Caveolin-1 Deficiency Inhibits the Basolateral $K^+$ Channels in the Distal Convoluted Tubule and Impairs Renal $K^+$ and $Mg^{2+}$ Transport

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ABSTRACT

$Kcnj10$ encodes the inwardly rectifying $K^+$ channel $Kir4.1$ in the basolateral membrane of the distal convoluted tubule (DCT) and is activated by c-Src. However, the regulation and function of this $K^+$ channel are incompletely characterized. Here, patch-clamp experiments in $Kcnj10$-transfected HEK293 cells demonstrated that c-Src-induced stimulation of $Kcnj10$ requires coexpression of caveolin-1 (cav-1), and immunostaining showed expression of cav-1 in the basolateral membrane of parvalbumin-positive DCT. Patch-clamp experiments detected a 40-pS inwardly rectifying $K^+$ channel, a heterotetramer of $Kir4.1/Kir5.1$, in the basolateral membrane of the early DCT (DCT1) in both wild-type (WT) and cav-1-knockout (KO) mice. However, the activity of this basolateral 40-pS $K^+$ channel was lower in KO mice than in WT mice. Moreover, the $K^+$ reversal potential (an indication of membrane potential) was less negative in the DCT1 of KO mice than in the DCT1 of WT mice. Western blot analysis demonstrated that cav-1 deficiency decreased the expression of the $Na^+$/Cl$^-$ cotransporter and Ste20-proline-alanine-rich kinase (SPAK) but increased the expression of epithelial $Na^+$ channel-$\alpha$. Furthermore, the urinary excretion of $Mg^{2+}$ and $K^+$ was significantly higher in KO mice than in WT mice, and KO mice developed hypomagnesemia, hypocalcemia, and hypokalemia. We conclude that disruption of cav-1 decreases basolateral $K^+$ channel activity and depolarizes the cell membrane potential in the DCT1 at least in part by suppressing the stimulatory effect of c-Src on $Kcnj10$. Furthermore, the decrease in $Kcnj10$ and $Na^+$/Cl$^-$ cotransporter expression induced by cav-1 deficiency may underlie the compromised renal transport of $Mg^{2+}$, $Ca^{2+}$, and $K^+$.

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Distal convoluted tubule (DCT) reabsorbs 5% filtered NaCl and plays an important role in the reabsorption of filtered $Ca^{2+}$ and $Mg^{2+}$. The transport of NaCl in the DCT is a two-step process: NaCl enters the cell through apical Na-Cl cotransporter (NCC), and Na$^+$ leaves the basolateral membrane by Na-K-ATPase while Cl$^-$ leaves the cells through the basolateral Cl$^-$ channels or KCl cotransporter. The $K^+$ channel in the basolateral membrane of the DCT participates in generating the cell membrane potential and recycles $K^+$ in the basolateral membrane for sustaining the Na-K-ATPase activity. Previous studies have identified a 40-pS inwardly rectifying $K^+$ channel in the basolateral membrane of the DCT. This 40-pS $K^+$ channel is a heterotetramer of $Kir4.1$ and $Kir5.1$ because the coexpression of $Kir4.1/5.1$ produced an inwardly rectifying $K^+$ channel with the same biophysical properties.

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properties as the 40 pS K⁺ channel expressed in the basolateral membrane of the native DCT.10–12 The role of Kir4.1 in forming the basolateral 40 pS K⁺ channel of the DCT was convincingly demonstrated by the observation that the disruption of Kcnj10 (Kir.4.1) completely eliminated the 40 pS K⁺ channel.13 Moreover, the basolateral K⁺ conductance was largely abolished in the early DCT (DCT1) of Kcnj10/2 mice, suggesting that Kcnj10 (Kir.4.1) is a major contributor to the basolateral K⁺ conductance in the DCT1.

Loss-of-function mutations of Kcnj10 cause SeSAME/EAST syndrome, and the renal phenotype of the disease is characterized by salt wasting, hypomagnesemia, metabolic alkalosis, and hypokalemia.14–16 Experiments performed in Kcnj10 knockout (KO) mice showed that the disruption of Kcnj10 decreased the expression of NCC and SPAK.13 This strongly suggests the critical role of basolateral K channels in the regulation of transepithelial membrane transport in the DCT. However, because homozygous Kcnj10/2 mice could not survive >2 weeks after the birth,17 this made it impossible to further characterize the membrane transport in those mice. To solve this problem, we took the advantage of caveolin-1/2 (cav-1 KO) mice to recapitulate the renal phenotypes of loss-of-function mutations of Kcnj10 in the DCT. Cav-1 has been demonstrated to serve as the structural protein of caveolae in a variety of tissues, including the kidney, and plays an important role in endocytosis and signaling transduction.18 Moreover, cav-1 KO mice are viable and fertile.18 Two initial observations of our experiments strongly suggest the justification of using cav-1 KO mice for studying the effect of Kcnj10 downregulation on the membrane transport in the DCT: (1) cav-1 was required for the stimulation of Kcnj10 by c-Src, and (2) patch-clamp experiments performed in cav-1 KO mice further demonstrated that the disruption of cav-1 lowered the basolateral K conductance in DCT1. Therefore, cav-1 KO mice are used in this experiment to prove the principal that Kir4.1/Kir5.1 in the DCT1 plays a role in determining SPAK and NCC.

RESULTS

To examine whether cav-1 is expressed in the basolateral membrane of the DCT, we carried out immunostaining in the wildtype (WT) and cav-1 KO mice. Figure 1A is a fluorescence microscope image at a low magnification showing the expression of cav-1 in the kidney of WT mice. The positive cav-1 staining was observed in both renal cortex and medullary region, and this staining was specific because no cav-1 immunostaining was observed in the KO mice (Figure 1B). We confirmed the previous report that cav-1 was expressed in the glomerulus, DCT, cortical collecting duct (CCD), and renal vessels.19,20 Microscopic examination with a high magnification revealed that cav-1 was expressed in the glomerulus (Figure 1C), DCT (Figure 1C), and CCD (Figure 1D), whereas the cav-1 staining was not visible in the KO mice (Figure 1E). Moreover, immunostaining image demonstrates that cav-1 is highly expressed in the basolateral membrane of the DCT. (A) Fluorescence microscope image showing the expression of cav-1 with a low magnification in the kidney of a WT mouse. (B) No positive immunostaining of cav-1 was observed in the kidney of a cav-1 KO mouse. The immunostaining was performed under identical conditions, and the kidney slices of both WT and KO mice were placed in the same slide. Original magnification, ×40 in A and B. (C) A fluorescence microscope image with a high magnification showing the positive cav-1 staining in the glomerulus (gl) and in the DCT (d). (D) A fluorescence microscope image illustrating the cav-1 staining in the CCD characterized by dotted staining (arrow) in the basolateral membrane. (E) An image with a high magnification showing the absence of cav-1 staining in the gl in a cav-1 KO mouse. Double immunostaining shows the expression of parvalbumin (F), cav-1 (G), and merged image (H), indicating that cav-1 is highly expressed in the basolateral membrane of the DCT. A strong cav-1 staining is also observed in small vessels indicated by *. The bar in the left corner from section C–H represents the size of 10 μm. Original magnification, ×400 in C–H.
membrane of the DCT (Figure 1, F–H), which is identified by positive immunostaining of parvalbumin, a Ca$^{2+}$-binding protein exclusively expressed in the DCT.21 Because previous study showed that Kir4.1 was expressed in the basolateral membrane of DCT,13 it is reasonable to assume that Kir4.1 and cav-1 are colocalized in the basolateral membrane of DCT cells. Our previous study demonstrated that c-Src phosphorylated Kcnj10 and that the inhibition of src-family protein tyrosine kinase (SFK), a family of c-Src-like tyrosine kinase,22 decreased the basolateral 40 pS K$^+$ channel activity in DCT1.9 Therefore, we next examined whether cav-1 was required for the stimulatory effect of c-Src on Kir.4.1 in Kcnj10-cotransfected HEK293T cells with the perforated whole-cell recording. Figure 2A shows a set of typical recordings of Ba$^{2+}$-sensitive K$^+$ currents measured in cells transfected with Kcnj10, Kcnj10+c-Src, Kcnj10+cav-1, and Kcnj10+cav-1+c-Src. Figure 2B is a Western blot demonstrating that the total expression of Kcnj10 protein was similar among four groups. From the inspection of Figure 2A, it is apparent that c-Src alone did not significantly increase K$^+$ currents without cav-1 cotransfection (224 =6 pA/pF at –60 mV in Kcnj10-transfected cells versus 230=21 pA/pF in Kcnj10+c-src-transfected cells) (n=6). In contrast, in cav-1 cotransfected HEK cells, c-Src significantly increased the K$^+$ currents to 360=26 pA/pF (n=6), whereas the expression of cav-1 had no significant effect on K$^+$ currents (210=23 pA/pF) (Figure 3A). Therefore, c-Src–induced stimulation of Kcnj10 requires cav-1 participation. Because Kcnj10 protein expression was similar with or without cav-1 (Figure 2B), the sympathetic effect of cav-1 on c-Src–induced stimulation of Kcnj10 channel activity was most likely caused by post-translational modulation.

Inhibition of SFK has been shown to decrease the basolateral K$^+$ channel activity in mouse DCT1.9 We next examined whether cav-1 was also required for the effect of PP1, an inhibitor of SFK, on Kcnj10. We performed the perforated whole-cell recording in HEK293T cells transfected with Kcnj10 or Kcnj10+cav-1. From the inspection of Figure 3B, it is apparent that the inhibition of SFK with PP1 had no effect on the Ba$^{2+}$-sensitive K$^+$ current in Kcnj10-transfected cells (210=15 pA/pF, n=5). In contrast, PP1 decreased the K$^+$ currents to 110=12 pA/pF (n=6) in cav-1 cotransfected cell (Figure 3, A and B). We also repeated the experiments in which the effect of SFK inhibition on K$^+$ currents was examined in HEK293T cells transfected with Kcnj10/16 alone or cav-1 +Kcnj10/16. Results are summarized in Figure 3C showing that PP1 inhibited Kcnj10/16 only in the cav-1 cotransfected cells but not in those without cav-1 transfection (Kcnj10/16: 280=12 pA/pF; Kcnj10/16+PP1: 260=10 pA/pF; Kcnj10/16+cav-1: 275=12 pA/pF; Kcnj10+cav-1+PP1: 150=15 pA/pF; n=4–6). Therefore, results suggest that cav-1 is critically involved in the modulation of SFK effect on Kcnj10. In addition to c-Src, with-no-lysine kinase 4 (WNK4) is expressed in the DCT and plays a role in the regulation of NCC.23,24 To determine whether the synergistic effect of cav-1 on c-Src-mediated stimulation of Kcnj10 was specific, we examined the effect of WNK4 on Kcnj10 in the cav-1 cotransfected cells with the perforated whole-cell recording (Figure 3D). The expression of WNK4 had no effect on Kir4.1 in Kcnj10-transfected cells or in the cells transfected with cav-1+Kcnj10 (control: 234=22 pA/pF; cav-1: 240=23 pA/pF; WNK4: 230=15 pA/pF; WNK4+cav-1: 230=20 pA/pF; n=6). Therefore, the expression of cav-1 specifically modulates the effect of c-Src on Kir.4.1.

Previous study showed that SFK activated Kcnj10 by stimulating tyrosine phosphorylation at Tyr.9 To examine whether the mutation of Tyr9 could affect cav-1–induced synergistic effect on SFK-induced stimulation of Kcnj10, we performed the experiments in the cells transfected with Kcnj10Y9F in which the tyrosine phosphorylation site was mutated. Figure 4 is of typical recordings showing that the K$^+$ currents in Kcnj10Y9F–cotransfected cells were significantly smaller (75=10 pA/pF; n=5) than those in the control cells (transfected with Kcnj10). Moreover, the expression of c-Src modestly increased the K$^+$ currents from 76=10 pA/pF to 105=10 pA/pF (n=6) in cav-1 cotransfected cells. Therefore, cav-1 modulates the SFK effect on
Kcnj10, at least in part, by facilitating the phosphorylation of Kcnj10 at Tyr.9

After demonstrating that cav-1 was required for the effect of SFK on Kcnj10 in a cell model, we further explored the role of cav-1 in the regulation of Kcnj10 in cav-1 KO mice. If cav-1 is required for facilitating the stimulatory effect of SFK on the basolateral 40 pS K+ channel, the disruption of cav-1 should mimic the effect of SFK inhibition and decrease the 40 pS K+ channel activity in the basolateral membrane of the DCT. Figure 5A is a typical single-channel recording made in a cell-attached patch of DCT1 of cav-1 KO mice. As in WT mice, we identified a 40 pS inwardly rectifying K+ channel in the DCT, and this 40 pS K+ channel was the only type of K+ channel detected in the basolateral membrane of DCT1 from total 66 patches in cav-1 KO mice. The absence of Kcnj10 homotetramer (20 pS K+ channel) in the DCT suggests that Kcnj10 prefers to interact with Kcnj16 (Kir.5.1)
in vivo. This notion was also supported by the report that the 20 pS K+ channel was abundant in the DCT of Kcnj16−/− mice.25 Moreover, the mean channel open probability in cav-1 KO mice (0.42±0.02, n=10) was not significantly different from those of WT animals (0.46±0.02, n=10) (Figure 5B). However, the probability of finding this 40 pS K+ channel was significantly lower in cav-1 KO mice (25 patches with the K channel from total 66 patches) than in WT mice (18 patches with K channel from total 35 patches) (Figure 5C). Therefore, the mean K+ channel number per patch

Figure 3. cav-1 is required for the effect of SFK inhibitor on Kcnj10. (A) A bar graph summarizing the results of experiments in which Ba2+-sensitive K+ currents were measured at –60 mV with perforated whole-cell recording in the cells transfected with KCNJ10. (B) A set of recordings showing K+ currents measured with the perforated whole-cell recording in KCNJ10 or KCNJ10+cav-1–transfected HEK293T cells under control conditions (no PP1) or treated with 1 μM PP1. (C) A bar graph summarizing the results of experiments in which the effect of 1 μM PP1 on Ba2+-sensitive K+ currents was examined at –60 mV with perforated whole-cell recording in the cells transfected with Kcnj10/Kcnj16 or Kcnj10/Kcnj16+cav-1. (D) A bar graph summarizing the results of experiments in which Ba2+-sensitive K+ currents were measured at –60 mV with perforated whole-cell recording in the cells transfected with KCNJ10 (control), KCNJ10+cav-1, KCNJ10+WNK4, and KCNJ10+WNK4+cav-1.
was significant less in DCT1 of cav-1 KO mice (1.17±0.23) than in WT mice (2.5±0.45) (Figure 5D). To examine whether the disruption of cav-1 reduced the expression of Kcnj10 in the kidney, we performed the immunostaining in WT and KO mice. Figure 5E shows immunostaining images with a low magnification in the kidney of WT and KO mice. From the inspection of Figure 5E, it is apparent that the intensity of Kcnj10 staining is lower in KO mice than WT mice. This is further confirmed by the microscopic examination, with a large magnification showing that the immunostaining intensity of the basolateral Kcnj10 in KO mice is weaker than in WT mice (Figure 5F). This suggests that the lack of cav-1 decreased the surface expression of the functional 40 pS K+ channel in the DCT1.

The notion that the lack of cav-1 decreased the basolateral K channel activity in the DCT was further supported by experiments in which whole-cell K+ currents were measured with the perforated patch. As discussed previously, the DCT1 cells have no apical K+ channel, and they have a lack of coupling with neighboring cells.9,13 Therefore, the whole-cell K currents were equal to the basolateral K+ conductance. Figure 6A is a set of whole-cell recording showing that K+ currents of DCT1 in cav-1 KO mice (513±72 pA) were significantly lower than those of WT mice (1,300±205 pA) (n=8). The inhibition of SFK decreased the K+ current of DCT1 in WT mice to 470±80 pA (n=7) (Figure 6B), whereas it had no significant effect on the basolateral K+ conductance of DCT in cav-1 KO mice (440±80 pA) (n=8). This suggests that the disruption of cav-1 significantly inhibits the basolateral K+ conductance in DCT1 and that the decrease in K+ conductance is most likely caused by the defective regulation of the 40 pS K+ channel in the basolateral membrane by SFK. Because Kcnj10 plays a dominant role in determining the cell membrane potential in DCT1,13 a decrease in the basolateral K+ conductance is expected to depolarize the cell membrane potential. This notion was supported by measuring K+ reversal potential using the perforated whole-cell recording with 5 mM K+ in the bath and 140 mM K+ in the pipette. The results summarized in Figure 6C from five measurements show that the K+ reversal potential was 65±3 mV in DCT1 of WT mice, and it depolarized to 49±2 mV in the presence of SFK inhibitor. In contrast, the K+ reversal potential was significantly lower in DCT1 of cav-1 KO mice (50±3 mV) than in WT mice, and it was also not significantly different from the value in the presence of SFK inhibitor (47±3 mV).

The basolateral K+ conductance plays a key role in providing the driving force for Cl− exit through the basolateral Cl− channel. We previously demonstrated that the disruption of Kcnj10 also inhibited the basolateral Cl− conductance in the DCT of Kcnj10−/− mice.13 Therefore, we used the perforated whole-cell recording to examine the Cl− conductance in the DCT of both WT and cav-1 KO mice. Figure 7A is a recording showing NPPB-sensitive Cl− currents in DCT1 of WT and cav-1 KO mice, and results from six experiments are summarized in Figure 7B. It is apparent that Cl− currents in the DCT1 of KO mice (−490±120 pA at −60 mV) were significantly lower than those of WT mice (−920±90 pA at −60 mV). Therefore, the disruption of cav-1 also inhibited the basolateral Cl− conductance in the DCT1, which should increase the intracellular Cl− concentration, thereby inhibiting WNK autophosphorylation and WNK-SPAK interaction.26,27 We previously demonstrated that the disruption of Kcnj10 decreased the expression of NCC and SPAK in Kcnj10 KO mice.13 Therefore, we examined the expression of SPAK in WT and cav-1 KO mice. Figure 7C is a Western blot showing that the lack of cav-1 inhibited the expression of full-length SPAK by 52%±10% (n=4). Because SPAK has been shown to stimulate NCC activity through phosphorylation,28−30 it is conceivable that the disruption of cav-1-induced inhibition of SPAK should reduce the NCC phosphorylation. Figure 7D is a Western blot showing the expression of Thr53-phosphorylated NCC, which is an index of activated NCC,31 and the total NCC in the kidney from the WT and cav-1 KO mice. In comparison with the control value,
the disruption of cav-1 decreased the expression of phosphorylated NCC and total NCC by 80% ± 10% and 45% ± 10% (n=5), respectively.

We also examined the expression of NCC with a fluorescence microscope. Figure 8A is an image with a low magnification showing that the expression of NCC is lower in the KO mice than in WT mice. This is further confirmed by the microscopic examination with a large magnification showing a sharp staining of NCC in the WT mice in comparison with the KO mice (Figure 8B). To examine whether the decrease in NCC expression was the result of suppressing transcription, we carried out real-time quantitative PCR to examine the mRNA level of NCC and SPAK, and Figure 8C shows an agarose gel of the PCR product of NCC and SPAK. The results from four experiments are summarized in Figure 8D, demonstrating that the disruption of cav-1 did not alter the mRNA of both full-length SPAK and kidney-specific SPAK, whereas it increased mRNA of NCC by 40% ± 10% (n=4). Therefore, the decrease in NCC and SPAK protein expression in cav-1 KO mice was caused by a post-translational regulation. The increase in NCC
mRNA in cav-1 KO mice may be caused by a compensation mechanism. The effect of the cav-1 disruption on NCC was specific because the expression of TRPV5, which is expressed in the apical membrane of the DCT, was the same between WT and KO mice ($n=4$) (Figure 8E).32

Because cav-1 is also expressed in the basolateral membrane of the CNT and CCD, we next examined whether the disruption of cav-1 affected basolateral K channel and apical epithelial sodium channel (ENaC) in the CNT. We conducted the perforated whole-cell recording to measure the K$^+$ reversal potential of the CNT, which is an indication of the basolateral K$^+$ conductance and the membrane potential. The results summarized in Figure 9A show that the K$^+$ reversal potential in the CNT of cav-1 KO mice ($61\pm 2$ mV, $n=4$) was slightly but significantly ($P<0.03$) lower than those of WT mice ($69\pm 3$ mV, $n=4$). However, the depolarization amplitude in the CNT was modest in comparison with those in DCT1, suggesting that Kcnj10 in the CNT might not play a dominant role in determining the basolateral membrane potential. We also examined the expression of ENaC in WT and cav-1 KO mice with Western blot and immunostaining. Figure 9B

Figure 6. Disruption of cav-1 decreased basolateral K$^+$ conductance in DCT1. (A) Whole-cell patch recording demonstrating the effect of PP1, an inhibitor of SFK, on the K$^+$ currents of the DCT1 in the WT and cav-1 KO mice, respectively. The pipette solution and bath solution contained a symmetrical 140 mM KCl, and the K$^+$ currents were measured from −60 to 60 mV at 20-mV steps (the protocol of the voltage clamp is included). (B) A bar graph summarizing the results of experiments in which Ba$^{2+}$-sensitive K$^+$ currents in the DCT1 of WT or cav-1 KO mice were measured at −60 mV with perforated whole-cell recording in the presence of or in the absence of SFK inhibitor (1 μM PP1). The pipette and bath solution were the same as previously described. (C) K$^+$ reversal potentials in the DCT1 of WT or cav-1 KO mice were measured with perforated whole-cell recording in the presence of or in the absence of SFK inhibitor (1 μM PP1). The DCT1 was bathed in the 140 mM Na$^+$/5 mM K$^+$ bath solution, and the pipette solution contained 140 mM KCl.
We also measured the plasma concentrations of Na\(^+\), K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) in WT and the KO mice. From the inspection of Figure 10, cav-1 KO mice have hypocalcemia (control: 7.8±0.6 mg/dl; KO: 4.0±0.6 mg/dl), hypomagnesemia (control: 2.13±0.11 mg/dl; KO: 1.27±0.09 mg/dl), and hypokalemia (control: 4.77±0.11 mEq/l; KO: 3.65±0.11 mEq/l). Therefore, cav-1 KO mice have a renal phenotype similar to the Gitelman’s syndrome, except plasma Ca\(^{2+}\).

**DISCUSSION**

In this study, we demonstrated that the disruption of cav-1 inhibited the basolateral 40 pS K\(^+\) channel in DCT1 and caused membrane depolarization. Therefore, cav-1 KO mice could be used as an alternative animal model to study the effects of loss-of-function mutations of Kir.4.1 on the electrolyte transport in the DCT. The mechanism by which the lack of cav-1 decreases the number of the 40 pS K\(^+\) channel in the basolateral membrane is not clear. cav-1 plays an important role in mediating a variety of cellular functions, such as mediating the signal transduction and ion channel trafficking.\(^{18,33}\) For instance, cav-1 has been shown to regulate the delivery of glutamate transporter in C6 glioma cells\(^{34}\) and to inhibit Kir.1.1 (renal outer medullary K\(^+\)).\(^{35}\) However, the observation that c-Src-induced stimulation of Kir.4.1 requires the involvement of cav-1 strongly suggests that a decrease in the basolateral 40 pS K\(^+\) channel activity in the DCT of cav-1 KO mice was, at least in part, the result of the defective regulation of Kir.4.1 by SFK.

Our previous study demonstrated that Kir.4.1 was the substrate of SFK and that tyrosine residue 9 was phosphorylated by c-Src.\(^{9}\) The role of SFK in the regulation of the basolateral 40 pS K\(^+\) channel was demonstrated by the observation that the inhibition of SFK decreased the basolateral 40 pS K\(^+\) channel activity and depolarized the basolateral membrane potential in DCT1. However, because inhibiting SFK decreased Kir.4.1 activity only in the cav-1 cotransfected cells, this strongly suggests that cav-1 is required for the effect of SFK on Kir.4.1. In this regard, cav-1 is highly expressed in the basolateral membrane of DCT and has been shown to be associated with Kir.4.1 and Ca\(^{2+}\)-sensing receptor (CaSR).\(^{12,19,36}\)

Also, it has been shown that cav-1 interacts directly with c-Src and the interaction domain is within residues 82–101.\(^{37}\) Therefore, it is possible that cav-1 provides a microenvironment for the interaction between SFK and Kir.4.1. Relevant to this
speculation is the report that cav-1 serves as a scaffolding protein to sequester Ras protein and heme oxygenase-1 in caveolae.9,38,39 Because SFK plays an important role in stimulating the 40 pS K+ channels in the basolateral membrane of the DCT, the disruption of cav-1 impairs the stimulatory effect of SFK on the basolateral K+ channels, thereby decreasing the basolateral K+ conductance.9 Therefore, the defective regulation of Kir4.1 by SFK is one of the possible mechanisms by which the disruption of cav-1 inhibits the basolateral K+ conductance in the DCT.

As in Kcnj10 KO mice, the disruption of cav-1 also leads to a decrease in NCC expression.13 Because cav-1 was not detected in the apical membrane of DCT, it is unlikely that cav-1 may directly be involved in regulating NCC membrane trafficking.19 Therefore, the disruption of cav-1–induced decrease in NCC expression may be the results of the depolarization of the DCT cell membrane potential. We have previously demonstrated the expression of NCC and SPAK were decreased in homozygous Kcnj10−/− and heterozygous Kcnj10+/− mice; however, heterozygous mice have a normal growth, as do WT mice.13 Moreover, the decrease in NCC and SPAK expression was closely correlated with the basolateral membrane potential. Because the disruption of cav-1 also depolarized the membrane potential of DCT1, this should be, at least in part, responsible for decreasing NCC and SPAK expression in cav-1 KO mice. The depolarization in the basolateral membrane potential is expected to decrease the Cl− exit by the basolateral Cl− channels, thereby increasing the intracellular Cl− concentration. In addition, the disruption of cav-1 may also directly inhibit the basolateral Cl− channel activity, thereby synergizing the effect of Kcnj10 inhibition on the intracellular Cl− level. An increase in the intracellular Cl− concentration leads to inhibit the WNK–SPAK interaction, thereby decreasing SPAK expression and the SPAK-induced stimulation of NCC phosphorylation, which is essential for the activity of NCC.28–30,40 Therefore, it is possible that a decrease in SPAK expression is expected to inhibit NCC phosphorylation, thereby decreasing NCC expression. It has been reported that the SPAK–induced phosphorylation of NCC inhibited ubiquitylation of the thiazide-sensitive NCC in the DCT.41 Although the disruption of cav-1 reduced the expression of NCC, the net renal Na+ excretion was not significantly larger in cav-1 KO mice than in WT mice. It is possible that a decrease in NCC expression in the DCT should increase Na+ delivery to the CNT/CCD, thereby stimulating Na+ absorption through ENaC in these nephron segments, thereby offsetting the effect of low NCC expression. This speculation was also supported by the observation that the expression of ENaC-α was upregulated in cav-1 KO mice. As a consequence of enhancing Na+ absorption in the CNT/CCD, K secretion in these nephron segments increased. This speculation is supported by the observation that the renal K+ excretion was significantly enhanced in cav-1 KO mice and that they developed hypokalemia.

In addition to hypokalemia, the disruption of cav-1 also induced hypocalcemia and hypomagnesemia. It has been previously reported that the disruption of cav-1 impaired renal calcium reabsorption and led to hypercalciuria and urolithiasis.42 The mechanism by which the disruption of cav-1 causes hypomagnesemia and hypocalcemia is not completely understood. However, a decrease in the basolateral K+ conductance in the DCT should partially contribute to increasing renal excretion of Mg2+ and Ca2+ in the DCT because both transcellular Mg2+ and Ca2+ transport processes are electrogenic processes. Therefore, a decrease in the basolateral K conductance in the DCT should partially contribute to increasing renal excretion of Mg2+ and Ca2+ in the DCT because both transcellular Mg2+ and Ca2+ transport processes are electrogenic processes. Therefore, a decrease in the basolateral K conductance in the DCT should partially contribute to increasing renal excretion of Mg2+ and Ca2+ across the apical membrane, thereby inhibiting their absorption in the DCT. Furthermore, the disruption of cav-1 may affect the function of Ca2+–sensing receptor (CaSR), thereby affecting electrolyte transport in the kidney. This possibility is further enhanced by the observation that Kcnj10 interacts with CaSR and cav-1 and
was inhibited by CaSR activation. However, further study is required to address the role of CaSR in decreasing the basolateral K+ conductance of the DCT in cav-1 KO mice.

Figure 11 is a cell scheme illustrating the mechanism by which the disruption of cav-1 impairs the transport in the DCT. The lack of cav-1 in the basolateral membrane impairs the stimulatory effect of SFK on Kir4.1, thereby decreasing the basolateral K+ conductance and depolarizing the cell membrane potential. A depolarization of the cell membrane potential decreases the driving force for Mg2+ and Ca2+ entry and increases their excretion. Also, a decrease in K conductance reduces the Cl− exit across the basolateral membrane and increases the intracellular Cl− concentration, which inhibits SPAK-induced stimulation of NCC and increases the Na+ delivery to the CNT/CCD. Therefore, the disruption of cav-1 could mimic the loss-of-function mutations of Kcnj10-induced tubulopathy. We conclude that cav-1 plays a role in the stimulation of the basolateral K+ channels by SFK in the DCT and that the basolateral K+ channel critically participates in regulating the expression of NCC and SPAK in the DCT. The disruption of cav-1–induced inhibition of the basolateral K+ channels and NCC in the DCT may be partially responsible for renal Mg2+ and K+ waste.

CONCISE METHODS

Cell Culture and Transient Transfection
We used HEK293T cells (American Type Culture Collection) for the transient expression of flag-tagged or GFP-tagged Kcnj10. The cells were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen), supplemented with 10% FBS (Invitrogen) in 5% CO2 and 95% air at 37°C. Cells were grown to 50–70% confluence for transfection, and the corresponding cDNA was simultaneously applied to the cells using TurboFect transfection reagent (Fermentas). Briefly, a cDNA cocktail (0.5 μg Kcnj10) was diluted with 200 μl serum-free DMEM and further mixed with 4 μl Turbofect transfection reagent for the transfection of cells cultured in a 35-mm Petri dish. Cells transfected with the vector alone were used as a control, and their background currents were subtracted from that of the experimental groups. After 15 minutes of incubation at room temperature, the mixture of the transfection agents was applied to the cells followed by an additional 24 hours of incubation before use.

Preparation of DCT1
C57BL/6 mice and cav-1 KO mice with C57BL/6 background (either sex, 4 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were fed with a control diet and had free access to water. After mice were euthanized by cervical dislocation, we perfused the left kidney with 5 ml collagenase type 2 (1 mg/1 ml) containing L-15 medium (Life Technology). The collagenase perfused kidney was removed, and the renal cortex was cut with a sharp razor. We followed the method described previously and in the Supplemental Material to dissect the DCT/CNT. Supplemental Figure 1 shows a typical DCT/CNT image.

Perforated Whole-Cell Recording
An Axon 200A patch-clamp amplifier was used for the measurement of the whole-cell recording, and K currents were low-pass filtered at 1 KHz, digitized by an Axon interface (Digidata 1322). Data were analyzed using the pCLAMP Software System 9 (Axon). We carried out the perforated whole-cell patch-clamp experiments on HEK293T cells or the DCT1 at room temperature as described previously. For measuring the whole cell
The whole-cell K current was determined by adding 1 mM Ba\(^{2+}\) in the bath solution.

**Single Patch-Clamp Study in DCT1**

An Axon200B patch-clamp amplifier was used to record the single channel current. The currents were low-pass filtered at 1 KHz and digitized by an Axon interface. Channel activity defined as NPo (a product of channel number and open probability) was calculated from data samples of 60 seconds duration in the steady state as follows:

\[
NPo = \sum (t_1 + 2t_2 + ... + it_i)
\]

where \(t_i\) is the fractional open time spent at each of the observed current levels. The composition of the pipette solution for the single-channel recording was the same as that for the whole-cell recording, whereas the bath solution was HEPES-buffered 140 mM NaCl.

**Immunostaining**

Mice were anesthetized with ketamine (100 mg/kg) and clonidine analogue (10 mg/kg), and the abdomens were cut open for perfusion of kidneys with PBS containing heparin (40 unit/ml) followed by 20 ml of 4% paraformaldehyde. After perfusion, the kidneys were removed and dehydrated and cut to 8\(\mu\)m thick slices with Leica1900 cryostat (Leica). The tissue slices were dried at 42°C for 1 hour. The slides were washed with 1× PBS for 15 minutes and permeabilized with 0.3% Triton dissolved in 1XPBS buffer containing 1% BSA and 0.1% lysine (pH=7.4) for 15 minutes. Kidney slices were blocked with 2% horse serum for 30 minutes at room temperature and were then incubated with primary antibodies (parvalbumin and cav-1) for 12 hours at 4°C. Slides were thoroughly washed with 1× PBS and followed by an addition of second antibodies mixture in 0.4% Triton for 2 hours at room temperature.

**Preparation of Protein Samples, Western Blot, and Quantitative Real-Time PCR**

The proteins for Western blot were obtained from HEK293T cells transfected with the corresponding DNA or mouse renal cortex. The tissues were prepared with ice-cold homogenization solution containing 250 mM sucrose, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% protease, and phosphatase inhibitor cocktails titrated to a pH of 7.6. Tissue samples were homogenized in ice-cold homogenization solution with PBS containing heparin (40 unit/ml) followed by 20 ml of 4% paraformaldehyde. After perfusion, the kidneys were removed and subjected to post-fixation with 4% paraformaldehyde for 12 hours. The kidneys were dehydrated and cut to 8\(\mu\)m thick slices with Leica1900 cryostat (Leica). The tissue slices were dried at 42°C for 1 hour. The slides were washed with 1× PBS for 15 minutes and permeabilized with 0.3% Triton dissolved in 1XPBS buffer containing 1% BSA and 0.1% lysine (pH=7.4) for 15 minutes. Kidney slices were blocked with 2% horse serum for 30 minutes at room temperature and were then incubated with primary antibodies (parvalbumin and cav-1) for 12 hours at 4°C. Slides were thoroughly washed with 1× PBS and followed by an addition of second antibodies mixture in 0.4% Triton for 2 hours at room temperature.
methods for quantitative PCR experiments and primers used for detecting NCC and SPAK mRNA are described in the Supplemental Material and Supplemental Table 1, respectively.

Metabolic Cage Study
We followed the protocol for the metabolic cage study described previously. Briefly, we used eight WT mice and eight cav-1 KO mice for the study, and both WT and KO mice were age and sex matched. Two mice were housed in a cage for the study, and they stayed in the cage for 3–4 days to allow them to be adapted with the environment. After training, mice were fed with a normal diet (1%K+0.3%Na) for 4 days to allow them to be adapted with the environment. Then, mice were fed with a low-sodium diet (0.3%Na) for an additional 3 days, and their 24-hour food intake, urine output, and urinary Na+,K+,Ca²⁺, and Mg²⁺ concentrations were measured at the end of the experiments. The urine samples were collected for measuring the electrolytes profile. We took the mean value from 3-day measurements for each cage. At the end of the experiments, the blood samples were collected through cardiac puncture. Plasma and urinary Na⁺, K⁺, Ca²⁺, and Mg²⁺ concentrations were measured at the Yale O’Brienne Kidney Center, and urinary electrolyte profiles were presented as milliequivalent per gram of body weight per 24 hours.

Experimental Materials and Statistics
cav-1 (sc-894) and parvalbumin (sc-7449) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We obtained Flag (f-3165) and ENaC-β (SAB5200106) antibodies from Sigma-Aldrich (St. Louis, MO), c-Src (#2123) antibody from Cell Signaling Technology (Danvers, MA), and actin antibody (ab3280) from Abcam, Inc. (Cambridge, MA). The antibodies for Kenj10 (APC-035), ENaC-α (ASC-030), and TRPV5 (ACC-035) were from Alomone Labs (Jerusalem, Israel). The antibody for NCC was a gift from Robert Hoover at Emory University, whereas antibodies of SPAK and Thr⁵³ phosphorylated NCC were kindly provided by David Ellison/James McCormick at Oregon Health & Science University. The data are presented as mean±SEM. We used the paired t test or one-way ANOVA to determine the statistical significance.

Figure 11. Cell scheme illustrating the mechanism by which the downregulation of cav-1 impairs the regulation of the basolateral Kir4.1 by SFK in the DCT1, thereby inhibiting NCC and decreasing the driving force for transcellular Mg²⁺/Ca²⁺ absorption in the DCT. Dotted lines represent a diminished signaling.

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