Differential parathyroid and kidney Ca^{2+}-sensing receptor activation in autosomal dominant hypocalcemia 1

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Summary

Background Parathyroid Ca^{2+}-sensing receptor (CaSR) activation inhibits parathyroid hormone (PTH) release, while activation of renal CaSRs attenuates Ca^{2+} transport and increases expression of the pore-blocking claudin-14. Patients with autosomal dominant hypocalcemia 1 (ADH1), due to activating *CASR* mutations, exhibit hypocalcemia but not always hypercalciuria (elevated Ca^{2+} in urine). The latter promotes nephrocalcinosis and renal insufficiency. Although CaSRs throughout the body including the kidney harbor activating *CASR* mutations, it is not understood why only some ADH1 patients display hypercalciuria.

Methods Activation of the CaSR was studied in mouse models and a ADH1 patient. *In vitro* CaSR activation was studied in HEK293 cells.

Findings *Cldn14* showed blood Ca^{2+} concentration-dependent regulation, which was absent in mice with kidney-specific *Casr* deletion, indicating *Cldn14* is a suitable marker for chronic CaSR activation in the kidney. Mice with a gain-of-function mutation in the *Casr* (Nuf) were hypocalcemic with low plasma PTH levels. However, renal CaSRs were not activated at baseline but only after normalizing blood Ca^{2+} levels. Similarly, significant hypercalciuria was not observed in a ADH1 patient until blood Ca^{2+} was normalized. *In vitro* experiments indicate that increased CaSR expression in the parathyroid relative to the kidney could contribute to tissue-specific CaSR activation thresholds.

Interpretation Our findings suggest that parathyroid CaSR overactivity can reduce plasma Ca^{2+} to levels insufficient to activate renal CaSRs, even when an activating mutation is present. These findings identify a conceptually new mechanism of CaSR-dependent Ca^{2+} balance regulation that aid in explaining the spectrum of hypercalciuria in ADH1 patients.

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Keywords: Hypercalciuria; CaSR; Kidney; Claudin-14; ADH1 case report

Abbreviations: ADH1, autosomal dominant hypocalcemia type 1; PTH, parathyroid hormone; CaSR, Ca^{2+}-sensing receptor; CLDN14, claudin-14

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Research in context

Evidence before this study

The calcium-sensing receptor (CaSR) is a key regulator of systemic calcium balance. Parathyroid CaSR activation reduces parathyroid hormone (PTH) secretion and activation of the renal CaSR reduces tubular calcium reabsorption. Patients with activating *CASR* mutations exhibit autosomal dominant hypocalcemia type 1 (ADH1), a disease characterized by low blood calcium concentrations and inappropriately low serum PTH concentrations, consistent with overactivation of the parathyroid CaSR. Intriguingly, despite robust CaSR expression in the kidney, increased urinary calcium excretion is sometimes, but not always observed in ADH1 patients. Importantly, calcium wasting in these patients can, if persistent, cause kidney stone formation, renal calcium deposits and kidney disease. This complicates clinical management. Although CaSRs throughout the body are activated by increased extracellular calcium concentrations, why ADH1 patients have reduced PTH but not always urinary calcium wasting is not clear, despite abundant CaSR expression in both the kidney and the parathyroid. It is generally assumed that signaling via CaSRs in ADH1 patients carrying gain-of-function CaSR mutations is increased both in the parathyroid and in the kidney.

Added value of this study

In the present study, we combine investigations in a preclinical mouse model of ADH1 with *in vitro* experiments and data from a patient case. In a gain-of-function CaSR mouse model and in mice treated with specific compounds to activate the CaSR, we show that renal CaSRs are not activated at baseline in contrast to what would be expected. We provide evidence from this mouse model that this occurs because CaSR overactivity in the parathyroid gland reduces PTH and circulating serum calcium levels, which is insufficient to activate the renal CaSR. Mechanistically this may occur, at least in part, due to a higher level of CaSR expression in the parathyroid relative to the kidney.

Implications of all the available evidence

Our findings suggest an intricate interplay between parathyroid and renal CaSRs and identify a conceptually new mechanism for CaSR regulated maintenance of calcium homeostasis. Furthermore, these findings are of relevance for understanding why renal calcium wasting is not consistently observed in all patients with ADH1 and may aid in their clinical management.

Introduction

Plasma ionized calcium (Ca^{2+}) is kept within a narrow concentration range of 1.10–1.35 mM (4.4–5.4 mg/dL).[1] Ca^{2+} activates the Ca^{2+}- sensing receptor (CaSR) in the parathyroid, thereby reducing the release of parathyroid hormone (PTH) which helps reduce plasma Ca^{2+} concentration.[2–4] Mechanistically this occurs as PTH is a key stimulator of Ca^{2+} reabsorption from the renal tubule, resorption from bone, and a main driver of active vitamin D metabolite $1,25(OH)_2D_3$ formation. $1,25(OH)_2D_3$ itself is a potent stimulator of intestinal Ca^{2+} absorption and increases renal tubular Ca^{2+} reabsorption and bone resorption.[1]

In addition to regulating PTH release, local CaSR activation in the kidney and intestine regulates Ca^{2+} transport through a direct response to changes in plasma Ca^{2+} levels.[1,5–7] In the kidney, the CaSR is expressed in select segments of the tubular system, with the highest expression on the basolateral membrane of the thick ascending limb of Henle's loop (TAL) and expressed at markedly lower levels at more distal sites and the early collecting system.[6] Inhibition of the CaSR in isolated microperfused TAL tubules increases paracellular Ca^{2+} reabsorption by increasing Ca^{2+} permeability of the TAL.[6] This suggests that the CaSR plays an important role in the regulation Ca^{2+} transport across this segment. While the direct mechanisms mediating these acute actions remains to be fully investigated, chronic CaSR activation is well known to increase expression of the pore-blocking claudin-14 (CLDN14), which is expressed at low levels under basal conditions.[8,9] In fact, CaSR activation in the kidney plays a crucial role in the defense against hypercalcemia.[10] *Cldn14* expression is greatly increased by chronic activation of the CaSR during chronic hypercalcemia,[8,9] while smaller changes in *Cldn14* expression are observed with increased dietary Ca^{2+} content.[8,11,12] siRNA-mediated *Casr* knockdown attenuates Ca^{2+}-dependent increases in CLDN14 expression *in vitro*,[11] suggesting that *Cldn14* expression increases with chronic CaSR activation.

Gain-of-function mutations in the *CASR* cause autosomal dominant hypocalcemia type 1 (ADH1). ADH1 is characterized by hypocalcemia with inappropriately low plasma PTH concentrations.[13,14] Frank hypercalciuria is not always encountered at presentation but occurs commonly after treatment.[13–18] In a study with nine ADH1 patients, only one was hypercalciuric before treatment.[18] In another report, two out of eleven patients were hypercalciuric at presentation.[14] This suggest that the majority of ADH1 patients do not have frank hypercalciuria at presentation. Hypercalciuria increases the risk of nephrolithiasis, nephrocalcinosis and thus chronic kidney disease in patients with ADH1.[19] It is not clear why some ADH1 patients present with reduced PTH levels which are indicative of CaSR activation, but not hypercalciuria. Furthermore, in a large family with ADH1, all having the same mutation, urinary Ca^{2+} excretion tended to be highest in those receiving vitamin D treatment to correct hypocalcemia.[20] Treatment of hypocalcemia is avoided in asymptomatic patients to prevent hypercalciuria and related sequale.[13] As the activating

mutation of the CaSR would be present in the kidney as well as the parathyroid, why these patients do not always have hypercalciuria is unclear.

We therefore studied the activation of the CaSR in a single mouse model of ADH1 (Nuf) and after administration of the calcimimetic, etelcalcetide hydrochloride.[21–23] We first validated renal *Cldn14* expression as a marker of renal CaSR activation by showing that *Cldn14* abundance time- and concentration-dependently increased with chronic changes in serum Ca^{2+}. This regulation was absent in hypercalcemic animals with targeted ablation of the *Casr* in the kidney. Surprisingly, we found that renal *Cldn14* expression was unchanged in Nuf mice, despite an activating CaSR mutation. However, when plasma Ca^{2+} levels were increased in Nuf mice, but still lower than wild-type animals, increased renal *Cldn14* expression was observed, indicating that chronic activation of the renal CaSR is not present until blood Ca^{2+} is elevated to sufficient levels to activate the receptor. Overall, our findings from this model delineate tissue-specific CaSR thresholds, where CaSR activity in the parathyroid reduces PTH secretion and hence circulating Ca^{2+} levels, which then are insufficient to activate renal CaSRs in the chronic setting, even when containing an activating mutation. These findings suggest a new mechanism of regulated Ca^{2+} balance by the interplay between parathyroid and renal CaSRs and may aid in the management of ADH1 patients and hypercalciuria-related sequelae.

Methods

Animal experiments

Nuf mice (C3;102-$Casr^{Nuf}$/H; RRID:MGI:3603347), which harbor an activating mutation (L723Q) in the fourth transmembrane region of the CaSR, have been described previously and were obtained through MRC Harwell via the European Mouse Mutant Archive.[23] Floxed $Casr^{tm1Mrpk}$/J mice[10] (RRID:IMSR_JAX:030647) and the Ksp-Cre driver line (B6.Cg-Tg(Cdh16-cre)91Igr/J) (RRID:IMSR_JAX:012237)[24] were both obtained via JAX and crossed to generate animals with CaSR deletion specifically in kidney.

Experimental protocol 1. Experiments were performed on 30 male WT C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France; 12 weeks old). Animals were divided randomly into groups receiving the control diet or a diet enriched with dihydrotachysterol (DHT; dihydrotachysterol (Sigma-Aldrich, St. Louis, MO, USA)), a synthetic vitamin D analog that is activated in the liver independently of renal hydroxylation.[25] The following dosing strategies were applied (n = 5 per group): 0.4% w/v DHT (per 100 g of standard rodent food) for 3 days, 0.08% w/v DHT for 3 days, 0.04% w/v DHT for 3 days, 0.4% w/v DHT for 2 days or 0.4% w/v DHT for 1 day or vehicle controls. The standard rodent maintenance diet contained 0.2% Mg^{2+}, 0.7% Ca^{2+} and 600 IU vitamin D3 (TD.00374; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany); animals had *ad libitum* access to food and drinking water. Animals were subsequently anesthetized with 1.5% isoflurane (Nicholas Piramal Ltd., London, UK) and blood was drawn from the vena cava. Mice were then euthanized by cervical dislocation and kidneys were isolated as previously.[26] Data are shown in Figure 1.

Experimental protocol 2. Sex and age-matched wild type (WT) (n = 4; 3 males and 1 female) and kidney-specific $Casr^{-/-}$ (n = 7; 5 males and 2 females) mice from the same litter (aged 12 weeks) were placed in individual metabolic cages in a temperature-controlled (25 °C) environment with *ad libitum* access to food and drinking water. After acclimation, all animals were switched to 0.4% w/v dietary DHT administration for 3 days as described above. Mice were subsequently anesthetized and euthanized as described in protocol 1. Data are shown in Figure 1.

Experimental protocol 3. Experiments were performed on sex-matched and age-matched (average age: 10 weeks) WT (n = 9 animals, 4 males and 5 females), heterozygous (Nuf/+) (n = 15 animals, 7 males and 8 females) and homozygous (Nuf/Nuf) (n = 10 animals, 7 males and 3 females). All animals were transferred to individual metabolic cages with *ad libitum* access to the standard rodent maintenance diet described above and drinking water. After acclimatization for 3 days, 24 h urine was collected. Animals were subsequently anesthetized and euthanized as described in protocol 1. Data are shown in Figure 2.

Experimental protocol 4. 8 WT (4 males, 4 females) and 8 Nuf/+ (4 males and 4 female) mice (age 16–17 weeks) were placed on a 2%-supplemented Ca^{2+} diet (Envigo, Indianapolis, IN, USA) for 7 days. First, they received the diet in their standard cages for 3 days and were subsequently transferred to individual metabolic cages, where they continued receiving the high Ca^{2+} diet. All animals had *ad libitum* access to food and water and were housed in a controlled environment, as described above. After 3 days of acclimatization, 24 h urine was collected. Mice were subsequently anesthetized and euthanized under 1.5% isoflurane as described in protocol 1. Data are shown in Figure S2.

Experimental protocol 5. 8 WT (4 males, 4 females) and 7 Nuf/+ (3 males, 4 females) mice (age 16–17 weeks) were housed in individual metabolic cages as

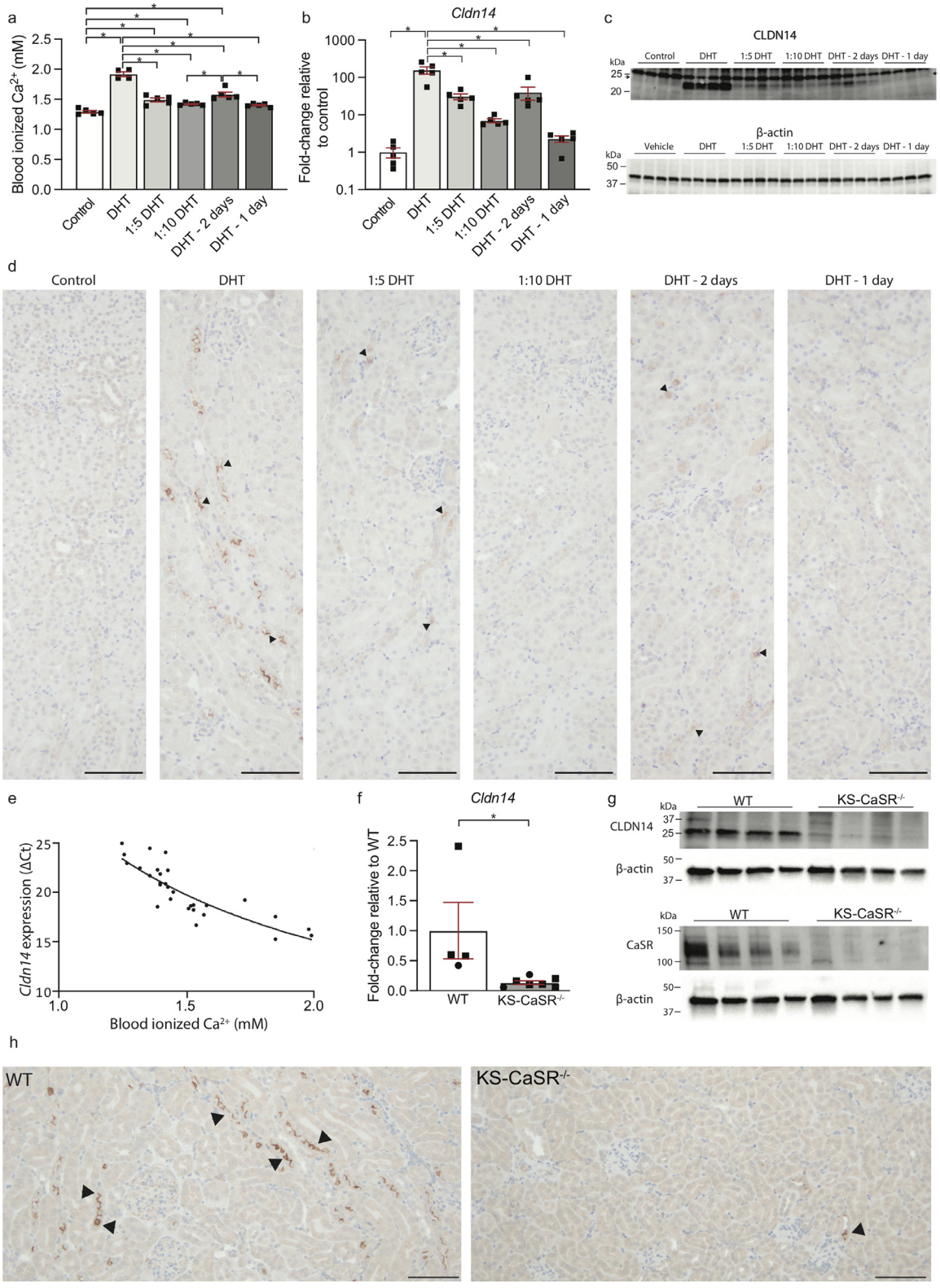

Figure 1. Renal *Cldn14* expression is dose-dependently regulated by blood Ca^{2+} levels via the CaSR. (a) With the exception of dietary DHT administration of 0.4% w/v DHT for 1 day, all DHT treatment groups exhibited increased blood Ca^{2+} levels compared to control

described above. After acclimatization for 3 days, baseline 24 h urine was collected. All mice were then switched to a diet supplemented with 0.4% w/v DHT as describe before for 3 days; 24 h urine was collected for all days.[25] Mice were anesthetized and euthanized as described above. Data are shown in Figure 3.

Experimental protocol 6. 5 WT and 7 Nuf/+ female mice (age 8–11 weeks) were used in the experiment. To increase plasma Ca^{2+} towards normal levels in Nuf mice, only Nuf/+ mice were placed on 0.04% w/v DHT-containing diet for 7 days, whereas WT mice received standard diet. Mice were first maintained in regular cages, then placed in individual metabolic cages for acclimatization for 3 days, after which 24 h urine was collected on the 7th day. Mice were anesthetized and euthanized as above. Data are shown in Figure 4.

Experimental protocol 7. 18 C57BL7/6J male mice (average age 10 weeks) were randomly divided into three groups with a similar average body weight. One group of animals served as controls, one group received infusion with 5 mg/ml etelcalcetide hydrochloride (Parsabiv; Amgen) via Alzet osmotic 2002 minipumps (Alzet, Cupertino, CA, USA) with a delivery rate of 0.5 µl/h (i.e. 2.5 µg/h), and one group received infusion with 20 mg/ml etelcalcetide hydrochloride via Alzet osmotic 2001 minipumps (Durect Corporation, Cupertino, CA, USA) with a delivery rate of 1.0 µl/h (e.g. 20 µg/h). Three days after insertion of minipumps, animals were housed in individual metabolic cages. After acclimatization for 3 days, baseline 24 h urine was collected on day 7. Mice were anesthetized and euthanized as above. Data are shown in Figure 5.

Biochemical measurements

Blood ionized Ca^{2+} concentrations were measured by means of an ABL835 FLEX analyzer (Radiometer, Copenhagen, Denmark). Plasma was isolated from the remaining blood by centrifuging at 4500 g for 10 min at 4 °C and subsequently stored at −80 °C for further analyses. Urine Ca^{2+} concentrations were measured using a colorimetric assay.[27] Plasma and urine creatinine concentrations were measured using an ABX Pentra Creatinine 120 CP kit (HORIBA ABX SAS, Montpellier, France) on a Microlab 300 analyzer (ELITechGroup, Puteaux, France), according to the manufacturer's protocol. For experimental protocol 6 and 7, plasma and urine creatinine concentrations were measured by the Department of Clinical Biochemistry and Pharmacology of Odense University Hospital. Intact plasma parathyroid hormone (PTH) levels were determined with a mouse PTH 1–84 ELISA kit (Immutopics International, San Clemente, CA).[8]

Fractional excretion of Ca^{2+}, the percentage of filtered Ca^{2+} that is excreted in the urine, was calculated as: $\frac{[\text{Creatinine}]_{\text{plasma}} \times [Ca^{2+}]_{\text{urine}}}{[Ca^{2+}]_{\text{ionized,blood}} \times [\text{Creatinine}]_{\text{urine}}} \times 100\%$[28].

RNA extraction and semi-quantitative PCR

Kidneys were snap-frozen in liquid nitrogen after isolation and stored at −80 °C for future RNA extraction. RNA was extracted from the tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and processed as previously.[26,29] In brief, after DNAse treatment (Invitrogen, Carlsbad, CA, USA), cDNA synthesis was performed using the SensiFAST cDNA Synthesis Kit (Bioline, London, UK). Then, qPCR was performed in triplicate for each sample on the QuantStudio 6 Pro Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using TaqMan Master Mix (Applied Biosystems, Waltham, MA, USA) and specific probes and primers (Table S1). Probes and primers were obtained from Applied Biosystems, Foster City, CA. For gene expression in the experiments employing the Nuf mice, analysis was performed using the $2^{-\Delta\Delta Ct}$ approach with *18S* rRNA as a reference gene. For the experiments with etelcalcetide hydrochloride, *Gapdh* was used as a

mice in a dose-dependent manner (n = 4–5). $P < 0.0001$ for vehicle vs DHT, vehicle vs DHT − 2 days, DHT vs 1:5 DHT, DHT vs 1:10 DHT, DHT vs DHT − 2 days and DHT vs DHT − 1 day; $P = 0.0004$ for vehicle vs 1:5 DHT; $P = 0.0301$ for vehicle vs 1:10 DHT; $P = 0.0050$ for 1:10 DHT vs DHT − 2 days; $P = 0.0014$ for DHT − 2 days vs DHT − 1 day. (b) Renal *Cldn14* expression was significantly increased in animals treated with 0.4% w/v DHT for 3 days compared to control animals (n = 5). $P < 0.0001$ for vehicle vs DHT, DHT vs 1:5 DHT, DHT vs 1:10 DHT and DHT vs DHT − 1 day; $P = 0.0002$ for DHT vs DHT − 2 days. (c) Western blotting for CLDN14 showed that CLDN14 protein expression in the kidney was highly abundant in animals treated with 0.4% w/v DHT for 3 days and to a lesser extent in the other treatment groups. Indicated by arrow. Note an unspecific band is also present above. β-actin confirmed equal loading (n = 4). (d) Immunohistochemical staining of kidney sections for CLDN14 as indicated by arrows. 3-day 0.4% w/v DHT-treatment increased CLDN14 expression in TAL compared to control animals. Scale bar indicates 200 µm (e) Correlation between blood Ca^{2+} levels and ΔCt values for *Cldn14* (calculated as *Cldn14* Ct values − *18S* Ct values) using a power trend line showed lower ΔCt values for *Cldn14*, and thus higher *Cldn14* expression, at higher blood Ca^{2+} levels. R^2=0.73. (f) After a 3-day 0.4% w/v DHT containing diet, *Cldn14* expression was significantly lower in kidney-specific *Casr*$^{-/-}$ (KS-*Casr*$^{-/-}$) mice compared to WT mice (n = 4–7). $P = 0.0326$. (g) CLDN14 and CaSR protein expression was detectable by western blotting in DHT-treated WT mice, but not in DHT-treated KS-*Casr*$^{-/-}$ mice. β-actin confirmed equal loading (n = 4). (h) CLDN14 expression increased in the TAL of WT mice treated with 0.4% w/v DHT for 3 days, but not in TALs of KS-*Casr*$^{-/-}$ mice. Scale bar indicates 100 µm. DHT, 0.4% w/v DHT for 3 days; 1:5 DHT, 0.08% w/v DHT for 3 days; 1:10 DHT, 0.04% w/v DHT for 3 days. Data are shown as mean ± SEM. *$P < 0.05$ by one-way ANOVA with Tukey's post hoc test.

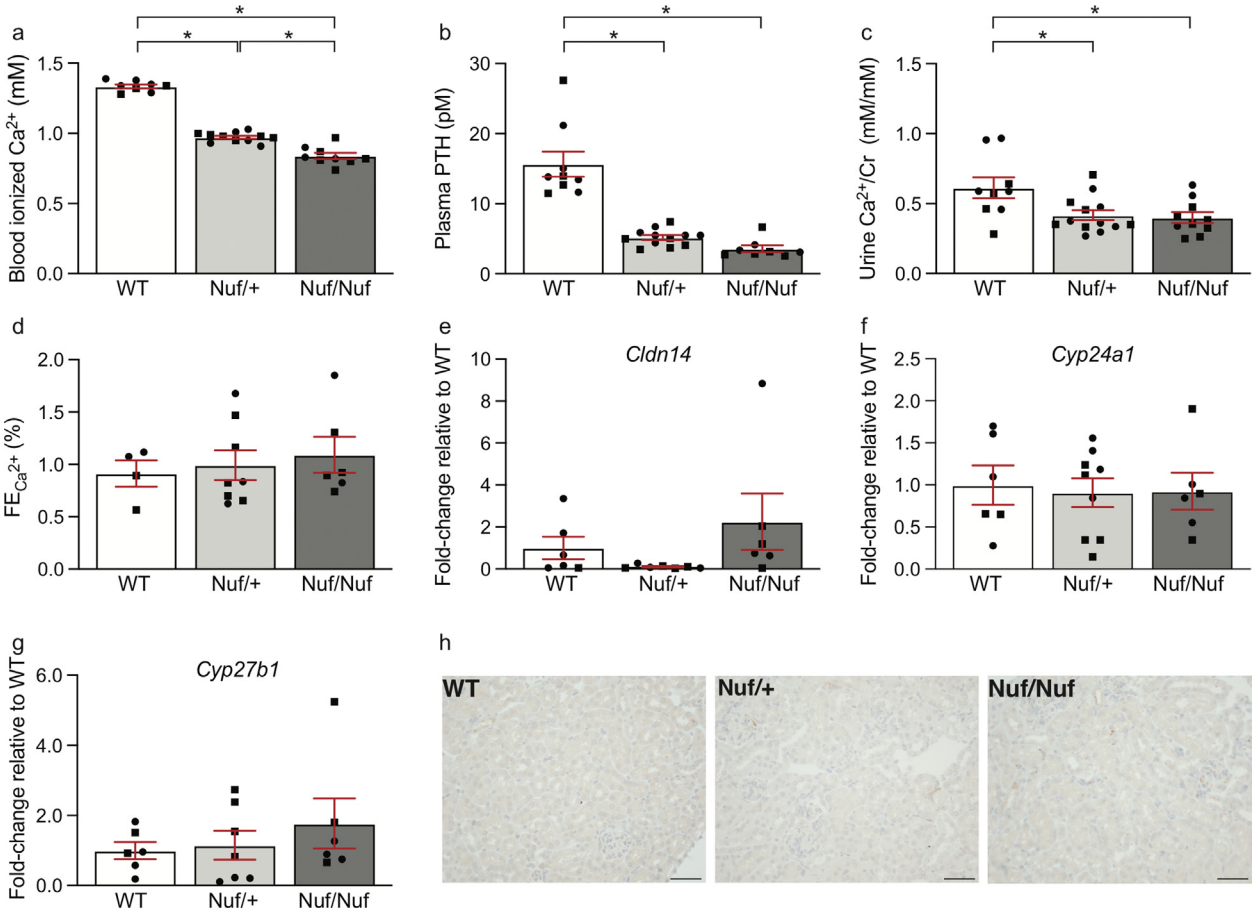

Figure 2. The renal CaSR is not activated under baseline conditions in Nuf mice. (a) Calcium levels in blood were significantly lower in Nuf/+ and Nuf/Nuf mice compared to WT animals. Ionized blood Ca^{2+} was also significantly lower in Nuf/Nuf mice compared to Nuf/+ mice (n = 8−11). P <0.0001 for WT *vs.* Nuf/+, WT *vs.* Nuf/Nuf and Nuf/+ *vs.* Nuf/Nuf. (b) Plasma intact PTH was significantly lower in Nuf/+ and Nuf/Nuf mice than in WT mice (n = 9−12). $P < 0.0001$ for WT *vs.* Nuf/+ and WT *vs.* Nuf/Nuf. (c) Urinary Ca^{2+} excretion was significantly lower in Nuf/+ mice and Nuf/Nuf mice compared to WT animals, but no significant differences were detected between Nuf/+ and Nuf/Nuf mice (n = 9−13). P = 0.0219 for WT *vs.* Nuf/+ and P = 0.0181 for WT *vs.* Nuf/Nuf. (d) Fractional urinary excretion of Ca^{2+} (FE_{Ca}^{2+}) was similar in all genotypes (n = 4−8). (e) The renal expression of *Cldn14*, a marker of CaSR activation in the kidney, was not significantly different between genotypes (n = 6−7). (f) The renal expression of *Cyp24a1*, involved in the inactivation of $1,25(OH)_2D_3$, was also unaltered in Nuf mice (n = 6−9). (g) The renal expression of *Cyp27b1*, involved in the activation of $1,25(OH)_2D_3$, was not statistically different between genotypes (n = 6−7). (h) Immunohistochemical staining of CLDN14 in the TAL was not observed in any of the genotypes. Scale bar indicates 50 µm. Data are shown as mean ± SEM. *$P < 0.05$ by one-way ANOVA with Tukey's post hoc test.

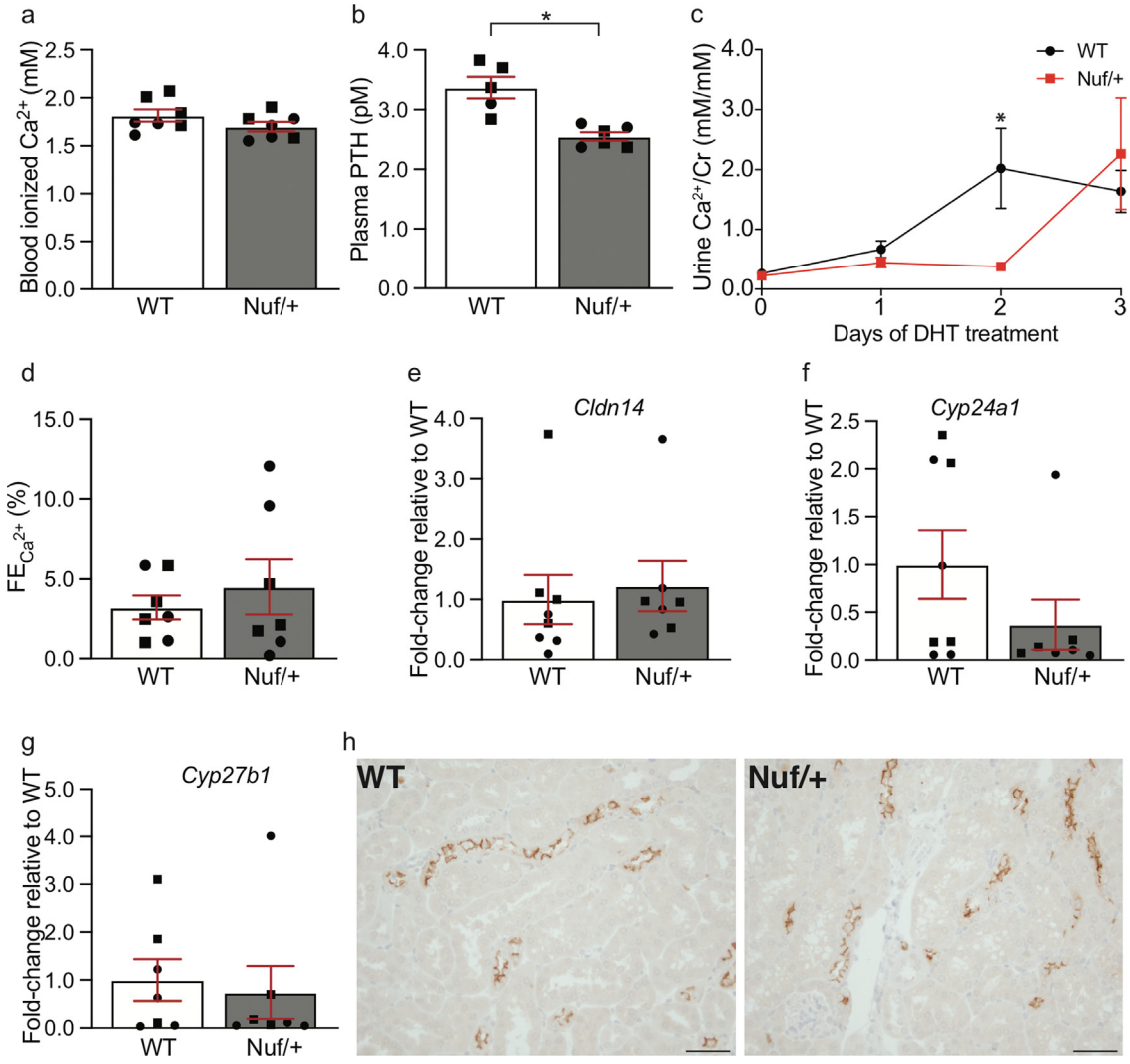

Figure 3. Effect of dietary DHT supplementation on Ca^{2+} balance. (a) No significant differences in blood Ca^{2+} between WT and Nuf/+ mice were present after 3 days of dietary DHT supplementation ($n = 7$). (b) Plasma PTH concentrations were significantly lower in Nuf/+ mice ($n = 5-6$). $P = 0.0016$. (c) Although DHT increased urinary Ca^{2+} excretion in both genotypes, this increase was delayed significantly, by one day, in Nuf/+ mice compared to WT mice ($n = 7-8$). $P = 0.0341$. (d) Fractional urinary excretion of Ca^{2+} (FE_{Ca}^{2+}) 3 days after DHT treatment was not significantly different between genotypes ($n = 7$). (e) No difference in renal *Cldn14* expression was present between Nuf/+ mice and WT mice after 3 days of DHT supplementation ($n = 7-8$). (f,g) The renal expression of *Cyp24a1* ($n = 7-8$) and *Cyp27b1* ($n = 7$) was also similar between Nuf/+ mice and WT mice. (h) Immunohistochemistry showed a marked increase in CLDN14 in both Nuf/+ and WT animals treated with DHT for 3 days. No apparent differences were observed between Nuf/+ and WT animals. Scale bar indicates 50 µm. *$P < 0.05$ for WT *vs.* Nuf/+ by Student's independent t-test (two-tailed) (b) or by repeated measurements two-way ANOVA with Bonferroni's *post hoc* test; interaction between time and genotype was significant ($P = 0.0352$) (c). Data are shown as mean ± SEM.

reference gene. This was because *18S* showed significant differences between groups.

Immunohistochemistry

Immunohistochemistry was performed as described before.[28] Kidneys were immersion-fixed in 10% formalin overnight at 4 °C. They were then stored in PBS until paraffin embedding. Embedding was performed using a Tissue-Tec Vacuum Infiltration Processor 6 (Sakura Finetek, Torrance, CA, USA) and then kidneys were cut into 2 μm thick sections with a HM 355S Automatic Microtome (Thermo Scientific, Kalamazoo, MI, USA). For immunostaining, sections were

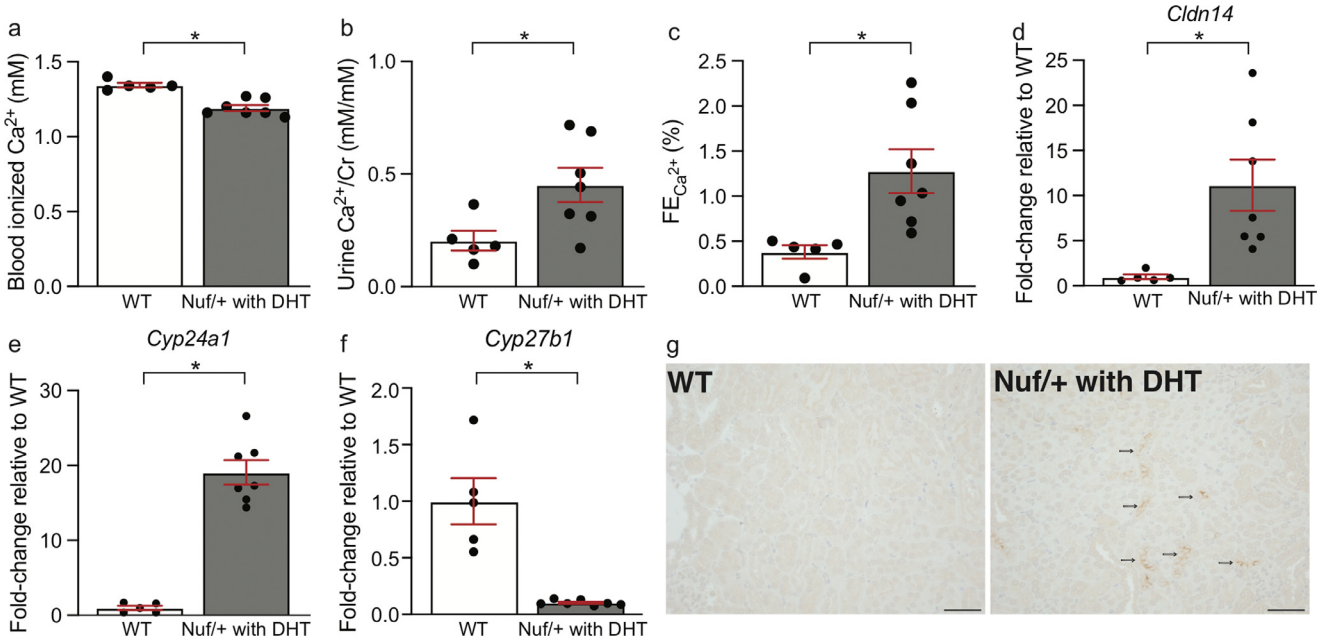

Figure 4. The renal CaSR becomes activated after DHT treatment in Nuf/+ mice. (a) Plasma Ca^{2+} remained significantly lower in Nuf/+ mice treated with DHT for 7 days compared to untreated WT mice ($n = 5–7$). $P = 0.0003$. (b) Urinary Ca^{2+} excretion was significantly higher in Nuf/+ mice following DHT treatment compared to untreated WT mice ($n = 5–7$). $P = 0.0311$ (c) Fractional urinary excretion of Ca^{2+} ($FE_{Ca}{}^{2+}$) was also significantly higher in DHT-treated Nuf mice than untreated WT mice ($n = 5–7$). $P = 0.0133$. (d) The expression of *Cldn14* in the kidney was significantly higher in Nuf/+ mice compared to untreated WT animals ($n = 5–7$). $P = 0.0139$. (e) In addition, increased renal *Cyp24a1* expression was present in Nuf/+ mice ($n = 5–7$). $P < 0.0001$. (f) In contrast, the expression of *Cyp27b1* was significantly reduced in Nuf/+ mice ($n = 5–7$). $P = 0.0004$. (g) Immunohistochemistry showed a marked increase of CLDN14 in TAL of DHT-treated Nuf/+ mice, whereas this was absent in WT animals. Scale bar indicates 50 µm. Data are shown as mean ± SEM. *$P < 0.05$ by Student's independent t-test (two-tailed).

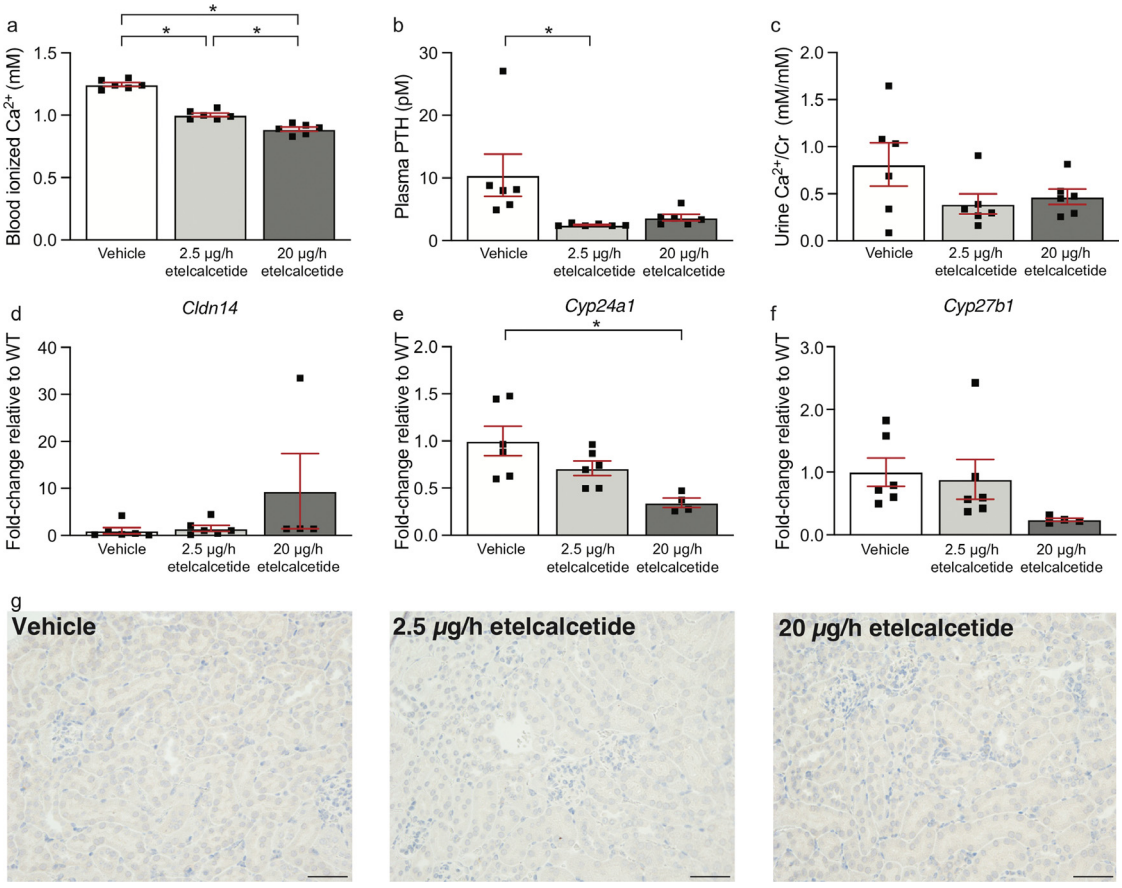

Figure 5. Etelcalcetide hydrochloride leads to hypocalcemia but does not activate the renal CaSR. (a) WT mice infused with etelcalcetide hydrochloride showed a dose-dependent reduction in blood Ca^{2+} concentrations after 7 days of treatment (n = 6). $P < 0.0001$ for vehicle *vs.* 2.5 µg/h etelcalcetide hydrochloride and vehicle *vs.* 20 µg/h etelcalcetide hydrochloride; $P = 0.0003$ for 2.5 µg/h etelcalcetide hydrochloride *vs.* 20 µg/h etelcalcetide hydrochloride. (b) Plasma intact PTH was significantly lower in 2.5 µg/h etelcalcetide hydrochloride-treated mice than vehicle treated mice. $P = 0.0314$ (c) No statistically significant differences in urinary Ca^{2+} excretion were found between the two groups receiving etelcalcetide hydrochloride compared to vehicle-treated animals (n = 6). (d) No changes in renal *Cldn14* were present between vehicle-treated animals and the two groups of animals that received a different dose of etelcalcetide hydrochloride (n = 4–6). (e) The renal expression of *Cyp24a1* was significantly lower in animals having received 20 µg/h etelcalcetide hydrochloride compared to vehicle-treated animals (n = 4–6). $P = 0.0064$ (f) No significant differences were present for *Cyp27b1* between any of the groups (n = 4–6). (e) CLDN14 was not observed by immunohistochemistry in mice treated with either vehicle, 2.5 µg/h etelcalcetide hydrochloride or 20 µg/h etelcalcetide hydrochloride. Scale bar indicates 50 µm. Data are shown as mean ± SEM (n = 4–6). *$P < 0.05$ by one-way ANOVA with Tukey's *post hoc* test.

processed with Tissue-Tek Tissue-clear (Sakura Finetek, Brøndby, Denmark) and a graded series of ethanol. Subsequently, they were boiled for 10 min in Tris-EGTA buffer (10 mM Tris, 0.5 mM EGTA, pH = 9.0). Endogenous peroxidase activity and free aldehyde groups were blocked by incubating in 0.6% H_2O_2 and 50 mM NH_4Cl in PBS. Then, sections were incubated overnight at 4 °C using a mouse monoclonal antibody against CLDN14 (Clone 5) produced in-house and extensively characterized[9]. After washing, sections were incubated with secondary HRP coupled antibodies followed by addition of DAB+ Substrate Chromogen System (Dako Omnis, Glostrup, Denmark). For each experiment, 6 mice were tested per genotype and representative images are shown. For the immunohistochemical analyses of the parathyroid and renal cortex, sections were processed as described above and stained using a rabbit polyclonal antibody against the CaSR (PB9924, Boster Biological Technology, Pleasanton, CA, USA).

Clinical data from ADH1 patient

Blood ionized Ca^{2+} and creatinine, urine Ca^{2+} and creatinine, and blood PTH were determined in the University of Alberta Hospital central laboratory by validated clinical biochemical protocols. Ionized blood Ca^{2+}

concentrations between 1.09 and 1.25 mM are considered to be normal, while hypercalciuria is considered to be a urinary $Ca^{2+}/Cr > 0.6$.[30] Normal plasma PTH is 1.4–6.8 pmol/L and normal serum creatinine is 50–120 μmol/L. We included data that had either paired blood ionized Ca^{2+} and parathyroid horomone levels or blood ionized Ca^{2+} and urine Ca^{2+} and creatinine levels. Due to the many samples collected over the life of this child, we averaged ionized Ca^{2+} and FECa or urine Ca^{2+}/Cr over a range of blood ionized Ca^{2+} and plotted them in Figure 6.

Cldn14 reporter assay in HEK293 cells

Cloning of the *Cldn14* promoter, which contains a CaSR-responsive element has been described previously.[31–33] HEK293 cells were purchased from the ATCC (Rockville, MD, USA; RRID:CVCL_0045) and cultured in DMEM with 10% FBS and 5% penicillin, streptomycin and glutamine as previously.[34] Cells were transfected via the calcium-phosphate method with the reporter construct, the pRL-TK renilla luciferase vector as an internal control and varying concentration of a vector encoding myc-tagged CaSR (Origene Inc.). After 24 h incubation with standard medium containing 1.5 mM calcium, the cells were assayed for *Cldn14* reporter expression using a luciferase assay system from Promega Corporation, according to the manufacturer's directions. Luciferase activity was assayed with 10 mL of extract on a DLR Ready, TD-20/20 Luminometer (Turner Design).

Western blotting

Samples were processed as described in detail previously.[9,35] Briefly, homogenized tissue was pelleted by centrifugation at 2000 *g* to remove nuclei and larger organelles and then at 17,000 *g*. Protein concentration was measured on the 17,000 *g* pellet using the DC protein assay (Bio-Rad, Copenhagen, Denmark) and samples loaded on polyacrylamide minigels (Bio-Rad). Samples were then electroeluted (25 mM Tris base, 192 mM glycine, 20% methanol, or 10% ethanol) to PVDF membranes (Merck, Darmstadt, Germany). Membranes were blocked in 5% skimmed milk in PBS with Tween (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween, pH 7.4) and incubated overnight at 4 °C with rabbit polyclonal antibodies against the CaSR (PB9924, Boster Biological Technology), mouse monoclonal anti-CLDN14 antibody[9] or rabbit anti-β-actin antibody (Abcam, Cambridge, UK; #Ab8227; RRID:AB_306371). Following washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 gamma 1 heavy chain antibodies (Rockland Immunohistochemicals, Limerick, PA, USA; #610-103-040; RRID:AB 2614833) for CLDN14 or secondary anti-rabbit

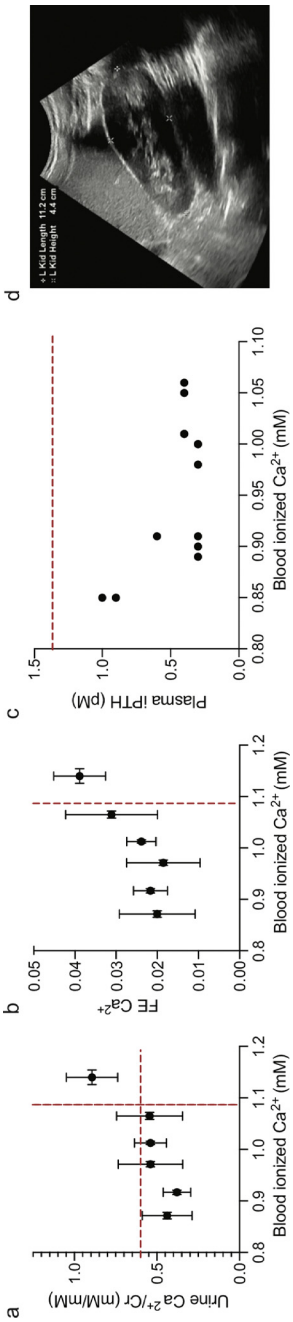

Figure 6. Ca^{2+} homeostasis in a patient with ADH1 (a) Treatment of a patient with ADH1 in order to normalize blood Ca^{2+} levels was associated with the occurrence of hypercalciuria when the patient was normocalcemic ($n = 6–19$ individual measurements per cluster). The vertical dashed red line represents the lower limit of normal for Ca^{2+} (ionized blood Ca^{2+} between 1.09 and 1.25 mM) and the horizontal red line represents the upper limit of urinary Ca^{2+} excretion considered normal (normal Ca^{2+}/Cr is > 0.6). (b) In addition, fractional urinary excretion of Ca^{2+} (FE Ca^{2+}) was increased after treatment leading to normocalcemia ($n = 6–19$ individual measurements per cluster). The horizontal dashed red line represents the lower limit of normal plasma Ca^{2+}. (c) Plasma intact PTH levels (iPTH) were suppressed during all collection periods ($n = 6–19$ individual measurements per cluster). The horizontal dashed red line represents the lower limit of normal PTH for the clinical laboratory (Normal range is 1.4–6.8 pmol/L). d) Medullary calcinosis, a complication of treatment-induced hypercalciuria in ADH1, was observed by ultrasound.

HRP conjugated antibodies for CaSR and β-actin (HRP, DakoCytomation) and visualized using an enhanced chemiluminescence system (ECL) on a Chemidoc XRS + system (Bio-Rad). For each sample, the intensity ratio of protein of interest/β-actin was determined and normalized to the average of the control animals.

Lysate from the luciferase assay was subjected to SDS-PAGE and immunoblotting after mixing 3:1 with RIPA buffer as previously[8]. A mouse primary anti-CaSR monoclonal antibody (1:2000; GTX19347, GeneTex, Zeeland, MI, USA; RRID:AB_423536) or a mouse primary anti-Myc (9B11) monoclonal antibody (1:1000; #2276, Cell Signaling Technology, Danvers, MA, USA) was applied followed by a secondary horseradish peroxidase-coupled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). This enabled detection of the CaSR at the appropriate molecular weight after incubation with Western Lightning Plus ECL reagents (PerkinElmer, Boston, MA) and visualized using a Kodak Image Station 440CF (Kodak, Rochester, NY).

Ethics

All animal experiments were conducted in accordance with Danish Law under animal experimental permits #2014-15-0201-00043 and #2019-15-0201-01629. With respect to the patient data, in consultation with the Research Ethics Board at the University of Alberta, formal ethical approval was not considered necessary, however, assent from the patient was provided as was formal written consent to include the clinical details of our subject by his guardian.

Statistical analyses

Sample size consisted of at least 5–6 animals per group and was based on our previous experience, as this was sufficient to previously find differences in renal *Cldn14* expression;[8,9] each experimental unit refers to a single animal. No inclusion or exclusion criteria were applied; all allocated animals were used and no data was excluded from analysis. Randomization was applied where relevant (i.e. in the allocation of WT mice to treatment or control groups in experimental protocols 1 and 7) by giving the individual animals different identification numbers and manually assigning these over treatment groups. No blinding was performed. For the metabolic cage experiments, cages for the different groups were placed alternatively on the shelf. All data are available upon request. For all graphs, circles indicate female animals and squares male animals. Given the small sample size, we assumed that all data were normally distributed.

Statistical analyses were performed using GraphPad Prism 8.3.0 (GraphPad software, San Diego, CA, USA). Comparisons between more than two groups were performed using one-way ANOVA with Tukey's *post hoc* test or repeated measurements two-way ANOVA with Bonferroni's *post hoc* test, as indicated in the figure legends. Analyses between two groups were performed using Student's independent t-test (two-tailed). For all analyses, a P value < 0.05 was considered statistically significant. Data are presented as mean ± SEM

Role of the funding sources

The funding sources were not involved in the study design, data collection, data analyses, data interpretation, in the writing of the manuscript or in the decision to submit the manuscript for publication.

Results

Renal *Cldn14* expression is dose-dependently regulated by blood Ca^{2+} levels via the CaSR

Previous studies found that CLDN14 is regulated by hypercalcemia and calcimimetics *in vivo*[8,9,11] and that *Casr* knockdown attenuates Ca^{2+}-dependent increases in CLDN14 expression *in vitro*.[11] To assess whether *Cldn14* expression increases in a dose dependent manner as plasma Ca^{2+} increases and thus can serve as a marker of kidney CaSR activity, we administered the synthetic vitamin D analog DHT at different dosages and durations to wildtype mice, as active vitamin D increases plasma Ca^{2+} by stimulating intestinal absorption, bone resorption and renal tubular Ca^{2+} reclamation.[36] Ca^{2+} levels were increased after a 3-day treatment with 0.4% w/v, 0.08% w/v and 0.04% w/v DHT compared to untreated animals, with the greatest increase in animals fed the highest dose of DHT (Figure 1a). Administration of 0.4% w/v DHT for 2 days, but not 1 day, also increased blood Ca^{2+} levels (Figure 1a). These changes were paralleled by proportionate increases in renal *Cldn14* gene (Figure 1b) and CLDN14 protein expression (Figures 1c and S1a), with the highest dosage (0.4% w/v DHT) of DHT resulting in the greatest increase compared to control mice. Similarly, immunohistochemical analysis of kidney sections showed a strong CLDN14 signal in animals treated for 3 days with 0.4% w/v DHT, while CLDN14 expression was also clearly increased in animals treated with 0.08% w/v DHT for 3 days or 0.4% w/v DHT for 2 days. CLDN14 expression was largely absent in control mice (Figure 1d). Curve fitting using a power trend found correlation (R^2 = 0.73) between blood Ca^{2+} levels and *Cldn14* gene expression, with the lowest ΔCt values (and hence the highest expression) at the highest blood Ca^{2+} concentrations (Figure 1e).

To determine whether hypercalcemia drives *Cldn14* expression via the CaSR, 0.4% w/v DHT was administered for 3 days to WT and kidney-specific *Casr*$^{-/-}$ mice (KS-*Casr*$^{-/-}$). After DHT treatment, renal *Cldn14* expression was significantly lower in kidney-specific

Casr$^{-/-}$ (KS-*Casr*$^{-/-}$) mice than in WT mice (Figure 1f). Similarly, both CLDN14 and CaSR were barely detectable by western blotting on kidney lysates from KS-*Casr*$^{-/-}$ mice compared to WT mice after DHT treatment (Figures 1g and S1b,c). Marked CLDN14 expression was observed using immunohistochemistry in hypercalcemic WT animals, but not KS-*Casr*$^{-/-}$ mice following DHT-treatment (Figure 1h).

Nuf mice are hypocalcemic with suppressed PTH but without altered renal *Cldn14* expression

Nuf/+ and Nuf/Nuf mice, which have overactivating mutations in the *Casr*, were used to study the role of the renal CaSR in ADH1. These mice are hypocalcemic as reported before,[23] with Nuf/Nuf mice exhibiting more severe hypocalcemia than Nuf/+ mice (Figure 2a). Plasma PTH concentrations were reduced in Nuf/+ and Nuf/Nuf mice in comparison to WT mice (Figure 2b). Nuf/+ and Nuf/Nuf mice both had reduced urinary Ca^{2+}/Cr relative to wild-type mice (Figure 2c). Despite the hypocalciuria, fractional urinary excretion of Ca^{2+} was unaltered (Figure 2d) as previously.[37]

To investigate if the renal CaSR is activated in the Nuf mice, we measured expression of *Cldn14*. However, *Cldn14* expression was unaltered in Nuf/+ and Nuf/Nuf mice compared to WT mice (Figure 2e). In addition, the renal expression of *Cyp24a1* (Figure 2f) and *Cyp27b1* (Figure 2g) was unchanged in Nuf mice. Immunohistochemistry showed low or absent expression of CLDN14 in all genotypes (Figure 2h). Given the similar phenotype of heterozygous and homozygous Nuf mice and that patients with ADH1 are heterozygous for activating mutations, we only used heterozygous Nuf mice for the remainder of experiments.

Activation of renal CaSRs is only achieved when blood Ca^{2+} levels are increased in Nuf mice

We next attempted to raise blood Ca^{2+} levels in Nuf/+ mice by administering a high (2% w/w) Ca^{2+} diet to WT and Nuf/+ mice for 6 days. However, as with ADH1 patients, this approach was unsuccessful at raising blood Ca^{2+} concentrations (Figure S2a). Urinary Ca^{2+}/Cr remained lower in Nuf/+ mice than in WT animals (Figure S2b) with similar fractional urinary excretion (Figure S2c), although fractional excretion was markedly higher for both genotypes compared to a normal diet (Figure 1d). Renal *Cldn14* expression was similar between the genotypes (Figure S2d) and so was *Cyp24a1* (Figure S2e) and *Cyp27b1* (Figure S2f). Immunohistochemistry revealed no CLDN14 expression in either Nuf/+ mice or WT mice (Figure 2e).

We next aimed to investigate the effects of hypercalcemia on Nuf mice by adding 0.4% w/v DHT to the food of WT and Nuf/+ mice for 3 days to increase blood Ca^{2+} levels.[25] The DHT-induced hypercalcemia was similar in both genotypes (Figure 3a). Plasma PTH concentrations remained slightly lower in Nuf/+ mice (Figure 3b). DHT increased urinary Ca^{2+} excretion in both genotypes (Figure 3C). Remarkably, the increase in urinary Ca^{2+} excretion following DHT supplementation was significantly delayed in Nuf/+ mice compared to WT animals (Figure 3c). On day 3, the fractional urinary excretion of Ca^{2+} was not significantly different between genotypes, indicating that the percentage of filtered Ca^{2+} that is excreted is unchanged in Nuf/+ mice (Figure 3d). Consistent with similar plasma Ca^{2+}, the expression of *Cldn14*, *Cyp24a1* and *Cyp27b1* was similar between Nuf/+ mice and WT mice (Figure 3e–g). Dietary DHT supplementation increased CLDN14 expression in both Nuf/+ and WT mice, with no obvious differences between the genotypes (Figure 3h).

Differential activation of the renal CaSR after normalization of plasma Ca^{2+} levels in Nuf/+ mice

Since DHT treatment induced hypercalcemia in both genotypes (Figure 3a), we next attempted to normalize the blood Ca^{2+} of Nuf/+ mice to WT levels. Therefore, we administered 0.04% w/v DHT only to Nuf/+ mice. Blood Ca^{2+} concentrations remained significantly lower in DHT-treated Nuf/+ mice compared to untreated WT mice, although the difference between Nuf/+ mice and wild-type animals was smaller compared to previous experiments (Figure 4a; see also 2a and S2a). However, despite lower plasma Ca^{2+}, urinary Ca^{2+} normalized to creatinine (Figure 4c) and fractional excretion of Ca^{2+} (Figure 4c) were significantly increased in Nuf/+ mice. Renal *Cldn14* expression was significantly higher in Nuf/+ mice compared to WT mice (Figure 4d). Furthermore, *Cyp24a1* expression was significantly higher in Nuf/+ mice (Figure 4e), while *Cyp27b1* expression was significantly decreased in Nuf/+ mice (Figure 4f). CLDN14 immunolocalization was detectable in the TAL of Nuf/+ mice, but not in the kidneys of untreated WT mice (Figure 4g).

Infusion of etelcalcetide hydrochloride leads to PTH suppression without activating the renal CaSR

The calcimimetic etelcalcetide hydrochloride is a novel therapeutic for secondary hyperparathyroidism, suitable for infusion. Using this calcimimetic, we sought to observe differing thresholds of activation of the CaSR in the parathyroid versus the kidney.[21,22] Infusion of 2.5 µg/h and 20 µg/h drug resulted in a dose-dependent decrease in blood Ca^{2+} concentrations in WT mice (Figure 5a). Plasma PTH concentrations were significantly lower in mice treated with 2.5 µg/h etelcalcetide hydrochloride compared to vehicle-treated animals (Figure 5b). No differences were present in urinary Ca^{2+} excretion (Figure 5c), while *Cldn14* expression was also unaltered by etelcalcetide hydrochloride (Figure 5d).

The renal expression of *Cyp24a1* was significantly decreased in the group that received 20 µg/h etelcalcetide hydrochloride compared to the vehicle-treated group (Figure 5e). No significant differences were present for *Cyp27b1* (Figure 5f). CLDN14 expression was not detectable in any of the treatment groups (Figure 5g). This data is consistent with pharmacological activation of the CaSR in the parathyroid and not the kidney at this dosage, using etelcalcetide hydrochloride.

Relationship between urinary Ca^{2+} excretion and blood Ca^{2+} concentrations in a patient with ADH1

To translate our findings to patients with ADH1, urinary Ca^{2+} excretion over time was plotted relative to blood Ca^{2+} concentrations in a patient with ADH1. This now 18-year-old boy presented at 2 years of age with muscle spasms secondary to hypocalcemia and was then found to have low plasma PTH levels, without hypercalciuria. Sequencing of the *CASR* revealed a novel mutation (c. C2351T, p.A784V). Given ongoing clinical symptoms including muscle spasms, a failure to gain appropriate weight and height and peripheral paresthesia, he was treated initially with calcitriol and Ca^{2+} supplementation. Ongoing symptoms despite this therapy, as well as persistent hypocalcemia, led to the decision to start subcutaneous synthetic PTH 1–34 (3.5 units t.i.d., teriparatide; Forteo) treatment at the age of 10. He remains on this currently at a dose of 20 µg, t.i.d. Strikingly, hypercalciuria (Ca^{2+}/Cr > 0.6)[30] was invariably present when he was normocalcemic (ionized blood Ca^{2+} between 1.09 and 1.25 mM) (Figure 6a). Moreover, when blood ionized Ca^{2+} concentrations were below the normal range he was much less likely to have hypercalciuria (Figure 6b). Fractional urinary excretion of Ca^{2+} was increased when blood Ca^{2+} concentrations were within the normal range, compared to most of the time when hypocalcemia was present (Figure 6b). Blood PTH concentrations were strongly suppressed regardless of blood Ca^{2+} levels (normal range 1.4–6.8 pmol/L) (Figure 6c). Unfortunately, he developed nephrocalcinosis at 9 years of age (Figure 6d). His renal function remains preserved with a plasma creatinine of 69 µM at last follow-up (normal range 50–120 µmol/L).

In vitro activation of the CaSR

Data from the Human Protein Atlas suggests that CaSR expression is higher in the parathyroid than in peripheral tissues, which could explain the different CaSR activation thresholds between the parathyroid and kidney.[38] To investigate this, we performed paired stainings of the CaSR in mouse kidney and parathyroid gland, which were in line with increased abundance of the CaSR in the parathyroid (Figure 7a) compared to renal cortical TAL (Figure 7b). To test the effect of increasing CaSR expression in the presence of a consistent extracellular Ca^{2+} concentration sufficient to activate the CaSR (1.5 mM Ca^{2+}), a CaSR-responsive *Cldn14* promoter reporter construct was transfected with increasing amounts of a myc-tagged CaSR into HEK293 cells.[33] Luminescence (and thus CaSR activity) significantly increased proportionately with increasing amounts of the CaSR construct transfected (Figure 7c). Western blotting confirmed increased CaSR expression after transfection with increased amount of construct (Figures 7d and S3).

Discussion

The CaSR plays important roles in maintaining systemic Ca^{2+} balance, exemplified by the marked perturbations observed in patients with ADH1 mutations in the CaSR. However, it remains incompletely understood why hypercalciuria is not always observed in patients with activating ADH1 mutations, which otherwise present with hypocalcemia due to CaSR-dependent suppression of PTH release. Using *Cldn14* as a gauge of chronic CaSR activation, we examined renal CaSR activation in a mouse model of ADH1 and following pharmacological CaSR activation. Our data suggest that differential activation thresholds for parathyroid and renal CaSRs exist, where activation of the CaSR in the parathyroid reduces circulating Ca^{2+} concentrations, which are insufficient to activate renal CaSRs, even in the presence of some activating ADH1 mutations. This is based on the following observations: *(i)* CaSR-dependent increases in the expression of *Cldn14* are absent in Nuf mice or following calcimimetic administration. *(ii)* *Cldn14* expression only increases following elevations in plasma Ca^{2+} concentrations above baseline levels in both WT and Nuf mice. *(iii)* A patient with ADH1 displays persistently suppressed blood intact PTH levels under hypocalcemic conditions, while hypercalciuria occurred when treatment succeeded in increasing blood ionized Ca^{2+} levels.

The underlying mechanism mediating a differential response of the parathyroid CaSR versus epithelial CaSRs at a given plasma Ca^{2+} concentration remains to be elucidated in detail. Immunohistochemical staining performed on paired samples from mouse kidney and parathyroid suggests the CaSR is more highly expressed in the parathyroid relative to other tissues. This is supported by data from the Human Protein Atlas.[38] Therefore, pharmacological activation or gain-of-function mutations in the CaSR may lead to greater total activation of the CaSR in the parathyroid. Consistent with this, *in vitro* transcription of *Cldn14* increased proportionately to the amount of CaSR expressed, at the same extracellular concentration of Ca^{2+}. Furthermore, tissue-specific signaling pathways may exist, resulting in activation of the CaSR in the parathyroid and the kidney by different extracellular Ca^{2+} concentrations. In fact, the CaSR-dependent signaling in renal TAL segments

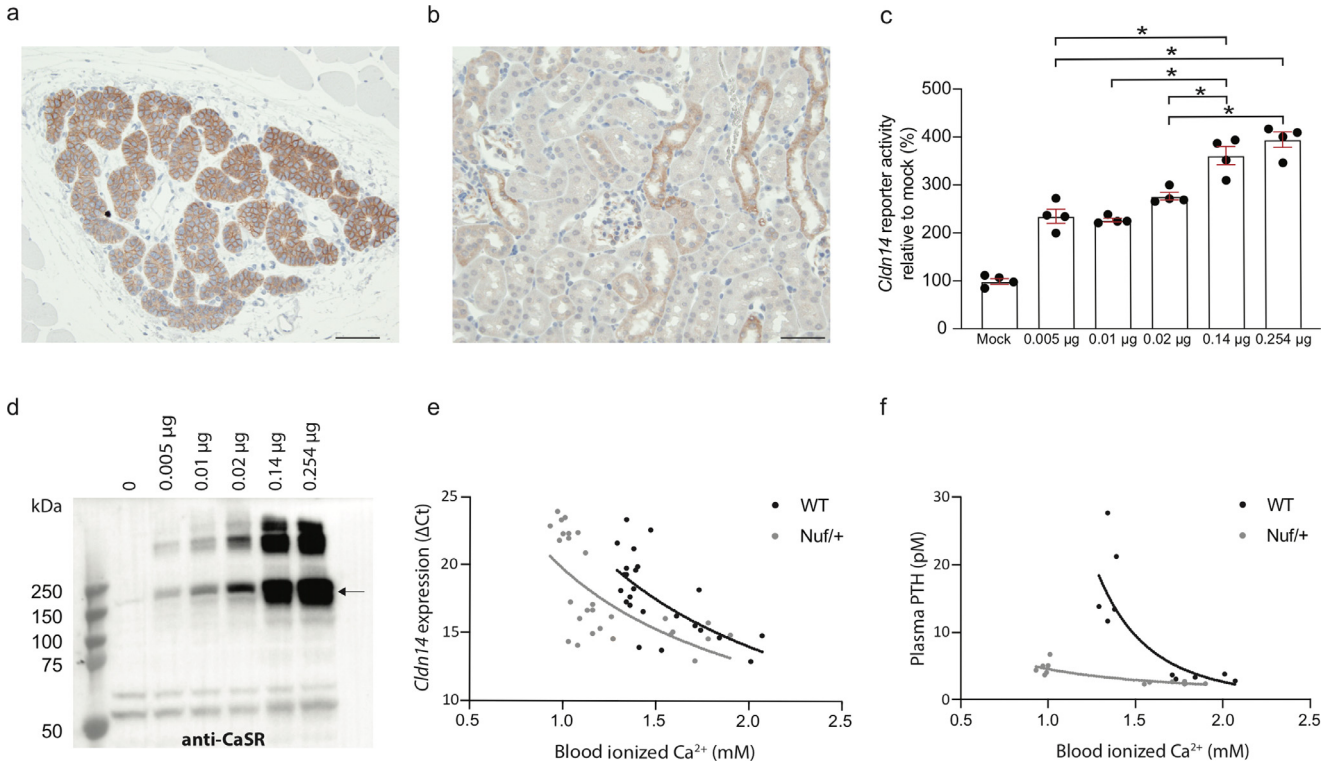

Figure 7. Relationship between CaSR abundance and CaSR activity. (a) Immunohistochemistry revealed a marked immunoreactivity of CaSR in the parathyroid of mice. (b) CaSR immunoreactivity was also detected in the thick ascending limb of Henle's loop in mouse kidney, albeit at a lower intensity. Scale bar indicates 50 µm. (c) After transfection of HEK293 cells with different amounts of a construct encoding the human CaSR (hCaSR) and a fixed amount of a CLDN14-promoter reporter construct, higher reporter signal was observed after transfection of cells with an increased amount of construct (n = 4 independent experiments). Data are shown as mean ± SEM. $P < 0.0001$ for 0.005 µg *vs.* 0.14 µg, 0.05 µg *vs*. 0.254 µg, 0.01 µg *vs*. 0.14 µg, 0.01 µg *vs*. 0.254 µg, 0.02 µg *vs*. 0.254 µg; $P = 0.002$ for 0.02 µg *vs*. 0.14 µg. *$P < 0.05$ by one-way ANOVA with Tukey's post hoc test. (d) Increased hCaSR expression was confirmed by western blotting with an anti-CaSR antibody. Representative immunohistochemical images and western blots are shown; arrows indicate dimeric CaSR signal. (e) Pooling blood Ca^{2+} concentrations and *Cldn14* gene expression (ΔCt; *Cldn14* Ct − *18S* Ct) data from all experiments revealed a non-linear relationship, which could be described with a power trend line that fitted the data points equally well for both genotypes (R^2 = 0.44 for WT and 0.46 for Nuf/+ mice). A leftward shift was observed for Nuf/+ mice, indicating lower ΔCt values for *Cldn14* (and thereby higher expression) at the same blood Ca^{2+} levels as WT mice. (f) A similar non-linear relationship was present between plasma PTH concentrations and blood Ca^{2+} concentrations (R^2 = 0.82 for WT and 0.78 for Nuf/+), yet PTH was already markedly suppressed in the Nuf/+ mice.

may differ from that reported in the parathyroid.[39] Furthermore, heterocomplex formation with other receptors, variance in G protein abundance or identity and direct interactions with signaling proteins may differ between sites.[40]

Importantly, when *Cldn14* expression is plotted against blood Ca^{2+} concentrations for all Nuf/+ experiments, curve fitting using a power trend line resulted in a similar relationship for WT (R^2 = 0.44) and Nuf/+ mice (R^2 = 0.46) (Figure 7e). However, this relationship is shifted leftwards for Nuf/+ mice. This is consistent with the renal CaSR being inactive at baseline in Nuf/+ mice, then becoming activated at a lower blood Ca^{2+} concentration in Nuf/+ than WT mice. A similar relationship is present for both genotypes between plasma PTH concentrations and blood ionized Ca^{2+} concentrations (R^2 = 0.82 and 0.78 for WT and Nuf/+, respectively) (Figure 7f). However, plasma PTH was already decreased in Nuf/+ mice at baseline blood Ca^{2+} levels, indicating baseline parathyroid CaSR activation in Nuf/+ mice. Based on the seemingly differential activation of the parathyroid and kidney CaSR observed in this study, we propose that alterations in the activity of the CaSR in the parathyroid and subsequent modulation of PTH secretion is a key response to maintain normocalcemia. In contrast, activation of renal CaSRs might be limited to states of hypercalcemia where a reduction in PTH fails to reduce plasma Ca^{2+} levels. Accordingly, $Pth^{-/-}$ mice, but not $Casr^{-/-}$ $Pth^{-/-}$ double knockout mice are still able to withstand hypercalcemic challenges.[41]

Nuf mice exhibit hypocalcemia and reduced plasma PTH concentrations similar to the ADH1 patients, reflecting activation of the parathyroid CaSR.[13] Hypocalcemia is not compensated by increased tubular reclamation of Ca^{2+} as evidenced by unaltered FECa between Nuf and WT mice at baseline. Thus, Nuf mice are unable to compensate for hypocalcemia by increasing renal Ca^{2+} reabsorption. This may be explained by lower circulating levels of PTH in Nuf mice, which is a known potent stimulator of Ca^{2+} reabsorption in the TAL and DCT. Similar mechanisms are likely to explain the observations from mice treated with etelcalcetide hydrochloride. In these models, the filtered Ca^{2+} load is expected to be decreased due to lower blood Ca^{2+} levels. This likely contributes to the reduced urinary Ca^{2+} levels observed. Importantly, renal CaSRs are not activated in this chronic setting as indicated by unaltered *Cldn14* expression and the absence of hypercalciuria as would be expected by the presence of a CaSR with an activating mutation in the kidney. A substantial fraction of ADH1 patients are not hypercalciuric, which could be explained by the absence of renal CaSR activation. Often such patients display hypercalciuria after treatment to correct symptomatic hypocalcemia.[13–18,20] This increase in urinary Ca^{2+} excretion observed after treatment may occur following direct CaSR activation in the kidney resulting in tubular Ca^{2+} wasting, in combination with an increased filtered load of Ca^{2+} and lower PTH levels. More severe gain-of-function mutations in the *CASR* causes ADH1 with Bartter-like features.[42–44] Accordingly, two mouse models with different gain-of-function mutations, based on human ADH1 mutations, displayed an increase in urinary Ca^{2+} excretion at baseline, despite having similar blood Ca^{2+} concentrations to the Nuf mice.[44–46] These differences illustrate that the phenotype resulting from genetic overactivation of the CaSR can be more severe depending on the exact mutation, which would allow the renal CaSR to become activated at even lower circulating Ca^{2+} concentrations. Consistent with differential renal and parathyroid CaSR activation, etelcalcetide hydrochloride-treated mice are hypocalcemic without activation of the renal CaSRs, while another calcimimetic, cinacalcet, potently activates the renal CaSR.[8,9] Etelcalcetide binds to the extracellular domain of the CaSR and cinacalcet to the seven-transmembrane domain,[47] the latter allowing for more potent modulation of the CaSR. Furthermore, differences in potency and dosage may also account for these observed effects.

Caveats and limitations

This study is not without limitations. First, *Cldn14* is used as an indirect marker of CaSR activity in kidney. While PTH has been considered a valid marker of parathyroid function and CaSR activity, the use of *Cldn14* to gauge renal CaSR activity is novel. Although we cannot fully exclude that other factors may also affect *Cldn14* expression, we clearly show that increases in blood Ca^{2+} drive expression of *Cldn14* and that this occurs via a CaSR-dependent process. This is consistent with our previous data showing that chronic hypercalcemia or CaSR activation increases renal *Cldn14* expression by some 10–170 times above baseline resulting in marked alterations in CLDN14 protein levels.[8,9] This marked increase in CLDN14 expression occurs irrespective of whether hypercalcemia is induced by active vitamin D derivatives or via PTH.[8,9] The only hypercalcemic model presented to date with no significant changes in CLDN14 expression in the chronic setting, is the transgenic model presented in this manuscript, where the CaSR is deleted specifically in the kidney. Furthermore, we have identified a *Cldn14* promoter element that is directly regulated by the CaSR when the receptor is activated.[31–33] Collectively, these findings are in line with *Cldn14* being a reasonable indicator of CaSR activity in the kidney. One should also consider that CaSR-dependent signaling in the TAL may differ from that reported in the parathyroid[39] so other surrogate markers for CaSR activity such as intracellular Ca^{2+} release, would not necessarily be a better indicator. The absence of changes in *Cldn14* expression in Nuf mice at baseline is consistent with the CaSR not being activated

in the kidney in these animals at baseline. While we cannot fully exclude that a yet-to-be-identified putative negative regulator of *Cldn14* exists, raising Ca^{2+} in the Nuf mice, even to levels lower than those observed in wildtype mice resulted in predictable increases in *Cldn14* expression. This is in line with renal CaSR activation only occurring when plasma Ca^{2+} levels are sufficiently high. Potentially hypocalcemia itself could drive compensatory mechanisms to increase tubular Ca^{2+} reclamation. However, this has not been described, aside from the well-known effects of PTH pathway activation, which is markedly reduced in these animals.

Nuf mice have a missense mutation in the fourth transmembrane domain of the CaSR,[23] while transmembrane mutations in human ADH1 patients, including the one described here, are usually present in transmembrane domains 5 and 6.[13] Nevertheless, the Nuf mice show classical ADH1 features with hypocalcemia and inappropriately low PTH levels suggesting that this is a reasonable mouse model to study ADH1. As outlined above, varying severity of the gain-of-function ADH1 mutations have been noted, with a few patients presenting with hypercalciuria even when hypocalcemic, which is different from the mouse model and patient investigated in our studies. However, other animal models with more severe gain-of-function ADH1 mutations have been generated to reflect this and highlights the fact that some mutations give rise to hypercalciuria at baseline, even though the animals show comparable blood Ca^{2+} concentrations to those found in the Nuf mice.[44–46] A limitation of our study is thus the use of a single genetic model for the mouse experiments and a single patient as an example case.

In conclusion, we demonstrate that renal CaSRs have different activation thresholds from the parathyroid and document an intricate interplay between the parathyroid and renal CaSRs in maintaining Ca^{2+} balance. These findings could aid in explaining why hypercalciuria, a significant risk factor for nephrocalcinosis and chronic kidney disease, is not consistently observed in patients with ADH1 as this may depend on ambient levels of Ca^{2+} in the blood of the patient and the severity of the ADH1 mutation. Furthermore, these findings may aid in the clinical management of patients to avoid aberrant renal CaSR activation when possible.

Declaration of interests

RTA has received consulting fees and a grant from Ardylex Inc to determine the mechanism of action of their drug Tenapanor, which is not related to this work. RTA has received honoraria for Ultragenyx for a lecture on FGF23 lowering therapies. WvM obtained an Erasmus+ grant for a graduate research stay in the lab of H. Dimke. The authors have declared that no conflict of interest exists.

Data sharing

All relevant data have been presented in the manuscript or in the supplemental information. Raw data is available upon reasonable request to the corresponding authors.

Author contributions

HD and RTA conception; WvM, HD and RTA designed research; WvM, RT, RTA and HD performed experiments; WvM, RT, RTA and HD analyzed data; WvM and HD have verified the underlying data; WvM, RT, RTA and HD interpreted results of experiments; WvM prepared figures; WvM and HD drafted the manuscript; WvM, RT, RTA and HD edited, revised and approved the final version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103947.

References

1 Peacock M. Calcium metabolism in health and disease. *Clin J Am Soc Nephrol CJASN*. 2010;5(Suppl 1):S23–S30.
2 Brown EM, Hurwitz S, Aurbach GD. Preparation of viable isolated bovine parathyroid cells. *Endocrinology*. 1976;99(6):1582–1588.
3 Conigrave AD. The calcium-sensing receptor and the parathyroid: past, present, future. *Front Physiol*. 2016;7:563.
4 Brown EM, Gamba G, Riccardi D, et al. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*. 1993;366(6455):575–580.
5 Diaz-Soto G, Rocher A, Garcia-Rodriguez C, Nunez L, Villalobos C. The calcium-sensing receptor in health and disease. *Int Rev Cell Mol Biol*. 2016;327:321–369.
6 Loupy A, Ramakrishnan SK, Wootla B, et al. PTH-independent regulation of blood calcium concentration by the calcium-sensing receptor. *J Clin Invest*. 2012;122(9):3355–3367.
7 Lee JJ, Liu X, O'Neill D, et al. Activation of the calcium sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI Insight*. 2019;5:1–13.
8 Dimke H, Desai P, Borovac J, Lau A, Pan W, Alexander RT. Activation of the Ca(2+)-sensing receptor increases renal claudin-14

expression and urinary Ca(2+) excretion. *Am J Physiol Ren Physiol*. 2013;304(6):F761–F769.
9 Frische S, Alexander RT, Ferreira P, et al. Localization and regulation of claudin-14 in experimental models of hypercalcemia. *Am J Physiol Renal Physiol*. 2021;320(1):F74–F86.
10 Toka HR, Al-Romaih K, Koshy JM, et al. Deficiency of the calcium-sensing receptor in the kidney causes parathyroid hormone-independent hypocalciuria. *J Am Soc Nephrol JASN*. 2012;23(11):1879–1890.
11 Gong Y, Renigunta V, Himmerkus N, et al. Claudin-14 regulates renal Ca(+)(+) transport in response to CaSR signalling via a novel microRNA pathway. *EMBO J*. 2012;31(8):1999–2012.
12 Plain A, Wulfmeyer VC, Milatz S, et al. Corticomedullary difference in the effects of dietary Ca(2)(+) on tight junction properties in thick ascending limbs of Henle's loop. *Pflug Arch*. 2016;468(2):293–303.
13 Roszko KL, Bi RD, Mannstadt M. Autosomal dominant hypocalcemia (hypoparathyroidism) types 1 and 2. *Front Physiol*. 2016;7:458.
14 Pearce SH, Williamson C, Kifor O, et al. A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *N Engl J Med*. 1996;335(15):1115–1122.
15 Cavaco BM, Canaff L, Nolin-Lapalme A, et al. Homozygous calcium-sensing receptor polymorphism R544Q presents as hypocalcemic hypoparathyroidism. *J Clin Endocrinol Metab*. 2018;103(8):2879–2888.
16 Tan YM, Cardinal J, Franks AH, et al. Autosomal dominant hypocalcemia: a novel activating mutation (E604K) in the cysteine-rich domain of the calcium-sensing receptor. *J Clin Endocrinol Metab*. 2003;88(2):605–610.
17 D'Souza-Li L, Yang B, Canaff L, et al. Identification and functional characterization of novel calcium-sensing receptor mutations in familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia. *J Clin Endocrinol Metab*. 2002;87(3):1309–1318.
18 Yamamoto M, Akatsu T, Nagase T, Ogata E. Comparison of hypocalcemic hypercalciuria between patients with idiopathic hypoparathyroidism and those with gain-of-function mutations in the calcium-sensing receptor: is it possible to differentiate the two disorders? *J Clin Endocrinol Metab*. 2000;85(12):4583–4591.
19 Alexander RT, Hemmelgarn BR, Wiebe N, et al. Kidney stones and kidney function loss: a cohort study. *BMJ*. 2012;345:e5287.
20 Sorheim JI, Husebye ES, Nedrebo BG, et al. Phenotypic variation in a large family with autosomal dominant hypocalcaemia. *Horm Res Paediatr*. 2010;74(6):399–405.
21 Alexander ST, Hunter T, Walter S, et al. Critical cysteine residues in both the calcium-sensing receptor and the allosteric activator AMG 416 underlie the mechanism of action. *Mol Pharmacol*. 2015;88(5):853–865.
22 Harada K, Fujioka A, Konno M, Inoue A, Yamada H, Hirota Y. Pharmacology of Parsabiv((R)) (etelcalcetide, ONO-5163/AMG 416), a novel allosteric modulator of the calcium-sensing receptor, for secondary hyperparathyroidism in hemodialysis patients. *Eur J Pharmacol*. 2019;842:139–145.
23 Hough TA, Bogani D, Cheeseman MT, et al. Activating calcium-sensing receptor mutation in the mouse is associated with cataracts and ectopic calcification. *Proc Natl Acad Sci U S A*. 2004;101(37):13566–13571.
24 Shao X, Somlo S, Igarashi P. Epithelial-specific Cre/lox recombination in the developing kidney and genitourinary tract. *J Am Soc Nephrol JASN*. 2002;13(7):1837–1846.
25 Qaw F, Calverley MJ, Schroeder NJ, Trafford DJ, Makin HL, Jones G. *In vivo* metabolism of the vitamin D analog, dihydrotachysterol. Evidence for formation of 1 alpha,25- and 1 beta,25-dihydroxy-dihydrotachysterol metabolites and studies of their biological activity. *J Biol Chem*. 1993;268(1):282–292.
26 Alexander RT, Beggs MR, Zamani R, Marcussen N, Frische S, Dimke H. Ultrastructural and immunohistochemical localization of plasma membrane Ca2+-ATPase 4 in Ca2+-transporting epithelia. *Am J Physiol Ren Physiol*. 2015;309(7):F604–F616.
27 van der Hagen EA, Lavrijsen M, van Zeeland F, et al. Coordinated regulation of TRPV5-mediated Ca(2)(+) transport in primary distal convolution cultures. *Pflug Arch*. 2014;466(11):2077–2087.
28 Beggs MR, Appel I, Svenningsen P, Skjodt K, Alexander RT, Dimke H. Expression of transcellular and paracellular calcium and magnesium transport proteins in renal and intestinal epithelia during lactation. *Am J Physiol Renal Physiol*. 2017;313(3):F629–FF40.
29 Rievaj J, Pan W, Cordat E, Alexander RT. The Na(+)/H(+) exchanger isoform 3 is required for active paracellular and transcellular Ca(2)(+) transport across murine cecum. *Am J Physiol Gastrointest Liver Physiol*. 2013;305(4):G303–G313.
30 Alexander RT, Lich C, Smoyer W, Rosenblum ND, Yu A, Chertow G, Luyckx V, Marsden P, Skorecki K, Taal M. Diseases of the kidney and urinary tract in children. editor. *Brenner and Rector's The Kidney*. 11 ed Elsevier; 2020.
31 van Megen WH, Beggs MR, An SW, et al. Gentamicin inhibits Ca(2+) channel TRPV5 and induces calciuresis independent of the calcium-sensing receptor-claudin-14 pathway. *J Am Soc Nephrol*. 2022;33(3):547–564.
32 Lee JJ, Alzamil J, Rehman S, Pan W, Dimke H, Alexander RT. Activation of the CaSR increases claudin-14 expression via a PLC-p38-Sp1 pathway. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2021;00:e21982.
33 Ferreira PG, van Megen WH, Tan R, et al. Renal claudin-14 expression is not required for regulating Mg(2+) balance in mice. *Am J Physiol Ren Physiol*. 2021;320(5):F897–F907.
34 Ure ME, Heydari E, Pan W, et al. A variant in a cis-regulatory element enhances claudin-14 expression and is associated with pediatric-onset hypercalciuria and kidney stones. *Hum Mutat*. 2017;38(6):649–657.
35 Dimke H, Flyvbjerg A, Bourgeois S, et al. Acute growth hormone administration induces antidiuretic and antinatriuretic effects and increases phosphorylation of NKCC2. *Am J Physiol Renal Physiol*. 2007;292(2):F723–F735.
36 Haussler MR, Whitfield GK, Kaneko I, et al. Molecular mechanisms of vitamin D action. *Calcif Tissue Int*. 2013;92(2):77–98.
37 Hannan FM, Walls GV, Babinsky VN, et al. The calcilytic agent NPS 2143 rectifies hypocalcemia in a mouse model with an activating calcium-sensing receptor (CaSR) mutation: relevance to autosomal dominant hypocalcemia type 1 (ADH1). *Endocrinology*. 2015;156(9):3114–3121.
38 Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. *Science*. 2015;347:(6220)1260419.
39 Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev*. 2001;81(1):239–297.
40 Chang W, Tu CL, Jean-Alphonse FG, et al. PTH hypersecretion triggered by a GABAB1 and Ca(2+)-sensing receptor heterocomplex in hyperparathyroidism. *Nat Metab*. 2020;2(3):243–255.
41 Kantham L, Quinn SJ, Egbuna OI, et al. The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. *Am J Physiol Endocrinol Metab*. 2009;297(4):E915–E923.
42 Vargas-Poussou R, Huang C, Hulin P, et al. Functional characterization of a calcium-sensing receptor mutation in severe autosomal dominant hypocalcemia with a Bartter-like syndrome. *J Am Soc Nephrol*. 2002;13(9):2259–2266.
43 Vezzoli G, Arcidiacono T, Paloschi V, et al. Autosomal dominant hypocalcemia with mild type 5 Bartter syndrome. *J Nephrol*. 2006;19(4):525–528.
44 Watanabe S, Fukumoto S, Chang H, et al. Association between activating mutations of calcium-sensing receptor and Bartter's syndrome. *Lancet*. 2002;360(9334):692–694.
45 Dong B, Endo I, Ohnishi Y, et al. Calcilytic ameliorates abnormalities of mutant calcium-sensing receptor (CaSR) knock-in mice mimicking autosomal dominant hypocalcemia (ADH). *J Bone Miner Res off J Am Soc Bone Miner Res*. 2015;30(11):1980–1993.
46 Hirai H, Nakajima S, Miyauchi A, et al. A novel activating mutation (C129S) in the calcium-sensing receptor gene in a Japanese family with autosomal dominant hypocalcemia. *J Hum Genet*. 2001;46(1):41–44.
47 Gao Y, Robertson MJ, Rahman SN, et al. Asymmetric activation of the calcium-sensing receptor homodimer. *Nature*. 2021;595(7867):455–459.