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Active Ca^{2+} reabsorption in the connecting tubule

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Abstract The kidney plays a crucial role in the maintenance of the body calcium (Ca^{2+}) balance. Ca^{2+} is an essential ion in all organisms and participates in a large variety of structural and functional processes. In mammals, active tubular Ca^{2+} reabsorption is restricted to the distal part of the nephron, i.e., the late distal convoluted (DCT2) and the connecting tubules (CNT), where approximately 10–15% of the total Ca^{2+} is reabsorbed. This active transcellular transport is hallmarked by the transient receptor potential vanilloid 5 (TRPV5) epithelial Ca^{2+} channel, regulated by an array of events, and mediated by hormones, including 1,25-dihydroxyvitamin D_3, parathyroid hormone, and estrogen. Novel molecular mechanisms have been identified, such as the direct regulatory effects of klotho and tissue kallikrein on the abundance of TRPV5 at the apical membrane. The newly discovered mechanisms could provide potential pharmacological targets in the therapy of renal Ca^{2+} wasting. This review discusses the three basic molecular steps of active Ca^{2+} reabsorption in the DCT/CNT segments of the nephron, including apical entry, cytoplasmic transport, and basolateral extrusion of Ca^{2+}. In addition, an overview of the recently identified mechanisms governing this active Ca^{2+} transport through the DCT2/CNT epithelial cells will be presented.

Keywords Transepithelial Ca^{2+} transport · TRPV5 · PTH · Vitamin D · Klotho · Calbindin

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Introduction

Calcium (Ca^{2+}) is essential for the physiological functioning of all living cells. In humans, 99% of total body Ca^{2+} resides in the skeleton. The remaining 1% is distributed in soft tissues and extracellular fluid, which is the prime target of the Ca^{2+} homeostatic systems. Three tightly controlled mechanisms, including bone resorption and formation, intestinal absorption, and renal reabsorption, maintain Ca^{2+} homeostasis. In the kidney, approximately 45% of the plasma Ca^{2+}, present in free ionized form, filters through the glomerulus and enters the proximal tubule segment of the nephron, where ~65% of the filtered Ca^{2+} is passively reabsorbed [56, 62]. In the thick ascending loop of Henle (TAL), an additional 20% is reabsorbed through this passive paracellular pathway, mediated by the tight junction protein claudin-16 [3, 43]. In these segments, Ca^{2+} reabsorption is not specifically regulated and depends on gradients established by NaCl and water reabsorption [61]. The final regulation of Ca^{2+} excretion, according to physiological needs, appears to occur primarily in two segments of the distal part of the nephron, namely in the late part of the distal convoluted tubule (known as DCT2) and the connecting tubule (CNT; Table 1) [16].

Morphologic characteristics of the CNT

The above discussed distal segments of the nephron exhibit distinct morphological, as well as functional features. In the superficial cortex, the DCT/CNT region is short and flows directly into the cortical collecting duct (CCD). Midcortical and juxtamedullar nephrons, on the other hand, have longer DCT/CNTs merging with other CNT segments into arcades before transitioning to the CCD [56]. DCTs consist of two short segments, DCT1 and DCT2, both comprising a

Table 1 Regulation of Ca^{2+} transporters

Refs		Apical membrane		Cytoplasm		Basolateral membrane	
		Transporter	Action	Transporter	Action	Transporter	Action
	Intracellular factors	TRPV5		Calbindin-D_{28K}		NCX1 PMCA1b Na^+/K^+-ATPase	
	Hormones						
[27]	1,25(OH)2D3		Gene expression ↑		Gene expression ↑	NCX1 PMCA1b	Gene expression ↑,=
[67]	Estrogen		Gene expression ↑		Gene expression ↑	NCX1 PMCA1b	Gene expression ↑
[41]	Insulin		Gene expression ↓ Gene expression ↑		Gene expression ↓		
[12, 68]	PTH		Plasma membrane abundance ↑		Gene expression ↑	NCX1	Gene expression ↑
	Transporter trafficking regulators						
[72]	S100A10–annexin-2		Plasma membrane abundance ↑				
[73]	Clathrin		Plasma membrane abundance ↑				
[12]	Caveolin		Plasma membrane abundance ↑				
[71]	Rab11a		Plasma membrane abundance ↑				
	Transporter activity regulators						
[50]	$[Ca^{2+}]_i$		Activity ↓				
[51]	$[Mg^{2+}]_i$		Activity ↓				
[42]	PIP_2		Activity ↑				
[23]	80K-H		Activity ↑				
	Extracellular factors						
[5, 40, 77]	pH		Plasma membrane retention ↑ Activity ↑				
[5]	$[Mg^{2+}]_{pro\text{-}urine}$		Activity ↓				
[24]	TK/bradykinin		Plasma membrane retention ↑				
[11, 14]	Klotho		Plasma membrane retention ↑			Na^+/K^+-ATPase, NCX1	Plasma membrane abundance ↑ Indirectly increased activity

Regulation of Ca^{2+} transporters in kidney by intracellular and extracellular factors. Data included in the table were based on listed references
↑ increased gene expression, increased channel activity, or increased plasma membrane abundance,↓ downregulation of gene expression or inhibition of channel activity, = no effect

uniform population of principal cells, whereas the CNT contains both principal and two types of intercalated cells [3, 16, 43, 56]. Furthermore, the principal cells in the CNT have less cell–cell contacts and mitochondria, and their apical membrane contains fewer projections than DCT cells. Unlike the polygonal-shaped CNT cells, intercalated cells appear to be round with an apical membrane densely adorned with microprojections [43, 56]. The proton secreting α-intercalated cells have extensive apical microvilli with abundant expression of the H^+/K^+ exchanger and vacuolar H^+-ATPase and numerous subapically localized small mitochondria, whereas the bicarbonate secreting β-intercalated cells have fewer apical microvilli, and their mitochondria tend to accumulate basolaterally, where the proton pump is also located [56]. Although the ratio of α- and β-intercalated cells vary depending on the actual physiological state, α-intercalated cells are more common in the CNT.

In addition to the ubiquitously expressed Na^+/K^+-ATPase, the Na^+/Ca^{2+} exchanger (NCX1) and the plasma membrane ATPase type 1b (PMCA1b) have been found along the DCT2/CNT region, whereas the apically localized thiazide-sensitive Na^+/Cl^- co-transporter (NCC) and the transient receptor potential melastin subtype 6 are present in the DCT (see Xi et al. in this issue). The DCT2 region also shares additional similarities with the CNT segment, as both segments express the transient receptor potential vanilloid type 5 (TRPV5) and the Ca^{2+}-binding protein calbindin-D_{28K}. The tight junctions in these segments are imperme-

able for Ca^{2+}, and transcellular Ca^{2+} transport occurs against an electrochemical gradient, supporting that Ca^{2+} reabsorption in the DCT2/CNT is mediated by active transepithelial transport.

Transepithelial Ca^{2+} reabsorption

Transepithelial transport of Ca^{2+} is a three-step process. It initiates with influx of Ca^{2+} across the apical membrane mediated by TRPV5 [29]. Subsequently, entered Ca^{2+} is sequestered by the specialized intracellular carrier protein calbindin-D_{28K} and diffuses to the basolateral membrane (Fig. 1). Finally, transporter proteins, such as NCX1 and PMCA1b, extrude Ca^{2+} from the epithelial cell into the circulation (Fig. 1). The identification and characterization of TRPV5 as the gatekeeper of renal epithelial Ca^{2+} transport [30] gave new momentum to the understanding of the molecular mechanisms underlying the process of active Ca^{2+} reabsorption.

TRPV5—the apical gate

TRPV5, also known as the epithelial Ca^{2+} channel, is a member of the TRP channel superfamily [29]. This channel comprises large and flexible intracellular amino- and carboxyl-terminal tails flanking six transmembrane segments (TM) and an additional hydrophobic stretch between TM5 and TM6, predicted to be the pore-forming region. The amino-terminal tail contains six ankyrin repeats [19, 54] that are important structural elements for both channel assembly and protein–protein interactions [13, 19]. Furthermore, the first extracellular loop between TM1 and TM2 contains an evolutionary conserved asparagine (N_{358}) crucial for complex-glycosylation and in turn for regulating channel activity [11, 13, 33]. The carboxyl-terminal tail harbors three potential protein kinase C (PKC) sites, which suggests an important role for phosphorylation in channel activity. Moreover, in cultured mammalian cell systems, as well as in oocytes, TRPV5 is assembled into large homotetramers in order to acquire the active conformation

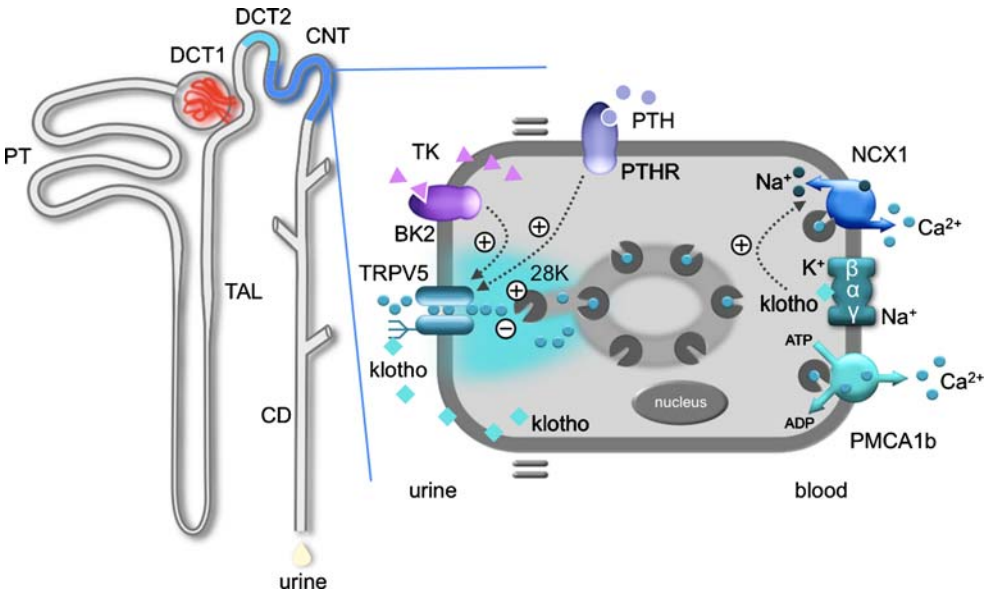

Fig. 1 Model of transcellular Ca^2 reabsorption in DCT2 and CNT. The renal distal tubule in the nephron comprises anatomically discrete segments, including the thick ascending limb of the loop of Henle (*TAL*) and the distal convoluted tubule (*DCT*) that ends in the connecting tubule (*CNT*). The late part of the DCT (*DCT2*) and CNT play an important role in fine-tuning renal excretion of Ca^{2+}. The epithelial Ca^{2+} channel (*TRPV5*) is primarily expressed apically in these segments and co-localizes with calbindin-D_{28K} (*28K*), Na^+/Ca^{2+} exchanger (*NCX1*), and the plasma membrane ATPase (*PMCA1b*). Upon entry via TRPV5, Ca^{2+} is buffered by 28K and diffuses to the basolateral membrane, where it is released and extruded by a concerted action of NCX1 and PMCA1b. In addition, the basolateral membrane exposes a parathyroid hormone receptor (*PTHR*) and the Na^+/K^+-ATPase consisting of the α-, β- and γ-subunit. PTHR activation by PTH stimulates TRPV5 activity, and entered Ca^{2+} can subsequently control the expression level of the Ca^{2+} transporters. At the apical membrane, there is a bradykinin receptor (*BK2*) that is activated by urinary tissue kallikrein (*TK*) to activate TRPV5-mediated Ca^{2+} influx. In the cell, entered Ca^{2+} acts as a negative feedback on channel activity, and 28K plays a regulatory role by association with TRPV5 under low intracellular Ca^{2+} concentrations. Extracellular urinary klotho directly stimulates TRPV5 at the apical membrane by modification of the *N*-glycan, whereas intracellular klotho enhances Na^+/K^+-ATPase surface expression that in turn activates NCX1-mediated Ca^{2+} efflux

state [26, 33]. Facing each other, the hydrophobic stretches between TM5 and TM6 in each subunit are postulated to form the aqueous pore centered at the fourfold symmetry axis.

Detailed electrophysiological studies have compellingly demonstrated the constitutive activity of TRPV5 at low intracellular Ca^{2+} concentrations and physiological membrane potentials [75]. The current–voltage relationship of TRPV5 shows strong inward rectification [29, 74, 75]. Another important functional feature of TRPV5 is the high Ca^{2+} selectivity, making this epithelial Ca^{2+} channel the most Ca^{2+}-selective member in the TRP superfamily [75]. Finally, the generation of a TRPV5 deficient mouse strain ($TRPV5^{-/-}$) provided compelling evidence for the physiological function of this channel. Active Ca^{2+} reabsorption in DCT2 and CNT is severely impaired in these animals as $TRPV5^{-/-}$ mice waste approximately six- to tenfold more Ca^{2+} than their wild-type littermates, which is in line with the postulated gatekeeper function of TRPV5 in active Ca^{2+} reabsorption [31].

Shortly after the identification of TRPV5, a homologous channel, sharing 75% amino acid identity with TRPV5, was cloned from intestine and named TRPV6 [53]. Although there are some functional differences between these channels, TRPV6 exhibits the same Ca^{2+} selectivity and current–voltage relationship [32, 47, 78]. Moreover, this latter channel has been implicated in intestinal Ca^{2+} absorption. Disturbances in the Ca^{2+} homeostasis were also reported in mice lacking TRPV6 ($TRPV6^{-/-}$) as these animals display reduced intestinal Ca^{2+} absorption and low bone mineral density [1]. Although it has been shown that TRPV6 is moderately expressed in adult kidney [59] and the $TRPV6^{-/-}$ mice have increased urinary Ca^{2+} excretion, the exact role of TRPV6 in the kidney is not yet fully understood.

Calbindin-D_{28K}—the intracellular shuttle

The principal cells of the DCT2/CNT segments are continuously challenged by a substantial Ca^{2+} influx through TRPV5, yet the cells manage to maintain a low intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Maintaining the free $[Ca^{2+}]_i$ at the basal level is essential for several reasons. High levels of free Ca^{2+} in the cytoplasm are known to induce apoptosis and protein precipitation. In addition, Ca^{2+} has an essential signaling function for many processes in the cell. More importantly, an increased $[Ca^{2+}]_i$ has been shown to inhibit the activity of TRPV5 [75]. Based on the currently available data, three different models have been postulated for transepithelial Ca^{2+} transfer from the apical to the basolateral membrane. The first model is based on a passive diffusion tunneling through the endoplasmatic reticulum, vesicular transport along the microtubules involving lysosomes, and finally, facilitated diffusion. According to the second model, Ca^{2+}-transporting cells utilize lysosomes to sequester Ca^{2+} and facilitate its movement to the basolateral membrane [35]. The apical Ca^{2+} influx through TRPV5 initiates the disruption of the actin cytoskeleton and the formation of Ca^{2+}-enriched endocytic vesicles, which are transported along the microtubules and some fuse with lysosomes [46]. In a third model, the intracellular diffusion of Ca^{2+} is facilitated by the vitamin D_3-dependent Ca^{2+}-binding protein, calbindin-D_{28K}, in the principal cells of DCT2 and CNT segments [7, 20]. Together with parvalbumin, calmodulin, and troponin C, calbindins are Ca^{2+}-binding proteins that form a family of proteins with Ca^{2+} affinity [15]. Calbindin-D_{28K} has three pairs of EF-hands that are the structural basis of the high Ca^{2+} affinity binding capacity [6]. Moreover, in the kidney, the expression of calbindin-D_{28K} is restricted to DCT2, CNT, and CCD regions. It has also recently been shown that calbindin-D_{28K} translocates to the TRPV5-containing plasma membranes upon a decrease in intracellular $[Ca^{2+}]$ and directly associates with this channel [39]. Due to the relatively slow Ca^{2+}-binding kinetics of calbindin-D_{28K}, hormone-induced Ca^{2+} signaling can also occur independently of the transcellular Ca^{2+} transport rate [37]. Bound to calbindin-D_{28K}, Ca^{2+} is shuttled to the basolateral membrane, where Ca^{2+} is discharged into the blood flow by the basolateral Ca^{2+} extrusion systems. Finally, some studies reported that calbindin-$D_{28K}{}^{-/-}$ mice fed a high Ca^{2+} diet have impaired renal Ca^{2+} handling as they excrete more Ca^{2+} in their urine than the wild-type control littermates [60], whereas other studies did not observe a difference that is probably due to the compensatory increase of renal calbindin-D_{9K} expression [22]. These data suggest that calbindin-D_{28K} facilitates the intracellular diffusion of Ca^{2+} in DCT2 and CNT.

NCX1 and PMCA1b—the basolateral extrusion system

The energy-consuming step of transcellular Ca^{2+} transport lies in the Ca^{2+} efflux process. In this step, intracellular Ca^{2+} is transported across the basolateral membrane against its electrochemical gradient, and the ions are extruded back to the blood flow. Two transporters have been implicated in this mechanism, PMCA1b and NCX1. Plasma membrane ATPases are high-affinity Ca^{2+} efflux pumps that maintain the resting Ca^{2+} concentration in virtually all cells [4]. The highest Ca^{2+}-ATPase activity in kidney was reported in the DCT segment. However, earlier studies have suggested that the capacity of this PMCA pump in CNT seems to be insufficient to keep pace with the absorptive flux of Ca^{2+} because it can transport only ~30% of the total Ca^{2+} efflux [2, 69]. In contrast to PMCA1b, the Na^+/Ca^{2+} exchanger has been shown to be a prerequisite for transepithelial Ca^{2+}

transport [2, 69]. NCX1 exchanges Ca^{2+} and Na^{+} generally in a 1:3 stoichiometric ratio. Moreover, NCX1 is a widely expressed protein as it can be found in several tissues, including the heart, brain, and skeletal muscle [45]. In the kidney, the expression of NCX1 is restricted to the distal part of the nephron, particularly the CNT segment, where it predominantly localizes along the basolateral membrane [3, 28, 43] and accounts for the remaining 70% of the Ca^{2+} efflux [2, 69].

Regulatory mechanisms of Ca^{2+} transport in DCT2/CNT

The aforementioned transporters comprise the machinery transporting Ca^{2+} from pro-urine to the blood in the DCT2/CNT. Several factors have been shown to contribute to the regulation of the Ca^{2+} transporting capacity of these particular nephron segments, which can be classified into four categories: (1) the control of the overall abundance of the transporter proteins by calciotropic hormones, (2) the rate of intracellular trafficking of the plasma membrane transporters, (3) alteration of activity of the transport proteins in the membrane by intracellular factors, and finally, (4) tuning apical Ca^{2+} influx by extracellular (luminal) factors.

Controlling the abundance of Ca^{2+} transporters

PTH

Parathyroid hormone (PTH) is an essential component of the Ca^{2+} homeostasis. The secretion of PTH from the parathyroid gland is triggered by changes in blood Ca^{2+} levels sensed by the parathyroid Ca^{2+}-sensing receptor [8]. PTH receptors have been detected in DCT2/CNT, enabling the direct control of active Ca^{2+} reabsorption by PTH [57].

PTH-mediated regulation of the Ca^{2+} transporters was studied in parathyroidectomized rats [68]. Parathyroidectomy reduced the expression of TRPV5, calbindin-D_{28K}, and NCX1. This decline in expression of Ca^{2+} transporters resulted in decreased active Ca^{2+} reabsorption and the development of hypocalcemia [68]. After PTH supplementation, the expression of Ca^{2+} transporters, as well as increased plasma Ca^{2+} concentration were normalized in these parathyroidectomized rats. In addition, the regulation by PTH was investigated in primary cultures of rabbit CNT cells. In these Ca^{2+}-transporting cells, PTH resulted in an elevated expression of the Ca^{2+} transport proteins TRPV5, calbindin-D_{28K}, NCX1, and PMCA1b. Taken together, these results indicate that PTH stimulates renal Ca^{2+} handling by co-regulating the expression of the Ca^{2+} transport proteins [68]. In addition, experiments in these primary CNT cell cultures supported a gatekeeper role of TRPV5 since a blockade of the apical Ca^{2+} influx by ruthenium red prevented the PTH-induced upregulation of the other Ca^{2+} transporters.

Vitamin D

The vitamin D endocrine system plays a pivotal role in Ca^{2+} homeostasis. In the recent years, it has become clear that the active form of vitamin D (1,25-dihydroxyvitamin D_3, or abbreviated $1,25(OH)_2D_3$) is a potent regulator of the Ca^{2+} transport proteins. Several groups have shown transcriptional regulation of TRPV5, calbindin-D_{28K}, and NCX1 by $1,25(OH)_2D_3$, whereas a $1,25(OH)_2D_3$-sensitivity for PMCA1b is not consistently reported. Studies in vitamin-D-deficient knockout models showed an impressive down-regulation of renal TRPV5, calbindin-D_{28K}, and NCX1 mRNA that could be normalized by $1,25(OH)_2D_3$ supplementation, whereas PMCA1b was not significantly affected [27]. On the other hand, several studies indicated that PMCA1b is positively regulated by $1,25(OH)_2D_3$ in the intestine to increase Ca^{2+} absorption. Northern blot analysis indicated that repletion of vitamin-D-deficient chickens with vitamin D increases PMCA mRNAs in the duodenum, jejunum, ileum, and colon [9]. Because these studies and the role of vitamin D in Ca^{2+} homeostasis have been reviewed extensively [29, 64, 70], detailed information is not included in this review.

Estrogen

Although estrogen is generally not considered as a calciotropic hormone, it is widely accepted that it plays a role in renal Ca^{2+} handling. In rats, estradiol has been suggested to enhance the expression of TRPV5, NCX1, PMCA1b, and calbindin-D_{28K} [67]. In line with these observations, a recent study with aromatase deficient mice lacking the aromatase enzyme (aromatase$^{-/-}$) and, therefore, estrogen deficient, also showed decreased expression of these transporters and concomitant renal Ca^{2+} wasting [52]. Additionally, estradiol treatment of these animals normalized the urinary Ca^{2+} excretion and gene expression. In agreement with these observations, increased renal Ca^{2+} wasting, as well as renal stone formation in women after menopause, is a well-known phenomenon [25], suggesting that estrogens may significantly contribute to the regulation of the transepithelial Ca^{2+} transport in the DCT2/CNT.

Controlling the intracellular trafficking

S100A10/annexin-2

S100A10 (also known as annexin-2 light chain) is an auxiliary protein for TRPV5 [72]. With two Ca^{2+}-

insensitive EF-hands, S100A10 is predominantly present as a heterotetrameric complex with annexin-2, which has been implicated in many cellular processes, including endocytosis and exocytosis [21]. An important regulatory role has been proposed for the S100A10–annexin-2 heteromer in TRPV5 functioning [72]. The binding of annexin-2 to TRPV5 through S100A10 was shown to facilitate the translocation of TRPV5 toward the plasma membrane. This association of S100A10 and TRPV5 takes place through a short conserved peptide sequence, located in the carboxyl-terminus of TRPV5. Moreover, co-expression of S100A10, annexin-2, and TRPV5 has been observed in DCT2/CNT [72]. Taken together, these findings show that the S100A10–annexin-2 complex is a significant component for trafficking of TRPV5 toward the plasma membrane.

Rab11a

The small GTPase Rab11a has also been identified as a novel TRPV5- associated protein [71]. Rab11a is one of the key regulatory proteins that controls the recycling of endosomes [10, 76]. Rab11a was found to co-localize with TRPV5 in the DCT2/CNT. Here, both TRPV5 and Rab11a are present in subapical vesicular structures [71]. In addition, a direct protein–protein interaction was observed between Rab11a and TRPV5, suggesting that TRPV5 channels, present on the apical plasma membrane, recycle from the intracellular (recycling) endosomes in a Rab11-dependent manner.

Clathrin and caveolin

Van de Graaf and coworkers observed that the extraction of TRPV5 from the cell surface takes place in a constitutive clathrin-dependent manner [73]. In addition, the same authors showed that following its internalization, TRPV5 is not immediately targeted to protein degradation. Instead, by entering a Ca^{2+}-dependent recycling pathway, TRPV5 remained stable in the subapical endosomal fraction [73]. Another recent study by Huang et al. suggested a caveolin-1-mediated internalization of TRPV5, which was inhibited by PKC [12]. Caveolin-1 is a structural component of caveolae and is crucial for the stabilization of the specialized membrane domains. Moreover, the caveolae-dependent internalization of TRPV5 was strongly inhibited by PTH-induced phosphorylation of TRPV5 via PKC, indicating that next to its genetic effect on expression, PTH also has a rapid effect on channel abundance [12]. These findings indicate that apical sorting of TRPV5 is likely to be mediated by several mechanisms that could be differentially controlled depending on physiological needs of the body.

Regulation of TRPV5 activity at the membrane

Intracellular Ca^{2+}, Mg^{2+}, and PIP_2

Although the Ca^{2+} concentration in the luminal compartment of DCT2/CNT is in the 1.0–1.5 mM range, the resting $[Ca^{2+}]_i$ in these cells is maintained around 100 nM by NCX1 and PMCA1b. TRPV5 has a high Ca^{2+} selectivity, and at physiological Ca^{2+} concentrations, its current is mainly carried by Ca^{2+}. In human embryonic kidney (HEK293) cells heterogeneously expressing TRPV5, currents can be activated under conditions of high intracellular Ca^{2+} buffering by hyperpolarizing voltage steps. Earlier, Nilius et al. suggested that intracellular Ca^{2+} acts as a negative feedback switch regulating TRPV5 activity. The Ca^{2+} current through TRPV5 is inhibited by the $[Ca^{2+}]_i$ with an IC_{50} of 82 nM [50]. Considering this high affinity of Ca^{2+}-dependent channel inhibition, the presence of the co-expressed Ca^{2+} buffer calbindin in DCT2/CNT plays an important role to maintain TRPV5 activity. Conclusively, the $[Ca^{2+}]_i$ itself directly regulates channel function in order to maintain optimal Ca^{2+} reabsorption without excessive influx of Ca^{2+}.

Single TRPV5 channel currents in cell-attached and inside-out patches were only detected in the absence of Ca^{2+} and had a conductance of ~75 pS [51]. So far, no reliable single channel measurements have been performed in the presence of extracellular Ca^{2+}. Another interesting feature of TRPV5 is the open pore blockage by intracellular Mg^{2+}. Currents through TRPV5 are small at physiological extracellular Mg^{2+} and Ca^{2+} concentrations, but sufficient to increase the $[Ca^{2+}]_i$ at hyperpolarized potentials. Therefore, block by Mg^{2+} and decrease of the current by extracellular Ca^{2+} might be physiologically important to prevent Ca^{2+} overload of TRPV5-expressing cells [51, 75]. In addition, Huang and colleagues reported that PIP_2 activates TRPV5 and that activation of the channel by PIP_2 reduces the sensitivity of TRPV5 to the inhibition by the intracellular $[Mg^{2+}]$ [17, 42]. In this model, hydrolysis of PIP_2 by receptor activation of PLC may increase the sensitivity for Mg^{2+} inhibition.

80K-H

Another protein called 80K-H has been shown to directly interact with TRPV5 in a Ca^{2+}-dependent manner [23]. The 80K-H protein contains two putative EF-hands and an ER-targeting signal. This TRPV5-linked protein was originally cloned as a PKC substrate and was subsequently associated with intracellular signaling [58]. Binding with Ca^{2+} abolished the inactivation of the two EF-hand motifs of 80K-H, and this, in turn, reduced the TRPV5-mediated Ca^{2+} current and increased the sensitivity of TRPV5 to the $[Ca^{2+}]_i$, accelerating the feedback inhibition of the channel [23].

Moreover, 80K-H also co-localizes with TRPV5 in the DCT2/CNT. Based on these findings, 80K-H has been hypothesized to act a Ca^{2+} sensor to regulate the activity of TRPV5 at the plasma membrane [23].

Pro-urinary factors stabilize TRPV5 in the apical membrane

Tissue kallikrein

Tissue kallikrein (TK) is a multifunctional serine protease that is primarily synthesized in the DCT2 and CNT and catalyzes the kininogen kinin conversion [55]. TK is secreted into the pro-urine, where it mediates the formation of bradykinin that binds to the type 2 bradykinin receptor (B2R) [18]. A striking effect of TK has been observed in primary rabbit CNT cells mediating transcellular Ca^{2+} transport [24]. Apical addition of TK or bradykinin (BK) significantly increased transcellular Ca^{2+} transport that was prevented by B2R antagonists, whereas basolateral application of either TK or BK had no effect [24]. This stimulatory effect of TK was mediated by the apical B2R signaling through the phospholipase C/diacylglycerol/PKC pathway, resulting in phosphorylation of TRPV5 and subsequent delay in its retrieval from the plasma membrane. Additionally, mice lacking TK ($TK^{-/-}$) waste a large amount of Ca^{2+} without any significant alterations in plasma Ca^{2+}, PTH, and vitamin D_3 levels or any detectable changes in the expression of Ca^{2+} transporters in the DCT2/CNT. These observations together highlight the importance of the regulation of the TRPV5 channel abundance in the membrane by the pro-urine TK.

Urinary pH and Mg^{2+}

The acid–base balance has long been known to affect Ca^{2+} homeostasis. For example, patients with chronic metabolic acidosis waste Ca^{2+}. Pioneering micropuncture studies have shown that chronic metabolic acidosis results in Ca^{2+} wasting [63]. Recent advances in total reflection fluorescent microscopy analysis allowed the identification of the molecular basis underlying these in vivo observations. Exposure of TRPV5-expressing cells to an alkaline extracellular environment (pH 8.0) caused rapid recruitment of TRPV5-containing vesicles to the cell surface and a consequent increase in TRPV5 activity [40]. In the reciprocal experiment, acidic extracellular milieu (pH 6.5) induced the internalization of TRPV5-containing vesicles from the plasma membrane, resulting in a reduced channel activity [40]. The extracellular acidity clearly affected the current kinetics resulting in diminished single channel conductance as shown by Yeh et al. [77]. Binding of protons to an extracellular glutamate near the pore helix of TRPV5 at position 522 (E_{522}) resulted in decreased channel activity, whereas substitution with a glutamine ($E_{522}\rightarrow Q_{522}$) abolished the proton sensitivity. This recognized E_{522} as the extracellular pH sensor in TRPV5. Based on these experiments, binding of protons to the sensor has been proposed to induce a conformational change of the TRPV5 pore helix, leading to a lowered channel activity. These observations clearly point out that urinary acidification results in decreased channel activity at the apical cell membrane, as well as in a rapid retrieval of TRPV5 from the apical membrane, both of which are likely to account for the renal Ca^{2+} wasting in metabolic acidosis. Also, urinary Mg^{2+} is known to modulate urinary Ca^{2+} excretion, but the mechanism underlying this relationship is unknown. In a recent study by Bonny et al., it was elegantly demonstrated that an alteration in urinary Ca^{2+} excretion is directly proportional to the change in Mg^{2+} excretion and inversely proportional to the adjustment in urine pH [5]. Because TRPV5 was inhibited by Mg^{2+}, these data are compatible with the hypothesis that urinary Mg^{2+} directly inhibits Ca^{2+} reabsorption in DCT2/CNT, which can be overruled by an alkaline luminal pH.

Klotho

Klotho is a type-I (single-pass) membrane protein predominantly expressed in tissues involved in Ca^{2+} homeostasis, such as kidney, choroid plexus, and the parathyroid gland [34]. The ablation of klotho causes severe multiple phenotypes in klotho-deficient ($klotho^{-/-}$) mice, such as short life span associated with infertility and sternly impaired Ca^{2+} and phosphate metabolism [38, 65]. There is a growing body of evidence that klotho controls active Ca^{2+} reabsorption in the DCT2/CNT segments through several mechanisms.

In kidney, klotho is exclusively expressed in DCT2/CNT, where following extracellular domain shedding, it is secreted into the circulation and the pro-urine [13]. The secreted form of klotho exerts β-glucuronidase activity [14]. More importantly, klotho was suggested to regulate the apical entry of Ca^{2+} in the DCT2/CNT region. The presence of extracellular klotho robustly increased the activity of TRPV5 in cultured rabbit primary CNT cells. Moreover, TRPV5-expressing HEK293 cells also showed a significant rise in channel activity after klotho treatment that was accompanied with concomitant increased plasma membrane channel abundance [14]. In addition, removal of the complete *N*-glycan tree by Endo-F was recently reported to result in a more pronounced increase in TRPV5 activity compared to that of observed upon klotho treatment [44]. Recently, Cha et al. suggested sialidase rather than β-glucuronidase activity for klotho, as they observed a klotho-mediated removal of terminal sialic acids from the *N*-glycan in TRPV5 [11]. This cleavage exposed the

underlying galactose-*N*-acetylglucoseamine disaccharides in TRPV5, which can directly interact with membrane-bound galectin-1, causing the subsequent plasma membrane retention of TRPV5. Interestingly, treatment with PNGaseF to hydrolyze the entire *N*-glycan of TRPV5 mimicked the stimulatory effect of klotho, suggesting additional mechanisms besides binding to membrane galectin-1. It should be noted that Cha et al. also reported a comparable increase in TRPV5 activity upon β-glucuronidase treatment; however, this effect could be observed only at much higher (0.1–1 μM) concentrations of the enzyme, suggesting that klotho exhibits primarily sialidase activity at physiologic concentrations (~20–200 pM) [11]. These observations confirmed the original conclusion that extracellular klotho hydrolyses oligosaccharide chains from the *N*-glycosylated TRPV5, causing channel retention at the membrane and a subsequent increase in TRPV5-mediated Ca^{2+} influx [14]. In line with these findings, microperfusion studies have shown that CNTs from klotho$^{-/-}$ mice fail to respond to PTH and, therefore, waste large amount of Ca^{2+} [66]. Interestingly, klotho was reported to interact and increase Na^+/K^+-ATPase activity at the plasma membrane and stimulating the Na^+/Ca^{2+} exchange through NCX1 [34]. Taken altogether, there is compelling evidence that klotho is a novel calciotropic factor that, amongst others, can exert its stimulatory effect on TRPV5 cell surface retention from the pro-urine in the DCT2 and the CNT.

Clinical relevance

Several clinical disorders, such as chronic renal failure or diabetes, are associated with the symptoms of dysregulating body Ca^{2+} homeostasis. Chronic renal failure (CRF) is frequently characterized by hypocalcemia, osteoporosis, growth retardation, and secondary hyperparathyroidism. Remarkably, the phenotype of the klotho$^{-/-}$ mice resembles most of these characteristics [36]. Moreover, CRF patients also have greatly reduced renal klotho levels [36]. Together with the fact that klotho can regulate the activity of TRPV5 and NCX1 via Na^+/K^+-ATPase, the involvement of klotho in the pathogenesis of Ca^{2+} abnormalities in CRF may be envisaged.

Hypercalciuria and nephrolithias, disorders with a high prevalence and socio–economic burden in the Western society, are often treated with thiazide diuretics. These drugs are known to affect the Ca^{2+} balance by inducing hypocalciuria. Over the last decade, it was speculated that thiazide-inhibited NCC activity stimulates active Ca^{2+} reabsorption in the DCT/CNT. However, recent studies indicated that paracellular Ca^{2+} transport in the proximal tubule due to extracellular volume contraction explains the hypocalciuria during chronic thiazide treatment [48, 49]. Hypercalciuria is also an early finding in diabetes mellitus patients. Similarly, rats with experimentally induced diabetes display a significant increase in the fractional excretion of Ca^{2+} [41]. However, these diabetic rats showed increased mRNA and protein levels of both TRPV5 and calbindin-D_{28K}. Additionally, insulin therapy corrected the hyperglycemia-associated hypercalciuria and abolished the upregulation of TRPV5, suggesting that the increased TRPV5 abundance in diabetic rats is due to a compensatory adaptation to an increased load of Ca^{2+} secondary to hyperglycemia. Although in many patho-physiological conditions the expression level of the Ca^{2+} transporters is out of balance, mutations in these proteins have not been discovered yet.

Concluding remarks and future perspectives

Ca^{2+} reabsorption in the kidney and particularly in the distal DCT2/CNT segments of the nephron is critical in the maintenance of the Ca^{2+} balance. Here, TRPV5 is the gatekeeper of the Ca^{2+} entry, and therefore, a tight control of its activity enables the organism to adjust Ca^{2+} reabsorption according to any demands of the body. The available experimental data summarized in this review highlights the most important mechanisms that can actually regulate active Ca^{2+} reabsorption. Of these, controlling the TRPV5 cell surface expression by extracellular factors in the pro-urine is a newly discovered mechanism. Klotho delays the retrieval of TRPV5 from the cell membrane by modifying the *N*-glycan composition, whereas the insertion of channels in the cell membrane is promoted by TK-induced phosphorylation or alkaline pH. All mechanisms result in increased TRPV5 abundance and, in turn, regulate the entry of Ca^{2+} at the gate. However, the canvas is far from complete. The molecular mechanism by which intracellular Ca^{2+} bound to calbindin-D_{28K} is transported to the basolateral extrusion transporters NCX1 and PMCA1b is largely unknown. It could be envisaged that the local Na^+ concentration may promote the release of Ca^{2+}; however, no experimental data are available to support this theory. Therefore, the Ca^{2+} transfer between calbindin-D_{28K} on one site and NCX1 and PMCA1b on the other site needs special attention. Another interesting question to be solved is the regulation of the basolateral trafficking of NCX1 and PMCA1b in the DCT2 and CNT cells. Certain players, such as the scaffold 14-3-3, phospholemman, ankyrin, or caveolin-3, have been proposed to play a role in the trafficking of the NCX transporters in neurons and cardiac cells. Nevertheless, the basolateral sorting of these transporters in the DCT2/CNT is essentially unknown. Whether there is a crosstalk between the apical Ca^{2+} entry and the basolateral Ca^{2+} extrusion regulatory apparatus is not known. The fact that klotho increases both

the channel abundance of TRPV5 in the apical membrane and the activity of NCX1 at the basolateral side certainly predicts the existence of such crosstalk [14, 34]. The main question for the coming years is how all of these Ca^{2+} transport proteins communicate with each other in order to facilitate optimal and regulated transcellular Ca^{2+} reabsorption in DCT2 and CNT under conditions of disturbed Ca^{2+} homeostasis.

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