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New Functional Aspects of the Extracellular Calcium-Sensing Receptor

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Abstract

Purpose of review—Variations in extracellular calcium level have a large impact on kidney function. Most of the effects seen are attributed to the calcium-sensing receptor (CaSR), a widely expressed G-protein-coupled cell surface protein with important function in bone mineral homeostasis. The purpose of this review is to recapitulate novel functional aspects of CaSR.

Recent findings—Results from mouse models surmise important functions for CaSR in various tissues. In the kidney, the main role of CaSR is the regulation of calcium reabsorption in the thick ascending limb, independently of its role on parathyroid hormone secretion. CaSR modulates claudin 14, the gatekeeper of paracellular ion transport in the thick ascending limb that is associated with urinary calcium excretion. One intracellular signaling pathway by which CaSR alters tight junction permeability is the calcineurin-NFAT1c-microRNA-claudin14 axis.

Summary—The main function of CaSR in the kidney is the regulation of calcium excretion in the thick ascending limb, independently of parathyroid hormone. CaSR modulates paracellular cation transport by altering expression of the tight junction protein claudin 14. Still more work is needed to fully understand all functions of CaSR in the kidney. Alternative pathways of calcium "sensing" in the kidney need to be investigated.

Keywords

calcium-sensing receptor; paracellular transport; claudin 14; thick ascending limb

Introduction

Calcium affects diverse biological processes in the body ranging from bone formation to blood pressure regulation. The tight control of serum calcium levels is crucial for the normal function of various organs, including the kidney. Changes in serum calcium levels directly affect renal tubular physiology. Acute hypercalcemia increases urinary calcium, magnesium and salt excretion, while urinary phosphate excretion and pH level are typically decreased in this setting (1). The exact mechanisms for these effects are not entirely understood. Chronic

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Conflicts of interest
There are none.

hypercalcemia has been associated with decreased urinary concentrating ability and decreased water reabsorption (2). Variations in extracellular calcium level also affect 1-α-hydroxylation of 25-hydroxy-vitaminD and thereby its activity (3). Several studies have demonstrated that these effects occur in part directly through calcium, acting as a “first messenger” in the kidney, independent of changes in hormone levels that regulate calcium homeostasis (4).

A key step towards understanding calcium actions as “first messenger” was the cloning of the extracellular calcium-sensing receptor (CaSR) from bovine parathyroid gland tissue (5). CaSR is a dimer-forming G-protein-coupled receptor (GPCR) consisting of three protein domains. The extracellular domain (ECD) binds the principal physiological ligand calcium and other polyvalent molecules (6). The seven-transmembrane domain (7TMD) couples the receptor to activating or inhibitory G-proteins, which transduce intracellular signals (7). The C-terminal tail can bind filamin-A, contribute to CaSR localization in caveolae, and link it to the actin-based cytoskeleton (8).

CaSR is highly expressed in the parathyroid gland where it maintains extracellular calcium levels by regulating parathyroid hormone (PTH) release. Numerous animal and *in vitro* studies have investigated CaSR function in several tissues, including cell types without direct roles in calcium homeostasis. Recent data suggest critical functions of CaSR also in the bone (9), intestine (10), developing lung (11), colon (12), epidermis (13) and mammary gland (14). However, individuals with inherited CaSR dysfunction do not typically display clinical findings consistent with these findings and they live a “relatively healthy” life when their abnormal PTH release is addressed (15). Explanations for this discrepancy include a) only “partial” CaSR dysfunction in reported inherited human cases, b) genetic redundancy for calcium-sensing, c) physiological differences in CaSR function among species, d) non-specific effects of allosteric CaSR modulators, and e) use of limited model systems to study CaSR functions (16).

The Role of CaSR in Human Disease

Inherited *CASR* alterations cause three distinct disorders of calcium homeostasis. Heterozygosity for inactivating mutations is responsible for familial hypocalciuric hypercalcemia (FHH), while bi-allelic loss-of-function mutations cause neonatal severe hyperparathyroidism (NSHPT) (17). Individuals with FHH have typically a life-long mild increase in serum calcium level, along with increased magnesium reabsorption (18). Interestingly, in contrast to individuals with primary hyperparathyroidism, FHH patients may have preserved urinary concentrating ability (19).

Not all FHH cases are caused by *CASR* mutations. Autoantibodies to the ECD can impair activation of CaSR, mimicking the FHH phenotype (20). Recently, loss-of-function mutations in *GNA11*, encoding for the G-protein Gα11, have been identified as cause for FHH type 2 (21)■. *In vitro* data suggested that *GNA11* mutations decrease the sensitivity of CaSR-expressing cells to extracellular calcium, presumably due to decreased signal transduction by altered Gα11 protein. Missense mutations in *AP2S1*, encoding for the σ-subunit of the adaptor protein-2 (AP2), have also been reported as cause for FHH cases

negative for *CASR* mutations (FHH type 3) (22)■. AP2 is a ubiquitously expressed protein with a central role in clathrin-mediated endocytosis and internalization of GPRCs in general. All documented mutations altered the amino acid arginine at position 15 (Arg15) of the AP2-σ-subunit. The investigators speculated that the Arg15 residue of AP2-σ-subunit is specific for recognizing the C-terminal dileucine motif of CaSR for its internalization, thereby "only" causing a FHH phenotype (22)■. They hypothesized mutations in other *AP2S1* codons could affect different tissues and result in different diseases.

By contrast, activating *CASR* mutations cause autosomal-dominant hypocalcemia (ADH type 1) with hypercalciuria and, in some cases, renal salt wasting, resembling Bartter's syndrome (23). Gain-of-function mutations in *GNA11* were recently reported as cause for ADH type 2 (21)■. If individuals with ADH type 2 are affected by salt wasting is unclear. Notably, a role for CaSR in renal salt handling was also suggested by a small study in parathyroidectomized individuals with CaSR loss-of-function mutations. These individuals showed a markedly reduced natriuretic response to calcium infusion (24).

Recent genetic population studies investigated the association of allelic *CASR* variants with various common diseases, including kidney stones (25), hypertension (26), coronary heart disease (27), diabetes mellitus (27), bone mineral density (28), Alzheimer disease (29), epilepsy (30), pancreatitis (31) and various cancers (27, 32). These studies showed either no association with allelic *CASR* variants (27, 28, 33), minor effects on the tested outcomes (25, 27, 34), or non-replicable results, which may be related to genetic heterogeneity of the tested populations (26, 27, 32, 35). More recently, we tested the association of rare allelic variants in 40 genes associated with urinary calcium excretion in 960 well-characterized individuals, including *CASR* (36). We found no association with allelic *CASR* variation, instead our data suggested association of urinary calcium excretion with claudin 14, which had been associated with nephrolithiasis and bone mineral density in a large genome-wide association study (37).

Mouse Models of CaSR

To study the importance of CaSR in tissues outside the parathyroid gland, various CaSR-deficient mouse models were generated, which are summarized in Table (9, 10, 13, 14, 38–42). Mice lacking both copies of *Casr* in all tissues, generated by "targeting" *Casr* exon 5, recapitulated human NSHPT (38). Crossing these Δ*Casr*E5 mice with animals lacking PTH expression markedly improved the NSHPT phenotype (43). However, the ability to increase calcium excretion in response to calcium loading remained decreased (44). Further studies in Δ*Casr*E5 mice revealed the expression of functional *Casr* splice variants lacking *Casr* exon 5 in some tissues (45, 46), rendering this germline CaSR-deficient mouse model incomplete. This discovery prompted the development of another CaSR-deficient mouse model (Δ*Casr*E7), which allows to "knockout" *Casr* in selected cell types by Cre/loxP recombination. This model was used to study CaSR in numerous tissues including chondrocytes (utilizing the collagen 2 alpha 1 promoter, Col2α1). Surprisingly Col2α1Δ*Casr*E7 mice developed complete embryonic lethality (41). This finding could be related to the intact intracellular expression of a truncated "Δexon7-CaSR" protein, resembling the ECD (encoded by *Casr* exons 2–6). Although the Δexon7-CaSR expression

was not toxic to HEK293 cells (41), truncated or mutated proteins have led to dominant-negative phenotypes in other transgenic animal models (47, 48). Additionally, the Δexon7-CaSR is likely secreted in considerable amounts. The ECD is known to get into the secretory pathway in the absence of the endoplasmic reticulum retention signal of full-length CaSR (49). Therefore Δexon7-CaSR could presumably be at high concentrations in restricted intercellular spaces that are hard to mimic by adding exogenous ECD. Therefore, both secreted as well as intracellular Δexon7-CaSR could have unwanted effects in target tissues of conditional Δ*Casr*E7 mice (Table).

We developed a conditional Δ*Casr*E3 mouse model, targeting the second translated *Casr* exon, that featured complete CaSR deficiency on both the RNA and protein levels, thereby avoiding possible limitations of the previous mouse models (40)■. Homozygous Δ*Casr*E3 null mice recapitulated human NSHPT. Increased perinatal lethality as previously reported in Δ*Casr*E5 was also present, probably due to severe hypercalcemia (38). Notably, “partial” embryonic lethality was observed in Δ*Casr*E3 probably occurring about E13. The expected Mendelian proportion of the null genotype (~25%) was significantly lower about E18 (~11%) (unpublished data). We also tested our model under the expression of the chrondrocyte-specific Col2α1 promoter that had been used in Δ*Casr*E7 mice (41). Our Col2α1Δ*Casr*E3 mice were born at expected Mendelian ratio with life expectancy of 3–5 weeks. Their phenotype was consistent with severe hyperparathyroidism (Table). Further studies, utilizing a Cre reporter mouse (mT/mG) to test the tissue specificity of Col2α1 expression (unpublished data), showed that Col2α1 is expressed not only in chondrocytes but also in the parathyroid gland and other tissues (42).

A mouse model with an activating *Casr* mutation (Leu723Gln) in the 7TMD featured hypoparathyroidism associated with hypocalcemia and hyperphosphatemia (Table) (39). This mutant mice displayed no evidence of salt wasting, in contrast to human *CASR* gain-of-function mutations (23). Species differences with regard to CaSR and electrolyte transport in the TAL could explain this discrepancy. Notably, rats treated with a CaSR antagonist can develop hypertension (50); however, the mechanism of blood pressure rise in these animals is unclear (51, 52).

Expression of CaSR in the Kidney

Several studies showed CaSR expression throughout the kidney with segment-specific polarization (53, 54). Expression was abundant on the basolateral membrane in the cortical (CTAL) and medullary TAL (MTAL). Distinct apical CaSR staining was detected in H+-ATPase-positive intercalated cells in the connecting tubule (CNT) and cortical collecting duct (CCD) as well as in cells of the inner medullary collecting duct (IMCD). In the proximal tubule (PT), CaSR was found in the apical brush border. Co-localization of CaSR expression with the TRPV5 calcium channel in the distal convoluted tubule (DCT) and CNT was also reported (55). In the developing kidney, CaSR expression was described in the TAL and distal tubule (56).

These reports prompted many *in vitro* and *in vivo* studies that suggested roles for CaSR in many tubular functions, including calcium reabsorption, phosphate homeostasis, urinary

acidification and concentration, and renin release (57). Recently, however, Houillier and colleagues applied quantitative PCR experiments to microdissected tubular segments from rodents kidneys (58). They found CaSR expression to be highest in MTAL and CTAL. Only faint expression was found in the CCD, while expression was undetectable in other tubular segments. Immunohistochemistry studies localized CaSR protein exclusively on the basolateral membrane of TAL cells with no detectable expression in the PT, DCT, CNT or CD. Our expression data showed that CaSR is heavily expressed in the TAL and absent in the distal nephron. Significantly increased *CYP27B1* (1-α-hydroxylase) mRNA levels from kidney tissue of renal tubule-specific Δ*Casr*E3 mice suggested that CaSR is expressed in the PT (40)■. Expression of CaSR in the glomerulus was also reported (59), but this finding could not be reproduced (58).

Based on the literature, there is consensus on CaSR expression on the basolateral membrane of the TAL, but the degree of expression on the apical aspects of the nephron and the glomerulus remain debated among different research groups (57).

Function of CaSR in the Thick Ascending Limb

Previous data suggested that CaSR activation in the TAL reduces the activity of apical potassium channels (60), leading to a decreased lumen-positive potential difference and thereby reducing (passive) paracellular cation transport (61). In contrast, Houillier and colleagues showed that parathyroidectomized rodents on stable PTH infusion develop hypocalciuria and increased serum calcium level when exposed to CaSR antagonist, suggesting PTH-independent functions of renal CaSR (58). In microperfusion studies of isolated TAL tubules, they found no alteration in sodium flux or transepithelial voltage despite increased paracellular calcium transport. This finding suggested that the increase in paracellular ion permeability is not due to altered transepithelial electrical gradient.

Renal tubule-specific Δ*Casr*E3 mice (rtCasr−/−) displayed normal serum concentrations of calcium, magnesium and PTH in the presence of relative hypocalciuria (40)■, which was more pronounced under high calcium diet (Figure 1A). RNA expression studies from renal tissue in these mice revealed ~80% decreased claudin 14 expression (*CLDN14*; Figure 1B) and a slight but significant increase in claudin 16 expression (*CLDN16*; Figure 1C). *In vitro* data demonstrated that CLDN14 inhibits paracellular ion permeability in the TAL by negative regulation of the tight junction CLDN16/CLDN19 heterodimer complex presumably via direct interaction with CLDN16 (62). This finding is supported by CLDN14-deficient mice, which feature hypocalciuria despite high calcium diet (62). These recent data suggested that CaSR controls paracellular calcium transport in the TAL by regulating CLDN14, the “watchdog” of paracellular ion permeability. The main role of CaSR in the kidney is the inhibition of calcium reabsorption (40)■.

CaSR activation was previously shown to regulate calcineurin activity, which is necessary for prostaglandin production in the TAL (63). The calcineurin-NFAT (Nuclear Factor of Activated T-cells) pathway is a widely studied canonical calcium-signaling pathway, regulating gene transcription. Gong *et al.* investigated the mechanism by which CaSR regulates CLDN14 *in vivo*, utilizing CLDN14-deficient mice and transgenic animals

overexpressing CLDN14 (64)■. By using pharmacological agents, they demonstrated that CaSR regulates CLDN14 via calcineurin-NFATc1-microRNA signaling pathway, thereby altering tight junction (TJ) permeability for calcium and magnesium in the TAL (Figure 2). Their data showed that CaSR inhibition can activate calcineurin, which then dephosphorylates NFATc1 (NFAT, cytoplasmic 1). Nuclear translocation of activated NFATc1 increases expression of the microRNAs miR-9-1 and miR-374, which decrease CLDN14 mRNA level (62). The resultant reduction in CLDN14 protein level leads to activation of the paracellular ion channel formed by the CLDN16/CLDN19 complex, increasing calcium reabsorption (62). The calcineurin inhibitor cyclosporine abolishes the effects of CaSR inhibition *in vitro*, potentially explaining how cyclosporine induces hypercalciuria (65). The potential importance of CaSR in regulating the TJ assembly was further demonstrated by recent *in vitro* studies in MDCK cells. CaSR activation induced plasmalemmal deposition of TJ components, such as ZO-1 (66)■, altering transepithelial electrical resistance and presumably TJ barrier function.

Renal effects of hypercalcemia are not fully reproduced by activating renal CaSR in the TAL (58, 67). This was shown by studying the effects of CaSR agonists in comparison to high calcium diet in parathyroidectomized and PTH-supplemented rats. CaSR agonist decreased calcium reabsorption, inducing a negative calcium balance and lowering serum calcium concentration. However, CaSR activation did not change urinary excretion of sodium, phosphate, magnesium, or urine volume. By contrast, high calcium diet raised blood calcium concentrations and elicited renal salt wasting and water losses. These findings suggest that extracellular calcium can act on the renal tubule through ‘sensors’ other than CaSR (67).

One of these calcium sensors in the kidney could be GPRC6A, which shares ~34% amino acid sequence identity with CaSR (also known as GPRC2A) (68). Interestingly, GPRC6A can be modulated by CaSR ligands, and even “responded” to CaSR modulators, albeit not in a specific fashion (69). Germline GPRC6A-deficient mice featured complex metabolic changes, including hypercalciuria (70). Similar to CaSR, GPRC6A is ubiquitously expressed (71). Its exact localization in the kidney remains uncertain, but it appeared to be heavily expressed in the CD (67). Recent data showed that GPRC6A, as previously reported for CaSR, can modulate acid secretion in the distal nephron segment (67). These data implied that GPRC6A could serve as another calcium sensor in the kidney, functioning independently from CaSR, and perhaps subserving distinct functions. Further investigations on other renal GPCRs that can bind calcium and could form heterodimers with CaSR or GPRC6A are necessary.

Function of CaSR in the Proximal Tubule

The PT plays a critical role in maintaining extracellular fluid volume, calcium excretion, citrate and phosphate transport, 1-α-hydroxylation of vitaminD, and urinary acidification. Despite the “controversial” CaSR expression data, there are several lines of functional data that suggest biological roles for CaSR in this tubule segment (72). CaSR has been shown to regulate PTH-suppressible phosphate reabsorption in the PT (73), modulate 1-α-hydroxylation of 25-hydroxy-vitaminD (3), and dampen the response to 1,25-dihydroxy-

vitaminD independently of PTH action (74). Nevertheless, the potential functions of CaSR in this nephron segment remain overall ill-defined. One contributing factor is that CaSR expression may not only be regulated by calcium, but also by PTH, 1,25-dihydroxy-vitaminD, dietary phosphate, and fibroblast growth factor 23 (FGF23), making CaSR function in the PT quite complex (75).

Recent data highlight a role for CaSR in fluid reabsorption and acid secretion in the PT (76)■. Geibel and colleagues utilized both *in vivo* micropuncture in rats and *in vitro* perfused mouse PT segments. In wildtype rodents, raising luminal calcium or adding a CaSR agonist caused increased proton secretion and fluid absorption, while no effect was seen in Δ*Casr*E5 mice. The investigators postulated that CaSR might regulate the luminal sodium-proton-exchanger (NHE), thereby increasing acid secretion. This could lead to increase ionization of luminal calcium, potentially enhancing its reabsorption in later tubule segments. In their studies, they also demonstrated that CaSR function might be required for transepithelial fluid flux in the PT in response to apical changes of divalent ion concentration (76)■.

Although the studies on CaSR expression in the PT are controversial, *in vitro* data support an intertwined role for CaSR in PT physiology with the sodium-phosphate cotransporter 2a (NaPi-2a), PTH and its receptor PHT1R, 1-α-hydroxylase, vitaminD, and FGF23. However, the relatively “mild” phenotype of rtCasr−/− mice (hypocalciuria) with normal PT anatomy and absence of significant changes in serum calcium, PTH, 1,25-dihydroxy-vitaminD, phosphate and FGF23 level suggest that CaSR may not have a major biological role in this tubular segment (40)■. Genetic redundancy of calcium-sensing in the whole organism may blunt tissue or cell-specific roles for CaSR, which could be a possible cause for inconsistent results.

Conclusion

Recent studies establish that CaSR in the kidney modifies paracellular calcium reabsorption in the TAL by regulating the tight junction protein CLDN14. The role of renal CaSR in net sodium chloride and water transport remains unclear due to inconsistent data. It seems likely that additional calcium-sensing mechanisms exist along the renal tubule responding to and regulating changes in blood and urine calcium levels. Further investigations examining all renal calcium-sensing mechanisms, including CaSR, will help elucidate the role of extracellular calcium-sensing in renal physiology.

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Key Points

1. Inherited syndromes caused by mutations in *CASR* can be mimicked by DNA sequence variations in at least two other genes, *GNA11* and *AP2S1*, which are involved in CaSR signal transduction and trafficking.
2. Renal CaSR regulates the paracellular ion permeability in the TAL, independently of PTH release. The role of CaSR in the kidney is the inhibition of calcium reabsorption.
3. CaSR alters the expression of the tight junction protein claudin14 in the TAL in response to changes in serum calcium levels. One of its intracellular signaling pathways involves NFATc1 and microRNAs.
4. The existence of other extracellular calcium sensors is likely.

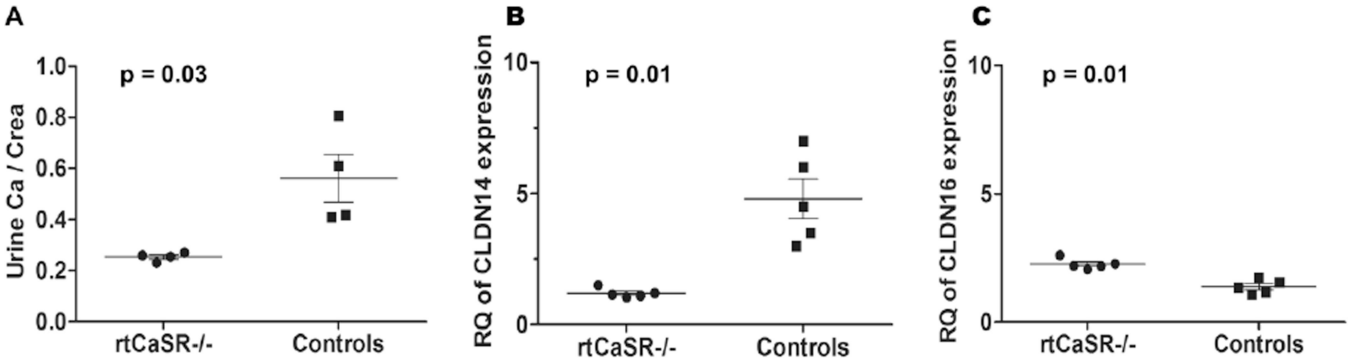

Figure 1.

Three-month-old renal tubule-specific CaSR-deficient mice (rtCasr−/−) compared to littermate controls. **A**. Urinary calcium to creatinine ratio (Uca/crea) in rtCasr−/− mice demonstrate relative hypocalciuria under high calcium diet (1.5% calcium chloride drinking water); 0.25±0.02 vs. 0.56±0.2. **B.** Relative quantitation (RQ) of claudin14 (CLDN14), a negative regulator of paracellular ion transport in the thick ascending limb (TAL), displays ~80% decreased expression levels in rtCasr−/− from whole kidney RNA; 1.2±0.18 vs. 4.8±1.7. **C.** On the contrary, expression level of claudin16 (CLDN16), which facilitates paracellular cation transport in the TAL (together with claudin19), is slightly but significantly increased; 2.3±0.2 vs. 1.4±0.8. Data were analyzed with non-parametric test.

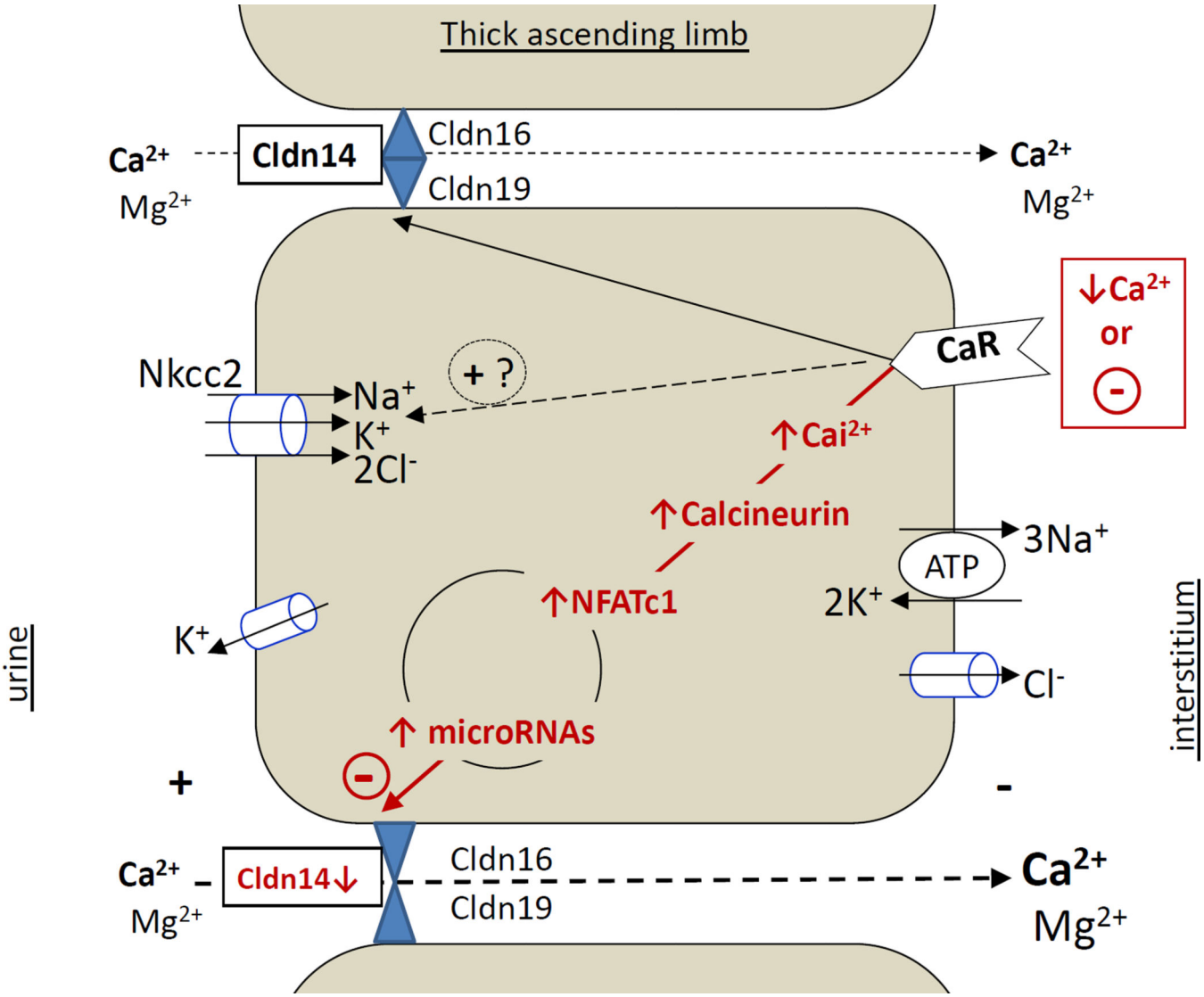

Figure 2.

Simplified schema of a renal tubular epithelial cell in the TAL illustrating the CaSR-NFATc1-microRNA-CLDN14 pathway. CaSR activity on the basolateral membrane determines intracellular calcium-signaling and calcineurin activity, thereby regulating NFATc1 via (de)phosphorylation. When NFATc1 is activated, it translocates to the nucleus and increases transcription of CLDN14-regulating microRNAs miR-9-1 and miR-374. These microRNAs decrease CLDN14 expression and thereby alter the cation permeability of the paracellular ion channel formed by the CLDN16/CLDN19 heterodimer complex. CaSR could regulate CLDN14 by additional mechanisms as well. Abbreviations: CaSR = calcium-sensing receptor, NFATc1 = Nuclear Factor of Activated T-cells, cytoplasmic 1, CLDN = claudin, TAL = thick ascending limb

Table

Phenotype of germline (Δ*Casr*E5, *Casr*Leu723Gln) and conditional (Δ*Casr*E3, Δ*Casr*E7) CaSR mouse models

Target tissue	**Δ*Casr*E5 (extracellular domain, ECD)**	***Casr*Leu723Gln (transmembrane domain, 7TMD)**	**Δ*Casr*E3 (extracellular domain, ECD)**	**Δ*Casr*E7 (7TMD & intracellular domain)**
Germline	hyperparathyroidism, hypercalcemia, osteomalacia, short lifespan (38) [Pgk]	hypoparathyroidism, hypocalcemia, hyperphosphatemia, hypercalciuria, ectopic calcifications, cataracts (39) *IPMS mutagenesis*	hyperparathyroidism, hypercalcemia, osteomalacia, short lifespan, "partial" embryonic lethality (40) [Prm1]	
Parathyroid gland [Pth]				hyperparathyroidism, hypercalcemia, hypercalciuria, growth retardation, short lifespan (41)
Chondrocyte [Col2α1]*			hyperparathyroidism, # hypercalcemia, hypercalciuria, hypophosphatemia, hyper-phosphaturia, short lifespan (42)	embryonically lethal (41)
Osteoblast			temporary growth delay [Osx] #	severely retarded bone development [Osx, 2.3Col, 3.6Col]; severe osteoporosis [Ocn, Dmp1] (9)
Renal tubules [Six2]			Hypocalciuria (40)	
Mammary gland epithelia [Blg]				hypoparathyroidism, hypercalcemia, hypercalciuria, decreased bone formation (14)
Keratinocyte [K41]				impaired keratinocyte differentiation and permeability barrier homeostasis (13)
Intestine[Vil1]				hyperproliferation of colon epithelia (10)

*Not chondrocyte-specific (also expressed in parathyroid gland).

#Unpublished data. Promoters used to generate germline / tissue-specific mice are shown in brackets:Pgk = 3-phosphoglycerate kinase, Prm1 = Protamine 1, Pth = Parathyroid hormone, Col2α1 = Collagen 2 α 1, Osx = Osterix, 2.3Col = Collagen 1, 3.6Co l= Collagen 1,Ocn = Osteocalcin, Dmp1 = Dentin Protein Matrix 1, Six2 = Sine oculis 2, Blg = Betalacto-globulin, K14 = type 1 Keratin, Vil1 = Villin 1; IMPS = isopropyl methanesulfonate