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The Calcium-sensing Receptor Increases Activity of the Renal NaCl Cotransporter Through the WNK4-SPAK Pathway

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Running title: NCC regulation by the CaSR

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Abstract

Extracellular Ca^{2+} inhibits NaCl reabsorption in the thick ascending limb of Henle's loop (TALH) through the basolateral Calcium-sensing Receptor (CaSR) to induce hypercalciuria. CaSR is also expressed in the apical membrane of the distal convoluted tubule (DCT), were we hypothesized that it could play a role in activating NCC via the WNK4-SPAK pathway to prevent NaCl loss. Here we demonstrate, using a combination of *in vitro* and *in vivo* models that activation of CaSR leads to phosphorylation and concomitant activation of NCC. First, functional expression of NCC (thiazide-sensitive $^{22}Na^{+}$ uptake) was assessed in *Xenopus laevis* oocytes where we found that NCC activity was increased in a WNK4-dependent manner when CaSR was activated with gadolinium. Second, in HEK293 cells, the calcimimetic R-568 stimulated SPAK phosphorylation only in the presence of WNK4. WNK4 inhibitor, WNK463, also prevented this effect. Furthermore, we found that CaSR activation leads to KLHL3 and WNK4 phosphorylation and consequently, increased WNK4 abundance and activity. Lastly, mice administered with R-568 showed NCC phosphorylation. Our results show that activation of CaSR can increase NCC activity via the WNK4-SPAK pathway. It is possible that activation of CaSR by Ca^{2+} in the apical membrane of DCT increases NaCl reabsorption via NCC, with the consequent well known decrease of calcium reabsorption, further promoting hypercalciuria.

Significance Statement

Extracellular calcium modulates calciuria by acting on the basolateral membrane calcium-sensing receptor (CaSR) of the thick ascending limb of Henle's loop (TALH), reducing calcium reabsorption at the expense of apical salt absorption. CaSR is also expressed in the apical membrane of the distal convoluted tubule. Here we show using *in vitro* and *in vivo* models, that stimulation of the CaSR induces activation of the Na-Cl cotransporter (NCC), by a pathway that involves a PKC-induced activation of the KELCH3-WNK4-SPAK pathway that ultimately phosphorylates NCC, increasing its activity. This study proposes a mechanism through which salt reabsorption is upregulated beyond the TALH to recover the salt, while calcium is excreted.

Introduction

The calcium-sensing receptor (CaSR) is a member of class C of the G-protein coupled receptors (GPCR) and its role is to constantly monitor Ca^{2+} in the extracellular environment[1]. In the kidney, CaSR is essential for sensing Ca^{2+} in both the urinary filtrate and interstitial fluid to adequately modulate calcium excretion. To achieve this, CaSR is expressed all along the nephron[2-5].

Ca^{2+} and salt (NaCl) handling in the kidney are particularly integrated in two segments of the nephron: the thick ascending limb of Henle's loop (TALH) and the distal convoluted tubule (DCT). In the TALH Ca^{2+} is reabsorbed by a paracellular route, a process which is largely dependent on NaCl reabsorption[6,7]. Apical NaCl influx via the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter (NKCC2) is accompanied by potassium recycling to the lumen, through the apical renal outer medullary K^{+} channel (ROMK, KCNJ1), and by the basolateral extrusion of NaCl by the Na^{+}/K^{+}-ATPase and the chloride channel (CLCNKB)[8]. The apical recycling of K^{+} generates a transepithelial voltage difference providing a driving force that drags paracellular reabsorption of cations, amongst them, Ca^{2+} [9]. Consequently, a positive correlation exists between NaCl and Ca^{2+} reabsorption in this nephron segment. For instance, patients with Bartter syndrome exhibit a salt loosing nephropathy and hypercalciuria[10]. Likewise, clinicians have taken advantage of this positive correlation phenomenon by using loop diuretics to treat hypercalcemia[11].

In the TALH, CaSR is expressed in the basolateral membrane[2,5] where it senses increased interstitial Ca^{2+} and promotes its urinary excretion by halting NKCC2 and ROMK activity[4,12-15]. In this manner, the increase in Ca^{2+} excretion is due to decreased NaCl reabsorption in the

TALH that must be reabsorbed beyond the macula densa. Indeed, gain-of-function mutations of CaSR have been reported to produce a Bartter-like syndrome[16,17].

The DCT reabsorbs approximately 5-10% of the filtered NaCl and Ca^{2+} [6,7,18]. Its impact on blood pressure and Ca^{2+} excretion is prominent since NaCl reabsorption beyond the macula densa is not regulated by tubuloglomerular feedback and no specific Ca^{2+} reabsorption pathways are present beyond this point[7]. In the DCT, reabsorption of NaCl occurs through the thiazide sensitive Na^{+}-Cl^{-} cotransporter (NCC), whereas that of Ca^{2+} through the apical transient receptor potential cation channel subfamily V (TRPV5)[18]. In this part of the nephron, NaCl and Ca^{2+} transport occurs in opposite directions; increased NaCl reabsorption is associated with decreased Ca^{2+} reabsorption[19]. For instance, patients with Gitelman syndrome present a salt losing nephropathy accompanied by hypocalciuria[20]. Clinicians have taken advantage of this by using thiazide diuretics to promote Ca^{2+} reabsorption in patients with urolithiasis [21]. The exact mechanism for this inverse relationship is still unclear. CaSR is expressed both in the basolateral and apical membranes of DCT cells[4,5,22]. However, the role CaSR might play in regulating NaCl reabsorption in this nephron segment is not known.

The activity of NCC is modulated by a kinase pathway consisting of the with-no-lysine-kinases (WNKs) acting upon the Ste20-related proline alanine-rich kinase (SPAK)[23]. Active WNK kinases phosphorylate SPAK[24], which subsequently phosphorylates and activates NCC[25]. Two proteins, Cullin 3 (CUL3) and Kelch-like 3 (KLHL3), are part of a E3-RING ubiquitin ligase complex that in turn regulate WNK kinases. KLHL3 specifically binds to WNKs marking them for degradation[26,27]. Disease-causing mutations in WNK4, KLHL3 or CUL3 result in impaired

degradation of WNK kinases leading to increased NCC activity that results in a syndrome called Pseudohypoaldosteronism type II (PHAII) [28,29].

Hormones that regulate NaCl reabsorption in the DCT do so by affecting the KLHL3-WNK-SPAK-NCC pathway. Angiotensin II (AngII) regulates NCC activity in a WNK4-dependent manner[30,31]. This regulation occurs via protein kinase C (PKC), which directly phosphorylates WNK4 in two main sites, S64 and S1196, increasing WNK4 activity[32]. PKC also promotes phosphorylation of KLHL3 in a serine residue (S433) that lays in the WNK4-binding domain preventing degradation of WNK4[33]. The effects of AngII in the DCT are mediated by the AT1 receptor, a pleiotropic GPCR whose intracellular signaling mechanisms are similar to that of CaSR[34]. Both receptors are preferentially coupled to Gαq and thus activate PLC transduction pathway increasing intracellular Ca^{2+} and activating PKC[14,35]. In the present work, using a combination of *in vitro* and *in vivo* approaches, we sought to test the hypothesis that activation of CaSR modulates NCC activity through the KLHL3-WNK4-SPAK pathway.

Concise Methods

In vitro Experiments

To test the effects of CaSR on NCC activity *in vitro* we assessed NCC activity in *Xenopus laevis* oocytes by measuring tracer $^{22}Na^{+}$ uptake when CaSR was stimulated with gadolinium chloride ($GdCl_3$), as described in complete methods (Supplemental Information, SI). In mammalian cells, the effect of CaSR activation was assessed in HEK-293 cells transiently transfected with CaSR wild-type (WT), CaSR mutants, WT mWNK4-HA, hSPAK-GFP-HA, with/without KELCH3 DNA and mWNK45A-HA mutant. Cells were stimulated with the calcimimetic NPS R-568

(Tocris Biosciences) and SPAK phosphorylation, as well WNK4 abundance and phosphorylation was assessed by Western blot analysis (Complete methods, SI).

In vivo **Experiments**

To test the effect of activating CaSR to KELCH3-WNK4SPAK-NCC pathway *in vivo* we used C57BL/6 male mice 12 to 16 weeks old exposed to vehicle or NPS R-568 (Tocris Biosciences) (3.0 µg/g of weight) by oral gavage[36,37]; or furosemide (Sigma) single IP dose of 15 mg/kg. 3 h later kidneys were extracted, and proteins were prepared for Western blot (Complete methods, SI). We also used *ex vivo* kidney preparations such as the Langendorff system, as previously described[38,39]. Kidneys were perfused with vehicle or the calcimimetic, NPS R-568, at a rate of 0.60 µg/ml/min for 30 min.

Statistical Analysis

Unpaired Student's t test (two tailed) was used for comparison between two groups. One-way ANOVA with Dunnett's multiple comparison test was performed for comparison between multiple groups. $p<0.05$ was considered significant. Values are reported as mean $\pm$ SEM.

Results

CaSR activates NCC in a WNK4-dependent manner in *X. laevis* oocytes

Xenopus oocytes were co-injected with wild-type (WT) CaSR and NCC cRNA with or without WNK4 or WNK1 cRNA, and subjected to thiazide-sensitive tracer $^{22}Na^{+}$ transport assays as previously reported[40]. Co-expressing NCC with WNK4 or WNK1 promoted a marked increase in basal NCC activity, 2- and 4-fold ($p<0.001$) respectively (Figure 1 A), as previously described[41,42]. However, this increase was not affected by the presence of CaSR (Figure 1A).

Thus, unstimulated CaSR by itself had no effect on NCC activity. We then tested the effect of CaSR stimulation in the absence or presence of WNK1 or WNK4 kinases. As Figure 1B shows, after exposing oocytes to the type 1 CaSR agonist, Gd^{3+}, NCC uptake increased 2-fold ($p<0.0001$) only in oocytes co-expressing both CaSR and WNK4 (Figure 1B). We observed no effect of Gd^{3+} in oocytes injected with NCC+CaSR or NCC+WNK1+CaSR. These results suggest that, similarly to the effects of AngII[30,31], WNK4 is required for the activation of CaSR to have an effect on NCC.

CaSR phosphorylates SPAK in a WNK4-dependent manner in HEK-293 cells

To test whether the CaSR-NCC effect could also be observed in a human cell model, we analyzed the effects of activating CaSR on SPAK phosphorylation (pSPAK), as a surrogate of SPAK-NCC activation by WNKs in HEK-293 cells[24]. Cells were transiently transfected with SPAK-HA-GFP, WNK4-HA and CaSR and then treated with the calcimimetic NPS R-568 (R-568)[43-45]. Results show that R-568 induced a time- and dose- dependent pSPAK increase in cells fasted in a serum-free medium (Supplemental Figure 2A and B). We next evaluated the role of WNK4 on SPAK phosphorylation by CaSR. HEK-293 cells were transfected with SPAK-HA-GFP, CaSR and/or WNK4-HA. In cells transfected with CaSR alone, pSPAK did not increase after treatment with the calcimimetic. Only in the presence of CaSR and WNK4 together, the calcimimetic promoted a significant increase in pSPAK ($p<0.05$) (Figure 2A and B). To further test that WNK4 is required for translating CaSR activation to SPAK phosphorylation, we assessed the effect of the highly specific WNK inhibitor, WNK463[46] on CaSR-induced SPAK phosphorylation. As shown in Figure 2C and D, the positive effect of R-568 on pSPAK was completely prevented by the presence of WNK463 inhibitor, confirming that in mammalian cells the effect of CaSR is

WNK4-dependent. It is known that CaSR activation leads to activation by phosphorylation of the mitogen-activated protein kinase ERK1,2[47]. Therefore, we analyzed ERK1,2 phosphorylation to verify CaSR activation in these experiments. As shown in Figure 2A, a clear functional activation of CaSR was achieved with R-568 in CaSR-transfected cells, as demonstrated by increased ERK1,2 phosphorylation, but SPAK phosphorylation by CaSR only increases in the presence of WNK4.

An activating mutation of CaSR increases WNK4 abundance

Mutations in the CaSR gene result in Mendelian disorders characterized by altered Ca^{2+} homeostasis[48]. Activating mutations of the receptor cause autosomal dominant hypocalcemia, while inactivating mutations cause dominant familial hypocalciuric hypercalcemia or recessive neonatal severe hyperparathyroidism[15,49,50]. We used two reported mutations, one activating, CaSR-E228K, and one inactivating, CaSR-R185Q, to assess their effects on the WNK4-SPAK-NCC pathway[51-53]. We transfected HEK-293 cells with the WT CaSR or the mutants with WNK4 and observed that CaSR-E228K increased WNK4 abundance (Fig 3A and 3C). We reasoned that if CaSR was acting by the same signal transduction pathway as the AT1 receptor, the presence of KLHL3 would enhance this effect on WNK4. As expected, co-transfection of KLHL3 induced a significant decrease of WNK4 abundance (Figure 3A and B) that was prevented by CaSR-E228K, but not by CaSR-R185Q, establishing a significant KLHL3-dependent increase in WNK4 total protein levels, only in the presence of the active mutant CaSR-E228K (Figure 3D). These results are consistent with the proposal that active

CaSR may elicit the same signal transduction pathway as that of AT1R, resulting in decreased degradation of WNK4, likely due to inhibition of KLHL3[33].

CaSR promotes KLHL3 and WNK4 phosphorylation by PKC

Two previous studies have demonstrated that AngII effects on WNK4 are due to a Gαq-PKC signaling transduction pathway[32,33]. To further determine whether CaSR activation elicited similar effects, we assessed if PKC phosphorylation of KLHL3 and WNK4 occurred after CaSR activation. KLHL3-Flag was immunoprecipitated from lysates of HEK-293 cells co-transfected with CaSR WT or CaSR mutants and subjected to immunoblotting with a monoclonal antibody that recognizes PKC phosphorylation site, pRRXS[32,33,54]. In the presence of the active mutant CaSR-E228K, KLHL3 pRRXS phosphorylation remarkably increased ($p>0.01$), while this was not observed with the inactive mutant CaSR-R185Q (Figure 4A). If PKC was responsible for these effects, we would expect that inhibition of PKC would prevent CaSR-induced pRRXS increase in KLHL3. As shown in Fig 4B, bisindolylmaleimide I (BIM), used at a concentration considered to be a specific inhibitor of PKC[55], significantly reduced KLHL3 pRRXS phosphorylation.

We next evaluated if CaSR-induced activation of PKC also promoted WNK4 phosphorylation. To this end we analyzed whether activating CaSR in HEK-293 cells with R-568 promoted phosphorylation of a key WNK4 PKC phosphorylation site, serine residue S1196[32]. Following transfection of WNK4-HA, SPAK-HA-GFP and CaSR, incubation with the calcimimetic resulted in a clear increase in S1196 phosphorylation (Fig 4C). Since the experiment was done with an acute CaSR activation of 30 min, no changes were seen in total WNK4 abundance, however, activation by phosphorylation of this site has been previously established, partially explaining why we can see an effect before WNK4 abundance increases. Furthermore, we used a WNK4

mutant that has all five serines of the PKC consensus sites (RRXS sites) mutated to alanines (WNK4-5A), which prevents PKC-induced phosphorylation[32]. The 5A mutation did not alter WNK4 abundance, but remarkably reduced CaSR effect on SPAK phosphorylation (Figure 4D), suggesting that phosphorylation of these sites, and the consequent activation of WNK4 by PKC, is necessary for the complete effect of CaSR on the WNK4-SPAK pathway.

CaSR promotes NCC phosphorylation *in vivo*

To define whether the CaSR effect on NCC occurred *in vivo*, we administered C57/BL6 male wild-type (WT) mice with an acute oral treatment of R-568 (3 µg/g of body weight) [36,37] and 3 h later, mice were euthanized to investigate the effects on NCC phosphorylation by immunoblotting. Calcimimetics directly target the TALH CaSR function[12], thereby decreasing NKCC2 activity (hence, phosphorylation) and promoting increased luminal Ca^{2+} and NaCl delivery to the distal nephron[12]. To test if this effect occurred in our *in vivo* model we assessed NKCC2 phosphorylation after the administration of the calcimimetic. Figure 5A and B shows that mice treated with the calcimimetic exhibited a significant decrease of NKCC2 phosphorylation.

Activation of NCC is associated with increased phosphorylation of three residues, T55, T60 and S73 in human NCC[25,56], therefore, phosphorylation of any of these residues has been extensively used as surrogate of NCC activation[56]. As expected, treatment with the calcimimetic induced a 1.5-fold increase in NCC phosphorylation ($p<0.05$) (Figure 5C and D), without promoting changes in total NCC (NCC/β-actin 1.00 vs. 0.96531, p=NS). Moreover, in concordance with our *in vitro* data, activation of CaSR resulted in a significant increase in total WNK4 protein (1.7-fold increase, $p<0.05$) (Figure 5C and D). To evaluate if the increased WNK4

protein was activated by PKC, we analyzed the phosphorylation of residue S64, as previously reported[32]. We found that most of the WNK4 protein in the calcimimetic-administered group was phosphorylated in S64 (Figure 5C). However, the pS64/WNK4 ratio between vehicle and R-568 groups remained similar (pS64/WNK4 1.00 vs. 1.3050, p=NS). The absence of significance between the vehicle- and R-568- administered groups could be due to the concurrent increase in WNK4 protein. Additionally, immunofluorescence microscopy of kidneys extracted from wild-type mice showed increased membrane abundance after an acute dose of the calcimimetic (Figure 5E). Interestingly, the increase in NCC phosphorylation was not present in a knock-in mice in which SPAK cannot be activated by WNKs (mutation S243A)[57] (pNCC/NCC 1.00 vs 0.99, p=NS) (Figure 5F and G).

CaSR is expressed at both the apical and basolateral membranes of DCT cells. To investigate if increasing Ca^{2+} delivery to the DCT, and therefore, only activation of the apical CaSR is sufficient to elicit NCC phosphorylation, we administered C57/BL6 male WT mice with an acute treatment of furosemide (15 mg/kg over 3h), as previously described[58]. This specific dosage and short time of treatment has been described to increase Ca^{2+} and NaCl delivery to the DCT, without promoting dehydration[58]. No changes in plasma potassium after 3 h were observed (vehicle 4.3 ± 73 vs. furosemide 4.3 ± 0.25, p=NS). As expected, furosemide administration increased NCC phosphorylation 4-fold ($p<0.05$) while not increasing total NCC (NCC/β-actin 1.00 vs 0.9456, p=NS) (Figure 6A and B). In addition, furosemide administration was associated with increased WNK4 total protein and increased phosphorylation of WNK4 at S64 (Figure 6C and D). Taken together, these results suggest that the acute inhibition of NKCC2 is associated with increased WNK4-NCC phosphorylation that was probably triggered by increased luminal Ca^{2+}.

CaSR promotes NCC phosphorylation *ex vivo*

The administration of the calcimimetic in the previous experiments could have promoted NCC activation either by a direct effect on the kidney, through the mechanism proposed in our hypothesis, or by a secondary effect due to activation/modification of any of the multiple hormonal systems that can activate NCC[59]. Acute calcimimetic administration is associated with decreased activity of the renin-angiotensin II system[60,61], making this possibility unlikely. Nevertheless, we studied NCC phosphorylation using an *ex vivo* system where intervention of the central nervous system and other extra renal hormonal systems are not expected to be present. Kidneys of WT male Wistar rats were perfused with physiological saline with vehicle or with R-568 (0.60 µg/ml/min). The concentration of R-568 used in these experiments did not change the perfusion pressure, arguing against the presence of an intrarenal AngII effect. As shown in Figure 7 A and B, NCC and SPAK phosphorylation levels were significantly higher in kidneys perfused with the calcimimetic.

Discussion

In the present study, we show that CaSR activation is associated with increased NCC activity *in vitro* and *in vivo*. This increase involves PKC activation of the WNK4-SPAK pathway, supporting the hypothesis that CaSR modulates NCC activity. As previously shown for AngII, modulation of NCC via WNK4-SPAK occurs by two different pathways. Phosphorylation and concurrent activation of WNK4, and prevention of WNK4 degradation by KELCH3 phosphorylation. CaSR induced activation of NCC has an implication in the physiological response to increased extracellular Ca^{2+}, which requires the kidney to promote its excretion at the apparent expense of reducing NaCl reabsorption in the TALH, thus increasing the

delivery of NaCl and Ca^{2+} to the distal nephron[14]. Integration of NaCl and Ca^{2+} homeostasis by CaSR in the DCT could prevent unwanted NaCl loss, while further permitting Ca^{2+} excretion. In this regard, CaSR expression in the apical membrane of the DCT has been clearly established by many groups and recent studies co-localize CaSR with NCC in human and mouse kidneys[5,22]. Taking together the observations in this study, we propose the existence of a mechanism in the DCT, where apical CaSR responds to increased intratubular Ca^{2+} concentration evoking a CaSR-Gαq-PKC-WNK4-SPAK signaling transduction pathway that promotes NCC activation to recover the NaCl that was not reabsorbed in the TALH, due to NKCC2 and ROMK inhibition (Fig 8). Because it is known that increased NaCl reabsorption in the DCT is associated with decreased Ca^{2+} absorption[14], this mechanism not only claims the NaCl, but also further promotes hypercalciuria. The controversy of whether the thiazide effect on Ca^{2+} excretion occurs directly in the DCT or elsewhere[62,63] does not contradict our findings.

We are aware of the possibility that the basolateral CaSR in DCT may also elicit a response to activate NCC, and our results do not rule out this possibility. In this scenario, increased extracellular Ca^{2+} could simultaneously reduce NKCC2 activity in the TALH but increase NCC activity in the DCT, by activating the basolateral receptor in both segments. However, due to the presence of CaSR and NCC in the apical membrane, is it likely that luminal Ca^{2+} is also involved in this response. NCC activation elicited by a single acute dose of furosemide, known to promote increased Ca^{2+} delivery to DCT, supports the fact that activation of apical CaSR is enough to provoke the proposed response. It is also worth mentioning that patients with Autosomal Dominant Hypocalcemia (due to CaSR activating mutations) may exhibit a Bartter-like syndrome (also known as Bartter syndrome type V) that has been described as mild in

most patients[17,64,65]. Perhaps, CaSR activation in the DCT helps to reduce natriuresis, as compared with other types of Bartter syndrome.

A similar mechanism prompted by CaSR in the nephron has been described before. It has been clinically recognized for many years that hypercalcemia induces polyuria[66,67]. Increasing urinary Ca^{2+} to the distal nephron could also promote precipitation of Ca^{2+} and phosphate salts. *Sands et al.* elegantly demonstrated that apical CaSR in the collecting duct responds to increased luminal Ca^{2+} to blunt vasopressin-induced insertion of AQP-2 water channels into the apical membrane[68]. The latter would prevent water reabsorption in the collecting duct, allowing the urine to be diluted and thus preventing Ca^{2+} precipitation and formation of renal stones. The authors also demonstrated that the signaling pathway and molecular mechanisms initiated by CaSR was also by Gαq and PKC proteins[68]. More recently, other groups have further established the association of active apical CaSR with decreased AQP2 abundance[69-71].

The observation that CaSR activation modulates NCC activity via WNK4-SPAK pathway may have further implications beyond the physiological mechanism of how NaCl is recovered in DCT when TALH NaCl reabsorption is decreased by Ca^{2+}. First, it is known that arterial hypertension is highly prevalent in primary hyperparathyroidism, ranging from 40 to 65%, which is much higher than the expected 25 to 30% of hypertension in general adult population[72]. Given our observations, a possible mechanism could be that increased Ca^{2+} in the tubular fluid, as it occurs in hypercalcemia, stimulates the activity of NCC promoting NaCl reabsorption and hence, the development of hypertension. Second, it has been recently demonstrated that glucose and other sugars act as type II calcimimetics, enhancing CaSR

affinity for Ca^{2+}[73]. This could be relevant in the apical membrane of the DCT since all the filtered glucose is reabsorbed in the proximal tubule and therefore these cells are not continuously exposed to glucose. In diabetic patients, the excess of filtered glucose often escapes reabsorption in the proximal tubule, allowing a significant amount of glucose in the tubular fluid that reaches the DCT. It is possible that the presence of glucose acting as a calcimimetic increases apical CaSR sensibility, enhancing NCC activity of thus NaCl reabsorption, which could help to explain the higher prevalence of hypertension in patients with diabetes[74]. These possibilities are speculative but can certainly be explored in future studies.

Authors contribution

S B-V, L R-V, M C-B, D H E, D R, N A B, and GG designed the study, planned experiments, interpreted data and edited the manuscript. S B-V, L R-V, J B-C, R B, L C-P, N V, A M-O, C P, L G-M performed experiments and reviewed the manuscript. S B-V and GG wrote the paper.

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Figure Legends

Fig. 1. CaSR activates NCC in a WNK4-dependent manner in *X. laevis* oocytes. A. The presence of none activated CaSR has no effect on WNK4 or WNK1-induced activation of NCC. Functional expression assay shows the thiazide-sensitive Na^+ uptake in groups of oocytes injected with NCC, NCC + hWNK4, NCC + WNK1D11 cRNA (black bars) or together with CaSR cRNA (grey bars), as stated. Uptake in oocytes injected with NCC cRNA alone was arbitrarily set to 100% and the corresponding groups were normalized accordingly. **p<0.005 vs. NCC. **B.** Activation of CaSR with GdCl3 increased the activity of NCC only in the presence of WNK4. Uptake was performed in control conditions (black bars) or after stimulation with $GdCl_3$ 80 µM for 15 min. Each group in control conditions (black bar) was arbitrarily set to 100% and the corresponding group with $GdCl_3$ was normalized accordingly (gray bars). ***p<0.0005 vs. its own control. Supplemental Figure 1 shows the same experiments but with data expressed as pmol/oocyte/h.

Fig. 2. CaSR phosphorylates SPAK in a WNK4-dependent manner in HEK-293 cells. A. Representative immunoblot of cells transfected with hSPAK-GFP-HA, mWNK4-HA and hCaSR in different combinations as stated. The day before the experiment cells were serum-starved in the normal growth medium and left overnight. The next day, cells were stimulated with R-568 (200 nM) for 30 min. **B.** Densitometric analysis of A. SPAK transfection alone in control conditions was arbitrarily set to 1 and the corresponding groups were normalized accordingly. Bars represent mean $\pm$ S.E.M, of at least 3 independent experiments. *p<0.05 vs. control. **C.** Representative immunoblot showing two experiments of cells transected with empty vector (EMPTY), hSPAK-GFP-HA, mWNK4-HA and hCaSR and treated as in A. The WNK inhibitor WNK463 was added to the medium for 2 h on the day of the experiment to a final concentration of 4 µM. **D.** Densitometric analysis of C. SPAK in control conditions was arbitrarily set to 1 and the corresponding groups were normalized accordingly. Bars represent mean $\pm$ S.E.M, of at least 3 independent experiments. *p<0.05 vs. Control (no stimulation with R-568 and no WNK463). ***p<0.005 vs. R-568.

Fig. 3. An activating mutation of CaSR phosphorylates SPAK and increases WNK4 abundance. A. Representative immunoblot of HEK-293 cells transfected with mWNK4-HA, hCaSR WT and CaSR mutants with or without *Klhl3* DNA (40 ng). For this set of experiments, cells were maintained in normal growth medium after transfection. **B.** Densitometric analysis of A. where the expression of WNK4 alone (WNK4) was set to 1 and the rest of the groups were normalized accordingly. Bars represent mean $\pm$ S.E.M, of at least 3 independent experiments.***p<0.0005 and **p<0.05 vs. WNK4. **C.** and **D.** Densitometric analysis where WNK4 (Control) without KLHL3 cotransfection (C.) or with KLHL3 (D.) were set to 1 and the

rest of the groups were normalized accordingly. Bars depict mean ± S.E.M, of at least 3 independent experiments ***p<0.0005 vs. WNK4+KLHL3 (Control of D.).

Fig. 4. CaSR promotes KLHL3 and WNK4 phosphorylation by PKC. A. Representative immunoblot of immunopurified KLHL3-Flag from HEK-293 cells transfected with KLHL3, WT hCaSR and CaSR mutants. Cells were maintained in normal growth medium after transfection. Graph depicts densitometric analysis of at least 3 independent experiments. KLHL3 immunopurified from transfection alone (Control) was set as 1 and the rest of the groups were normalized accordingly. Bars represent mean ± S.E.M. **p<0.005 vs. Control. **B.** Representative image of immunopurified KLHL3-Flag from HEK-293 cells transfected with KLHL3, CaSR-E228K and treated with a PKC inhibitor (BIM). 4 µM of BIM was added to the normal growth medium and left overnight. The next day, cells were lysed and immunoblotted. Graph shows densitometric analysis of 3 experiments. Bars represent mean ± S.E.M. *p<0.05 vs KLHL3 CaSR-E228K without BIM. **C.** Representative immunoblot of cells transfected with SPAK-GFP-HA, mWNK4-HA and WT hCaSR, serum-starved and stimulated with R-568 (200 nM) for 30 min. Lysates were blotted with the indicated antibodies. The graph depicts densitometric analysis. *p<0.05 vs. Control (no stimulation with R-568). **D.** Cells were transfected with SPAK-GFP-HA, mWNK4-HA and WT hCaSR or the mutant mWNK45A, which has all PKC-phosphorylation sites mutated to alanines, and then stimulated as in C. The graph represents densitometric analysis of at least 3 independent experiments for the mWNK45A mutant. Bars are mean ± S.E.M. ***p<0.0005 vs. its own control (Data for SPAK-mWNK4-CaSR are shared with Figure 2 D).

Fig. 5. CaSR promotes NCC phosphorylation *in vivo.* Animals were administered with vehicle or with R-568 3 µg/g of body weight through oral gavage. 3 h later, kidneys were harvested and processed for immunoblot. Each column of the representative immunoblot represents the kidneys from one animal. **A and C.** Representative immunoblot of the effect of oral R-568 administration on NCC and NKCC2 phosphorylation, WNK4 abundance and phosphorylation in S64 in WT mice (Upper image). pS64/WNK4 1.00 vs. 1.3050, p=NS. **E.** Immunofluorescent staining of kidney sections from WT mice treated with Vehicle or R-568. (Scale bars, 20 µm). **F.** Representative immunoblot of the effect of R-568 on NCC phosphorylation in SPAK knock-in mice ($SPAK^{243A/243A}$). **B., D. and G.** Densitometric analysis of representative immunoblots. Bars represent mean ± S.E.M. *p<0.05 vs. Vehicle.

Fig. 6. An acute furosemide treatment promotes NCC phosphorylation *in vivo.* Animals were administered with vehicle or with furosemide 15 mg/kg of body weight through IP injection. 3 h later, kidneys were harvested and processed for immunoblot. Each column of the

representative immunoblot represents the kidney from one animal. **A. and C.** Representative immunoblots of the effect of the acute administration of furosemide on NCC phosphorylation, WNK4 abundance and phosphorylation in S64 in WT mice. pS64/WNK4 1.00 vs. 1.53, p=NS. **B. and D.** Densitometric analysis of n=8 controls and n=7 furosemide administered mice. Bars represent mean ± S.E.M *p<0.05 vs. Vehicle.

Fig. 7. CaSR promotes NCC phosphorylation *ex vivo.* A. Representative immunoblot of protein extracts from *ex vivo* perfused rat kidneys. The kidneys were perfused with physiological saline with vehicle or with R-568 at a rate of 0.60 µg/ml/min. Each column of the immunoblot represents one kidney. **B.** Bars represent mean ± S.E.M of the densitometric analysis of A. n=6 Vehicles and n=7 R-568. **p<0.005 vs. vehicle. *p<0.05 vs. vehicle.

Fig. 8. Proposed model for CaSR effect on NCC*.* Increased extracellular Ca^{2+} leads to CaSR-mediated inhibition of NKCC2 and ROMK, halting the transepithelial voltage difference that drags paracellular reabsorption of Ca^{2+} ions. Reduction in Ca^{2+} reabsorption in the TALH causes increased NaCl and Ca^{2+} delivery to the distal nephron. In the DCT, integration of calcium and NaCl homeostasis by the CaSR must respond to prevent unwanted NaCl loss. We propose the existence of a mechanism in the DCT where apically expressed CaSR responds to increased intratubular Ca^{2+} concentration evoking a CaSR-Gαq-PKC-WNK4 signaling transduction pathway that promotes NCC activation.