclamide and Mg-ATP sensitivity of the ROMK-type 30 pS K channel present in TAL cells. Since CFTR and ROMK-type 30 pS K channels were expressed in principal cells of the CCD, the functional interaction between both channels likely also occurs in this renal epithelial cell type. This possibility is supported by the similar effects of PKA phosphorylation on Mg-ATP sensitivity of the K channels we observed in both TAL and principal cells, as shown in Figure 10. However, CFTR had no effect on basic 30 pS K channel properties: single-channel conductance and kinetics, gating by low pH, activation by micromolar Mg-ATP, and inhibition by external ATP via purinergic receptors. Thus, the effect of CFTR on ROMK is apparently specific for gating of the 30 pS K channel by millimolar cytosolic Mg-ATP.

The functional interaction between CFTR and ROMK is similar to that previously observed for KATP channels, in which another ABC protein, the sulfonylurea receptor SUR1/2, conferred glibenclamide sensitivity and enhanced ATP interaction with the Kir6.x subunits (12, 13, 26). These observations suggest a general model for ATP sensitivities of inwardly rectifying K channels involving ABC-Kir interactions. The ABC-Kir interactions of CFTR and ROMK are, however, quite distinct from those for classical KATP channels in 4 respects. First, while KATP channels are formed by a stable 4:4 interaction between SUR1/2 and Kir6.x subunits (12, 13), the interaction between CFTR and ROMK may require a third partner. The scaffolding protein NHERF has been shown to stabilize the CFTR-ROMK interaction by binding to both channel proteins via their COOH-terminal PDZ domains (22, 54, 55). In turn, NHERF can function as a scaffolding protein to link the 30 pS K channel complex to ezrin, a PKA scaffolding protein (56, 57). Second, while SUR1/2-Kir6.x interactions are required for surface expression of KATP channels via mutual shielding of ER retention signals (29),
CFTR is not required for surface expression of ROMK. The latter was shown by NPo not being reduced, but rather increased, in CFTR–/– mice (Table 2). Third, the CFTR-ROMK stoichiometry is unknown. SUR1-Kir6.2 exhibits a fixed 4:4 stoichiometry due to mutual shielding of ER retention signals on both Kir6.x and SUR subunits (29). In contrast, ROMK, which also contains a C-terminal ER retention signal, uses a phosphorylation mechanism to promote forward trafficking (58), that is, it does not require interaction with an ABC protein. However, the Hill coefficients of approximately 4 for glibenclamide and Mg-ATP suggest that the CFTR-ROMK may also be 4:4. Fourth, the functional interaction between CFTR and ROMK was not static as in KATP channels but dynamic, as shown by the abrogation of Mg-ATP sensitivity of the 30 pS K channels in wild-type mice by increasing the activity of cAMP/PKA (Figure 10). In this regard, Foskett and coworkers (55) have suggested that the magnitude of PKA-stimulated CFTR channel activity is regulated by bivalent PDZ-mediated interactions with NHERF and modulated by the stoichiometry of NHERF/CFTR (54). A recent study (59) supports the dimeric form of CFTR in plasma membranes in cultured cells with a fraction of CFTR dimer formed with NHERF. Thus, it is possible that PKA-mediated activation of CFTR may lead to increased bivalent association of CFTR with NHERF and thereby compete with NHERF-ROMK interactions. The latter would reduce or eliminate CFTR-ROMK interactions critical for Mg-ATP sensitivity.

The gating of the 30 pS K channel by Mg-ATP may be due to interaction of this nucleotide with ROMK and/or CFTR. In the absence of CFTR, weak sensitivity of the 30 pS K channel to Mg-ATP was observed at the more acidic cytosolic pH of 7.2 (Table 2 and Figure 8C), which is the resting pH of both TAL and principal cells in isolated tubules bathed in CO2/HCO3–-containing media (60). This suggests that ATP may directly interact with ROMK to gate the channel, a possibility supported by our previous observations that ROMK2 expressed without CFTR in X. laevis oocytes exhibited weak sensitivity to Mg-ATP at a pH of 7.2 (48) and that proteins containing soluble cytosolic components of ROMK channels can bind TNP-ATP (49, 50). Thus, CFTR may enhance the sensitivity of the 30 pS K channel to ATP by increasing its affinity to bind and gate the channel, a pattern similar to that for KATP channels (61). Alternatively or additionally, the nucleotide binding domains (NBD1 and/or NBD2) on CFTR may provide the critical binding site for gating of ROMK by Mg-ATP. In support of this latter possibility, gating of the 30 pS K channel in TAL or

Figure 9
Acute effect of cytosolic curcumin on ROMK in inside-out patches from TAL apical membrane of wild-type mice and in giant patches from X. laevis oocytes. (A) Representative single-channel recording of an inside-out patch from wild-type mouse TAL treated with 10 μM curcumin. Mg-ATP (2 mM) reversibly and completely inhibited channel activity. (B) Average normalized NPo after treatment with curcumin and curcumin + 2 mM Mg-ATP. (C) pH sensitivity of ROMK in excised inside-out giant patches from X. laevis oocytes before (squares) and after exposure (circles) to 10 μM curcumin (pH at 50% inhibition of current [pH50] 6.69 ± 0.01; n = 3 per group). I/Imax, measured current normalized to maximal current.

Figure 10
Effect of PKA phosphorylation on Mg-ATP sensitivity of ROMK in inside-out patches from TAL and CCD principal cells of wild-type mice. Pretreatment of TAL (A) or principal cells from the CCD (B) with 2 μM forskolin and 300 μM IBMX prior to patching abolished the inhibition of single-channel activity (normalized NPo) by 1–2 mM Mg-ATP. (C) Representative recording of the reversible inhibition of ROMK activity by cytosolic 1.2 mM Mg-ATP in an inside-out patch from the apical membrane of a wild-type mouse CCD principal cell. (D) In contrast, following pretreatment with 2 μM forskolin and 300 μM IBMX, 1–2 mM Mg-ATP had no effect on ROMK activity.
principal cells requires Mg-ATP, rather than Na-ATP (62, 63), suggesting that ATP hydrolysis may be involved in K channel gating by nucleotides. Moreover, NBD1 on CFTR has been found to be essential for glibenclamide sensitivity, and by inference for ATP sensitivity (20), of ROMK expressed in X. laevis oocytes (23, 24).

Curcumin treatment of ΔF508-CFTR mice (3–7 days) increased the Mg-ATP sensitivity of the 30 pS K channel in TAL cells. This result is in general agreement with the observations of Egan and coworkers (43) on the ability of oral administration of curcumin in ΔF508-CFTR mice to partially correct defects associated with homozygous expression of ΔF508-CFTR. In contrast, others have failed to rescue abnormal ΔF508-CFTR processing in cultured cells or to rescue the functional defects in homozygous ΔF508-CFTR mice (44). While the reasons for these differences in curcumin effects in ΔF508-CFTR mice are unclear, the genetic backgrounds of the mice previously used by the Egan (mixed C57b/128; ref. 43) and Verkman (C57b; ref. 44) groups were different which could affect curcumin pharmacokinetics, and thereby responses to this natural product. The dosage, route, and duration of curcumin administration and the ΔF508-CFTR mice used in the present study were the same as used in the previous study by Egan et al. (43).

Since curcumin had no direct effect on 30 pS or ROMK channel activity or gating, it seems reasonable to suggest that the effect of this treatment on Mg-ATP sensitivity was due to enhanced functional interaction between CFTR and ROMK. The latter could be due to increased availability of ΔF508-CFTR in the apical membranes of TAL cells, to modulation of the functional state of CFTR, or to some other undefined effect. While our experiments did not address which of these possibilities accounted for the effect of curcumin on the ATP sensitivity of the 30 pS K channels, an increase in apical surface availability of ΔF508-CFTR would be expected to enhance the probability of CFTR-ROMK interactions and thereby Mg-ATP sensitivity. Alternatively, since acute exposure to curcumin has been shown to reduce the phosphorylation state of CFTR (42), it is possible that this could enhance ROMK and ΔF508-CFTR interactions and thereby increase Mg-ATP sensitivity of the K channel even in the absence of increased trafficking of the mutant CFTR to the apical membrane. In this regard, we showed that increasing the cAMP/PKA activity in TAL and principal cells abolished the sensitivity of 30 pS K channels to 2 mM ATP; an observation consistent with our previous studies of X. laevis oocytes coexpressing CFTR and ROMK2 (21). Thus, either increased surface delivery of CFTR, altered phosphorylation states of ROMK, CFTR, and/or other associated proteins, or both could account for the increased Mg-ATP sensitivity of the 30 pS K channel after curcumin treatment.

What is the physiological role of CFTR-ROMK interactions? A functional model incorporating our results is depicted in Figure 11. Two observations suggest this model for CFTR-ROMK interactions: the functional expression of apical 30 pS K channels was increased in the absence of CFTR (Table 2), and the effect of CFTR on ROMK ATP sensitivity was abrogated by cAMP/PKA (Figure 10). In addition, in vitro experiments suggest that NHERF may stabilize the interaction between CFTR and ROMK (22) and that NHERF interacts with ezrin, which contains a binding site for PKA (57). Accordingly, 2 functional populations of ROMK can exist in apical membranes of TAL and principal cells. ROMK channels associated with CFTR via their respective interactions with NHERF are sensitive to normal millimolar ATP and therefore inactive (Figure 11A). Another fraction of ROMK channels that does not associate with CFTR is insensitive to ATP and is thereby fully active at normal cytosolic concentrations of ATP (Figure 11A). The distribution between inactive and active populations of ROMK is determined by the state of PKA phosphorylation. The model shown in Figure 11 provides a mechanism for rapidly varying the number of functional channels in the apical plasma membrane by PKA-dependent phosphorylation.

In both TAL and principal cells of the CCD, the peptide hormone arginine vasopressin (AVP, or antidiuretic hormone), is a major regulator of cell cAMP and therefore PKA activity. AVP was shown to increase the Ba2+-sensitive apical K conductance of mouse TAL (65), and in the rat this hormone increased fractional K excretion by 77% despite a 7-fold reduction in urine flow rate (66). In addition, in the rat AVP increased the NP, of small-conductance K channels in principal cells from the CCD (66) and enhanced the Ba2+-sensitive Rb flux from bath to lumen in the isolated perfused CCD (67). Although the driving force for K secretion in TAL and CCD may be modified by exposure to AVP under certain conditions (68), it is clear that apical 30 pS K channel activity is increased following exposure to this cAMP-generating hormone.

What are the physiological implications of this model for renal potassium handling? In the water diuretic state, when AVP levels are low, CFTR and ROMK interact, producing a functional complex wherein ROMK is highly sensitive to cell ATP and the 30 pS K channels would therefore be inactive (low cAMP; Figure 11B). In contrast, in the antidiuretic state, when AVP is high, CFTR no lon-
ger interacts with ROMK, and the 30 pS K channel is insensitive to cytosolic ATP and therefore fully active (high CAMP; Figure 11B). Thus CFTR acts as a functional switch that modulates K channel activity between the water diuretic and antidiuretic states. In the water diuretic state, when urinary flow is high, the reduced apical K conductance would limit K secretion and urinary K loss. In contrast, in cystic fibrosis, when plasma membrane CFTR is absent, K secretion would be maintained during water diuresis, leading to increased urinary K loss. Consistent with this notion, individuals with cystic fibrosis may be more prone to the development of hypokalemic metabolic alkalosis (69, 70).

Methods

CFTR transgenic mice. The cftr<sup>−/−</sup>-cftr<sup>−/−</sup> transgenic mouse (mCFTR<sup>−/−</sup>; ref. 27) lacks expression of mouse CFTR but expresses human CFTR, which is driven by the intestinal specific promoter for the rat fatty acid binding protein (FABP). This rescues the severe gastrointestinal phenotype of the cftr<sup>−/−</sup> mouse (27, 71). We also used the cftr<sup>−/−</sup> transgenic mouse (28, 43), in which CFTR contains the ΔF508 mutation (AF508-CFTR), on a genetic background (approximately 25% 129 and 75% C57BL/6). The ΔF508-CFTR transgenic mice have a high mortality and demonstrate poor weight gain due to abnormal intestinal function. To improve survival, all mice were fed with Harlan Teklad 9F food and drinking water that was supplemented with 17.5 g/250 ml of Colyte (SCHWARZ PHARMA).

In both CFTR transgenic mouse models, mouse CFTR expression in kidney is absent. C57BL/6 mice (20–30 g, Charles River Laboratories) were used as wild-type controls for ROMK and CFTR channel activity and were supplemented with 17.5 g/250 ml of Colyte (SCHWARZ PHARMA). The Curcumin treatment was administered to orally administered curcumin at 45 mg/kg daily for 3–7 days. Curcumin powder was mixed into liquid formula (Alimentim; Ross Laboratories), and the NPD of the mice was measured using a high-impedance voltage meter as previously described (43). The measurement was performed twice on each mouse, once before treatment and another after treatment. Briefly, a probing bridge—PE10 tubing was inserted into a heated perfusion line and was placed into a nostril of the mouse. A reference electrode (27-gauge butterfly needle filled with 4% agar in Ringers solution) was placed subcutaneously. A microperfusion pump controlled the saline flow through the probing electrode (0.15 ml/h). During the procedure the mice were maintained on a warming pad, and saline drops were used to keep their eyes moist. Since there is some discomfort with the procedure, the mice were anesthetized with ketamine (100 mg/kg; and Xylazine, 10 mg/ml i.p.). They were continually observed throughout the procedure as well as after until they fully recovered.

Preparation and patch clamping of kidney tubules. Transverse kidney slices were cut and placed in chilled dissection solution, and cortical TAL or cortical collecting tubules were dissected under microscope at room temperature with forceps. The individual tubules were immobilized on a 5-mm × 5-mm cover glass coated with Cell-Tak (Biopolymers) and transferred to a chamber mounted on the stage of an inverted microscope (Olympus IMT-2). The tubules were opened lengthwise with a sharpened micropipette to expose the luminal surface of the cells, and patch clamping was carried out at room temperature (22–24°C). Tubules were dissected and bathed in Ringers solution containing 140 mM NaCl, 5 KCl, 1.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 10 mM HEPES and adjusted to pH 7.4 or 7.2 with NaOH. Glass pipettes were pulled from borosilicate glass capillaries (Daegan Corporation) using a 2-step Narishige PP83 puller (Narishige Scientific Instrument Lab). Pipette tips were polished with Narishige MF-83, and the pipette resistance ranged from 6 to 8 MΩ when filled with 140 mM KCl pipette solution. The pipette contained 140 mM KCl, 1.8 mM MgCl<sub>2</sub>, and 10 mM HEPES and was adjusted to pH 7.4 with KOH. When patches were excised, the bath solution was replaced with a Ca<sup>2+</sup>-free bath solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM MgCl<sub>2</sub>, 2 mM EDTA, 10 mM HEPES, and 0.2 mM Mg-ATP to prevent channel rundown and adjusted to pH 7.4 with NaOH.

Preparation and patch clamping of X. laevis oocytes. Oocytes were anesthetized in 0.02% 3-aminobenzoic acid ethyl ester (tricaine titrated to pH 7.4 with 5 mM HES using NaOH) for 3–7 minutes. After partial ovariectomy, oocytes were defolliculated in a Ca<sup>2+</sup>-free solution (96 mM NaCl, 2 mM KCl, 2 mM MgCl<sub>2</sub>, and 5 mM HEPES, adjusted to pH 7.4 with NaOH) containing 2 mg/ml type IA collagenase (Sigma-Aldrich). Oocytes were washed and kept in ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, and 5 mM HEPES, adjusted to pH 7.4 with NaOH) for injection. ROMK2 cDNA was subcloned into pSPORT-1 vectors and linearized with Not-I restriction endonuclease to generate full-length complementary RNA (cRNA) transcripts. Oocytes were injected with 50 nl of cRNA (5 ng) or water (control) using an Inject + Matic (Genovex) injector no later than 12 hours after defolliculation. Oocytes were then incubated in ND-96 solution containing 500 U/ml penicillin and 500 μg/ml streptomycin (Invitrogen Corp.) at 18°C for 48 hours. Before patch clamping oocytes, the vitelline membrane was removed with a forceps following immersion in a hyperosmotic solution for 1–2 minutes (200 mM N-methyl-d-glucamine, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM EDTA, and 10 mM HEPES, adjusted to pH 7.4 with HCl). Oocyte bath solution contained 150 mM KCl, 2 mM EDTA, and 10 mM Mes/Tris and was adjusted to pH 7.4 with Mes or Tris buffer. A multivariable quick-exchange solution system (model SF-77B, Warner Instruments) was used in oocyte giant patches. Glibenclamide, Mg-ATP, forskolin, and IBMX were purchased from Sigma-Aldrich. Curcumin (Sigma-Aldrich) stock solution was solved in DMSO. The final concentration of DMSO in the experimental solution was less than 1:1,000, which has no effect on channel activity. All the experimental solutions were made freshly on the day of experiment.

Statistics. Single-channel currents were amplified by an EPC-7 amplifier (List Electronics) and low-pass filtered at 1 kHz by an 8-pole Bessel filter (902LPF; Frequency Devices). Signals were digitized at a sampling rate 4 kHz (DigiData 1200; Axon Instruments) and saved in a PC for future analysis. Patch data were analyzed using pCLAMP software (version 6.0.4; Axon Instruments) at a digital filter frequency of 500 Hz. Channel activity was calculated by the following equation during sampling periods of 10–30 seconds.

\[
NP_o = \sum (t_i + t_{i+1} + \ldots + t_{N_i})
\]

where \(N_o\) is the number of observed current levels in the patch, \(P_o\) is the single-channel open probability, and \(t_i\) is the fractional open time spent
at each of the current levels. Results are given as means ± SEM. Statistical significance was determined using 2-tailed Student’s t test; a P value less than 0.05 was considered significant. Channel conductance was estimated from linear regression analysis of single-channel current-voltage curves. Voltage applied to the pipette was referenced to the bath potential; holding potential (V-) was −40 mV unless indicated otherwise in the figures.

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