Activation of the Ca\(^{2+}\)-sensing receptor increases renal claudin-14 expression and urinary Ca\(^{2+}\) excretion

Henrik Dimke, Prajakta Desai, Jelena Borovac, Alyssa Lau, Wanling Pan, and R. Todd Alexander

Department of Physiology and Membrane Protein Disease Research Group, University of Alberta, Edmonton, Alberta, Canada; and Department of Pediatrics, University of Alberta, Edmonton, Alberta, Canada

Submitted 7 May 2012; accepted in final form 26 December 2012

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 Renal Physiol 304: F761–F769, 2013. First published January 2, 2013; doi:10.1152/ajprenal.00263.2012.—Kidney stones are a prevalent clinical condition imposing a large economic burden on the healthcare system. Hypercalciuria remains the major risk factor for development of a Ca\(^{2+}\)-containing stone. The kidney’s ability to alter Ca\(^{2+}\) excretion in response to changes in serum Ca\(^{2+}\) is in part mediated by the Ca\(^{2+}\)-sensing receptor (CaSR). Recent studies revealed renal claudin-14 (Cldn14) expression localized to the thick ascending limb (TAL) and its expression to be regulated via coimmunolocalization and microdissection experiments Cldn14 was a matter of debate (3, 9, 19) until very recently with CaSR expression restricted to the TAL (12).

We therefore set out to ascertain the potential role of Cldn14 in regulating renal Ca\(^{2+}\) excretion. We found that renal Cldn14 expression is strongly upregulated via activation of the CaSR. Thus how Cldn14 contributes to the formation of kidney stones was not clear. Even the renal localization of Cldn14 was a matter of debate (3, 9, 19) until very recently when coimmunolocalization and microdissection experiments demonstrated expression restricted to the TAL (12).

The PREVALENCE OF KIDNEY STONES is increasing (33). In some populations, kidney stones have been observed in as many as 12% of men and 5% of women (5). The occurrence of symptomatic stones is strongly dependent on age and race (33), and the recurrence rate is high. Stone disease is expensive to treat due to frequent emergency room visits, hospitalizations, and surgeries. Treatment costs 5 billion dollars annually in the United States alone (30). Moreover, stone disease is directly associated with an increase in the likelihood of adverse renal outcomes, including end-stage renal disease (1).

The greatest risk factor for the development of a Ca\(^{2+}\)-containing stone is hypercalciuria (22). Notably, ~80% of stones are calcium (Ca\(^{2+}\)) containing (5). Increased urinary Ca\(^{2+}\) excretion contributes to Ca\(^{2+}\) crystal growth (5). Conversely, a reduction in urinary Ca\(^{2+}\) excretion slows stone formation (10, 21). Hypercalciuria may result from disturbed transport of Ca\(^{2+}\) in several organs. Increased intestinal absorption or resorption from bone promotes hypercalciuria because renal Ca\(^{2+}\) transporters adjust the urinary excretion to maintain serum Ca\(^{2+}\) within normal limits (24). Similarly, reduced reabsorption of Ca\(^{2+}\) due to disturbances in renal Ca\(^{2+}\) reabsorption may also lead to hypercalciuria.

Genetic defects resulting in abnormal renal electrolyte transport can cause hypercalciuria (7). Importantly, mutations in proteins mediating paracellular transport, specifically claudins (Cldn) 16 or Cldn19, cause renal Ca\(^{2+}\) wasting (7, 20, 32). These claudins form a cation-permeable paracellular pore in the renal thick ascending limb (TAL) (17, 20, 32). Electrolyte transport in this segment is coupled to the reabsorption of filtered Ca\(^{2+}\). Consequently, ion flux in the TAL is regulated by calcitropic hormones and Ca\(^{2+}\) itself (6, 8). Although Ca\(^{2+}\)-sensing receptor (CaSR) activation plays a role in this process, the details of downstream signaling remain incompletely elucidated (11).

A large genome-wide association study recently found single nucleotide polymorphisms (SNPs) in human CLDN14 that strongly associate with kidney stones and lower bone mineral density, inferring a role for Cldn14 in the pathogenesis of idiopathic hypercalciuria (34). Homozygous carriers of the synonymous coding SNP, rs219780[C], had a 1.64-fold increased risk of developing kidney stones. Mutations in the CLDN14 gene have been identified in two large consanguineous families. Surprisingly, affected individuals have nonsyndromic deafness but do not demonstrate symptoms consistent with Ca\(^{2+}\) wasting. Similarly, Cldn14/Cldn11 double knockout mice do not display significantly disturbed urinary Ca\(^{2+}\) excretion (9). Thus how Cldn14 contributes to the formation of kidney stones was not clear. Even the renal localization of Cldn14 was a matter of debate (3, 9, 19) until very recently when coimmunolocalization and microdissection experiments demonstrated expression restricted to the TAL (12).

Address for reprint requests and other correspondence: R. T. Alexander, Dept. of Pediatrics, 4-585 Edmonton Clinic Health Academy, 11405-87 Ave., Univ. of Alberta, Edmonton, Alberta, Canada T6G 2R7 (e-mail: todd2@ualberta.ca).

http://www.ajprenal.org 1931-857X/13 Copyright © 2013 the American Physiological Society
dysregulation provides a likely molecular explanation as to why SNPs in the CLDN14 gene correlate with kidney stones and lower bone mineral density (34).

**MATERIALS AND METHODS**

**Experimental protocol 1.** FVB/N mice (Jackson Laboratories, Bar Harbor, ME) were fed a low (0.01%, TD.95027), normal (0.6%, TD.97191), or high (2%, TD.00374)-Ca\(^{2+}\) diet for either 10 or 21 days (n = 48, 8/group). Diets were custom made by Harlan Laboratories (Madison, WI). Mice were housed in metabolic cages at the end of the experimental period for a 24-h urine collection. On the last day, animals were anesthetized using pentobarbital sodium, and blood was withdrawn by perforating the orbital vessels and 1) used to measure electrolytes (with a VetScan i-STAT 1 Analyzer, Abaxis, Union City, CA) and 2) processed into serum. Kidneys were removed and snap frozen in liquid nitrogen. All experimental procedures were approved by the Animal Care and Use Committee for Health Sciences at the University of Alberta (protocol 576).

**Experimental protocol 2.** FVB/N mice (n = 8/group) were placed on a standard diet with ad libitum access to food and water. 1,25-Dihydroxyvitamin D\(_3\) [1,25(OH)\(_2\)D\(_3\); Sigma-Aldrich, Oakville, ON] was dissolved in absolute ethanol and diluted to 5% in phosphate-buffered saline. Animals received 1,25(OH)\(_2\)D\(_3\) (500 pg/g body weight, n = 8) or vehicle (n = 8) by intraperitoneal injections for 5 days. During the last 48 h of the experimental period, mice were placed in metabolic cages. Mice were maintained and processed exactly as described in experimental protocol 1.

**Experimental protocol 3.** FVB/N mice (n = 6/group) were given cinacalcet (Sensipar, Amgen) in food at a dose of 1 mg/g body weight, n = 8) or vehicle (n = 8) by intraperitoneal injections for 5 days. The last 48 h of the experimental period, mice were processed into serum. Kidneys were removed and snap frozen in liquid nitrogen. All experimental procedures were approved by the Animal Care and Use Committee for Health Sciences at the University of Alberta (protocol 576).

**Determination of solutes, creatinine, and hormones.** Urinary Ca\(^{2+}\) was determined using a colorimetric assay kit (Quantichrom TM Ca\(^{2+}\) Assay Kit, BioAssay System, Hayward, CA). Urinary creatinine was measured using a Creatinine Parameter Assay Kit (R&D Systems, Minneapolis, MN). Intact plasma parathyroid hormone (PTH) levels were determined by a mouse PTH ELISA kit (Immutopics International, San Clemente, CA), and serum 1,25(OH)\(_2\)D\(_3\) concentrations were determined with a mouse PTH ELISA kit (Immutopics International, San Clemente, CA).

**Urinary Ca\(^{2+}\) flux studies.** 45CaCl\(_2\) tracer (PerkinElmer) flux was determined in Ussing chambers with the transepithelial potential difference clamped to 0 mV and equimolar Ca\(^{2+}\) and Cl\(^{-}\) ratio. The Snapwell inserts with confluent OK monolayers were washed three times using buffer A and then mounted between the two hemichambers, both of which were filled with 10 ml of buffer A. Current clamps were performed using a DVC 1000 IV Clamp (World Precision Instruments, Sarasota, FL), and electrodes containing an agarose bridge with 3 M KCl. Data were acquired as a trace and recorded using PowerLab (ADInstruments, Colorado Springs CO) running Chart 4.0 software. To determine the transepithelial resistance (TER) and permeability properties of the epithelia, a 90-μA current was applied across each monolayer and a dilution potential was induced by replacing buffer A in the apical hemichamber with buffer B (80 mM NaCl, 130 mM mannitol, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.4). The Snapwell inserts with confluent OK monolayers were determined by a reference measurement with buffer A, and then mounted between the two hemichambers, both of which were filled with 10 ml of buffer A. Current clamps were performed using a DVC 1000 IV Clamp (World Precision Instruments, Sarasota, FL), and electrodes containing an agarose bridge with 3 M KCl. Data were acquired as a trace and recorded using PowerLab (ADInstruments, Colorado Springs CO) running Chart 4.0 software. To determine the transepithelial resistance (TER) and permeability properties of the epithelia, a 90-μA current was applied across each monolayer and a dilution potential was induced by replacing buffer A in the apical hemichamber with buffer B (80 mM NaCl, 130 mM mannitol, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.4). The dilution potential and voltage of each filter were determined following removal of the cells by trypsinization (30 min, at 37°C), and this measurement was subtracted from the values generated by the filter containing cells. Goldman-Hodgkin-Katz and Kimizu-Koketsu (18) equations were used as described previously (16) to calculate the absolute permeability of Na\(^+\) and Cl\(^{-}\) and to determine the relative permeability of Na\(^+\)-to-Cl\(^{-}\) (P\(_{Na}\)/P\(_{Cl}\)) ratio.

Ca\(^{2+}\) flux studies. 45Ca\(^{2+}\) tracer (PerkinElmer) flux was determined in Ussing chambers with the transepithelial potential difference clamped to 0 mV and equimolar Ca\(^{2+}\) in each half of the hemichamber to prevent generating an electrochemical gradient for Ca\(^{2+}\) flux across the monolayer. Empty vector (mock)-transfected and cells expressing Myc-tagged Cldn14 were seeded onto Transwell permeable supports (Corning, Corning, NY) and grown for 7 days. 45CaCl\(_2\) (25 μCi/ml) was applied to the apical hemichamber, and samples were
taken sequentially from both the basolateral and apical compartments. Ca\textsuperscript{2+} flux was calculated as the rate of \textsuperscript{45}Ca\textsuperscript{2+} appearance in the basolateral side (cpm/min) divided by the specific activity of radioactivity in the apical side (cpm/mol of Ca\textsuperscript{2+}). Radioactivity of the samples was measured with a LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter).

Statistical analysis. Values are presented as means ± SE. Comparisons between two groups were made using an unpaired Student's t-test with Bonferroni correction for multiple comparisons.

RESULTS

High dietary Ca\textsuperscript{2+} intake increases Cldn14 expression. FVB/N mice were placed on a low (0.01%)-, normal (0.6%)-, or high (2%)-Ca\textsuperscript{2+} diet for 10 days. Another group of mice was placed on a low-Ca\textsuperscript{2+} diet for 10 days. We found no difference in renal Cldn14 expression in mice maintained on a low-Ca\textsuperscript{2+} diet vs. a normal diet for either period of time (Fig. 1C). Mice maintained on a low-Ca\textsuperscript{2+} diet had a 2.5-fold increase in renal expression of Cldn14 after 10 days and a similar increase after 21 days. We found no difference in renal Cldn14 expression in mice maintained on a low-Ca\textsuperscript{2+} diet vs. a normal diet for either period of time (Fig. 1G).

Active 1,25(OH)\textsubscript{2}D\textsubscript{3} increases Cldn14 expression. To determine the potential role of 1,25(OH)\textsubscript{2}D\textsubscript{3} on the expression of Cldn14, animals received daily injections of 1,25(OH)\textsubscript{2}D\textsubscript{3} or vehicle for 5 days. 1,25(OH)\textsubscript{2}D\textsubscript{3} promotes intestinal hyperab-

Fig. 1. High dietary Ca\textsuperscript{2+} increases claudin (Clhn) 14 expression. A and B: serum ionized Ca\textsuperscript{2+} (A) and urinary Ca\textsuperscript{2+}/creatinine (Crea) ratio (B) in mice receiving a diet containing low (0.01%), normal (0.6%), or high (2%) Ca\textsuperscript{2+} content for either 10 or 21 days. Also shown are parathyroid hormone (PTH; C) and 1,25-dihydroxyvitamin D\textsubscript{3} [1,25(OH)\textsubscript{2}D\textsubscript{3}; D] levels measured in serum, renal mRNA expression of 1α-hydroxylase (1α-OHase; E) and D-24-hydroxylase (24-OHase; F) in mice on different dietary amounts of Ca\textsuperscript{2+}, and renal Cldn14 mRNA expression (G). Values are means ± SE. *P < 0.05 relative to animals maintained on the control diet. #P < 0.05 relative to animals maintained on low dietary Ca\textsuperscript{2+}.
sorption and renal transport of Ca\(^{2+}\). Animals injected with 1,25(OH)\(_2\)D\(_3\) developed significantly increased levels of ionized Ca\(^{2+}\) in the blood (Fig. 2A). The urinary Ca\(^{2+}\)/Crea ratio was also elevated, but did not reach statistical significance (Fig. 2B). Serum PTH was undetectable in the mice administered 1,25(OH)\(_2\)D\(_3\), and 1,25(OH)\(_2\)D\(_3\) levels were increased (Fig. 2, C and D). The renal expression of 1\(\alpha\)-OHase significantly decreased and 24-OHase was elevated (Fig. 2, E and F). Renal Cldn14 mRNA expression increased ~10-fold in animals injected with 1,25(OH)\(_2\)D\(_3\) (Fig. 2G).

Cldn14 expression is stimulated by calcimimetics in vivo. Renal Cldn14 expression was elevated by a high-Ca\(^{2+}\) diet, a condition suppressing PTH and 1,25(OH)\(_2\)D\(_3\). In contrast, administration of 1,25(OH)\(_2\)D\(_3\) increased Cldn14 expression 10-fold while a low-Ca\(^{2+}\) diet had no effect on Cldn14 expression, even though it increased 1,25(OH)\(_2\)D\(_3\) levels. Taken together, the data suggest that elevated serum Ca\(^{2+}\) is responsible for stimulating renal Cldn14 expression. To test this hypothesis, mice were administered the calcimimetic cinacalcet hydrochloride (cinacalcet). In these animals, ionized free Ca\(^{2+}\) in the blood was significantly reduced, as expected from CaSR hyperactivation (Fig. 3A). Consistent with this, animals receiving cinacalcet developed tetany by the end of the experimental period. The urinary Ca\(^{2+}\)/Crea ratio was significantly elevated in animals treated with cinacalcet (Fig. 3B). PTH levels were undetectable, and 1,25-(OH)\(_2\)D\(_3\) levels were unaltered (Fig. 3, C and D). Renal expression of 1\(\alpha\)-OHase was decreased, and the expression of 24-OHase was increased (Fig. 3, E and F). Renal mRNA expression of Cldn14 was increased 40-fold in animals administered cinacalcet (Fig. 3G), consistent with the hypothesis that CaSR activation increases renal Cldn14 expression.

Cldn14 forms a preferential cation barrier. We hypothesized that overexpressing Cldn14 would increase TER. As such, we chose to overexpress it in a renal epithelial cell culture model with a very low TER, such as OK cells (4). OK cells were therefore stably transfected with Cldn14 bearing a C-terminal Myc-tag. Dilution potential measurements were performed in confluent monolayers of cells expressing Myc-tagged Cldn14 or cells expressing the empty vector alone (Mock). Consistent with Cldn14 forming a preferential cation barrier, overexpression markedly reduced Na\(^+\) permeability, while the permeability to Cl\(^-\) did not decrease significantly (Fig. 4, A and B). Together, this resulted in a decreased \(P_{Na}/P_{Cl}\) ratio (Fig. 4C), and a pronounced decrease in the transepithelial flux of Ca\(^{2+}\) (Fig. 4D). In line with this, overexpression of

![Fig. 2. 1,25(OH)\(_2\)D\(_3\) administration increases renal Cldn14 abundance. A and B: serum ionized Ca\(^{2+}\) (A) and urinary Ca\(^{2+}\)/Crea ratio (B) in mice injected ip with 1,25(OH)\(_2\)D\(_3\) for 5 days. Also shown are serum levels of PTH (C) and 1,25(OH)\(_2\)D\(_3\) (D), renal mRNA expression of 1\(\alpha\)-OHase (E) and 24-OHase (F) in animals treated with 1,25(OH)\(_2\)D\(_3\) or vehicle (Con), and expression of renal Cldn14 mRNA in response to chronic administration of 1,25(OH)\(_2\)D\(_3\) (G). Values are means ± SE. PTH was not detectable (ND) in mice administered 1,25(OH)\(_2\)D\(_3\). *P < 0.05 relative to animals receiving vehicle injection (control).](http://ajprenal.physiology.org/)
Cldn14 caused a clear increase in TER (Fig. 4E). Immunoblotting of whole cell lysate for Myc detected a single band of ~25 kDa, the predicted size of Cldn14, which was absent in mock-transfected cells (Fig. 4F). Expression of Cldn14 mRNA was dramatically increased in the OK cells transfected with the Myc-tagged Cldn14 construct (Fig. 4G). Immunofluorescence staining with α-Myc revealed expression of Cldn14 at cell-cell contacts, where it colocalized with ZO-1 (Fig. 4H), consistent with localization to the tight junction. In an effort to determine whether Cldn14 expression affected the expression of other claudins, we evaluated the mRNA abundance of claudins known to be expressed in the OK cell line (4). We found that overexpression of Cldn14 did not alter the expression of claudins -1, -4, -9, -12, -15, or -20. However, it induced a threefold increase in Cldn6 expression (Fig. 4I).

To verify the functional role of Cldn14 in the tight junction, we also investigated the role of Cldn14 in MDCK II cells using the Tet-off system. The results are listed in Table 1. Overexpression of Cldn14 in this system yielded changes in the P_{Na}\!//P_{Cl} ratio, P_{Ca}, and TER comparable to those observed in OK cells, suggesting that preferential blockade of cations is a direct effect of increased Cldn14 expression. This is in line with data obtained previously by Ben-Yosef et al. (3).

**DISCUSSION**

Renal regulation of Ca^{2+} excretion is central to maintaining the serum Ca^{2+} concentration within a tight range. Alterations of this process can cause hypercalciuria, leading to the formation of Ca^{2+}-containing kidney stones (22). The CaSR, which is expressed in the basolateral membrane of the TAL, plays a central role in this process. However, the downstream mechanisms after CaSR activation remain incompletely elucidated. Here, we report that CaSR stimulation prevents paracellular Ca^{2+} flux by increasing renal Cldn14 expression. This is based on the following four observations. 1) Renal Cldn14 expression is increased with a high-Ca^{2+} diet, while unaltered when dietary Ca^{2+} is low or normal, suggesting that elevated free Ca^{2+} could be the main stimulator of Cldn14 expression. 2) Increased systemic concentrations of ionized Ca^{2+} observed after chronic 1,25(OH)_{2}D_{3} administration also leads to marked increases in Cldn14 expression. 3) Administration of the calcimimetic cinacalcet, which acts on the CaSR to increase its sensitivity to Ca^{2+}, potently induces a 40-fold increase in the abundance of Cldn14. 4) Overexpression of Cldn14 markedly increases transcellular resistance and decreases paracellular Ca^{2+} flux. Taken together, these data suggest a mechanism...
whereby increased circulating $\text{Ca}^{2+}$ activates the CaSR, causing increased renal Cldn14 expression. This in turn blocks $\text{Ca}^{2+}$-permeable paracellular pores, preventing the increased amount of filtered $\text{Ca}^{2+}$ from being reabsorbed back into the blood. Ultimately, a $\text{Ca}^{2+}$ load is excreted, returning circulating $\text{Ca}^{2+}$ to normal levels.

Multiple studies have described the renal localization of Cldn14 with conflicting results (3, 9, 19). Recently, a detailed
expression induced by high dietary Ca²⁺ neuvers markedly upregulated renal Cldn14 mRNA expression (12). We found that CaSR activation by different dietary or pharmacological manipulations markedly upregulated renal Cldn14 mRNA expression induced by high dietary Ca²⁺. This suggests that CaSR activation is responsible for altered levels of expression. However, the major pathway responsible for altered Cldn14 expression is likely via the CaSR, as knockdown of the receptor in vitro ablates the ability of Ca²⁺ to increase Cldn14 expression (12). We found that CaSR activation by different dietary or pharmacological maneuvers markedly upregulated renal Cldn14 mRNA expression. Gong et al. (12) found that increased renal Cldn14 mRNA expression induced by high dietary Ca²⁺ translates into increased renal Cldn14 protein abundance. We expect therefore that a similar relationship is present in our experiments.

Paracellular Ca²⁺ transport across the TAL depends on a lumen-positive transepithelial potential. This gradient appears to be generated by two interdependent mechanisms. The first is the result of asymmetrical secretion of electrolytes, after their influx into TAL epithelial cells. This contributes to a lumen-positive voltage ranging from 5 to 10 mV (14, 15). The second mechanism is the consequence of Na⁺ backflux into the lumen of the cortical TAL, a process potentially further increasing the transepithelial potential difference to values as high as 30 mV (13, 15, 16, 17, 29). Backflux of Na⁺ occurs via the Cldn16/Cldn19 complex, which forms a cation-permeable pore (17). This same complex likely also permits the paracellular reabsorption of divalent cations down their electrochemical gradient. Mutations in Cldn16 or Cldn19 reduce cation selectivity of this complex and cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC). The pathogenesis of this disease has largely been attributed to a loss of Na⁺ backflux, which decreases the lumen-positive driving force (17, 20, 32).

Recently, Gong et al. (12) demonstrated that Cldn14 can interact with Cldn16, but not Cldn19 (12). However, Cldn16 has the ability to bind both claudins. Therefore, the three claudins could potentially exist as a complex. Consistent with this, the coexpression of all three claudins significantly reduced Na⁺ permeation relative to overexpression of just Cldn16 and Cldn19 together. Our data are in agreement with this observation. Overexpression of Cldn14 in OK and MDCK II cells dramatically reduces the $P_{Na}/P_{Cl}$ ratio and $P_{Ca}$, in line with previous reports suggesting that Cldn14 acts as a preferential cation barrier (3, 12). We also observed a threefold increase in the expression of Cldn6, when we overexpressed Cldn14 in OK cells. This contrasts with the ~200-fold increase in Cldn14. As Cldn6 has been shown to decrease $P_{Na}$, $P_{Cl}$, and TER, we cannot exclude the possibility that some of the effect of Cldn14 in OK cells is mediated by increased Cldn6 expression (31). Thus increased Cldn14 expression in the cortical TAL would prevent Ca²⁺ reabsorption by 1) reducing the permeability of the pore to Ca²⁺ and 2) blocking backflux of Na⁺, thereby decreasing the electrochemical gradient driving paracellular Ca²⁺ flux across this segment.

Estimates of the concentration of Ca²⁺ at the bend of the loop of Henle suggest it is almost double (~3 mM) that of superficial proximal puncture sites (~1.8 mM) (2). A high reabsorptive capacity for Ca²⁺ from the TAL is evinced by significantly lower concentrations of Ca²⁺ in fluid obtained from more distal puncture sites (2). Dietary Ca²⁺ loading is expected to increase the distal delivery of Ca²⁺ by several-fold (~3-fold in our dietary Ca²⁺ experiments). During such conditions, modification of the lumen-positive voltage would likely not be enough to prevent significant paracellular reabsorption across the TAL due to the chemical gradient present (lumen to blood). Consequently, altered paracellular $P_{Ca}$ likely contributes significantly to decreased TAL Ca²⁺ reabsorption after activation of the CaSR.

SNPs in the CLDN14 gene correlate with kidney stone formation, osteopenia, and hypercalciuria. How Cldn14 could cause these abnormalities was not apparent, since a correlation between the risk variants and mRNA expression of Cldn14 in adipose and peripheral blood samples was not observed (34). Very recent data (12) and this study together suggest that significant renal CaSR activation is strongly dependent on activation of the CaSR. Low baseline renal expression of Cldn14 explains why patients lacking CLDN14 do not demonstrate evidence of altered Ca²⁺ homeostasis (i.e., stone formation or osteopenia) (38), although detailed physiological characterization may reveal that they are unable to effectively excrete a Ca²⁺ load. Moreover, the urinary excretion of Ca²⁺ in Cldn11/Cldn14 double knockout mice was not altered, providing further evidence that the absence of Cldn14 does not impact renal Ca²⁺ handling under normal dietary conditions (9). A critical experiment confirming a role for Cldn14 in renal Ca²⁺ handling was recently performed (12). Cldn14-deficient mice were placed on a high-Ca²⁺ diet and found to have relative hypomagnesuria and hypocalciuria compared with wild-type controls (12). This response would be expected in the absence of the CaSR-Cldn14 axis, as an increased lumen-to-blood Ca²⁺ concentration gradient would favor increased paracellular Ca²⁺ reabsorption from the TAL.

In some patients, autosomal dominant hypocalcemic hypercalciuria is a result of activating mutations in the CASR gene (26, 27). Mild asymptomatic hypocalemia is generally observed in these patients (26). These mutations cause the maximal activation of the receptor to increase (26, 27). More severe activating mutations in the CaSR cause an autosomal dominant form of Bartter’s syndrome (27, 35, 37). These individuals have a classic Bartter-like phenotype but differ clinically from individuals with classic Bartter as they have decreased PTH, hypocalemia, renal Ca²⁺ wasting, and nephrocalcinosis. Discovery of the CaSR-Cldn14 axis provides further insight into these symptoms. Activating mutations in

### Table 1. MDCK type II cells expressing Cldn14 controlled by the Tet-Off system

<table>
<thead>
<tr>
<th>Measurement</th>
<th>+Dox (Control)</th>
<th>−Dox (Cldn14 Expressed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TER, 11°cm²</td>
<td>65 ± 8</td>
<td>102 ± 14*</td>
</tr>
<tr>
<td>$P_{Na}$, 10⁻³ cm/s</td>
<td>2.35 ± 0.4</td>
<td>1.27 ± 0.1*</td>
</tr>
<tr>
<td>$P_{Cl}$, 10⁻³ cm/s</td>
<td>1.20 ± 0.2</td>
<td>0.88 ± 0.2</td>
</tr>
<tr>
<td>$P_{Na/P_{Cl}}$</td>
<td>2.18 ± 0.4</td>
<td>1.50 ± 0.1*</td>
</tr>
<tr>
<td>$P_{Ca}$</td>
<td>47.2 ± 1.7</td>
<td>31.8 ± 3.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. MDCK, Madin-Darby canine kidney; Cldn, claudin; Dox, doxycycline; TER, transepithelial resistance; $P$, permeability. *$p < 0.05$; $n = 3$ condition.
the CaSR would increase Cldn14 expression inappropriately, causing renal Ca2+ wasting and nephrocalcinosis. Polymorphisms in the CaSR have also been implicated in idiopathic hypercalciuria (36). As both Cldn14 and the CaSR are now part of a common pathway, association studies may increase their power by grouping CaSR and Cldn14 together. Finally, whether cinacalcet poses a potential risk for the development of kidney stones in patients with primary hyperparathyroidism remains to be established.

In conclusion, our results extend recently published data suggesting that Cldn14 is regulated via a novel CaSR-dependent mechanism (12). Based on these findings, it is likely that Cldn14 plays a key role in the regulation of renal Ca2+ excretion. Elegant work from Gong et al. (12) suggests that micro-RNAs binding to the 3′-untranslated region of Cldn14 are regulated by CaSR signaling and in turn alter Cldn14 expression. Whether changes in the micro-RNA recognition sites are responsible for hypercalciuria in humans remains to be determined. Regardless, alterations in the CaSR-Cldn14 axis likely contribute importantly to the development of hypercalciuria and the formation of kidney stones.

ACKNOWLEDGMENTS

We thank Drs. J. Casey and S. Frische for insightful comments on the manuscript.

GRANTS

H. Dimke is supported by the Danish Medical Research Council (Forskningsrådet for Sundhed og Sygdom). This work was funded by grants from the Kidney Foundation of Canada and the Canadian Institute of Health Research (To R. T. Alexander). R. T. Alexander is supported by a Clinician Scientist Award from the Canadian Institutes of Health Research and an Alberta Innovates Health Solutions Clinical Investigator Award.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

CaSR activation increases Cldn14 expression.


