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Thick ascending limb: the Na^+:K^+:2Cl^- co-transporter, NKCC2, and the calcium-sensing receptor, CaSR

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Abstract

The thick ascending limb of Henle's loop is a nephron segment that is vital to the formation of dilute and concentrated urine. This ability is accomplished by a consortium of functionally coupled proteins consisting of the apical Na^+:K^+:2Cl^- co-transporter, the K^+ channel, and basolateral Cl^- channel that mediate electroneutral salt absorption. In thick ascending limbs, salt absorption is importantly regulated by the calcium-sensing receptor. Genetic or pharmacological disruption impairing the function of any of these proteins results in Bartter syndrome. The thick ascending limb is also an important site of Ca^{2+} and Mg^{2+} absorption. Calcium-sensing receptor activation inhibits cellular Ca^{2+} absorption induced by parathyroid hormone, as well as passive paracellular Ca^{2+} transport. The present review discusses these functions and their genetic and molecular regulation.

Keywords

Diuretics; Hypertension; Salt transport; Regulation; Phosphorylation; Isoforms

The function of the thick ascending limb of Henle's loop (TAL) is critical for salt absorption, for the regulation of divalent mineral cation, and acid–base metabolism. The TAL is also essential for the generation and maintenance of the countercurrent multiplication mechanism that allows the kidney to produce urine that can be more diluted or concentrated than plasma, a functional capacity that is essential for the survival of mammals that live on land, including human beings. Pioneering studies by Burg and Green [22] and Rocha and Kokko [128] were the first to suggest that chloride was absorbed by the TAL and that this process was inhibited by loop diuretics such as furosemide or bumetanide. Then, in the early 1980s, studies by Greger and co-workers [64, 65] and Hebert et al. [76, 77] established that the major salt transport pathway in apical membranes of TAL is an electroneutral Na^+:K^+:2Cl^- co-transporter that is specifically inhibited by loop diuretics and activated by hormones

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This review is dedicated to the memory of Dr. Steven C. Hebert. Dr. Hebert, our colleague and friend, was a leader in elucidating the mechanism and regulation of salt and water balance by the kidney. He embodied the foresight to anticipate many of the elements contributing to renal homeostasis, an infectious enthusiasm that inspired fellows and colleagues alike, and an open and welcoming collegiality that represents all the best traits of academia.

acting through Gαs-coupled receptors such as vasopressin. Ten years later, work by Hebert and collaborators was crucial to define the molecular nature of ion transport mechanisms in TAL by isolating the complementary DNA (cDNA) encoding the renal-specific, apically expressed, bumetanide-sensitive Na^+:K^+:2Cl^- co-transporter, NKCC2 [30]; the inward-rectifier potassium channel, ROMK [5]; and the basolateral calcium-sensing receptor, CaSR [18, 123]. In the present work, we review some of the knowledge that has become available during the last 15 years as a consequence of cloning NKCC2, ROMK, and CaSR.

The molecular mechanisms of salt absorption by TAL are depicted in Fig. 1. As in many epithelia, the Na^+:K^+-ATPase, polarized to basolateral membranes, generates the gradient for sodium entry across apical membranes [66] in which most of the sodium movement occurs though NKCC2 [64, 65, 67, 68, 76]. Salt absorption by TAL, however, requires the simultaneous operation of several transport proteins (Fig. 1). Sodium and chloride ions traversing at the apical cell surface by way of NKCC2 leave the cell at the basolateral membrane through the Na^+:K^+-ATPase and Cl^- channels (CLC-KB). These membrane proteins are composed of two subunits, one that mediate the transport function and another that is necessary to chaperon the protein to the plasma membrane [56, 88, 96, 122, 134, 135, 151]. The chaperon subunits are known as β-subunit for the Na^+:K^+-ATPase and Barttin for CLC-KB. Potassium ions entering across apical plasma membranes are returned to the tubular lumen via ROMK. The potassium concentration of glomerular ultrafiltrate (4 mEq/L) is much lower than that of sodium (145 mEq/L) or chloride (110 mEq/L). Without recirculation, the K^+ concentration in the TAL lumen would be rapidly reduced, stopping the function of NKCC2. Thus, K^+ recycling ensures that its concentration within the TAL lumen remains constant in order to allow proper function of NKCC2. Additionally, the lumen-positive voltage of TAL resulting from K^+ recycling drives absorption of a second cation (Na^+, Ca^{2+}, Mg^{2+}) through the paracellular pathway. Therefore, the coordinated function between NKCC2, ROMK, and CLC-KB, on the one hand, renders TAL epithelial cells thermodynamically more efficient because two cations are reabsorbed at the expense of ATP needed to pump one and, on the other hand, promotes the absorption not only of Na^+ ions but also of divalent cations [141].

Molecular physiology of NKCC2

NKCC2 belongs to solute carrier family 12 (SLC12; Human Genome Organization), the electrically silent, cation-coupled chloride co-transporter family [27, 54]. *SLC12A1* located on human chromosome 15 encodes the Na^+:K^+:2Cl^- co-transporter that is exclusively expressed on apical membranes of the TAL, whereas *SLC12A2* located on human chromosome 5 encodes the Na^+:K^+:2Cl^- co-transporter that is expressed on basolateral membranes of several epithelial cells and in many non-epithelial cells. The cDNA encoding these isoforms was simultaneously identified by two groups in 1994. Hebert and coworkers identified the apical renal isoform that was named BSC1 for bumetanide-sensitive co-transporter 1 [30] and then the basolateral isoform that was thus named BSC2 [35], while Forbush and coworkers first identified the basolateral isoform that was named NKCC1 [161] and then the renal-specific isoform, NKCC2 [111].

NKCC2 has been cloned and sequenced from human [136], rat [55], mouse [84, 100], rabbit [111], shark [53], and eel kidney [32]. It is a protein of 1,095 amino acid residues with a proposed topology featuring a central hydrophobic domain made up of 12 putative transmembrane (TM) spanning regions (Fig. 2a). A long hydrophilic loop connects TM segments 7 and 8 and contains two glycosylation sites. The hydrophobic domain is flanked by a short amino- and a long carboxyl-terminal domain that are located within the cell. Anti-NKCC2 polyclonal antibodies were used to demonstrate that NKCC2 is specifically expressed in the apical membrane of TAL (Fig. 2a) [38, 86]. NKCC2 is able to form

dimmers, and it is likely that it functions as dimers [138]. NKCC2 is glycosylated at the two sites located in the long extracellular loop [109]. The absence of glycosylation is associated with decreased co-transporter activity and increased affinity for extracellular chloride [109]. Thus, the long glycosylated loop between TM 7 and 8 must be oriented toward the extracellular space.

Alternative spliced isoforms of NKCC2

The molecular and functional diversity of NKCC2 is enriched by the existence of at least six alternatively spliced variants that arise from the combination of two independent splicing mechanisms. The first is the existence of three mutually exclusive 96-bp cassette exon 4, denoted A, B, and F, that encode part of transmembrane 2 (TM2) and the interconnecting segment between TM2 and TM3 (Fig. 2a,c). These exons were originally described in rabbit [111] and later identified in human [136], mouse [100], and rat kidney [163]. Thus, NKCC2 can be expressed as NKCC2-A, NKCC2-B, or NKCC2-F.

Knowledge of the axial distribution of NKCC2 isoforms along TAL comes from molecular analysis using isoform-specific probes for Northern blots (Fig. 2d) [111] or in situ hybridization [84], as well as from isoform-specific polymerase chain reaction primers applied to single nephron segments [163]. Variant F is present only in the inner stripe of the outer medulla, that is, at the beginning of the medullary thick ascending limb (MAL), whereas variant B is present only toward the end of the cortical thick ascending limb (CAL) in the renal cortex where macula densa cells are located. Variant A is present in all TAL, suggesting that it is probably the default isoform, and that specific splicing mechanisms take place in MAL and CAL to ensure the formation of F and B isoforms, respectively. The A, B, and F variants exhibit different ion transport kinetics [61, 113] that explain the axial capacity of TAL for solute transport observed many years before [21]. The affinity for the three co-transported ions is B>A>F (Fig. 2e) in both mouse [113] and rabbit [61] NKCC2. Thus, the F isoform, located at the beginning of the MAL (Fig. 2d) where solutes are highly concentrated, is the low-affinity co-transporter, whereas the B-isoform, located at the end of CAL (Fig. 2d) where tubular fluid has been diluted, is the high-affinity isoform, thereby ensuring continuous salt absorption despite lower ion concentrations. The A variant, located throughout the TAL, exhibits an intermediary affinity and is the isoform with the greatest intrinsic transport capacity [113]. Experiments using chimeric constructs [52] or site-directed mutagenesis [60] of NKCC2 expressed in *Xenopus laevis* oocytes point to only six residues as responsible for kinetic differences between B and F isoforms (Fig. 2c).

A second splicing mechanism involves utilization of an alternative polyadenylation site in exon 16 that produces two distinct C terminal domains: a long isoform composed of 457 amino acid residues generating the NKCC2 protein of 1095 residues (L-NKCC2) and a truncated one of 129 residues producing a shorter NKCC2 isoform of 770 residues (S-NKCC2), which is lacking the last 329 residues of L-NKCC2 but contains 55 residues at the end, not present in L-NKCC2 (Fig. 2b). The expression S-NKCC2 at the protein level was corroborated using polyclonal antibodies raised against the unique 55-residue fragment [100]. Since this splicing mechanism is independent of A, B, and F exons, at least in mouse kidney, six isoforms are potentially present: L-NKCC2 A, B, and F and S-NKCC2 A, B, and F (Fig. 2a,b) [100]. S-NKCC2 exhibits functional properties of a co-transporter and as a regulator of L-NKCC2. As a co-transporter, S-NKCC2 functions as a K^+-independent but bumetanide-sensitive Na^+:Cl^- co-transporter that is active only during cell swelling and is inhibited by cAMP [112]. Consistent with the size of S-NKCC2 and L-NKCC2 [100], Hass et al. [69] isolated 75- and 150-kDa proteins from mouse outer medulla membranes using a photosensitive bumetanide analogue [^{3}H]BSTBA.

The S-NKCC2 isoform provides the molecular explanation for physiological observations conducted in rabbit and mouse TAL cells suggesting the presence of two operating modes of salt transport [41, 42, 141]. Under hypotonic conditions or in the absence of vasopressin (i.e., absence of cAMP), the furosemide-sensitive salt transporter behaves as a K^+-independent, Na^+:Cl^- co-transporter (S-NKCC2). In contrast, when cells were exposed to hypertonicity or to vasopressin (i.e., cAMP) the furosemide-sensitive transport became K^+-dependent, turning the transport mode to a Na^+: K^+:$2Cl^-$ co-transporter (L-NKCC2). It remains to be defined how the K^+ transport ability is lost by the SNKCC2 variant, especially since it has been demonstrated in L-NKCC2 that information required for Na^+, K^+, and Cl^- transport is located within the central hydrophobic domain [146]. Therefore, it is possible that the 55 amino acid residues play a role in reducing the K^+ transport ability of S-NKCC2. Interestingly, there are many positively charged residues within the unique 55-residue segment of S-NKCC2 (Fig. 2c) that could function in preventing translocation of a positively charged ion such as K^+.

The role of A and B isoforms in TAL physiology in vivo has been analyzed by elegant studies in which isoform-specific knockout mice were generated [24, 106]. Complete deletion of NKCC2 in mice resulted in a very severe salt-losing nephropathy, leading to early death of all pups in the first or second week of life [143]. In contrast, heterozygous deletion of one allele (NKCC2$^{+/-}$ mice) had no apparent consequences upon renal function [142]. Thus, it is not surprising that elimination of a single NKCC2 isoform, either A or B, had no appreciable effects upon salt and water balance [24, 106]. These studies, however, revealed that the A variant operates better under high perfusion flow rates, supporting previous observations that this isoform mediates high capacity transport [113]. In addition, in NKCC2-A$^{-/-}$ mice, the expected reduction in renin secretion by intravenous infusion of saline solution was not observed, whereas in NKCC2-B$^{-/-}$ mice, the renin secretion reduction was similar to wild-type mice, suggesting that because of its high capacity for Cl^- transport, the A isoform may be required for the macula-densa-induced decrease in renin secretion during high flow rates. The double knockout A-B or null mice with specific deletions of F isoforms have not been generated.

Regulation of NKCC2

Modulation of NKCC2 activity by cAMP-generating hormones such as vasopressin, glucagon, isoproterenol, and parathyroid hormone is a fundamental mechanism regulating salt transport in TAL [77, 78]. Before NKCC2 cDNA was identified, the most studied of these hormones was vasopressin (Fig. 1). Experiments using isolated perfused tubules showed that vasopressin increases TAL NaCl absorption [70, 77, 131] by what appeared to involve trafficking of NKCC2 to apical plasma membrane. Surprisingly, however, since NKCC2 cDNA was identified, few studies have analyzed the regulation of NKCC2 by cAMP or vasopressin. As mentioned above, regulation of LNKCC2 is another function that has been suggested for the S-NKCC2 isoform. Although it is well known that the apical Na^+:K^+:$2Cl^-$ co-transporter in TAL is activated by vasopressin–cAMP, when expressed in oocytes, L-NKCC2 activity is not affected either by increasing cAMP or by inhibiting PKA activity. However, when oocytes were injected with both L-NKCC2 and S-NKCC2, the activity of L-NKCC2 was reduced and fully restored by adding cAMP [114]. Colchicine prevents the cAMP-induced increase of L-NKCC2 activity, suggesting that it is due to an increase in L-NKCC2 translocation to the plasma membrane [97]. Thus, it is possible that S-NKCC2 has a dominant negative effect upon L-NKCC2, which is released when cAMP is increased. The distribution of S-NKCC2 in TAL is consistent with its regulatory function (Fig. 2b). S-NKCC2 exhibits a predominant location in sub-apical membrane space; it is expressed in some, but not in all MAL cells, and is present in the medullary but not in the

cortical portion of TAL [100]. In this regard, vasopressin has no effect upon salt absorption in the cortical portion of mouse TAL [76].

Compelling evidence indicates that less than 2% of NKCC2 in isolated TAL tubules is located at the apical membrane under basal conditions and that this percentage increased remarkably after exposing the tubules to cAMP [107]. This was also demonstrated in vivo in another study using polyclonal antibodies that recognize NKCC2 when phosphorylated at threonine residues 96 and 101 of the amino-terminal domain [58]. Treatment of mice with vasopressin increased phosphorylation of NKCC2 at these threonines and transporter translocation to the apical membrane. Another study showed that growth hormone also activates TAL salt absorption. Growth hormone, which acts through a tyrosine-kinase-associated receptor, induces phosphorylation of NKCC2 at the same threonines as vasopressin [37], thus suggesting that the same residues can be phosphorylated by a non-cAMP-dependent pathway. Finally, recent studies suggest that vasopressin-induced trafficking of NKCC2 is mediated by lipid rafts [160] and that NKCC2 is also regulated by direct interaction between the co-transporter and aldolase B [10].

Few studies have addressed acute regulation of NKCC2 by pathways other than cAMP-PKA. The metabolic sensing AMP kinase, AMPK, directly interacts with and phosphorylates NKCC2 at serine 126, resulting in activation of the co-transporter [47]. Such an action could be a coupling signal between the metabolic state of the cell and the activity of NKCC2. Cells shrinkage activates NKCC2 and is associated with phosphorylation of threonines 96 and 101 [59]. NKCC2 is a target of the recently discovered kinase family known as WNKs (with no lysine kinases) implicated in the genesis of hereditary hypertension [93]. WNK3 is expressed in TAL cells and is a positive regulator of NKCC2 [127]. In this study, it was observed that co-injection of *Xenopus* oocytes with NKCC2 and WNK3 increased the $^{86}Rb^{+}$ uptake induced by NKCC2. The observed increase was greater than twofold over control-injected oocytes with NKCC2 complementary RNA (cRNA) alone. It was also observed in NKCC2 + WNK3 cRNA-injected oocytes that the presence of WNK3 promotes phosphorylation of threonines 96 and 101. Interestingly, elimination of WNK3 catalytic activity, by introducing the D294A mutation in WNK3 that is known to turn WNK3 into a catalytically inactive form, not only prevented the positive effect of the kinase upon NKCC2 but also turned WNK3 into a negative regulator [127]. Additionally, recent studies suggest that NKCC2 is activated by intracellular chloride depletion and that two type of kinases, WNK1/WNK3 and the STE-20 kinases SPAK/OSR1, are implicated [98, 117]. These studies suggest that intracellular chloride depletion activates WNK1/WNK3 that in turn interact with and activate SPAK/OSR1, which could be the kinase phosphorylating NKCC2.

Since different stimuli induce NKCC2 activation and phosphorylation of threonines 96 and 101, it is possible that these threonines are part of a common mechanism to activate NKCC2 rather than a specific cAMP-PKA phosphorylation site. Thus, the molecular mechanisms for vasopressin- or cAMP-induced activation of NKCC2 remain to be uncovered.

Salt transport in TAL can also be modulated by changing the level of NKCC2 gene expression. Vasopressin, for instance, induces a long-term increase of NaCl transport in TAL in isolated perfused tubule studies in Brattleboro rats [11] presumably due to upregulation of NKCC2 protein expression in TAL cells [90]. Similarly, consistent with the observation that arachidonic acid metabolites inhibits NKCC2 activity in rabbit MALs [40], increased NKCC2 expression induced by the cyclooxygenase inhibitors indomethacin or diclofenac was reversed by the prostaglandin E_2 analog misoprostol [43]. Long-term expression of NKCC2 is also regulated by acid–base status. NKCC2 expression increases during acidosis [6, 7] by augmenting the stability of NKCC2 messenger RNA (mRNA)

without affecting *SLC12A1* transcription rate [87]. Under physiologic conditions, most of the ammonium produced by proximal tubules is reabsorbed by TAL to be later secreted in medullary collecting ducts and excreted into the urine [63, 91]. Thus, during acidosis in which production of ammonium by proximal tubules increases, NKCC2 expression increases as a compensatory mechanism to enhance ammonium absorption.

Bartter syndrome type I

In 1962, Frederick Bartter described a salt-losing nephropathy that featured hypokalemia, metabolic alkalosis, polyuria, and hypertrophy of the juxtaglomerular apparatus [9]. The clinical manifestation suggested that the affected segment of the nephron was the TAL. It is now known that Bartter syndrome is a heterogenous but nevertheless monogenic autosomal-recessive disorder that has been characterized at the molecular level, mostly in consanguineous families. Up to five genes have been shown to cause Bartter syndrome: Inactivating mutations of NKCC2, the apical K^+ channel ROMK, the basolateral Cl^- channel CLC-KB, and its chaperon subunit Barttin result in Bartter syndrome types I, II, III, and IV, respectively, whereas activating mutations of the calcium sensing receptor, CaSR, produce Bartter syndrome type V. That all these five genes when mutated produce the same disease strongly supports the molecular model of TAL salt absorption depicted in Fig. 1 in which simultaneous operation of all these proteins is required for proper function of this nephron segment. Types I and II Bartter syndrome are the more severe forms since the clinical picture is usually present during the antenatal period as excessive accumulation of amniotic fluid (polyhydramnios). After birth, most of the patients present with low blood pressure, metabolic alkalosis with hypokalemia, hyperreninemia, secondary aldosteronism, hypercalciuria, and nephrocalcinosis [132]. Consistent with reports of mutations in *SLC12A1* as the cause of Bartter's syndrome type I, targeted disruption of this gene in mice produces a profound salt-wasting nephropathy with elevated mortality during the first 2 weeks of life [143].

Figure 3 shows the proposed secondary structure of NKCC2 to indicate the specific missense mutations and location within the protein that have been defined as the cause of Bartter syndrome type I. In addition, ten different small deletions (not shown in Fig. 3) or mutations producing frame shifts, and thus truncated proteins, have been described [2, 12, 51, 92, 136, 137, 148]. Mutations are distributed throughout the co-transporter. Analysis of the functional consequences of mutations G193R, A267S, G319R, A508T, Δ526N, and Y998X in *Xenopus laevis* oocytes resulted in severely reduced activity of proteins that were correctly routed to the plasma membrane, suggesting that intrinsic activity was diminished [137]. One boy with a mild phenotype was found to have a G224D mutation affecting only isoform B [147], thus producing an isoform-specific disease. More recently, two brothers with late-onset manifestations of Bartter syndrome type I were described [118]. Both were compound heterozygous, harboring a frame shift, D918fs, and a missense mutation, F177Y, that reduced NKCC2 activity by half compared with wild-type NKCC2. It was suggested that in these cases, the disease did not begin until the second decade of life due to the residual activity of F177Y.

As discussed in the section on CaSR, Ca^{2+} directly regulates salt transport by TAL, and this occurs by interaction with the CaSR in the basolateral membrane. Activation of this sensor by Ca^{2+} decreases salt reabsorption by TAL, thus reducing the paracelluar calcium reabsorption. Consistent with this, two studies show that activating mutations of the CaSR results in a Bartter phenotype [149, 159]. Thus, mutations in the CaSR causing Bartter syndrome are the gain-of-function type. Watanabe et al. [159] described two unrelated patients with activating mutations in the CaSR (A843E and C131W) and a Bartter phenotype. In the second study, Vargas-Poussou et al. [149] described another pediatric patient with an L125P mutation in the CaSR that also exhibited a Bartter phenotype.

NKCC2 and arterial hypertension

The fact that mutations in *SLC12A1* cause a monogenic disease that features arterial hypotension due to decreased NKCC2 activity indicates that this is a fundamental gene defending normal blood pressure, and thus, single nucleotide polymorphisms (SNPs) or mutations within *SLC12A1* potentially could be implicated in heightening or diminishing the risk for developing arterial hypertension. In this regard, a recent study [85] analyzed the presence and distribution of rare independent *SLC12A1* mutations encoding NKCC2, *SLC12A3* encoding the thiazide-sensitive Na^+:Cl^- co-transporter NCC, and *KCNJ1* encoding the apical ROMK potassium channel in 3125 subjects of the Framingham Heart Study who have been studied for 35 years with frequent periodic cardiovascular evaluation and for which DNA was available. Thirty different mutations were observed in 49 subjects. Most of them were missense mutations and were predicted to impair protein function. Of these, ten were mutations in *SLC12A1* (Fig. 3), 15 in *SLC12A3*, and five in *KCNJ1*. The clinical and epidemiological analysis of this cohort of subjects revealed that carriers of these mutations were located within the lower deciles of blood pressure, with significantly lower systolic and diastolic blood pressures when compared with non-carriers. In addition, carriers of these mutations were significantly protected against the development of arterial hypertension, since prevalence of this disease was significantly lower at all ages when compared with non-carriers. The results of this study strongly suggest that in the open population, NKCC2 contributes to normal variation in blood pressure and some mutations are protective against hypertension. It remains to be determined if there are mutations or SNPs in *SLC12A1* that increases the activity and/or expression levels of NKCC2, which could be implicated in enhancing the risk for hypertension.

CaSR regulation of NKCC2, ROMK, and TAL calcium absorption

The role of calcium in regulating renal tubular transport processes and metabolism had been investigated and described and was generally accepted phenomenologically, though no convincing explanation was advanced to clarify the mechanism of such regulation. The concept and existence of a calcium receptor was proposed and its features presciently predicted [15]. Several years later, Brown, Hebert, and their colleagues succeeded in the molecular cloning of the CaSR, the features of which were entirely consistent with its anticipated characteristics [19, 126]. A number of comprehensive reviews discuss the general physiology, pathophysiology, and pharmacology of the CaSR [16, 20, 25, 83]. Here, we focus on the role of the CaSR in modulating Na^+, Ca^{2+}, and K^+ transport in TALs.

Molecular physiology of the CaSR

The CaSR is a member of the class C superfamily of G-protein-coupled membrane receptors (GPCR). Class C also includes pheromone and odorant receptors, to which the CaSR is most structurally related, as well as metabotropic glutamate and γ-aminobutyric (GABA) receptors [57]. The distinguishing structural feature of these GPCRs is that the ligand-binding sites are contained within the large (≈600 amino acid) extracellular receptor domain. The primary ligand-binding pocket, or orthosteric site,[1] is formed by two prominent three-dimensional lobes that characterize class C receptors. This structure has been compared to a venus flytrap [29]. The CaSR constitutively forms homodimers, which is essential to its action [8, 108].

Except for two cysteines forming a putative disulfide bridge, class C receptors do not have any of the key features that characterize class A (rhodopsin, β-adrenergic) or class B (PTH, calcitonin, secretin) receptors [57].

[1]Binding to the same recognition site as an endogenous agonist.

Ligand bias

Although ionized calcium (Ca^{2+}) is the cognate physiological CaSR ligand, its selectivity is not absolute. Mg^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Pb^{2+}, Gd^{3+}, La^{3+}, Eu^{3+}, Tb^{3+}, and Yt^{3+} activate the CaSR [17, 73, 103]. A comprehensive pharmacological evaluation with concentrations for half-maximal activation for comparable activation or signaling parameters remains to be performed. Nonetheless, these inorganic di- and trivalent cations, along with polycations such as spermine, aminoglycosides (e.g., streptomycin, gentamicin, and neomycin), and polybasic amino acids (e.g., polylysine) are full agonists and are referred to as type I calcimimetics. Allosteric modulators that require the presence of Ca^{2+} or other full agonists that enhance the sensitivity of activation without altering the maximal response also regulate the CaSR and are designated type II calcimimetics. Allosteric modulators include organic polycations [NPS R467, NPS R568, and cinacalcet (AMG R073)] [74, 104] and L-amino acids [28]. The venus flytrap extracellular domain is required for amino acid binding [101] as it is for activation by cationic agonists Ca^{2+}, Gd^{3+}, and neomycin [72]. Though both amino acids and type II calcimimetics allosterically activate the CaSR, they do so through different mechanisms [164]. CaSR activity is also modulated by ionic strength [73] and by pH [121]. These actions could have significant regulatory effects especially for TALs, which are found in a high-ionic strength environment, and collecting tubules where urinary acidification proceeds.

The CaSR is abundantly expressed in the kidneys. CaSR mRNA transcripts are present essentially throughout the nephron, viz., glomerulus, proximal convoluted and straight tubules, medullary and cortical TALs, distal convoluted tubules, cortical and inner medullary collecting ducts [125]. CaSR protein expression is prominently found in proximal tubules, TALs, and cortical collecting tubules [124]. Notably, the membrane domain on polarized renal tubular epithelial cells on which the CaSR is found differs between nephron segments. In proximal tubules, the CaSR is expressed at the base of apical brush-border membranes. Expression decreases from S1 to S3 proximal tubule segments. By contrast, in TALs, the CaSR is found on basolateral cell membranes [23, 124]. In cortical and inner medullary collecting ducts, the CaSR is localized to apical plasma membranes [23, 124, 130]. The CaSR is expressed only in some of the type A intercalated cells of the cortical collecting duct [124]. The trafficking motifs responsible for directed membrane targeting of the CaSR have not been identified (Fig. 4).

G proteins, CaSR signaling

G protein binding—Signaling by the CaSR is remarkably complex and incompletely defined. Studies of isolated parathyroid cells established that raising extracellular Ca^{2+} activated phospholipase C (PLC) with attendant inositol phosphate formation and transient elevations of intracellular Ca^{2+} and inhibited adenylyl cyclase with decreased cAMP accumulation [15, 79, 157]. Initial characterization of the cloned and heterologously expressed CaSR demonstrated that these properties were attributable to the receptor itself and not an epiphenomenon [19]. The CaSR is expressed by Madin–Darby canine kidney (MDCK) cells where it couples to $G_{\alpha q/11}$ and $G_{\alpha i2,3}$ [4]. These and similar findings prove that CaSR signaling is mediated by the G protein families $G_{q/11}$ and G_i, although $G_{q/11}$ is believed to be the more relevant signaling pathway. Recent evidence suggests that G protein signaling by the CaSR may change from $G_{q/11}$ to G_i following malignant transformation [94].

An overview of major CaSR signaling pathways is shown in Fig. 5. Calcium binding to the CaSR results in G-protein-dependent stimulation of PLC with attendant inositol trisphosphate (IP_3) formation and rapid but transient release of Ca^{2+} from intracellular stores. In dispersed parathyroid cells, PLC activation and PTH secretion is inhibited by

treatment with pertussis toxin [26, 45] as is CaSR activation in MAL cells [1], indicating that it is mediated by $G_{\alpha i/o}$, whereas in MDCK cells [4], PLC activation is refractory to pertussis toxin, suggesting that $G_{\alpha q/11}$ mediates PLC activation. Thus, different G proteins may mediate CaSR activation in distinct cell types where in some stimulation proceeds through a mechanism involving G_i as opposed to releasing $G\beta\gamma$ subunits from pertussis toxin-insensitive $G_{q/11}$ or through PLC-mediated increases in Ca^{2+}, which then inhibits, respectively, $G\beta\gamma$- or Ca^{2+}-sensitive isoforms of adenylate cyclase.

PLC activation is a direct consequence of CaSR occupancy and is mediated by $G_{q/11}$ [150]. Other CaSR signaling pathways including activation of G_i, phospholipase A_2 (PLA_2), phospholipase D (PLD), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 and phosphatidylinositol-4 kinases (PI3K and PI4K, respectively) have been described but are less well characterized [19, 81, 89, 158]. Activation of PLD and Rho is mediated by $G\alpha_{12/13}$ [82].

CaSR signaling in TAL

Elevated extracellular Ca^{2+} in MALs inhibits vasopressin-stimulated cAMP accumulation, whereas in CALs, raising extracellular Ca^{2+} suppresses PTH-stimulated cAMP formation while not impairing PTH-induced cAMP accumulation by proximal tubules. Takaichi and Kurokawa [144, 145] noted that in dissected nephron segments, increasing extracellular calcium from 1 to 5 mM inhibited cAMP production in response to PTH and calcitonin in cortical TALs of Henle, but not in proximal convoluted tubules.

Although CaSR signaling is primarily effected by PLC in most cell types, PLC activation in TAL does not appear to mediate increases of intracellular Ca^{2+}, which has its origin in extracellular fluid rather than from release from cytoplasmic stores [34, 154]. Moreover, increases of intracellular Ca^{2+} reduce cyclic AMP formation by inhibiting the type 6 adenylate cyclase and increasing cAMP metabolism by activating a Ca^{2+}-dependent phosphodiesterase as noted above.

An alternative mechanism involved in CaSR signaling in TAL involves Ca^{2+} modulation of phospholipase A_2 (PLA_2) activity. Ca^{2+} activation of PLA_2 increases cytosolic arachidonic acid, which is rapidly metabolized by a P450 ω-hydroxylase (CYP4) to 20-hydroxyeicosatetraenoic acid (20-HETE) or by COX2 to prostaglandins [154]. Because P450 is more highly expressed than COX2, 20-HETE formation is normally favored. CaSR activation, however, increases TNFα stimulation of COX2 [44], which diverts arachidonic acid metabolism toward prostaglandin synthesis at the expense of 20-HETE.

Increasing extracellular calcium stimulates cyclooxygenase-2 (COX2) and PGE_2 synthesis by MAL cells in a dose- and time-dependent manner [152, 153]. These effects, as discussed below, are important for the regulatory effect of CaSR activation on NaCl absorption.

Regulation of NKCC2, ROMK, and other proteins in TAL by the CaSR

CaSR activation has two prominent physiological actions to regulate electrolyte absorption in TALs. Moreover, mutations of the CaSR cause profound disruption of renal sodium and calcium ion homeostasis. As noted earlier, in the TAL, the CaSR is expressed on basolateral cell membranes. As such, it monitors extracellular calcium concentrations in the renal interstitium. Importantly, variations of interstitial calcium occur under normal conditions, to an extent permitting physiological regulation of CaSR modulated functions [102].

Activation of the TAL CaSR directly affects NaCl absorption mediated by NKCC2 and indirectly regulates NaCl absorption by modulating ROMK (renal outer medullary K^+ channel; Kir 1.1) channels. Likewise, CaSR activation directly affects Ca^{2+} transport.

Whether or not effects on Na^+ transport indirectly affect Ca^{2+} is uncertain and controversial. The mechanisms of Na^+, K^+, and Ca^{2+} transport in TAL are outlined at the beginning of this review (cf. Fig. 1) and serve as a general background for understanding the regulatory influences of CaSR actions. It should be borne in mind that in addition to passive paracellular Ca^{2+} movement that occurs along the entire length of the TAL, hormone-regulated active cellular Ca^{2+} absorption occurs selectively in MAL, which is regulated by calcitonin, and in CAL, where it is governed by PTH [139, 140].

NKCC2 and CaSR—Luminal membrane Na^+ entry in TALs is mediated mostly by the electroneutral, furosemide-sensitive Na^+–K^+–$2Cl^-$ co-transport, NKCC2 (SLC12A1). The observation that Ca^{2+} regulates TAL Na^+ transport predates the molecular cloning of either NKCC2 or the CaSR. Calcium infusion increases both calcium and sodium excretion [95], in part due to inhibition of iso-osmotic solute absorption by proximal tubules [39]. However, urinary calcium excretion increases to a greater extent than does sodium excretion, suggesting both a specific inhibitory effect of hypercalcemia on calcium transport and, by inference, that this action occurs in distal tubules where calcium and sodium absorption is inversely related and can be dissociated [48]. Evidently, in intact animals, elevations of serum calcium are accompanied by suppression of PTH secretion [39], which decreases calcium absorption at hormone-sensitive sites in cortical TALs and distal convoluted tubules. However, hypercalcemia also directly inhibits calcium absorption by TALs of Henle's loop [119, 133].

Hypercalcemia suppresses PTH-stimulated cAMP formation specifically in TALs while not impairing PTH-induced cAMP accumulation by proximal tubules. Takaichi and Kurokawa [144] noted that in dissected nephron segments, increasing extracellular calcium from 1 to 5 mM inhibited cAMP production in response to PTH and calcitonin in cortical TALs of Henle, but not in proximal convoluted tubules. High ambient calcium also inhibited cAMP production stimulated by forskolin, indicating that a post-receptor mechanism additionally contributes to Ca^{2+} regulatory effects. These additional mechanisms are likely to be mediated by the type 6, Ca^{2+}-inhibitable adenylyl cyclase and the Ca^{2+}-activated phosphodiesterase.

Subsequent studies established that Ca^{2+} directly inhibits NaCl absorption in TALs [33] and that elevated peritubular calcium (the location of the CaSR), but not luminal calcium or magnesium, decreases the absorption of both Ca^{2+} and Mg^{2+} [120]. Contrary to these observations, Desfleurs et al. [36] found that elevating extracellular calcium stimulated cAMP formation but did not affect NaCl absorption or vasopressin-stimulated NaCl transport in mouse CAL. These results are difficult to interpret because of the stimulatory action of Ca^{2+} on cAMP given that the CaSR is not known to couple to $G\alpha_s$. Indeed, CAL express the type 6 Ca^{2+}-sensitive adenylyl cyclase, which is inhibited upon raising extracellular calcium [34]. Enhanced phosphodiesterase activity also contributes to diminished cAMP formation. The previous findings pointing to an action of vasopressin effects on CAL are at odds with the segment-specific actions of vasopressin on NaCl, which proceeds in MAL but not CAL of the mouse [75].

ROMK—ROMK2[2] (Kir1.1b), as noted above, mediates K^+ ion recycling across apical plasma membranes to the luminal fluid of the TAL (Fig. 1). This action is largely responsible for generating the lumen-positive voltage that serves as a driving force for the

[2]TAL apical membranes express 30- and 70-pS channels, and high-conductance, Ca^{2+}-activated maxi K^+ channels. ROMK, the 30-pS channel is absent from apical membranes of ROMK-null mice. The 70-pS channel mediates 80% of the apical K^+ conductance. Current thinking suggests that the 70-pS K^+ channel is a heterotetramer that includes ROMK, which may be a pore-containing subunit of the 70-pS K^+ channels. ROMK1 is expressed in cortical collecting ducts, whereas ROMK2 is present in TAL [13].

cation-selective paracellular movement of Ca^{2+}, Mg^{2+}, and of additional Na^+ absorption by the TAL.

CaSR regulation of ROMK—The regulation of TAL K^+ channels by eicosanoids and P450 metabolites has been elegantly delineated [155]. As mentioned above, CaSR activation in TALs increases 20-HETE formation in the short term and prostaglandin synthesis after longer times [153]. 20-HETE potently inhibits NKCC2, ROMK, and the basolateral Na^+/K^+-ATPase and, by this means, disrupts NaCl absorption at multiple cellular sites and through independent mechanisms [3, 40, 156]. CaSR stimulation also induces TNFα expression, activating COX2, thereby generating PGE_2 which contributes further to inhibition of NKCC2 [31, 152]. Notably, increasing extracellular Ca^{2+} reduces the activity of the 70-pS ROMK. The 30-pS ROMK per se is insensitive to 20-HETE or CaSR activation. Yet, 20-HETE and CaSR activation clearly inhibit NaCl absorption by TALs. It is possible that 20-HETE may regulate another subunit of the 70-pS K^+ channel, thereby explaining how CaSR activation modulates apical K^+ channels activity and, thereby, NKCC2-mediated Na^+ uptake.

CaSR regulation of TAL Ca^{2+} absorption—An extensive literature testifies to the inhibitory effect of hypercalcemia on renal calcium absorption [110]. The physiological relevance of CaSR regulation of renal calcium transport is underscored by hyper- and hypocalcemic disorders resulting from CaSR mutations: Familial hypocalciuric hypercalcemia and neonatal severe hyper-parathyroidism are caused by inactivating CaSR mutations [115], whereas autosomal dominant hypocalcemia is caused by activating mutations [116].

Calcium absorption in the TAL proceeds by parallel routes and mechanisms. Passive calcium absorption occurs across the lateral intercellular space that forms the para-cellular pathway. Transport is proportional to the net electrical and chemical driving forces. PTH in the CAL and calcitonin in the MAL activates active transcellular calcium movement. Thus, in contrast to proximal tubules, where calcium absorption is entirely passive and parallels that of sodium, or distal tubules, where only active calcium absorption occurs and is inversely related to the rate and magnitude of sodium transport, the TAL represents a hybrid situation in which calcium and sodium movement may occur in parallel or may be dissociated depending on the prevailing hormonal status and other ambient conditions affecting mineral ion and salt homeostasis.

Hypercalcemia directly inhibits basal calcium absorption by TALs of Henle's loop [119, 133]. The participation of the CaSR in regulating basal and PTH-dependent Ca^{2+} transport was analyzed in single perfused in CAL [99]. CaSR activation suppressed passive paracellular calcium absorption, thereby confirming the findings obtained by Desfleurs et al. [36]. The effects of CaSR activation were specific for calcium absorption and had no effect on sodium transport. These findings are consistent with the finding that hypercalcemia exerts a profound inhibitory action on calcium absorption by TALs in thyroparathyroidectomized rats but only a minor reduction of sodium absorption [119]. Further studies determined that CaSR activation modulated PTH-dependent Ca^{2+} absorption. Gd^{3+}, the prototype of a non-calcium type I CaSR agonist, was chosen for these experiments so as to maintain equal concentrations of calcium at both apical and basolateral surfaces, thereby avoiding a transepithelial calcium gradient that would alter passive calcium diffusion[3]. PTH increased Ca^{2+} absorption without an accompanying change of the transepithelial voltage. Gd^{3+} decreased Ca^{2+} absorption to control levels, again without altering the transepithelial voltage. The type II calcimimetic NPS R-467 exerted comparable actions to inhibit PTH-dependent Ca^{2+} transport. Thus, CaSR activation inhibited PTH-dependent Ca^{2+} transport. Moreover, these effects occurred in the absence of a change of

electromotive driving force, i.e., the reduction of Ca^{2+} absorption was due to inhibition of active, transcellular Ca^{2+} absorption and not to a change in passive, paracellular calcium movement. The use of non-Ca^{2+} ligands to activate the CaSR were not due to blockade of ATP-permeable channels [129], Ca^{2+}-sensitive K^+ channels, the nonselective Ca^{2+}-permeable cation channel, polycystin-2 [62], the Ca^{2+}-selective Trp3 channel [105], or other non-selective cation channels [46, 162] or mechano-sensitive channels [71]. Indeed, the precise molecular target of PTH-dependent, CaSR-regulated Ca^{2+} absorption by CALs is not known. TRPV5, which mediates vitamin-D-dependent Ca^{2+} transport in distal tubules, is not expressed in TAL. Yet, as noted above, PTH clearly stimulates cellular calcium absorption by CAL.

CaSR activation with Gd^{3+} or NPS R-467 dissociated Na^+ and Ca^{2+} movement in CAL insofar as it inhibited Ca^{2+} absorption without affecting Na^+ transport or the transepithelial voltage [99]. Addition of bumetanide to the luminal fluid virtually abolished Na^+ absorption and transepithelial voltage.

The mechanism by which CaSR activation inhibits PTH-stimulated Ca^{2+} absorption in CAL has not been examined. However, it is known that stimulation of Ca^{2+} transport by PTH in CAL cells requires parallel activation of both protein kinase A and of protein kinase C; interfering with either pathway is sufficient to inhibit Ca^{2+} movement [49, 50]. Based on these observations, we imagine that by activating G_i, cAMP formation is attenuated, thereby interfering with PTH action.

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[3]Such a problem may contribute to or account for the findings of Desfleurs, who found that increasing basolateral calcium inhibited net calcium absorption without altering Na, Cl, K, or Mg transport by single perfused mouse CALs, [36]. CaSR activation was achieved by increasing basolateral calcium. The CAL, however, is highly permeable to calcium and elevating calcium asymmetrically at the serosal surface increases calcium backflux and results in diminished net calcium absorption regardless of an effect on the CaSR [14]. Under these conditions, it is not possible to distinguish a non-specific effect of diminished driving force from a specific action that can be attributed to the CaSR.

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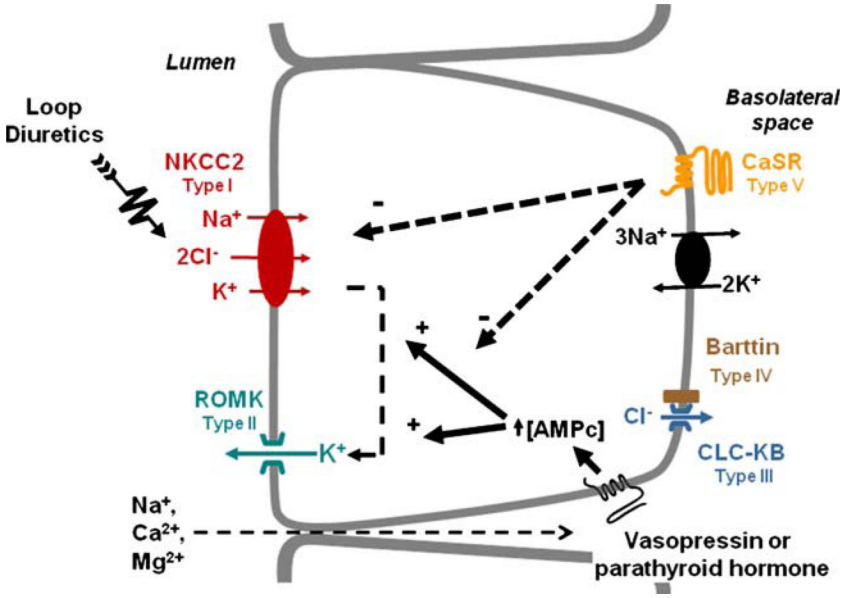
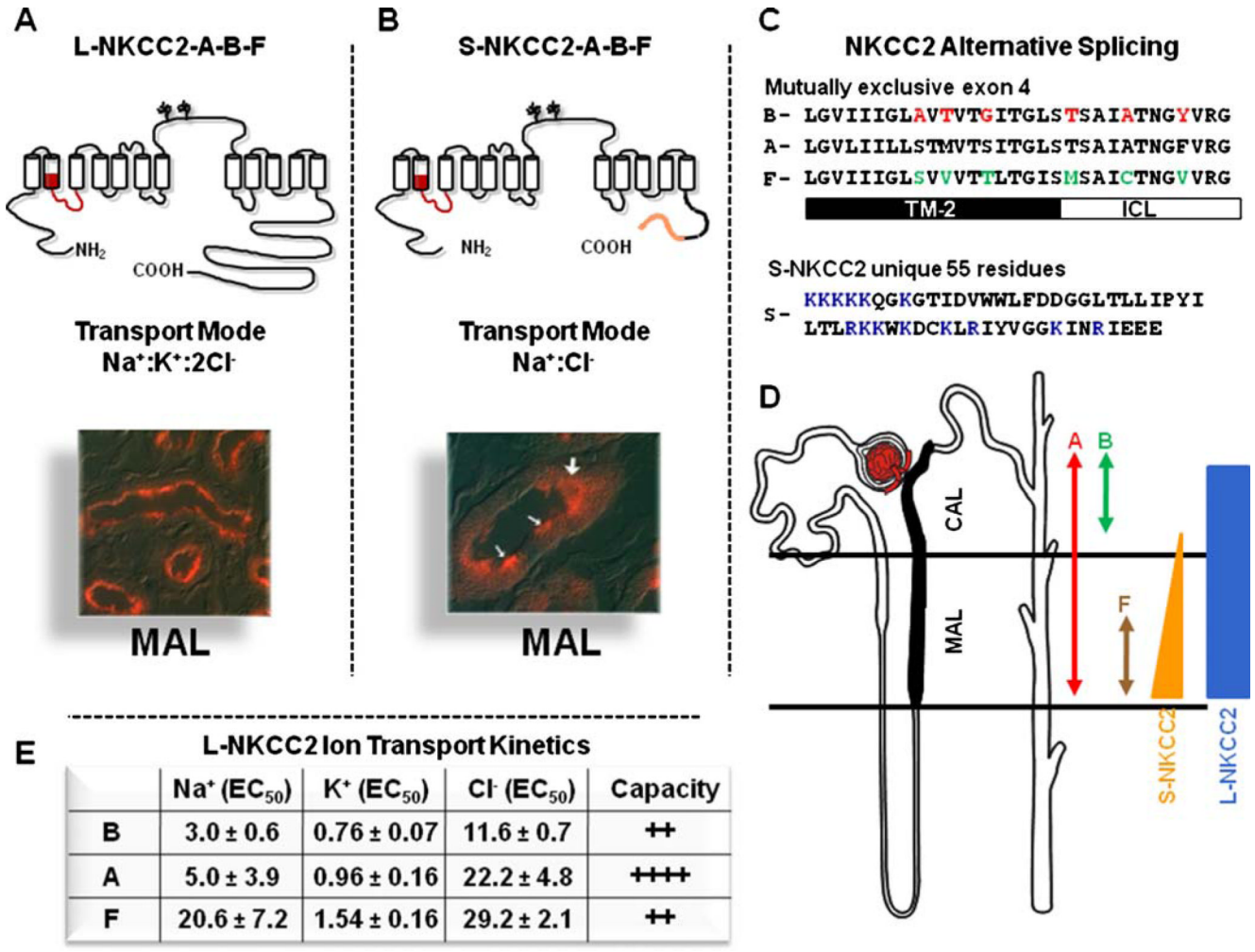


Fig. 1.

Molecular physiology of salt transport in TAL. Five genes are known to be the cause of Bartter syndrome type I to type V as stated. By acting in a Gαs-coupled receptor, vasopressin or parathyroid hormone increases cAMP production which in turns increases the activity of NCCC2 and ROMK, thereby augmenting salt reabsorption. In contrast, by acting in a Gαq-coupled receptor, extracellular Ca^{2+} inhibits, both NKCC2 and ROMK, decreasing salt reabsorption



E

L-NKCC2 Ion Transport Kinetics

	Na^+ (EC_{50})	K^+ (EC_{50})	Cl^- (EC_{50})	Capacity
B	3.0 ± 0.6	0.76 ± 0.07	11.6 ± 0.7	++
A	5.0 ± 3.9	0.96 ± 0.16	22.2 ± 4.8	++++
F	20.6 ± 7.2	1.54 ± 0.16	29.2 ± 2.1	++

Fig. 2.

Molecular physiology of the Na^+:K^+:2Cl^- co-transporter, NKCC2. **a** Topology, transport mode, and immunolocalization of the long isoform of NKCC2 (L-NKCC2). There are 12 transmembrane spanning segments and a long hydrophilic loop between TM 7 and 8, with two glycosylation sites. Location of the mutually exclusive cassette exons is shown in *red*. **b** Topology, transport mode, and immunolocalization of the short isoform of NKCC2 (L-NKCC2). Location of the mutually exclusive cassette exons is shown in *red* and the unique 55 piece at the end is shown in *orange*. *White arrows* in the picture show positive cells. **c** Sequence and alignment of the alternative splicing of SLC12A1. The 31 residues of the three exons are shown. Switching the red or green residues between B and F isoforms is enough to switch their ion transport kinetics between each other. *TM-2* transmembrane domain 2. *ICL* Interconnecting segment between TM2 and TM3. The 55 unique piece of S-NKCC2 is shown. Residues in *blue* are positively charged. **d** Distribution of L-NKCC2 and S-NKCC2, as well as exons A, B, and F along TAL, as stated. **e** Ion transport kinetics and capacity of transport for L-NKCC2 A, B, and F variants, as informed by Plata et al. [113]

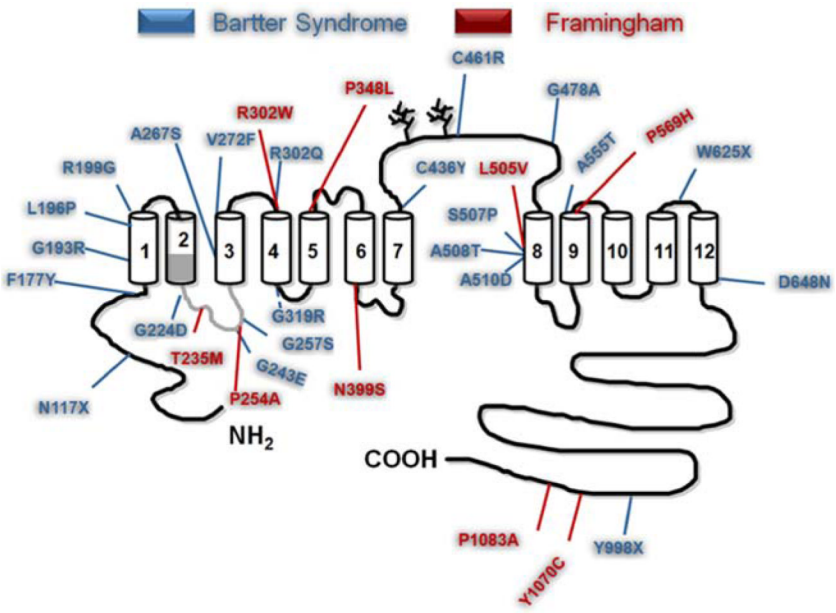


Fig. 3.

Missense mutations informed for NKCC2. Mutations in *blue* were informed as harbored by patients with Bartter syndrome [2, 12, 51, 92, 136, 137, 148]. Mutations in *red* were informed in normal subjects of the Framingham Heart Study [85]

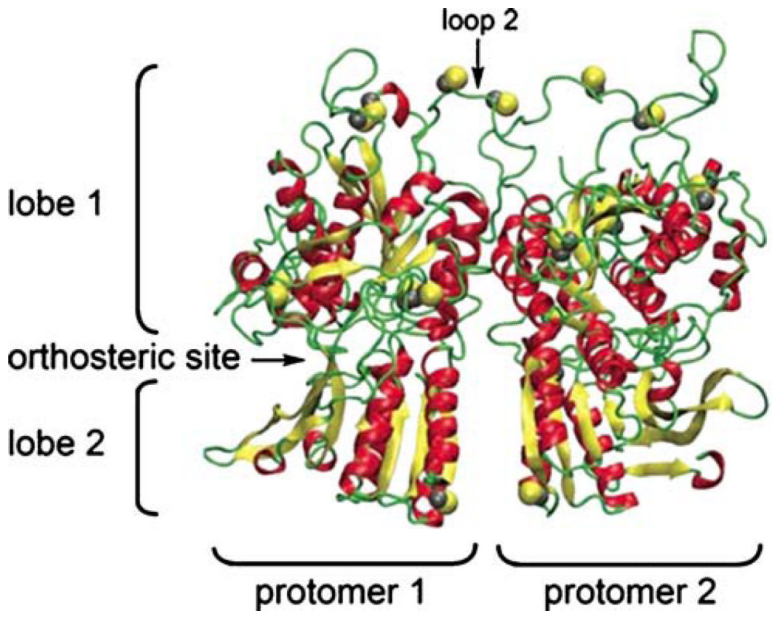


Fig. 4.

Three-dimensional structure of the dimerized CaSR. α-Helices are shown in red, β-sheets are *yellow*, and loops and turns in *green*. Cysteines are shown in *yellow*. The putative orthosteric site in protomer 1 is labeled. The dimer interface runs along the vertical axis between the two protomers (from [80])

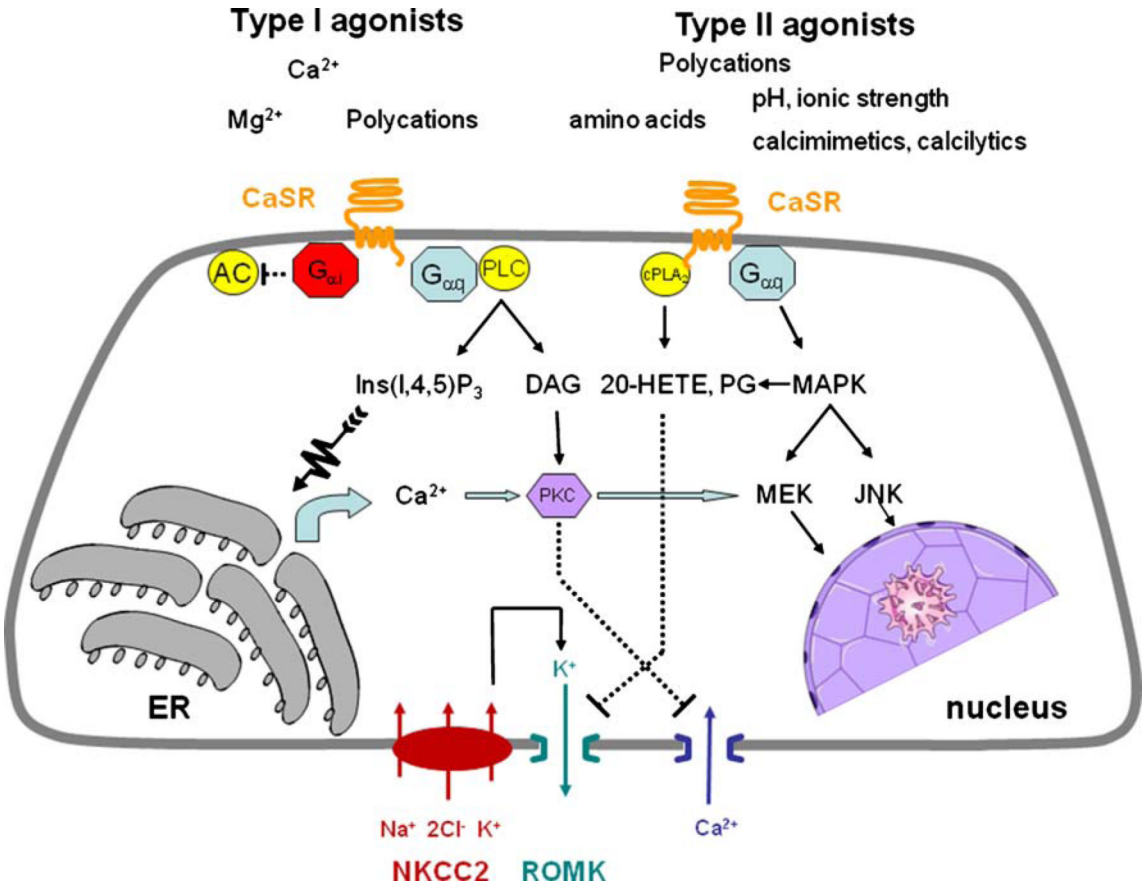


Fig. 5.

CaSR signaling in TAL. Simplified model of CaSR by representative type I and type II agonists. The CaSR is located on basolateral cell membranes of TAL. Its activation inhibits cAMP formation mediated by Gαi and formation of lipid second messengers and prostanoids through phospholipase C (*PLC*) and cytoplasmic phospholipase A2 (*cPLA2*), respectively. Inhibition of Na^+:K^+:$2Cl^-$ co-transport on apical cell membranes is indirect and mediated by blockade of ROMK by 20-HETE or PGE2. CaSR activation inhibits both PTH-stimulated calcium absorption and passive paracellular calcium absorption (not shown). Further details are provided in the text