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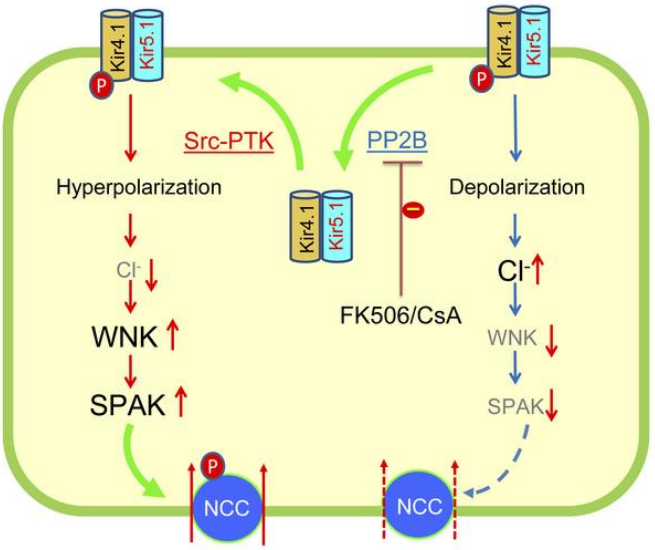
Graphical abstract



Calcineurin-inhibitors stimulate Kir4.1/Kir5.1 of distal-convoluted-tubule to increase Na-Cl cotransporter (NCC)

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Abstract

We examine whether calcineurin or protein-phosphatase-2B (PP2B) regulates the basolateral Kir4.1/Kir5.1 in distal-convoluted-tubule (DCT). Application of tacrolimus (FK506) or cyclosporine-A (CsA) increased whole-cell-Kir4.1/Kir5.1-mediated-K^+-currents and hyperpolarized DCT-membrane. Moreover, FK506-induced-stimulation of Kir4.1/Kir5.1 was absent in kidney-tubule-specific 12-kDa-FK506-binding-protein knockout-mice (Ks-FKBP-12-KO). In contrast, CsA still stimulated Kir4.1/Kir5.1 of the DCT in Ks-FKBP-12-KO mice, suggesting that FK506-induced stimulation of Kir4.1/Kir5.1 was due to inhibiting PP2B. Single-channel-patch-clamp experiments demonstrated that FK506 or CsA stimulated the basolateral Kir4.1/Kir5.1-activity of DCT, defined by NP_o (a product of channel-**N**umber- and-**O**pen-**P**robability). However, this effect was absent in the DCT treated with Src-family-protein-tyrosine-kinase (SFK) inhibitor or hydroxyl-peroxide which stimulates SFK. Fluorescence-image demonstrated that CsA-treatment increased membrane-staining-intensity of Kir4.1 in the DCT of *Kcnj10*$^{flox/flox}$ mice. Moreover, CsA-treatment has no obvious effect on pNCC expression in Ks-Kir4.1-KO mice. Immunoblotting showed that acute FK506 treatment increased pNCC-expression in *Kcnj10*$^{flox/flox}$ mice, but this effect was attenuated in Ks-Kir4.1-KO mice. *In vivo* measurement of thiazide-induced-renal-Na^+ excretion demonstrated that FK506 enhanced thiazide-induced natriuresis. This effect was absent in Ks-FKBP-12-KO mice and blunted in Ks-Kir4.1-KO mice. We conclude that inhibition of PP2B stimulates Kir4.1/Kir5.1 of DCT and NCC, and that PP2B-inhibition-induced stimulation of NCC is partially achieved by stimulation of the basolateral Kir4.1/Kir5.1.

Key Words: *KCNJ10*, *KCNJ16*, Hyperkalemia, tacrolimus, cyclosporin A

Introduction

Expression and activity of calcineurin (protein phosphatase 2B, PP2B) are detected in the renal tubules including proximal tubule, thick ascending limb (TAL), DCT and cortical collecting duct (CCD)(1-4). Acute inhibition of PP2B with cyclosporine A (CsA) or tacrolimus (FK506), two frequently used immunosuppressive drugs after organ transplantation (5), has been shown to stimulate cation-coupled Cl^- cotransporters such as NKCC2 and NCC (1, 2). Moreover, Hoorn et al have suggested that the stimulation of NCC may be, in part, responsible for PP2B-inhibition-induced hypertension and hyperkalemia (2), two common side effects of using CsA or FK506 (6, 7). This notion was supported by the finding that the inhibition of NCC with thiazide was able to reverse the effect of tacrolimus on hypertension (2). Several studies have suggested that PP2B may play a role in the regulation of renal K^+ excretion and K^+ homeostasis (2, 8, 9). Our previous study demonstrated that decreased dietary K^+ intake suppressed PP2B catalytic subunits expression in the rat kidney (8). Moreover, Uchida et al have shown that the acute inhibition of PP2B with tacrolimus abolished acute high K^+ intake (HK)-induced inhibition of NCC (9). It is now well established that the NCC activity is responsible not only for the reabsorption of 5% filtered Na^+ load but also plays a critical role in the regulation of epithelial-Na^+-channel (ENaC)-dependent renal K^+ excretion by controlling Na^+ delivery to the aldosterone sensitive distal nephron (ASDN) (10, 11), because Na^+ deliver rate to the ASDN is an important factor determining ENaC-dependent renal K^+ excretion (12, 13). Therefore, PP2B may play a physiological role in regulating renal K^+ excretion by controlling NCC expression/activity. A large body of evidence has demonstrated that the basolateral Kir4.1/Kir5.1 of the DCT determines the expression and activity of NCC (14-17). For instance, high Kir4.1/Kir5.1 activity is associated with increased NCC expression/activity during decreased dietary K^+ or Na^+ intakes whereas low Kir4.1/Kir5.1 activity is associated with decreased NCC expression/activity during increased dietary K^+ or Na^+ intakes (14-17). Thus,

the aim of the present study is to test whether PP2B activity may also regulate the basolateral Kir4.1/Kir5.1 activity in the DCT and whether acute calcineurin-inhibition induced stimulation of NCC activity is, in part, achieved by the augmentation of the basolateral Kir4.1/Kir5.1 activity.

Results

We first examined the effect of tacrolimus (FK506) and cyclosporine A (CsA) on the basolateral K^+ channel activity of the DCT using whole-cell path-clamp recording technique by measuring Ba^{2+}-sensitive whole-cell K^+ currents in the early part of the DCT (DCT1). The reason to conduct experiments in the DCT1 is due to the fact that no K^+ channel other than Kir4.1/Kir5.1 heterotetramer is detected in DCT1 and Kir4.1/Kir5.1 heterotetramer is the predominant form of K^+ channel in the basolateral membrane (18-20). Thus, the whole-cell K^+ currents of DCT1 represent Kir4.1/Kir5.1 activity. Fig 1A is a set of whole-cell recordings showing the Ba^{2+}-sensitive K^+ currents measured with step protocol from -100 to 60 mV in the DCT1 treated with vehicle, FK506 (10 μM) or CsA (200 nM) for 10 min.. Fig 1B is a scatter plot summarizing the results in three male (m) and three female (f) mice (measured at -60 mV) demonstrating that acute addition of FK506 or CsA increases the whole-cell K^+ currents from 1283±62 pA to 2340±135 pA and 2240±140 pA, respectively. We next examined the effect of FK506 on the basolateral K^+ channels in the DCT1 of Ks-FKBP12 KO and *Fkbp1a*$^{flox/flox}$ mice (control) treated in vivo with FK506 or CsA. The reasoning is to examine whether in vivo treatment of CsA or FK506 can also stimulate Kir4.1/Kir5.1 of the DCT and to examine whether FK506-induced stimulation of Kir4.1/Kir5.1 was due to the inhibition of PP2B. The male control and male Ks-FKBP12 KO mice were treated with FK506 (0.75 mg/Kg BW) by peritoneal injection (p.i) 30 min before experiments. Fig. 1C is a recording showing the Ba^{2+}-sensitive whole-cell K^+ currents in the DCT1 of the control mice and FKBP12-deficient mice

treated with vehicle or FK506 and the results are summarized in Fig.1D. It is apparent that Kir4.1/Kir5.1-mediated K^+ currents of the DCT1 were significantly larger (2220±160 pA, n=6) in FK506-treated group than vehicle-treated *Fkbp1a*$^{flox/flox}$ mice (1200±70 pA, n=4). However, K^+ currents in FK506-treated Ks-FKBP12 KO mice (1280±70 pA, n=5) were similar to the vehicle-treated group (1270±100 pA, n=6), suggesting that FK506-induced inhibition of PP2B is achieved by binding to FKBP12. In contrast, CsA treatment (3 mg/Kg BW by p.i 30 min before experiments) still significantly increases the whole-cell K^+ currents in both control (2100±80 pA, n=6) and Ks-FKBP12 KO mice (2020±80 pA, n=6) (Fig. 1C and 1D). This suggests that the affect CsA-induced stimulation of Kir4.1/Kir5.1 of the DCT was not affected by deleting FKBP12 because CsA inhibits PP2B through binding to cyclophilin (21).

The notion that the inhibition of PP2B stimulates Kir4.1/Kir5.1 of the DCT is also suggested by testing the effects of FK506 and CsA on the 40 pS K^+ channels (a Kir4.1/Kir5.1 heterotetramer) of the isolated DCT using single channel recording. Fig.2A shows a single channel recording showing the effect of FK506 (10 μM) on the basolateral 40 pS K^+ channel (Kir4.1/Kir5.1). Acutely addition of FK506 stimulates the 40 pS K^+ channel activity in the DCT and increases the NP_o from 1.38 ±0.20 to 2.39 ±0.26 (3m/3f mice). We have also tested effect of CsA on the 40 pS K^+ channels of the DCT and Fig. 2B is a single channel recording showing that adding CsA (200 nM) also stimulates Kir4.1/Kir5.1 of the DCT and increases channel activity from 1.40 ±0.24 to 2.56±0.25 (3m/3f mice). Previous study demonstrated that Kir4.1/Kir5.1 activity was modulated by SFK which phosphorylated Kir4.1 protein thereby increasing Kir4.1/Kir5.1 activity in the DCT (19). To test whether the stimulatory effect of FK506 or CsA on Kir4.1/Kir5.1 was due to enhancing SFK-induced stimulation of Kir4.1/Kir5.1 by inhibiting dephosphorylation of the K^+ channel, we examined the effect of FK506 or CsA on Kir4.1/Kir5.1 in the DCT treated with PP1 (22), a specific SFK inhibitor. Fig.3A is a single channel recording demonstrating that the inhibition of SFK with PP1 not

only decreases the 40 pS K^+ channel NP_o (from 1.55 ±0.10 to 0.38 ±0.06, n=4) but also abolishes the effect of FK506 (NP_o, 0.34±0.06, n=4) or CsA (NP_o, 0.30±0.06, n=4) on the Kir4.1/Kir5.1 activity in the DCT. This suggests the possibility that PP2B may be involved in regulating the Kir4.1/Kir5.1 activity of the DCT by dephosphorylating Kir4.1/Kir5.1 induced by SFK. This notion is further supported by experiments in which we have examined whether CsA or FK506 is able to stimulate Kir4.1/Kir5.1 in the DCT treated with H_2O_2 which is known to stimulate SFK (23). Fig. 3B is a single channel recording made in a cell-attached patch showing that adding 100 μM H_2O_2 increases the Kir4.1/Kir5.1 channel activity (NP_o) from 1.45±0.2 to 2.7±0.27 (n=4 male mice). Moreover, adding 10 μM FK506 or CsA (200 nM) has no additional effect on the 40 pS K^+ channel (FK506, 2.6±0.24 and CsA, 2.4±0.17, n=4). This supports the hypothesis that PP2B-inhibitor induced activation of Kir4.1/Kir5.1 of the DCT is related to directly or indirectly modulate SFK-induced phosphorylation of the K^+ channel of the DCT.

Because Kir4.1/Kir5.1 is the predominant form of K^+ channels in the basolateral membrane of the DCT (20), we expect that the stimulation of Kir4.1/Kir5.1 by calcineurin inhibitors should increase the negativity of DCT membrane potential (hyperpolarization). Thus, we next examined the effect of FK506 and CsA on the reversal potential of inward-to-outward current ([I]-reversal-potential), which is an index of the membrane potential (In our previous study we also refer it as I_K reversal potential). Fig 4A shows two whole-cell recordings demonstrating the [I]-reversal-potential of the DCT treated with vehicle, FK506 (0.75 mg/Kg. BW) or CsA (3 mg/Kg BW) by p.i 30 min before experiments. Fig 4B is a scatter plot summarizing the results of experiments performed in both male and female mice. FK506 treatment increased negativity of [I]-reversal-potential of the DCT from -62±1 mV to -72.5±1 mV (3m/3f mice). Whereas CsA treatment increased negativity of [I]-reversal-potential of the DCT from -62±1 mV to -73±1 mV (3m/3f mice). Thus, the inhibition of PP2B hyperpolarizes

the membrane potential of the DCT. We have also examined the effect of high dose FK506 (3 mg/Kg BW by p.i 30 min before experiments) on [I]- reversal- potential in *Fkbp1a*$^{flox/flox}$ mice and Ks-FKBP12-KO mice. From the inspection of Fig.4C and 4D, it is apparent that high dose FK506 hyperpolarized the DCT membrane (control value, -63±1 mV; FK506, -73±1 mV, n=4 male mice) to the same extend as the low dose. Moreover, this effect was abolished in Ks-FKBP12 KO mice (control value, -61±1 mV; FK506, -61±1 mV, n=4 male mice), suggesting FK506-induced hyperpolarization was due to the inhibition of PP2B because it required FKBP12. In contrast, CsA hyperpolarizes the DCT membrane not only in the control mice (-72.5±1mV, n=5 male mice) but also in FKBP12 KO mice (-72±1mV, n=4 male mice)

The inhibition of PP2B has been demonstrated to increase the abundance of pNCC and tNCC (2). Since a high basolateral K^+ channel activity is associated with increased NCC function (15, 24), we speculate that PP2B-inhibition-induced stimulation of Kir4.1/Kir5.1 in the DCT may contribute partially to PP2B-inhibition-induced stimulation of NCC. Thus, we next examined the effect of FK506 treatment (0.75 mg/Kg BW by p.i. 30 min before experiments) on the expression of pNCC and tNCC in the control (*Kcnj10*$^{flox/flox}$) and in Ks-Kir4.1 KO mice. Fig. 5A shows two Western blots from six male mice demonstrating the expression of pNCC and tNCC in the control (*Kcnj10*$^{flox/flox}$) and Ks-Kir4.1 KO mice treated with FK506 or vehicle (uncut Western blot is shown in Fig. s1). Fig.5B is a Western blot showing the Kir4.1 expression to validate the deletion of Kir4.1. Fig. 5C shows two scatter plots summarizing the normalized band density of pNCC and tNCC. We confirmed the previous finding that FK506 treatment robustly increases the expression of pNCC (by 66±5%) (25). Moreover, FK506 treatment also slightly increased tNCC (by 33±6%), an effect possibly induced by inhibiting NCC ubiquitination after increasing NCC phosphorylation (26). We confirmed our previous observations that pNCC and tNCC expression in Kir4.1-KO mice decreased to 39±3% and 34±2% of the control value observed in *Kcnj10*$^{flox/flox}$ mice,

respectively (14, 16). Moreover, we observed that the effect of FK506-treatment on pNCC expression was either attenuated or even largely abolished in Kir4.1 deficient mice. By calculation of these results, we observed that mean pNCC expression of Kir4.1 KO mice treated with FK506 was only 53±5% of the control value. Thus, FK506 treatment increased pNCC expression in Kir4.1 KO mice only by 35% compared to vehicle whereas it was 66% in $Kcnj10^{flox/flox}$ mice. Thus, calcineurin-inhibitors induced stimulation of pNCC was significantly attenuated in Kir4.1 KO mice. This suggests that the effect of PP2B-inhibition-induced stimulation of pNCC was at least in part through activation of the basolateral Kir4.1/Kir5.1 of the DCT.

The finding that acute FK506 treatment increased NCC activity in the control mice but to a lesser degree in Ks-Kir4.1 KO mice was also confirmed by *in vivo* measurement of HCTZ (30 mg/kg body weight)-induced urinary Na^+ excretion (E_{Na}) after initial saline perfusion. Fig. 6A summarizes results of each individual experiment in which HCTZ-induced E_{Na} was measured in the control mice (3 male $Kcnj10^{flox/flox}$ and 3 male $Fkbp1a^{flox/flox}$), Ks-Kir4.1 KO mice (n=5 male) and Ks-FKBP12 KO mice (n=4 male) treated with FK506 (0.75 mg/Kg BW by p.i) or vehicle 30 min before experiments. Fig. 6B is a scatter plot summarizing the delta value of HCTZ-induced E_{Na} in the control, Ks-Kir4.1 KO and Ks-FKBP12 KO mice treated with vehicle or FK506. HCTZ-induced natriuresis in FK506-treated control mice (0.49±0.08 to 4.01±0.16 μEq/min/100g BW, n=6 male mice) was significantly larger than in vehicle treated control (0.80±0.06 to 2.45±0.10 μEq/min/100g BW, n=6 male mice). Deletion of Kir4.1 inhibited NCC as evidenced by the fact that baseline E_{Na}, which was measured after initial saline infusion (0.3 ml), was significantly higher than the control mice (14). Moreover, HCTZ-induced E_{Na} was largely absent (2.0±0.15 to 2.1±0.15 μEq/min/100g BW) in 4 male Ks-Kir4.1 KO mice treated with vehicle. However, FK506 treatment was still able to slightly increase HCTZ-induced E_{Na} (2.13±0.07 to 2.95±0.20 μEq/min/100g BW, n=5 male mice). But, the delta

value (0.82±0.10 μEq/min/100g BW) in the FK506 treated Ks-Kir4.1 KO mice was significantly smaller than those in FK506 treated control mice (3.49±0.17 μEq/min/100g BW). Again, the stimulatory effect of FK506 on HCTZ-induced E_{Na} (FK506, 1.10±0.08 to 2.91±0.13 μEq/min/100g BW, n=4 male mice) was completely absent in Ks-FKBP12 KO mice (vehicle, 1.11±0.03 to 2.94±0.08 μEq/min/100g BW, n=4 male mice). Thus, data suggest that FK506-induced stimulation of E_{Na} requires FKBP12 and is partially achieved by activation of Kir4.1/Kir5.1 of the DCT. Acute FK506 treatment did not affect the baseline renal K^+ excretion (E_K) in comparison to vehicle (Fig.s1A). However, FK506 treatment increased HCTZ-dependent renal E_K, from 0.57±0.03 μEq/min/100g BW (vehicle) to 0.8±0.06 μEq/min/100g BW (n=5 male mice) (Fig.s2B). This is presumably induced by increasing Na^+ delivery to the late distal tubule thereby enhancing ENaC-dependent E_K. However, HCTZ-induced E_{Na}/E_K ratio in FK506-treated mice is higher than vehicle-treated group (Fig.s3C). This suggests that acute inhibition of calcineurin mainly stimulates NCC of the DCT but it had lesser effect of ENaC.

We have next used immunofluorescence microscopy to examine the basolateral surface expression of Kir4.1 and the luminal surface expression of pNCC (Ser^{71}) in *Kcnj10*$^{flox/flox}$ (control) treated with vehicle (Fig.7A) or CsA (3 mg/Kg BW by p.i. 30 min before perfusion fixation) (Fig.7B) and in Ks-Kir4.1-KO mice treated with vehicle (Fig.7C) or CsA (Fig. 7D). Semi-quantification of Kir4.1 image shows that CsA-treatment increased basolateral Kir4.1-flourescence intensity compared to vehicle-treated animals by 45% ($P<0.05$) from 76±10 to 110±11 (arbitrary units) (Fig.s3C). Although apical pNCC immunostaining in Kir4.1 KO mice is faint, a clear luminal staining of pNCC is still obvious in the DCT cells showing remained Kir4.1 expression (due to incomplete deletion). In contrast, Kir4.1-deficent DCT cells virtually showed no obvious pNCC fluorescence staining. The results are consistent with the western blot and in vivo measurement of HCTZ-induced natriuresis. It is possible that the increase in

pNCC may occur only in the DCT cells in which Kir4.1 is still present. This suggests that Kir4.1 activity is required for calcineurin-inhibition-induced stimulation of NCC.

Discussion

The main finding of the present study is that acute application of calcineurin inhibitors increases the basolateral Kir4.1/Kir5.1 activity of the DCT. Two lines of evidence have strongly suggested that the FK506 or CsA-induced stimulation of Kir4.1/Kir5.1 activity is due to the inhibition of PP2B: 1) CsA and FK506, two calcineurin inhibitors with different structures, had the same stimulatory effect on the Kir4.1/Kir5.1; 2) the effect of FK506 on the basolateral Kir4.1/Kir5.1 was completely absent in the FKBP12 deficient mice while CsA was still able to stimulate the K^+ channel, suggesting that the effect of FK506 but not CsA was achieved by binding to FKBP12 thereby inhibiting PP2B (21, 27). The notion that PP2B regulates the basolateral Kir4.1/Kir5.1 of the DCT is supported by four lines of evidence: 1) Application of FK506 and CsA increased the basolateral 40 pS K^+ channel activity defined by NP_o; 2) The Kir4.1/Kir5.1-mediated whole-cell K^+ currents were larger in the DCT treated with FK506 and CsA than with vehicle treated tubule; 3) CsA treatment increased the membrane staining intensity of Kir4.1; 4) FK506 and CsA treatment increased the negativity of the [I]-reversal-potential of the DCT, an indication of hyperpolarization. The role of PP2B in the regulation of membrane transport of the DCT has been well established by the finding that the acute inhibition of PP2B increased the NCC expression/activity (1, 2, 9). Thus, our present results have suggested that PP2B regulates the membrane transport not only by modulating NCC but also by regulating the basolateral K^+ channels of the DCT. Although the present study has demonstrated that calcineurin inhibitors stimulate the basolateral Kir4.1/Kir5.1 activity in the DCT, it is not known whether CsA or FK506 is also able to regulate Kir4.1 homotetramer because Kir4.1 homotetramer, a 20-25 pS K^+ channel activity, is hard to be detected under control conditions (18, 28, 29). Thus, a separated study may be required using

Kir5.1 KO mice to determine the effect of CsA or FK506 on Kir4.1 homotetramer.

Although the mechanism by which PP2B regulates Kir4.1/Kir5.1 is not completely understood, two lines of evidence suggest the possibility that PP2B regulates Kir4.1/Kir5.1 by targeting SFK-mediated tyrosine phosphorylation. First, the inhibition of SFK abolished the effect of FK506 or CsA on the basolateral Kir4.1/Kir5.1 activity. Second, CsA or FK506 has no additional effect on Kir4.1/Kir5.1 of the DCT treated with H_2O_2. Our previous study has demonstrated that Kir4.1 protein is a substrate of SFK which phosphorylates Kir4.1 at its N-terminus (19). The role of SFK in regulating Kir4.1/Kir5.1 was also supported by in *vitro* and in *vivo* studies in which we have demonstrated that SFK increased Kir4.1/Kir5.1-mediated K^+ currents in the DCT and the surface expression (19, 30). Thus, we speculate that CsA or FK506-induced activation of Kir4.1/Kir5.1 may be achieved by inhibiting PP2B-dependent dephosphorylation of Kir4.1/Kir5.1 thereby enhancing the effect of SFK on the basolateral K^+ channels of the DCT. Although calcineurin is considered to be protein serine/threonine phosphatase, a previous study has demonstrated that PP2B was also able to dephosphorylate tyrosine phosphorylation although K_m and V_{max} values were lower for phosphotyrosyl substrates than phosphoseryl substrates (31). Moreover, PP2B has been reported to regulate tyrosine phosphorylation of a Cl^- channels in renal epithelial cells (32). Further experiments are required to explore whether PP2B is able to modulate tyrosine-phosphorylation of Kir4.1/Kir5.1.

We have also confirmed the previous finding that the acute inhibition of PP2B increased the NCC expression/activity (2, 33). However, three lines of evidence strongly suggest that the PP2B-inhibition induced stimulation of NCC is at least in part by stimulation of Kir4.1/Kir5.1: 1) FK506-induced stimulation of both pNCC and tNCC expression was attenuated in Ks-Kir4.1 KO mice; 2) HCTZ-induced natriuresis was also smaller in Ks-Kir4.1 KO mice than

Kcnj10$^{flox/flox}$ mice; 3) CsA-induced stimulation of apical pNCC expression was more obvious in the DCT cells where Kir4.1 expression was still visible than other DCT cells where Kir4.1 staining was absent. We speculate that PP2B may regulate NCC expression/activity by Kir4.1/Kir5.1-dependent and independent mechanism. It is now well established that the basolateral Kir4.1/Kir5.1 determines the expression and activity of NCC by modulating the basolateral membrane potential (34, 35). Because the basolateral membrane potential provides the driving force for Cl^- ions movement across the basolateral membrane (36), a hyperpolarization should stimulate Cl^- exit thereby decreasing the intracellular Cl^- [Cl^-_i] concentrations whereas a depolarization is expected to have an opposite effect on [Cl^-_i]. Decreased [Cl^-_i] concentrations should stimulate Cl^--sensitive with-no-lysine kinase (WNK) such as WNK1 and WNK4 (37). WNKs are upstream protein kinases which stimulate Ste20-related protein proline/alanine-rich kinase (SPAK) and oxidative responsive kinase (OSR) (38-42). Because SPAK and OSR are responsible to stimulate NCC activity by promoting serine/threonine phosphorylation, the stimulation of WNKs induced by the activation of Kir4.1/Kir5.1 is expected to increase the NCC activity/expression. PP2B may regulate the basolateral Kir4.1/Kir5.1 activity by inhibiting SFK-mediated tyrosine phosphorylation thereby decreasing the negativity of the DCT membrane potential (depolarization). Thus, the acute inhibition of PP2B with FK506 or CsA should hyperpolarizes DCT membrane potential thereby decreasing [Cl^-_i] concentrations. The finding that acute effect of FK506 on NCC expression/activity is attenuated in Kir4.1 KO mice supports this argument. However, the finding that inhibition of PP2B was still able to increase NCC expression/activity in Kir4.1 KO mice indicates that PP2B may also regulate NCC phosphorylation directly by Kir4.1/Kir5.1 independent mechanism.

Although the role of PP2B in the regulation of NCC and renal K^+ excretion has been strongly suggested by previous studies, these results were obtained using pharmacological

approaches (1, 2, 9). A recent elegant study has employed a mouse model with specific deletion of calcineurin-regulatory-subunit B-alpha (CnB1) in the DCT to examine the role of PP2B in regulating NCC and renal K^+ excretion (43). While confirming that the inhibition of PP2B with FK506 stimulated pNCC expression in the control mice, Banki *et al.* have demonstrated that the expression of pNCC and tNCC was actually decreased in the mice with DCT-specific disruption of CnB1 in comparison to the corresponding control mice (43). Moreover, although the urinary K^+ and Na^+ excretions were compromised in DCT-specific CnB1 knockout mice treated with acute (60 min) K^+ loading in comparison to the corresponding control mice, the different response to K^+ loading between two genotypes were absent during a prolonged K^+ loading. This suggests that PP2B signaling in the DCT may not be indispensable for stimulating renal K^+ excretion during long-term HK intake. The discrepancy between Banki's study and previous studies may be a time dependent effect because Banki's study was performed in the mice 3 or 10 weeks after the disruption of CnB1 whereas previous studies were performed in the animals with relatively acute treatment of calcineurin inhibitors (1, 2, 9, 33). Considering the physiological significance for maintaining a proper renal K^+ excretion and K^+ homeostasis, it is conceivable that factors other than PP2B may be able to compensate the function of calcineurin of the DCT through a tubule remodeling process.

The finding that acute inhibition of PP2B stimulates the basolateral Kir4.1/Kir5.1 of the DCT suggests that calcineurin may be involved in determining the baseline activity of Kir4.1/Kir5.1. Fig. 8 is a scheme illustrating the role of PP2B in regulating the basolateral Kir4.1/Kir5.1 activity in the DCT. We speculate that PP2B may play a role in fast stimulation of renal K^+ excretion by targeting Kir4.1/Kir5.1 in response to an acute K^+ loading because the basolateral Kir4.1/Kir5.1 in the DCT is a key component of "K-sensor" mechanism (11, 14-16, 24). Since Kir4.1/Kir5.1 is also a major type of basolateral K^+ channels in the CCD (44-46), the inhibition of calcineurin with FK506 or CsA is expected to hyperpolarize the membrane

potential of the CCD thereby increasing the driving force of Na^+ absorption through ENaC. Thus, it is possible that FK506 or CsA-induced increase in renal Na^+ absorption is due to not only stimulating NCC activity but also enhancing ENaC function in the CCD. Accordingly, the selective inhibition of Kir4.1/Kir5.1 in the kidney would be a potential approach to treat CsA or FK506-induced hypertension. We conclude that the acute inhibition of calcineurin stimulates the basolateral Kir4.1/Kir5.1 activity of the DCT and CsA or FK506-induced stimulation of Kir4.1/Kir5.1 is, at least in part, contributes to the calcineurin-inhibition-induced stimulation of NCC.

Methods

All supporting data and detailed methods including animal preparation, electrophysiology, western blot, fluorescence immunostaining and in vivo measurement of HCTZ-induced natriuresis are available within the article and its online supplementary file.

Generating KS-Kir4.1 knockout (KO) and Ks-FKBP12 KO mice

Mice expressing Pax8-rtTA and tet-on LC-1 transgene were crossed with *Kcnj10*-floxed or *FKBP1a*-floxed mice to generate inducible kidney-tubule-specific Kir4.1 KO (Ks-Kir4.1 KO) and Ks-FKBP12 KO mice, respectively. *Kcnj10* or *Fkbp1a* deletion was conducted in 8-week-old male and female mice homozygous for floxed *Kcnj10* or *Fkbp1a* gene and heterozygous for Pax8-rtTA/LC-1 transgene by providing doxycycline (5mg/ml, 5% sucrose) in the drinking water for 2 weeks. This was followed by at least 2 additional weeks without doxycycline treatment before performing experiments. Littermate mice of the same age and genetic background drinking 5% sucrose were used as controls ($Kcnj10^{flox/flox}$ or $Fkbp1a^{flox/flox}$). Tail DNA was PCR amplified and the primers for genotyping are shown in table 1s.

Preparation of the DCT

Mice were sacrificed by CO_2 inhalation plus cervical dislocation. The abdomen of the mice was quickly opened to expose the left kidney which was then perfused with 2 ml L-15 medium (Life Technology) containing type 2 collagenase (250 unit/ml). After the perfusion, the left kidney was removed for harvesting the renal cortex which was further cut into small pieces and incubated in collagenase-containing L-15 media for 30-50 min at 37ºC. The tissue was then washed three times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated DCT tubules were placed on a small cover glass coated with poly-lysine and the cover glass was placed on a chamber mounted on an inverted microscope.

Patch-clamp experiments

A Narishige electrode puller (Narishige, Japan) was used to make the patch-clamp pipettes from Borosilicate glass (1.7-mm OD). The resistance of the pipette was 5 MΩ (for single channel recording) or 2 MΩ (for whole-cell recording) when it was filled with solution containing (in mmol/L) 140 KCl, 1.8 $MgCl_2$ and 10 HEPES (titrated with KOH to pH 7.4). We have used the single channel recording to examine Kir4.1/Kir5.1 channel activity, defined as NP_o (a product of channel number and open probability) and used the perforated whole-cell recording to measure Ba^{2+}-sensitive Kir4.1/Kir5.1-mediated K^+ currents and [I]-reversal-potential in the isolated DCT. The detailed method for the patch-clamp experiment is described in the supplementary material.

Immunoblotting

Whole kidney protein extract was obtained from frozen kidney homogenized in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1mM EGTA, 1 mM DTT supplemented with phosphatase and protease inhibitor cocktails (Sigma). Protein (40-60 μg) was separated on 4-12% (wt/vol) Tris-Glycine gel (NovexTM, TheromoFisher Scientific) and transferred to nitrocellulose membrane. The membranes were incubated 1 hour with LI-

COR blocking buffer (PBS) and then incubated overnight at 4ºC with anti-NCC (1:2000), anti-pNCC at threonine-53 (1:2000) and anti-Kir4.1antibodies (1:1000). All antibodies used in the experiments are validated previously (47, 48). An Odyssey infrared imaging system (LI-COR) was used to capture the images at a wavelength of 680 or 800 nM.

Perfusion fixation and tissue processing

Adult (10-12 weeks) *Kcnj10*$^{flox/flox}$ (control) and Ks-Kir4.1 KO mice (n=4) were anesthetized with isoflurane and pentobarbital sodium and perfused via the abdominal aorta with phosphate buffered saline (PBS) for 30 sec followed by 3% paraformaldehyde (PFA)/PBS for 5 min to fix the kidneys. Kidney tissue was proceeded for paraffin embedding and sectioning (4 μm). Kidney sections were dewaxed and boiled in citrate buffer (pH 6) for 5 min for antigen retrieval followed by incubation with blocking medium (5% skim milk/PBS for 30 minutes). For double labeling of Kir4.1 and phosphorylated NCC (pS71-NCC), the antibodies to Kir4.1 (guinea pig anti-Kir4.1; Alomone labs) and phosphorylated NCC (rabbit anti-pS71-NCC (1), generated in Mutig laboratory) were sequentially applied for 1 h, separated by a washing step. Fluorescent Cy2-, Cy3-, or Cy5-conjugated antibodies (DIANOVA) were used for detection. The signal was evaluated in a Leica DMRB or a Zeiss confocal microscope (LSM 5 Exciter). Kir4.1 fluorescence signal was semi-qualified using ZEN and ImageJ software as described previously (1).

In vivo measurement of HCTZ-induced natriuresis

Animals were anesthetized by peritoneal injection of Inactin (100 mg/Kg body weight). The mice were placed on a heated small blanket to maintain body temperature at 37ºC. The trachea was cannulated to clear any mucus that may be produced during the experiment. A carotid artery was catheterized with PE10 tubing for blood collection, jugular vein was also cannulated for iv infusion. The bladder was exposed and catheterized via a suprapubic incision with a 10

cm piece of PE-10 tubing for urine collections. After completion of surgery, isotonic saline was given intravenously for 4 hr (0.25-0.3 ml/1 hr and total 1.0-1.2 ml 0.9% saline) to replace surgical fluid losses and to maintain hemodynamics. Urine collections started one hr after infusion of 0.3 ml saline and total 6 collections (every 30 minutes) were performed (2 for controls and 4 for experiments). After six times of urine collections, the mice were sacrificed by IV somnasol.

Material Chemicals including inactin, cyclosporin-A, PP1 and HCTZ were purchased from Sigma-Aldrich (St. Louis, MO) whereas tacrolimus was obtained from Alfa Aesar (Tewksbury, MA). We purchased GAPDH antibody from Sigma-Aldrich, NCC-antibody from Millipore, pNCC(Thr^{53}) antibody from PhosphoSolutions and Kir4.1-antibody from Alomono Labs. Antibody for pNCC (Ser71) was developed in Kerim Mutig laboratory.

Statistical analysis We used software (Sigma plot 14) for the statistical analysis. For analyzing the values between two groups we used t-test, and for comparisons of the values within the same group, we used paired t-test. We used one-way or two-way ANOVA for analyzing results of more than two groups, and Holm-Sidak test was used as post-hoc analysis. *P*-values <0.05 were considered statistically significant. Data are presented as the mean ± SEM.

Author contributions

DDZ, XPD, KM, FR, YX, JYZ and DHL conducted the experiments and analyzed data. DDZ, XPD, DHL and WHW designed the study. DDZ, XPD, KM, LDH and WHW drafted the manuscript.

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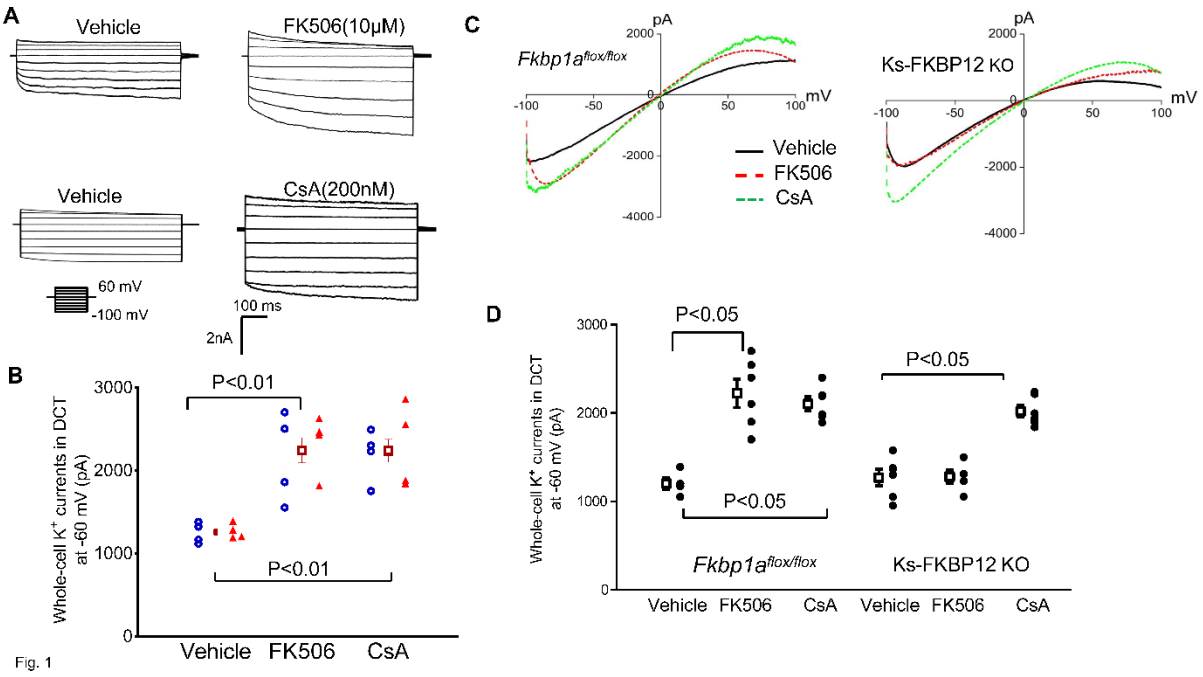

Fig. 1

Fig. 1 Inhibition of calcineurin (PP2B) increases the basolateral K^+ currents in DCT. (A) A set of whole-cell recordings shows the effect of FK506 (10μM) and CsA (200nM) on Kir4.1/Kir5.1-mediated K^+ currents in the DCT. The Kir4.1/Kir5.1-mediated whole-cell K^+ currents were measured with a step protocol from −100 to 60 mV. The vehicle, FK506 or CsA was added in the bath for ten minutes. **(B)** A scatterplot summarizing the results of above experiments in which Ba^{2+}-sensitive K^+ currents (Kir4.1/Kir5.1) of the DCT were measured at −60 mV. Each data point from male (blue circles) or female mice (red triangles) is presented for two separate columns and the mean value + SEM (including data from male and female mice) is shown in the middle. Significance is determined by one-way ANOVA. **(C)** A set of recordings shows Kir4.1/Kir5.1-mediated whole-cell K^+ currents in DCT of male *Fkbp1a*$^{flox/flox}$ and male Ks-FKBP12 KO mice treated with FK506 (0.75 mg/Kg BW) or CsA (3 mg/Kg BW) by peritoneal injection 30 min before the experiment, respectively. The whole-cell K^+ currents were measured with a ramp protocol from −100 to 100 mV. **(D)** A scatterplot summarizing the results of experiments in which Kir4.1/Kir5.1-mediated whole-cell K^+ currents of the DCT were measured at −60 mV. Mean values and SEM are shown on the left of each column. A symmetric 140 mM KCl solution was used for the bath and the pipette. Significant difference as determined by two-way ANOVA.

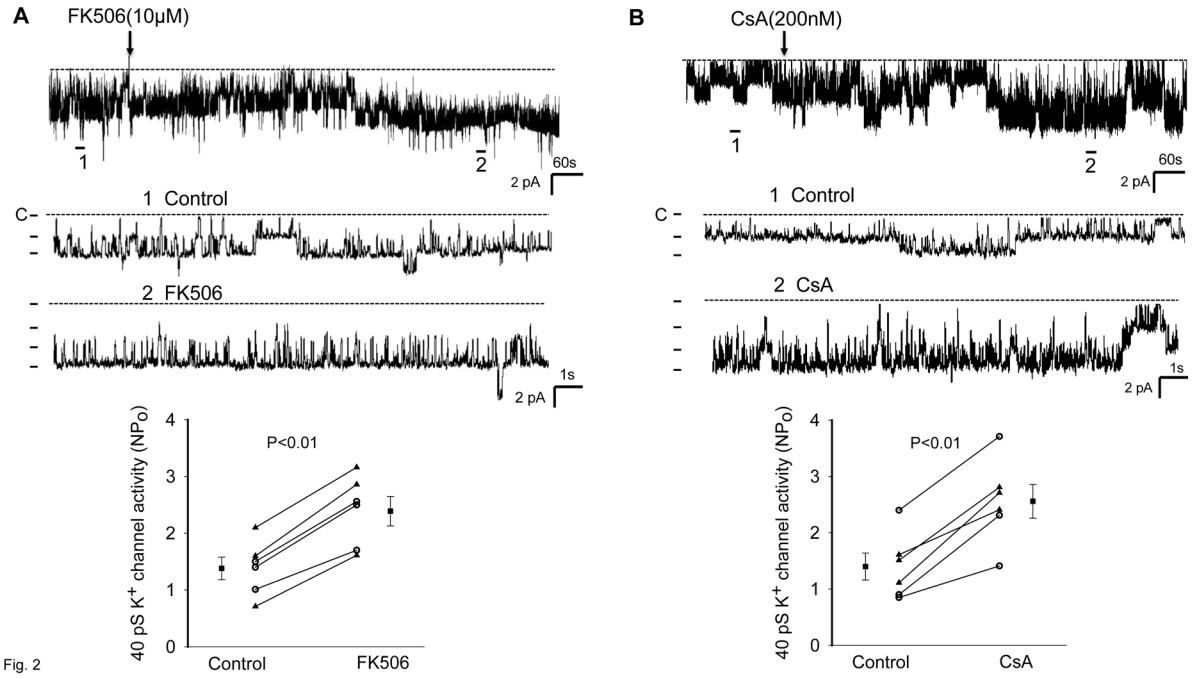

Fig. 2

Fig. 2 Inhibition of calcineurin (PP2B) stimulates the basolateral Kir4.1/5.1 (40pS K^+) channels in the DCT. A single-channel recording shows the effect of 10μM FK506 (A) or 200 nM CsA (B) on the basolateral 40pS K^+ channels in DCT. Two parts of the trace (indicated by numbers) are extended to show the fast resolution. The experiments were performed in cell-attached patches. The results of the experiments are summarized in two scatter plots at the bottom of each panel. The DCT was bathed in a solution containing 140 mM NaCl/5 mM KCl and the pipette solution contained 145 mM K^+. Significance is determined by a paired student *t* test. Data from male and female mice are indicated by circles and triangles, respectively. Mean value (including male and female mice) is indicated by a square.

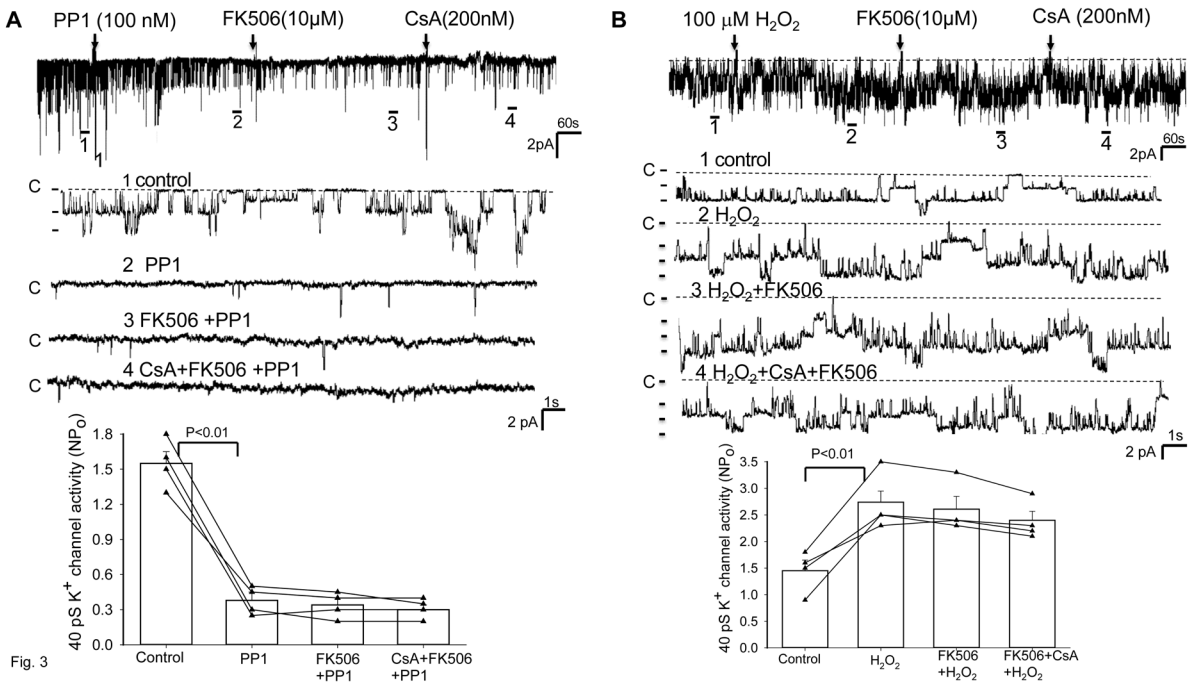

Fig. 3

Fig.3 The effect of calcineurin inhibitors on Kir4.1/Kir5.1 is absent in the DCT treated with PP1 or H_2O_2. A single channel recording demonstrates the effect of FK506 and CsA on the 40 pS K^+ channels in the DCT pretreated with 100 nM PP1, an inhibitor of SFK (A) or pretreated with 100 μm H_2O_2 (B). Four parts of the trace (indicated by numbers) are extended to show the fast resolution. The experiments were performed in cell-attached patches (male mice). The results of the experiments are summarized in two bar graphs with scatter plotting at the bottom of each panel. The DCT was bathed in a solution containing 140 mM NaCl/5 mM KCl and the pipette solution contained 145 mM K^+. Significance is determined by one-way ANOVA.

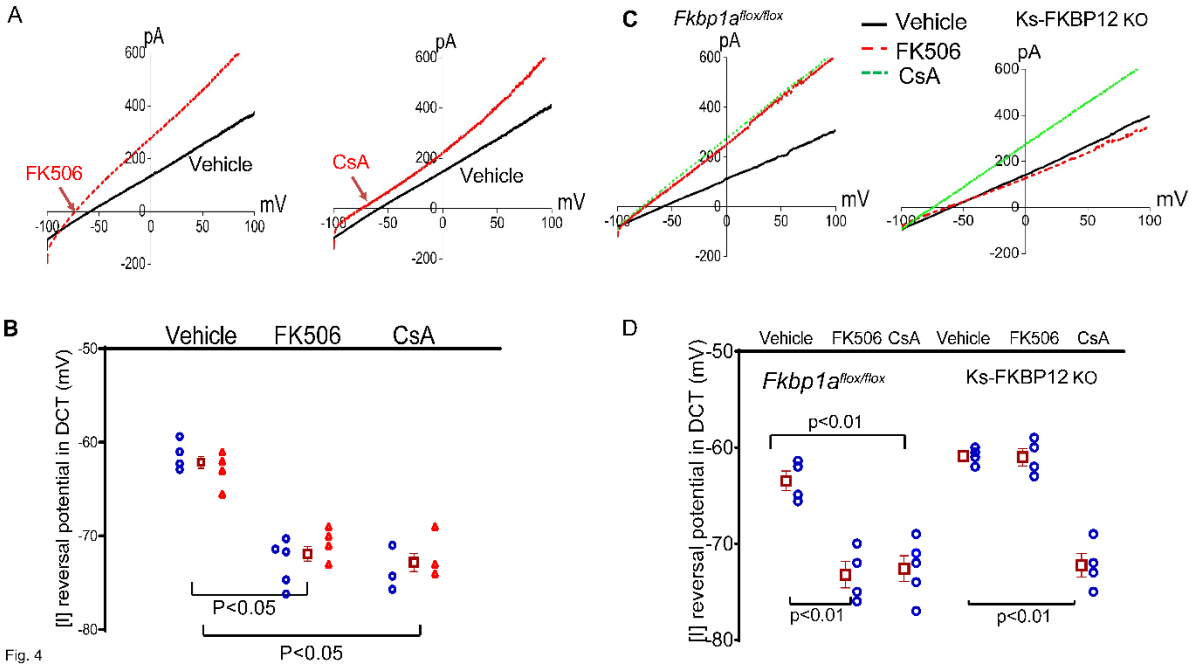

Fig. 4 **Inhibition of calcineurin (PP2B) hyperpolarizes DCT membrane**. (A) A set of traces of the whole-cell voltage-clamp shows the effect of FK506 and CsA on [I]-reversal-potential in the DCT of the mice treated with vehicle, FK506 (0.75 mg/Kg. BW) or CsA (3 mg/Kg BW) by p.i 30 min before experiments. **(B)** A scatter plot summarizing the results of the experiments in which I_K reversal potential of the DCT were measured with whole-cell recording. Each data point from male (blue circles) or female mice (red triangles) is presented for two separate columns and the mean value + SEM (including data from male and female mice) is shown in the middle. Significance is determined by one-way ANOVA. (C) A set of traces of the whole-cell voltage-clamp shows the effect of FK506 and CsA on [I]-reversal-potential in the DCT of male *Fkbp1a*$^{flox/flox}$ mice or Ks-FKBP12 KO mice treated with vehicle, FK506 (3 mg/Kg. BW) or CsA (3 mg/Kg BW) by p.i 30 min before experiments. **(D)** A scatterplot summarizing the results of above experiments (male mice), and mean values and SEM are shown on the left of each column. Significance is determined by two-way ANOVA. The DCT was bathed in a solution containing 140 mM NaCl/5 mM KCl and the pipette solution contained 145 mM K^+.

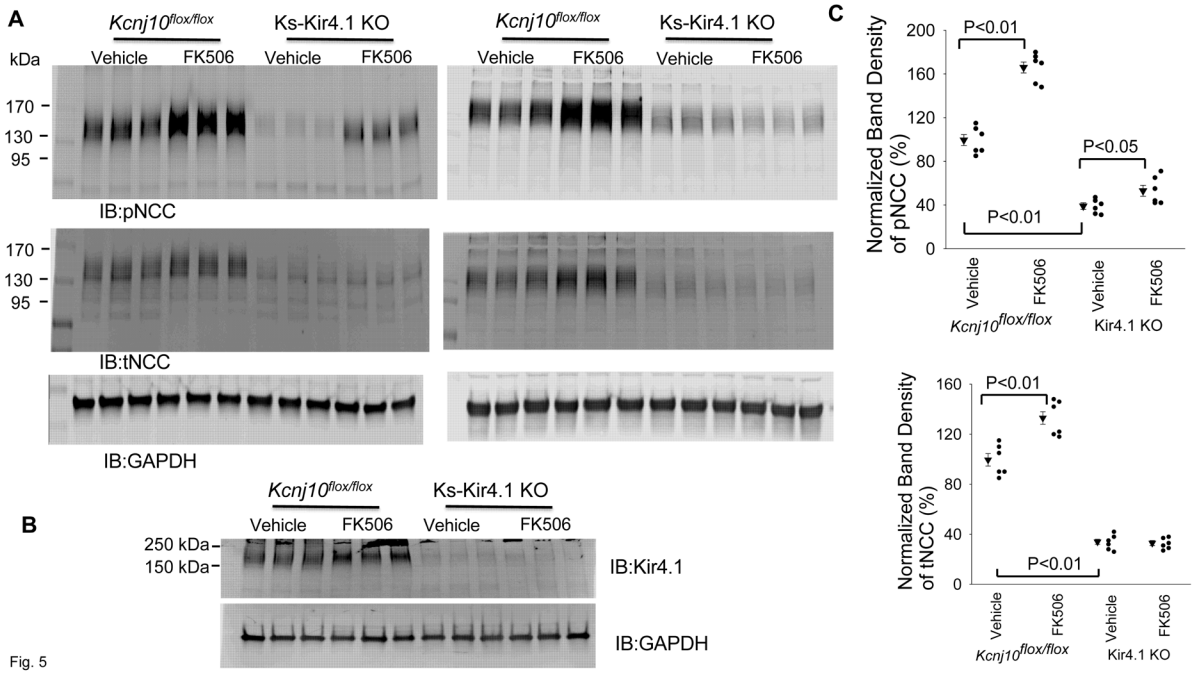

Fig. 5

Fig. 5 FK506 treatment-induced stimulation of pNCC expression is attenuated in Ks-Kir4.1 KO mice. (A) Two western blots show the abundance of pNCC^{T53} and tNCC in male *Kcnj10$^{flox/flox}$* mice and in Ks-Kir4.1 KO mice treated with vehicle (control) and FK506 (0.75 mg/Kg BW). FK506 or vehicle was applied by peritoneal injection 30 min before the experiment. (B) A Western blot shows the expression of Kir4.1 in the control and Ks-Kir4.1 KO mice to validate the deletion of Kir4.1. Kir4.1 band represents a Kir4.1 homotetramer and Kir4.1/Kir5.1 heterotetramer. (C) The normalized band density of pNCC and tNCC expression from the experiments is summarized in two scatter plots. For the western blot experiments, the lysate obtained from the renal cortex tissue was unheated. Significance was determined by two-way ANOVA.

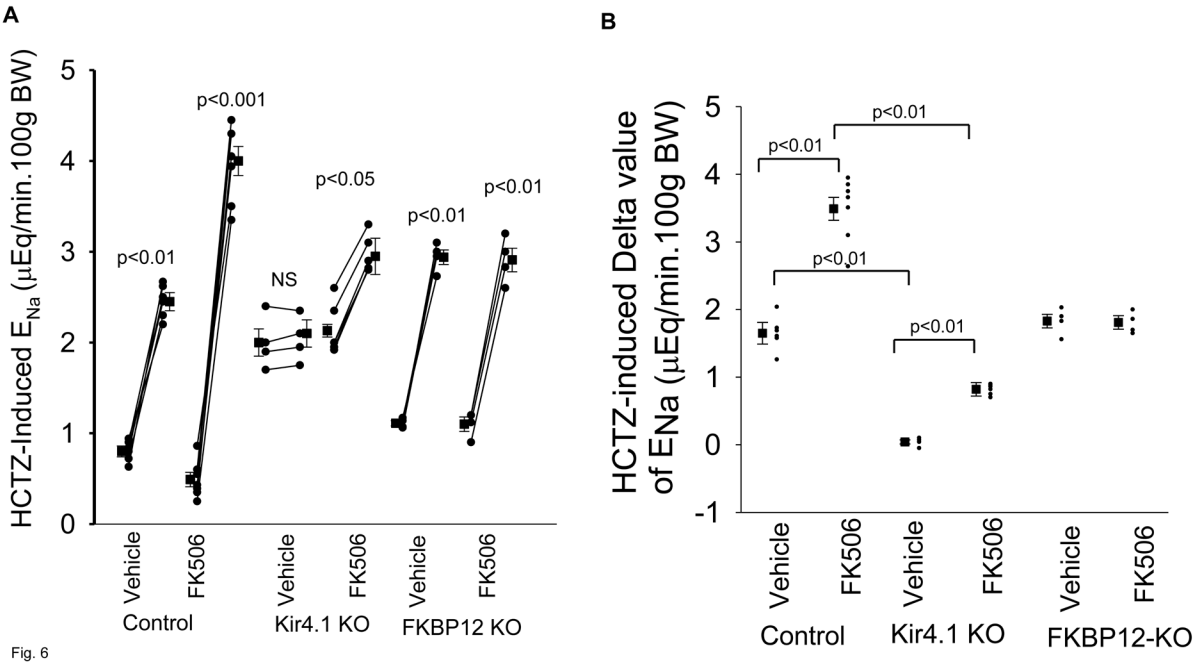

Fig. 6 FK506-induced stimulation of HCTZ-induced natriuresis is attenuated in Ks-Kir4.1 KO mice and is absent in Ks-FKBP12 KO mice. (A) A line graph shows the results of each experiment in which urinary sodium excretion (E_{Na}) was measured before and after a single dose of hydrochlorothiazide (HCTZ; 30 mg/kg BW) in control mice (*Kcnj10*$^{flox/flox}$ and *Fkbp1a*$^{lox/flox}$), Ks-Kir4.1 KO mice and Ks-FKBP12 KO mice with or without FK506 (0.75 mg/Kg BW) injection for 30min before the experiment. The significance is determined by paired *t* test. (B) A scatter plot shows the mean value and each single data point of the net HCTZ-induced E_{Na} in different groups. Significance was determined by two-way ANOVA.

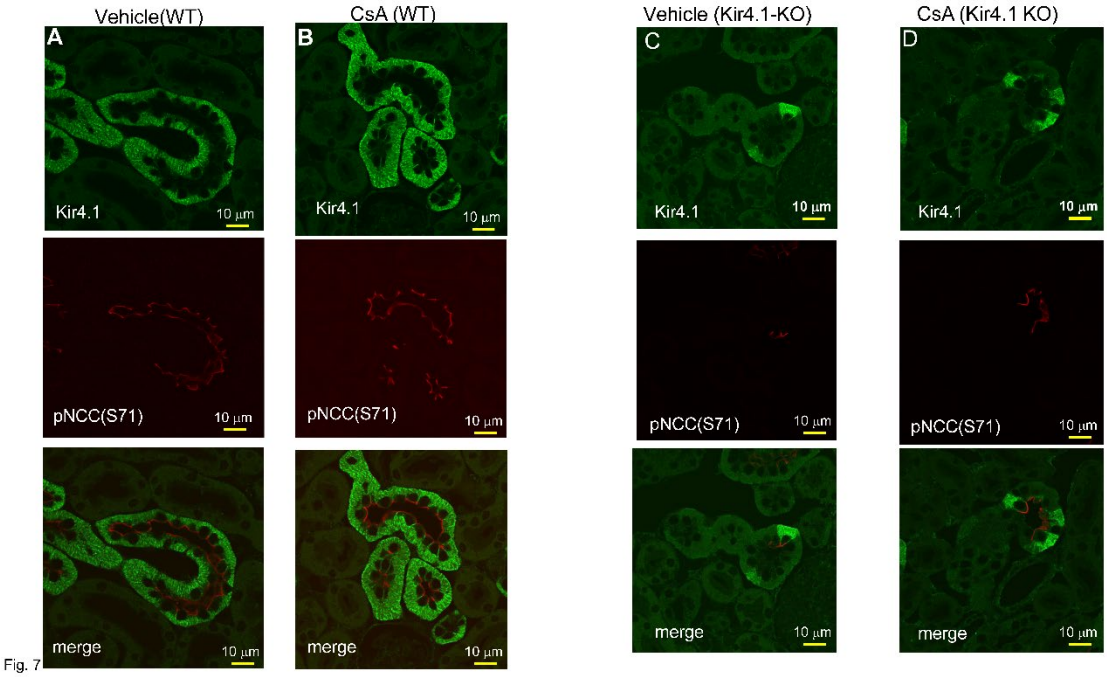

Fig. 7

Fig. 7 CsA treatment increases immunostaining intensity of Kir4.1. Immunostaining image shows the expression of Kir4.1 and phospho-NCC (S^{71}) in the DCT of *Kcnj10*$^{flox/flox}$ mice treated with the vehicle (A) or CsA (B) and Ks-Kir4.1 KO mice treated with the vehicle (C) or CsA (D). The mice were treated with the vehicle or CsA (3 mg/Kg BW) by peritoneal injection 30 min before the perfusion fixation of the kidneys.

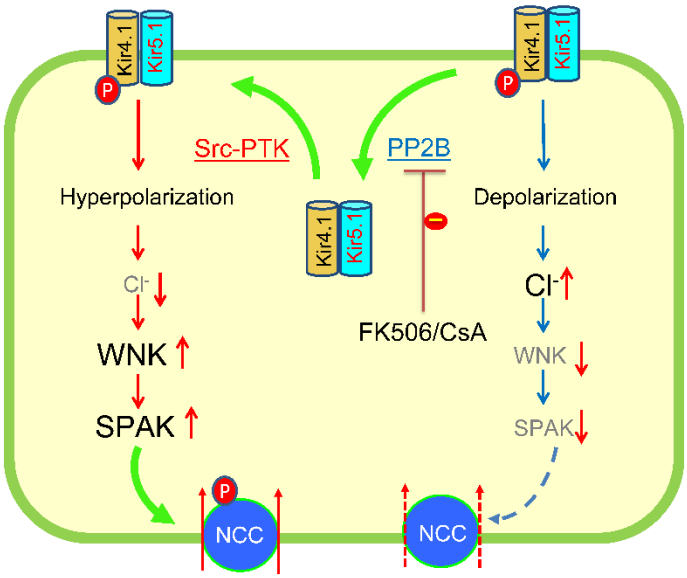

Fig.8

Fig. 8 A cell scheme illustrating the role of PP2B in regulating the basolateral Kir4.1/Kir5.1 in the DCT. The large and bold font size represents the stimulation whereas grayed and small font size represents the inhibition. Abbreviations: Src-PTK, Src family protein tyrosine kinase; WNK, with-no-lysine kinase, SPAK, ste20 proline and alanine-rich protein kinase.