Unraveling the primary principles of renal transcellular calcium transport

Eline van der Hagen

Unraveling the primary principles of renal transcellular calcium transport

Eline van der Hagen

Unraveling the primary principles of renal

transcellular calcium transport

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 12 december 2014
om 12.30 uur precies

door

Eline Adriana Elisabeth van der Hagen
geboren op 26 maart 1986
te Tilburg

Table of contents

Introduction

1

Molecular regulation of the Ca²⁺ balance

Calcium (Ca²⁺) plays a major role in numerous important physiological processes. It has an important function in the generation and preservation of bone. As a second messenger it functions in intracellular signal transduction pathways in a variety of processes, from muscle contraction and neuronal transmission to fertilization, blood coagulation and cell proliferation.[1,2] Consequently, tight regulation of total body Ca²⁺ levels and intracellular Ca²⁺ concentrations is of major importance. Disturbances in Ca²⁺ homeostasis have large implications for health, as demonstrated by heart failure, seizures, osteoporosis and kidney stones.[3-5] In plasma, free Ca²⁺ concentrations are kept within a range of 1.1-1.3 mM,[6,7] while cytosolic Ca²⁺ concentrations are only ~100 nM at resting state.[2] This results in a large chemical gradient across the plasma membrane. Intracellular concentrations are kept at this low level by the actions of the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) transporting cytosolic Ca²⁺ into the organelle and the plasma membrane Ca²⁺-ATPase (PMCA) and Na⁺/Ca²⁺ exchanger (NCX) responsible for Ca²⁺ transport out of the cell.[2]

Overall the Ca²⁺ balance is maintained by the concerted action of intestinal Ca²⁺ absorption, storage in bone and reabsorption in the kidney.[8] To ensure Ca²⁺ homeostasis, tight regulation of Ca²⁺ excretion via the urine is crucial. Of all Ca²⁺ filtered by the glomerulus, the majority (99%) is reabsorbed. Reabsorption of Ca²⁺ largely takes place through paracellular transport in the proximal part of the nephron (65-70%) and the thick ascending limb (TAL, 20-25%).[9,10] The final amount of Ca²⁺ excretion via the urine is determined in the distal part of the nephron, through a highly regulated process of transcellular Ca²⁺ reabsorption.[9,11] Transcellular Ca²⁺ transport is specifically located in the second part of the distal convoluted tubule (DCT2) and connecting tubule (CNT), also known as the distal convolution. Here, at the apical membrane Ca²⁺ is taken up from the pro-urine via the epithelial Ca²⁺ channel transient receptor potential vanilloid 5 (TRPV5).[11,12] After entry, Ca²⁺ is buffered by the Ca²⁺-binding protein calbindin-D$_{28k}$ and the complex diffuses intracellularly towards the basolateral side.[13,14] Finally, Ca²⁺ is extruded towards the blood compartment by the ATP-dependent Ca²⁺-ATPase (PMCA1) and the Na⁺-Ca²⁺ exchanger type 1 (NCX1) (Figure 1).[1,15-17]

Coordinated control of renal transcellular Ca²⁺ transport

The process of transcellular Ca²⁺ transport is tightly regulated by several hormones and other calciotropic factors. The most important regulators of Ca²⁺ homeostasis are the hormones 1,25-dihydroxy vitamin D$_3$ (1,25-(OH)$_2$D$_3$) and parathyroid hormone (PTH). In response to low Ca²⁺ levels in the blood, PTH is released into the circulation by the parathyroid gland, which senses blood Ca²⁺ levels via its Ca²⁺-sensing receptor (CaSR). Subsequently, PTH stimulates Ca²⁺ reabsorption in the kidney, both in TAL and distal convolution.[5] In addition, PTH as well as low plasma Ca²⁺ levels, trigger the renal synthesis of 1,25-(OH)$_2$D$_3$ from its precursor, thereby enhancing intestinal Ca²⁺ absorption.[18] In the kidney, 1,25-(OH)$_2$D$_3$ specifically increases transcellular Ca²⁺ transport in the distal convolution. 1,25-(OH)$_2$D$_3$ acts through the nuclear vitamin D receptor (VDR), which upon activation binds to vitamin D-responsive elements (VDREs) in target gene promoter regions.[5,19] 1,25-(OH)$_2$D$_3$ elevates the expression levels of all Ca²⁺ transport proteins identified in the distal convolution including TRPV5, calbindin-D$_{28k}$,

1
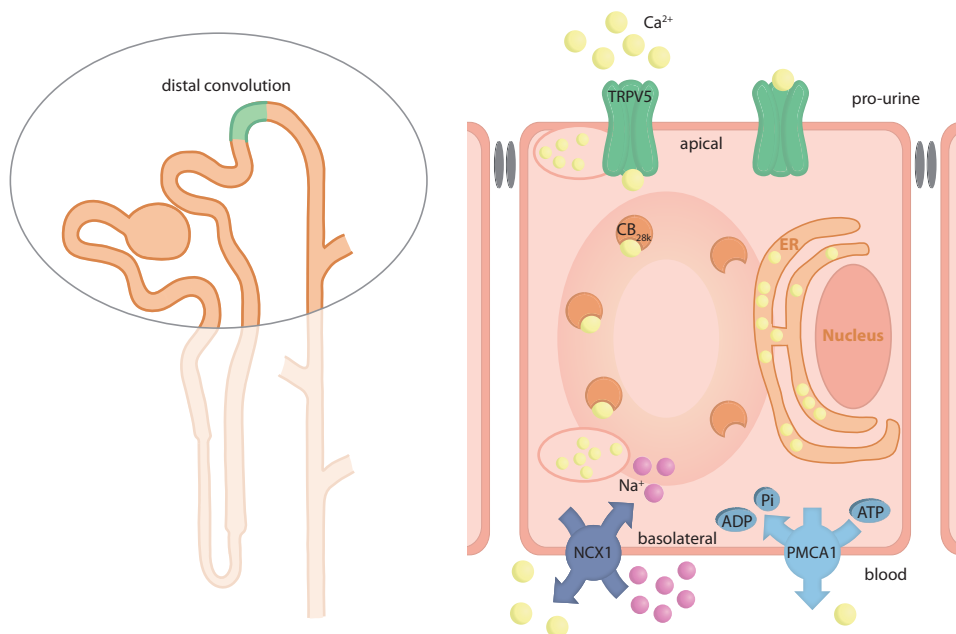
Figure 1 - Transcellular Ca²⁺ transport in the distal convolution. Ca^{2+} entry from the pro-urine is facilitated by the Ca^{2+} channel TRPV5. NCX1 and PMCA1 are responsible for the extrusion of Ca^{2+} from the cell towards the blood against a steep concentration gradient, being driven by the Na^+ gradient and hydrolysis of ATP, respectively. Intracellularly, Ca^{2+} can be transported via vesicles, through the endoplasmic reticulum (ER) or by binding to calbindin-D_{28k} (CB_{28k}).

PMCA1 and NCX1, resulting in enhanced transcellular Ca^{2+} transport.[20,21] PTH elevates expression levels of TRPV5, calbindin-D_{28k} and NCX1, but not PMCA1.[22] In addition to 1,25-$(OH)_2D_3$ and PTH, other non-typical calciotropic hormones are implicated in the regulation of transcellular Ca^{2+} transport as well. Estrogen increases expression levels of TRPV5, calbindin-D_{28k}, PMCA1 and NCX1, through the nuclear estrogen receptor detected in the distal convolution.[23] In contrast, testosterone reduces expression levels.[24] Furthermore, pH and dietary Ca^{2+} influence Ca^{2+} transport, as both metabolic acidosis and a high Ca^{2+} diet result in reduced expression levels of TRPV5 and calbindin-D_{28k}.[20,25,26] Finally, recent evidence suggests that high levels of dietary Na^+ increase the expression levels of TRPV5, calbindin-D_{28k} and NCX1, to compensate for the accompanying reduced proximal Ca^{2+} reabsorption.[27]

Hence, the main Ca^{2+} transport proteins in the distal convolution are tightly regulated at the transcriptional level. Two mechanisms are responsible for this observation. In addition to direct stimulation of expression levels, studies using PTH and TRPV5 knockout animals suggest that Ca^{2+} itself, via influx through TRPV5, controls the expression of other Ca^{2+} transport proteins.[26] Moreover, activity and function of individual components of the Ca^{2+} transport machinery, comprising Ca^{2+} entry through TRPV5, intracellular shuttling and subsequently extrusion by PMCA1 and NCX1, could be directly regulated as well.

Apical Ca²⁺ entry: TRPV5

Within the TRPV subfamily, the highly homologous and Ca^{2+}-selective TRPV5 and TRPV6 channels both play an important role in Ca^{2+} homeostasis. TRPV5 is the predominant isoform in kidney epithelial cells, and shows a distinct localization in the distal convolution segment of the kidney.[28] The channel is localized at the apical membrane facing the tubule lumen and in vesicles observed in close proximity to the plasma membrane.[29] The key role of TRPV5 in transcellular Ca^{2+} transport was confirmed in TRPV5 knockout (TRPV5$^{-/-}$) mice, showing significantly reduced Ca^{2+} transport levels in the distal convolution and severe hypercalciuria.[30] Recently, a different transgenic TRPV5 mouse model also exhibited hypercalciuria. These mice harbor a missense mutation (S682P) resulting in significantly decreased TRPV5 channel Ca^{2+} permeability. Both mice models demonstrate high levels of 1,25-$(OH)_2D_3$ to compensate for urinary Ca^{2+} loss by increasing absorption of Ca^{2+} in the intestine.[30,31] Thus, the epithelial Ca^{2+} channel TRPV5 enables the entry of Ca^{2+} into the cells lining the distal convolution, and is, thereby, the postulated gatekeeper of transcellular Ca^{2+} transport in the kidney.

Molecular characteristics and basic interactions of the TRPV5 channel
As a member of the TRP superfamily, TRPV5 consists of six putative transmembrane segments flanked by large cytoplasmic N- and C-terminal tails (Figure 2). TRP channels are suggested to assemble into tetramers with the hydrophobic loop between transmembrane 5 and 6 acting as a pore domain, together forming a cation selective channel.[32,33] The TRPV5 channel exists in a constitutively open state, with the pore residue D542 being crucial for Ca^{2+} permeability.[34] Although structure and function of the cytosolic N- and C-terminal domains is generally quite diverse between TRP subfamilies,[35] in the TRPV5 N-terminal tail six highly conserved ankyrin repeats have been identified. The ankyrin repeats comprise multiple protein/ligand interaction domains of ~33 residues with helix-loop-helix structures.[36-38] Several factors, such as ATP and calmodulin, have been demonstrated to bind TRP ankyrin repeats. Although the TRPV5 channel N-terminus is involved in channel oligomerization[39] and binding of calmodulin,[40] the role of ankyrin repeats within this domain has not been identified so far.[37,41] In the TRPV5 C-terminal tail a highly conserved TRP domain region is present, which could play a role in the formation of the closed channel conformation.[42] Residue R606 within this TRP domain of TRPV5 has been implicated in sensing of phosphatidylinositol 4,5-bisphosphate (PIP_2), inactivating the channel when PIP_2 levels are depleted.[42]

Trafficking of the TRPV5 channel
Next to the conserved elements, the function of TRPV5 is highly dependent on several specific regulatory sites especially within its C-terminus. For example, the C-terminal residue H712 is crucial for channel internalization.[43] As a plasma membrane protein, TRPV5 is dependent on its trafficking towards the cell surface. Presence of TRPV5 on the plasma membrane is highly dynamic. TRPV5-containing vesicles reside in close proximity to the plasma membrane, ready to be inserted.[29] Moreover, channels are constitutively internalized through processes mediated by clathrin-coated vesicles and caveolar vesicles, the latter being facilitated by caveolin-1.[44,45] After endocytosis, the majority of internalized TRPV5 is recycled, and transported back to the cell surface via interaction with Rab11a in recycling endosomes.[45,46] Several other interactions that could play a role in TRPV5 trafficking have been identified as well. A heterotetrameric

1
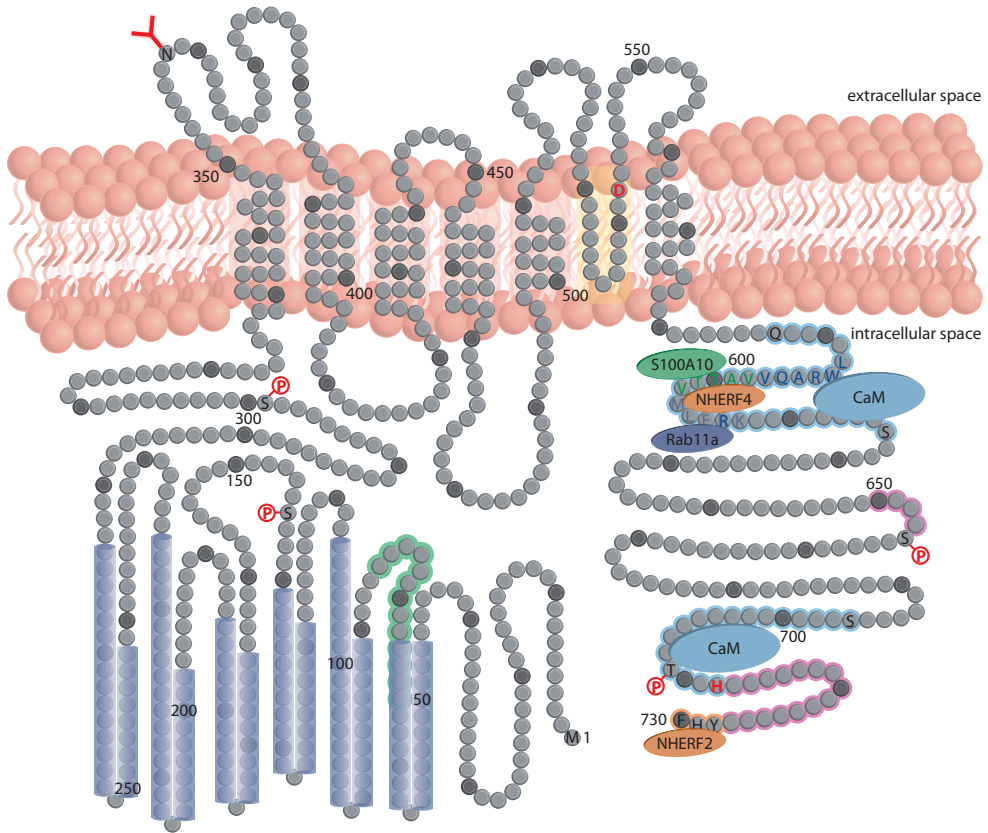
Figure 2 – Schematic representation of TRPV5. The TRPV5 channel consists of six putative transmembrane domains, a pore domain (in yellow) with the critical residue D542, and large cytoplasmic N- and C-termini. An N-glycosylation site is present at residue N358. The N-terminal region contains six highly conserved ankyrin repeats (in purple) and harbors two phosphorylation sites: S144 and S299. In green, the region implicated in channel oligomerizaton is indicated. The C-terminus is important in many regulatory processes. It contains the conserved TRP box region (LWRAQV) and TRP domain involved in sensing of PIP_2 (key residue R606), which also is a hot spot for binding of several other factors: S100A10, Rab11a, NHERF4 and calmodulin (CaM). Two calmodulin binding sites have been identified at positions S696-H712 and Q587-S616. The regions A650-C653 and G701-F730 (in pink) are implicated in Ca^{2+}-dependent inactivation of the channel. At the end of the tail a PDZ motif is present, an interaction site for NHERF2. Two phosphorylation sites are localized in the C-terminus: S654 and T709.

complex of S100A10-annexin 2 is crucial for TRPV5 trafficking by binding to a conserved VATTV sequence in the C-terminus, of which the residue T599 is essential for binding.[47] Other auxiliary proteins are the Na^+/H^+ exchanger regulatory factor 2 (NHERF2) (together with serum- and glucocorticoid-induced kinase 1, SGK1 and With No lysine (K) kinase 4, WNK4) and NHERF4, both interact with the PDZ motifs within the TRPV5 C-terminus.[48-50] NHERF2 is suggested to link plasma membrane proteins such as the TRPV5 channel to the cytoskeleton,[51] and plays a role in regulation of TRPV5 plasma membrane abundance. NHERF4 is a putative membrane scaffold protein for TRPV5 as well.[48] Trafficking of TRPV5 is a highly regulated process, and is influenced by many factors, such as Ca^{2+} itself, pH, PIP_2, Mg^{2+}, tissue kallikrein and hormones such as PTH and klotho.[29]

Differential regulation of the TRPV5 channel

The TRPV5 channel plays a key role in transcellular Ca^{2+} transport in the kidney. Therefore, TRPV5 activity, as controlled by the number of channels on the plasma membrane and single channel activity, is regulated by numerous factors.

Regulation by Ca^{2+}/calmodulin - The TRPV5 C-terminus plays an important role in the regulation of channel activity via its sensitivity for intracellular Ca^{2+} levels. The regions A650-C653 and G701-F730 are implicated in Ca^{2+}-dependent inactivation of the channel.[52] Furthermore, the Ca^{2+}-sensor calmodulin regulates TRPV5 activity by binding to the region S696-H712, while the region Q587-S616 is likely also involved in Ca^{2+}-dependent TRPV5 regulation by calmodulin.[53-55] *In vitro* peptide binding studies indentified three additional putative calmodulin binding sites in TRPV5: V133-R154, Q310-F329 and P405-I428.[55] By sensing intracellular Ca^{2+} levels, binding of calmodulin can provide the channel with an important negative feedback mechanism to protect the cells from elevated intracellular Ca^{2+} levels.[54] In addition, TRPV5-mediated Ca^{2+} uptake is regulated by extracellular Ca^{2+} concentrations through activation of the CaSR on the plasma membrane. Activation of the CaSR eventually leads to protein kinase C (PKC)-mediated phosphorylation of the residues S299 and S654 on the TRPV5 N- and C-termini and subsequently to an enhanced Ca^{2+} transport rate.[56] In contrast, both intra- and extracellular Mg^{2+}, a divalent cation with physical properties most closely to Ca^{2+}, were reported to inhibit TRPV5 function.[34,57]

Regulation by pH - TRPV5 channels are also highly regulated by protons (H^+). Both TRPV5 trafficking and single channel activity are decreased by an acidic pH.[58-60] Firstly, TRPV5 membrane abundance is reduced upon extracellular acidification, due to the retrieval of TRPV5-containing vesicles from the plasma membrane.[58] Secondly, channel activity is decreased by both intra- and extracellular acidification. The TRPV5 residues E522 and K607 are implicated in pH sensing. Residue E552 is present in the extracellular loop close to the pore region, while K607 is located in the intracellular C-terminus. Binding of protons to these residues decreases channel activity due to conformational changes of the pore region, reducing the channel's open probability, the probability of a channel being in the open state.[59,60] The open probability of a channel together with channel conductance determines, the amount of current through the channel in the open state, the total single channel activity.[59,60]

Regulation by hormones - In addition to the regulation of TRPV5 gene expression, different hormones regulate TRPV5 channel activity by modulating channel trafficking and intrinsic channel activity.[22,44,61] Firstly, PTH increases plasma membrane expression by delaying channel retrieval from the plasma membrane, via a pathway similar to the response reported for CaSR activation. Both the CaSR and the parathyroid hormone receptor type 1 (PTH1R) are G-protein-coupled receptors (GPCRs), and seem to act through identical pathways leading to PKC-mediated phosphorylation of the residues S299/S654 in the TRPV5 channel.[44] Secondly, PTH increases TRPV5 channel activity by a very fast response. By binding to the PTH receptor, a cAMP-dependent cascade is activated, resulting in phosphorylation of residue T709 by protein kinase A (PKA).[61] Through phosphorylation of residue T709, the inhibitory binding of calmodulin is reduced, which decreases the negative feedback regulation and subsequently increases channel activity.[54] Additionally, TRPV5 is regulated via its N-linked glycan tree at

1

residue N358. The hormone klotho, specifically produced and secreted by DCT cells, enhances TRPV5-mediated Ca^{2+} entry through stabilization of the channel on the plasma membrane via this N-glycan.[62]

Regulation by proteases - Many proteases have been identified in urine, especially in patients suffering from kidney damage reflected by leakage of large plasma proteins through the disrupted glomerular basement membrane.[63] Proteases can, however, also be produced along the nephron.[64] Tissue kallikrein, a serine protease produced in the CNT, stimulates TRPV5 activity. Binding of tissue kallikrein to the GPCR bradykinin receptor subtype 2 (B2R) increases abundance of TRPV5 channels on the plasma membrane in a process identical to activation of CaSR and PTH1R.[64] In contrast, TRPV5 activity is inhibited by plasmin, a protease present in the urine of patients with Nephrotic Syndrome.[63] Plasmin activates the protease-activated receptor-1 (PAR-1), a GPCR, inducing PKC-mediated phosphorylation of residue S144. In similarity to PTH, plasmin appears to modulate calmodulin binding through phosphorylation of a residue within a potential calmodulin binding site, although in this case leading to channel inhibition rather than activation.[63]

Intracellular Ca^{2+} shuttling: calbindin-D_{28k}

After TRPV5-mediated Ca^{2+} entry at the apical membrane of the epithelial cell, Ca^{2+} is shuttled intracellularly towards the basolateral membrane. Since Ca^{2+} is an important second messenger, chronic elevations in intracellular Ca^{2+} levels should be prevented in order to avoid disturbances of signal transduction pathways. Mathematical models indicated that the observed rate of transcellular Ca^{2+} transport is ~70 times higher than the capacity of free Ca^{2+} diffusion while maintaining normal physiological cytosolic concentrations.[5,65,66] Therefore, several possible mechanisms have been proposed to facilitate intracellular Ca^{2+} shuttling. Firstly, Ca^{2+} can be transported via endocytic vesicles, which are formed due to disruption of local actin filaments upon Ca^{2+} influx (Figure 1). The vesicles fuse with lysosomes and are subsequently transported by microtubules.[5] Secondly, Ca^{2+} can be taken up by the endoplasmic reticulum and thereby tunneled towards the basolateral membrane.[67] Thirdly, Ca^{2+} diffuses towards the basolateral membrane bound to the Ca^{2+}-binding protein calbindin-D_{28k}, according to the so-called "facilitated diffusion model". Hereby, the free Ca^{2+} concentration is kept low while total cellular Ca^{2+} increases, enlarging the capacity for Ca^{2+} diffusion.[5,66,67]

Calbindin-D_{28k} (gene name *CALB1*) is a ~28 kDa protein of the CALB family of proteins with six EF-hand domains. Of the six EF-hand domains in calbindin-D_{28k}, four are functional and bind Ca^{2+} with different affinities. The high affinity site has a slow rate of Ca^{2+} binding (K_D ~180-240 nM, K_{on} $1 \cdot 10^7$ M^{-1} s^{-1}), whereas the low affinity EF hands (K_D ~410-510 nM) bind Ca^{2+} 8 times faster.[68,69] In general, calbindin-D_{28k} is a freely diffusible cytosolic protein, but it is also reported to bind several proteins including TRPV5. Thereby, calbindin-D_{28k} is thought to function both as a Ca^{2+}-buffer and sensor.[13,69,70] Calbindin-D_{28k} is generally expressed in cells handling high levels of Ca^{2+} influx, such as neurons (especially Purkinje cells) and TRPV5-expressing tissues.[13] Calbindin-D_{28k} knockout (calbindin-$D_{28k}^{-/-}$) mice display normal serum concentrations for Ca^{2+}, phosphate and PTH and normal urinary Ca^{2+} excretion. These mice only show a mild impairment in motor coordination/motor learning, most probably due to a disturbed calbindin-D_{28k} function in

Purkinje cells in the brain.[69,71,72] In mice, calbindin-D_{9k} is expressed together with calbindin-D_{28k} in the distal convolution. In other organisms as well as humans, calbindin-D_{9k} is expressed mainly in the intestine where it is implicated in an analogous process of intestinal transcellular Ca^{2+} absorption together with the epithelial Ca^{2+} channel TRPV6.[14,70,73] Renal expression of calbindin-D_{9k} in mice probably compensates for the loss of calbindin-D_{28k} in calbindin-$D_{28k}^{-/-}$ mice. Calbindin-$D_{28k}^{-/-}$/calbindin-$D_{9k}^{-/-}$ double knockout animals showed reduced bone length on a low Ca^{2+} diet. Urinary Ca^{2+} and PTH levels were not measured.[74] In addition, double knockout animals for calbindin-D_{28k} and vitamin D receptor (VDR), which express no calbindin-D_{28k} and only 10% of calbindin-D_{9k} in the kidney, demonstrate an aggravated rachitic skeletal phenotype, higher urinary Ca^{2+} excretion, more severe secondary hyperparathyroidism and thus higher PTH levels when compared to VDR$^{-/-}$ mice.[72] Thus, calbindin-D_{28k} plays an important role in renal transcellular Ca^{2+} transport.

In the distal convolution of the kidney, calbindin-D_{28k} co-localizes with TRPV5 on the luminal side of the membrane, where it buffers Ca^{2+} entering the cell through the channel. Fast buffering of Ca^{2+} by calbindin-D_{28k} at the channel mouth can slow down the Ca^{2+}-dependent inactivation of TRPV5, allowing higher rates of Ca^{2+} influx. A similar mechanism was observed for voltage-gated Ca^{2+} channels in nerve cells.[13,69] Calbindin-D_{28k} translocates to the luminal membrane when intracellular Ca^{2+} levels are low, but only in the presence of TRPV5. Thereby TRPV5 and calbindin-D_{28k} could coordinately facilitate Ca^{2+} transport. However, the mode of interaction between calbindin-D_{28k} and TRPV5 remains to be elucidated.[13] Phenotypes of hypercalciuria as observed in TRPV5$^{-/-}$ mice are not aggravated in TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice, confirming the role of TRPV5 as gatekeeper the process of renal transcellular Ca^{2+} transport.[75]

Basolateral Ca^{2+} extrusion: PMCA1 and NCX1

At the basolateral side of the distal convolution cell, the Ca^{2+} transporters PMCA1 and NCX1 are present to extrude Ca^{2+} towards the blood compartment. NCX1 is the predominant mechanism of extrusion, accounting for ~70% of total Ca^{2+} transported over the basolateral membrane.[15,16,28] NCX1 has a lower affinity for Ca^{2+} as compared to PMCAs, but a much higher transport capacity (turnover number between 2,500 and 5,000 s^{-1} versus 10-50 s^{-1} for PMCAs).[76,77] Hence, PMCA1 is probably responsible for fine-tuning of cytosolic Ca^{2+} levels.[15] In contrast to the entry step, extrusion of Ca^{2+} takes place across a steep concentration gradient, making it very energy consuming. PMCA1 directly consumes ATP through hydrolysis in its catalytic center, while NCX1 function is dependent on the Na^+ gradient established by the Na^+-K^+ ATPase. A concerted action of PMCA and NCX in maintaining intracellular Ca^{2+} levels has been proven in other cells as well, such as neurons and vascular endothelial cells.[78,79]

Physiological role of PMCAs
PMCAs are present in all eukarytic cells where they function to maintain low intracellular Ca^{2+} levels. Four mammalian PMCA isoforms exist (PMCA1-4), as well as over 20 splice variants. PMCA1 and PMCA4 are ubiquitously expressed, while PMCA2 and PMCA3 are expressed almost exclusively in excitable tissues.[80-82] In kidney, primarily PMCA1b and PMCA4b are present.[16,17] PMCA1b is the principal efflux mechanism for intestinal transcellular Ca^{2+} transport mediated by TRPV6 and calbindin-D_{9k}. Therefore, PMCA1b is considered as the major PMCA in

1

distal convolution transcellular Ca^{2+} transport as well.[83,84] Of all nephron segments, the distal convolution shows the highest levels of PMCA protein, implicating its key role in transcellular Ca^{2+} transport.[17] The physiological role of the PMCAs was studied by the generation of different knockout animals. Homozygous PMCA1 knockout (PMCA1$^{-/-}$) mice are embryonic lethal, whereas heterozygous mutants do not show any disturbances, suggesting a crucial housekeeping role. PMCA4 mutant mice do not show any apparent phenotype, although male PMCA4$^{-/-}$ mice show infertility, which suggests a more specified role for this isoform.[85] As far as we know there is no data available on urine and serum Ca^{2+} levels in PMCA1 or PMCA4 knockout mice.

Molecular characteristics of the PMCA pump

PMCAs are part of the large superfamily of P-type ATPases and consist of ten transmembrane domains, two large intracellular loops (between TM2-3 and TM4-5), and cytosolic N- and C-termini (Figure 3). The N-terminus is the most variable region between the four isoforms. In analogy to the SERCA nomenclature, the intracellular loop between TM2 and TM3 together with the N-terminus, forms the so-called actuator "A-domain", which is one of the two binding sites for activator acidic phospholipids. The intracellular loop between TM4 and TM5 contains the catalytic "P-domain" that contains the aspartate residue forming the acyl phosphate intermediate and "N-domain" containing the conserved lysine crucial for binding of ATP. PMCAs transport one Ca^{2+} for every hydrolyzed ATP, with a K_D of 10-20 µM in resting state to 0.2-0.5 µM upon activation.[80,81,86]

All four PMCA isoforms are subjected to alternative splicing, which occurs at site A and C. Splice site A lies within the first intracellular loop within the "A-domain" and site C in the C-terminal
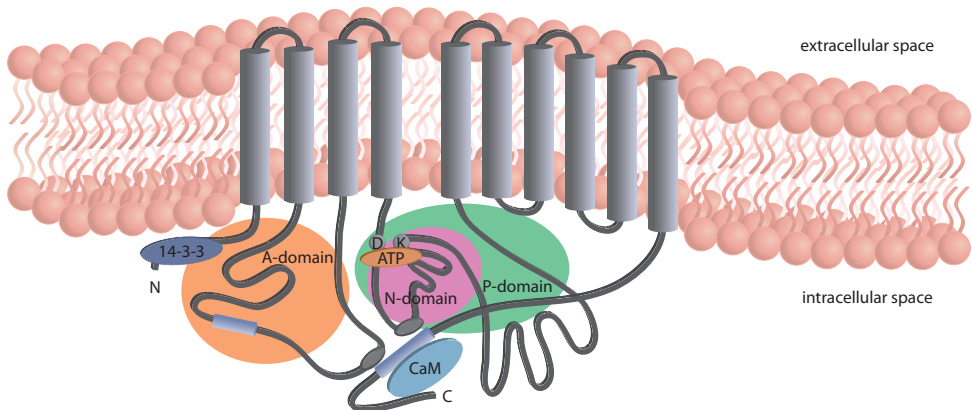
Figure 3 - Topology of the plasma membrane Ca^{2+}-ATPase (PMCA1). PMCA1 contains ten transmembrane domains, intracellular N- and C-termini and two large cytoplasmic domains between TM2-3 and TM4-5. The N-terminus and cytoplasmic domain between TM2-3 comprise the actuator A-domain for binding of activating acidic phospholipids. The cytoplasmic loop between TM4-5 contains both the catalytic P-domain with the aspartate (D) for formation of the acyl phosphate intermediate and the N-domain containing the lysine (K) for binding of ATP. The N-terminus is involved in binding of the 14-3-3 protein. Calmodulin (CaM) binds to a variable region due to alternative splicing (in purple) in the C-terminus, thereby releasing inhibitory interactions with the two cytosolic loops. Another splice region is present in the first cytosolic loop, however for PMCA1 alternative splicing has never been observed at this site.

calmodulin binding site, although PMCA1 is never spliced at site A. Splice variants have specific distributions during development and in different tissues of the body and are dedicated for their function there.[77,81] Differences between splice variants are demonstrated for PMCA4a and PMCA4b (differentially spliced at site C), the latter being much slower in activation and inactivation rates, indicating it is specialized for handling slow changes in Ca^{2+} levels in non-excitable cells.[82,87] Therefore the so-called "b" variant of PMCA1 as found in kidney is well suited for its continuous role in Ca^{2+} extrusion.

Regulation of the PMCA pump
In order to function, PMCAs need to be activated, as Ca^{2+} affinities in resting state are too low to initiate transport. The main regulator of PMCA function is calmodulin. The calmodulin binding region has an auto-inhibitory function on the PMCA pump via its binding to the two large cytoplasmic loops. Upon binding, calmodulin removes the calmodulin binding domain from the cytoplasmic loops, thereby releasing the catalytic domain from auto-inhibition. Binding of calmodulin decreases the K_D from 10-20 μM to less than 1 μM.[15,81,82] Activation by calmodulin is slow and differs among the splice variants. In general the so-called "a" splice variants show lower calmodulin affinities and higher calmodulin-independent basal activities as compared to the "b" variants.[15,81,82] In addition to calmodulin, PMCAs are activated by acidic phospholipids and polyunsaturated fatty acids, which can bind to two regions within the PMCA pump, one in the calmodulin binding domain and the other in the first cytosolic loop. This way the K_D for Ca^{2+} is decreased to ~100 nM.[81] Other processes regulating PMCA function are PKA/PKC-mediated phosphorylation of residues in the C-terminus, proteolytic cleavage of part of the C-terminus and dimerization via the C-terminal tails.[15,82,88] Regulatory mechanisms are strongly dependent on the specific PMCA isoform. For example, some isoforms are activated by PKC (PMCA4b) whereas others are unaffected (PMCA4a)[82].

Physiological role of NCX1
While PMCAs are considered to fine-tune cytosolic Ca^{2+} concentrations, NCX1 is the main extruder of Ca^{2+} in the process of transcellular Ca^{2+} transport. NCX1 belongs to the SLC8 superfamily of cation/Ca^{2+} exchangers, next to NCX consisting of YRBG, CAX, NCKX and CCK proteins.[89] Na^+/Ca^{2+} exchangers comprise a fast and high-capacity Ca^{2+} transporting system. They transport Ca^{2+} in a rate of three Na^+ ions for one Ca^{2+} ion using the physiological Na^+ gradient over the plasma membrane.[15] Under specific circumstances, NCX1 also functions in the reverse-mode, allowing influx of Ca^{2+}.[90,91] The NCX family consists of three members: NCX1, NCX2 and NCX3, of which NCX1 is the main isoform expressed in kidney. NCX1 is highly expressed in cardiac myocytes as well, where its role and regulation in Ca^{2+} extrusion during relaxation and diastole have been extensively studied.[15,92] NCX1 knockout (NCX1$^{-/-}$) mice are embryonic lethal,[93] prohibiting the study of the physiological effects of NCX1 in the kidney.

Molecular characteristics of the NCX1 exchanger
The three NCX proteins are structurally very similar. NCX1 is predicted to consist of ten transmembrane helices (Figure 4). The first five transmembrane domains are separated from the last five transmembrane domains by a large cytosolic loop of ~500 residues. The short N- and C-termini are directed towards the extracellular space. NCX1 has a single N-linked glycosylation site.[90,94,95] In addition, NCX1 proteins contain two highly conserved internal

1

repeats, the so-called α-repeats that are implicated in ion binding and transport. In the large intracellular loop two Ca^{2+} binding domains are present (CBD1 and CBD2).[90] The second Ca^{2+} binding domain contains a region that is subjected to alternative splicing of six exons named A, B, C, D, E and F, leading to the generation of many splice variants. Exons A and B are mutually exclusive. Exon A is generally expressed in excitable tissues, whereas exon B is present in other tissues including kidney. The other four exons are cassette exons that can all be transcribed or spliced out, although all identified isoforms so far contain exon D.[90,96-98] In kidney three splice variants have been identified, NCX1.2 (exons B, C and D), NCX1.3 (exons B and D) and NCX1.7 (exons B, D, and F). NCX1.3 is considered the main NCX present in the kidney.[96,97,99] A second alternative splice region was found in the 5'-untranslated region of the gene, regulating protein expression.[90,99,100]

Regulation of the NCX1 exchanger

By providing the driving force for Ca^{2+} transport, the Na^+ gradient is an important regulator of NCX1 function. Mathematical models indicate that amiloride enhances Ca^{2+} transport by increasing the driving force for NCX1, as intracellular Na^+ levels are reduced due to inhibition of the epithelial Na^+ channel ENaC at the apical membrane.[90,101,102] In addition to Na^+, also intracellular Ca^{2+} regulates NCX1 function. The Ca^{2+} binding domain 1 (CBD1) is the primary Ca^{2+}-sensor, detecting subtle differences in cytosolic Ca^{2+} and subsequently undergoing large structural changes causing activation of the exchanger. The CBD2 undergoes only small structural changes upon Ca^{2+} binding at high intracellular Ca^{2+} concentrations.[103,104] Due to the splice region within CBD2, regulation by Ca^{2+} is isoform specific. The renal NCX1.3 splice variant is more responsive to intracellular Ca^{2+} than the cardiac isoform NCX1.1.[105-107] Another regulatory element within the NCX1 intracelullar loop domain is the so-called XIP (exchanger inhibitor peptide) region, which is localized close to the fifth transmembrane segment. This XIP region is implicated in the regulation by Na^+ and acidic phospholipids and might also be involved in the regulation by Ca^{2+} via calmodulin binding.[108-110] However, calmodulin interaction with NCX1 has not been demonstrated.

Also required for NCX1 function is ATP, by stimulating NCX1 via the formation of PIP_2 from phosphatidylinositol (PI).[102,111-113] PIP_2 enhances the activity of many plasma membrane proteins and ion channels, as was also observed for PMCA and TRPV5.[42,81] In contrast to these transporters, regulation of NCX through phosphorylation processes remains controversial. NCX1 proteins are reported to be stimulated through phosphorylation via PKA- and PKC-mediated pathways, but results are variable, suggesting that regulation could be indirect, being mediated by other proteins in multi-protein complexes.[114-116] In addition, phosphorylation was shown to have different effects on the different NCX1 splice variants. Isoforms containing exon A showed significantly increased Ca^{2+} transport after activation of PKA, whereas expression levels of exon B containing isoforms were not affected.[105]

Basolateral transporters: Trafficking and interactions with cytoskeletal proteins

The process of PMCA and NCX1 trafficking is not studied in great detail. However, interactions with several auxiliary proteins have been demonstrated. The large cytosolic loop of NCX interacts with several proteins including the 14-3-3 protein,[117] ankyrin,[118] the filamentous actin network,[119] caveolin 3 (in the heart),[120,121] and caveolin 1 and 2 (in neuronal cells).[122]
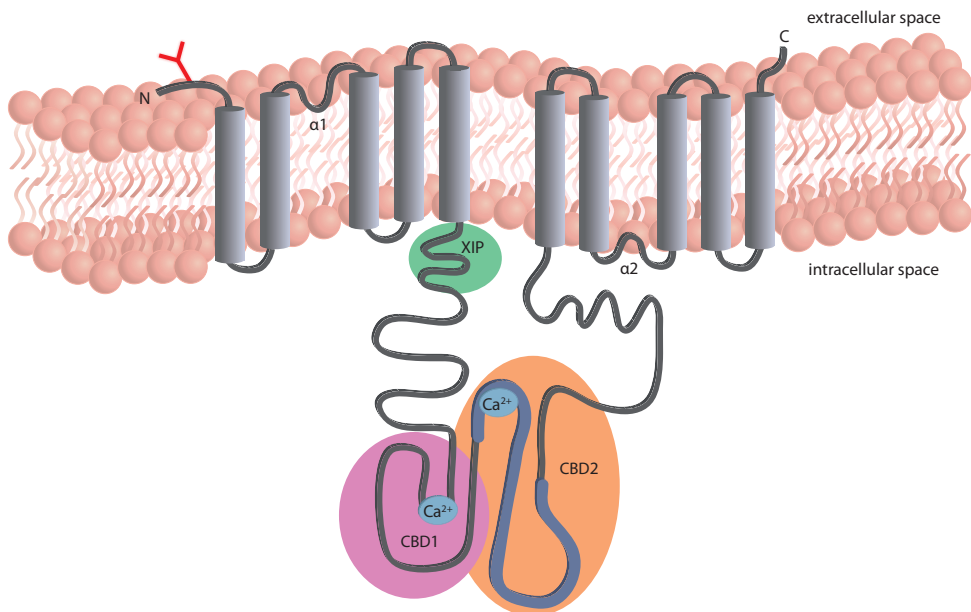
Figure 4 - Topology of the Na$^+$-Ca^{2+} exchanger (NCX1). NCX1 consists of ten transmembrane domains, extracellular N- and C-termini and a large cytoplasmic domain between TM5-6. Two highly conserved α-repeats are present. The N-terminus contains a single glycosylation site. The cytoplasmic loop consists of two Ca^{2+} binding domains (CBD1 and CBD2), of which part of CBD2 is an alternative splicing region. In addition, the so-called XIP region is located close to TM5.

These interactions suggest the formation of multi-protein complexes within specific micro-domains.[15,123] Interestingly, the XIP peptide, a sequence present in the NCX1 protein, was found to inhibit PMCAs, which might implicate cross-regulatory functions on NCX and PMCA regulation.[124] PMCAs have also been shown to interact with components of the cytoskeleton. The 14-3-3 protein plays an inhibitory role on pump activity by binding to the PMCA1 N-terminus and might also be implicated in chaperoning and trafficking of the PMCA Ca^{2+}-pump.[125] Furthermore, a PDZ domain-binding motif is located at the C-terminus of all "b"-splice variants, but none of the "a"-splice variants. As for NCX1, interactions with the cytoskeleton allow PMCAs to cluster into functional multi-protein complexes.[82]

Outline of this thesis

Transcellular Ca^{2+} transport is regulated by the concerted action of entry through the TRPV5 channel, buffering by calbindin-D$_{28k}$ and extrusion via PMCA1 and NCX1. Being considered the gatekeeper of transcellular Ca^{2+} transport, the TRPV5 channel has been intensively studied. Although functional aspects of most regions within the TRPV5 channel have been identified, the role of the ankyrin repeats abundantly present in the N-terminal domain has not been clarified to date. Therefore, the role of the TRPV5 N-terminus on channel function, tetramerization and trafficking has been studied in **chapter 2**. In addition to regulation by its structural features, several hormonal factors have been identified to regulate TRPV5

1

function. These are the classical calciotropic hormones 1,25-$(OH)_2D_3$ and PTH, as well as more atypical hormones such as estrogen and testosteron. TRPV5 function is also regulated by other factors, such as proteases. Thus, many other yet unknown factors could play a role in the regulation of transcellular Ca^{2+} transport via the TRPV5 channel as well. In **chapter 3** a novel potential regulator of transcellular Ca^{2+} transport and TRPV5 activity, the β-adrenergic agonist dobutamine, was studied.

To understand the effects of regulation of the individual components in the highly coordinated process of transcellular Ca^{2+} transport, the molecular interplay of the various elements needs to be studied in detail. Comprehensive studies of renal transcellular Ca^{2+} transport are enabled by a novel primary distal convolution cell model as presented in **chapter 4**. Distal convolution tubule fragments were isolated from mice specifically expressing enhanced Green Fluorescent Protein (EGFP) in these nephron segments, rendering a unique distal convolution cell model with the Ca^{2+} transport characteristics and regulatory pathways remained intact. By being responsible for Ca^{2+} extrusion, also PMCAs and NCX1 play a key role in determining total flux of Ca^{2+} in the distal convolution. The role and regulation of basolateral extrusion of Ca^{2+} in transcellular Ca^{2+} transport in the kidney is poorly described so far. Therefore in **chapter 5,** NCX1 was cloned from mice kidney and its general function and regulation were characterized. In addition, calmodulin was studied as a regulator of renal NCX1 function. Finally, the results and implications of the studies reported in **chapters 2 to 5** are summarized and discussed in **chapter 6**.

References

1.   Hoenderop JG, Bindels RJ: Epithelial Ca^{2+} and Mg^{2+} channels in health and disease. *J Am Soc Nephrol* 16: 15-26, 2005

2.   Berridge MJ, Lipp P, Bootman MD: The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11-21, 2000

3.   Hoorn EJ, Zietse R: Disorders of calcium and magnesium balance: a physiology-based approach. *Pediatr Nephrol* 28: 1195-1206, 2013

4.   Berridge MJ, Bootman MD, Roderick HL: Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4: 517-529, 2003

5.   van de Graaf SF, Bindels RJ, Hoenderop JG: Physiology of epithelial Ca^{2+} and Mg^{2+} transport. *Rev Physiol Biochem Pharmacol* 158: 77-160, 2007

6.   Pekelharing JM, Blankenstein MA, van Haard PMM: Farmacotherapeutisch Kompas - Referentiewaarden klinische chemie, 2013

7.   Toffaletti JG, edited by McClatchey (Lippincott Williams & Wilkins, 2002)

8.   Bushinsky DA: Contribution of intestine, bone, kidney, and dialysis to extracellular fluid calcium content. *Clin J Am Soc Nephrol* 5 Suppl 1: S12-22, 2010

9.   Dimke H, Hoenderop JG, Bindels RJ: Hereditary tubular transport disorders: implications for renal handling of Ca^{2+} and Mg^{2+}. *Clin Sci (Lond)* 118: 1-18, 2010

10.   Friedman PA: Renal calcium transport - Sites and insights. *News in Physiological Sciences* 3: 17-21, 1988

11.   Markadieu N, Bindels RJ, Hoenderop JG: The renal connecting tubule: Resolved and unresolved issues in Ca^{2+} transport. *Int J Biochem Cell Biol* 43: 1-4, 2011

12.   Hoenderop JG, van der Kemp AW, Hartog A, van de Graaf SF, van Os CH, Willems PH, Bindels RJ: Molecular identification of the apical Ca^{2+} channel in 1, 25-dihydroxyvitamin D_3-responsive epithelia. *J Biol Chem* 274: 8375-8378, 1999

13.   Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, Voets T, Nilius B, Clapham DE, Hoenderop JG, Bindels RJ: Calbindin-D_{28K} dynamically controls TRPV5-mediated Ca^{2+} transport. *EMBO J* 25: 2978-2988, 2006

14.   Hoenderop JG, Nilius B, Bindels RJ: Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol* 64: 529-549, 2002

15.   Brini M, Carafoli E: The plasma membrane Ca^{2+} ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb Perspect Biol* 3, 2011

16.   Kip SN, Strehler EE: Characterization of PMCA isoforms and their contribution to transcellular Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol*

284: F122-132, 2003

17. Magyar CE, White KE, Rojas R, Apodaca G, Friedman PA: Plasma membrane Ca²⁺-ATPase and NCX1 Na⁺/Ca²⁺ exchanger expression in distal convoluted tubule cells. *Am J Physiol Renal Physiol* 283: F29-40, 2002

18. Moe SM: Disorders involving calcium, phosphorus, and magnesium. *Prim Care* 35: 215-237, v-vi, 2008

19. Brown AJ, Dusso A, Slatopolsky E: Vitamin D. *Am J Physiol* 277: F157-175, 1999

20. Hoenderop JG, Dardenne O, van Abel M, van der Kemp AW, van Os CH, St -Arnaud R, Bindels RJ: Modulation of renal Ca²⁺ transport protein genes by dietary Ca²⁺ and 1,25-dihydroxyvitamin D₃ in 25-hydroxyvitamin D₃-1alpha-hydroxylase knockout mice. *FASEB J* 16: 1398-1406, 2002

21. Kip SN, Strehler EE: Vitamin D₃ upregulates plasma membrane Ca²⁺-ATPase expression and potentiates apico-basal Ca²⁺ flux in MDCK cells. *Am J Physiol Renal Physiol* 286: F363-369, 2004

22. van Abel M, Hoenderop JG, van der Kemp AW, Friedlaender MM, van Leeuwen JP, Bindels RJ: Coordinated control of renal Ca²⁺ transport proteins by parathyroid hormone. *Kidney Int* 68: 1708-1721, 2005

23. van Abel M, Hoenderop JG, Dardenne O, St Arnaud R, van Os CH, van Leeuwen HJ, Bindels RJ: 1,25-dihydroxyvitamin D₃-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. *J Am Soc Nephrol* 13: 2102-2109, 2002

24. Hsu YJ, Dimke H, Schoeber JP, Hsu SC, Lin SH, Chu P, Hoenderop JG, Bindels RJ: Testosterone increases urinary calcium excretion and inhibits expression of renal calcium transport proteins. *Kidney Int* 77: 601-608, 2010

25. Nijenhuis T, Renkema KY, Hoenderop JG, Bindels RJ: Acid-base status determines the renal expression of Ca²⁺ and Mg²⁺ transport proteins. *J Am Soc Nephrol* 17: 617-626, 2006

26. Johnson CM, Hill CS, Chawla S, Treisman R, Bading H: Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. *J Neurosci* 17: 6189-6202, 1997

27. Yatabe MS, Yatabe J, Takano K, Murakami Y, Sakuta R, Abe S, Sanada H, Kimura J, Watanabe T: Effects of a high-sodium diet on renal tubule Ca²⁺ transporter and claudin expression in Wistar-Kyoto rats. *BMC Nephrol* 13: 160, 2012

28. Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia. *Physiol Rev* 85: 373-422, 2005

29. de Groot T, Bindels RJ, Hoenderop JG: TRPV5: an ingeniously controlled calcium channel. *Kidney Int* 74: 1241-1246, 2008

30. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ: Renal Ca²⁺ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest* 112: 1906-1914, 2003

31. Loh NY, Bentley L, Dimke H, Verkaart S, Tammaro P, Gorvin CM, Stechman MJ, Ahmad BN, Hannan FM, Piret SE, Evans H, Bellantuono I, Hough TA, Fraser WD, Hoenderop JG, Ashcroft FM, Brown SD, Bindels RJ, Cox RD, Thakker RV: Autosomal dominant hypercalciuria in a mouse model due to a mutation of the epithelial calcium channel, TRPV5. *PLoS One* 8: e55412, 2013

32. Venkatachalam K, Montell C: TRP channels. *Annu Rev Biochem* 76: 387-417, 2007

33. Nilius B, Voets T: TRP channels: a TR(I)P through a world of multifunctional cation channels. *Pflugers Arch* 451: 1-10, 2005

34. Nilius B, Vennekens R, Prenen J, Hoenderop JG, Droogmans G, Bindels RJ: The single pore residue Asp542 determines Ca²⁺ permeation and Mg²⁺ block of the epithelial Ca²⁺ channel. *J Biol Chem* 276: 1020-1025, 2001

35. Gaudet R: TRP channels entering the structural era. *J Physiol* 586: 3565-3575, 2008

36. Phelps CB, Huang RJ, Lishko PV, Wang RR, Gaudet R: Structural analyses of the ankyrin repeat domain of TRPV6 and related TRPV ion channels. *Biochemistry* 47: 2476-2484, 2008

37. Gaudet R: A primer on ankyrin repeat function in TRP channels and beyond. *Mol Biosyst* 4: 372-379, 2008

38. Owsianik G, D'Hoedt D, Voets T, Nilius B: Structure-function relationship of the TRP channel superfamily. *Rev Physiol Biochem Pharmacol* 156: 61-90, 2006

39. Chang Q, Gyftogianni E, van de Graaf SF, Hoefs S, Weidema FA, Bindels RJ, Hoenderop JG: Molecular determinants in TRPV5 channel assembly. *J Biol Chem* 279: 54304-54311, 2004

40. Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ: Regulation of the mouse epithelial Ca²⁺ channel TRPV6 by the Ca²⁺-sensor calmodulin. *J Biol Chem* 279: 28855-28861, 2004

41. Lishko PV, Procko E, Jin X, Phelps CB, Gaudet R: The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* 54: 905-918, 2007

42. Rohacs T, Lopes CM, Michailidis I, Logothetis DE: PI(4,5)P₂ regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci* 8: 626-634, 2005

43. de Groot T, Verkaart S, Xi Q, Bindels RJ, Hoenderop JG: The identification of Histidine 712 as a critical residue for constitutive TRPV5 internalization. *J Biol Chem* 285: 28481-28487, 2010

44. Cha SK, Wu T, Huang CL: Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294: F1212-1221, 2008

45. van de Graaf SF, Rescher U, Hoenderop JG, Verkaart S, Bindels RJ, Gerke V: TRPV5 is internalized via clathrin-dependent endocytosis to enter a Ca²⁺-controlled recycling pathway. *J Biol Chem* 283: 4077-4086, 2008

46. van de Graaf SF, Chang Q, Mensenkamp AR, Hoenderop JG, Bindels RJ: Direct interaction with Rab11a targets the epithelial Ca²⁺ channels TRPV5 and TRPV6 to the plasma membrane. *Mol Cell Biol* 26: 303-312, 2006

47. van de Graaf SF, Hoenderop JG, Gkika D, Lamers D, Prenen J, Rescher U, Gerke V, Staub O, Nilius B, Bindels RJ: Functional expression of the epithelial Ca^{2+} channels (TRPV5 and TRPV6) requires association of the S100A10-annexin 2 complex. *EMBO J* 22: 1478-1487, 2003

48. van de Graaf SF, Hoenderop JG, van der Kemp AW, Gisler SM, Bindels RJ: Interaction of the epithelial Ca^{2+} channels TRPV5 and TRPV6 with the intestine- and kidney-enriched PDZ protein NHERF4. *Pflugers Arch* 452: 407-417, 2006

49. Jing H, Na T, Zhang W, Wu G, Liu C, Peng JB: Concerted actions of NHERF2 and WNK4 in regulating TRPV5. *Biochem Biophys Res Commun* 404: 979-984, 2011

50. Palmada M, Poppendieck S, Embark HM, van de Graaf SF, Boehmer C, Bindels RJ, Lang F: Requirement of PDZ domains for the stimulation of the epithelial Ca^{2+} channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase SGK1. *Cell Physiol Biochem* 15: 175-182, 2005

51. Takeda T, McQuistan T, Orlando RA, Farquhar MG: Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. *J Clin Invest* 108: 289-301, 2001

52. Nilius B, Weidema F, Prenen J, Hoenderop JG, Vennekens R, Hoefs S, Droogmans G, Bindels RJ: The carboxyl terminus of the epithelial Ca^{2+} channel ECaC1 is involved in Ca^{2+}-dependent inactivation. *Pflugers Arch* 445: 584-588, 2003

53. Holakovska B, Grycova L, Bily J, Teisinger J: Characterization of calmodulin binding domains in TRPV2 and TRPV5 C-tails. *Amino Acids* 40: 741-748, 2011

54. de Groot T, Kovalevskaya NV, Verkaart S, Schilderink N, Felici M, van der Hagen EA, Bindels RJ, Vuister GW, Hoenderop JG: Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. *Mol Cell Biol* 31: 2845-2853, 2011

55. Kovalevskaya NV, Bokhovchuk FM, Vuister GW: The TRPV5/6 calcium channels contain multiple calmodulin binding sites with differential binding properties. *J Struct Funct Genomics* 13: 91-100, 2012

56. Topala CN, Schoeber JP, Searchfield LE, Riccardi D, Hoenderop JG, Bindels RJ: Activation of the Ca^{2+}-sensing receptor stimulates the activity of the epithelial Ca^{2+} channel TRPV5. *Cell Calcium* 45: 331-339, 2009

57. Lee J, Cha SK, Sun TJ, Huang CL: PIP$_2$ activates TRPV5 and releases its inhibition by intracellular Mg^{2+}. *J Gen Physiol* 126: 439-451, 2005

58. Lambers TT, Oancea E, de Groot T, Topala CN, Hoenderop JG, Bindels RJ: Extracellular pH dynamically controls cell surface delivery of functional TRPV5 channels. *Mol Cell Biol* 27: 1486-1494, 2007

59. Yeh BI, Kim YK, Jabbar W, Huang CL: Conformational changes of pore helix coupled to gating of TRPV5 by protons. *EMBO J* 24: 3224-3234, 2005

60. Yeh BI, Sun TJ, Lee JZ, Chen HH, Huang CL: Mechanism and molecular determinant for regulation of rabbit transient receptor potential type 5 (TRPV5) channel by extracellular pH. *J Biol Chem* 278: 51044-51052, 2003

61. de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG: Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J Am Soc Nephrol* 20: 1693-1704, 2009

62. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG: The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 310: 490-493, 2005

63. Tudpor K, Lainez S, Kwakernaak AJ, Kovalevskaya NV, Verkaart S, van Genesen S, van der Kemp A, Navis G, Bindels RJ, Hoenderop JG: Urinary plasmin inhibits TRPV5 in nephrotic-range proteinuria. *J Am Soc Nephrol* 23: 1824-1834, 2012

64. Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ: Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *EMBO J* 25: 4707-4716, 2006

65. Costanzo LS, Windhager EE: Calcium and sodium transport by the distal convoluted tubule of the rat. *Am J Physiol* 235: F492-506, 1978

66. Bronner F, Stein WD: CaBPr facilitates intracellular diffusion for Ca pumping in distal convoluted tubule. *Am J Physiol* 255: F558-562, 1988

67. Larsson D, Nemere I: Vectorial Transcellular Calcium Transport in Intestine: Integration of Current Models. *J Biomed Biotechnol* 2: 117-119, 2002

68. Nagerl UV, Novo D, Mody I, Vergara JL: Binding kinetics of calbindin-D$_{28k}$ determined by flash photolysis of caged Ca^{2+}. *Biophys J* 79: 3009-3018, 2000

69. Schwaller B: The continuing disappearance of "pure" Ca^{2+} buffers. *Cell Mol Life Sci* 66: 275-300, 2009

70. Schwaller B: Cytosolic Ca^{2+} buffers. *Cold Spring Harb Perspect Biol* 2: a004051, 2010

71. Airaksinen MS, Eilers J, Garaschuk O, Thoenen H, Konnerth A, Meyer M: Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. *Proc Natl Acad Sci U S A* 94: 1488-1493, 1997

72. Zheng W, Xie Y, Li G, Kong J, Feng JQ, Li YC: Critical role of calbindin-D28k in calcium homeostasis revealed by mice lacking both vitamin D receptor and calbindin-D28k. *J Biol Chem* 279: 52406-52413, 2004

73. Sim JY, Jung EM, Yoo YM, Choi KC, Jeung EB: Transcriptional and translational expression of calbindin-D9k in the duodenum, kidney and uterus of a female canine model. *J Vet Sci* 11: 15-19, 2010

74. Ko SH, Choi KC, Oh GT, Jeung EB: Effect of dietary calcium and 1,25-(OH)$_2$D$_3$ on the expression of calcium transport genes in calbindin-D$_{9k}$ and -D$_{28k}$ double knockout mice. *Biochem Biophys Res Commun* 379: 227-232, 2009

75. Gkika D, Hsu YJ, van der Kemp AW, Christakos

S, Bindels RJ, Hoenderop JG: Critical role of the epithelial Ca^{2+} channel TRPV5 in active Ca^{2+} reabsorption as revealed by TRPV5/calbindin-D_{28K} knockout mice. *J Am Soc Nephrol* 17: 3020-3027, 2006

76. Carafoli E, Santella L, Branca D, Brini M: Generation, control, and processing of cellular calcium signals. *Crit Rev Biochem Mol Biol* 36: 107-260, 2001

77. Strehler EE, Zacharias DA: Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol Rev* 81: 21-50, 2001

78. Sedova M, Blatter LA: Dynamic regulation of $[Ca^{2+}]i$ by plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange during capacitative Ca^{2+} entry in bovine vascular endothelial cells. *Cell Calcium* 25: 333-343, 1999

79. Roome CJ, Knopfel T, Empson RM: Functional contributions of the plasma membrane calcium ATPase and the sodium-calcium exchanger at mouse parallel fibre to Purkinje neuron synapses. *Pflugers Arch* 465: 319-331, 2013

80. Strehler EE, Filoteo AG, Penniston JT, Caride AJ: Plasma-membrane Ca^{2+} pumps: structural diversity as the basis for functional versatility. *Biochem Soc Trans* 35: 919-922, 2007

81. Di Leva F, Domi T, Fedrizzi L, Lim D, Carafoli E: The plasma membrane Ca^{2+} ATPase of animal cells: structure, function and regulation. *Arch Biochem Biophys* 476: 65-74, 2008

82. Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi A: Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. *Ann N Y Acad Sci* 1099: 226-236, 2007

83. Hoenderop JG, Willems PH, Bindels RJ: Toward a comprehensive molecular model of active calcium reabsorption. *Am J Physiol Renal Physiol* 278: F352-360, 2000

84. Howard A, Legon S, Walters JR: Human and rat intestinal plasma membrane calcium pump isoforms. *Am J Physiol* 265: G917-925, 1993

85. Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE: Targeted ablation of plasma membrane Ca^{2+}-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *J Biol Chem* 279: 33742-33750, 2004

86. Brini M, Carafoli E: Calcium pumps in health and disease. *Physiol Rev* 89: 1341-1378, 2009

87. Caride AJ, Filoteo AG, Penniston JT, Strehler EE: The plasma membrane Ca^{2+} pump isoform 4a differs from isoform 4b in the mechanism of calmodulin binding and activation kinetics: implications for Ca^{2+} signaling. *J Biol Chem* 282: 25640-25648, 2007

88. Niggli V, Adunyah ES, Carafoli E: Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. *J Biol Chem* 256: 8588-8592, 1981

89. Cai X, Lytton J: The cation/Ca^{2+} exchanger superfamily: phylogenetic analysis and structural implications. *Mol Biol Evol* 21: 1692-1703, 2004

90. Lytton J: Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem J* 406: 365-382, 2007

91. Larbig R, Torres N, Bridge JH, Goldhaber JI, Philipson KD: Activation of reverse Na^+-Ca^{2+} exchange by the Na^+ current augments the cardiac Ca^{2+} transient: evidence from NCX knockout mice. *J Physiol* 588: 3267-3276, 2010

92. Shigekawa M, Iwamoto T: Cardiac Na^+-Ca^{2+} exchange: molecular and pharmacological aspects. *Circ Res* 88: 864-876, 2001

93. Wakimoto K, Kobayashi K, Kuro OM, Yao A, Iwamoto T, Yanaka N, Kita S, Nishida A, Azuma S, Toyoda Y, Omori K, Imahie H, Oka T, Kudoh S, Kohmoto O, Yazaki Y, Shigekawa M, Imai Y, Nabeshima Y, Komuro I: Targeted disruption of Na^+/Ca^{2+} exchanger gene leads to cardiomyocyte apoptosis and defects in heartbeat. *J Biol Chem* 275: 36991-36998, 2000

94. Liao J, Li H, Zeng W, Sauer DB, Belmares R, Jiang Y: Structural insight into the ion-exchange mechanism of the sodium/calcium exchanger. *Science* 335: 686-690, 2012

95. Szerencsei RT, Kinjo TG, Schnetkamp PP: The topology of the C-terminal sections of the NCX1 Na^+/Ca^{2+} exchanger and the NCKX2 Na^+/Ca^{2+}-K^+ exchanger. *Channels (Austin)* 7: 109-114, 2013

96. Kim I, Koh GY, Lee CO: Identification of alternatively spliced Na^+-Ca^{2+} exchanger isoforms expressed in the heart. *Comp Biochem Physiol B Biochem Mol Biol* 119: 157-161, 1998

97. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 269: 5145-5149, 1994

98. Schulze DH, Polumuri SK, Gille T, Ruknudin A: Functional regulation of alternatively spliced Na^+/Ca^{2+} exchanger (NCX1) isoforms. *Ann N Y Acad Sci* 976: 187-196, 2002

99. Lee SL, Yu AS, Lytton J: Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 269: 14849-14852, 1994

100. Nicholas SB, Yang W, Lee SL, Zhu H, Philipson KD, Lytton J: Alternative promoters and cardiac muscle cell-specific expression of the Na^+/Ca^{2+} exchanger gene. *Am J Physiol* 274: H217-232, 1998

101. Bonny O, Edwards A: Calcium reabsorption in the distal tubule: regulation by sodium, pH, and flow. *Am J Physiol Renal Physiol* 304: F585-600, 2013

102. Hilgemann DW, Collins A, Matsuoka S: Steady-state and dynamic properties of cardiac sodium-calcium exchange. Secondary modulation by cytoplasmic calcium and ATP. *J Gen Physiol* 100: 933-961, 1992

103. Hilge M, Aelen J, Vuister GW: Ca^{2+} regulation in the Na^+/Ca^{2+} exchanger involves two markedly different Ca^{2+} sensors. *Mol Cell* 22: 15-25, 2006

104. Nicoll DA, Sawaya MR, Kwon S, Cascio D, Philipson KD, Abramson J: The crystal structure of the primary Ca^{2+} sensor of the Na^+/Ca^{2+} exchanger

reveals a novel Ca²⁺ binding motif. *J Biol Chem* 281: 21577-21581, 2006

105. Ruknudin A, He S, Lederer WJ, Schulze DH: Functional differences between cardiac and renal isoforms of the rat Na⁺-Ca²⁺ exchanger NCX1 expressed in Xenopus oocytes. *J Physiol* 529 Pt 3: 599-610, 2000

106. Dunn J, Elias CL, Le HD, Omelchenko A, Hryshko LV, Lytton J: The molecular determinants of ionic regulatory differences between brain and kidney Na⁺/Ca²⁺ exchanger (NCX1) isoforms. *J Biol Chem* 277: 33957-33962, 2002

107. Hurtado C, Prociuk M, Maddaford TG, Dibrov E, Mesaeli N, Hryshko LV, Pierce GN: Cells expressing unique Na⁺/Ca²⁺ exchange (NCX1) splice variants exhibit different susceptibilities to Ca²⁺ overload. *Am J Physiol Heart Circ Physiol* 290: H2155-2162, 2006

108. Maack C, Ganesan A, Sidor A, O'Rourke B: Cardiac sodium-calcium exchanger is regulated by allosteric calcium and exchanger inhibitory peptide at distinct sites. *Circ Res* 96: 91-99, 2005

109. Matsuoka S, Nicoll DA, He Z, Philipson KD: Regulation of cardiac Na⁺-Ca²⁺ exchanger by the endogenous XIP region. *J Gen Physiol* 109: 273-286, 1997

110. Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, Philipson KD: Identification of a peptide inhibitor of the cardiac sarcolemmal Na⁺-Ca²⁺ exchanger. *J Biol Chem* 266: 1014-1020, 1991

111. Hilgemann DW, Ball R: Regulation of cardiac Na⁺,Ca²⁺ exchange and KATP potassium channels by PIP₂. *Science* 273: 956-959, 1996

112. Hilge M: Ca²⁺ regulation of ion transport in the Na⁺/Ca²⁺ exchanger. *J Biol Chem* 287: 31641-31649, 2012

113. He Z, Feng S, Tong Q, Hilgemann DW, Philipson KD: Interaction of PIP₂ with the XIP region of the cardiac Na/Ca exchanger. *Am J Physiol Cell Physiol* 278: C661-666, 2000

114. Wanichawan P, Louch WE, Hortemo KH, Austbo B, Lunde PK, Scott JD, Sejersted OM, Carlson CR: Full-length cardiac Na⁺/Ca²⁺ exchanger 1 protein is not phosphorylated by protein kinase A. *Am J Physiol Cell Physiol* 300: C989-997, 2011

115. Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M: Protein kinase C-dependent regulation of Na⁺/Ca²⁺ exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* 37: 17230-17238, 1998

116. Morad M, Cleemann L, Menick DR: NCX1 phosphorylation dilemma: a little closer to resolution. Focus on "Full-length cardiac Na⁺/Ca²⁺ exchanger 1 protein is not phosphorylated by protein kinase A". *Am J Physiol Cell Physiol* 300: C970-973, 2011

117. Pulina MV, Rizzuto R, Brini M, Carafoli E: Inhibitory interaction of the plasma membrane Na⁺/Ca²⁺ exchangers with the 14-3-3 proteins. *J Biol Chem* 281: 19645-19654, 2006

118. Li ZP, Burke EP, Frank JS, Bennett V, Philipson KD: The cardiac Na⁺-Ca²⁺ exchanger binds to the cytoskeletal protein ankyrin. *J Biol Chem* 268: 11489-11491, 1993

119. Condrescu M, Reeves JP: Actin-dependent regulation of the cardiac Na⁺/Ca²⁺ exchanger. *Am J Physiol Cell Physiol* 290: C691-701, 2006

120. Camors E, Charue D, Trouve P, Monceau V, Loyer X, Russo-Marie F, Charlemagne D: Association of annexin A5 with Na⁺/Ca²⁺ exchanger and caveolin-3 in non-failing and failing human heart. *J Mol Cell Cardiol* 40: 47-55, 2006

121. Bossuyt J, Taylor BE, James-Kracke M, Hale CC: The cardiac sodium-calcium exchanger associates with caveolin-3. *Ann N Y Acad Sci* 976: 197-204, 2002

122. Cha SH, Shin SY, Jung SY, Kim YT, Park YJ, Kwak JO, Kim HW, Suh CK: Evidence for Na⁺/Ca²⁺ exchanger 1 association with caveolin-1 and -2 in C6 glioma cells. *IUBMB Life* 56: 621-627, 2004

123. Lencesova L, O'Neill A, Resneck WG, Bloch RJ, Blaustein MP: Plasma membrane-cytoskeleton-endoplasmic reticulum complexes in neurons and astrocytes. *J Biol Chem* 279: 2885-2893, 2004

124. Enyedi A, Penniston JT: Autoinhibitory domains of various Ca²⁺ transporters cross-react. *J Biol Chem* 268: 17120-17125, 1993

125. Rimessi A, Coletto L, Pinton P, Rizzuto R, Brini M, Carafoli E: Inhibitory interaction of the 14-3-3{epsilon} protein with isoform 4 of the plasma membrane Ca²⁺-ATPase pump. *J Biol Chem* 280: 37195-37203, 2005

1

Chapter

2

Role of the transient receptor potential vanilloid 5 (TRPV5) protein N-terminus in channel activity, tetramerization, and trafficking

Eline A.E. van der Hagen, Theun de Groot,
Sjoerd Verkaart, Veronika A.M. te Boekhorst,
René J.M. Bindels, Joost G.J. Hoenderop

Department of Physiology, Radboud university
medical center, Nijmegen, the Netherlands

2

Abstract

The epithelial Ca^{2+} channel transient receptor potential vanilloid 5 (TRPV5) constitutes the apical entry site for active Ca^{2+} reabsorption in the kidney. The TRPV5 channel is a member of the TRP family of cation channels, which are composed of four subunits together forming a central pore. Regulation of channel activity is tightly controlled by the intracellular amino (N)- and carboxy (C)-termini. The TRPV5 C-terminus regulates channel activity by various mechanisms, but knowledge regarding the role of the N-terminus remains scarce. To study the role of the N-terminus in TRPV5 regulation, we generated different N-terminal deletion constructs. We found that deletion of the first 32 residues did not affect TRPV5-mediated $^{45}Ca^{2+}$ uptake, whereas deletion up to residue 34 and 75 abolished channel function. Immunocytochemistry demonstrated that these mutant channels were retained in the endoplasmic reticulum and in contrast to wild-type TRPV5 did not reach the Golgi apparatus, explaining the lack of complex glycosylation of the mutants. A limited amount of mutant channels escaped the endoplasmic reticulum and reached the plasma membrane, as shown by cell surface biotinylation. These channels did not internalize, explaining the reduced but significant amount of these mutant channels at the plasma membrane. Wild-type TRPV5 channels, despite significant plasma membrane internalization, showed higher plasma membrane levels compared with the mutant channels. The assembly into tetramers was not affected by the N-terminal deletions. Thus, the N-terminal residues 34-75 are critical in the formation of a functional TRPV5 channel because the deletion mutants were present at the plasma membrane as tetramers, but lacked channel activity.

Introduction

The epithelial Ca^{2+} channel TRPV5, the gatekeeper of active Ca^{2+} reabsorption in the kidney, is a member of the TRP superfamily. TRP channels are involved in many different biological processes ranging from sensory physiology, contributing to taste, olfaction, and vision to muscle proliferation and Ca^{2+} and Mg^{2+} homeostasis of the body.[1,2] Based on their sequence and structural homology, the mammalian TRP channel superfamily is divided into six subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPV (vanilloid). All TRP proteins have a common topology, including six transmembrane segments flanked by large cytoplasmic N- and C-terminal domains. They are suggested to assemble into tetramers, the hydrophobic loop between transmembrane 5 and 6 forming a putative core domain, ultimately leading to the formation of a cation-selective channel.[1,3]

Despite this common topology, the structure and function of the cytosolic N- and C-terminal domains between the different TRP subfamilies are quite diverse.[4] The N-terminus of the TRPM subfamily holds four TRPM homology regions (MHRs), which are essential for proper functioning of these channels.[5-8] Highly conserved ankyrin repeats are present in the N-termini of TRPC, TRPV and TRPA channels. Ankyrin repeats are composed of multiple protein/ligand interaction domains of ~33 residues each with a highly conserved helix-loop-helix structure.[9,10] ATP and calmodulin, have been demonstrated to bind TRP ankyrin repeats; however, more binding factors remain to be identified.[10,11] Within the C-terminus, a highly conserved short hydrophobic stretch, called the TRP box region, has been identified in TRPC, TRPM, and TRPV families.[12] The TRP box region is located just after the sixth transmembrane segment and might serve as a coiled-coil zipper keeping the channel in a closed conformation.[13] The C-terminus of the TRPM subfamily harbors a coiled-coil region, which is involved in channel tetramerization and is critical for channel activation.[7,8,14-16] The members TRPM6 and TRPM7 contain a unique α-kinase domain at the far end of the C-terminus, which regulates channel activity by a variety of molecular mechanisms.[17,18]

From the subfamily of TRPV channels, TRPV1-4 play a role in sensory signaling, whereas the Ca^{2+}-selective and highly homologous TRPV5 and TRPV6 channels are crucial players in Ca^{2+} homeostasis. Like the other members of this TRPV subfamily the C-terminus of TRPV5 is relatively short as compared with the N-terminus. However, various molecular mechanisms regulate TRPV5 channel activity via the C-terminus. In contrast, information regarding the role of the N-terminus in TRPV5 function is more limited. One report revealed a critical role for a N-terminal protein kinase C (PKC) phosphorylation site in tissue kallikrein-mediated TRPV5 stimulation.[19] This site was also necessary for PKC phosphorylation triggered by parathyroid hormone (PTH).[20] Additionally, calmodulin has been demonstrated to bind the TRPV5 N-terminus;[21] however, the role in channel function has not been identified. Finally, residues 64-77 appeared essential for oligomerization of different N-termini within the TRPV5 tetramer.[22] Because TRPV5 is a rate-limiting factor in active Ca^{2+} reabsorption, understanding the molecular regulation of this Ca^{2+} channel is essential to elucidate the mechanisms of renal Ca^{2+} handling. The aim of our study was to gather more information regarding the role of the TRPV5 N-terminus in channel regulation.

Experimental Procedures

Molecular biology - Constructs containing N-terminally HA-tagged rabbit TRPV5 in pCB6 were obtained as described previously.[23] This vector was used to develop HA- and FLAG-tagged N-terminal deletion constructs. First, using *in vitro* mutagenesis the restriction site *Bsp*EI was inserted directly after the HA-tag. Second, a FLAG-tagged TRPV5 construct was generated by replacing the HA-tag by the FLAG tag using restriction sites *Kpn*I and *Bsp*EI. Third, using PCR the different N-terminal TRPV5 truncants were subcloned in the original vectors containing HA- and FLAG-tagged TRPV5 via restriction sites *Bsp*EI and *Xba*I. The tapasin-EGFP construct was a kind gift from the lab of Dr. K. Jalink (Amsterdam).

Cell culture and transfection - HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker Europe, Vervier, Belgium) containing 10% v/v fetal calf serum (PAA, Linz, Austria), 2 mM L-glutamine, 10 µl/ml non-essential amino acids at 37°C in a humidity-controlled incubator with 5% v/v CO_2. For biochemical experiments cells were transiently transfected with the appropriate plasmids using polyethyleneimine (PEI, PolySciences Inc) with a DNA:PEI ratio of 6:1. For patch clamp experiments, cells were transiently transfected with the appropriate plasmids using Lipofectamine® 2000 (Invitrogen). Transfected cells were used after 24 h.

$^{45}Ca^{2+}$ uptake - Radioactive Ca^{2+} uptake was determined using TRPV5-expressing HEK293 cells seeded on poly-L-lysine (Sigma) coated 24-well plates. Cells were pretreated for 20 min with 25 µM BAPTA-AM, subsequently washed with PBS and incubated for 5 min in KHB buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM sodium acetate, 2 mM NaH_2PO_4, 20 mM HEPES pH 7.4, adjusted with NaOH) and finally incubated for 10 min with $^{45}CaCl_2$ (1 µCi/ml) in KHB buffer with voltage-gated Ca^{2+} channel inhibitors (10 µM felodipine, 10 µM verapamil). To block TRPV5-mediated $^{45}Ca^{2+}$ uptake, cells were incubated with 10 µM ruthenium red during the 5 and 10 min incubation steps. After multiple washing steps with ice-cold stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM Na-acetate, 0.5 mM $CaCl_2$, 1.5 mM $LaCl_3$ and 20 mM HEPES pH 7.4, adjusted with NaOH) the uptake of $^{45}Ca^{2+}$ was measured.

Electrophysiology - Patch clamp experiments were performed as described previously[24] in the tight seal, whole cell configuration at room temperature using an EPC-9 patch-clamp amplifier computer controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany).

Immunocytochemistry - TRPV5-expressing cells were grown on coverslips. Culture medium was removed, and cells were washed with cold PBS and subsequently fixed for 30 min at room temperature with 4% w/v formaldehyde in PBS. After washing twice with PBS, cells were treated with permeabilization buffer (0.2% v/v Triton X-100 in PBS with 0.1% w/v BSA) for 15 min. Then, cells were treated with 50 mM NH_4Cl for 15 min and washed with PBS and once with permeabilization buffer. Goat serum dilution buffer (16% v/v donkey or goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.2% v/v Triton X-100 in PBS) was applied before incubations with primary and secondary antibodies. Primary antibodies were diluted 1:200 in goat serum dilution buffer and incubated at 4°C overnight. Secondary antibodies, also diluted 1:200 in goat serum dilution buffer, were conjugated to Alexa Fluor dyes (Invitrogen). Secondary antibody incubations were for 1 h at 37°C. Cells were imaged by confocal microscopy (Olympus FV1000).

Cell surface biotinylation and internalization assay - HEK293 cells were transfected with different TRPV5 constructs in pCB6 using PEI. After 2 days cell surface proteins were biotinylated for 30 min at 4°C using sulfo-NHS-LC-LC-biotin or sulfo-NHS-SS-biotin (0.5 mg/ml; Pierce, Etten-Leur, the Netherlands) as described previously.[25] In short, cells were kept at 4°C or incubated for 30 min at 37°C to allow endocytosis from the plasma membrane. TRPV5 internalization was measured by treating cells with fresh 100 mM 2-mercaptoethanesulfonic acid sodium salt (mesna) for 3 times 20 min at 4°C. After treatment with 120 mM iodoacetic acid to quench mesna, cells were lysed in 150 mM NaCl, 5 mM EGTA, 50 mM Tris, pH 7.5 adjusted with HCl, 1% v/v Triton and protease inhibitors at 4°C and centrifuged at 14,000 x g for 10 min. Finally, biotinylated proteins in the supernatant were precipitated using neutravidin-coupled beads (Pierce), and analyzed by immunoblotting using anti-HA antibodies (2367, Cell Signaling Technology). Anti-β-actin (A5441, Sigma) was used to detect non-specific labeling of intracellular proteins.

Co-immunoprecipitation - HEK293 cells expressing both HA-TRPV5 and FLAG-TRPV5 were lysed in 150 mM NaCl, 5 mM EGTA, 50 mM Tris, pH 7.5 adjusted with HCl, 0.5% v/v Nonidet P-40, and protease inhibitors: 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin at 4°C. After centrifugation at 16,000 x g for 15 min at 4°C, the supernatant containing 3 μg of total protein was added to 30 μl equivalents of protein A/G plus-agarose beads (Santa Cruz Biotechnology), which were preincubated for 3 h at room temperature with 2 μl of polyclonal anti-FLAG antibody raised in rabbit (F7425, Sigma) in 0.7 ml of IPP500 (500 mM NaCl, 10 mM Tris pH 8.0 adjusted with HCl, 0.5% v/v Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin). As a control for non-specific antibody binding 2 μl of rabbit anti-GFP antibody (home-made) was bound to protein A/G plus-agarose beads. The beads were washed three times with the lysis buffer described above. Cell lysate and antibody-bound beads were incubated overnight at 4°C. Subsequently, the beads were washed with lysis buffer and proteins were eluted in Laemmli as described previously.[26]

Oligomerization assay - HA-tagged TRPV5, TRPV5-Δ1-38 and TRPV5-Δ1-75 were expressed in HEK293 cells. Cell lysates were mixed with 2x perfluorooctanoic acid (PFO) sample buffer (100 mM Tris, 20% v/v glycerol, 8% w/v PFO (Sigma-Aldrich), 0.005% w/v bromophenol blue, pH 8.0, adjusted with NaOH) and run on a 4-12% Tris-glycine acrylamide gel (Invitrogen) in 25 mM Tris, 192 mM glycine, 0.5% w/v PFO (pH 8.5, adjusted with NaOH) at 4°C. Subsequently TRPV5 proteins were detected by immunoblotting using anti-HA antibodies.

Molecular modeling - The crystal structure of delta endotoxin Cry8Ea1 (Protein Data Base ID code 3EB7) was used as a template to generate a model of the beginning of the TRPV5 N-terminus (residues 10 to 46). Sequence homology for this region was 35%. YASARA was employed for modeling.

Statistical analysis - In all experiments, data are expressed as mean ± S.E. Statistical comparison was determined using a one-way ANOVA followed by the Newman-Keuls multiple comparison test. p values < 0.05 were considered significant.

Results

TRPV5 lacking the first 38 N-terminal residues is not functional

To analyze the role of the N-terminus in the regulation of the TRPV5 channel, several channel deletion constructs were generated each missing parts of the N-terminus. To study the role of the ankyrin domains in TRPV5 function, deletion constructs TRPV5-Δ1-75 and TRPV5-Δ1-161 were developed missing ankyrin domains 1 and 1-3, respectively (Figure 1A). Additionally, three constructs lacking the first 25, 32 and 38 N-terminal residues, but with intact ankyrin domains were generated (Figure 1A). Human embryonic kidney (HEK) 293 cells were transfected with these constructs to study protein expression and function using $^{45}Ca^{2+}$ uptake assays. Immunoblotting of wild-type TRPV5 (TRPV5-WT) demonstrated a double band (Figure 1B), representing a core and complex glycosylated form.[23] Removal of the first 38 or 75 amino acids completely prevented complex glycosylation, whereas this was only slightly reduced for TRPV5-Δ1-32 (Figure 1B). Unfortunately, deletion mutants TRPV5-Δ1-25 and TRPV5-Δ1-161 showed severely reduced protein expression compared with TRPV5-WT, so these constructs were not included in the functional analysis (data not shown). The activity of the deletion mutants was measured by the influx of radioactive $^{45}Ca^{2+}$ into TRPV5-expressing HEK293 cells. Removal of the first 32 amino acids of the N-terminus did not affect TRPV5-mediated $^{45}Ca^{2+}$ influx (Figure 1C). In contrast, removal of the first 38 or 75 amino acids abolished TRPV5 activity because the $^{45}Ca^{2+}$ influx levels were not different from the TRPV5 blocker ruthenium red (Figure 1C). To investigate further the mechanism by which removal of the N-terminus abolishes TRPV5 function, we used TRPV5-Δ1-38 because this was the minimal deletion eliminating channel activity. Patch clamp analysis was implemented to study whether TRPV5-Δ1-38 was permeable to Na^+. In contrast to TRPV5-WT, the TRPV5-Δ1-38 mutant did not exhibit Na^+ currents different from mock-transfected cells (Figure 1D,E).

TRPV5-Δ1-38 is retained in the endoplasmic reticulum

Because a lack of complex glycosylation can be a direct result of retention in the endoplasmic reticulum, the localization of TRPV5-Δ1-38 was visualized using immunocytochemistry. In short, HEK293 cells coated on coverslips were transfected with TRPV5-WT or TRPV5-Δ1-38. To investigate the presence in the Golgi apparatus, cells were stained with the Golgi marker α-giantin. TRPV5 localization was determined using an antibody against the HA-tag. TRPV5-WT appeared mainly in vesicles and specifically co-localized with α-giantin (Figure 2A). In contrast, TRPV5-Δ1-38 was not localized to vesicles and did not specifically overlap with the Golgi marker (Figure 2A). To study whether TRPV5-Δ1-38 was retained in the endoplasmic reticulum, EGFP-fused tapasin, an endoplasmic reticulum resident protein was co-expressed. In contrast to TRPV5-WT, TRPV5-Δ1-38 co-localized with the endoplasmic reticulum protein tapasin-EGFP (Figure 2B).

Plasma membrane regulation of TRPV5-Δ1-38 is affected

The presence of TRPV5-WT channels at the plasma membrane can not be visualized by immunocytochemistry. Therefore, other biochemical experiments like cell surface biotinylation were used to investigate the expression of TRPV5 at the plasma membrane.[27] Because TRPV5-Δ1-38 was retained in the endoplasmic reticulum we investigated whether this
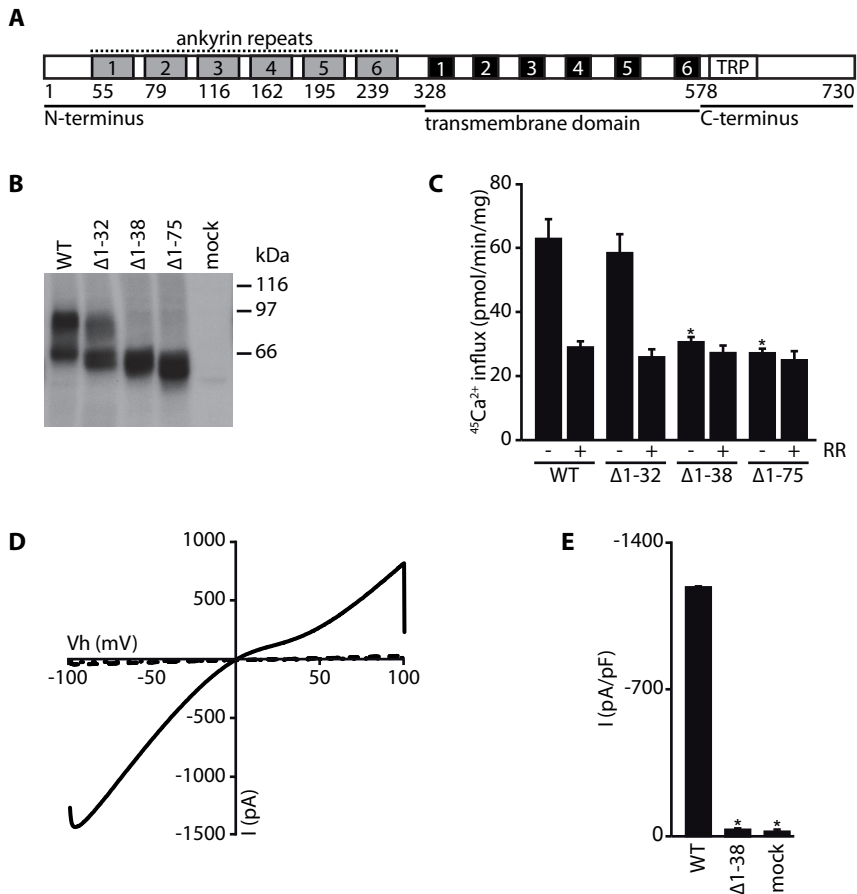
Figure 1 – Channel activity of TRPV5-WT and N-terminal deletion mutants. A) Schematic overview of the TRPV5 protein domains. First residue of the different predicted ankyrin domains is depicted. **B)** Protein expression of TRPV5-WT and mutants. **C)** $^{45}Ca^{2+}$ influx in HEK293 cells transiently expressing TRPV5-WT (n=11), TRPV5-Δ1-32 (n=11), TRPV5-Δ1-38 (n=11) and TRPV5-Δ1-75 (n=9). TRPV5-mediated $^{45}Ca^{2+}$ uptake was blocked with 10 μM ruthenium red (RR). *,$p < 0.05$ versus TRPV5-WT control. **D)** I-V relationship measured from 400-ms voltage ramps in the whole-cell configuration in nominally divalent-free solution for TRPV5-WT (solid line), TRPV5-Δ1-38 (dashed line) and mock (dotted line). **E)** Average current densities at 80 mV in TRPV5-WT (n=8), TRPV5-Δ1-38 (n=11) and mock (n=8) transfected HEK293 cells. *,$p < 0.05$ versus TRPV5-WT.

mutant still reached the plasma membrane. Cell surface proteins of cells expressing TRPV5-WT or TRPV5-Δ1-38 were biotinylated and subsequently precipitated with neutravidin-agarose beads. TRPV5 protein expression in whole cell lysate and biotinylated fraction was determined using immunoblotting. Strikingly, both TRPV5-WT and TRPV5-Δ1-38 were present at the plasma membrane, although the latter to a lower extent (Figure 3A). This was confirmed by quantification of protein levels (Figure 3B). Previous studies indicated a key role of the N-glycan at residue N358 in TRPV5 retrieval from the plasma membrane.[28] To study whether the lack of complex glycosylation of TRPV5-Δ1-38 affected channel plasma membrane trafficking, we performed an internalization assay. Consequently, all proteins at the cell surface were probed

2
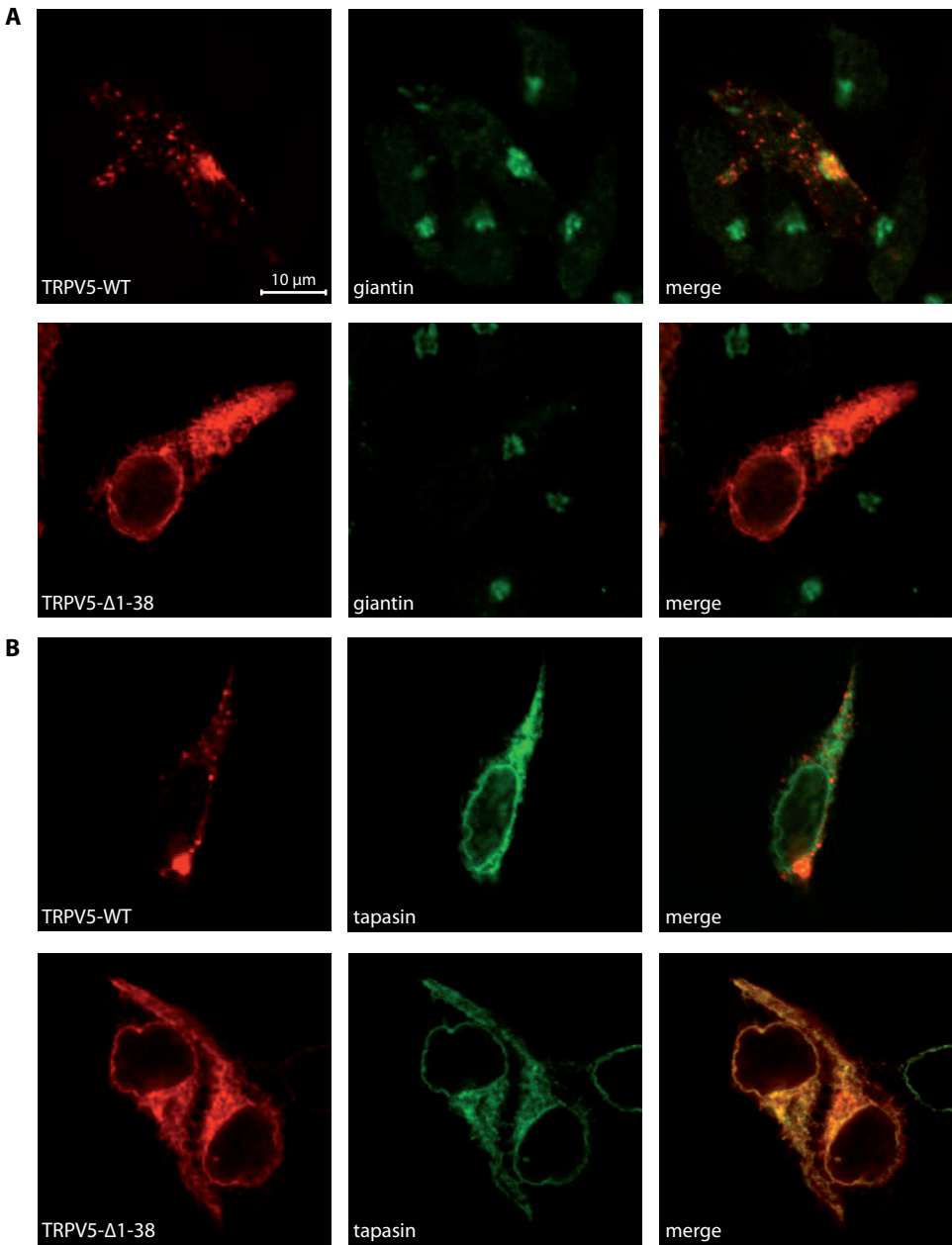
Figure 2 – Subcellular localization of TRPV5-WT and the N-terminal deletion mutant TRPV5-Δ1-38. A) Immunofluorescence staining of HA-tagged TRPV5-WT and TRPV5-Δ1-38, respectively, in the presence of the Golgi marker α-giantin. **B)** Co-expression with the endoplasmic reticulum marker tapasin fused to EGFP.

with sulfo-NHS-SS-biotin, and cells were incubated for 30 min at 37°C to allow internalization of biotinylated proteins. Next, cells were treated with the membrane-impermeant mesna to
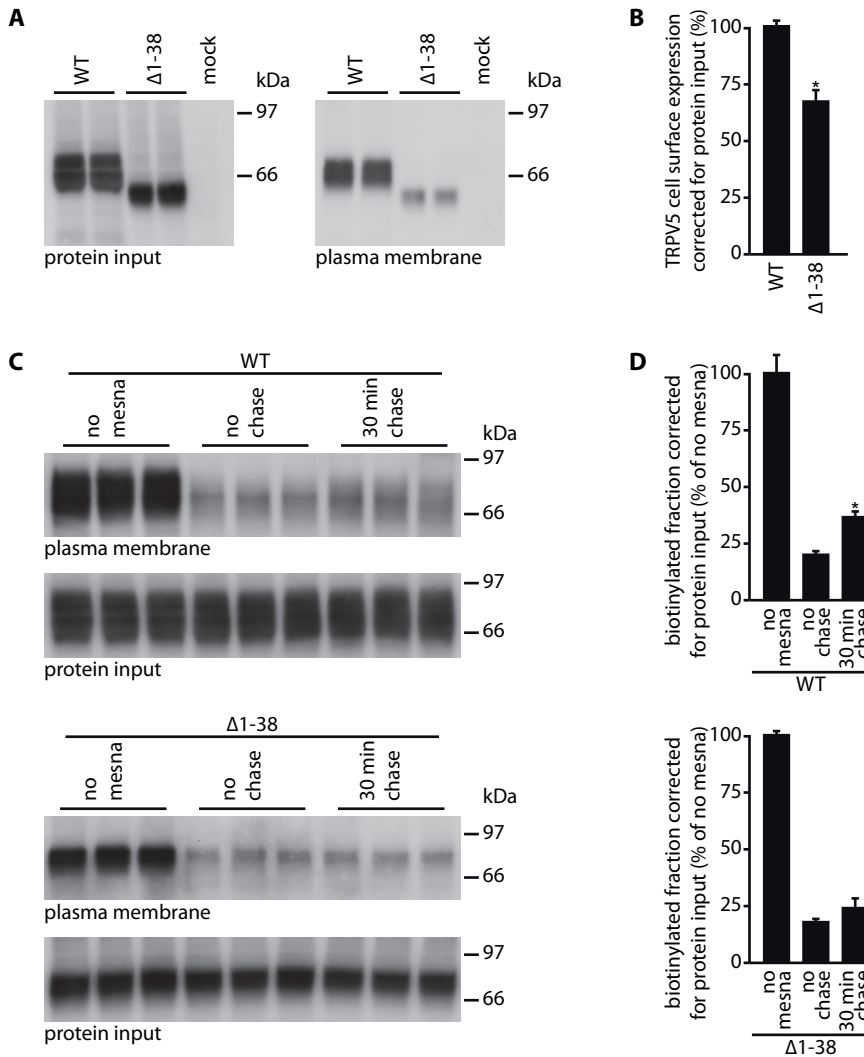
Figure 3 – Plasma membrane expression and internalization of TRPV5-WT and -Δ1-38. A) Plasma membrane expression of TRPV5-WT and TRPV5-Δ1-38 in transiently transfected HEK293 cells assessed by cell surface biotinylation. **B)** Quantification of cell surface expression of TRPV5-WT (n=7) and TRPV5-Δ1-38 (n=7). *,p < 0.05 versus TRPV5-WT. **C)** Internalization of TRPV5-WT and TRPV5-Δ1-38 as measured by the biotinylated fraction after mesna cleavage with and without a 30-min chase. **D)** Quantified amounts of TRPV5-WT (n=5) and TRPV5-Δ1-38 (n=5) channels internalized after a 30-min chase normalized against TRPV5 levels in whole cell lysate (no mesna). *,p < 0.05 versus no chase.

remove all biotin bound to remaining cell surface proteins. Treatment with mesna removed ~80% of all biotin for both TRPV5-WT and TRPV5-Δ1-38 (Figure 3C,D). A 30-min chase at 37℃ resulted in a significant elevation of TRPV5-WT protein amounts (Figure 3C,D). These channels were internalized and, therefore, resistant to mesna treatment. In contrast, a 30-min chase for cells expressing TRPV5-Δ1-38 did not affect the biotinylated fraction, indicating that these channels did not internalize (Figure 3C,D).

Tetramerization of TRPV5-Δ1-38

Tetrameric assembly of TRPV5 is necessary to establish a functional channel because the central Ca^{2+}-selective pore is formed by the hydrophobic pore-forming loops of the four subunits and is located between transmembrane domain 5 and 6.[23] To study whether the retention of TRPV5-Δ1-38 in the endoplasmic reticulum and the absence of function at the plasma membrane are due to the inability to tetramerize, co-immunoprecipitation was performed. Therefore, we transfected HEK293 cells with HA-tagged and FLAG-tagged N-terminal deletion mutants (Figure 4A). Subsequently, FLAG-tagged proteins were precipitated using anti-FLAG antibody-bounded beads (Figure 4A). Then, these immune complexes were examined for the presence of HA-tagged TRPV5 proteins. TRPV5-WT and all deletion mutants were co-precipitated (Figure 4A), demonstrating that oligomerization of the TRPV5 channels was not affected by deletion of the first 75 residues. Next PFO-PAGE was implemented to investigate whether TRPV5-Δ1-38 forms a tetrameric complex at the plasma membrane. Perfluorooctanoic acid (PFO) is a mild detergent, retaining oligomeric structures, and was successfully applied on other TRP channels.[29] Biotinylated fractions and whole cell lysates of TRPV5-WT, TRPV5-Δ1-38 and TRPV5-Δ1-75 were analyzed by PFO-PAGE and subsequent immunoblotting. All subjected proteins were present in four different oligomeric states, demonstrating tetramerization of the truncated channels at the plasma membrane.

Residues Q31 and D34 are essential for proper TRPV5 function

To identify the precise part of TRPV5 critical for proper channel functioning, the residues surrounding amino acid 32 were investigated (Figure 5A). First, two additional deletion
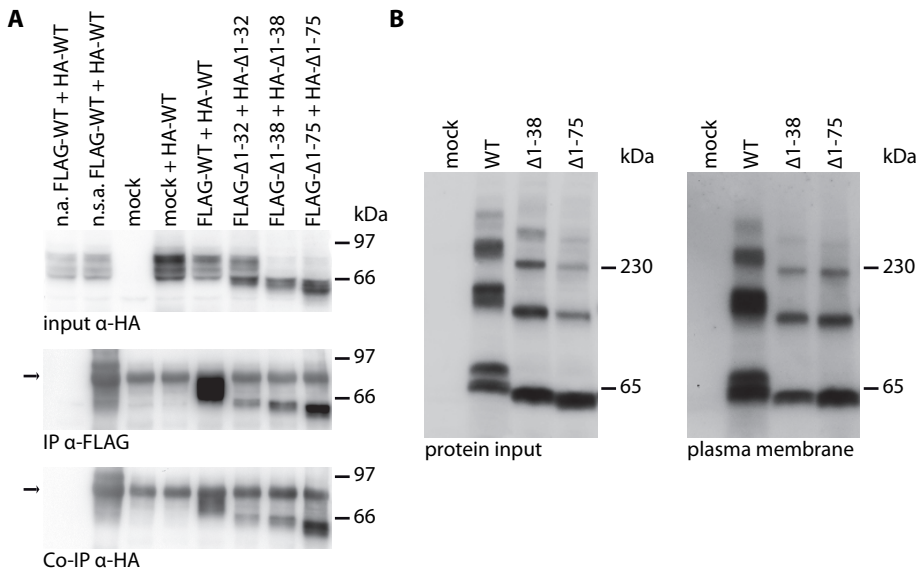
Figure 4 – Tetramerization of the TRPV5 Δ1-38 N-terminal deletion mutant. A) FLAG-tagged and HA-tagged TRPV5-WT and N-terminal deletion mutants were immunoprecipitated (IP) using the anti-FLAG antibody and subsequently co-immunoprecipitated TRPV5 channels were determined using the anti-HA antibody. Of note, antibodies used for precipitation are visible on immunoblot as depicted by the arrows. n.a., no antibody; n.s.a., control antibody for non-specific binding. **B)** Cell surface expression of TRPV5 tetramers was determined by submitting biotinylated fractions to PFO-PAGE analysis.

mutants were generated, TRPV5-Δ1-34 and TRPV5-Δ1-36. TRPV5 function was determined by introducing TRPV5-WT and the different deletion mutants into HEK293 cells, and subsequent the amount of $^{45}Ca^{2+}$ influx was measured. Significant $^{45}Ca^{2+}$ influx was observed for TRPV5-WT and TRPV5-Δ1-32 (Figure 5B). In contrast, deletion mutants TRPV5-Δ1-34, -Δ1-36 and -Δ1-38 were not permeable for Ca^{2+} because they did not exhibit any TRPV5-mediated $^{45}Ca^{2+}$
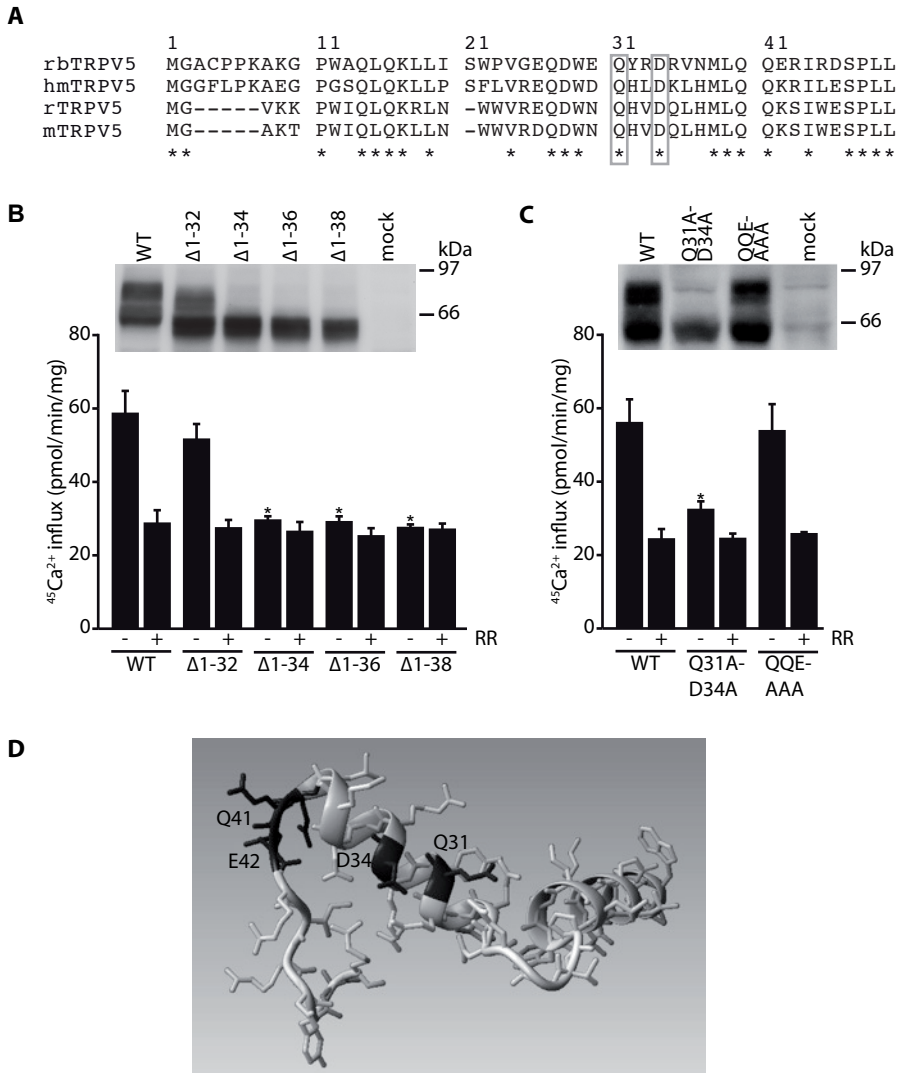
Figure 5 – Identification of key residues in N-terminal region of TRPV5. A) Multiple alignment of rabbit, human, rat and mouse TRPV5 protein sequences, respectively. Conserved residues Q31 and D34 are indicated. **B)** $^{45}Ca^{2+}$ influx in HEK293 cells transiently expressing TRPV5-WT (n=11), TRPV5-Δ1-32 (n=11), TRPV5-Δ1-34 (n=11), TRPV5-Δ1-36 (n=11) and TRPV5-Δ1-38 (n=11). TRPV5-mediated $^{45}Ca^{2+}$ uptake is blocked by addition of 10 μM ruthenium red (RR). *,p < 0.05 versus TRPV5-WT control. Protein expression was determined by immunoblotting (insert) **C)** $^{45}Ca^{2+}$ influx by TRPV5-WT (n=12), the double point mutant TRPV5-Q31A-D34A (n=11) and triple point mutant TRPV5-QQE-AAA (n=12). *,p < 0.05 versus TRPV5-WT control. **D)** Model of TRPV5 N-terminus showing residues 10-46 using YASARA. Mutated residues are indicated.

influx (Figure 5B). Immunoblotting demonstrated the absence of complex glycosylation of TRPV5-Δ1-34, -Δ1-36 and -Δ1-38 (Figure 5B). Next, single point mutations were introduced in the TRPV5 protein to identify the residues critical for channel function. Based on alignments of the rabbit TRPV5 sequence with other species the conserved residues Q31 and D34, located around the N-terminal region essential for TRPV5 function, were selected (Figure 5A). To determine the effect of these residues in TRPV5 function the amino acids were mutated into an alanine and subsequently studied by assessing the $^{45}Ca^{2+}$ influx. $^{45}Ca^{2+}$ influx was significantly reduced in the double point mutant Q31A-D34A (Figure 5C). Furthermore, this mutant lacked complex glycosylation. TRPV5 modeling using the YASARA bioinformatics program suggested the presence of these critical residues within a helix (Figure 5D). Alanine substitution of three similar residues outside the helix (Q40, Q41 and E42), rendering a triple point mutant (QQE-AAA), did not affect TRPV5 function (Figure 5C).

30°C incubation recovers function of TRPV5-Δ1-38 and TRPV5-Q31A-D34A

Retention of proteins at the endoplasmic reticulum, as was observed for TRPV5-Δ1-38, is often a result of protein misfolding.[30] Incubation at lower temperatures is known to rescue proteins that are retained in the endoplasmic reticulum, allowing them to pass the endoplasmic quality control system.[31,32] To investigate whether this procedure can recover the function of TRPV5-Δ1-38 and TRPV5-Q31A-D34A, HEK293 cells were transfected with these constructs and subsequently cultured at 30°C for 18 h. TRPV5 channel activity was measured by the influx of radioactive $^{45}Ca^{2+}$. Activity of the TRPV5-Δ1-38 mutant channel was nearly recovered (Figure 6A). Remarkably, $^{45}Ca^{2+}$ influx was restored in the Q31A-D34A mutant, and immunoblotting demonstrated a double band (Figure 6A,B), indicating recovery of complex glycosylation.
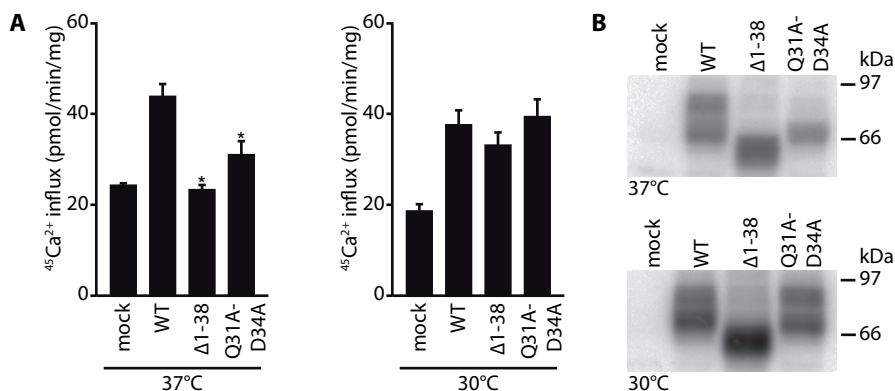
Figure 6 – Activity of TRPV5-WT, TRPV5-Δ1-38 and TRPV5-Q31A-D34A in cells cultured at reduced temperature. A) $^{45}Ca^{2+}$ influx in HEK293 cells transiently expressing mock construct (n=9), TRPV5-WT (n=9), TRPV5-Δ1-38 (n=9) and TRPV5-Q31A-D34A (n=9) cultured for 18 h at 37°C and 30°C respectively. *,p < 0.05 versus TRPV5-WT. B) Protein expression of TRPV5-WT, TRPV5-Δ1-38 and TRPV5-Q31A-D34A as determined by immunoblotting.

Discussion

This study demonstrated the instrumental role of the TRPV5 N-terminus in channel activity based on the following findings. Deletion of the first 38 residues of the N-terminus abolished

TRPV5 activity. The TRPV5-Δ1-38 protein exhibited no complex glycosylation and appeared to be retained in the endoplasmic reticulum. Hence, plasma membrane expression of TRPV5-Δ1-38 was strongly reduced. However, the TRPV5-Δ1-38 mutant is still able to form tetramers at the plasma membrane. Thus, although TRPV5-Δ1-38 channels reach the plasma membrane, they are not active, suggesting that the first 38 residues of the N-terminus are critical for the formation of a functional channel.

Mutation or removal of N-terminal regions abrogates function of many TRP channels studied,[8,33-36] however the underlying mechanism remains poorly understood. Within the TRPV subfamily, N-terminal deletion mutants or splice variants of TRPV1, TRPV4 and TRPV6 were investigated. Deletion of ankyrin repeats 1 and 3 in TRPV4 and ankyrin repeat 3 in TRPV6 disabled channel function. These channels could not tetramerize anymore.[34,35] This suggests that certain ankyrin domains within the N-terminus are critical for proper channel tetramerization. Assembly dysfunction of TRPV4 resulted in retention in the endoplasmic reticulum, whereas the intracellular localization of the N-terminal TRPV6 mutant was not different from the wild-type channel.[34,35] In TRPV1, deletion of the first 114 and more residues abrogated channel function.[33] Interestingly, this is exactly the start of the first ankyrin repeat.[9] Plasma membrane expression was not different from the wild-type channel.[33] Our study demonstrated that N-terminal deletion mutants were retained in the endoplasmic reticulum. Removal of the first ankyrin repeat (TRPV5-Δ1-75) did not affect channel tetramerization. Thus, elimination of essential ankyrin repeats disables the formation of tetramers, whereas deletion of other parts of the N-terminus abrogates channel function due to a different molecular mechanism.

Functional and biochemical experiments revealed that removal of the first 38 residues of the TRPV5 N-terminus resulted in loss of channel function and lack of complex glycosylation. Furthermore, most of the TRPV5-Δ1-38 mutant was retained in the endoplasmic reticulum and did not reach the Golgi apparatus, which is in line with the lack of complex glycosylation. Quality control within the endoplasmic reticulum recognizes and retains misfolded proteins or protein complexes.[30] Strikingly, some TRPV5-Δ1-38 complexes were not retained by the endoplasmic reticulum quality control and reached the plasma membrane. PFO-PAGE demonstrated that these mutants formed tetramers at the plasma membrane, however functional analysis did not show any current for these TRPV5-Δ1-38 tetramers. The absence of activity of TRPV5-Δ1-38 tetramers at the plasma membrane together with the retention of the majority of protein in the endoplasmic reticulum indicates that the TRPV5-Δ1-38 subunits were probably not folded correctly. Thus, an intact TRPV5 N-terminus is not required for channel tetramerization, but the N-terminal residues 34 to 75 are probably essential for correct protein folding and thus normal channel function. The assembly of misfolded proteins into oligomers has been observed by others.[37,38]

Besides the role of the TRPV5 N-terminus in channel function, we made some other interesting observations during this study. First, the co-occurrence of TRPV5-Δ1-38 tetramerization and retention in the endoplasmic reticulum indicates that tetramerization of TRPV5 channels takes place in the endoplasmic reticulum. Oligomerization of proteins is indeed known to take place in the endoplasmic reticulum and is a prerequisite to pass quality control of the endoplasmic

reticulum.[30,39] Second, several TRPV5-Δ1-38 mutants escaped the endoplasmic reticulum quality control eventually appearing at the plasma membrane. These channels did not display any complex glycosylation, indicating that this small subset of channels reached the plasma membrane without being processed by the Golgi apparatus. Protein targeting to the plasma membrane independent from the Golgi apparatus has been observed by others.[40] Finally, we observed that the plasma membrane retrieval of TRPV5-Δ1-38 mutant was delayed. This could be related to the absence of complex glycosylation, which has been implicated in the retrieval of TRPV5 channels from the plasma membrane.[28] This delayed retrieval overestimates the amount of TRPV5-Δ1-38 proteins that reaches the plasma membrane.

In 2003, it was demonstrated that TRPV5 has a tetrameric stoichiometry, which results in a functional channel with an assumed ring-like structure around a central pore.[23] A few years later, Chang *et al.* demonstrated interactions between GST-fused TRPV5 N-termini and between GST-fused TRPV5 N- and C-termini.[22] The N-terminal residues 64-77 were necessary for these interactions and were therefore described as a multimerization domain. We demonstrated that deletion of residues 1-75 abolished channel function and that these channels were still able to form tetramers. Thus, although the TRPV5 N-termini interact, they are not critical for tetramerization of the channel. The TRPV5-Δ1-75 mutant lacks the multimerization domain 64-77, possibly hindering proper folding into a functional channel. The role of the multimerization domain 64-77 in a TRPV5 tetramer remains to be investigated.

In addition, we pinpointed the N-terminal residues essential for proper channel function. Therefore, TRPV5 protein sequences from several species were aligned and within the first 38 N-terminal residues only several residues appeared to be conserved. Residues Q31 and D34 were close or within the N-terminal region essential for TRPV5 function. Furthermore, modeling with delta endotoxin Cry8Ea1, displaying low sequence homology with the TRPV5 N-terminus, suggested that these residues were located in the center of a helix. Alanine substitution of Q31 and D34 rendered a non-functional TRPV5 mutant, lacking complex glycosylation. Mutation of three similar residues, Q40, Q41 and E42, just outside the putative helix did not affect channel function. This indicates that the region around residues 31-34 might be essential for correct folding of the channel or binding of other factors.

Finally, TRPV5-Δ1-38 and TRPV5-Q31A-D34A mutants were rescued at reduced temperature, pointing to misfolding as the reason for retention at the endoplasmic reticulum and inactivity at the membrane. For the TRPV5-Q31A-D34A mutant complex glycosylation was restored as well, suggesting recovery of trafficking through the Golgi apparatus. Thus, the putative helix around residues Q31A-D34A is of major structural importance, affecting both channel processing and function.

This report demonstrated the essential role of the TRPV5 N-terminus in channel function. Deletion of the N-terminus from residue 34 and further abrogated channel function and resulted in endoplasmic retention. Interestingly, we found that the first 75 residues of TRPV5, containing the first ankyrin repeat, are not necessary for channel tetramerization. The role of other ankyrin repeats in TRPV5 tetramerization remains to be established.

Acknowledgements

References

1. Venkatachalam K, Montell C: TRP channels. *Annu Rev Biochem* 76: 387-417, 2007

2. Pedersen SF, Owsianik G, Nilius B: TRP channels: an overview. *Cell Calcium* 38: 233-252, 2005

3. Nilius B, Voets T: TRP channels: a TR(I)P through a world of multifunctional cation channels. *Pflugers Arch* 451: 1-10, 2005

4. Gaudet R: TRP channels entering the structural era. *J Physiol* 586: 3565-3575, 2008

5. Schlingmann KP, Waldegger S, Konrad M, Chubanov V, Gudermann T: TRPM6 and TRPM7-- Gatekeepers of human magnesium metabolism. *Biochim Biophys Acta* 1772: 813-821, 2007

6. Perraud AL, Schmitz C, Scharenberg AM: TRPM2 Ca^{2+} permeable cation channels: from gene to biological function. *Cell Calcium* 33: 519-531, 2003

7. Launay P, Cheng H, Srivatsan S, Penner R, Fleig A, Kinet JP: TRPM4 regulates calcium oscillations after T cell activation. *Science* 306: 1374-1377, 2004

8. Phelps CB, Gaudet R: The role of the N terminus and transmembrane domain of TRPM8 in channel localization and tetramerization. *J Biol Chem* 282: 36474-36480, 2007

9. Phelps CB, Huang RJ, Lishko PV, Wang RR, Gaudet R: Structural analyses of the ankyrin repeat domain of TRPV6 and related TRPV ion channels. *Biochemistry* 47: 2476-2484, 2008

10. Gaudet R: A primer on ankyrin repeat function in TRP channels and beyond. *Mol Biosyst* 4: 372-379, 2008

11. Lishko PV, Procko E, Jin X, Phelps CB, Gaudet R: The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* 54: 905-918, 2007

12. Valente P, Garcia-Sanz N, Gomis A, Fernandez-Carvajal A, Fernandez-Ballester G, Viana F, Belmonte C, Ferrer-Montiel A: Identification of molecular determinants of channel gating in the transient receptor potential box of vanilloid receptor I. *Faseb J* 22: 3298-3309, 2008

13. Gaudet R: in *TRP ion channel function in sensory transduction and cellular signaling cascades*, edited by W. B. Liedtke and S. Heller (CRC Press, 2007)

14. Tsuruda PR, Julius D, Minor DL, Jr.: Coiled coils direct assembly of a cold-activated TRP channel. *Neuron* 51: 201-212, 2006

15. Erler I, Al-Ansary DM, Wissenbach U, Wagner TF, Flockerzi V, Niemeyer BA: Trafficking and assembly of the cold-sensitive TRPM8 channel. *J Biol Chem* 281: 38396-38404, 2006

16. Mei ZZ, Xia R, Beech DJ, Jiang LH: Intracellular coiled-coil domain engaged in subunit interaction and assembly of melastatin-related transient receptor potential channel 2. *J Biol Chem* 281: 38748-38756, 2006

17. Cao G, Thebault S, van der Wijst J, van der Kemp A, Lasonder E, Bindels RJ, Hoenderop JG: RACK1 inhibits TRPM6 activity via phosphorylation of the fused alpha-kinase domain. *Curr Biol* 18: 168-176, 2008

18. Takezawa R, Schmitz C, Demeuse P, Scharenberg AM, Penner R, Fleig A: Receptor-mediated regulation of the TRPM7 channel through its endogenous protein kinase domain. *Proc Natl Acad Sci U S A* 101: 6009-6014, 2004

19. Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ: Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *Embo J* 25: 4707-4716, 2006

20. Cha SK, Wu T, Huang CL: Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294: F1212-1221, 2008

21. Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ: Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. *J Biol Chem* 279: 28855-28861, 2004

22. Chang Q, Gyftogianni E, van de Graaf SF, Hoefs S, Weidema FA, Bindels RJ, Hoenderop JG: Molecular determinants in TRPV5 channel assembly. *J Biol Chem* 279: 54304-54311, 2004

23. Hoenderop JG, Voets T, Hoefs S, Weidema F, Prenen J, Nilius B, Bindels RJ: Homo- and heterotetrameric architecture of the epithelial Ca^{2+} channels TRPV5 and TRPV6. *Embo J* 22: 776-785, 2003

24. Nilius B, Vennekens R, Prenen J, Hoenderop JG, Bindels RJ, Droogmans G: Whole-cell and single channel monovalent cation currents through the novel rabbit epithelial Ca^{2+} channel ECaC. *J Physiol* 527 Pt 2: 239-248, 2000

25. van de Graaf SF, Rescher U, Hoenderop JG, Verkaart S, Bindels RJ, Gerke V: TRPV5 is internalized via clathrin-dependent endocytosis to enter a Ca^{2+}-controlled recycling pathway. *J Biol Chem* 283: 4077-4086, 2008

2

26. Kamsteeg EJ, Wormhoudt TA, Rijss JP, van Os CH, Deen PM: An impaired routing of wild-type aquaporin-2 after tetramerization with an aquaporin-2 mutant explains dominant nephrogenic diabetes insipidus. *Embo J* 18: 2394-2400, 1999

27. Lambers TT, Oancea E, de Groot T, Topala CN, Hoenderop JG, Bindels RJ: Extracellular pH dynamically controls cell surface delivery of functional TRPV5 channels. *Mol Cell Biol* 27: 1486-1494, 2007

28. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG: The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 310: 490-493, 2005

29. Dragoni I, Guida E, McIntyre P: The cold and menthol receptor TRPM8 contains a functionally important double cysteine motif. *J Biol Chem* 281: 37353-37360, 2006

30. Ellgaard L, Helenius A: Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* 4: 181-191, 2003

31. Wang X, Koulov AV, Kellner WA, Riordan JR, Balch WE: Chemical and biological folding contribute to temperature-sensitive DeltaF508 CFTR trafficking. *Traffic* 9: 1878-1893, 2008

32. van den Berghe PV, Stapelbroek JM, Krieger E, de Bie P, van de Graaf SF, de Groot RE, van Beurden E, Spijker E, Houwen RH, Berger R, Klomp LW: Reduced expression of ATP7B affected by Wilson disease-causing mutations is rescued by pharmacological folding chaperones 4-phenylbutyrate and curcumin. *Hepatology* 50: 1783-1795, 2009

33. Jung J, Lee SY, Hwang SW, Cho H, Shin J, Kang YS, Kim S, Oh U: Agonist recognition sites in the cytosolic tails of vanilloid receptor 1. *J Biol Chem* 277: 44448-44454, 2002

34. Erler I, Hirnet D, Wissenbach U, Flockerzi V, Niemeyer BA: Ca^{2+}-selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. *J Biol Chem* 279: 34456-34463, 2004

35. Arniges M, Fernandez-Fernandez JM, Albrecht N, Schaefer M, Valverde MA: Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J Biol Chem* 281: 1580-1586, 2006

36. Engelke M, Friedrich O, Budde P, Schafer C, Niemann U, Zitt C, Jungling E, Rocks O, Luckhoff A, Frey J: Structural domains required for channel function of the mouse transient receptor potential protein homologue TRP1beta. *FEBS Lett* 523: 193-199, 2002

37. Schulteis CT, Nagaya N, Papazian DM: Subunit folding and assembly steps are interspersed during Shaker potassium channel biogenesis. *J Biol Chem* 273: 26210-26217, 1998

38. Green WN, Millar NS: Ion-channel assembly. *Trends Neurosci* 18: 280-287, 1995

39. Farhan H, Freissmuth M, Sitte HH: Oligomerization of neurotransmitter transporters: a ticket from the endoplasmic reticulum to the plasma membrane. *Handb Exp Pharmacol*: 233-249, 2006

40. Nickel W, Seedorf M: Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annu Rev Cell Dev Biol* 24: 287-308, 2008

2

β1-Adrenergic receptor signaling activates the epithelial calcium channel, transient receptor potential vanilloid type 5 (TRPV5), via the protein kinase A pathway

Eline A.E. van der Hagen, Kukiat Tudpor, Sjoerd Verkaart,
Marla Lavrijsen, Annemiete van der Kemp, Femke van Zeeland,
René J.M. Bindels, Joost G.J. Hoenderop

Department of Physiology, Radboud university medical center,
Nijmegen, the Netherlands

Abstract

Epinephrine (Epi) and norepinephrine (NE) are present in the pro-urine. β-adrenergic receptor (β-AR) blockers administered to counteract sympathetic overstimulation in patients with congestive heart failure (CHF) have a negative inotropic effect, resulting in reduced cardiac contractility. Positive inotropes, β1-AR agonists, are used to improve cardiac functions. Active Ca^{2+} reabsorption in the late distal convoluted and connecting tubules (DCT2/CNT) is initiated by Ca^{2+} influx through the transient receptor potential vanilloid type 5 (TRPV5) Ca^{2+} channel. Although it was reported that β-ARs are present in the DCT2/CNT region, their role in active Ca^{2+} reabsorption remains elusive. Here we revealed that β1-AR, but not β2-AR is localized with TRPV5 in DCT2/CNT. Subsequently, treatment of TRPV5-expressing mouse DCT2/CNT primary cell cultures with the β1-AR agonist dobutamine showed enhanced apical-to-basolateral transepithelial Ca^{2+} transport. In human embryonic kidney (HEK293) cells, dobutamine was shown to stimulate cAMP production, signifying functional β1-AR-expression. Fura-2 experiments demonstrated increased activity of TRPV5 in response to dobutamine, which could be prevented by the PKA inhibitor H89. Moreover, non-phosphorylable TRPV5-T709A and phosphorylation-mimicking TRPV5-T709D mutants were unresponsive to dobutamine. Surface biotinylation showed that dobutamine did not affect plasma membrane abundance of TRPV5. In conclusion, activation of β1-AR stimulates active Ca^{2+} reabsorption in DCT2/CNT; an increase in TRPV5 activity via PKA phosphorylation of residue T709 possibly plays an important role. These data explicate a calciotropic role in addition to the inotropic property of β1-AR.

Introduction

Ca^{2+} plays a pivotal role in bone skeletal development and acts as a second messenger in excitatory cells; thus maintenance of Ca^{2+} homeostasis is vital for the body.[1] Ca^{2+} balance is tightly regulated by three primary organs: the gastrointestinal tract, bone, and the kidney.[1] Ca^{2+} absorbed from the intestine is stored mostly in bone (99%) whereas the rest is either conjugated with other charged molecules or freely circulating in blood.[2] The latter portion of Ca^{2+} is filtered in the glomerulus of the kidney and is reabsorbed to the circulation by the proximal tubule (PT, 65%), thick ascending limb of Henle's loop (TAL, 20%), and DCT2/CNT (14%).[3] Mechanisms of Ca^{2+} reabsorption in these three segments are of different origin: passive Ca^{2+} reabsorption through the paracellular space in the PT and TAL, dependent on the electrochemical gradient, whereas active transcellular Ca^{2+} reabsorption in the DCT2/CNT is energetically driven by ATP hydrolysis.[1] Ca^{2+} transport in the TAL and DCT2/CNT is subject to regulation by several factors including G protein-coupled receptors (GPCRs).[1] Agonists of two members of GPCRs i.e., Ca^{2+}-sensing receptor (CaSR) and parathyroid hormone (PTH) receptor type 1, inhibit and stimulate Ca^{2+} reabsorption in TAL and in DCT2/CNT, respectively.[4-6]

Active Ca^{2+} reabsorption in the DCT2/CNT is a crucial fine-tuning event determining final urinary Ca^{2+} excretion and consists of three consecutive steps: apical entry through the transient receptor potential vanilloid type 5 (TRPV5) Ca^{2+} channel, intracellular buffering and translocation to basolateral membrane by calbindin-D_{28k}, and extrusion into the blood by the Na^+/Ca^{2+} exchanger 1 (NCX1) and plasma membrane Ca^{2+} ATPase type 1b (PMCA1b).[1,7,8] TRPV5-mediated Ca^{2+} influx is the rate-limiting step for the active renal Ca^{2+} reabsorption as shown by hypercalciuria and osteopenia in TRPV5-deficient mice.[3] Among six members of the vanilloid TRP family, TRPV5 is the most Ca^{2+}-selective channel possessing a constitutive inward rectifying property at low intracellular Ca^{2+} concentrations and physiological membrane potentials.[9-11] Therefore, the amount of Ca^{2+} influx through the channel depends on channel activity and plasma membrane abundance.[3] TRPV5 activity and plasma membrane abundance are regulated by various factors, including GPCRs. For example, activation of bradykinin receptor type 2 (B2R) and PTH receptor type 1 (PTH1R) initiate phosphorylation of TRPV5 through PLC/DAG/PKC and adenylyl cyclase/cAMP/PKA signaling cascades, respectively.[6,12]

Epinephrine (Epi) and norepinephrine (NE) have diverse hormonal and neurotransmitter functions in the body. Epi and NE were shown to be present in pro-urine filtered from blood, but were also found to be synthesized by renal glomeruli/tubules and released from renal sympathetic nerves.[13] Epi and NE can act through several members of GPCR adrenergic receptors: α1-AR, α2-AR, and three β-ARs. In kidney, α1-AR is expressed in arterioles whereas α2-AR is located predominantly in proximal tubules.[14] α1-AR and α2-AR are responsible for stimulation of renal vasoconstriction and Na^+ reabsorption, respectively.[14] β-ARs can be divided into three subtypes: β1-AR, β2-AR, and β3-AR.[15,16] β1 and β2-ARs are reportedly expressed in rat and mouse DCT, whereas β3-AR is not detectable in the kidney.[17-20] The roles of β1- and β2-ARs are well known respectively for myocardial contraction and vasodilation, whereas β3-AR is important for lipolysis.[18,21] β-AR blockers (β-blockers) are frequently administered to counteract sympathetic overstimulation in patients with congestive heart failure (CHF), resulting in reduced cardiac contractility.[22] Positive inotropes, β1-AR agonists, are used to improve cardiac

functions.[22] Upon stimulation by Epi and NE, β-ARs activate Gα by the exchange of GDP for GTP, which can further enhance the activity of adenylyl cyclase (AC) and phospholipase C (PLC), mediators of cAMP/protein kinase A (cAMP/PKA)- and diacyl glycerol/protein kinase C (DAG/PKC)-dependent phosphorylation, respectively.[20]

Even though Epi and NE are secreted in the pro-urine, to our knowledge, no effects of these hormones through signaling via β-ARs on renal active Ca^{2+} transport have been reported. We hypothesized that β-ARs regulate active Ca^{2+} reabsorption in DCT2/CNT. Thus, the present study aims to investigate *i)* co-localization of β1- and β2-AR with TRPV5 in DCT2/CNT; *ii)* the effect of β-AR activation by a β-AR agonist on active Ca^{2+} reabsorption; and *iii)* the molecular mechanism of TRPV5 activation by β-AR.

Experimental Procedures

Immunohistochemistry - Mouse kidney sections were incubated for 16 h at 4°C with rabbit polyclonal antibody against β1-AR (1:100) or β2-AR (1:300) (NB100-92439 and NBP1-68227, Novus Biologicals). To visualize the receptors, an enhancer step was performed using a biotinylated goat anti-rabbit antibody. Biotin was then coupled to streptavidin-HRP and visualized with Thyramid (TSA Fluorescein System, NEL701A001KT, Perkin Elmer). TRPV5 staining was described previously.[4] Negative controls, i.e. conjugated antibodies solely, were devoid of any staining.

Isolation of DCT2/CNT using COPAS sorting and primary cell culture - Transgenic mice expressing EGFP under the TRPV5 promoter were generated as described.[23] Mice were maintained on a SSNIFF rodent complete diet (SSNIFF, Soest, Germany) with free access to water. The animal ethics board of Radboud University Nijmegen approved all of the experimental procedures. The process of renal tubular sorting by COPAS (Union Biometrica) has been described.[24] Approximately 4,000-10,000 tubules from one mouse were pelleted (800 x *g*, 5 min, 4°C) prior to culture or mRNA isolation as described below. For primary culture 2,000 fluorescent tubules, a mixture of tubules from two mice, were resuspended into warmed cell culture medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F12, Invitrogen) with supplements as described[24] and seeded onto 0.33 cm^2 polycarbonate Transwell® inserts (Corning Costar) previously coated with rat-tail collagen (16 μl per insert of 0.75 mg/ml collagen in 95% v/v ethanol with 0.25% v/v acetic acid). Volumes used in the apical and basolateral compartments were 100 μl and 600 μl, respectively. Cells were cultured at 37°C in 5% v/v CO_2-95% v/v atmospheric air and the medium was refreshed every day.

$^{45}Ca^{2+}$ transport measurement - For radioactive $^{45}Ca^{2+}$ transport experiments, primary cells cultured on Transwell® inserts as described above were used. Cells were used 7 to 8 days after seeding; the day prior to the experiment transepithelial electrical resistance (TEER) was measured using an epithelial volt-ohmmeter (EVOM, World Precision Instruments). Cells were pretreated by adding 5 μM indomethacin to the culture medium for 30 min. Culture medium was removed and cells were washed once with physiological salt solution (PSS: 140 mM NaCl, 2 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 5 mM L-alanine, 5 μM indomethacin, 10 mM HEPES/Tris, pH 7.4.). The apical compartment contained 100 μl and the

basolateral compartment contained 600 μl. Physiological salt solution was replaced with the same volumes of the pre-warmed identical solution with and without 10 μM dobutamine hydrochloride (sc-203031, Santa Cruz Biotechnology); the apical medium contained 3 μCi/ml $^{45}Ca^{2+}$. Ten microliters of basolateral medium was collected at time points 0, 15, 30, 60, 120 and 180 min and analyzed for radioactivity in a PerkinElmer Life Sciences liquid scintillation counter. Unidirectional flux from the apical side to the basolateral side ($J_{A \to B}$) was calculated as described previously.[25]

Semi-quantitative real-time PCR - To evaluate mRNA expression, RNA was extracted from pellets of 1,000 tubules isolated by COPAS using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. The obtained RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen). cDNA was used to determine mRNA expression levels by real-time PCR of *Adrb1* (β1-AR) and *Adrb2* (β2-AR). As controls *Trpv5* and *Slc12a1* (Na-K-Cl cotransporter 2, NKCC2) were included. The housekeeping gene *Gapdh* was used as an endogenous control. Primers targeting the genes of interest were designed using Primer3 and are listed in Table 1. Normal PCR using AmpliTaq Gold® (Invitrogen) was performed on HEK293 cDNA to check for β1-AR and β2-AR expression (primers: *ADRB1* F, CCCAGAAGCAGGTGAAGAAG R, CCCAGCCAGTTGAAGAAGAC, *ADRB2* F, GGCAGCTCCAGAAGATTGAC, R, TGGAAGGCAATCCTGAAATC), products were visualized on a 1.5% w/v agarose gel.

Table 1 - *Mus Musculus* oligonuceotide sequences used in real-time PCR		
Gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Adrb1	GATCTGGTCATGGGATTGCT	AAGTCCAGAGCTCGCAGAAG
Adrb2	CCTTAACTGGTTGGGCTACG	GCTCTTGAAAGGCAATCCTG
Trpv5	CTGGAGCTTGTGGTTTCCTC	TCCACTTCAGGCTCACCAG
Slc12a1	GGCTTGATCTTTGCTTTTGC	CCATCATTGAATCGCTCTCC
Gapdh	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC

Adrb1, β1-adrenergic receptor gene; *Adrb2*, β2-adrenergic receptor gene; *Trpv5*, transient receptor potential vanilloid type 5 gene; *Slc12a1*, Na-K-Cl cotransporter 2 gene (NKCC2); *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase gene.

Cell culture and transfection - HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker) containing 10% v/v fetal calf serum (PAA), 2 mM L-glutamine, 10 μL/mL non-essential amino acids at 37°C in a humidity controlled incubator with 5% CO_2. Cells were transiently transfected with the appropriate plasmids using polyethyleneimine (PEI, PolySciences Inc) with a DNA:PEI ratio of 1:6 for biochemical or live-cell imaging experiments.

Cell surface biotinylation and immunoblotting - HEK293 cells (9×10^4 cells/cm^2) were plated and transfected with 5 μg TRPV5-HA pCINeo/IRES-EGFP or pCINeo/IRES-EGFP in 10-cm dishes. At 24 h after transfection cells were re-seeded on poly-L lysine (Sigma) coated 6-well plates. At 48 h after transfection, cells were incubated for 1 h with 10 μM dobutamine or vehicle. Cells were homogenized in 1 ml of lysis buffer as described previously using the NHS-LC-LC-biotin (Pierce).[6] Finally, biotinylated proteins were precipitated using neutravidin beads (Pierce). TRPV5 expression was analyzed by immunoblotting for the precipitates (plasma membrane

fraction) and for the total cell lysates using the anti-HA antibody.[6]

Intracellular Ca²⁺ and cAMP measurements using Fura-2 AM and EPAC-EGFP-mCherry - For combined $[Ca^{2+}]_i$ and cAMP measurements, HEK293 cells were seeded onto coverslips (Ø 25 mm) and co-transfected with the cAMP sensor (exchange protein directly activated by cAMP, (EPAC)-EGFP-mCherry,[26] kindly provided by Dr. K. Jalink) for cAMP measurements, and the appropriate TRPV5 pCINeo/IRES-EGFP construct from which the sequence encoding EGFP was deleted. After 24 h, cells were loaded with 3 µM Fura-2 AM (Molecular Probes) and 0.01% v/v Pluronic F-129 (Molecular Probes) in DMEM medium at 37°C for 20 min. After loading, cells were washed with PBS and allowed to equilibrate at 37°C for another 10 min in HEPES-Tris buffer (in mM: 132.0 NaCl, 4.2 KCl, 1.4 CaCl₂, 1.0 MgCl₂, 5.5 D-glucose and 10.0 HEPES/Tris, pH 7.4). Changes in $[Ca^{2+}]_i$ and cAMP were simultaneously monitored, using a modified Fura-2 protocol allowing simultaneous measurements of Ca²⁺ and cAMP.[10] Briefly, the cAMP sensor EPAC EGFP-mCherry was excited at 488 using a monochromator. Fluorescence emission light was directed by a 525DRLP dichroic mirror (Omega Optical Inc.) through a 535af26 emission filter (EGFP fluorescence) and a 565ALP emission filter (mCherry fluorescence) onto a CoolSNAP HQ monochrome CCD-camera. The integration time of the CCD-camera was set at 200 ms with a sampling interval of 5 s. All measurements were performed at room temperature. Quantitative image analysis was performed with Metamorph 6.0 (Molecular Devices Corporation). For each wavelength, the mean fluorescence intensity was monitored in an intracellular region and, for purpose of background correction, an extracellular region of identical size. After background correction, the fluorescence emission ratio of 340 nm and 380 nm excitation was calculated to determine the $[Ca^{2+}]_i$ while the fluorescence emission ratio of the red and green fluorescence of the cAMP sensor was used to determine changes in cellular cAMP content.

Statistical analysis - If not specified otherwise, the data are expressed as mean ± S.E. The significant differences between the means of two groups were analyzed by unpaired Student's *t* test using the measurements per cell/sample (n≥9) of at least three independent experiments. The level of statistical significance is $p < 0.05$. All data were analyzed using GraphPad Prism.

Results

β1-AR and β2-AR are expressed in DCT2/CNT
To investigate the role of β1-AR and β2-AR on active Ca²⁺ reabsorption in DCT2/CNT, immunohistochemical staining was performed on frozen mouse kidney sections. Stainings showed co-localization of β1-AR with TRPV5 in DCT2/CNT segments, but not β2-AR (Figure 1A,C). β1-AR and β2-AR antibody specificity was evaluated by negative controls of secondary antibody only (Figure 1B, neg ctrl panel) and staining of liver tissue (Figure 1B, liver panel) reported to be negative for both β1-AR and β2-AR.[27,28] mRNA expression of β1-AR (*Adrb1*) and β2-AR (*Adrb2*) in DCT2/CNT cells isolated from pTRPV5-EGFP mice using the COPAS Biosorter, was determined by real-time PCR. Results showed that both β1-AR and β2-AR are enriched in DCT2/CNT compared with total kidney cortex material, although the expression level of β1-AR is considerably higher (Figure 2A), especially compared with the negative control NKCC2 *(Slc12a1)* normally expressed in TAL. TRPV5 expression was used as a positive control. Altogether, these results indicated that β1-AR is expressed at the DCT2/CNT part of the
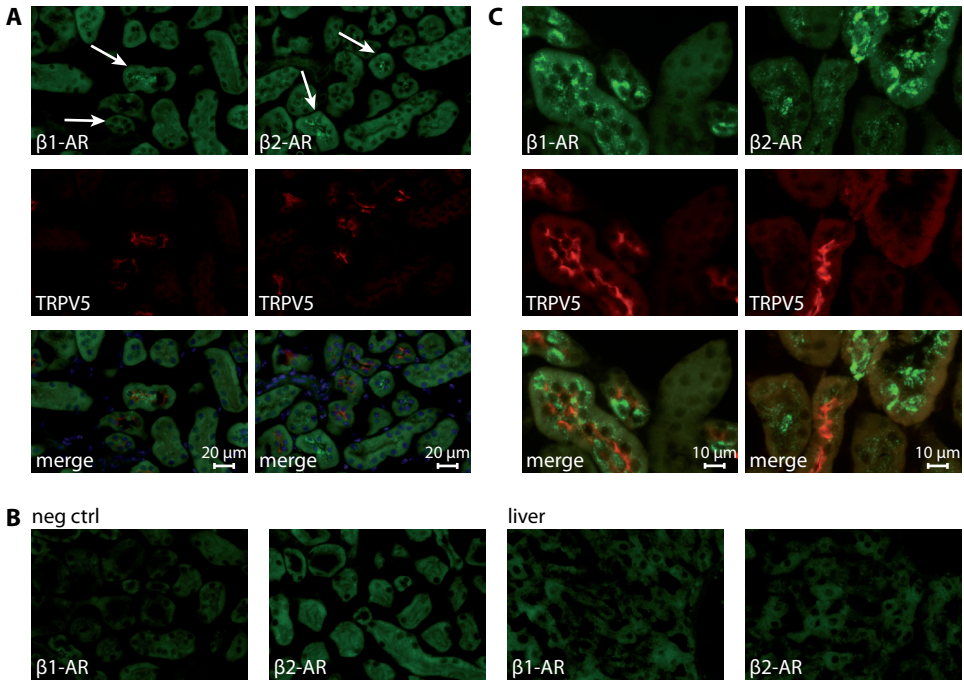
Figure 1 – β1-AR and β2-AR expression in mouse kidney. A) Localization of β1-AR, β2-AR, and TRPV5 is depicted by immunohistochemistry on mouse kidney tissue. **B)** Negative controls of secondary antibody only and stainings of liver tissue are shown. **C)** Higher magnification immunohistochemistry pictures of the localization of β1-AR, β2-AR, and TRPV5 are shown.

nephron together with TRPV5. At the cellular level, only part of the β1-AR signal did co-localize with TRPV5, suggesting expression in both apical and basolateral areas (Figure 1C).

Dobutamine stimulates transcellular Ca^{2+} transport in mouse DCT2/CNT

To examine the role of β1-AR stimulation on Ca^{2+} reabsorption in the distal part of the nephron, primary DCT2/CNT tubules were cultured on Transwell inserts for 7/8 days allowing the formation of tight monolayers (transepithelial electrical resistance of 603±80 Ω·cm^2 1 day prior to the experiment). Transepithelial transport of Ca^{2+} from the apical to basolateral compartment was measured by apical addition of ^{45}Ca^{2+} in the presence or absence of 10 µM dobutamine, a specific β1-AR agonist, in both the apical and basolateral solution. Transport of Ca^{2+} was significantly increased by 10 µM dobutamine (19±2 and 30±4 nmol·hr^{-1}·cm^{-2} for control and dobutamine, respectively, $p < 0.05$) after 60 min (Figure 2B).

β1-AR agonist stimulates cAMP production in HEK293 cells

To study the role of the Ca^{2+} channel TRPV5 in β1-AR-mediated activation of transcellular Ca^{2+} transport, we examined the effect of dobutamine in HEK293 cells, which were reported to express β1-AR.[29] First, the expression of β1- and β2-AR in HEK293 cells was confirmed (Figure 3). Identity of the observed bands was verified by sequencing of the respective PCR products. Because β1-AR activation leads to cAMP production[18] the β1-AR-mediated TRPV5 Ca^{2+} influx
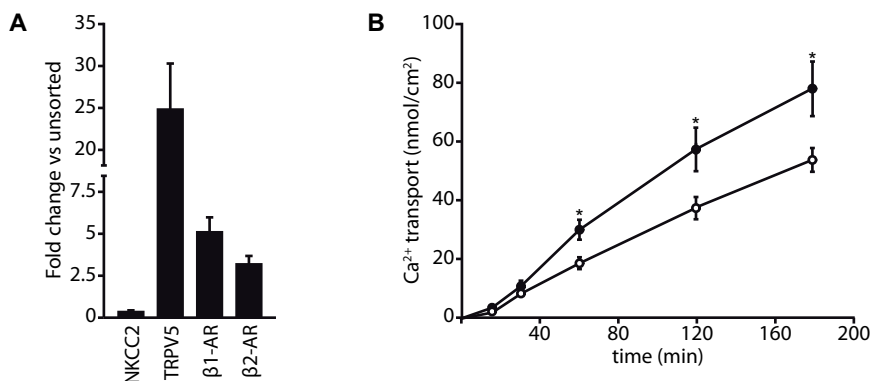
Figure 2 – Effect of β1-AR agonist (10 μM dobutamine) on transepithelial Ca²⁺ transport in mouse DCT2/CNT primary cultured monolayers. A) mRNA enrichment of β1-AR (*Adrb1*) and β2-AR (*Adrb2*) in isolated mouse DCT2/CNT cells compared with total kidney cortex; TRPV5 (*Trpv5*) was used as positive control, NKCC2 (*Slc12a1*) as negative control (n=6). **B)** Ca²⁺ transport under control (open circles) and dobutamine (closed circles) conditions, respectively. t_{15}, t_{30}, t_{60}, t_{120} and t_{180} indicate time points of sample collections after 15-, 30-, 60-, 120- and 180-min incubation. *,$p < 0.05$ in comparison to control at the same time point (n=7-8).

in HEK293 cells transfected with the cAMP sensor EPAC and TRPV5 was studied as described under "Experimental Procedures". The EPAC and Fura-2 ratios were measured before (t=0 min, t_0) and after (t=3 min, t_3) dobutamine treatment (10 μM) (Figure 4A,D). The EPAC ratio plotted at t_0 and t_3 indicated a significant increase in cAMP levels after a 2-min incubation with dobutamine as measured by a 5.1±0.1% increase in the EGFP:mcherry ratio due to loss of the FRET signal upon binding of cAMP (Figure 4B). Fura-2 ratios were increased by 19.5±0.3% upon stimulation by dobutamine (Figure 4E). The dobutamine-induced

Figure 3 – Expression of β1- and β2-AR in HEK293 cells. + and - signify PCR products of reverse-transcribed and non-reverse-transcribed mRNA, respectively. Product sizes were 346 bp for β1-AR and 328 bp for β2-AR.

increase in EPAC and Fura-2 ratio was dose-dependent (Figure 4C,F), implying that β1-AR signaling can functionally stimulate TRPV5 activity in HEK293 cells.

Dobutamine does not change TRPV5 protein abundance at the plasma membrane
Ca²⁺ influx through TRPV5 is regulated by plasma membrane abundance and activity of the channel.[3] The effect of dobutamine on TRPV5 plasma membrane expression was assessed in HEK293 cells transiently transfected with TRPV5 using cell surface biotinylation. A 60-min incubation with 10 μM dobutamine did not affect plasma membrane abundance of TRPV5 (Figure 5A,B). Total expression of TRPV5 in the dobutamine-treated cells did not differ from control as well (Figure 5A).

Dobutamine stimulates TRPV5-mediated Ca²⁺ uptake in HEK293 cells via PKA phosphorylation of T709 residue
TRPV5 channel activity is known to be stimulated by cAMP/PKA-mediated pathways.[6] HEK293 cells were transfected with TRPV5 and loaded with the Fura-2 Ca²⁺-sensor to measure Ca²⁺
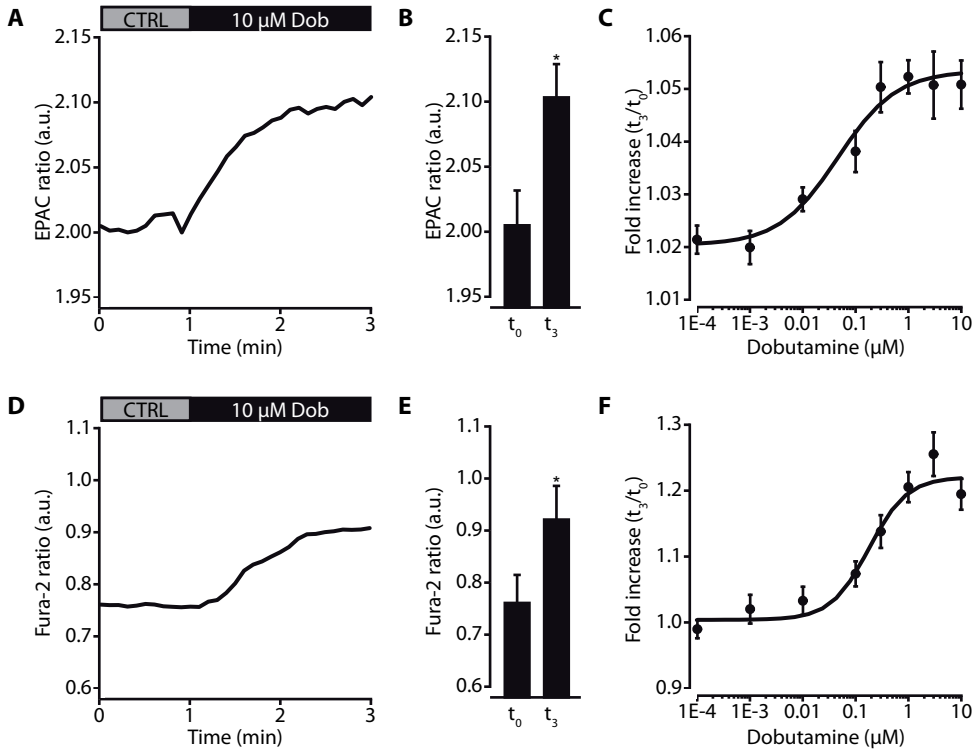
Figure 4 – Effect of β1-AR agonist dobutamine on cAMP production and Ca^{2+} uptake in HEK293 cells transiently transfected with TRPV5 and EPAC sensor. A) Averaged EPAC trace upon the addition of 10 μM dobutamine (Dob) (n=60 cells). **B)** EPAC ratios before (t_0) and after (t_3) dobutamine stimulation. *,p < 0.05 compared with t_0 (n=60 cells). **C)** Dose-response curve of the effect of dobutamine treatment on cAMP production as measured by EPAC ratios (t_3 compared with t_0) (n=28-76 cells/condition). **D)** Averaged Fura-2 trace upon the addition of 10 μM dobutamine (Dob) (n=59 cells). **E)** Fura-2 ratios demonstrated before (t_0) and after (t_3) dobutamine stimulation. *,p < 0.05 compared with t_0 (n=59 cells). **F)** Dose-response curve of the effect of dobutamine treatment on Ca^{2+} uptake as measured by Fura-2 ratios (t_3 compared with t_0) (29-70 cells/condition).
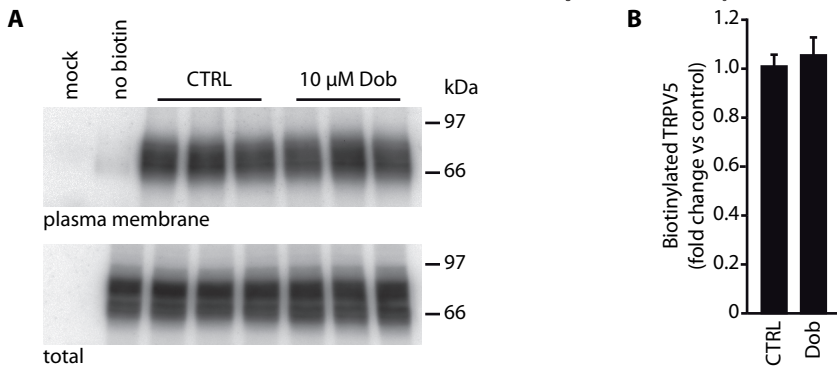
Figure 5 – Effect of β1-AR agonist on plasma membrane abundance of TRPV5. A) TRPV5-transfected HEK293 cells were treated for 60 min with or without 10 μM dobutamine. NHS-LC-LC-biotin was added to medium for 30 min before the cells were lysed, and the biotinylated (plasma membrane) fraction was pulled down using neutravidin-agarose beads. Representative immunoblot for TRPV5 analyzed for plasma membrane and total expression. CTRL, control; Dob, dobutamine-treated. **B)** Calculated immunoblot densities (n=9).

uptake. After a 1-min incubation with dobutamine, Ca^{2+} uptake reached maximal activation, ~2 times elevated compared with basal uptake levels (Figure 6A). Interestingly, these stimulatory effects were prevented when the cells were preincubated (30 min) with the PKA inhibitor, H89 (Figure 6A,B), indicating that β1-AR activation is mediated by PKA phosphorylation of the TRPV5 channel. Previously, threonine residue 709 on the C-terminus of TRPV5 was identified as the sole TRPV5 PKA phosphorylation site.[10] To study the effects of dobutamine on phosphorylation of TRPV5 at this residue, TRPV5-WT, non-phosphorylated mutant TRPV5-T709A, and the TRPV5-T709D mutant mimicking phosphorylation at this residue were used. The stimulatory effect of dobutamine was absent in the non-phosphorylable TRPV5-T709A mutant and the phosphorylation-mimicking TRPV5-T709D mutant, the latter already showing activation, indicating that the T709 residue is crucial for β1-AR-mediated PKA phosphorylation (Figure 6C,D).
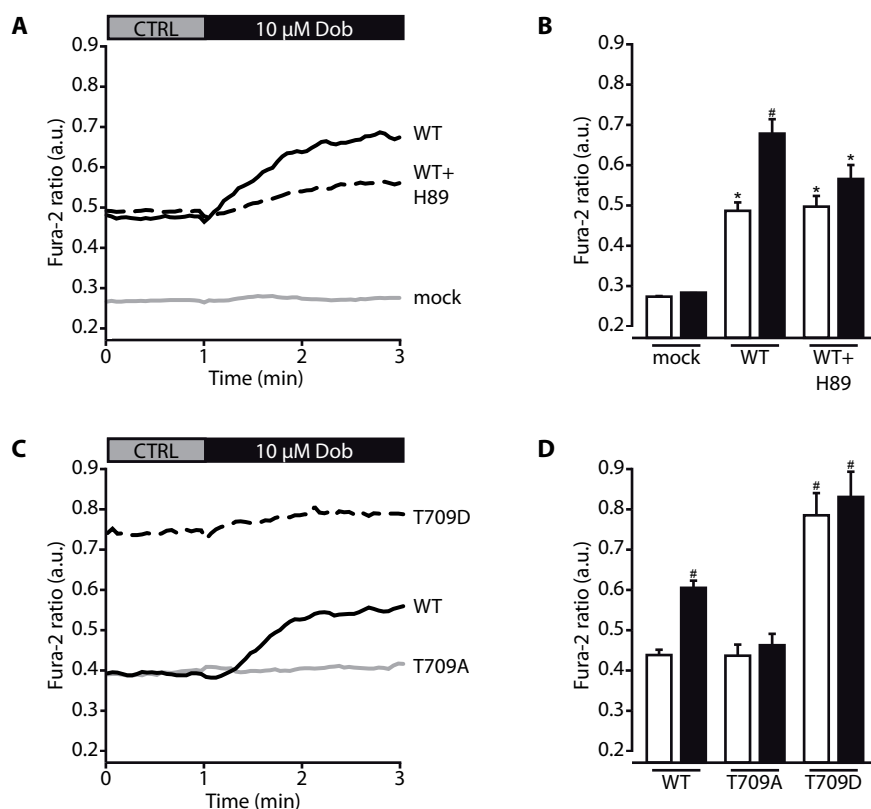
Figure 6 – Effect of β1-AR agonist on TRPV5-dependent Ca^{2+} uptake. A) Averaged Fura-2 traces of mock (n=10 cells), TRPV5-WT transfected preincubated with PKA inhibitor, H89, 10 μM (n=26) and without (n=12) upon the addition of 1 μM dobutamine. **B)** Fura-2 levels before (open bars) and after (filled bars) dobutamine stimulation of mock-transfected (n=10 cells), TRPV5-WT (n=12), and H89-treated TRPV5-WT (n=26) *,$p < 0.05$ compared with untreated mock. #,$p < 0.05$ compared with the respective dobutamine untreated (t_0). **C)** Averaged Fura-2 traces of TRPV5-T709A (n=21) and TRPV5-T709D (n=20 cells) mutants upon the addition of dobutamine compared with TRPV5-WT (n=12). **D)** Fura-2 levels demonstrated before (open bars) and after (filled bars) dobutamine stimulation of TRPV5-WT (n=12 cells), TRPV5-T709A (n=21), and TRPV5-T709D (n=20) #,$p < 0.05$ compared with untreated WT.

Discussion

The present study indicates that activation of β1-AR stimulates TRPV5-mediated Ca^{2+} transepithelial transport via PKA phosphorylation of the Ca^{2+} channel. Our conclusion relies on the following findings: *i*) β1-AR co-localizes with TRPV5 in mouse DCT2/CNT; *ii*) the β1-AR agonist dobutamine stimulates cAMP production and TRPV5-mediated Ca^{2+} uptake in HEK293 cells; *iii*) dobutamine increases the TRPV5 channel activity which can be prevented by the PKA inhibitor H89 and mutation of the channel at the T709 residue; *iv*) dobutamine stimulates the apical-to-basolateral transepithelial Ca^{2+} flux in the mouse primary DCT2/CNT culture.

To our knowledge, the expression profile and the consequences of activation of β-ARs in mouse DCT2/CNT have not been characterized. Boivin and colleagues elaborately investigated β1- and β2-ARs in rat kidney by immunohistochemistry.[17] In their study, β2-AR is faintly present in DCT and undetectable in CNT,[17] while in our study β2-AR did not co-localize with TRPV5 in the mice DCT2/CNT. For β1-AR, Boivin *et al.* showed co-localization with calbindin-D_{28k} on the apical membrane[17] in line with our finding that β1-AR co-localizes with TRPV5, although we did not observe a clear apical localization.

Gesek and White demonstrated that the immortalized mouse DCT cell line expresses both β1- and β2-ARs.[30] In the present study, both receptors were detected by PCR in material from DCT2/CNT as isolated by the COPAS biosorter. Considering the lower amounts of β2-AR detected in highly enriched DCT2/CNT material, combined with the absence of β2-AR in immunohistological stainings in DCT2/CNT, we conclude that β2-AR seems not present in the DCT2/CNT. Gesek and Friedman showed that isoproterenol stimulated both cAMP production and Na^{+} uptake, but not Ca^{2+} influx into the cells.[31] However, they selected thiazide-sensitive DCT1 cells, which contain no TRPV5, resulting in very low Ca^{2+} transport rates.[20,24,31] In contrast, in the present study, DCT2/CNT tubules were selectively sorted from TRPV5-expressing cells. In the polarized mouse primary DCT2/CNT culture we demonstrated that dobutamine stimulates apical-to-basolateral transepithelial Ca^{2+} transport. Due to generally lower expression levels of β1-AR, in primary cultures compared with the *in vivo* situation, relatively high concentrations of dobutamine were applied (10 µM) to stimulate transepithelial Ca^{2+} transport maximally.

HEK293 cells are generally used as a model for studying molecular mechanism of TRPV5-mediated Ca^{2+} uptake because they lack endogenous expression of this Ca^{2+} channel,[32] allowing transfection of exogenous wild-type or residue-specific TRPV5 mutants. In addition, due to overexpression of TRPV5 faster and stronger effects are to be expected compared with primary cultures. Moreover, the cells were reported to express β1-AR, which could be stimulated by a non-specific β-AR agonist isoproterenol, showing enhanced cAMP synthesis.[29] Here, we show that the intracellular level of cAMP is increased within minutes after addition of dobutamine, indicating that β1-AR was functionally expressed in HEK293 cells and that the cells are suitable for studying β1-AR-initiated intracellular signaling. Accordingly, dobutamine was demonstrated to stimulate Ca^{2+} uptake in TRPV5-expressing HEK293 cells with maximal activation at 2 min. This rapid action of the agonist is likely nongenomic as exemplified by the hormonal action of PTH on TRPV5 activity that exerted similar maximal stimulation within 2 min. It is reported that TRPV5 activation is stimulated by the cAMP-dependent PTH1R signaling pathway leading to PKA-mediated phosphorylation of TRPV5 on the C-terminus

3

of the channel.[6] Moreover, Topala and colleagues showed that the activation of the Ca^{2+}-sensing receptor (CaSR) induces TRPV5-mediated Ca^{2+} peak current in <1 s.[4] They elaborated that the CaSR-agonist neomycin mediated PKC phosphorylation of the residues S299 and S654. Interestingly, Gkika and co-workers demonstrated that long-term (1-h) stimulation of bradykinin receptor type 2 (B2R) with tissue kallikrein resulted in increased plasma membrane accumulation of TRPV5.[12] In the present study, however, 1-h dobutamine incubation did not affect plasma membrane abundance or total expression of TRPV5. Altogether, activation of β1-AR enhanced TRPV5 activity via cAMP-dependent PKA phosphorylation of the TRPV5 channel at residue T709 in the intracellular C-terminus.

Apart from the classic cAMP-dependent signaling mechanism of β-ARs, β1-AR has been reported to be PLC-dependent.[20] In the study by Kang and colleagues, a non-specific β-AR agonist isoproterenol stimulated Mg^{2+} uptake in isolated mouse DCT1 cell culture.[20] Isoproterenol is known to bind preferentially to β1-AR and to a lesser extent to β2-AR. In the latter study, both PKA and PLC inhibitors inhibited the stimulatory effects of isoproterenol. Phorbol 12-myristate-13-acetate (PMA), a DAG analog, had no effect on Mg^{2+} reabsorption, but potentiated effects of isoproterenol.[20] There is no report of dobutamine action on signaling mechanisms other than the cAMP-dependent pathway and the PKA inhibitor H89 completely blocked the dobutamine action. Thus, the β1-AR signaling pathway in the present study seems to be mediated solely by the cAMP/PKA.

The present study indicates a novel calciotropic role of dobutamine in renal DCT2/CNT in addition to the positive inotropic effect in cardiac myocytes. In addition, β-blockers widely used in treatment of CHF might have adverse effects on maintaining body Ca^{2+} homeostasis. CHF initiates when the heart fails to supply sufficient blood to match the body demand due to reduced cardiac contractility.[21,33] Heart rate is accelerated,[34] leading to a decreased effective circulating volume, which in turn triggers the renin-angiotensin-aldosterone system in the kidney.[35] As a result of CHF, patients develop secondary aldosteronism leading to increased fecal and urinary Ca^{2+} excretion.[36] In addition, patients are generally treated with loop diuretics and often suffer from vitamin D deficiency.[36,37] Moreover, hypercalciuria and osteopenia have been implicated in patients with advanced CHF awaiting cardiac transplantation.[36,38] Hence, potential calciotropic effects of β-blockers might further increase chances of developing hypocalcemia in these patients. In addition, β-blockers are used to resolve hypercalcemia among other symptoms in hyperthyroidism.[39,40] The mechanism of action of β-blockers in hyperthyroidism is unclear, but principally appears to antagonize overstimulated β-AR-mediated signaling. Interestingly, the Ca^{2+}-lowering effect of propranolol (nonselective β-blocker) was proposed to be caused by a direct effect on bone or renal Ca^{2+} handling.[40]

In conclusion, the present study demonstrates that dobutamine, a β1-AR agonist, stimulates TRPV5 activity via a PKA-dependent pathway. PKA activation results in phosphorylation of the T709 residue and consequently enhances TRPV5 activity. Epi and NE, by activating TRPV5 activity, could potentially be involved in normal physiological renal Ca^{2+} handling in the DCT2/CNT. Therefore, β-adrenergic receptor agonists and blockers, increasingly administered to patients, potentially exert adverse calciotropic effects.

Acknowledgements

References

1. Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia. *Physiol Rev* 85: 373-422, 2005

2. Nordin BE: Calcium and osteoporosis. *Nutrition* 13: 664-686, 1997

3. Hoenderop JG, Bindels RJ: Calciotropic and magnesiotropic TRP channels. *Physiology (Bethesda)* 23: 32-40, 2008

4. Topala CN, Schoeber JP, Searchfield LE, Riccardi D, Hoenderop JG, Bindels RJ: Activation of the Ca^{2+}-sensing receptor stimulates the activity of the epithelial Ca^{2+} channel TRPV5. *Cell Calcium* 45: 331-339, 2009

5. Motoyama HI, Friedman PA: Calcium-sensing receptor regulation of PTH-dependent calcium absorption by mouse cortical ascending limbs. *American journal of physiology. Renal physiology* 283: F399-406, 2002

6. de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG: Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *Journal of the American Society of Nephrology : JASN* 20: 1693-1704, 2009

7. Hoenderop JG, Nilius B, Bindels RJ: ECaC: the gatekeeper of transepithelial Ca^{2+} transport. *Biochim Biophys Acta* 1600: 6-11, 2002

8. Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, Voets T, Nilius B, Clapham DE, Hoenderop JG, Bindels RJ: Calbindin-D$_{28K}$ dynamically controls TRPV5-mediated Ca^{2+} transport. *EMBO J* 25: 2978-2988, 2006

9. Nilius B, Owsianik G, Voets T, Peters JA: Transient receptor potential cation channels in disease. *Physiol Rev* 87: 165-217, 2007

10. de Groot T, Kovalevskaya NV, Verkaart S, Schilderink N, Felici M, van der Hagen EA, Bindels RJ, Vuister GW, Hoenderop JG: Molecular Mechanisms of Calmodulin Action on TRPV5 and Modulation by Parathyroid Hormone. *Molecular and cellular biology* 31: 2845-2853, 2011

11. Nilius B, Prenen J, Vennekens R, Hoenderop JG, Bindels RJ, Droogmans G: Modulation of the epithelial calcium channel, ECaC, by intracellular Ca^{2+}. *Cell Calcium* 29: 417-428, 2001

12. Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ: Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *Embo J* 25: 4707-4716, 2006

13. Ziegler MG, Aung M, Kennedy B: Sources of human urinary epinephrine. *Kidney Int* 51: 324-327, 1997

14. Snavely MD, Ziegler MG, Insel PA: Subtype-selective down-regulation of rat renal cortical alpha- and beta-adrenergic receptors by catecholamines. *Endocrinology* 117: 2182-2189, 1985

15. Furchgott RF: The receptors for epinephrine and norepinephrine (adrenergic receptors). *Pharmacol Rev* 11: 429-441; discussion 441-422, 1959

16. Bylund DB: Alpha- and beta-adrenergic receptors: Ahlquist's landmark hypothesis of a single mediator with two receptors. *Am J Physiol Endocrinol Metab* 293: E1479-1481, 2007

17. Boivin V, Jahns R, Gambaryan S, Ness W, Boege F, Lohse MJ: Immunofluorescent imaging of beta 1- and beta 2-adrenergic receptors in rat kidney. *Kidney Int* 59: 515-531, 2001

18. Evans BA, Papaioannou M, Anastasopoulos F, Summers RJ: Differential regulation of beta3-adrenoceptors in gut and adipose tissue of genetically obese (ob/ob) C57BL/6J-mice. *Br J Pharmacol* 124: 763-771, 1998

19. Elalouf JM, Buhler JM, Tessiot C, Bellanger AC, Dublineau I, de Rouffignac C: Predominant expression of beta 1-adrenergic receptor in the thick ascending limb of rat kidney. Absolute mRNA quantitation by reverse transcription and polymerase chain reaction. *The Journal of clinical investigation* 91: 264-272, 1993

20. Kang HS, Kerstan D, Dai LJ, Ritchie G, Quamme GA: beta-Adrenergic agonists stimulate Mg^{2+} uptake in mouse distal convoluted tubule cells. *American journal of physiology. Renal physiology* 279: F1116-1123, 2000

21. Jessup M, Abraham WT, Casey DE, Feldman AM, Francis GS, Ganiats TG, Konstam MA, Mancini DM, Rahko PS, Silver MA, Stevenson LW, Yancy CW: 2009 focused update: ACCF/AHA Guidelines for the Diagnosis and Management of Heart Failure in Adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. *Circulation* 119: 1977-2016, 2009

22. Bollano E, Tang MS, Hjalmarson A, Waagstein F, Andersson B: Different responses to dobutamine

3

in the presence of carvedilol or metoprolol in patients with chronic heart failure. *Heart* 89: 621-624, 2003

23. Hofmeister MV, Fenton RA, Praetorius J: Fluorescence isolation of mouse late distal convoluted tubules and connecting tubules: effects of vasopressin and vitamin D_3 on Ca^{2+} signaling. *American journal of physiology. Renal physiology* 296: F194-203, 2009

24. Markadieu N, San-Cristobal P, Nair AV, Verkaart S, Lenssen E, Tudpor K, van Zeeland F, Loffing J, Bindels RJ, Hoenderop JG: A primary culture of distal convoluted tubules expressing functional thiazide-sensitive NaCl transport. *American journal of physiology. Renal physiology* 303: F886-892, 2012

25. Charoenphandhu N, Tudpor K, Pulsook N, Krishnamra N: Chronic metabolic acidosis stimulated transcellular and solvent drag-induced calcium transport in the duodenum of female rats. *American journal of physiology. Gastrointestinal and liver physiology* 291: G446-455, 2006

26. van der Krogt GN, Ogink J, Ponsioen B, Jalink K: A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. *PLoS One* 3: e1916, 2008

27. Hellgren I, Sylven C, Magnusson Y: Study of the beta1 adrenergic receptor expression in human tissues: immunological approach. *Biol Pharm Bull* 23: 700-703, 2000

28. Erraji-Benchekroun L, Couton D, Postic C, Borde I, Gaston J, Guillet JG, Andre C: Overexpression of beta2-adrenergic receptors in mouse liver alters the expression of gluconeogenic and glycolytic enzymes. *Am J Physiol Endocrinol Metab* 288: E715-722, 2005

29. Lavoie C, Mercier JF, Salahpour A, Umapathy D, Breit A, Villeneuve LR, Zhu WZ, Xiao RP, Lakatta EG, Bouvier M, Hebert TE: Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. *The Journal of biological chemistry* 277: 35402-35410, 2002

30. Gesek FA, White KE: Molecular and functional identification of beta-adrenergic receptors in distal convoluted tubule cells. *The American journal of physiology* 272: F712-720, 1997

31. Gesek FA, Friedman PA: Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. *The Journal of clinical investigation* 90: 429-438, 1992

32. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG: The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science (New York, N.Y* 310: 490-493, 2005

33. Taylor SH: Congestive heart failure. Towards a comprehensive treatment. *European heart journal* 17 Suppl B: 43-56, 1996

34. Malliani A, Pagani M: The role of the sympathetic nervous system in congestive heart failure. *European heart journal* 4 Suppl A: 49-54, 1983

35. Zannad F, Dousset B, Alla F: Treatment of congestive heart failure: interfering the aldosterone-cardiac extracellular matrix relationship. *Hypertension* 38: 1227-1232, 2001

36. Alsafwah S, Laguardia SP, Arroyo M, Dockery BK, Bhattacharya SK, Ahokas RA, Newman KP: Congestive heart failure is a systemic illness: a role for minerals and micronutrients. *Clinical medicine & research* 5: 238-243, 2007

37. Borkowski BJ, Cheema Y, Shahbaz AU, Bhattacharya SK, Weber KT: Cation dyshomeostasis and cardiomyocyte necrosis: the Fleckenstein hypothesis revisited. *European heart journal* 32: 1846-1853, 2011

38. Shane E, Mancini D, Aaronson K, Silverberg SJ, Seibel MJ, Addesso V, McMahon DJ: Bone mass, vitamin D deficiency, and hyperparathyroidism in congestive heart failure. *The American journal of medicine* 103: 197-207, 1997

39. Hayes JR, Ritchie CM: Hypercalcaemia due to thyrotoxicosis. *Irish journal of medical science* 152: 422-423, 1983

40. Geffner DL, Hershman JM: Beta-adrenergic blockade for the treatment of hyperthyroidism. *The American journal of medicine* 93: 61-68, 1992

3

3

Coordinated regulation of TRPV5-mediated Ca²⁺ transport in primary distal convolution cultures

Eline A.E. van der Hagen[1], Marla Lavrijsen[1], Femke van Zeeland[1],
Jeppe Praetorius[2], Olivier Bonny[3], René J.M. Bindels[1],
Joost G.J. Hoenderop[1]

[1] Department of Physiology, Radboud university medical center,
Nijmegen, the Netherlands
[2] Department of Biomedicine, Health, Aarhus University,
Aarhus, Denmark
[3] Department of Pharmacology and Toxicology, University of
Lausanne, Lausanne, Switzerland

Abstract

Fine-tuning of renal calcium ion (Ca^{2+}) reabsorption takes place in the distal convoluted and connecting tubules (distal convolution) of the kidney via transcellular Ca^{2+} transport, a process controlled by the epithelial Ca^{2+} channel transient receptor potential vanilloid 5 (TRPV5). Studies to delineate the molecular mechanism of transcellular Ca^{2+} transport are seriously hampered by the lack of a suitable cell model. The present study describes the establishment and validation of a primary murine cell model of the distal convolution. Viable kidney tubules were isolated from mice expressing enhanced Green Fluorescent Protein (EGFP) under the control of a TRPV5 promoter (pTRPV5-EGFP), using Complex Object Parametric Analyser and Sorting (COPAS) technology. Tubules were grown into tight monolayers on semi-permeable supports. Radioactive $^{45}Ca^{2+}$ assays showed apical-to-basolateral transport rates of 13.5 ± 1.2 nmol/hr/cm^2, which were enhanced by the calciotropic hormones parathyroid hormone and 1,25-dihydroxy vitamin D_3. Cell cultures lacking TRPV5, generated by crossbreeding pTRPV5-EGFP with TRPV5 knockout mice (TRPV5$^{-/-}$), showed significantly reduced transepithelial Ca^{2+} transport (26% of control), for the first time directly confirming the key role of TRPV5. Most importantly, using this cell model, a novel molecular player in transepithelial Ca^{2+} transport was identified: mRNA analysis revealed that ATP-dependent Ca^{2+}-ATPase 4 (PMCA4) instead of PMCA1 was enriched in isolated tubules and downregulated in TRPV5$^{-/-}$ material. Immunohistochemical stainings confirmed co-localization of PMCA4 with TRPV5 in the distal convolution. In conclusion, a novel primary cell model with TRPV5-dependent Ca^{2+} transport characteristics was successfully established, enabling comprehensive studies of transcellular Ca^{2+} transport.

Introduction

Ca²⁺ reabsorption in the kidney is a critical step in maintaining total body Ca²⁺ homeostasis. Most of the Ca²⁺ filtered through the glomerulus is reabsorbed along the nephron, since only 1-2% of the Ca²⁺ is excreted in urine. Ca²⁺ reabsorption occurs largely through paracellular transport in the proximal part of the nephron (65-70%) and the thick ascending limb (TAL, 20-25%).[1] However, in the distal part of the nephron, the final concentration of Ca²⁺ in the urine is determined by the regulated process of active transcellular Ca²⁺ reabsorption.[1-3]

The distal convolution comprises the second part of the distal convoluted tubule (DCT) and connecting tubule (CNT). Here, the epithelial Ca²⁺ channel transient receptor potential vanilloid 5 (TRPV5) is located at the apical cell membrane and allows influx of Ca²⁺ from the pro-urine into the cell.[4] After entry into the cell, Ca²⁺ is transported towards the basolateral side, via the Ca²⁺-binding protein calbindin-D$_{28k}$ and is extruded into the blood by the ATP-dependent Ca²⁺-ATPase (PMCA1) and the Na⁺-Ca²⁺ exchanger (NCX1).[5,6] NCX1 is considered to be the predominant mechanism of extrusion, accounting for ~70% of the extruded Ca²⁺.[7-9]

Ca²⁺ influx through the Ca²⁺ channel TRPV5 is tightly regulated by many factors, most importantly the calciotropic hormones 1,25-dihydroxy vitamin D$_3$ (1,25-(OH)$_2$D$_3$) and parathyroid hormone (PTH), both regulating TRPV5 expression.[10,11] PTH directly stimulates activity of TRPV5 via cAMP signaling and subsequent phosphorylation of the channel by Protein Kinase A (PKA).[12] TRPV5 activity is inhibited through Protein Kinase C-dependent phosphorylation by plasmin, present in the urine of patients with Nephrotic Syndrome.[13] Other factors regulate TRPV5 plasma membrane abundance and subsequent activity, such as the hormone klotho and tissue kallikrein. Klotho is co-expressed in the distal part of the nephron, regulating the function of TRPV5 by modifying its N-glycan, leading to stabilization of the channel on the plasma membrane.[14,15] Calciotropic hormones also regulate other components of transcellular Ca²⁺ transport. Calbindin-D$_{28k}$ and NCX1 levels are upregulated by PTH and 1,25-(OH)$_2$D$_3$ in vivo[10,11] and PMCA1 expression was found to be increased by 1,25-(OH)$_2$D$_3$ in a Madin-Darby canine kidney (MDCK) cell model[16] as well as in vivo.[10]

The exact mechanism of the interplay between the apical influx, intracellular movement and basolateral extrusion in transcellular Ca²⁺ transport has not been clarified to date and is difficult to study due to the lack of a polarized cell model with Ca²⁺ transporting characteristics closely resembling the in vivo situation. Available cell models such as the MDCK cell line show very low Ca²⁺ transport rates.[16,17] Other cell models which have higher Ca²⁺ transport levels, are the murine distal convoluted tubule cell line (mpkDCT),[18] primary DCT/CNT cells isolated by microdissection[18] and the rabbit kidney connecting tubule and cortical collecting duct primary culture.[19] However, the utility of these cell models is limited. Firstly, the existing mouse mpkDCT cell line is not responsive to the important calciotropic hormone PTH, indicating that essential pathways might be missing.[18] Second, microdissection of tubular fragments is too laborious for isolation of large numbers of tubule fragments to perform extensive primary cell culture studies. Thirdly, a mouse model is preferable over rabbit, in part because of the ready availability of primers and knockout animals. In the current study, we present a novel primary cell culture of the mouse distal convolution, showing TRPV5-dependent transcellular Ca²⁺ transport. By a combined approach of transgenic mice expressing enhanced Green Fluorescent

Protein (EGFP) under the control of a TRPV5 promoter,[20] and the Complex Object Parametric Analyser and Sorter (COPAS) technology, distal convolution segments could be automatically sorted. This approach enables us to obtain large amounts of high quality and functional tubule segments. The primary cell model facilitates the study of different key players in transcellular Ca^{2+} transport. For example, we here exploit the model to demonstrate that PMCA4, and not PMCA1, may be the key Ca^{2+} ATPase involved in renal hormone regulated transcellular Ca^{2+} reabsorption.

Experimental Procedures

Transgenic mice - Transgenic mice expressing EGFP under control of the TRPV5 promotor (pTRPV5-EGFP) were generated as described by Hofmeister *et al.*, 2009.[20] TRPV5$^{-/-}$ mice were crossbred with pTRPV5-EGFP mice in order to generate pTRPV5-EGFP/TRPV5$^{-/-}$ mice. Mice were housed with free access to an SSNIFF rodent complete diet (SSNIFF, Soest, Germany) and water. The animal ethics board of Radboud University Nijmegen approved all the experimental procedures. Genotype was assessed by PCR, using primers: Forward: ATGGTGAGCAAGGGCGAGGAGCT and Reverse: GCCGAGAGTGATCCCGGCCGCGGT to select for EGFP expression. Mice homozygous or heterozygous for EGFP could not be distinguished so both were selected. TRPV5$^{-/-}$ mice were genotyped as described previously.[21]

Immunohistochemistry - Immunohistochemistry for TRPV5 was performed as described.[22] Mouse kidney sections were incubated for 16 h at 4°C with guinea pig anti-TRPV5 antiserum (1:50). Anti-guinea pig secondary Alexa Fluor® 594 conjugated antibodies (1:300, A-11076, Molecular Probes, Eugene, USA) were used to visualize TRPV5 proteins. For PMCA4 co-stainings sections were treated as described with the addition of anti-PMCA4 (1:200 Abcam, ab2783). The PMCA4 proteins were visualized with a secondary antibody anti-mouse Alexa Fluor® 488 (1:300, A-11029, Molecular Probes, Eugene, USA). Negative controls of conjugated antibodies solely, were devoid of any staining.

Isolation of distal convolution using COPAS biosorter - Distal convolution segments were isolated using the COPAS Biosorter (Union Biometrica, Holliston, MA) as described by Markadieu *et al.* and optimized for the distal convolution.[23] In short: mice aged 4-6 weeks were anesthetized (ketamine 0.1 mg/g body weight and xylazine 0.01 mg/g) and perfused with ice-cold KREBS buffer. The kidney cortex was dissected, minced, and subjected to three subsequent digestion steps: incubation in 7 ml of digestion solution composed of 1 mg/ml collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) and 0.9 mg/ml hyaluronidase (H3884, Sigma) in KREBS buffer, for 15 min, 6-8 min and 5-7 min at 37°C respectively,[23] with each digestion followed by filtration. The tubule suspension was filtered through a 100-µm sieve and collected on a 40-µm sieve (BD Biosciences, Breda, the Netherlands). The material remaining on the 100-µm sieve was subjected to additional digestion steps as described above. Tubules collected from the three digestions were mixed, kept on ice and sorted by the COPAS Biosorter. Tubules were sorted using the enhanced mode configuration, sort region parameters: vertex: x 100 a.u., y 14 a.u.; polygon: x 100 a.u., y 14 a.u.. Using this protocol, an estimated 2,000 EGFP-expressing tubules were sorted per hour, and between 4,000 and 10,000 tubules per mouse were collected. Isolated tubules were kept on ice until all were sorted, after which the tubules

were pelleted (800 x *g*, 5 min, 4°C).

Real-time PCR - mRNA from distal convolution tubules was extracted using TRIzol® Reagent (Invitrogen). Briefly, pellets of 1,000 COPAS sorted tubules were treated with 100 µl TRIzol®, and mRNA was extracted using chloroform and isopropanol as described in the manufacturer's protocol. Reverse Transcription reaction was performed using M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's protocol, except that RNasin® (Promega) was used as an RNase inhibitor. Real-time PCR was performed with the use of iQ SYBR Green Supermix (Bio-rad) using exon-overlapping primer sets (Table 1). *Gapdh* was used as a housekeeping gene.

Table 1 - Primers sets used for real-time PCR analysis			
Gene	**Position within ORF**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
Gapdh	293-312, 453-434	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC
Slc12a1	1001-1020, 1103-1084	GGCTTGATCTTTGCTTTTGC	CCATCATTGAATCGCTCTCC
Pvalb	46-65, 146-127	CGCTGAGGACATCAAGAAGG	CCGGGTTCTTTTTCTTCAGG
Slc23a3	1070-1089, 1191-1172	CTTCGGCCACTGGCATTCTG	GATGGCAAGGTAGGAGATGG
Trpv5	924-943, 1011-993	CTGGAGCTTGTGGTTTCCTC	TCCACTTCAGGCTCACCAG
Calb1	211-230, 308-289	GACGGAAGTGGTTACCTGGA	ATTTCCGGTGATAGCTCCAA
Kl	914-933, 1018-999	GGTTGCCCACAACCTACTTT	TGGGAGCTTAAGGCGATAGA
Slc8a1	1857-1876, 2052-2033	CTCCCTTGTGCTTGAGGAAC	CAGTGGCTGCTTGTCATCAT
Atp2b1	2454-2473, 2546-2527	GTCACCGGCCTTACGTGTAT	TCCAGCCCTCTGACATTTCT
Atp2b4	541-560, 682-663	CTTAATGGACCTGCGAAAGC	ATCTGCAGGGTTCCCAGATA
Scnn1a	1585-1604, 1712-1693	CATGCCTGGAGTCAACAATG	CCATAAAAGCAGGCTCATCC
Aqp2	154-173, 511-492	CACGCTCCTTTTCGTCTTCT	GGTCAGGAAGAGCTCCACAG

Slc12a1, NKCC2; *Pvalb*, parvalbumin; *Slc12a3*, NCC; *Calb1*, calbindin-D$_{28k}$; *Kl,* klotho; *Slc8a1*, NCX1 (exon B specific primers); *Atp2b1*, PMCA1; *Atp2b4*, PMCA4; *Scnn1a*, ENaC.

Primary cell culture - Two thousand fluorescent tubules were pelleted, and suspended into a pre-warmed cell culture medium (DMEM/Ham's F12, Invitrogen, #11039) supplemented with 60 nM sodium selenite, 5 µg/ml transferrin, 2 mM L-glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 2% v/v fetal calf serum, 6 µg/ml insulin, 13 mM D-glucose, 0.5 mM sodium pyruvate, 20 µg/ml ciproxin, and 5 mM HEPES/NaOH pH 7.3.[23] Tubules were seeded onto 0.33 cm² polycarbonate Transwell® inserts (Corning Costar, cat. #3413) previously coated with rat tail collagen (16 µl per insert of 0.75 mg/ml collagen in 95% v/v ethanol with 0.25% v/v acetic acid). Volumes used in apical and basolateral compartments were 100 µl and 600 µl, respectively, according to the manufacturer's recommendations. Tubules were cultured at 37°C in 5% v/v CO$_2$ and the medium was refreshed every day.

Ca²⁺ concentration measurements - Medium from apical and basolateral compartments was collected and diluted 4 times in Milli-Q water. Standards were prepared using 0.5 mM CaCl$_2$ standard (Hach, 21277-16). Five microlitres of sample was mixed with 200 µl freshly mixed buffer: 10% v/v Ca²⁺ buffer (3.5 M 2 amino-2-methylpropan-1-ol, pH 10.7 with HCl) and 10% v/v chromogen (0.15 mM phthalein purple, 6.89 mM 8-hydroxyquinoline, 0.06 M HCl) in Milli-Q water. Absorbance at 570 nm was measured using a microplate spectrophotometer (Bio-rad,

Benchmark Plus).

$^{45}Ca^{2+}$ *transport assays* - The epithelial resistance was measured one day prior to the assay. Seven to eight days after seeding, monolayers were preincubated with 5 µM indomethacin in culture medium for 30 min. Assays were conducted at 37°C, all buffers were pre-warmed. Cells were washed with physiological salt solution (PSS) containing 140 mM NaCl, 2 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 5 mM L-alanine, 5 µM indomethacin, 10 mM HEPES/Tris, pH 7.4. Subsequently, apical buffer was replaced with PSS (100 µl) containing 3 µCi/ml $^{45}Ca^{2+}$. Basolateral buffer (600 µl) was replaced with PSS. Buffers were supplemented with either the desired compounds or solvent: 10 µM forskolin (Sigma), 100 nM PTH (Sigma). To study transport in the absence of Na^+, in PSS 140 mM NaCl was replaced by equal amounts of NMDG. Cells were incubated at 37°C. Ten microlitre samples were taken apically and basolaterally at time-point 0; basolateral samples were taken at subsequent time points: 15, 30, 60, 120 and 180 min and analysed for radioactivity using a liquid scintillation counter. Transport rate was calculated from the slope of the linear part of the time range, t=0-120 min.

Immunoblotting - Immunoblotting of TRPV5 protein was performed on kidney lysates from wild-type and TRPV5$^{-/-}$ mice (100 µg total protein per sample). The membrane was incubated in 1:50 diluted rabbit anti-TRPV5 antibody (generated by O. Bonny *et al.*) in PBS overnight at 4°C. Next the membrane was incubated with anti-rabbit secondary antibody conjugated with HRP and visualized with ECL plus. The membrane was then stripped and exposed to anti-actin antibody (1:500, Sigma).

Immunocytochemistry - Tubules were cultured on Transwell® inserts as described above. At day 7, monolayers were pre-treated for 10 h with 100 nM 1,25-$(OH)_2D_3$ and prepared for immunocytochemistry as described.[23] Filters were incubated overnight at 4°C in 1:250 diluted anti-TRPV5 (raised in rabbit, generated by O. Bonny *et al.*) in goat serum dilution buffer (16% v/v goat serum in PBS pH 7.4). Next, filters were washed with PBS and incubated for 1 hr at room temperature in 1:300 diluted Alexa Fluor® 594 conjugated anti-rabbit IgG (Molecular Probes, A11012) in goat serum dilution buffer in the dark. Filters were mounted on coverslips using Mowiol (Polysciences Europe GmbH, Eppelheim, Germany). Images were obtained using an Olympus FV1000 Confocal Laser Scanning Micropscope using a x60 oil-immersion objective.

Statistical analysis - Results are expressed as mean ± S.E. of at least three independent experiments and at least three different sortings from different mice. Significance of the results was determined by one-way ANOVA, followed by Bonferroni post-test in case of significance. Except for $^{45}Ca^{2+}$ experiments and quantification of PMCA4 stainings, these results were evaluated by the unpaired Student's *t* test. *p* values < 0.05 were considered significant.

Results

Isolation of EGFP-expressing distal convolution segments
To isolate distal convolution tubule fragments, kidneys from mice expressing EGFP under the control of the TRPV5 promoter were used.[20] Expression of EGFP in the distal convolution was confirmed by the co-localization of EGFP with immunohistochemical staining of TRPV5 (Figure 1A). To collect substantial amounts of EGFP-expressing distal convolution fragments, mice

kidneys were isolated, digested and applied to the COPAS Biosorter. Tubule fragments were selected based on EGFP expression levels (fluorescence) and particle size (Figure 1B).

RNA was isolated from 1,000 sorted fragments to evaluate purity, which was assessed by real-time PCR measurements of mRNA expression levels of specific tubular markers. Control fragments, representing total cortex material, were sorted based on size only. Expression levels of the distal convolution markers TRPV5, calbindin-D_{28k} (CB28k) and NCX1 were markedly enriched (17-25 fold) in the fluorescence-sorted fragments when compared to the control samples of the total cortex material (Figure 2A). Expression of klotho, a regulator of TRPV5-dependent Ca^{2+} transport in the distal convolution,[14] was 7-fold higher in the sorted distal convolution tubules. Interestingly, PMCA1 was not significantly enriched in our distal convolution material, whereas expression of PMCA4 was found to be increased 18-fold. Impurity of the isolated preparation with other tubular segments was assessed using markers for neighbouring segments. The Na-K-Cl cotransporter (NKCC2), a marker for TAL, was not enriched. The specific DCT markers parvalbumin (PV) and the Na-Cl co-transporter (NCC) were expressed 3- and 7-fold higher, respectively, in the sorted tubules compared to the control material (Figure 2A).

Transepithelial Ca²⁺ transport across distal convolution monolayers

Functional characteristics of Ca^{2+} transport in the isolated distal convolution cells were studied by generating polarized primary cell monolayers. Primary cultures were obtained by seeding 2,000 sorted tubule fragments onto 0.33 cm^2 transwell inserts. The basolateral membrane of the cells will attach on the transwell surface and subsequently the tubule structure will open and grow into a tight cell monolayer. Transepithelial electrical resistance (TEER) of the cell monolayer increased to 755±62 $\Omega \cdot cm^2$ on day 6 (Figure 2B) and remained stable for at

4
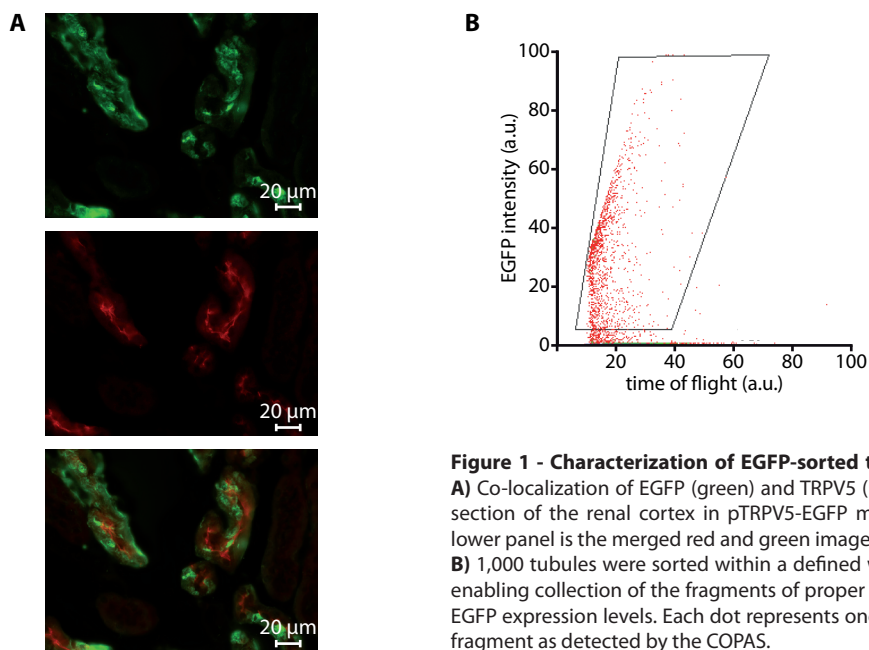
Figure 1 - Characterization of EGFP-sorted tubules. A) Co-localization of EGFP (green) and TRPV5 (red) in a section of the renal cortex in pTRPV5-EGFP mice. The lower panel is the merged red and green images above. **B)** 1,000 tubules were sorted within a defined window, enabling collection of the fragments of proper size and EGFP expression levels. Each dot represents one tubule fragment as detected by the COPAS.
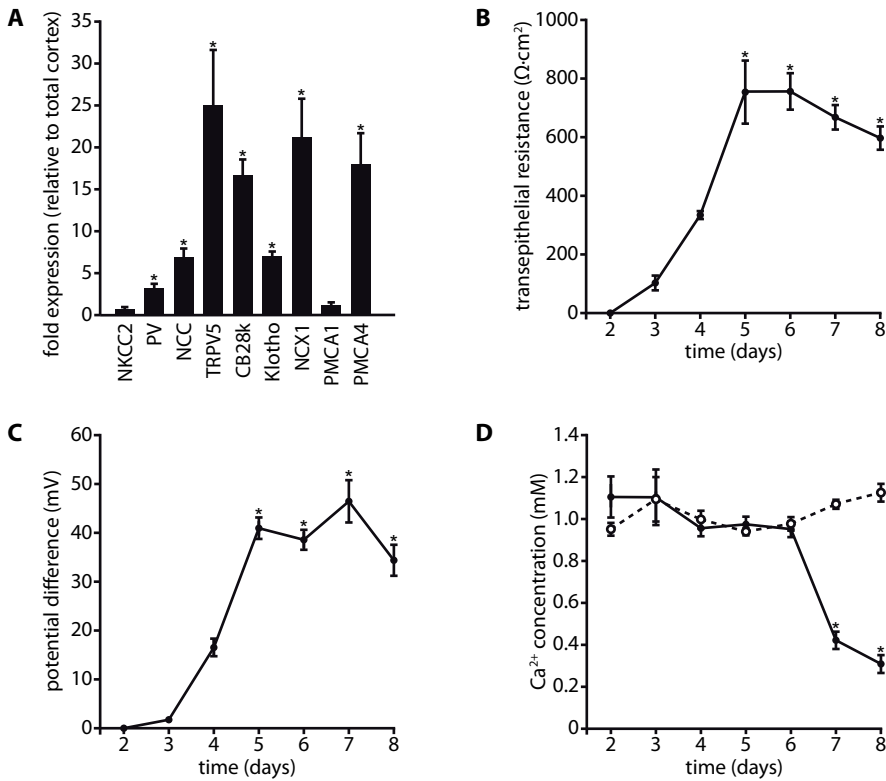
Figure 2 – Characterization of distal convolution cell monolayers. A) Real-time PCR was performed on mRNA samples extracted from 1,000 fluorescently sorted tubules as compared to tubules sorted based on size only (total cortex material). Results are normalized to *Gapdh* housekeeping gene and presented relative to control of non-fluorescently sorted material. Results are given as mean ± SE, n=5. *,p < 0.0001, p < 0.01 for PV as compared to total cortex material. **B)** Development of transepithelial resistance (x cm^2) was assessed over 8 days of culturing. Resistance was measured after every 24 h using an epithelial Volt-Ohm meter. Results are given as mean ± SE, n=6-44 for days 2 to 8 respectively. **C)** Potential differences (mV, lumen negative) were measured in parallel to transepithelial resistance every 24 h. Results are expressed as mean ± SE, n=3-31 for days 2 to 8 respectively. **D)** During culturing medium containing 1 mM of Ca^{2+} was changed and collected every 24 h. Ca^{2+} concentrations (mM) in 24 h conditioned medium were determined for apical (closed circles) and basolateral compartments (open circles). Results are expressed as mean ± SE, n=5-16, for days 2 to 8. *,p < 0.001 as compared to day 2.

least 3 days. Within the same time frame, a lumen negative potential difference of -38±2 mV developed across the monolayer (Figure 2C). During culturing, apical and basolateral media were refreshed and collected every 24 h. After 7 days the Ca^{2+} concentration in the apical compartment dropped from 1.0 mM to 0.3 mM (Figure 2D).

Stimulation of transepithelial Ca^{2+} transport by calciotropic hormones

As the cultured monolayers demonstrate transcellular Ca^{2+} transport, we tested the responsiveness of the primary cultures to calciotropic hormones. A ^{45}Ca^{2+} flux assay was used to study the regulation of transepithelial Ca^{2+} transport in our primary distal convolution cultures. After 7-8 days of culturing, monolayers were pre-treated with 5 μM indomethacin for

30 min, in order to inhibit prostaglandin synthesis.[24] Next, cell cultures were washed once with physiological salt solution (PSS) and the apical side was subsequently exposed to PSS containing 3 µCi/ml $^{45}Ca^{2+}$, all in the presence of 1 mM Ca^{2+}. An apical-to-basolateral Ca^{2+} transport rate of 13.5 ± 1.2 nmol/hr/cm² was measured (Figure 3A) during the first 2 h. Ca^{2+} transport was significantly enhanced in the presence of forskolin (10 µM, in apical and basolateral medium) (Figure 3B). The calciotropic hormone PTH (100 nM) had a similar stimulatory effect on Ca^{2+} transport across the distal convolution cultures. Furthermore, cell monolayers treated for 24 h with $1,25\text{-}(OH)_2D_3$ (100 nM) showed over 2 times more Ca^{2+} transport compared to untreated cultures.
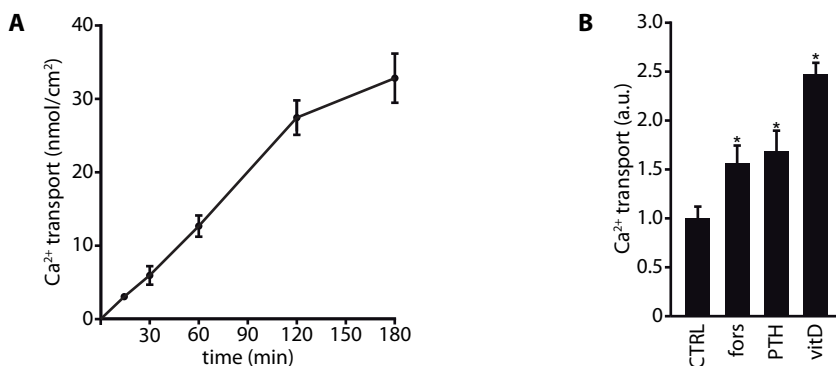
Figure 3 – Stimulation of Ca²⁺ transport in distal convolution cultures by PTH and 1,25-OH₂D₃. A) The Ca²⁺ transport rate was assessed in tubules cultured for 7-8 days by measuring transport (nmol/cm²) of $^{45}Ca^{2+}$ from the apical side to the basolateral compartment in the presence of 1 mM Ca²⁺ at time points up to 180 min. Results are presented as mean ± SE, n=23-32 respectively for time points 0-180 min. **B)** Ca²⁺ transport was assessed in the presence of 10 µM forskolin (n=11) and 100 nM PTH (n=13) as well as in cells treated for 24 h with 100 nM 1,25-(OH)₂D₃ (n=5), all both in apical and basolateral medium. Results were normalized for cell monolayers treated with solvent control and presented as mean ± SE. *,p < 0.05 as compared to untreated monolayers.

Transepithelial Ca²⁺ transport across distal convolution monolayers is mediated by TRPV5
In order to assess whether Ca^{2+} transport in the distal convolution derived monolayers is mediated by TRPV5, TRPV5 knockout (TRPV5$^{-/-}$) mice were crossbred with pTRPV5-EGFP mice in order to generate pTRPV5-EGFP/TRPV5$^{-/-}$ mice. Kidneys were subsequently processed, and tubules sorted and cultured in parallel with pTRPV5-EGFP expressing fragments. Real-time PCR analysis showed that the expression levels of the key players in Ca^{2+} transport, namely calbindin-D$_{28k}$, klotho, and NCX1 were significantly reduced in TRPV5$^{-/-}$ distal convolution tubule fragments compared to wild-type tubules (Figure 4A). Interestingly, PMCA1 expression levels were not changed, whereas PMCA4 levels were significantly lower in kidneys of TRPV5$^{-/-}$ mice. As a control for sorting of identical distal convolution tubule fragments for TRPV5$^{+/+}$ and TRPV5$^{-/-}$, mRNA levels of PV, NCC, the epithelial sodium channel (ENaC) and aquaporin 2 (AQP2) were analysed and were found unchanged (Figure 4A). Absence of TRPV5 protein in TRPV5$^{-/-}$ mice was confirmed by immunoblot (Figure 4B). After 7 days of culturing, TRPV5 protein expression could be detected on the apical membranes of cell monolayers derived from wild-type tubules by immunocytochemistry. Despite pre-treatment for 10 h with 1,25-(OH)₂D₃, positive staining for TRPV5 was absent in TRPV5$^{-/-}$ derived cell monolayers (Figure 4C). Expression of EGFP from
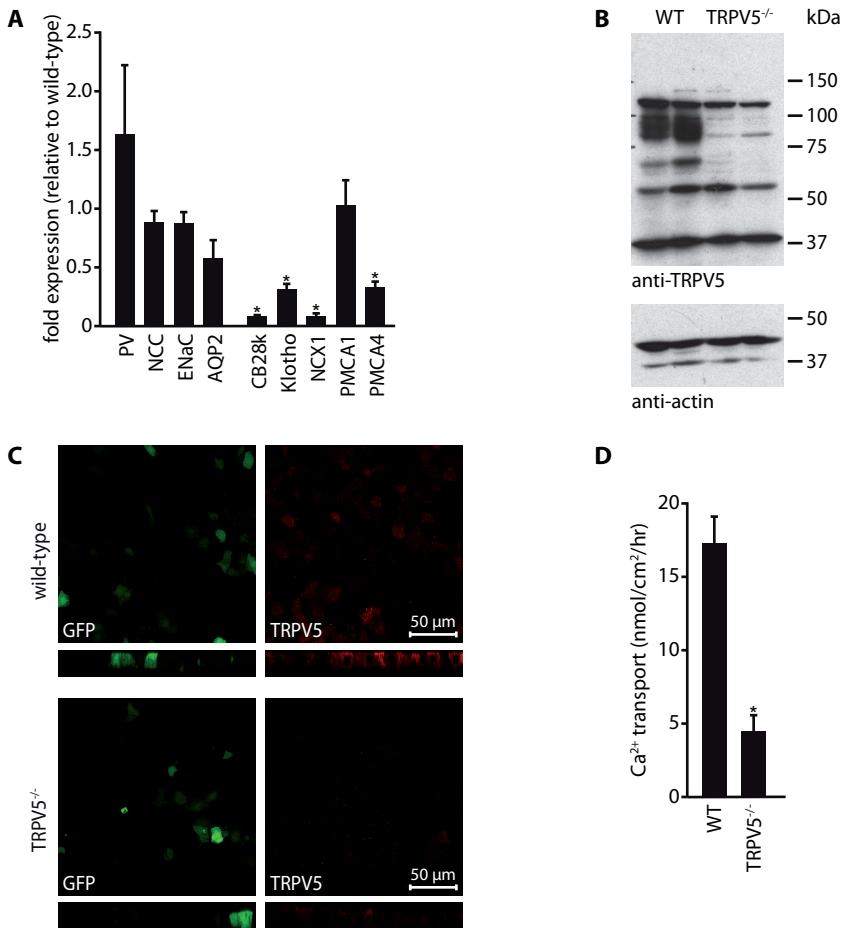
Figure 4 – Ca^{2+} transport in distal convolution cultures is TRPV5-mediated. A) pTRPV5-EGFP mice were crossbred with TRPV5$^{-/-}$ mice, generating pTRPV5-EGFP/TRPV5$^{-/-}$ mice. In parallel with wild-type tubules, 1,000 tubules from the knockout were sorted and mRNA was isolated. Results were normalized to *Gapdh* and expression levels were calculated against wild-type. Results are expressed as mean ± SE, n=5. *,p < 0.01 as compared to wild-type. **B)** TRPV5 protein expression in wild-type and TRPV5$^{-/-}$ kidney lysates; actin was used as a loading control. **C)** Tubules from pTRPV5-EGFP and pTRPV5-EGFP/TRPV5$^{-/-}$ mice were isolated and cultured in parallel. TRPV5 protein expression (red) was detected by immunocytochemistry in monolayers cultured on transwell inserts for 7 days. pTRPV5-EGFP expression (green) was visualized using excitation at 488 nm. **D)** Ca^{2+} transport (nmol/cm^2/hr) in monolayers derived from wild-type and TRPV5$^{-/-}$ tubules was measured by apically applying ^{45}Ca^{2+}. Results are presented as mean ± SE, n=5. *,p < 0.001 as compared to wild-type.

the TRPV5 promoter region was similar for wild-type and TRPV5$^{-/-}$ cultures. Most importantly, a marked reduction in transepithelial Ca^{2+} transport was observed in TRPV5$^{-/-}$ primary cultures compared to wild-type cultures (4±1 nmol/hr/cm^2 versus 17±2 nmol/hr/cm^2, respectively, *p* < 0.05) (Figure 4D). Moreover, Ca^{2+} concentrations in the apical culture medium did not decrease during culturing of TRPV5$^{-/-}$ tubules as was previously described above for wild-type (data not shown).

PMCA4 is proposed to be the key Ca²⁺-ATPase responsible for transepithelial Ca²⁺ transport in the distal convolution

PMCA4, but not PMCA1 mRNA, was demonstrated to be enriched in distal convolution tubule material. In addition, PMCA4 mRNA levels were significantly reduced in TRPV5$^{-/-}$ tubules similar to the identified key players of transepithelial Ca²⁺ transport. To confirm the role of PMCA4 in transepithelial Ca²⁺ transport in the distal convolution, expression of PMCA4 protein was assessed by immunohistochemical staining of wild-type and TRPV5$^{-/-}$ kidney tissues. PMCA4 exclusively co-localized with TRPV5 in the distal convolution, where it was expressed at the basolateral side (Figure 5A). In TRPV5$^{-/-}$ kidney tissues, PMCA4 expression levels were significantly reduced, as determined by quantification of the PMCA4 signal compared to wild-type mice (Figure 5A,B). To study the basolateral extrusion mechanism in the distal convolution, ^{45}Ca²⁺ transport was measured in the presence and absence of Na⁺, thereby disrupting efflux of Ca²⁺ through NCX1 and leaving transport by PMCAs intact. In the absence of Na⁺ (replaced by NMDG⁺) a significant drop of 49% in Ca²⁺ transport was observed as compared to an identical physiological buffer with NaCl (Figure 5C).
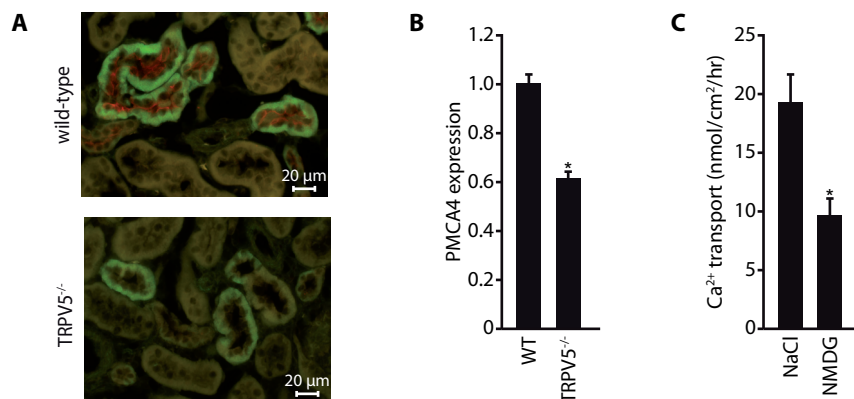
Figure 5 - PMCA4 is proposed as the key Ca²⁺ATPase in the distal convolution. A) Co-localization of PMCA4 (green) and TRPV5 (red) in a section of the renal cortex of both wild-type and TRPV5$^{-/-}$ mice. **B)** Quantification of the PMCA4 protein expression levels as assessed by immunohistochemistry in TRPV5$^{-/-}$ mice under equal conditions of exposure and compared to wild-type. Results are expressed as mean ± SE, n=47 different tubules for wild-type and n=39 for TRPV5$^{-/-}$. *,p < 0.0001 as compared to wild-type. **C)** Ca²⁺ transport was assessed in the presence (NaCl) and absence (NMDG) of Na⁺ (n=11), all both in apical and basolateral medium. Results are expressed as mean ± SE. *,p < 0.01 as compared cell monolayers assessed in the presence of Na⁺.

Discussion

By the combined use of the COPAS technology and pTRPV5-EGFP mice, we established a novel primary cell model of the distal convolution that allows examination of the molecular aspects of transcellular Ca²⁺ transport. Distal convolution characteristics were confirmed in the sorted tubules, since mRNA expression levels of all known key players in transcellular Ca²⁺ transport were significantly enriched. Secondly, when seeded on semi-permeable transwell inserts, distal convolution fragments developed tight monolayers and demonstrated robust transepithelial Ca²⁺ transport after 7 days, which could be enhanced by the calciotropic hormones PTH and

1,25-$(OH)_2D_3$. Thirdly, expression of TRPV5 protein was demonstrated at the apical membranes after 7 days of culturing and its role as gatekeeper of Ca^{2+} transport was confirmed by using cell cultures derived from TRPV5^{-/-} animals, demonstrating greatly reduced Ca^{2+} transport levels. Finally, making use of the unique properties of this primary cell system, we propose PMCA4, but not PMCA1, as the main PMCA isoform playing a role in hormone-regulated transcellular Ca^{2+} transport.

Using the COPAS Biosorter in combination with mice expressing EGFP under the control of a TRPV5 promoter region, viable distal convolution tubule fragments were easily and reproducibly collected, especially when compared to tubules obtained by manual microdissection.[18] Contamination with TAL was negligible, as observed by low expression levels of NKCC2. Based on the expression of PV and NCC, only minor contribution of DCT1 fragments was anticipated. Furthermore, our distal convolution cell model showed a Ca^{2+} transport rate noticeably higher than in microdissected primary DCT cultures and the mpkDCT cell lines.[18]

Importantly, key regulatory pathways remained intact in the cultured primary tubules, as was observed from stimulation of Ca^{2+} transport by the calciotropic hormones 1,25-$(OH)_2D_3$ and PTH. This is in contrast to the pre-existing mpkDCT cell lines, in which no response to PTH treatment was observed.[18] Short-term responses to PTH have been reported to be mediated through a cAMP/PKA and PKC phosphorylation pathway[25,26] and are likely to act on the TRPV5 channel. It has been reported that PTH can act on the TRPV5 channel through both pathways.[12,27] Hence, the observed increase in transepithelial Ca^{2+} transport in response to PTH is probably caused by activation of the TRPV5 channel. Moreover, our finding that forskolin and PTH equally enhanced Ca^{2+} transport in our primary culture, suggests a role for the cAMP pathway in the response to PTH. Furthermore, regulation of Ca^{2+} transport by 1,25-$(OH)_2D_3$ suggests that regulatory mechanisms on a transcriptional level are also preserved. 1,25-$(OH)_2D_3$ is known to control mRNA expression levels of TRPV5, calbindin-D_{28k} and NCX1, as was demonstrated in 25-hydroxyvitamin D_3-1α-hydroxylase knockout mice, which are not able to synthesize 1,25-$(OH)_2D_3$ from its inactive metabolite.[10] The conservation of the machinery for regulated transepithelial Ca^{2+} transport in our culture model, i.e. the relevant receptors, second messenger coupling, as well as effector transport proteins, allows for detailed studies of transcellular Ca^{2+} transport and its regulation. It can be expected that other, yet unknown, regulatory pathways will be preserved in the primary cells as well. Hence, we obtained a highly responsive novel primary distal convolution culture exhibiting robust Ca^{2+} transport rates, with the advantage of using the genetically well-defined and easily maintainable mouse models enabling us to make new discoveries pertaining to transcellular Ca^{2+} transport.

By using distal convolution tubules isolated from TRPV5^{-/-} mice, we demonstrated for the first time that Ca^{2+} transport in the distal part of the nephron is mainly dependent on the presence of TRPV5, substantiating the claim that TRPV5 is the gatekeeper of transcellular Ca^{2+} transport in the distal convolution. In addition, the absence of TRPV5 greatly affects expression of other known key players in Ca^{2+} transport, including calbindin-D_{28k} and NCX1[21,28] and the novel candidate PMCA4.

The basolateral extrusion mechanism was studied using Na^+-free conditions, in order to block NCX1 function. A 49% drop in Ca^{2+} transport, as compared to a 74% drop in TRPV5^{-/-}

tubules is in perfect agreement with the previously proposed ratio in which two-thirds of Ca^{2+} is extruded by NCX1 and one-third by PMCAs.[7-9] Interestingly, PMCA1, generally thought to be the major Ca^{2+} transporting ATPase in the distal convolution,[7,9] was not enriched in our distal convolution tubule fragments. Previously, PMCA1 and PMCA4 proteins were reported to be expressed in murine DCT cells.[29] The other two PMCA proteins, PMCA2 and PMCA3, are present in low amounts in kidney, but not in DCT cells.[29] In our DCT2/CNT tubule fragments, PMCA4 expression was enhanced in an order similar to TRPV5 and NCX1, suggesting a functional role of this transporter in transepithelial Ca^{2+} transport in the distal convolution. Moreover, immunohistochemistry demonstrated PMCA4 expression on the basolateral side of TRPV5-expressing cells. The proposed role of PMCA4 in transepithelial Ca^{2+} transport is supported by mRNA expression levels in tubule fragments from TRPV5$^{-/-}$ mice and protein levels in immunohistochemical stainings, where PMCA4 expression was significantly decreased, similar to the other key players in renal transcellular Ca^{2+} transport (calbindin-D_{28k} and NCX1). In contrast, PMCA1 mRNA levels were not changed in these tubules. Expression levels of components of transepithelial Ca^{2+} transport have previously been shown to be co-regulated. Our findings are in agreement with previous studies showing that calbindin-D_{28k} and NCX1 were concomitantly downregulated in TRPV5$^{-/-}$ animals.[21] These animals lack high levels of Ca^{2+} influx through absence of TRPV5, suggesting that Ca^{2+} influx regulates expression of the other Ca^{2+} transporting proteins, including PMCA4. This is not due to altered PTH and 1,25-$(OH)_2D_3$ levels since PTH levels were unchanged and 1,25-$(OH)_2D_3$ levels were even higher in TRPV5 knockout animals (which would lead to increased expression levels of Ca^{2+} transport proteins, rather than the decreased levels found in this study).[21] Furthermore, whereas independent animal studies (e.g. in klotho knockout mice, 25-hydroxyvitaminD_3-1α-hydroxylase knockout mice, and parathyroidectomized rats) confirmed that TRPV5, calbindin-D_{28k} and NCX1 expression levels were all co-regulated, PMCA1 levels were not regulated under these conditions.[10,11,30] Kip *et al.* demonstrated the importance of the PMCA4 gene in Ca^{2+} transport in an MDCK cell model by PMCA4 anti-sense knockdown.[9] Characterization of PMCA1 and PMCA4 knockout animals indicated that knockout of PMCA1 is embryonic lethal, whereas PMCA4 knockout mice appear healthy but have more subtle abnormalities, such as male infertility and osteopenia due to increased osteoclasts on the bone surface.[31,32] As far as we know there is no data available on urine and serum Ca^{2+} levels in PMCA4 knockout mice. Altogether, PMCA4 appears to be the major Ca^{2+}-ATPase in the distal convolution involved in transcellular Ca^{2+} transport, whereas PMCA1 has a more general housekeeping function. Further studies using knockout mice models are necessary to elucidate the precise interplay of NCX1, PMCA4 and PMCA1 in transcellular Ca^{2+} transport. However, compensatory mechanisms should be taken into account when using these mice models.

In conclusion, the use of the pTRPV5-EGFP mice and the COPAS to sort EGFP-expressing distal convolution, repeatedly and consistently led to the establishment of highly responsive novel primary cultures with many characteristics of the intact distal convolution cells. In addition, by crossbreeding pTRPV5-EGFP mice with knockout animals, high-quality transgenic cell models can be established relatively easily, as we have done for TRPV5. This enables more detailed studies of transcellular Ca^{2+} transport and regulation, such as the mechanisms of Ca^{2+} entry by TRPV5 and Ca^{2+} extrusion. As we now discovered PMCA4, and not PMCA1, as a co-regulated component of transepithelial Ca^{2+} transport in the distal convolution.

4

Acknowledgements

References

1. Dimke H, Hoenderop JG, Bindels RJ: Hereditary tubular transport disorders: implications for renal handling of Ca^{2+} and Mg^{2+}. *Clin Sci (Lond)* 118: 1-18, 2010

2. Markadieu N, Bindels RJ, Hoenderop JG: The renal connecting tubule: Resolved and unresolved issues in Ca^{2+} transport. *Int J Biochem Cell Biol* 43: 1-4, 2011

3. Bonny O, Edwards A: Calcium reabsorption in the distal tubule: regulation by sodium, pH, and flow. *Am J Physiol Renal Physiol* 304: F585-600, 2013

4. Hoenderop JG, van der Kemp AW, Hartog A, van de Graaf SF, van Os CH, Willems PH, Bindels RJ: Molecular identification of the apical Ca^{2+} channel in 1, 25-dihydroxyvitamin D3-responsive epithelia. *J Biol Chem* 274: 8375-8378, 1999

5. Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, Voets T, Nilius B, Clapham DE, Hoenderop JG, Bindels RJ: Calbindin-D$_{28K}$ dynamically controls TRPV5-mediated Ca^{2+} transport. *EMBO J* 25: 2978-2988, 2006

6. Hoenderop JG, Nilius B, Bindels RJ: Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol* 64: 529-549, 2002

7. Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia. *Physiol Rev* 85: 373-422, 2005

8. Brini M, Carafoli E: The plasma membrane Ca^{2+} ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb Perspect Biol* 3, 2011

9. Kip SN, Strehler EE: Characterization of PMCA isoforms and their contribution to transcellular Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol* 284: F122-132, 2003

10. Hoenderop JG, Dardenne O, van Abel M, van der Kemp AW, van Os CH, St -Arnaud R, Bindels RJ: Modulation of renal Ca^{2+} transport protein genes by dietary Ca^{2+} and 1,25-dihydroxyvitamin D$_3$ in 25-hydroxyvitamin D$_3$-1alpha-hydroxylase knockout mice. *FASEB J* 16: 1398-1406, 2002

11. van Abel M, Hoenderop JG, van der Kemp AW, Friedlaender MM, van Leeuwen JP, Bindels RJ: Coordinated control of renal Ca^{2+} transport proteins by parathyroid hormone. *Kidney Int* 68: 1708-1721, 2005

12. de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG: Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J Am Soc Nephrol* 20: 1693-1704, 2009

13. Tudpor K, Lainez S, Kwakernaak AJ, Kovalevskaya NV, Verkaart S, van Genesen S, van der Kemp A, Navis G, Bindels RJ, Hoenderop JG: Urinary plasmin inhibits TRPV5 in nephrotic-range proteinuria. *J Am Soc Nephrol* 23: 1824-1834, 2012

14. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG: The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 310: 490-493, 2005

15. Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ: Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *EMBO J* 25: 4707-4716, 2006

16. Kip SN, Strehler EE: Vitamin D$_3$ upregulates plasma membrane Ca^{2+}-ATPase expression and potentiates apico-basal Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol* 286: F363-369, 2004

17. Arthur JM: The MDCK cell line is made up of populations of cells with diverse resistive and transport properties. *Tissue Cell* 32: 446-450, 2000

18. Diepens RJ, den Dekker E, Bens M, Weidema AF, Vandewalle A, Bindels RJ, Hoenderop JG: Characterization of a murine renal distal convoluted tubule cell line for the study of transcellular calcium transport. *Am J Physiol Renal Physiol* 286: F483-489, 2003

19. Bindels RJ, Hartog A, Timmermans J, Van Os CH: Active Ca^{2+} transport in primary cultures of rabbit kidney CCD: stimulation by 1,25-dihydroxyvitamin D$_3$ and PTH. *Am J Physiol* 261: F799-807, 1991

4

20. Hofmeister MV, Fenton RA, Praetorius J: Fluorescence isolation of mouse late distal convoluted tubules and connecting tubules: effects of vasopressin and vitamin D₃ on Ca²⁺ signaling. *Am J Physiol Renal Physiol* 296: F194-203, 2009

21. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ: Renal Ca²⁺ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest* 112: 1906-1914, 2003

22. Hoenderop JG, Hartog A, Stuiver M, Doucet A, Willems PH, Bindels RJ: Localization of the epithelial Ca²⁺ channel in rabbit kidney and intestine. *J Am Soc Nephrol* 11: 1171-1178, 2000

23. Markadieu N, San-Cristobal P, Nair AV, Verkaart S, Lenssen E, Tudpor K, van Zeeland F, Loffing J, Bindels RJ, Hoenderop JG: A primary culture of distal convoluted tubules expressing functional thiazide-sensitive NaCl transport. *Am J Physiol Renal Physiol* 303: F886-892, 2012

24. van Baal J, de Jong MD, Zijlstra FJ, Willems PH, Bindels RJ: Endogenously produced prostanoids stimulate calcium reabsorption in the rabbit cortical collecting system. *J Physiol* 497 (Pt 1): 229-239, 1996

25. Hoenderop JG, De Pont JJ, Bindels RJ, Willems PH: Hormone-stimulated Ca²⁺ reabsorption in rabbit kidney cortical collecting system is cAMP-independent and involves a phorbol ester-insensitive PKC isotype. *Kidney Int* 55: 225-233, 1999

26. Lau K, Bourdeau JE: Evidence for cAMP-dependent protein kinase in mediating the parathyroid hormone-stimulated rise in cytosolic free calcium in rabbit connecting tubules. *J Biol Chem* 264: 4028-4032, 1989

27. Cha SK, Wu T, Huang CL: Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294: F1212-1221, 2008

28. Renkema KY, Nijenhuis T, van der Eerden BC, van der Kemp AW, Weinans H, van Leeuwen JP, Bindels RJ, Hoenderop JG: Hypervitaminosis D mediates compensatory Ca²⁺ hyperabsorption in TRPV5 knockout mice. *J Am Soc Nephrol* 16: 3188-3195, 2005

29. Magyar CE, White KE, Rojas R, Apodaca G, Friedman PA: Plasma membrane Ca²⁺-ATPase and NCX1 Na⁺/Ca²⁺ exchanger expression in distal convoluted tubule cells. *Am J Physiol Renal Physiol* 283: F29-40, 2002

30. Alexander RT, Woudenberg-Vrenken TE, Buurman J, Dijkman H, van der Eerden BC, van Leeuwen JP, Bindels RJ, Hoenderop JG: Klotho prevents renal calcium loss. *J Am Soc Nephrol* 20: 2371-2379, 2009

31. Kim HJ, Prasad V, Hyung SW, Lee ZH, Lee SW, Bhargava A, Pearce D, Lee Y, Kim HH: Plasma membrane calcium ATPase regulates bone mass by fine-tuning osteoclast differentiation and survival. *J Cell Biol* 199: 1145-1158, 2012

32. Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE: Targeted ablation of plasma membrane Ca²⁺-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *J Biol Chem* 279: 33742-33750, 2004

4

Regulation of the renal Na$^+$/Ca^{2+} exchanger 1 (NCX1) variant by calmodulin

Eline A.E. van der Hagen, Ellen P.M. van Loon,
Sjoerd Verkaart, Femke Latta, René J.M. Bindels,
Joost G.J. Hoenderop

Department of Physiology, Radboud university
medical center, Nijmegen, the Netherlands

Abstract

Fine-tuning of renal calcium (Ca^{2+}) reabsorption takes place in the late distal convoluted and connecting tubules (DCT2/CNT) of the kidney via transcellular Ca^{2+} transport. The Na^+/Ca^{2+} exchanger 1 (NCX1) is responsible for ~70% of basolateral Ca^{2+} extrusion. Since the other major Ca^{2+} transporters in the process of renal transcellular Ca^{2+} transport, TRPV5, and the plasma membrane Ca^{2+} ATPases PMCA1/4, are regulated by the Ca^{2+}-sensor calmodulin and as multiple calmodulin binding sites were predicted in the renal splice variant NCX1.3 *in silico*, we investigated whether calmodulin is a regulator of NCX1.3. In this study, we demonstrated a stimulatory action of calmodulin on the renal isoform of NCX1 (NCX1.3), resulting in enhanced exchanger activity as studied by uptake of Ca^{2+} by function of the exchanger in the reverse-mode. In addition, overexpression of calmodulin increased NCX1.3 protein levels, indicating stabilization of NCX1.3 by binding of calmodulin. Interestingly, this process appeared to be largely Ca^{2+}-independent, while the effect on exchanger activity was dependent of Ca^{2+} binding to calmodulin. Mutation of key residue L696 within a potential calmodulin binding site abolished stimulation of NCX1.3 activity, whereas the positive effect of calmodulin on NCX1 protein levels and binding of calmodulin to NCX1.3 remained intact. Hence, calmodulin increases NCX1.3 activity through binding to the region in close proximity to residue L696.

5

Introduction

The Na$^+$/Ca^{2+} exchanger 1 (NCX1) is widely expressed in the body and generally controls intracellular calcium (Ca^{2+}) levels by extruding Ca^{2+} from the cell. However, in the kidney NCX1 is also part of a highly regulated process of transcellular Ca^{2+} reabsorption, thereby regulating final loss of Ca^{2+} via the urine and thus overall Ca^{2+} balance.[1,2] In the late distal convoluted and connecting tubules (DCT2/CNT) of the nephron, the epithelial Ca^{2+} channel transient receptor potential vanilloid 5 (TRPV5) is responsible for entry of Ca^{2+} from the tubular lumen.[3] Thereafter, Ca^{2+} is shuttled towards the basolateral side,[4,5] where it is extruded towards the blood by the ATP-dependent plasma membrane Ca^{2+}-ATPases (PMCA1/4) and the Na$^+$-Ca^{2+} exchanger (NCX1).[6] NCX1 is considered to be the predominant extrusion mechanism, accounting for ~70% of the extruded Ca^{2+},[7-9] thereby playing a key role in the process of renal transcellular Ca^{2+} reabsorption.

Three NCX isoforms exist: NCX1, NCX2 and NCX3, which are structurally highly similar.[8,10] NCX consists of 10 transmembrane segments and a large regulatory cytosolic loop. The cytosolic loop contains two Ca^{2+}-binding domains (CBD1 and CBD2), of which the CBD2 region is subjective to alternative splicing.[10,11] NCX1 splice variants have a tissue-specific distribution. NCX1 was first cloned from cardiac tissue (NCX1.1), where its crucial role in myocyte contraction has been extensively studied.[12,13] However, studies on the function and regulation of the kidney specific variants are limited. In kidney, different NCX variants have been identified, NCX1.2 (exons BCD), NCX1.3 (exons BD) and NCX1.7 (exons BDF).[14-16] For NCX1.2 and NCX1.3 a role in Ca^{2+} transport has been shown in immortalized mouse distal convoluted tubule (DCT) cells.[16] NCX1.3 is considered to be the predominant variant and is often described as the renal NCX1.[11,17,18]

Transcellular Ca^{2+} transport in the kidney is a tightly regulated process, modulated by several hormones and other factors implicated in Ca^{2+} homeostasis. The calciotropic hormones 1,25-dihydroxy vitamin D$_3$ (1,25-(OH)$_2$D$_3$) and parathyroid hormone (PTH) being the most important.[19] Both were found to elevate expression levels of the identified Ca^{2+} transporting proteins: TRPV5, calbindin-D$_{28k}$ and NCX1, resulting in increased transcellular Ca^{2+} transport.[20-22] Hence, expression levels of the different components appear to be coordinately regulated. In addition, extensive research led to the identification of many factors directly regulating TRPV5 channel function.[23] The Ca^{2+}-sensing protein calmodulin was found to be a crucial regulator of TRPV5 by providing it with a negative feedback mechanism in case of high intracellular Ca^{2+} levels.[24] In addition, calmodulin is well known to release the PMCA pump from auto-inhibition, thereby being essential for PMCA1/4 functioning.[25] Li *et al.* identified a predicted calmodulin binding site in the so-called XIP (exchanger inhibitory peptide) region of NCX1. However, this region was shown to bind calmodulin as a peptide only, and interestingly XIP peptides were shown to inhibit NCX and PMCA function. Interaction of calmodulin with NCX1 has never been observed so far.[26,27]

The aim of the present study was to investigate which NCX1 variants are expressed in Ca^{2+} transporting epithelia in the kidney and to gain more insight in the role of calmodulin on renal NCX1 function by determining the binding of calmodulin to NCX1 and identification of potential calmodulin binding sites.

5

Experimental Procedures

Cloning of NCX1 variants from mice kidney and generation of NCX1 mutant constructs - To generate NCX1 cDNA, total mice kidney mRNA was extracted using TRIzol® (Invitrogen) and processed using RQ1 DNase and M-MLV Reverse Transcriptase (Invitrogen) using RNasin® (Promega, Madison, WI, USA) as a RNase inhibitor, according to the manufacturer's protocols. NCX primers (Table 1) were designed based on NCBI Reference Sequence: NM_011406.2, with the addition of a sequence for restriction digest using *Mlu*I and *Asc*I respectively. PCR was performed using Phusion® High-fidelity DNA polymerase (Invitrogen). Products were isolated from agarose gel, *Mlu*I and *Asc*I digested and ligated into the pCINeo-IRES-EGFP vector. Subsequently, pCINeo-mNCX1.2-IRES-EGFP, pCINeo-mNCX1.3-IRES-EGFP and pCINeo-mNCX1.7-IRES-EGFP plasmid DNAs were recovered from transformed *E.coli* clones. pCINeo-mNCX1.3-IRES-EGFP constructs with specific mutations were obtained using the QuikChange site-directed mutagenesis kit (Agilent, Amstelveen, the Netherlands) according to the manufacturer's protocol. All obtained constructs were verified by sequencing of the NCX1 open reading frame and applied restriction sites.

Table 1 - Oligonucleotide sequences applied in (real-time) PCR

Gene	Application	Forward primer (5'-3')	Reverse primer (5'-3')
Slc8a1	Cloning	ATGCTTCGATTAAGTCTCCCA	TTAGAAGCCCTTTATGTGGCA
Slc8a1	PCR splice site	AGAGCATTGGCATCATGGA	CAGTGGCTGCTTGTCATCAT
GAPDH	Real-time PCR	GGAGTCAACGGATTTGGTCGTA	GGCAACAATATCCACTTTACCAGAGT
Slc8a1	Real-time PCR	CTCCCTTGTGCTTGAGGAAC	CAGTGGCTGCTTGTCATCAT

Slc8a1, murine Na^+/Ca^{2+} exchanger 1 gene (NCX1); *GAPDH,* human glyceraldehyde-3-phosphate dehydrogenase gene.

PCR/gel electrophoresis - PCR was performed using homemade Taq polymerase (1 µl) in 10 mM Tris-HCl, 50 mM KCl, 0.01% v/v gelatin, 2.5 mM $MgCl_2$, 1 mM dNTPs and 0.2 µM of each primer (Table 1). Late DCT and connecting tubule (DCT2/CNT) cDNA was isolated by fluorescent sorting.[28] PCR products were run on a 2% w/v agarose gel.

Cell culture and transfection - HEK293(T) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker Europe, Vervier, Belgium) containing 10% v/v fetal calf serum (PAA, Linz, Austria), 2 mM L-glutamine, 10 µl/ml non-essential amino acids at 37°C and 5% v/v CO_2. Cells were transiently transfected using Lipofectamine® 2000 (Invitrogen, 2 µl/µg DNA). For co-transfection of pCINeo-NCX1.3-IRES-EGFP with pCINeo-IRES-EGFP or pCINeo-rCaM-IRES-EGFP, constructs were used in a ratio of 1:1.5. pCINeo-rCaM-IRES-EGFP constructs containing the rat calmodulin (CaM) and calmodulin with all four Ca^{2+} binding EF-hands being mutated, Δcalmodulin (ΔCaM), were obtained by subcloning from the pBF vector.[29-31]

Intracellular Ca^{2+} measurements using Fura-2 - After 24 hour transfection, HEK293 cells were re-seeded onto fibronectin-coated coverslips (Ø 25 mm). Twenty-four hours later, cells were loaded with 3 µM Fura-2 AM (Molecular Probes, Eugene, OR, USA) and 0.01% v/v Pluronic F-129 (Molecular Probes, Eugene, OR, USA) in KREBS buffer (in mM: 5.5 KCl, 147 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 D-glucose, 10 HEPES-NaOH, pH 7.4) at 37°C for 20 min. Then cells were washed with

KREBS, allowed to equilibrate for 10 min and placed on a 37°C stage of an inverted microscope (Axiovert 200 M, Zeiss, Jena, Germany), continuously being perfused with 37°C KREBS buffer. Sixty seconds after start of the measurements, perfusion buffer was changed to KREBS containing 1 µM thapsigargin (Sigma). At 220 s buffer was changed to KREBS with NaCl replaced by 147 mM N-methyl-d-glucamine (NMDG) in presence of thapsigargin. Details of monitoring $[Ca^{2+}]_i$ using Fura-2 and quantitative image analysis have been described previously.[32] In short, the Fura-2 probe is excited at 340 and 380 nm, with a sampling interval of 3 s. After background correction, the fluorescence emission ratio of 340 and 380 nm excitation was calculated.

$^{45}Ca^{2+}$ *uptake assays* - HEK293T cells were re-seeded into poly-L-lysine (Sigma) coated 24-well plates. Na^+-dependent uptake of $^{45}Ca^{2+}$ was measured generally using the assay as described by Iwamoto et al.[33] Cells were preincubated for 30 min with physiological buffer (in mM: 4 KCl, 147 NaCl, 2 $MgCl_2$, 1.0 $CaCl_2$, 10 D-glucose, 0.1% w/v BSA, 10 HEPES, 10 Tris-HCl, pH 7.4) containing 1 mM ouabain (MP Biomedicals, Santa Ana, CA, USA), 48 h after transfection. Finally, cells were incubated in identical buffer (37°C) with NaCl replaced by 147 mM NMDG containing trace amounts of $^{45}Ca^{2+}$ (0.8 µCi/ml) for 30 s. Then, cells were washed 3 times with ice-cold stop buffer (in mM: 120 NMDG, 10 $LaCl_3$, 10 HEPES, 10 Tris-HCl, pH 7.4), subsequently cells were lysed with 0.05% w/v SDS. Radioactivity was measured and uptake of $^{45}Ca^{2+}$ was calculated.

Real-time PCR - cDNA was prepared as described above. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-rad, Veenendaal, the Netherlands). Human GAPDH was used as a housekeeping gene. Primer sequences are presented in Table 1.

Calmodulin precipitation assay - Calmodulin-coupled agarose beads (Sigma) were incubated for overnight rotating at 4°C with transiently transfected HEK293T lysates (lysis buffer in mM: 150 NaCl, 50 Tris-HCl, pH 7.5, 0.5% v/v Triton X-100 and protease inhibitors with addition of either 1 $CaCl_2$ or 2.5 EGTA). After extensive washing using the lysis buffer indicated above but with 0.1% v/v Triton X-100, proteins were eluted from the beads using 2x Laemmli sample buffer and subsequently analyzed using immunoblotting.

Immunoblotting - NCX1 protein expression was analyzed using transiently transfected HEK293T cell lysates (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5 using HCl, 1% v/v Triton X-100 and protease inhibitors), immunoblotted using 1:500 diluted mouse anti-NCX1 antibody (ab6495, Abcam, Cambridge, UK) for overnight at 4°C. Secondary anti-mouse antibodies conjugated with HRP were used and visualized using femto ECL (Thermo scientific, Waltham, MA, USA).

Statistical Analysis - Results are expressed as mean ± S.E. Significance of the results was determined by one-way ANOVA using the measurements per cell/sample (n≥6) of at least three independent experiments, followed by Bonferroni post-test in case of significance. *p* values < 0.05 were considered significant. All data were analyzed using GraphPad Prism.

Results

Cloning of NCX1.2, NCX1.3 and NCX1.7 from mice kidney
In the kidney three different splice variants were reported to be expressed: NCX1.2 (exons B,C,D), NCX1.7 (exons B,D,F) and NCX1.3 (exons B,D), the latter being considered the predominant

variant. An overview of the renal splice variants is presented in Figure 1A. All three reported renal variants were obtained when cloning NCX1 from mouse kidney: NCX1.2, NCX1.3 and NCX1.7 as identified by complete sequencing. Sequences correspond to GenBank accession numbers XM_006523942, XM_006523944, XM_006523940, respectively. By comparing PCR products using primers flanking the alternative splice region, NCX1.3 was confirmed as the predominant NCX1 variant in the kidney DCT2/CNT segments (Figure 1B), while other variants could be observed as well. The identity of the NCX1.3 band was further confirmed by a *Ssp*I restriction digest, specifically digesting exon B (data not shown).
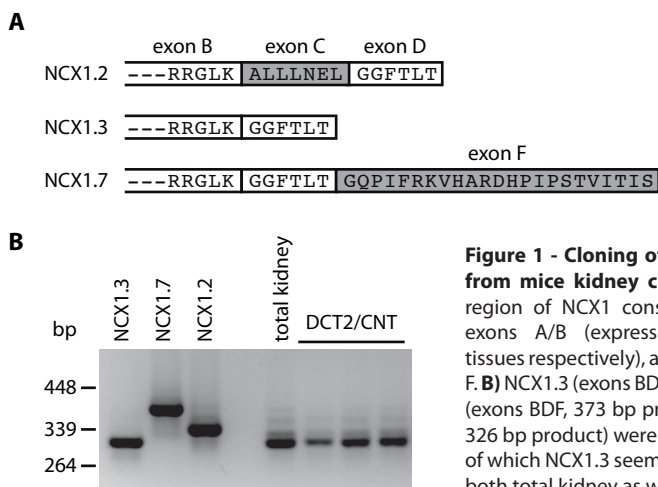
Figure 1 - Cloning of NCX1.2, NCX1.3 and NCX1.7 from mice kidney cDNA. A) The alternative splice region of NCX1 consists of the mutually exclusive exons A/B (expressed in excitable/non-excitable tissues respectively), and the cassette exons C, D, E and F. **B)** NCX1.3 (exons BD, PCR product of 304 bp), NCX1.7 (exons BDF, 373 bp product) and NCX1.2 (exons BCD, 326 bp product) were cloned from mice kidney cDNA, of which NCX1.3 seems to be the predominant form in both total kidney as well as DCT2/CNT material.

NCX1.3 cloned from mice kidney is functional in HEK293 cells

To study the function of NCX1.3 cloned from mice kidney, HEK293 cells were transiently transfected with the pCINeo-mNCX1.3-IRES-EGFP construct. Depending on the electrochemical gradient, NCX can either extrude Ca^{2+} from the cell (forward-mode) or take up extracellular Ca^{2+} (reverse-mode). The intracellular Ca^{2+}-sensor Fura-2 was used to demonstrate reverse-mode NCX1.3 activity. First, Ca^{2+} was released from the intracellular stores by perfusing the cells with KREBS buffer containing the SERCA inhibitor thapsigargin (1 µM). As a result, untransfected cells showed a clear rise in intracellular Ca^{2+} concentration, whereas NCX1.3-expressing cells did not demonstrate this response (Figure 2A,B). In order to study NCX1 activity, at 220 s, the reverse-mode function was induced by switching the perfusion buffer to Na^+-depleted medium, in which Na^+ was replaced by NMDG. In NCX1.3 overexpressing cells a clear rise in $[Ca^{2+}]_i$ was observed while no effect was observed in untransfected cells, demonstrating reverse-mode functioning of NCX1.3 (Figure 2A,B).

Regulation of NCX1.3 by PKA/PKC phosphorylation and intracellular Ca^{2+}

Regulation of NCX1.3 activity was studied by the uptake of radioactive $^{45}Ca^{2+}$ during reverse-mode function induced by switching to Na^+-depleted medium for 30 s. Firstly, general characteristics of the murine NCX1.3 variant were studied. The NCX1 inhibitor for the reverse-mode function SN-6 (50 µM)[34,35] inhibited NCX1.3 function by 32±8% when cells were pre-
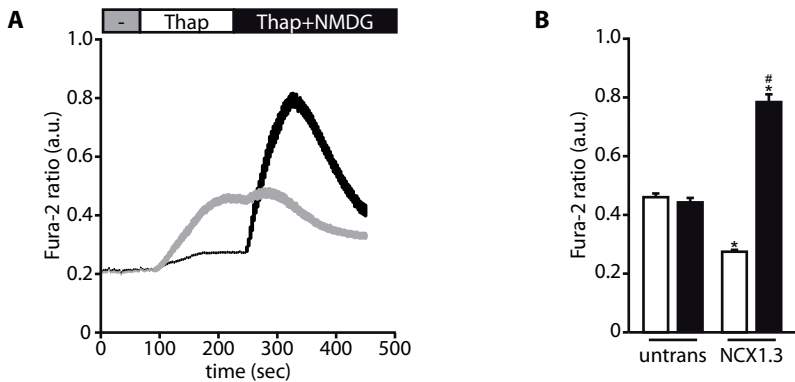
Figure 2 - Fura-2 analysis of NCX1.3 shows Ca²⁺ influx under reverse-mode conditions. A) Average Fura-2 traces in arbitrary units (a.u.) of HEK293 cells untransfected (in grey) and transfected (in black) with NCX1.3. At 60 seconds the perfusing buffer was switched to thapsigargin (Thap, 1 µM) containing buffer in order to release intracellular Ca²⁺ stores. At 220 s buffer was switched from NaCl to NMDG still in the presence of thapsigargin, inducing reverse-mode functioning of the NCX. **B)** Averaged Fura-2 levels at t=220 (open bars) and t=320 s (closed bars). *,$p < 0.0001$ in comparison to t=220 (n=93-94). Thap, thapsigargin; a.u., arbitrary units.

treated for 30 min. Pre-treatment with TPA (100 nM) or forskolin (10 µM), to induce activation of PKC and cAMP/PKA-mediated pathways, respectively, did not affect NCX1.3 activity (Figure 3A). Since, NCX1 is known to be regulated by intracellular Ca²⁺ levels through its two Ca²⁺-binding domains, we investigated the effect of lowering intracellular Ca²⁺ using BAPTA-AM (25 µM) or EGTA-AM (25 µM).[10,36,37] Application of either buffer significantly decreased NCX1.3 activity with 54±8%; or 25±9%, respectively (Figure 3B).
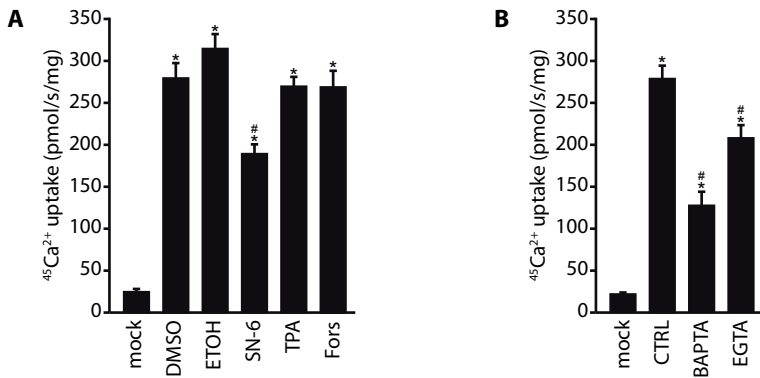
Figure 3 - Regulation of NCX1.3 as measured by influx of ⁴⁵Ca²⁺. A) HEK293T cells were transfected with mock construct or NCX1.3 (DMSO/ETOH controls). Uptake of ⁴⁵Ca²⁺ was measured in cells pre-treated for 30 min with 50 µM SN-6, 100 nM TPA (both in DMSO) or 10 µM forskolin (in ETOH) in physiological buffer containing NaCl and ouabain. Reverse-mode of NCX1.3 was induced upon switching to NMDG containing medium for 30 s. **B)** NCX1.3 transfected HEK293T cells were pre-treated for 30 min with BAPTA-AM and EGTA-AM (25 µM). *,$p < 0.0001$ as compared to mock, #,$p < 0.01$ as compared to DMSO/ETOH control (n=9). CTRL, control.

Overexpression of calmodulin results in higher NCX1.3 mediated Ca²⁺ uptake

Calmodulin is an important regulator of both TRPV5 and PMCA, together with NCX1 the key components of transcellular Ca²⁺ transport. In order to study the effect of calmodulin on

NCX1.3 function, calmodulin was transiently overexpressed in HEK293T cells together with NCX1.3. A significant increase in Ca^{2+} influx (from 165 ± 5 to 216 ± 8 pmol/s/mg) was observed due to overexpression of calmodulin. Interestingly, the calmodulin mutant, that is not able to bind Ca^{2+} (Δcalmodulin), had a significantly reduced stimulatory effect with a Ca^2 influx of 193 ± 7 pmol/s/mg (Figure 4A). NCX1.3 mRNA levels were not affected by overexpression of calmodulin or Δcalmodulin as shown by real-time PCR analysis (Figure 4B). Immunoblotting of lysates from HEK293T cells cultured in parallel to the cells used for $^{45}Ca^{2+}$ uptake studies, did demonstrate almost two times higher levels of NCX1.3 protein in response to overexpression of both calmodulin and Δcalmodulin (Figure 4C). When HEK293T cells overexpressing NCX1.3 were lysed in the presence of Ca^{2+} (1 mM $CaCl_2$), we clearly observed binding of NCX1.3 to
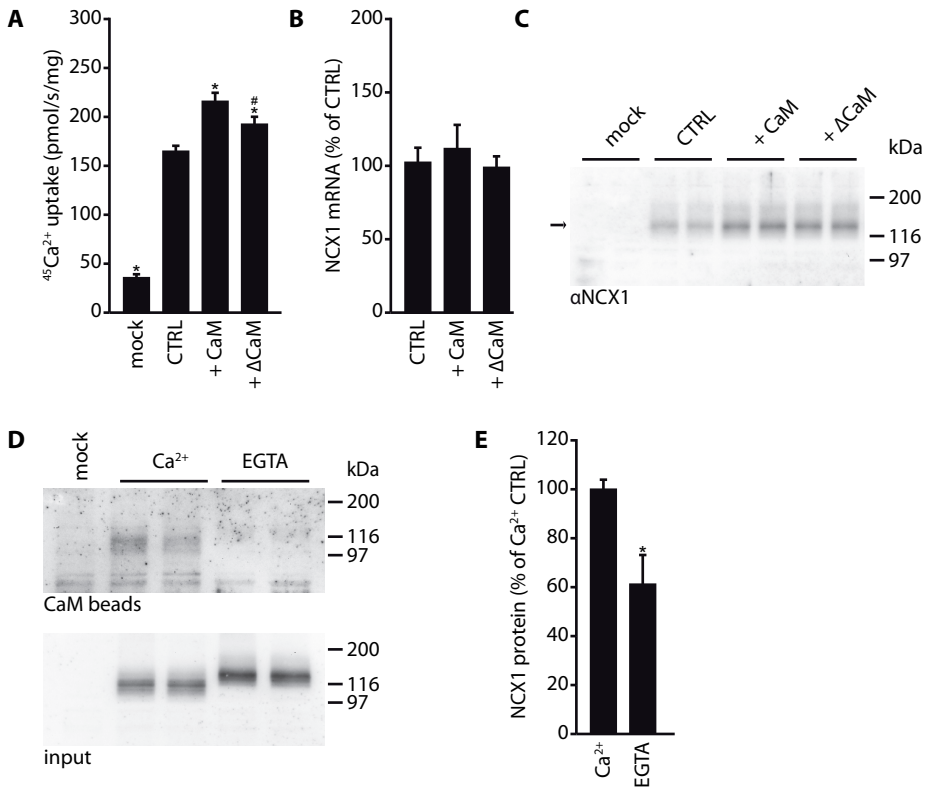
Figure 4 – Calmodulin overexpression enhances NCX1.3 activity and protein abundance. A) HEK293T cells were co-transfected with mock/NCX1.3 and mock/calmodulin/mutated calmodulin (mock/CaM/ΔCaM) expressing constructs. NCX1.3 activity was measured by uptake of $^{45}Ca^{2+}$ in reverse-mode function. *,$p < 0.05$ as compared to CTRL, #,$p < 0.05$ as compared to + CaM (n=15). **B)** Real-time PCR analysis of NCX1.3 mRNA levels in HEK293T cells co-expressing NCX1.3 (CTRL) and mock/CaM/ΔCaM constructs (n=6). **C)** Representative immunoblot analysis for NCX1 on protein lysates isolated from HEK293T cells co-expressing mock/NCX1.3 (CTRL) and mock/CaM/ΔCaM constructs as cultured in parallel to cells used in $^{45}Ca^{2+}$ uptake assays (n=10). **D)** Representative immunoblot for NCX1, showing binding of NCX1.3 to CaM as analyzed using CaM-coupled agarose beads. Mock/NCX1.3 expressing HEK293T cells were lysed in the presence of Ca^{2+} (1 mM) or EGTA (2.5 mM) and subsequently applied to the beads, protein lysates were used as input controls. **E)** Quantification of immunoblot densities for NCX1 binding to CaM beads, corrected for input and normalized versus Ca^{2+} containing control. *,$p < 0.01$ as compared to in 1 mM Ca^{2+} (n=9). CaM, calmodulin.

calmodulin-coupled agarose beads (Figure 4D). Co-expressed EGFP was used as a negative control and could not be detected (data not shown). Binding was decreased to 61±12% in the presence of EGTA (2.5 mM) as calculated by quantification of immunoblot densities (Figure 4D,E). Additionally, in the presence of Ca^{2+} a ~115 kDa band was observed for NCX1.3, whereas in the presence of EGTA a band was shown at ~140 kDa. Samples of protein lysates before application to the beads were used as input control.

Mutation of key residues within the putative calmodulin binding sites reduces NCX1.3 activity

Five calmodulin binding sites were predicted within the mouse NCX1.3 with varying
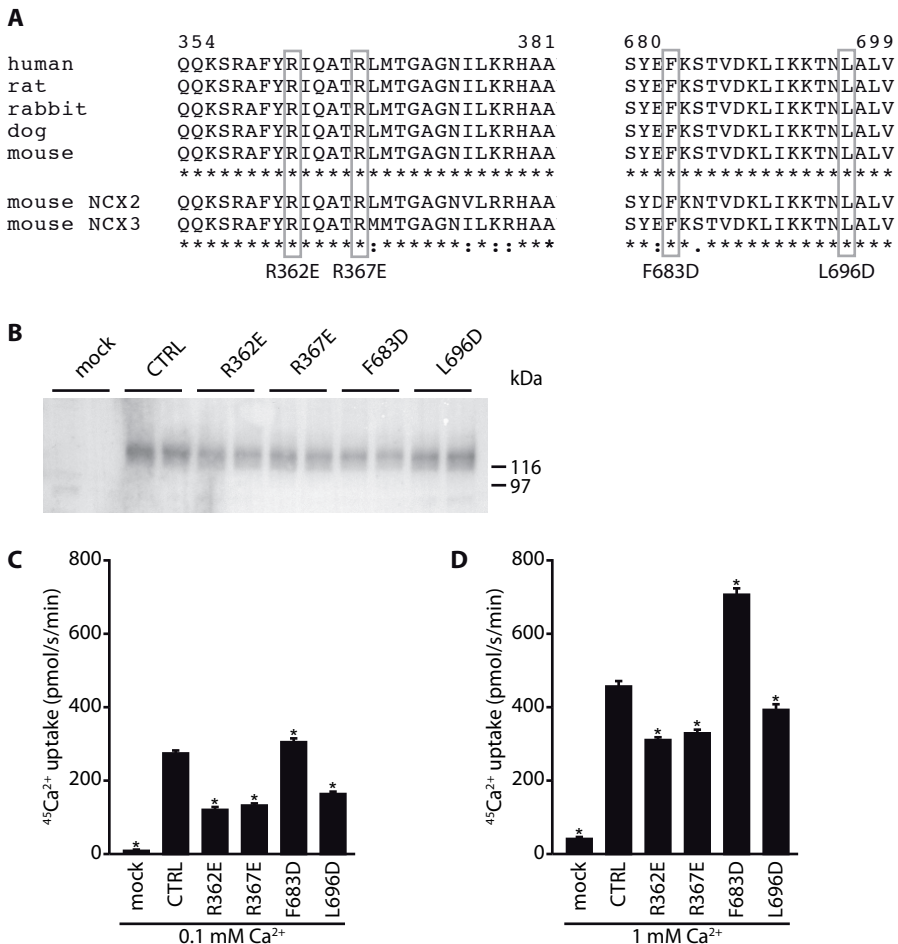
Figure 5 – Potential calmodulin binding sites within NCX1.3. A) Two calmodulin binding sites were predicted around key residues R362/R367 and F683/L696. Using site directed mutagenesis the indicated NCX1.3 mutants were generated: R362E, R367E, F683D and L696D. **B)** Representative immunoblot for NCX1, showing protein expression levels of the different NCX1.3 mutants (n=6). **C,D)** Reverse-mode activity of NCX1.3 (CTRL) and NCX1.3 constructs containing the specific mutations as measured using $^{45}Ca^{2+}$ influx at physiological (1 mM) and low (0.1 mM) concentrations of Ca^{2+} during preincubation and uptake. *,p < 0.001 as compared to CTRL (n=9).

prediction scores by *in silico* analysis using the Calmodulin Target Database.[38] Since binding of calmodulin to the XIP region within NCX1 has never been demonstrated, this region was not further investigated.[12,26] Two other sites were excluded, as these were localized in predicted transmembrane segments. Both remaining potential sites are conserved among species and NCX isoforms (Figure 5A). To study the role of these latter two potential calmodulin binding sites, predicted key residues were mutated as indicated in Figure 5A. NCX1.3 protein levels were slightly reduced by the different mutations (Figure 5B). Mutation of residues R362, R367 and L696 reduced Ca^{2+} influx when compared to wild-type NCX1.3 at both low (0.1 mM) and physiological (1 mM) extracellular Ca^{2+} concentrations (Figure 5C,D). Interestingly, mutation of F683 into an aspartic acid significantly increased exchanger activity by $12\pm3\%$ and $56\pm6\%$ at 0.1 and 1.0 mM Ca^{2+}, respectively.

NCX1.3-L696D is unresponsive to calmodulin overexpression

To test whether the observed decrease in exchanger activity is indeed caused by (endogenous) calmodulin, we studied whether the observed stimulatory effect of calmodulin overexpression was affected by mutation of two of these potential key residues. NCX1.3-R362E still showed increased activity due to overexpression of calmodulin at both low and physiological concentrations of Ca^{2+} (Figure 6A,B). Ca^{2+} influx was increased with $53\pm19\%$ when overexpressing calmodulin, this as compared to $65\pm18\%$ for wild-type at physiological Ca^{2+} concentrations. In contrast, stimulation of NCX1.3-L696D by calmodulin overexpression increased influx of Ca^{2+} with only $31\pm8\%$ and $24\pm8\%$ at Ca^{2+} concentrations of 0.1 and 1.0 mM, respectively. Interestingly, the stabilizing effect of calmodulin on NCX1.3 protein levels was maintained for both mutants (Figure 6C). In addition, all four mutants were still able to bind calmodulin as assessed using calmodulin-coupled agarose beads (Figure 6D). No significant differences in binding of NCX1.3 mutants to calmodulin beads were observed from quantifications of immunoblots (Figure 6E).

Discussion

In this study, NCX1.2, NCX1.3 and NCX1.7 were cloned from mice kidney cDNA. NCX1.3 was confirmed as the predominant NCX1 variant in murine kidney and DCT2/CNT nephron segments. Most importantly, we identified calmodulin as a positive regulator of NCX1.3 function as based on the following observations. Firstly, overexpression of calmodulin in HEK293T cells enhanced NCX1.3 protein abundance and increased exchanger activity as measured by influx of Ca^{2+} via reverse-mode function of the exchanger. Secondly, overexpression of calmodulin with mutations in its Ca^{2+}-binding sites demonstrated that the observed effect is only partly dependent on Ca^{2+} binding to calmodulin. Thirdly, binding of calmodulin to NCX1.3 was demonstrated using calmodulin-coupled agarose beads. Finally, a potential calmodulin binding site was identified in the NCX1.3 cytosolic loop in close proximity to residue L696. Interestingly, calmodulin was still able to increase protein levels of NCX1.3-L696D, suggesting that calmodulin also interacts with another binding site, which is responsible for the stabilizing effect of the NCX1.3 protein.

Murine NCX1.3 was cloned previously by Li *et al.* from mice osteoclasts but functional studies were never performed using this construct.[39] Next to NCX1.3, being considered the main NCX1

in kidney, we also cloned NCX1.2 and NCX1.7 from mice kidney. This is in agreement with previous reports that NCX1.2 (BCD), NCX1.7 (BDF) and especially NCX1.3 (BD) are expressed in the kidney.[15,16] For NCX1.2, not much is known about its role and regulation. NCX1.3 was previously reported to have a higher buffering capacity[40] and is less susceptible to oxidative
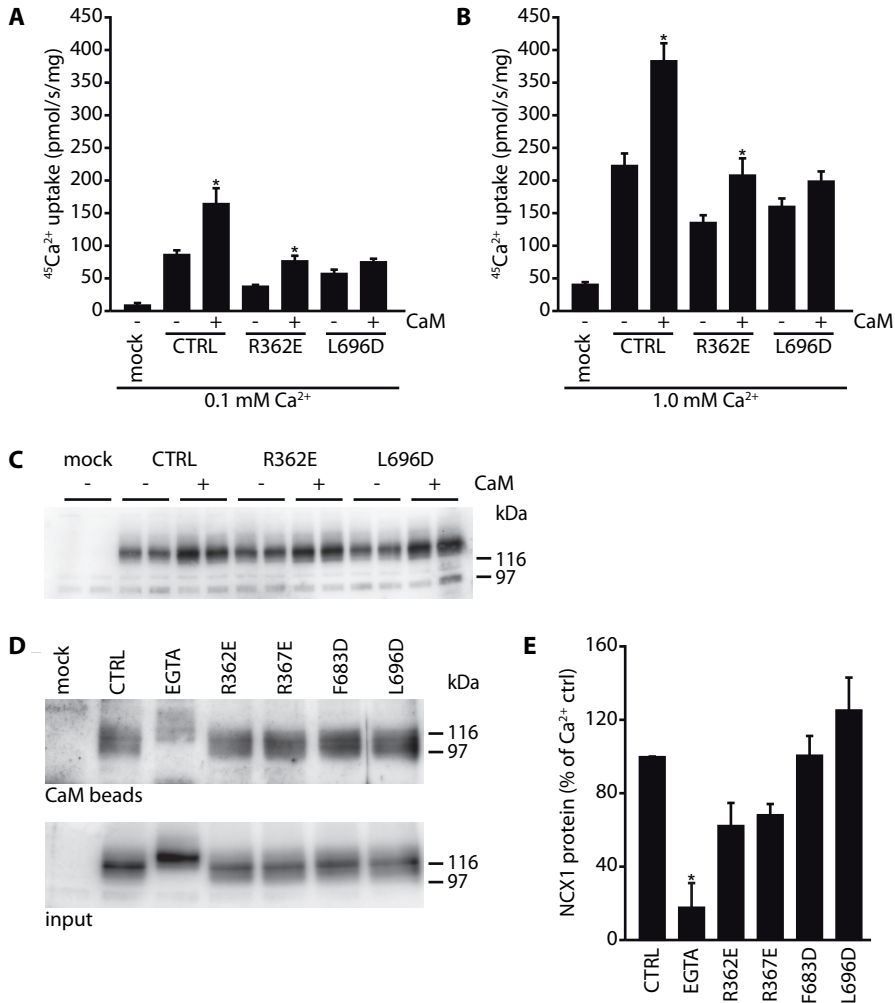
Figure 6 – Identification of calmodulin binding sites within NCX1.3. A,B) NCX1.3 activity is determined using the $^{45}Ca^{2+}$ reverse-mode assay in HEK293T cells co-expressing mock/NCX1.3 CTRL/NCX1.3-R362E/NCX1.3-L696D together with mock/calmodulin (CaM) expressing constructs in the presence of physiological (1 mM) and low (0.1 mM) concentrations of Ca^{2+}. *,$p < 0.05$ as compared to no CaM overexpression (n=12). **C)** Representative immunoblot for NCX1, showing protein expression levels of the different NCX1.3 mutants in the absence/ presence of overexpressed CaM from HEK293T cells cultured in parallel to the cells used for $^{45}Ca^{2+}$ uptake analysis (n=6). **D)** Binding of the different NCX1.3 mutants to CaM-coupled agarose beads as compared to wild-type (CTRL). NCX1.3 (CTRL) expressing HEK293T were lysed in the presence of Ca^{2+} (1 mM) or EGTA (2.5 mM), mock and NCX1.3 mutant expressing cells were all lysed in the presence of Ca^{2+}. Protein lysates were used as input controls. **E)** Quantification of immunoblot densities for NCX1 binding to CaM beads, corrected for input and normalized versus Ca^{2+} containing control. *,$p < 0.001$ as compared to wild-type (CTRL) in 1 mM Ca^{2+} (n=4).

stress as compared to NCX1.7.[41] Thereby NCX1.3 appears to be well suited as a high capacity extruder in the process of transcellular Ca^{2+} transport in the kidney. Also, NCX1.3 activity was reported to be elevated in response to PKC phosphorylation, which was not observed for NCX1.7.[40,42] In contrast, the present results demonstrated no effect of PKC signaling on NCX1.3 activity when activated using TPA. However, phosphorylation of NCX remains controversial and seems largely dependent on specific circumstances. For example, PKA phosphorylation of cardiac NCX1 was observed in both oocytes and rat ventricular cardiomyocytes, but this isoform was unresponsive to PKA activation when expressed in HEK293 cells.[43] In addition, despite mutation of all PKC consensus sites within NCX1, the protein still appeared to be phosphorylated.[33,43] Presumably, NCX1 is part of a macromolecular complex, containing kinases and phosphates, which might differ among tissues and different circumstances. Also, phosphorylation processes might indirectly affect NCX1 activity.[43,44] Activation of NCX1 by PKA was only reported for NCX1.1 and not NCX1.3, consistent with the present results.[45]

Although calmodulin was demonstrated to be involved in the regulation of renal transcellular Ca^{2+} transport by directly regulating the activity of TRPV5, which is responsible for Ca^{2+} influx,[24] as well as by activating the PMCAs enabling extrusion of Ca^{2+},[25] regulation of NCX1.3 through calmodulin remained unknown. By applying the intracellular Ca^{2+} buffers BAPTA-AM and EGTA-AM, we further substantiated that intracellular Ca^{2+} is of key importance for NCX1.3 activity.[37,46] Intracellular Ca^{2+} can potentially directly affect NCX1.3 function via its Ca^{2+}-binding domains and indirectly via binding to calmodulin. Chelation of Ca^{2+} significantly decreased binding of NCX1 to calmodulin-coated agarose beads as observed in the presence of EGTA. Binding of calmodulin to NCX1.3 appeared to be largely Ca^{2+}-dependent, although some binding was observed under Ca^{2+}-free conditions. Increased binding in the presence of Ca^{2+} could be due to Ca^{2+}-binding to calmodulin and/or Ca^{2+} binding to the CBDs of NCX1 itself. Additionally, presence of Ca^{2+} in the protein lysate resulted in the NCX1 protein to run lower (~115 kDa) on a SDS-gel. NCX1 is reported to be cleaved by Ca^{2+}-dependent calpain or other Ca^{2+}-sensitive proteases.[10,47] Calpain, cleavage however, results in a much smaller band of about 80 kDa for NCX1.[47,48] Also, no changes were observed upon application of high concentrations of protease inhibitors (leupeptin and calpeptin, data not shown). This observation can possibly be explained by the CBD1 being partially unstructured in the absence of Ca^{2+},[49] therefore resulting in fully denatured NCX1.3 protein in Ca^{2+}-free conditions only. Similar sizes were observed by Wanichawan *et al.*, showing ~140 kDa bands in boiled samples and both sizes in unboiled samples.[48]

Most importantly, NCX1 activity was increased by co-expressing calmodulin in HEK293T cells. In addition, NCX1.3 protein levels were elevated, while mRNA levels were unaltered, suggesting that binding of calmodulin stabilizes the NCX1.3 protein. Calmodulin binding might structurally stabilize the NCX1.3 protein or could reduce accessibility for proteases such as calpain.[10,47] The observed stabilizing effect on NCX1.3 protein levels was also observed for Δcalmodulin, a calmodulin mutant not able to bind any Ca^{2+} due to mutations in all four EF-hands.[30] Activity of NCX1.3 was only partly enhanced by Δcalmodulin. This suggests that regulation of NCX1.3 exchanger activity by calmodulin is a partly Ca^{2+}-dependent and partly Ca^{2+}-independent process, the latter probably due to the effect on protein stability. Similar Ca^{2+}-independent/dependent effects of calmodulin have been observed for Small Conductance

Ca^{2+}-Activated Potassium Channels (SK channels).[30,31] These channels show Ca^{2+}-independent binding of calmodulin to the channel and a Ca^{2+}-dependent effect on channel gating.[30] Ca^{2+}-free calmodulin was also shown to bind L-, N-, and P/Q-type voltage-gated Ca^{2+} channels.[31,50,51]

The so-called XIP region was previously identified as a potential calmodulin binding site, but calmodulin binding to this region within NCX1 could never be demonstrated.[26] In contrast, the XIP peptide is now considered as an inhibitor of NCX as well as PMCA function.[27] Therefore, we studied two additional predicted calmodulin binding sites. The site around residues F683/L696, seems to contain important regulatory properties and probably is involved in binding of calmodulin. Mutation of NCX1.3 residue L696 into aspartic acid decreased transport activity, which was not stimulated by overexpression of calmodulin at physiological concentrations. However, since stabilization of the NCX1.3 protein by calmodulin was not impaired, the Ca^{2+}-independent process appears to remain intact, probably accounting for the small albeit not significant increase in NCX1.3-L696D activity in presence of calmodulin. The F683D mutation within the same predicted site increased NCX1.3 activity, suggesting that this site might also be a binding site for other interacting partners. This concept is in agreement with the previously proposed macromolecular complex, where depending on specific circumstances several factors could compete for similar binding sites. Comparable to L696D, mutation of residues R362 and R367 decreased NCX1.3 activity in the presence of endogenous calmodulin. However, NCX1.3-R362E activity could still be stimulated by calmodulin overexpression. Also, after mutation of the four individual residues, NCX1.3 was still able to bind calmodulin. This leads to the understanding that multiple calmodulin binding sites are involved in the process of enhancing NCX1.3 activity and protein stability. The predicted binding site involving residues R362/R367 could be a second calmodulin binding site, as possibly the introduced mutations were not sufficient to prevent binding of calmodulin to this site.

In conclusion, the present study identifies NCX1.3 as the main NCX1 expressed in DCT2/CNT tubules facilitating transcellular Ca^{2+} reabsorption. For the first time NCX1.3 was demonstrated to bind the Ca^{2+}-sensor calmodulin. Interestingly, the effects of calmodulin on NCX1.3 occur in both a Ca^{2+}-dependent and Ca^{2+}-independent fashion. We propose that binding of Ca^{2+}-free calmodulin stabilizes the NCX1.3 protein, whereas Ca^{2+}-calmodulin is required for activation of the NCX1.3 exchanger. Furthermore, stimulatory action of calmodulin on reverse-mode exchanger activity involves the predicted calmodulin binding site within the NCX1.3 cytosolic loop around residue L696.

Acknowledgements

References

1. Berridge MJ, Bootman MD, Roderick HL: Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4: 517-529, 2003

2. Hoenderop JG, Bindels RJ: Epithelial Ca^{2+} and Mg^{2+} channels in health and disease. *J Am Soc*

5

Nephrol 16: 15-26, 2005

3. Hoenderop JG, van der Kemp AW, Hartog A, van de Graaf SF, van Os CH, Willems PH, Bindels RJ: Molecular identification of the apical Ca^{2+} channel in 1, 25-dihydroxyvitamin D_3-responsive epithelia. *J Biol Chem* 274: 8375-8378, 1999

4. Lambers TT, Mahieu F, Oancea E, Hoofd L, de Lange F, Mensenkamp AR, Voets T, Nilius B, Clapham DE, Hoenderop JG, Bindels RJ: Calbindin-D28K dynamically controls TRPV5-mediated Ca^{2+} transport. *EMBO J* 25: 2978-2988, 2006

5. Hoenderop JG, Nilius B, Bindels RJ: Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol* 64: 529-549, 2002

6. Lambers TT, Bindels RJ, Hoenderop JG: Coordinated control of renal Ca^{2+} handling. *Kidney Int* 69: 650-654, 2006

7. Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia. *Physiol Rev* 85: 373-422, 2005

8. Brini M, Carafoli E: The plasma membrane Ca^{2+} ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb Perspect Biol* 3, 2011

9. Kip SN, Strehler EE: Characterization of PMCA isoforms and their contribution to transcellular Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol* 284: F122-132, 2003

10. Lytton J: Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem J* 406: 365-382, 2007

11. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 269: 5145-5149, 1994

12. Shigekawa M, Iwamoto T: Cardiac Na^+-Ca^{2+} exchange: molecular and pharmacological aspects. *Circ Res* 88: 864-876, 2001

13. Nicoll DA, Longoni S, Philipson KD: Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* 250: 562-565, 1990

14. Kim I, Koh GY, Lee CO: Identification of alternatively spliced Na^+-Ca^{2+} exchanger isoforms expressed in the heart. *Comp Biochem Physiol B Biochem Mol Biol* 119: 157-161, 1998

15. Lee SL, Yu AS, Lytton J: Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 269: 14849-14852, 1994

16. White KE, Gesek FA, Reilly RF, Friedman PA: NCX1 Na/Ca exchanger inhibition by antisense oligonucleotides in mouse distal convoluted tubule cells. *Kidney Int* 54: 897-906, 1998

17. Dyck C, Omelchenko A, Elias CL, Quednau BD, Philipson KD, Hnatowich M, Hryshko LV: Ionic regulatory properties of brain and kidney splice variants of the NCX1 Na^+-Ca^{2+} exchanger. *J Gen Physiol* 114: 701-711, 1999

18. Dunn J, Elias CL, Le HD, Omelchenko A, Hryshko LV, Lytton J: The molecular determinants of ionic

19. van de Graaf SF, Bindels RJ, Hoenderop JG: Physiology of epithelial Ca^{2+} and Mg^{2+} transport. *Rev Physiol Biochem Pharmacol* 158: 77-160, 2007

20. Hoenderop JG, Dardenne O, van Abel M, van der Kemp AW, Van Os CH, St -Arnaud R, Bindels RJ: Modulation of renal Ca^{2+} transport protein genes by dietary Ca^{2+} and 1,25-dihydroxyvitamin D_3 in 25-hydroxyvitamin D_3-1alpha-hydroxylase knockout mice. *FASEB J* 16: 1398-1406, 2002

21. Kip SN, Strehler EE: Vitamin D_3 upregulates plasma membrane Ca^{2+}-ATPase expression and potentiates apico-basal Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol* 286: F363-369, 2004

22. van Abel M, Hoenderop JG, van der Kemp AW, Friedlaender MM, van Leeuwen JP, Bindels RJ: Coordinated control of renal Ca^{2+} transport proteins by parathyroid hormone. *Kidney Int* 68: 1708-1721, 2005

23. de Groot T, Bindels RJ, Hoenderop JG: TRPV5: an ingeniously controlled calcium channel. *Kidney Int* 74: 1241-1246, 2008

24. de Groot T, Kovalevskaya NV, Verkaart S, Schilderink N, Felici M, van der Hagen EA, Bindels RJ, Vuister GW, Hoenderop JG: Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. *Mol Cell Biol* 31: 2845-2853, 2011

25. Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi A: Plasma membrane Ca^{2+} ATPases as dynamic regulators of cellular calcium handling. *Ann N Y Acad Sci* 1099: 226-236, 2007

26. Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, Philipson KD: Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J Biol Chem* 266: 1014-1020, 1991

27. Enyedi A, Penniston JT: Autoinhibitory domains of various Ca^{2+} transporters cross-react. *J Biol Chem* 268: 17120-17125, 1993

28. van der Hagen EA, Lavrijsen M, van Zeeland F, Praetorius J, Bonny O, Bindels RJ, Hoenderop JG: Coordinated regulation of TRPV5-mediated Ca transport in primary distal convolution cultures. *Pflugers Arch*, 2014

29. Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ: Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. *J Biol Chem* 279: 28855-28861, 2004

30. Keen JE, Khawaled R, Farrens DL, Neelands T, Rivard A, Bond CT, Janowsky A, Fakler B, Adelman JP, Maylie J: Domains responsible for constitutive and Ca^{2+}-dependent interactions between calmodulin and small conductance Ca^{2+}-activated potassium channels. *J Neurosci* 19: 8830-8838, 1999

31. Lee WS, Ngo-Anh TJ, Bruening-Wright A, Maylie J, Adelman JP: Small conductance Ca^{2+}-activated K^+ channels and calmodulin: cell surface expression and gating. *J Biol Chem* 278: 25940-25946, 2003

32. de Groot T, Verkaart S, Xi Q, Bindels RJ, Hoenderop

5

JG: The identification of Histidine 712 as a critical residue for constitutive TRPV5 internalization. *J Biol Chem* 285: 28481-28487, 2010

33. Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M: Protein kinase C-dependent regulation of Na$^+$/Ca^{2+} exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* 37: 17230-17238, 1998

34. Kita S, Iwamoto T: Inhibitory mechanism of SN-6, a novel benzyloxyphenyl Na$^+$/Ca^{2+} exchange inhibitor. *Ann N Y Acad Sci* 1099: 529-533, 2007

35. Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T: The exchanger inhibitory peptide region-dependent inhibition of Na$^+$/Ca^{2+} exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol* 66: 45-55, 2004

36. Dargan SL, Parker I: Buffer kinetics shape the spatiotemporal patterns of IP$_3$-evoked Ca^{2+} signals. *J Physiol* 553: 775-788, 2003

37. Linck B, Qiu Z, He Z, Tong Q, Hilgemann DW, Philipson KD: Functional comparison of the three isoforms of the Na$^+$/Ca^{2+} exchanger (NCX1, NCX2, NCX3). *Am J Physiol* 274: C415-423, 1998

38. Ikura: Calmodulin Target Database, 2013

39. Li JP, Kajiya H, Okamoto F, Nakao A, Iwamoto T, Okabe K: Three Na$^+$/Ca^{2+} exchanger (NCX) variants are expressed in mouse osteoclasts and mediate calcium transport during bone resorption. *Endocrinology* 148: 2116-2125, 2007

40. Hwang EF, Williams I, Kovacs G, Peti-Peterdi J, Siroky B, Rice WC, Bates E, Schwiebert EM, Unlap MT, Bell PD: Impaired ability of the Na$^+$/Ca^{2+} exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium. *Am J Physiol Renal Physiol* 284: F1023-1031, 2003

41. Unlap MT, Williams C, Morin D, Siroky B, Fintha A, Fuson A, Dodgen L, Kovacs G, Komlosi P, Ferguson W, Bell PD: Amyloid beta peptide 1-40 stimulates the Na$^+$/Ca^{2+} exchange activity of SNCX. *Curr Neurovasc Res* 2: 3-12, 2005

42. Unlap MT, Peti-Peterdi J, Bell PD: Cloning of mesangial cell Na$^+$/Ca^{2+} exchangers from Dahl/Rapp salt-sensitive/resistant rats. *Am J Physiol Renal Physiol* 279: F177-184, 2000

43. Ruknudin AM, Wei SK, Haigney MC, Lederer WJ, Schulze DH: Phosphorylation and other conundrums of Na/Ca exchanger, NCX1. *Ann N Y Acad Sci* 1099: 103-118, 2007

44. Morad M, Cleemann L, Menick DR: NCX1 phosphorylation dilemma: a little closer to resolution. Focus on "Full-length cardiac Na$^+$/Ca^{2+} exchanger 1 protein is not phosphorylated by protein kinase A". *Am J Physiol Cell Physiol* 300: C970-973, 2011

45. Schulze DH, Polumuri SK, Gille T, Ruknudin A: Functional regulation of alternatively spliced Na$^+$/Ca^{2+} exchanger (NCX1) isoforms. *Ann N Y Acad Sci* 976: 187-196, 2002

46. Sanders L, Rakovic S, Lowe M, Mattick PA, Terrar DA: Fundamental importance of Na$^+$-Ca^{2+} exchange for the pacemaking mechanism in guinea-pig sino-atrial node. *J Physiol* 571: 639-649, 2006

47. Shaikh S, Samanta K, Kar P, Roy S, Chakraborti T, Chakraborti S: m-Calpain-mediated cleavage of Na$^+$/Ca^{2+} exchanger-1 in caveolae vesicles isolated from pulmonary artery smooth muscle. *Mol Cell Biochem* 341: 167-180, 2010

48. Wanichawan P, Louch WE, Hortemo KH, Austbo B, Lunde PK, Scott JD, Sejersted OM, Carlson CR: Full-length cardiac Na$^+$/Ca^{2+} exchanger 1 protein is not phosphorylated by protein kinase A. *Am J Physiol Cell Physiol* 300: C989-997, 2011

49. Breukels V, Touw WG, Vuister GW: Structural and dynamic aspects of Ca^{2+} and Mg^{2+} binding of the regulatory domains of the Na$^+$/Ca^{2+} exchanger. *Biochem Soc Trans* 40: 409-414, 2012

50. Erickson MG, Alseikhan BA, Peterson BZ, Yue DT: Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. *Neuron* 31: 973-985, 2001

51. Pitt GS, Zuhlke RD, Hudmon A, Schulman H, Reuter H, Tsien RW: Molecular basis of calmodulin tethering and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels. *J Biol Chem* 276: 30794-30802, 2001

5

General discussion

Introduction

Maintenance of calcium (Ca^{2+}) homeostasis is of key importance for the human body. Ca^{2+} is a major constituent of bone, while intracellular Ca^{2+} is involved as second messenger in many physiological processes including muscle contraction and nerve excitation.[1,2] Therefore, the Ca^{2+} balance must be tightly regulated. Ca^{2+} homeostasis is maintained via uptake in the intestine, storage in bone and reabsorption in the kidney.[3] In the kidney, Ca^{2+} is reabsorbed along the nephron through paracellular transport in the proximal and thick ascending limb (TAL) segments. Final excretion of Ca^{2+} is determined by transcellular Ca^{2+} reabsorption in the distal convolution, comprising the second part of the distal convoluted tubule (DCT2) and connecting tubule (CNT).[4] Here, the epithelial Ca^{2+} channel transient receptor potential vanilloid 5 (TRPV5) is expressed, allowing the influx of Ca^{2+} into the cell.[5] As a result, the TRPV5 channel is considered to be the "gatekeeper" of transcellular Ca^{2+} reabsorption. TRPV5 expression, plasma membrane abundance and channel activity are regulated by many factors including Ca^{2+}, pH, several hormones, glycosidases and proteases.[5-8] After apical entry via TRPV5, with the help of the Ca^{2+}-buffer calbindin-D_{28k} Ca^{2+} is shuttled towards the basolateral membrane, where it is extruded towards the blood compartment by the concerted action of the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger type 1 (NCX1).[5]

This thesis aimed to elucidate the molecular regulation of transcellular Ca^{2+} reabsorption in the distal convolution. Firstly, regulation of Ca^{2+} entry by the key player TRPV5 was further investigated. Secondly, a novel murine primary cell model for the distal convolution was developed, allowing studies of different components involved in transcellular Ca^{2+} transport. Finally, NCX1 was cloned from murine kidney to unravel its role and regulation as the main basolateral Ca^{2+} extruder.

The role and regulation of the epithelial Ca^{2+} channel TRPV5

Regulation of TRPV5 function by its N-terminal tail
As the gatekeeper of renal transcellular Ca^{2+} reabsorption,[5,9] structure and regulation of the TRPV5 Ca^{2+} channel have been extensively studied. The TRPV5 C-terminal region and in particular its TRP domain has been implicated in the regulation of TRPV5 intrinsic activity as determined by channel open probability together with channel conductance, and plasma membrane abundance as regulated by trafficking processes.[6,10,11] However, the role of the N-terminal region and especially its multiple ankyrin repeats remains largely elusive (Figure 1). Ankyrin repeats are highly conserved helix-loop-helix structures of ~33 residues, that are involved in binding of several proteins/ligands, such as calmodulin and ATP.[12-15] The TRPV5 N-terminus is known to bind calmodulin,[16] participates in the oligomerization of TRPV5 through the region L64-A77[17] and is phosphorylated at the residues S144 and S299.[7,18,19] In **chapter 2** the role of the TRPV5 N-terminus was studied in the regulation of plasma membrane abundance and channel activity. Several N-terminal deletion mutants were generated, demonstrating an abolished TRPV5-mediated influx of Ca^{2+} when the first 38 amino acids were deleted. The well-conserved residues Q31 and D34 were critical in this effect. Mutated channels were retained in the endoplasmic reticulum and lacked complex glycosylation. Some TRPV5 channels were present on the plasma membrane but proved non-functional and could not be internalized.

6
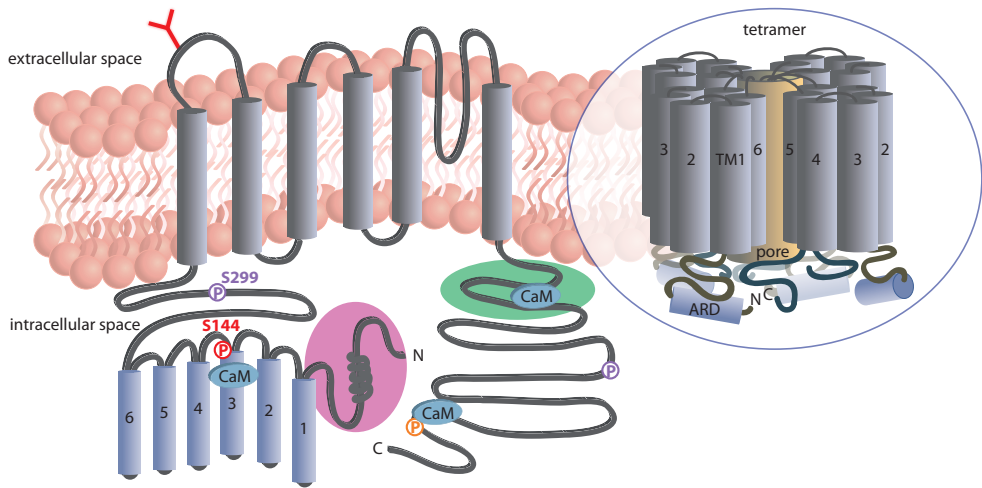
Figure 1 - Focus on the TRPV5 N-terminus and the channel tetramer. The TRPV5 monomer consists of six putative transmembrane domains, a pore domain and large cytoplasmic N- and C-termini. An N-glycosylation site is present at residue N358. The N-terminal domain contains six highly conserved ankyrin repeats, the ankyrin repeat domain (ARD), of which ankyrin repeat 3 contains a PKC phosphorylation site (S114) and is involved in regulation by calmodulin (CaM). A second phosphorylation site (S299) is present in the linker region between the ARD and TM1. The N-terminal domain depicted in pink is crucial for proper channel folding, and contains a predicted helix structure implicated in this process. The TRPV5 tetramer is arranged around a central pore comprising the four individual pore domains, together allowing influx of Ca^{2+}. The linker region between the ARD and TM1 interacts with ankyrin repeats 3/4 and the C-terminal domain of an adjacent TRPV5 protein.

In the endoplasmic reticulum, misfolded proteins are recognized by the quality control machinery, retained and targeted for degradation.[20,21] Our data suggest that some channels escaped from quality control and reached the plasma membrane, resulting in detectable amounts of TRPV5 channels on the plasma membrane as channels could not be properly internalized. Deletion of this N-terminal domain is associated with lack of glycosylation, which might cause inhibition of proper channel internalization. Glycosylation at residue N358 is involved in channel internalization, a process that is modulated by klotho.[22] Misfolding of TRPV5, due to deletion of residues M1-M38 or mutation of Q31/D34, was further evidenced by the rescue of TRPV5 activity in cells cultured at a lower temperature (30°C). In these conditions, proteins likely evade the quality control system and resume trafficking via the Golgi system. This in accordance to observations made for the cystic fibrosis transmembrane conductance regulator (CFTR), where misfolding and endoplasmic reticulum retention of the mutated chloride channel leads to cystic fibrosis.[23] For TRPV5 Q31/D34 mutants, in addition to channel activity, complex glycosylation was restored as well, indicating that Golgi trafficking was indeed resumed. Moreover, the residues Q31/D34 are located within a predicted helix structure, suggesting that this helix is essential for folding of TRPV5 into a functional channel. Mutation of similar residues just after the predicted helix did not affect channel function. Hence, TRPV5 N-terminal residues M1-M38 and in particular the residues Q31/D34 are essential for protein folding and thus a functional channel. The predicted helix structure within this N-terminal region appears to be a crucial structural feature within the TRPV5 channel.

Interestingly, despite impaired protein folding, TRPV5 mutant channels were able to form tetramers, even when residues M1-R75 including the first ankyrin repeat were deleted. Assembly of misfolded ion channels has been demonstrated before, for example for the voltage-gated K^{+} channel.[24] Since Golgi trafficking is abrogated, our results suggest that TRPV5 channel tetramerization takes place in the endoplasmic reticulum as is most commonly observed for plasma membrane proteins.[25] Channel assembly in the endoplasmic reticulum was demonstrated earlier for TRPV4.[26] The TRPV5 residues L64-A77 are essential for interactions between channel N-termini,[17] indicating a role in channel oligomerization. These interactions, however, were demonstrated between bacterial derived and *in vitro* translated TRPV5 N-terminal domains, therefore not taking into account total protein structures.[17] Moreover, structural studies of TRPV6 ankyrin repeats did not demonstrate oligomerization,[27] while channel oligomerization was shown by interactions of transmembrane domains, N- and C-termini.[28] This suggests that TRPV channel tetramerization is not solely dependent of N-terminal interactions, but an interplay of several domains.[27] This is supported by recent structural data of TRPV1, demonstrating interactions between the ankyrin repeat domain (ARD, comprising all 6 ankyrin repeats), β-sheet structures within the linker region between the ARD and first transmembrane segment (TM1), and the C-terminus of an adjacent subunit within the channel tetramer.[29] Hence, channel oligomerization probably is a result of several interactions within and between adjacent channel subunits. The present studies indicated that the region M1-R75 is not essential for channel tetramerization, but N-terminal interactions were not directly investigated. Therefore, ankyrin repeat 1 could still play a role in channel-channel interactions and thus quaternary protein folding.

The role of the six highly conserved ankyrin repeats in TRPV channels appears to be quite diverse.[13] TRPV ankyrin repeats show an atypical sequence deviating from the consensus,[13] thereby making their function less predictable. As the first TRP channel, TRPV1 structural topology has been determined recently by electron cryo-microscopy.[29] These data identify TRPV1 ankyrin repeats 3 and 4 as important structural features in overall channel folding and oligomerization, while ankyrin repeats 1 and 2 could not be detected probably due to high flexibility.[29] In addition, ankyrin repeats 1 and 3 in TRPV4 and repeat 3 in TRPV6 are involved in channel tetramerization,[26,30] suggesting a similar role for any of the ankyrin repeats within the TRPV5 N-terminus. Although it could be involved in channel-channel interactions,[17] ankyrin repeat 1 was now demonstrated not to be essential for TRPV5 channel tetramerization. Due to high homology between TRPV5 and TRPV6, ankyrin repeat 3 might be a good candidate. Furthermore, TRPV1 ankyrin repeats are involved in binding of ATP and calmodulin,[12] which is not conserved among other TRPV channels including its close homolog TRPV2.[12,14] For TRPV5, ankyrin repeat 3 has recently been reported to bind calmodulin (region V133-R154).[7,16,31] The N-terminal region preceding the ankyrin repeats is even less conserved among TRPV channels, predicting more divergence between the TRPV family members. In contrast to what we observed for the region M1-M38 in TRPV5, deletion of the first ~100 residues of TRPV1 had only small effects on channel function.[32] Deletion of TRPV1 residues 1-114 abrogated channel function, but as opposed to TRPV5, its cellular localization was not changed. Hence, TRPV5 residues M1-M38, preceding the first ankyrin repeat are required for channel folding, which is not conserved among TRPV channels. Function of the abundant ankyrin repeats and their role in channel oligomerization needs further investigation and probably involves interactions

6

with other structural aspects of the TRPV5 channel and interactions with auxiliary proteins.

Regulation of TRPV5 activity by β-adrenergic signaling

In addition to its many topological features and accompanying interactions, the TRPV5 channel is also heavily regulated by several systemic factors such as hormones, glycosidases and proteases.[6-8] The hormone epinephrine (adrenaline) has major effects in the body and in particular on the cardiovascular system.[33] Cardiovascular effects are mediated by increases in intracellular Ca^{2+} levels, leading to vasoconstriction and increased heart rate.[33] Epinephrine is present in the pro-urine due to filtration from blood, but a major fraction is secreted by the kidney itself.[34] Epinephrine acts through binding to α- or β-adrenergic receptors.[35] β1-(β1-AR) and β2-adrenergic receptors (β2-AR) are expressed in the distal convolution.[36-38] The third β-adrenergic receptor (β3-AR) has not been detected in kidney and is mainly located in adipose tissue.[39,40] The α-adrenergic receptors are expressed in kidney, but mainly in arterioles, proximal tubules and TAL.[41-43] Thus, adrenergic agonists are present in pro-urine and blood, while β-ARs are expressed in the distal convolution. However, the role of adrenergic signaling in transcellular Ca^{2+} reabsorption in the kidney was not known. Therefore in **chapter 3**, the effect of β-AR signaling on TRPV5-mediated Ca^{2+} transport was studied. Expression of the β1-AR protein, but not β2-AR, was detected in the distal convolution. Furthermore, the synthetic β1-AR agonist dobutamine enhanced transcellular Ca^{2+} transport in a murine distal convolution primary culture.

The β-ARs are G-protein-coupled receptors (GPCRs),[44] together with the Ca^{2+}-sensing receptor (CaSR), parathyroid hormone receptor type 1 (PTH1R) and bradykinin receptor subtype 2 (B2R), all reported to enhance the plasma membrane abundance and/or activity of the TRPV5 channel.[18,19,45,46] β1-AR stimulation activates the cAMP/protein kinase A (PKA) pathway, resulting in phosphorylation of several proteins involved in cardiac contraction, such as L-type Ca^{2+} channels, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the ryanodine receptor (RyR),[36,47,48] although adrenergic signals can also be mediated by PKC activation.[49,50] When treating TRPV5-expressing HEK293 cells with dobutamine, cAMP production and TRPV5 activity were increased. No changes in TRPV5 membrane abundance were observed. Hence, β1-AR signaling increases TRPV5 activity, mediated via a cAMP/PKA dependent pathway. As observed for PTH1R activation, this involves the phosphorylation of residue T709 in the C-terminal tail of the TRPV5 channel.[45] In contrast, stimulation of the GPCRs CaSR and B2R (activated by extracellular Ca^{2+} and tissue kallikrein, respectively) activates PKC, which subsequently phosphorylates residues S299/S654. This leads to increased plasma membrane abundance through delayed channel retrieval.[19,46] Activation of PTH1R was shown to induce both PKC- and PKA-dependent pathways, subsequently phosphorylating the residues S299/S654 and T709 respectively.[18,45] The different GPCRs enhance TRPV5 function by increasing plasma membrane abundance through activation of PKC and subsequent phosphorylation of residues S299/S654 and/or by increasing intrinsic channel activity through phosphorylation of residue T709 by PKA. The plasmin activated protease-activated receptor-1 (PAR-1) is a GPCR as well. Here, activation of PAR-1 inhibits TRPV5 function via phosphorylation of residue S144 by PKC.[7] Thus, depending on the specific GPCR and respective G-protein signaling pathways, activation of the respective receptors could have similar or entirely different effects on TRPV5 intrinsic channel activity and membrane abundance (Figure 2).
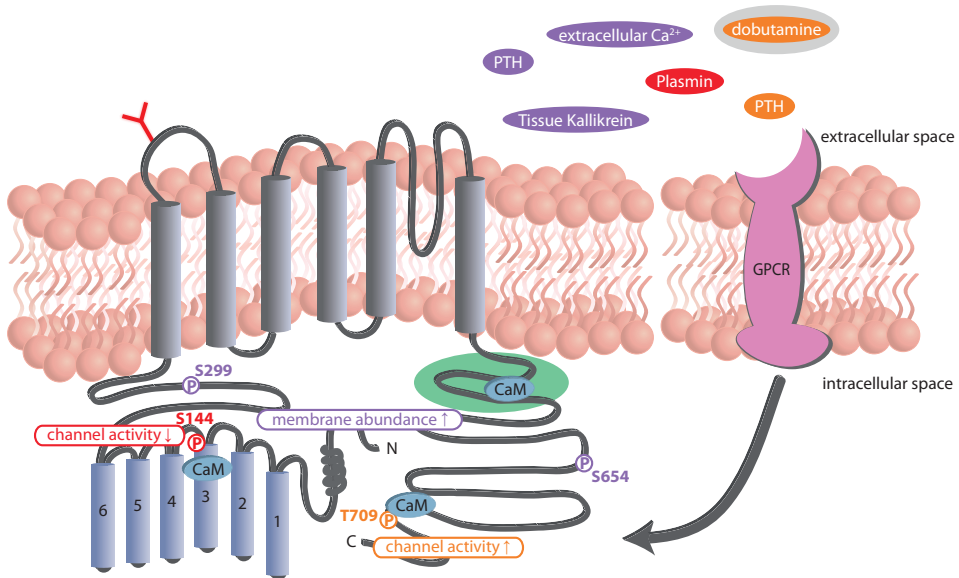
Figure 2 - Regulation of the TRPV5 channel. The TRPV5 C-terminus, important in many regulatory processes, contains the conserved TRP domain (in green) involved in regulation of channel activity and trafficking by binding of several factors, such as PIP_2, S100A10, Rab11a, NHERF4 and calmodulin (CaM). Two calmodulin binding sites have been identified in the C-terminal domain and one within the N-terminus. The TRPV5 channel is regulated by PKA/PKC phosphorylation processes, so far all being initiated via signaling through several G-protein-coupled receptors (GPCRs). Depending on the specific site(s), TRPV5 phosphorylation leads to either increased or decreased intrinsic channel activity or enhanced membrane abundance.

The observed increase of TRPV5 activity upon dobutamine stimulation highly suggests that β-adrenergic signaling enhances transcellular Ca^{2+} reabsorption in the distal convolution through activation of the TRPV5 channel. Activation of β1-AR, however, might also influence activity of other components involved in transcellular Ca^{2+} transport. β-AR signaling has been implicated in the regulation and expression of NCX1 in cardiomyocytes,[47,51-54] where it is reported to increase NCX1 activity via phosphorylation by PKA[47] and/or PKC[50]. However, due to the variability of the effects, the activation of NCX1 by β-AR signaling as well as the process of phosphorylation of NCX1 remains controversial.[50,55] Action of β-AR signaling on renal NCX1 might be dependent on specific environmental factors in different species or tissues and could be indirectly regulated via the participation of NCX1 in macromolecular complexes.[50,55] Further studies are required to determine the role of NCX1 in increases of renal transcellular Ca^{2+} transport as a result of β-AR signaling. In addition to the interplay between TRPV5 and NCX1 with respect to β-AR signaling, the effect of β-AR agonists and possibly antagonists on transcellular Ca^{2+} reabsorption should be studied *in vivo*. The β-AR agonist isoproterenol was demonstrated to enhance Ca^{2+} reabsorption in TAL,[56] together with our results on the distal convolution this suggests significant effects on total renal Ca^{2+} handling in response to the different endogenous and pharmacological β-AR agonists.

The present study identified a novel calciotropic role of dobutamine in transcellular Ca^{2+} transport, proposing an effect of the different endogenously released and clinically applied

6

β1-AR agonists and antagonists on overall body Ca^{2+} balance. In addition to the agonist dobutamine generally used in treatment of acute heart failure, β-AR antagonists (β-blockers) are widely used in the clinic for treatment of congestive heart failure (CHF).[57,58] CHF patients already commonly suffer from increased fecal and urinary Ca^{2+} excretion due to secondary aldosteronism.[59] Thereby, the potential calciotropic effects of β-blockers might enhance the development of hypocalcaemia in these patients. β-blockers have already been used in the treatment of hyperthyroidism to resolve hypercalcaemia,[60,61] and although the mechanism of action is unclear, effects of propranolol (nonselective β-blocker) on plasma Ca^{2+} levels are suggested to be caused by effects on Ca^{2+} handling in bone and/or kidney.[61,62] Therefore, the increasingly used β-AR agonists and antagonists might have diverse and potentially adverse effects on the Ca^{2+} balance due to (dys)regulation of transcellular Ca^{2+} transport in the kidney and should be investigated further.

Coordinated control of renal transcellular Ca^{2+} transport

Regulation of TRPV5 function has mainly been studied in human embryonic kidney (HEK293) cells transiently expressing TRPV5. In order to study the role and regulation of TRPV5 in the process of transcellular Ca^{2+} transport and to study the interplay of TRPV5-mediated apical Ca^{2+} influx with intracellular shuttling and basolateral extrusion mechanisms, a polarized cell model harboring all characteristics of the distal convolution cell is required. Several cell models have been used in the past.[63-66] Unfortunately, all exhibited significant limitations. Cell lines, such as the Madin-Darby canine kidney (MDCK) and murine distal convoluted tubule (mpkDCT), generally display low levels of Ca^{2+} transport indicating poor preservation of Ca^{2+} transport characteristics.[63,65] On the other hand, manual microdissection of tubule segments provides a limited amount of material. Moreover, a model derived from mice would be much more valuable than the existing primary rabbit culture[66] as mouse models are genetically much more defined, allowing detailed mRNA expression studies and the use of the several knockout animals to investigate the different components of Ca^{2+} transport. As demonstrated in **chapter 4** we developed a novel and unique murine primary distal convolution culture, demonstrating transcellular Ca^{2+} transport and preservation of important regulatory pathways. Distal convolution tubule fragments obtained from mice expressing enhanced Green Fluorescent Protein (EGFP) under the control of the TRPV5 promoter,[67] were sorted based on fluorescence intensity using the Complex Object Parametric Analyzer and Sorter (COPAS), subsequently grown on permeable filter supports to maintain cell polarity and to enable transcellular Ca^{2+} transport. Interestingly, the sorting of tubule fragments, as opposed to single cells, proved crucial in the preservation of distal convolution characteristics. Likewise, for pancreatic cells it was demonstrated that sorting of a particular cell cluster size is essential to obtain viable cells, as smaller cell clusters showed many dead cells and reduced viability, while bigger particles suffer from necrosis in its central part.[68] Also, for hepatocytes, extracellular matrix interactions have been demonstrated to be essential to regain cell polarity.[69] Therefore, the favorable effects of isolation of tubule parts are probably due to maintenance of three-dimensional structures including extracellular matrices and cell polarity.

Initially, the novel distal convolution model was exploited to substantiate the role of TRPV5 as gatekeeper in transcellular Ca^{2+} transport, by generating TRPV5^{-/-} primary distal convolution

cultures through crossbreeding of EGFP-expressing mice with TRPV5$^{-/-}$ animals.[9] Subsequently, Na$^+$-dependent basolateral Ca^{2+} extrusion was studied to assess the role of NCX1 in transcellular Ca^{2+} transport. Our data support the current understanding that NCX1 accounts for ~70% of Ca^{2+} efflux and PMCAs for the remaining fraction.[5,70,71] Interestingly, mRNA expression levels of the key components of transcellular Ca^{2+} transport suggest that PMCA4, but not PMCA1, plays a key role in Ca^{2+} transport in the distal convolution. PMCA1 was previously considered to be the primary PMCA involved in this process.[5] The components of renal transcellular Ca^{2+} transport are tightly co-regulated at the transcriptional level. Using the sorted distal convolution material we demonstrated enrichment of TRPV5, calbindin-D$_{28k}$, NCX1 and PMCA4 in this nephron segment, as well as reduced expression levels for all of these components in TRPV5$^{-/-}$ tubules. 1,25-dihydroxy vitamin D$_3$ (1,25-(OH)$_2$D$_3$) is known to enhance the expression of the Ca^{2+} transport proteins identified in the distal convolution including TRPV5, calbindin-D$_{28k}$, NCX1 and PMCA1.[64,72] However, in 1α-OHase$^{-/-}$ mice mRNA expression levels of PMCA1 were unaltered, while the expression levels of the other Ca^{2+} transporters were significantly reduced.[72] This suggests differential regulation of the PMCA1 gene by 1,25-(OH)$_2$D$_3$ as compared to the other key components. Interestingly, PTH has similar effects on the Ca^{2+} transporter expression levels as 1,25-(OH)$_2$D$_3$, but did not affect levels of PMCA1.[73] Other factors regulating protein transcription in the distal convolution are estrogen, testosterone, pH (metabolic acidosis/alkalosis), and dietary Na$^+$ and Ca^{2+} [72,74-77]. For estrogen, an increase in PMCA1 levels was reported as well.[74] Currently, PMCA4 levels under influence of these factors are unknown and require further study in order to determine the role and interplay of PMCA1 and PMCA4.

Thus, the Ca^{2+} transport proteins responsible for renal transcellular Ca^{2+} transport are tightly and coordinately regulated at the transcriptional level. Expression levels could be modulated by direct stimulation of the different promoter regions. Vitamin D-responsive elements (VDREs) have been identified in the calbindin-D$_{28k}$ promoter region and putative VDREs have been demonstrated for regulation of TRPV5.[78-80] To our knowledge for PMCA and NCX1 no VDREs have been identified so far.[81] Absence of specific promoter elements could also mean that transcriptional regulation is mediated via different pathways. In contrast to TRPV6 (responsible for Ca^{2+} influx in the intestine), estrogen response elements were not detected in the promoter region of TRPV5.[80] However, the nuclear estrogen receptor is also known to regulate gene transcription via activator protein 1 (AP-1) binding sites or GC-rich stimulatory protein (Sp1) binding sites, which were identified in the TRPV5 promoter region.[74] In addition to direct regulation of the different components, expression levels of calbindin-D$_{28k}$, PMCAs and NCX1 could also be directly regulated by influx of Ca^{2+} via TRPV5, again pointing to the significance of TRPV5 as key regulator of renal transcellular Ca^{2+} transport. Studies using PTH and TRPV5$^{-/-}$ animals suggest that Ca^{2+} itself controls the expression of other Ca^{2+} transport proteins.[9,72,73,82] Gene expression can be regulated by Ca^{2+} in several ways. For example, in the promoter region of the Ca^{2+}-regulated gene c-*fos*, the cyclic AMP response element (CRE) and serum response element (SRE) were identified as Ca^{2+}-responsive elements.[82] In Purkinje cells, although cell specific, calbindin-D$_{28k}$ was regulated by a Ca^{2+}-sensitive element (PCE1).[83]

By using the novel primary distal convolution cell model we were able to demonstrate the role of TRPV5 as gatekeeper, as well as to support the importance of NCX1 in the process of transcellular Ca^{2+} transport in the distal convolution. Also, we identified PMCA4 as the probable

6

PMCA involved in this process. The role of PMCA4 and possibly PMCA1 in transcellular Ca^{2+} transport requires further study. The novel primary cell model provides an excellent tool to investigate the functional role of the different PMCAs, for example by using different specific inhibitors or most favorably by crossbreeding with different knockout animals as was currently performed for TRPV5. After confirming the role of the individual components in Ca^{2+} transport, the interplay of these key transporters in the handling of Ca^{2+} needs to be elucidated on a functional level.

Basolateral extrusion of Ca^{2+}: regulation of renal NCX1

In addition to apical influx, basolateral extrusion of Ca^{2+} is crucial for transcellular Ca^{2+} transport as well. The importance of NCX1 was confirmed using the primary distal convolution culture, showing reduced levels of Ca^{2+} transport in the absence of extracellular Na^+. Also, NCX1 was enriched ~20 times in distal convolution as compared to total kidney. In comparison, TRPV5 is expressed specifically in the distal convolution and was enriched ~25 times. Therefore, in **chapter 5,** NCX1 was cloned from kidney cDNA and studied for its role and regulation in transcellular Ca^{2+} transport. We successfully cloned three NCX1 splice variants: NCX1.2 (exons B,C,D), NCX1.3 (exons B,D) and NCX1.7 (exons B,D,F). The NCX1 protein consists of ten transmembrane domains and a large cytosolic loop with two Ca^{2+} binding domains (CBD1, CBD2), the latter containing the alternative splice region (Figure 3).[50] Six exons (A-F) are present of which the first (A/B) are mutually exclusive, exon A generally being expressed in excitable tissues and exon B in non-excitable tissues such as the kidney. The other exons can all be spliced in or out, depending on the splice variant, although exon D was found to be present in all identified variants.[50,84] In kidney NCX1.2, NCX1.3 and NCX1.7 variants are expressed.[85-87] NCX1.3 is the main NCX1 isoform in the kidney and is therefore often referred to as "the renal NCX1 variant".[86,88]

Given its high kidney expression and enrichment in distal convolution segments, we focused our studies on the role and regulation of NCX1.3. NCX1.3 activity was studied by the uptake of Ca^{2+} via the reverse-mode function of the exchanger in transiently transfected HEK293 cells.[50,89] In our study, NCX1.3 activity was not regulated by PKA- and PKC-dependent phosphorylation. In agreement, phosphorylation by PKA was reported for the cardiac NCX1 (NCX1.1) but not for NCX1.3.[84,88] In contrast, phosphorylation of NCX1.3 by PKC was demonstrated before.[90,91] Phosphorylation of NCX1 remains controversial, since it appears to be highly dependent on the specific circumstances. For example, cardiac NCX1 was activated by PKA phosphorylation in rat ventricular cardiomyocytes and *Xenopus* oocytes, but did not respond to PKA activation when expressed in HEK293 cells.[51] Therefore, possibly NCX1 is not activated by phosphorylation in the kidney or the HEK293 model cell system lacks certain regulatory pathways or auxiliary proteins involving NCX1 activation. The current understanding is that NCX1 is part of a macromolecular complex, containing kinases and phosphatases, which arrangement differs among tissues and environment. Also, phosphorylation could affect NCX1 function indirectly by phosphorylation of interacting proteins.[50,92] Further research is needed to identify the role of phosphorylation of NCX1 in the distal convolution. This is of particular interest when considering the possible role of β-AR signaling and subsequent PKA/PKC activation on NCX1 function as discussed earlier. When studying NCX1 activity in reverse-mode function as compared to its normal forward-
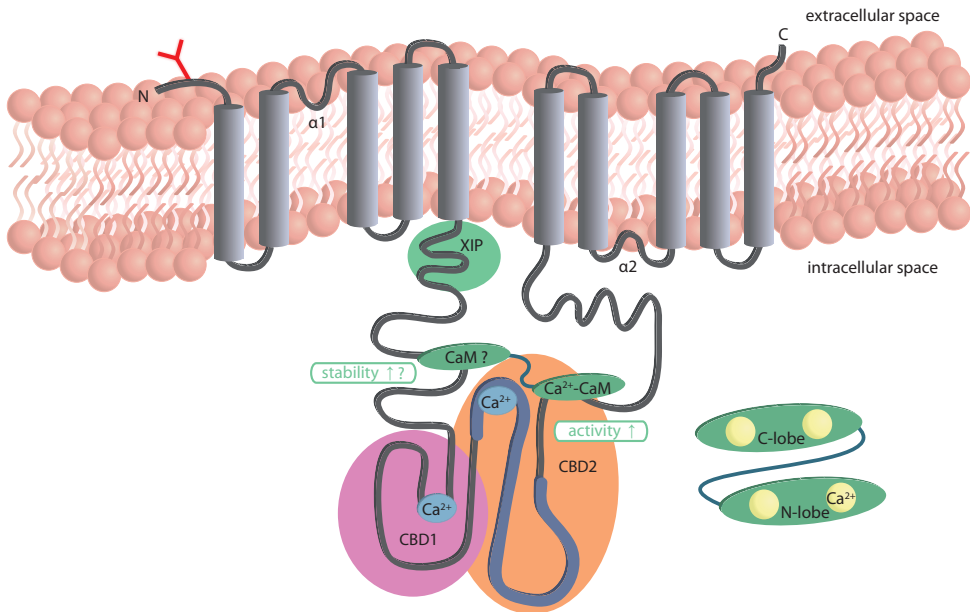
Figure 3 - Topology of NCX1 and the binding of calmodulin. NCX1 contains ten transmembrane domains, extracellular N- and C-termini and a large cytoplasmic domain between TM5-6. The cytoplasmic loop consists of two Ca^{2+} binding domains (CBD1 and CBD2), of which part of CBD2 is an alternative splicing region. A so-called XIP region, previously indicated as a potential calmodulin (CaM) binding site, is located close to TM5 (in green). The present studies suggest that Ca^{2+}-calmodulin binds to a region in proximity to CBD2 a process often mediated by the N-lobe of calmodulin, resulting in increased exchanger activity. Protein stability is increased Ca^{2+}-independently, probably mediated by the C-lobe of Ca^{2+}-free calmodulin

mode in Ca^{2+} extrusion, potential dissimilarities in regulatory mechanism have to be taken into account as differences in the regulation of the reverse- and forward-mode of the exchanger have been observed earlier. For example, all current specific NCX1 inhibitors affect the function of the exchanger in the reverse-mode only.[50,93]

As NCX1.3 is likely part of a large macromolecular complex and as both TRPV5 and PMCAs are regulated by calmodulin, we hypothesized that calmodulin also binds to and regulates NCX1.3. The so-called exchanger inhibitory peptide (XIP) region located within the NCX1 cytosolic loop, was previously identified as a potential calmodulin binding site.[94] The XIP peptide was shown to directly bind calmodulin, but an interaction of calmodulin with this NCX1 region was never demonstrated.[94,95] Interestingly, XIP has later been characterized as an inhibitor of both NCX1 and PMCA function.[94,95] Using the Calmodulin Target Database,[96] four additional potential binding sites were identified, of which two were excluded from further analysis given their significant overlap with transmembrane segments. We demonstrated binding of calmodulin to NCX1.3 in a Ca^{2+}-dependent manner, as significantly less binding was observed in absence of Ca^{2+}. Interestingly, calmodulin binding appeared to stabilize the NCX1.3 protein in a Ca^{2+}-independent manner, whereas NCX1.3 activity was enhanced primarily Ca^{2+}-dependently. The region F683/L696 was confirmed as a potential calmodulin binding site, as calmodulin over-expression was not able to enhance activity of NCX1.3-L696D as was observed for wild-

6

type. Interestingly, although calmodulin no longer affected the activity of this NCX1.3 mutant, calmodulin was still able to bind and stabilize mutant proteins. These results suggest that calmodulin binding to NCX1.3 involves at least two binding sites, one comprising the residues F683/L696. In this regard, the other potential binding site surrounding residues R362/R367 could not be confirmed as a calmodulin binding site.

Regulation of transport activity in a Ca^{2+}-dependent and Ca^{2+}-independent manner was shown previously for the small conductance Ca^{2+}-activated potassium channels (SK) and voltage-gated sodium channels.[97,98] Here, the C-terminal lobe of calmodulin, containing two of the four Ca^{2+} binding EF-hands, is responsible for Ca^{2+}-independent interactions, while binding of the calmodulin N-terminal lobe is required for Ca^{2+}-dependent channel gating. The Ca^{2+}-calmodulin binding domains are mostly found to bind to the canonical 1-8-14 calmodulin binding motif, while Ca^{2+}-independent binding generally is to IQ motifs.[97] Ca^{2+}-independent calmodulin interactions have also been observed for L-type, P/Q-type and R-type voltage-dependent Ca^{2+} channels.[99] For L-type channels similar differential interactions of calmodulin lobes have been observed as well.[100] Hence, Ca^{2+}-independent and dependent interactions of calmodulin with NCX1.3 could be mediated by binding of the calmodulin C- and N-lobes respectively, thereby involving multiple calmodulin binding sites. Of the five predicted calmodulin binding sites within NCX1.3 (including the XIP region),[96] the region R362/R367 is the only to contain a motif resembling the IQ motif implicated in Ca^{2+}-independent binding and should be investigated further.

Surprisingly, the potential calmodulin binding region F683/L696 likely plays a role in a different Ca^{2+}-dependent regulatory mechanism as well, since NCX1.3-F683D demonstrates increased exchanger activity when compared to wild-type, which is much lower at low (0.1 mM) extracellular Ca^{2+} concentrations. Interestingly, this region in NCX1 contains several predicted PKA phosphorylation sites. In rat NCX1.1, phosphorylation of residue T731 (T694 in mouse NCX1.3) has been confirmed *in vitro*.[92] For TRPV5, phosphorylation sites within calmodulin binding domains have been described twice: the region S696-H729 with residue T709 being phosphorylated in response to PTH and β-AR and the region V133-R154 where residue S144 is phosphorylated in response to PAR-1 activation by plasmin.[7,10] Since phosphorylation processes in NCX1 are still controversial, the role of phosphorylation on NCX1.3 function in the distal convolution has to be investigated first.

Conclusions and future perspectives

The aim of this thesis was to further characterize the regulation of transcellular Ca^{2+} transport in the distal convolution. The presented studies illustrate the coordinated regulation of the molecular components of renal transcellular Ca^{2+} transport, as well as the role and regulation of the individual elements, mainly focusing on the "gatekeeper" TRPV5 and the main extruder NCX1 (Figure 4). Deeper insight was obtained in the control of Ca^{2+} influx through TRPV5, by clarifying the role of its N-terminal region in channel folding. In addition, we demonstrated regulation of TRPV5 channel activity by the β1-AR agonist dobutamine, previously unknown as a regulator of renal transcellular Ca^{2+} transport. By modulating renal Ca^{2+} reabsorption, pharmacological β-AR agonists and antagonists (β-blockers) could greatly influence overall

Ca^{2+} homeostasis. The effect of these compounds on renal Ca^{2+} handling and total body Ca^{2+} balance needs to be studied *in vivo*. These studies would predict whether possible implications for treatment of patients with β-AR agonists and β-blockers should be investigated.

Furthermore, regarding the characterization of basolateral Ca^{2+} extrusion, we cloned and characterized murine renal NCX1 (NCX1.3), and identified an important interacting partner: the Ca^{2+}-sensor calmodulin, which regulates NCX1.3 protein stability and exchanger activity. In order to understand functioning of NCX1 in relation to transcellular Ca^{2+} transport, it is important to identify all key regulators of NCX1.3 activity. As a result, it is essential to determine the role of NCX1.3 phosphorylation, a process remaining controversial for NCX for a long time already. In addition, to understand functioning of NCX1 in renal transcellular Ca^{2+} transport, the role of the two other NCX variants, NCX1.2 and NCX1.7, in the distal convolution requires further investigations.
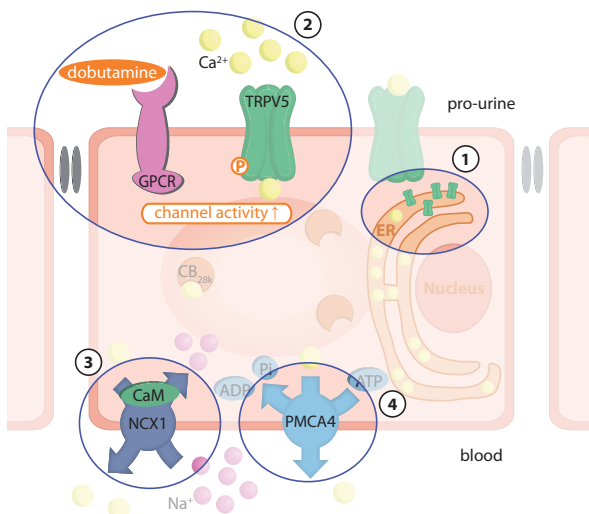
Figure 4 - Overview of the key findings in renal transcellular Ca^{2+} transport. Firstly, the TRPV5 N-terminal region (M1-M38) is implicated in channel folding, as absence of this segment results in endoplasmic reticulum (ER) retention. Secondly, TRPV5 channel activity is enhanced by the β1-AR (a GPCR) agonist dobutamine, a process mediated by channel phosphorylation. Thirdly, calmodulin (CaM) was demonstrated to bind NCX1 and to enhance exchanger activity and protein stability. Fourthly, based on transcriptional regulation PMCA4 and not PMCA1 was indicated as the key PMCA involved in transcellular Ca^{2+} transport.

6

In addition to studies on the regulation of the individual components TRPV5, NCX1 and possibly in the future PMCAs, to fully understand the process of transcellular Ca^{2+} transport it is essential to characterize the interplay of the different elements. Therefore, a highly innovative distal convolution cell model was developed. We confirmed the importance of TRPV5 in renal transcellular Ca^{2+} transport, as well as substantiated the role of NCX1 in this process. In addition, using the fluorescently sorted distal convolution material, we identified PMCA4 as an important and possibly the main PMCA involved in renal transcellular Ca^{2+} transport. The role and regulation of the different PMCAs in this process require further investigation. Additionally, the role, regulation and interplay of NCX1 and the PMCAs in basolateral extrusion of Ca^{2+} remain largely unknown. In this matter, the newly developed primary murine distal convolution cell model will enable several opportunities to study the regulation and interaction of the different components of renal transcellular Ca^{2+} transport, allowing study of the interplay of influx, shuttling and extrusion of Ca^{2+}. Knowledge on regulation of the individual elements as well

as their interplay will enable the construction of a comprehensive model of renal transcellular Ca^{2+} transport. This will allow detailed computational studies and valuable predictions on total renal Ca^{2+} handling. New insights in the regulation of renal Ca^{2+} reabsorption will help to understand and solve disorders that are characterized by disturbances in the Ca^{2+} balance, such as idiopathic hypercalciuria. This is a condition displaying increased urinary Ca^{2+} excretion due to so far unknown causes, often leading to the development of kidney stones.[101]

References

1. Hoenderop JG, Bindels RJ: Epithelial Ca^{2+} and Mg^{2+} channels in health and disease. *J Am Soc Nephrol* 16: 15-26, 2005

2. Berridge MJ, Lipp P, Bootman MD: The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11-21, 2000

3. Bushinsky DA: Contribution of intestine, bone, kidney, and dialysis to extracellular fluid calcium content. *Clin J Am Soc Nephrol* 5 Suppl 1: S12-22, 2010

4. Dimke H, Hoenderop JG, Bindels RJ: Hereditary tubular transport disorders: implications for renal handling of Ca^{2+} and Mg^{2+}. *Clin Sci (Lond)* 118: 1-18, 2010

5. Hoenderop JG, Nilius B, Bindels RJ: Calcium absorption across epithelia. *Physiol Rev* 85: 373-422, 2005

6. de Groot T, Bindels RJ, Hoenderop JG: TRPV5: an ingeniously controlled calcium channel. *Kidney Int* 74: 1241-1246, 2008

7. Tudpor K, Lainez S, Kwakernaak AJ, Kovalevskaya NV, Verkaart S, van Genesen S, van der Kemp A, Navis G, Bindels RJ, Hoenderop JG: Urinary plasmin inhibits TRPV5 in nephrotic-range proteinuria. *J Am Soc Nephrol* 23: 1824-1834, 2012

8. Leunissen EH, Nair AV, Bull C, Lefeber DJ, van Delft FL, Bindels RJ, Hoenderop JG: The epithelial calcium channel TRPV5 is regulated differentially by klotho and sialidase. *J Biol Chem* 288: 29238-29246, 2014

9. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ: Renal Ca^{2+} wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest* 112: 1906-1914, 2003

10. de Groot T, Kovalevskaya NV, Verkaart S, Schilderink N, Felici M, van der Hagen EA, Bindels RJ, Vuister GW, Hoenderop JG: Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. *Mol Cell Biol* 31: 2845-2853, 2011

11. Yeh BI, Kim YK, Jabbar W, Huang CL: Conformational changes of pore helix coupled to gating of TRPV5 by protons. *EMBO J* 24: 3224-3234, 2005

12. Lishko PV, Procko E, Jin X, Phelps CB, Gaudet R: The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* 54: 905-918, 2007

13. Gaudet R: A primer on ankyrin repeat function in TRP channels and beyond. *Mol Biosyst* 4: 372-379, 2008

14. Gaudet R: TRP channels entering the structural era. *J Physiol* 586: 3565-3575, 2008

15. Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY: The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* 13: 1435-1448, 2004

16. Lambers TT, Weidema AF, Nilius B, Hoenderop JG, Bindels RJ: Regulation of the mouse epithelial Ca^{2+} channel TRPV6 by the Ca^{2+}-sensor calmodulin. *J Biol Chem* 279: 28855-28861, 2004

17. Chang Q, Gyftogianni E, van de Graaf SF, Hoefs S, Weidema FA, Bindels RJ, Hoenderop JG: Molecular determinants in TRPV5 channel assembly. *J Biol Chem* 279: 54304-54311, 2004

18. Cha SK, Wu T, Huang CL: Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294: F1212-1221, 2008

19. Topala CN, Schoeber JP, Searchfield LE, Riccardi D, Hoenderop JG, Bindels RJ: Activation of the Ca^{2+}-sensing receptor stimulates the activity of the epithelial Ca^{2+} channel TRPV5. *Cell Calcium* 45: 331-339, 2009

20. Ellgaard L, Helenius A: Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* 4: 181-191, 2003

21. Hurtley SM, Helenius A: Protein oligomerization in the endoplasmic reticulum. *Annu Rev Cell Biol* 5: 277-307, 1989

22. Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG: The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 310: 490-493, 2005

23. Wang X, Koulov AV, Kellner WA, Riordan JR, Balch WE: Chemical and biological folding contribute to temperature-sensitive DeltaF508 CFTR trafficking. *Traffic* 9: 1878-1893, 2008

24. Schulteis CT, Nagaya N, Papazian DM: Subunit folding and assembly steps are interspersed during Shaker potassium channel biogenesis. *J Biol Chem* 273: 26210-26217, 1998

25. 25 Green WN, Millar NS: Ion-channel assembly. *Trends Neurosci* 18: 280-287, 1995

26. Arniges M, Fernandez-Fernandez JM, Albrecht N, Schaefer M, Valverde MA: Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *J Biol Chem* 281: 1580-1586, 2006

27. Phelps CB, Huang RJ, Lishko PV, Wang RR, Gaudet R: Structural analyses of the ankyrin repeat domain of TRPV6 and related TRPV ion channels. *Biochemistry* 47: 2476-2484, 2008

28. Hellwig N, Albrecht N, Harteneck C, Schultz G, Schaefer M: Homo- and heteromeric assembly of TRPV channel subunits. *J Cell Sci* 118: 917-928, 2005

29. Liao M, Cao E, Julius D, Cheng Y: Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504: 107-112, 2013

30. Erler I, Hirnet D, Wissenbach U, Flockerzi V, Niemeyer BA: Ca^{2+}-selective transient receptor potential V channel architecture and function require a specific ankyrin repeat. *J Biol Chem* 279: 34456-34463, 2004

31. Kovalevskaya NV, Bokhovchuk FM, Vuister GW: The TRPV5/6 calcium channels contain multiple calmodulin binding sites with differential binding properties. *J Struct Funct Genomics* 13: 91-100, 2012

32. Jung J, Lee SY, Hwang SW, Cho H, Shin J, Kang YS, Kim S, Oh U: Agonist recognition sites in the cytosolic tails of vanilloid receptor 1. *J Biol Chem* 277: 44448-44454, 2002

33. Overgaard CB, Dzavik V: Inotropes and vasopressors: review of physiology and clinical use in cardiovascular disease. *Circulation* 118: 1047-1056, 2008

34. Ziegler MG, Aung M, Kennedy B: Sources of human urinary epinephrine. *Kidney Int* 51: 324-327, 1997

35. Bylund DB: Alpha- and beta-adrenergic receptors: Ahlquist's landmark hypothesis of a single mediator with two receptors. *Am J Physiol Endocrinol Metab* 293: E1479-1481, 2007

36. Kang HS, Kerstan D, Dai LJ, Ritchie G, Quamme GA: beta-Adrenergic agonists stimulate Mg^{2+} uptake in mouse distal convoluted tubule cells. *Am J Physiol Renal Physiol* 279: F1116-1123, 2000

37. Boivin V, Jahns R, Gambaryan S, Ness W, Boege F, Lohse MJ: Immunofluorescent imaging of beta 1- and beta 2-adrenergic receptors in rat kidney. *Kidney Int* 59: 515-531, 2001

38. Gesek FA, White KE: Molecular and functional identification of beta-adrenergic receptors in distal convoluted tubule cells. *Am J Physiol* 272: F712-720, 1997

39. Sawa M, Harada H: Recent developments in the design of orally bioavailable beta3-adrenergic receptor agonists. *Curr Med Chem* 13: 25-37, 2006

40. Evans BA, Papaioannou M, Anastasopoulos F, Summers RJ: Differential regulation of beta3-adrenoceptors in gut and adipose tissue of genetically obese (ob/ob) C57BL/6J-mice. *Br J Pharmacol* 124: 763-771, 1998

41. Canessa LM, Piccio MM, Vachvanichsanong P, Sidhu A, Porter CC, Robillard JE, Felder RA, Jose PA: Alpha 1B-adrenergic receptors in rat renal microvessels. *Kidney Int* 48: 1412-1419, 1995

42. Sundaresan PR, Fortin TL, Kelvie SL: Alpha- and beta-adrenergic receptors in proximal tubules of rat kidney. *Am J Physiol* 253: F848-856, 1987

43. Meister B, Dagerlind A, Nicholas AP, Hokfelt T: Patterns of messenger RNA expression for adrenergic receptor subtypes in the rat kidney. *J Pharmacol Exp Ther* 268: 1605-1611, 1994

44. Dror RO, Arlow DH, Maragakis P, Mildorf TJ, Pan AC, Xu H, Borhani DW, Shaw DE: Activation mechanism of the beta2-adrenergic receptor. *Proc Natl Acad Sci U S A* 108: 18684-18689, 2011

45. de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG: Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J Am Soc Nephrol* 20: 1693-1704, 2009

46. Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ: Tissue kallikrein stimulates Ca^{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *EMBO J* 25: 4707-4716, 2006

47. Wei SK, Ruknudin A, Hanlon SU, McCurley JM, Schulze DH, Haigney MC: Protein kinase A hyperphosphorylation increases basal current but decreases beta-adrenergic responsiveness of the sarcolemmal Na^+-Ca^{2+} exchanger in failing pig myocytes. *Circ Res* 92: 897-903, 2003

48. Weiss S, Oz S, Benmocha A, Dascal N: Regulation of cardiac L-type Ca^{2+} channel CaV1.2 via the beta-adrenergic-cAMP-protein kinase A pathway: old dogmas, advances, and new uncertainties. *Circ Res* 113: 617-631, 2013

49. Wang S, Xu D, Wu TT, Guo Y, Chen YH, Zou JG: beta1adrenergic regulation of rapid component of delayed rectifier K^+ currents in guineapig cardiac myocytes. *Mol Med Rep* 9: 1923-1928, 2014

50. Lytton J: Na^+/Ca^{2+} exchangers: three mammalian gene families control Ca^{2+} transport. *Biochem J* 406: 365-382, 2007

51. Ruknudin AM, Wei SK, Haigney MC, Lederer WJ, Schulze DH: Phosphorylation and other conundrums of Na/Ca exchanger, NCX1. *Ann N Y Acad Sci* 1099: 103-118, 2007

52. Mani SK, Egan EA, Addy BK, Grimm M, Kasiganesan H, Thiyagarajan T, Renaud L, Brown JH, Kern CB, Menick DR: beta-Adrenergic receptor stimulated Ncx1 upregulation is mediated via a CaMKII/AP-1 signaling pathway in adult cardiomyocytes. *J Mol Cell Cardiol* 48: 342-351, 2010

53. Fan J, Shuba YM, Morad M: Regulation of cardiac sodium-calcium exchanger by beta-adrenergic agonists. *Proc Natl Acad Sci U S A* 93: 5527-5532, 1996

54. Golden KL, Fan QI, Chen B, Ren J, O'Connor J, Marsh JD: Adrenergic stimulation regulates Na^+/Ca^{2+} Exchanger expression in rat cardiac myocytes. *J Mol Cell Cardiol* 32: 611-620, 2000

55. Morad M, Cleemann L, Menick DR: NCX1 phosphorylation dilemma: a little closer to resolution. Focus on "Full-length cardiac Na^+/Ca^{2+} exchanger 1 protein is not phosphorylated by protein kinase A". *Am J Physiol Cell Physiol* 300: C970-973, 2011

56. Bailly C, Imbert-Teboul M, Roinel N, Amiel C: Isoproterenol increases Ca, Mg, and NaCl

reabsorption in mouse thick ascending limb. *Am J Physiol* 258: F1224-1231, 1990

57. Goldhaber JI, Hamilton MA: Role of inotropic agents in the treatment of heart failure. *Circulation* 121: 1655-1660, 2010

58. Lowes BD, Simon MA, Tsvetkova TO, Bristow MR: Inotropes in the beta-blocker era. *Clin Cardiol* 23: III11-16, 2000

59. Alsafwah S, Laguardia SP, Arroyo M, Dockery BK, Bhattacharya SK, Ahokas RA, Newman KP: Congestive heart failure is a systemic illness: a role for minerals and micronutrients. *Clin Med Res* 5: 238-243, 2007

60. Hayes JR, Ritchie CM: Hypercalcaemia due to thyrotoxicosis. *Ir J Med Sci* 152: 422-423, 1983

61. Geffner DL, Hershman JM: Beta-adrenergic blockade for the treatment of hyperthyroidism. *Am J Med* 93: 61-68, 1992

62. Bonnet N, Pierroz DD, Ferrari SL: Adrenergic control of bone remodeling and its implications for the treatment of osteoporosis. *J Musculoskelet Neuronal Interact* 8: 94-104, 2008

63. Arthur JM: The MDCK cell line is made up of populations of cells with diverse resistive and transport properties. *Tissue Cell* 32: 446-450, 2000

64. Kip SN, Strehler EE: Vitamin D_3 upregulates plasma membrane Ca^{2+}-ATPase expression and potentiates apico-basal Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol* 286: F363-369, 2004

65. Diepens RJ, den Dekker E, Bens M, Weidema AF, Vandewalle A, Bindels RJ, Hoenderop JG: Characterization of a murine renal distal convoluted tubule cell line for the study of transcellular calcium transport. *Am J Physiol Renal Physiol* 286: F483-489, 2003

66. Bindels RJ, Hartog A, Timmermans J, Van Os CH: Active Ca^{2+} transport in primary cultures of rabbit kidney CCD: stimulation by 1,25-dihydroxyvitamin D_3 and PTH. *Am J Physiol* 261: F799-807, 1991

67. Hofmeister MV, Fenton RA, Praetorius J: Fluorescence isolation of mouse late distal convoluted tubules and connecting tubules: effects of vasopressin and vitamin D_3 on Ca^{2+} signaling. *Am J Physiol Renal Physiol* 296: F194-203, 2009

68. Kikugawa R, Katsuta H, Akashi T, Yatoh S, Weir GC, Sharma A, Bonner-Weir S: Differentiation of COPAS-sorted non-endocrine pancreatic cells into insulin-positive cells in the mouse. *Diabetologia* 52: 645-652, 2009

69. Musat AI, Sattler CA, Sattler GL, Pitot HC: Reestablishment of cell polarity of rat hepatocytes in primary culture. *Hepatology* 18: 198-205, 1993

70. Brini M, Carafoli E: The plasma membrane Ca^{2+} ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. *Cold Spring Harb Perspect Biol* 3, 2011

71. Kip SN, Strehler EE: Characterization of PMCA isoforms and their contribution to transcellular Ca^{2+} flux in MDCK cells. *Am J Physiol Renal Physiol*

284: F122-132, 2003

72. Hoenderop JG, Dardenne O, van Abel M, van der Kemp AW, van Os CH, St -Arnaud R, Bindels RJ: Modulation of renal Ca^{2+} transport protein genes by dietary Ca^{2+} and 1,25-dihydroxyvitamin D_3 in 25-hydroxyvitamin D_3-1alpha-hydroxylase knockout mice. *FASEB J* 16: 1398-1406, 2002

73. van Abel M, Hoenderop JG, van der Kemp AW, Friedlaender MM, van Leeuwen JP, Bindels RJ: Coordinated control of renal Ca^{2+} transport proteins by parathyroid hormone. *Kidney Int* 68: 1708-1721, 2005

74. van Abel M, Hoenderop JG, Dardenne O, St Arnaud R, van Os CH, van Leeuwen HJ, Bindels RJ: 1,25-dihydroxyvitamin D(3)-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. *J Am Soc Nephrol* 13: 2102-2109, 2002

75. Hsu YJ, Dimke H, Schoeber JP, Hsu SC, Lin SH, Chu P, Hoenderop JG, Bindels RJ: Testosterone increases urinary calcium excretion and inhibits expression of renal calcium transport proteins. *Kidney Int* 77: 601-608, 2010

76. Nijenhuis T, Renkema KY, Hoenderop JG, Bindels RJ: Acid-base status determines the renal expression of Ca^{2+} and Mg^{2+} transport proteins. *J Am Soc Nephrol* 17: 617-626, 2006

77. Yatabe MS, Yatabe J, Takano K, Murakami Y, Sakuta R, Abe S, Sanada H, Kimura J, Watanabe T: Effects of a high-sodium diet on renal tubule Ca^{2+} transporter and claudin expression in Wistar-Kyoto rats. *BMC Nephrol* 13: 160, 2012

78. Hoenderop JG, Muller D, van der Kemp AW, Hartog A, Suzuki M, Ishibashi K, Imai M, Sweep F, Willems PH, van Os CH, Bindels RJ: Calcitriol controls the epithelial calcium channel in kidney. *J Am Soc Nephrol* 12: 1342-1349, 2001

79. Gill RK, Christakos S: Identification of sequence elements in mouse calbindin-D_{28k} gene that confer 1,25-dihydroxyvitamin D_3- and butyrate-inducible responses. *Proc Natl Acad Sci U S A* 90: 2984-2988, 1993

80. Weber K, Erben RG, Rump A, Adamski J: Gene structure and regulation of the murine epithelial calcium channels ECaC1 and 2. *Biochem Biophys Res Commun* 289: 1287-1294, 2001

81. Glendenning P, Ratajczak T, Prince RL, Garamszegi N, Strehler EE: The promoter region of the human PMCA1 gene mediates transcriptional downregulation by 1,25-dihydroxyvitamin D_3. *Biochem Biophys Res Commun* 277: 722-728, 2000

82. Johnson CM, Hill CS, Chawla S, Treisman R, Bading H: Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. *J Neurosci* 17: 6189-6202, 1997

83. Arnold DB, Heintz N: A calcium responsive element that regulates expression of two calcium binding proteins in Purkinje cells. *Proc Natl Acad Sci U S A* 94: 8842-8847, 1997

84. Schulze DH, Polumuri SK, Gille T, Ruknudin A: Functional regulation of alternatively spliced Na^+/Ca^{2+} exchanger (NCX1) isoforms. *Ann N Y*

Acad Sci 976: 187-196, 2002

85. Kim I, Koh GY, Lee CO: Identification of alternatively spliced Na⁺-Ca²⁺ exchanger isoforms expressed in the heart. *Comp Biochem Physiol B Biochem Mol Biol* 119: 157-161, 1998

86. Kofuji P, Lederer WJ, Schulze DH: Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na/Ca exchanger. *J Biol Chem* 269: 5145-5149, 1994

87. Lee SL, Yu AS, Lytton J: Tissue-specific expression of Na⁺-Ca²⁺ exchanger isoforms. *J Biol Chem* 269: 14849-14852, 1994

88. Ruknudin A, He S, Lederer WJ, Schulze DH: Functional differences between cardiac and renal isoforms of the rat Na⁺-Ca²⁺ exchanger NCX1 expressed in Xenopus oocytes. *J Physiol* 529 Pt 3: 599-610, 2000

89. Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M: Protein kinase C-dependent regulation of Na⁺/Ca²⁺ exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation. *Biochemistry* 37: 17230-17238, 1998

90. Unlap MT, Peti-Peterdi J, Bell PD: Cloning of mesangial cell Na⁺/Ca²⁺ exchangers from Dahl/Rapp salt-sensitive/resistant rats. *Am J Physiol Renal Physiol* 279: F177-184, 2000

91. Hwang EF, Williams I, Kovacs G, Peti-Peterdi J, Siroky B, Rice WC, Bates E, Schwiebert EM, Unlap MT, Bell PD: Impaired ability of the Na⁺/Ca²⁺ exchanger from the Dahl/Rapp salt-sensitive rat to regulate cytosolic calcium. *Am J Physiol Renal Physiol* 284: F1023-1031, 2003

92. Wanichawan P, Louch WE, Hortemo KH, Austbo B, Lunde PK, Scott JD, Sejersted OM, Carlson CR: Full-length cardiac Na⁺/Ca²⁺ exchanger 1 protein is not phosphorylated by protein kinase A. *Am J Physiol Cell Physiol* 300: C989-997, 2011

93. Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T: The exchanger inhibitory peptide region-dependent inhibition of Na⁺/Ca²⁺ exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. *Mol Pharmacol* 66: 45-55, 2004

94. Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, Philipson KD: Identification of a peptide inhibitor of the cardiac sarcolemmal Na⁺-Ca²⁺ exchanger. *J Biol Chem* 266: 1014-1020, 1991

95. Enyedi A, Penniston JT: Autoinhibitory domains of various Ca²⁺ transporters cross-react. *J Biol Chem* 268: 17120-17125, 1993

96. Ikura: Calmodulin Target Database, (2013)

97. Keen JE, Khawaled R, Farrens DL, Neelands T, Rivard A, Bond CT, Janowsky A, Fakler B, Adelman JP, Maylie J: Domains responsible for constitutive and Ca²⁺-dependent interactions between calmodulin and small conductance Ca²⁺-activated potassium channels. *J Neurosci* 19: 8830-8838, 1999

98. Kovalevskaya NV, van de Waterbeemd M, Bokhovchuk FM, Bate N, Bindels RJ, Hoenderop JG, Vuister GW: Structural analysis of calmodulin binding to ion channels demonstrates the role of its plasticity in regulation. *Pflugers Arch* 465: 1507-1519, 2013

99. Erickson MG, Alseikhan BA, Peterson BZ, Yue DT: Preassociation of calmodulin with voltage-gated Ca²⁺ channels revealed by FRET in single living cells. *Neuron* 31: 973-985, 2001

100. Peterson BZ, DeMaria CD, Adelman JP, Yue DT: Calmodulin is the Ca²⁺ sensor for Ca²⁺-dependent inactivation of L-type calcium channels. *Neuron* 22: 549-558, 1999

101. van Abel M, Hoenderop JG, Bindels RJ: The epithelial calcium channels TRPV5 and TRPV6: regulation and implications for disease. *Naunyn Schmiedebergs Arch Pharmacol* 371: 295-306, 2005

6

Summary
Samenvatting

Summary

Chapter 1: Introduction

Calcium (Ca^{2+}) plays a key role in many of our body's physiological processes, such as bone formation, muscle contraction and blood coagulation. As a result, it is essential that Ca^{2+} levels are tightly regulated, both in blood and within cells. The overall Ca^{2+} balance is regulated by obtaining Ca^{2+} from food in the intestine, storage of Ca^{2+} in bone and by controlling Ca^{2+} excretion via the urine in the kidneys. Each kidney contains ~1 million functional elements, the nephrons. Each nephron in turn is composed of a glomerulus where the blood is filtered, followed by a long tubule where the filtered pro-urine is concentrated and important nutrients are reabsorbed back into the blood. To prevent loss of Ca^{2+} via the urine, ~99% of all filtered Ca^{2+} is reabsorbed along the nephron. The majority is taken up via the paracellular route, where Ca^{2+} is transported to the blood via so-called tight junctions. The final Ca^{2+} excretion via urine, however, is controlled by transcellular Ca^{2+} transport. This transcellular Ca^{2+} transport takes place in the distal part of the kidney tubule, the distal convolution. Here, tubular cells absorb Ca^{2+} from the pro-urine to extrude it again on the opposite side of the cell, towards the blood. Ca^{2+} entry into the cell is controlled by the epithelial Ca^{2+} channel transient receptor potential vanilloid 5 (TRPV5), after which the Ca^{2+}-buffer calbindin-D_{28k} guides it to the other end of the cell. There Ca^{2+} is transported to the blood by the Na^+/Ca^{2+} exchanger, NCX1, and by the plasma membrane Ca^{2+}-ATPase, PMCA.

To preserve the Ca^{2+} balance within our body, transcellular Ca^{2+} transport in the kidney is tightly regulated. Many different factors are involved, among which the calciotropic hormones such as vitamin D_3 and parathyroid hormone (PTH). These hormones stimulate Ca^{2+} reabsorption in the distal convolution, by increasing mRNA expression levels of TRPV5, calbindin-D_{28k} and NCX1, resulting in a higher production of the corresponding proteins. In addition to the coordinated regulation of the mRNA and consequently protein expression levels, these transport proteins are also individually controlled by several local factors. These may for example specifically influence the intrinsic transport activity of TRPV5, NCX1 and PMCA, but could also affect their transport route within the cell, resulting in altered protein levels on the plasma membrane.

Regulation of TRPV5 channel activity on the plasma membrane has been extensively investigated. TRPV5 acts as a gatekeeper by regulating the uptake of Ca^{2+} into the cell and is therefore considered as the most critical element in transcellular Ca^{2+} transport. The key role of TRPV5 in this process was revealed by studying mice lacking functional TRPV5 protein: TRPV5$^{-/-}$ mice. These animals reabsorb less Ca^{2+} in the distal convolution and therefore excrete more Ca^{2+} via their urine. The TRPV5 protein is a selective Ca^{2+} channel, comprising six transmembrane domains and large intracellular amino (N)- and carboxy (C)-termini. Four TRPV5 proteins, a tetramer, are required to form a functional Ca^{2+} channel. TRPV5 channel activity is controlled by many factors, among which are the intracellular and extracellular Ca^{2+} concentration, pH, hormones such as PTH, as well as many other factors present in the pro-urine. The C-terminal domain of TRPV5, and especially the conserved TRP box domain, has an important role in these regulatory processes, as it binds many different proteins, such as the Ca^{2+}-sensor calmodulin. Moreover, specific amino acids in the TRPV5 C-terminal domain can be phosphorylated, thereby raising the number of channels on the plasma membrane or

7

increasing the intrinsic channel activity. Both processes result in increased Ca^{2+} reabsorption from the pro-urine. After being taken up by TRPV5, Ca^{2+} is excreted on the other side of the cell by the interplay of NCX1 and PMCA. In contrast to the passive uptake of Ca^{2+} by TRPV5, being driven by the Ca^{2+}-gradient over de plasma membrane, the transport of Ca^{2+} out of the cell is very energy consuming. The naturally occurring Na^+-gradient that is important for NCX1 function, is created by Na^+-K^+ ATPases, while the PMCAs are directly driven by ATP hydrolysis. Due to its high transport capacity, NCX1 is responsible for ~70% of Ca^{2+} excretion. PMCAs have a lower capacity, but do have a higher affinity for Ca^{2+}, making them important for fine-tuning total Ca^{2+} export. In contrast to the TRPV5 dependent Ca^{2+} uptake, there is still only limited knowledge on the role and regulation of NCX1 and PMCA in renal Ca^{2+} transport.

In this thesis the regulation of transcellular Ca^{2+} transport in the kidney is further investigated. Firstly, the regulation of the TRPV5 channel by i) the largely unknown TRPV5 N-terminal domain and ii) the effects of stress hormones via β-adrenergic receptor signaling, is studied. Secondly, by using an innovative approach, a novel primary distal convolution cell model was developed for detailed studies on the interplay of the different factors involved in transcellular Ca^{2+} transport. And finally, the regulation of the kidney-specific variant of the NCX1 protein was investigated.

Chapter 2: Role of the TRPV5 protein N-terminus in channel activity, tetramerization and trafficking

Unlike the TRPV5 C-terminus, the role of the N-terminal tail in the regulation of Ca^{2+} channel activity is still largely unknown. Like the other members of the TRPV family, the TRPV5 N-terminus contains six ankyrin domains. These are repeating domains consisting of conserved helix-loop-helix structures of ~33 amino acids, which can bind different factors, as for example ATP. The first ankyrin domain is implicated in mutual interactions between different TRPV5 N-termini. Furthermore, it has been shown that calmodulin is able to bind the TRPV5 N-terminus. And finally, the N-terminus is phosphorylated by protein kinase C (PKC) on amino acids S144 and S299. To investigate the role of the N-terminus in TRPV5 function, different mutants were generated that lacked part of the N-terminus. When the first 38 amino acids are absent, more specifically when amino acids Q31/D34 are mutated, the TRPV5 channel lost its activity, remained unglycosylated and was localized primarily in the endoplasmic reticulum. Most likely this indicates that the protein is misfolded and therefore retained in the endoplasmic reticulum by the quality control mechanism, thereby blocking further transport to the Golgi apparatus. Moreover, the small amount of TRPV5 channels that escaped quality control and did reach the plasma membrane, could not be internalized. Interestingly, despite problems with protein folding, the TRPV5 channels were still able to form tetramers, even when the first 75 amino acids and therefore the whole first ankyrin domain were missing. The critical amino acids Q31/D34 in the first part of the N-terminus are located in a possible helix structure, which appears to be essential for protein folding and thus functioning of the TRPV5 Ca^{2+} channel. Further investigations are required to clarify the role of the first ankyrin domain in mutual TRPV5 channel interactions.

Chapter 3: β1-Adrenergic receptor signaling activates the epithelial Ca^{2+} channel TRPV5, via the protein kinase A pathway

The stress hormones epinephrine and norepinephrine have different functions in the body, but mainly exert their effects on the cardiovascular system. They operate by binding to both α-adrenergic and β-adrenergic receptors (β-AR). Both hormones are not only present in the blood, but also in pro-urine. Additionally, β1-AR and β2-AR are located in the distal convolution, where TRPV5-mediated Ca^{2+} transport occurs. However, a possible effect of β-AR signaling on renal transcellular Ca^{2+} transport has never been described. The presence of β1-AR in the distal convolution was confirmed using immunohistochemistry, while β2-AR could not be detected. When primary distal convolution cultures were treated with the β1-AR agonist dobutamine, Ca^{2+} transport was clearly increased. β-AR receptors are G-protein coupled receptors, or GPCRs. Activation of other GPCRs such as the PTH receptor 1, the Ca^{2+} sensing receptor and the bradykinin receptor type 2, results in enhanced activity of the TRPV5 channel and/or an increased number of channels on the plasma membrane. Dobutamine-mediated activation of the β1-AR receptor in HEK293 cells transiently expressing TRPV5, resulted in increased TRPV5 activity, while there was no increase in the presence of the channel on the plasma membrane. This effect was caused by phosphorylation of amino acid T709 by protein kinase A (PKA), in similarity to the response to PTH receptor activation. Endogenous and synthetic β1-AR agonists, and possibly antagonists such as the commonly used β-blockers, are likely to have an important role in maintaining the total body Ca^{2+} balance through their potential effect on total Ca^{2+} reabsorption in the kidney.

Chapter 4: Coordinated regulation of TRPV5-mediated Ca^{2+} transport in primary distal convolution cultures

Studies into the molecular mechanism of transcellular Ca^{2+} transport in the distal convolution and especially the interplay of the different transport proteins, are seriously hindered by a suitable cell model. Several models have been developed in the past, but all harbor considerable limitations. Using a very innovative technique a novel primary cell model was developed to investigate the function of the distal convolution. The cell model described in this thesis makes use of transgenic mice expressing green fluorescent protein (GFP) from the TRPV5 promotor sequence. This allowed the use of the COPAS sorter to specifically isolate TRPV5 expressing kidney tubules (the distal convolution). Isolated tubular fragments were subsequently cultured on filters, to preserve the specific cellular characteristics. After seven days of culturing, the cells developed into a tight monolayer with all the typical Ca^{2+} transporting properties of the distal convolution. It is important to note that crucial regulatory pathways remained intact, such as regulation of gene expression by vitamin D_3 or the increase in Ca^{2+} transport after PTH treatment. By crossbreeding the GFP mice with TRPV5$^{-/-}$ mice, lacking any functional TRPV5, and subsequently culturing isolated TRPV5$^{-/-}$ tubule fragments, for the first time the key role of TRPV5 in transcellular Ca^{2+} transport was directly confirmed. Moreover, in the absence of the natural Na^+-gradient, Ca^{2+} transport was decreased, confirming the role of NCX1 in this process. Furthermore, mRNA levels of the different proteins crucial for transcellular Ca^{2+} transport, TRPV5, calbindin-D_{28K} and NCX1, were clearly enriched in the sorted kidney tubule fragments as compared to total kidney material before sorting. The same held true for PMCA4, but surprisingly not for PMCA1, which was previously considered the most important PMCA for transcellular Ca^{2+} transport. In addition, coordinated regulation of all these components was observed in the TRPV5$^{-/-}$ distal convolution, in which mRNA levels of calbindin-D_{28K}, NCX1 and

7

PMCA4 were decreased. These results indicate an important role for PMCA4, but not PMCA1, in renal transcellular Ca^{2+} transport. The findings illustrate that this innovative primary cell model will allow the detailed study of the interplay between all the different components involved in renal transcellular Ca^{2+} transport.

Chapter 5: Regulation of the renal NCX1 variant by calmodulin

The Na^+/Ca^{2+} exchanger 1 (NCX1) is responsible for excreting the majority of Ca^{2+} that has entered the cell via the TRPV5 channel. NCX1 consists of ten transmembrane domains, small extracellular N- and C-terminal domains and one large intracellular loop. This loop contains two Ca^{2+} binding domains (CBDs). Alternative splicing of NCX1 mRNA results in proteins with distinguishing characteristics that are expressed in different tissues. In the kidney, and especially in the distal convolution, the variants NCX1.2, NCX1.3 and NCX1.7 are present, of which NCX1.3 is commonly regarded as the most important NCX1 variant in the kidney. Both TRPV5 and PMCAs are regulated by calmodulin. Also, five calmodulin binding sites have been predicted in the NCX1 protein, among which the XIP domain. Previous studies have looked into the XIP domain as a potential calmodulin binding site, but actual binding has never been demonstrated. Two of the five potential binding sites are located within predicted transmembrane domains and were therefore not further pursued. The study described in this thesis demonstrates binding of calmodulin to NCX1.3. The strength of calmodulin binding was markedly increased in the presence of Ca^{2+}, but occurred in Ca^{2+}-free conditions as well. Overexpression of calmodulin together with NCX1.3 in HEK293T cells resulted in an increase in NCX1.3 activity and protein levels. The effect of calmodulin on NCX1.3 activity was largely dependent on Ca^{2+} binding to calmodulin, whereas the effect on protein levels seemed independent of Ca^{2+}. The two remaining possible calmodulin binding sites were investigated by mutating critical amino acids within these regions. Mutation of amino acid R362 had little effect on stimulation of NCX1.3 by calmodulin, yet when amino acid L696 was mutated, calmodulin could no longer increase NCX1.3 activity. The effect on protein levels however was preserved, as was the binding of calmodulin to NCX1.3. In addition to the probable binding site around amino acid L696 that is involved in regulating NCX1.3 activity, a second binding site possibly exists, controlling NCX1.3 stability. Further studies are required to elucidate the Ca^{2+}-independent regulation of NCX1.3 by calmodulin.

Chapter 6: General discussion

The different studies described in this thesis provide additional insight into two essential components of transcellular Ca^{2+} transport in the kidney: the Ca^{2+} channel TRPV5 and the Na^+/Ca^{2+} exchanger NCX1.3. In addition, the novel primary cell model provides a very valuable basis to characterize the interplay of all the different components involved. The investigation into the regulation of the TRPV5 channel, has led to important insights into the structural role of its N-terminal tail and how it is involved in TRPV5 folding, and even more importantly to the finding that stress hormones are able to stimulate channel activity. The pharmacological compound dobutamine, part of the family of stress hormones such as epinephrine, has for the first time been implicated in the regulation of the TRPV5 channel and transcellular Ca^{2+} transport. *In vivo* studies are necessary to confirm the calciotropic role of dobutamine and other β-adrenergic agonists, but also antagonists (β-blockers), on renal Ca^{2+} resorption and

7

the total body Ca^{2+} balance.

On top of these findings, this thesis has made an important start in studying the regulation of the renal NCX1 variant. This protein, NCX1.3, has been cloned from kidney tissue in order to characterize its function in renal Ca^{2+} transport. For the first time it has been shown that calmodulin interacts with NCX1.3. Calmodulin has a crucial role in regulating TRPV5 and PMCA. It will be interesting to determine whether all other factors that are known to regulate TRPV5 in different ways, also affect NCX1.3 in a similar fashion. A prime example would be the role of β-adrenergic signaling, since regulation of NCX1 in the heart (splice variant NCX1.1) has previously been shown. To proceed in this direction, it is important to first clarify the controversial issue of NCX1 phosphorylation processes. Future investigations into the role and regulation of NCX1.3 will contribute significantly to the understanding of its function in renal transcellular Ca^{2+} transport.

In addition to studying TRPV5, NCX1.3 and in the future PMCA, individually, it will be essential to investigate how these different components function together. The development of the primary distal convolution cell model serves as an excellent basis for further studies. Crossbreeding with different mouse models will allow detailed study of all the different components involved in trancellular Ca^{2+} transport. Moreover, the primary cell model provides an opportunity to investigate the role of many known and unknown factors on Ca^{2+} reabsorption in an *"ex vivo"* setting. All together this should lead to a complete image on the interplay between all the different components, TRPV5, calbindin-D$_{28k}$, PMCA4 and NCX1, and their regulation by different calciotropic factors, both known and unknown. Detailed insights into the regulation of renal transcellular Ca^{2+} transport will help to understand and treat several diseases where the Ca^{2+} balance has been disturbed, such as idiopathic hypercalciuria, for which the cause of the renal Ca^{2+} loss is still unknown.

7

Samenvatting

Hoofdstuk 1: Introductie

Calcium (Ca^{2+}) speelt een sleutelrol in vele fysiologische processen in het lichaam, zoals bij de vorming van onze botten, spiercontracties en bloedstolling. Daarom is het essentieel dat de concentratie van Ca^{2+}, zowel in het bloed als in de cel, nauwkeurig wordt gereguleerd. De Ca^{2+} balans wordt in evenwicht gehouden door een samenspel van verschillende organen. De darm is verantwoordelijk voor Ca^{2+} opname uit de voeding, het bot is belangrijk voor de opslag van Ca^{2+}, terwijl de nier de uitscheiding van Ca^{2+} via de urine regelt. De nieren bestaan ieder uit ~1 miljoen functionele elementen, de nefronen. Ieder nefron is opgebouwd uit een glomerulus waar het bloed wordt gefiltreerd en een lange nierbuis waar de gefiltreerde voorurine vervolgens wordt geconcentreerd en belangrijke stoffen weer worden opgenomen in het bloed. Om verlies van Ca^{2+} via de urine te voorkomen, wordt in de nier ~99% van het Ca^{2+} uit de voorurine geresorbeerd. Het grootste deel wordt opgenomen via de paracellulaire route, waarbij Ca^{2+} tussen de cellen door via de zogenaamde "tight junctions" richting het bloed wordt getransporteerd. De uiteindelijke Ca^{2+} uitscheiding via de urine wordt echter bepaald door het transcellulaire transport. Hierbij wordt het Ca^{2+} uit de voorurine door de cel opgenomen om het aan de bloedkant weer af te geven. Dit proces van transcellulair transport vindt plaats in het laatste (distale) segment van de nierbuis, het zogenaamde distaal convoluut en de verbindingsbuis. De binnenkomst van Ca^{2+} in de cel wordt gereguleerd door het epitheliale Ca^{2+} kanaal transient receptor potential vanilloid 5 (TRPV5), waarna het door de Ca^{2+}-buffer calbindine-D_{28k} naar de andere kant van de cel wordt geleid. Hier wordt het Ca^{2+} door de Na^+/Ca^{2+} uitwisselaar, NCX1, en door de plasmamembraan Ca^{2+}-ATPase, PMCA, richting het bloed getransporteerd.

Om de Ca^{2+} balans in het lichaam te behouden wordt het transcellulaire Ca^{2+} transport in de nier nauwkeurig geregeld. Vele factoren spelen hierbij een rol waaronder de calciotrope hormonen zoals vitamine D_3 en parathormoon (PTH). In het distaal convoluut/verbindingsbuis van de nierbuis, stimuleren deze hormonen de Ca^{2+} resorptie door het verhogen van de mRNA expressieniveaus van TRPV5, calbinindine-D_{28k} en NCX1, hetgeen resulteert in een hogere productie van de betreffende eiwitten. Naast het reguleren van de eiwitniveaus worden de elementen ook afzonderlijk gereguleerd door allerlei locale factoren. Deze factoren kunnen specifiek de intrinsieke activiteit van TRPV5, NCX1 en PMCA beïnvloeden, maar ook het transport en daarmee de hoeveelheid van deze eiwitten op de plasmamembraan.

Regulatie van de activiteit van het epitheliale Ca^{2+} kanaal TRPV5 op de plasmamembraan is uitgebreid onderzocht. TRPV5 regelt de opname van Ca^{2+} in de cel, vervult een poortwachtersfunctie, en wordt daarom gezien als het meest kritische element in het transcellulaire Ca^{2+} transport. De sleutelrol van TRPV5 in dit proces is duidelijk geworden door onderzoek in muizen die geen functioneel TRPV5 eiwit bezitten, de TRPV5$^{-/-}$ muizen. Deze muizen hebben een verminderde Ca^{2+} resorptie in het distale segment van de nierbuis en verliezen daardoor meer Ca^{2+} via de urine. Het TRPV5 eiwit is een kanaal dat bestaat uit zes transmembraan segmenten en grote intracellulaire amino (N)- en carboxyl (C)- domeinen. Vier van deze TRPV5 eiwitten, de zogenaamde tetrameer, vormen samen een functioneel Ca^{2+} kanaal. De activiteit van het TRPV5 kanaal wordt bepaald door vele factoren, zoals de Ca^{2+} concentratie,

pH, verschillende hormonen waaronder PTH en allerlei andere stoffen die aanwezig zijn in de voorurine. Het C-terminale domein in TRPV5 en dan met name het geconserveerde TRP box domein speelt een belangrijke rol in deze regulatoire processen, doordat het domein allerlei eiwitten zoals bijvoorbeeld de Ca^{2+}-sensor calmoduline bindt. Daarnaast kunnen specifieke aminozuren in het C-terminale domein van TRPV5 gefosforyleerd worden, waardoor het aantal kanalen op de plasmamembraan toeneemt of de intrinsieke kanaalactiviteit wordt verhoogd. Beiden leiden tot een hogere opname van Ca^{2+} uit de voorurine. Na opname door TRPV5, wordt het Ca^{2+} aan de andere kant van de cel uitgescheiden door een samenspel van NCX1 en PMCAs. In tegenstelling tot passieve opname van Ca^{2+} door het TRPV5 kanaal met de Ca^{2+}-gradiënt mee, kost het transport van Ca^{2+} de cel uit juist veel energie. De van nature aanwezige Na^+-gradiënt die van belang is voor het functioneren van NCX1 wordt gecreëerd door de Na^+-K^+ ATPases, terwijl de PMCAs direct worden aangedreven door hydrolyse van ATP. Vanwege zijn hoge transportcapaciteit is NCX1 verantwoordelijk voor ~70% van het uitgaande Ca^{2+} transport. PMCAs hebben een lagere capaciteit, maar een hoge affiniteit voor Ca^{2+}, en zijn daarom belangrijk voor de fijnregeling van de Ca^{2+} uitscheiding. In tegenstelling tot opname van Ca^{2+} via TRPV5, is de functionele rol en regulatie van NCX1 en PMCA in het renale Ca^{2+} transport nog weinig bestudeerd.

In dit proefschrift is de regulatie van het transcellulaire Ca^{2+} transport in de nier verder onderzocht. Daarbij is eerst de regulatie van het TRPV5 kanaal bestudeerd door *i*) het nog grotendeels onbekende TRPV5 N-terminale domein en *ii*) de effecten van stresshormonen via β-adrenerge receptor signalering. Daarnaast is met behulp van een zeer innovatieve techniek een primair celmodel ontwikkeld om het samenspel van de verschillende componenten van het transcellulaire Ca^{2+} transport te kunnen bestuderen. Als laatste is onderzoek verricht naar de regulatie van de nier-specifieke variant van het NCX1 eiwit.

Hoofdstuk 2: De rol van de N-terminus in TRPV5 activiteit, tetramerisatie en transport
In tegenstelling tot de C-terminus, is de rol van de TRPV5 N-terminus in de regulatie van het Ca^{2+} kanaal nog weinig onderzocht. Net als de andere leden van de TRPV familie, bevat de TRPV5 N-terminus zes ankyrine-domeinen. Dit zijn repeterende domeinen van geconserveerde helix-lus-helix structuren, ieder ~33 aminozuren groot, die een rol spelen in het binden van allerlei factoren, zoals bijvoorbeeld ATP. Het eerste ankyrine-domein is betrokken bij onderlinge interacties tussen verschillende TRPV5 N-terminus domeinen. Daarnaast is binding van calmoduline aan de N-terminus aangetoond. Verder wordt de N-terminus door proteine kinase C (PKC) gefosforyleerd op de aminozuren S144 and S299. Om de functie van de N-terminus te onderzoeken, werden verschillende mutanten gegenereerd die een gedeelte van de N-terminus misten. In afwezigheid van de eerste 38 aminozuren en specifieker na mutatie van de aminozuren Q31/D34, was het TRPV5 kanaal niet actief, niet geglycosyleerd en was het voornamelijk aanwezig in het endoplasmatisch reticulum. Zeer waarschijnlijk betekent dit dat het eiwit niet goed kan worden gevouwen en door de kwaliteitscontrole van het endoplasmatisch reticulum wordt tegengehouden. Verder transport naar het Golgi-apparaat en uiteindelijk de plasmamembraan is hierdoor verstoord. Daarnaast konden de enkele TRPV5 kanalen die ontsnapten aan de kwaliteitscontrole en dus toch op de plasmamembraan aanwezig waren, niet meer op de gebruikelijke manier worden geïnternaliseerd. Ondanks dat de eiwitvouwing verstoord was, konden de TRPV5 kanalen nog wel tetrameren vormen, zelfs

als de eerste 75 aminozuren en dus ook het eerste ankyrine-domein ontbraken. De kritische aminozuren Q31/D34 in het eerste deel van de N-terminus bevinden zich in een mogelijke helix structuur, deze helix is dus waarschijnlijk essentieel voor de vouwing en dus het functioneren van het TRPV5 Ca^{2+} kanaal. Verder onderzoek is nodig om de rol van het eerste ankyrine-domein in interacties tussen TRPV5 kanalen te verduidelijken.

Hoofdstuk 3: β1-Adrenerge signalering activeert het epitheliale Ca^{2+} kanaal TRPV5

De stresshormonen epinefrine en norepinefrine hebben verschillende functies in het lichaam, en reguleren met name de werking van het hart en de bloedvaten. De hormonen werken door te binden aan α-adrenerge receptoren en β-adrenerge receptoren (β-AR). Beide hormonen zijn niet alleen in het bloed, maar ook in de voorurine aanwezig. Daarnaast zijn de β1-AR en β2-AR aanwezig in het distale gedeelte van de nierbuis, waar TRPV5-gemedieerd transcellulair Ca^{2+} transport plaatsvindt. Echter, een mogelijke rol van β-AR signalering op renaal transcellulair Ca^{2+} transport is nooit eerder beschreven. Met behulp van immunohistochemie is de aanwezigheid van β1-AR in het distaal convoluut/verbindingsbuis bevestigd, terwijl β2-AR niet kon worden aangetoond. Wanneer primaire niercellen van het distaal convoluut/verbindingsbuis met de β1-AR agonist dobutamine werden behandeld, was het Ca^{2+} transport duidelijk verhoogd. De β-AR receptoren zijn G-proteïne gekoppelde receptoren (G-protein coupled receptors, GPCRs). Activatie van verschillende GPCRs zoals de PTH receptor 1, de Ca^{2+} sensing receptor en de bradykinin receptor type 2, resulteert in TRPV5 kanaalactivatie of verhoogde expressie van het kanaal op de plasmamembraan. Activatie van de β1-AR receptor door dobutamine in een HEK293 cel systeem dat TRPV5 tot expressie brengt, leidde tot verhoogde TRPV5 activiteit terwijl het aantal kanalen op de plasmamembraan onveranderd bleef. Dit werd veroorzaakt door fosforylatie van aminozuur T709 door proteine kinase A (PKA); een effect dat ook optreedt door activatie van de PTH receptor. Endogene en farmacologische β1-AR agonisten en mogelijk ook antagonisten zoals de veelgebruikte β-blokkers hebben door hun potentiële effect op de totale Ca^{2+} resorptie in de nier dus zeer waarschijnlijk een belangrijk effect op de Ca^{2+} balans in het lichaam.

Hoofdstuk 4: Gecoördineerde regulatie van TRPV5-gemedieerd Ca^{2+} transport in primaire cultures van distaal convoluut/verbindingsbuis

Studies naar het moleculaire mechanisme van transcellulair Ca^{2+} transport in het distale deel van het nefron, en dan met name het samenspel van de betrokken componenten, worden ernstig belemmerd door het gebrek aan een geschikt celmodel. In het verleden zijn verschillende modellen ontwikkeld, maar deze hebben allen significante beperkingen. Met behulp van een zeer innovatieve techniek is een nieuw celmodel ontwikkeld voor studies naar de werking van het distaal convoluut/verbindingsbuis. Het in dit proefschrift beschreven celmodel maakt gebruik van muizen die een groen fluorescerend eiwit (green fluorescent protein, GFP) tot expressie brengen vanaf de TRPV5 promotersequentie. Hierdoor konden met gebruik van de COPAS sorteerder specifiek de nierbuisjes die TRPV5 bevatten (distaal convoluut/verbindingsbuis) worden geïsoleerd. Deze nierbuisfragmenten werden vervolgens gekweekt op speciale filters, zodat de specifieke eigenschappen van de cellen bewaard bleven. Op deze manier werd na zeven dagen kweken, een celmonolaag met de typische Ca^{2+}-transporterende karakteristieken van het distaal convoluut/verbindingsbuis verkregen. Cruciaal is ook dat de belangrijkste regulatoire routes intact waren, zoals regulatie

7

van genexpressie door vitamine D_3 en de snelle verhoging van het Ca^{2+} transport als reactie op behandeling met PTH. Door het kruisen van de GFP-muizen met TRPV5$^{-/-}$ muizen die geen functioneel TRPV5 bevatten, en vervolgens het kweken van de gesorteerde TRPV5$^{-/-}$ nierbuis fragmenten, werd voor de eerste keer de sleutelrol van het TRPV5 kanaal in het transcellulaire Ca^{2+} transport bevestigd. Daarnaast was het Ca^{2+} transport verlaagd in de afwezigheid van de natuurlijke Na^+-gradient, hetgeen de rol van NCX1 in dit proces aantoont. De mRNA niveaus van de verschillende componenten die een rol spelen in het transcellulaire Ca^{2+} transport, namelijk TRPV5, calbindine-D_{28k} en NCX1, waren duidelijk verrijkt in gesorteerde tubulussegmenten wanneer dit werd vergeleken met het niermateriaal voor sorteren. Dit geldt ook voor PMCA4, maar niet voor PMCA1 dat voorheen werd gezien als de belangrijkste PMCA in het proces van transcellulair Ca^{2+} transport. Soortgelijke co-regulatie van deze componenten werd waargenomen in TRPV5$^{-/-}$ distaal convoluut/verbindingsbuis, waar de mRNA niveaus van calbindine-D_{28k}, NCX1, PMCA4 zijn verlaagd. Deze resultaten duiden op een belangrijke rol van PMCA4, en niet PMCA1, in renaal transcellulair Ca^{2+} transport. De bevindingen illustreren dat met behulp van dit innovatieve celmodel in de toekomst de regulatie, en voor het eerst ook het samenspel van de verschillende componenten, in het transcellulaire Ca^{2+} transport nauwkeurig zal kunnen worden bestudeerd.

Hoofdstuk 5: Regulatie van de renale Na^+/Ca^{2+} uitwisselaar 1 (NCX1) door calmoduline

De Na^+/Ca^{2+} uitwisselaar 1 (NCX1) is verantwoordelijk voor het uitscheiden van het grootste deel van het via TRPV5 opgenomen Ca^{2+}. NCX1 bestaat uit tien transmembraan domeinen, kleine extracellulaire N- en C-terminale domeinen en een grote intracellulaire lus. Deze lus bevat twee Ca^{2+}-bindende domeinen (CBDs). De tweede CBD is een plaats voor alternatieve splicing, waardoor verschillende NCX1 varianten bestaan. Alternatieve splicing van NCX1 leidt tot eiwitten met onderscheidende eigenschappen die in verschillende weefsels in het lichaam tot expressie komen. In de nier, en met name in het distaal convoluut/verbindingsbuis, zijn de varianten NCX1.2, NCX1.3 en NCX1.7 aanwezig, waarvan NCX1.3 het meest tot expressie komt en dan ook vaak als de belangrijkste NCX1 variant in de nier wordt beschouwd. Zowel TRPV5 als de PMCAs worden gereguleerd door calmoduline. Voor NCX1 werden vijf mogelijke bindingsplaatsen voor calmoduline voorspeld, waaronder het zogenaamde XIP domein. Dit domein is al eerder onderzocht als potentiële calmoduline bindingsplaats, maar binding is nooit aangetoond. Twee andere voorspelde bindingsplaatsen vielen samen met transmembraan domeinen en zijn daarom niet verder onderzocht. De in dit proefschrift beschreven studie laat binding van calmoduline aan NCX1.3 zien. Deze calmoduline binding was veel sterker in de aanwezigheid van Ca^{2+}, maar trad ook op onder Ca^{2+}-vrije condities. Overexpressie van calmoduline samen met NCX1.3 in een HEK293T celmodel, resulteerde in een verhoogde NCX1.3 activiteit en eiwitexpressie. Het effect van calmoduline op de NCX1.3 activiteit was grotendeels afhankelijk van het binden van Ca^{2+} aan calmoduline, terwijl het effect op de eiwitniveaus Ca^{2+}-onafhankelijk was. De twee mogelijke calmoduline bindingsplaatsen werden onderzocht door het muteren van kritische aminozuren binnen deze regio's. Mutatie van het aminozuur R362 had weinig effect op de stimulatie van NCX1 door calmoduline, echter wanneer het aminozuur L696 werd gemuteerd, kon calmoduline de NCX1.3 activiteit niet verder verhogen. Het effect op de eiwitniveaus bleef wel bestaan, evenals binding van calmoduline aan NCX1.3. Naast de waarschijnlijke bindingsplaats rond aminozuur L696, die een rol speelt in de regulatie van

NCX1.3 activiteit is er dus vermoedelijk een tweede bindingsplaats die zorgt voor stabilisatie van het NCX1.3 eiwit. Nader onderzoek is nodig om het mechanisme van Ca^{2+}-onafhankelijke regulatie van NCX1.3 door calmoduline te verhelderen.

Hoofdstuk 6: Algemene discussie

De studies beschreven in dit proefschrift geven meer inzicht in twee essentiële componenten van het transcellulaire Ca^{2+} transport in nier: het Ca^{2+} kanaal TRPV5 en de Na^+/Ca^{2+} uitwisselaar NCX1.3. Daarnaast leggen zij een belangrijke basis om het samenspel van de verschillende componenten nauwkeurig te kunnen karakteriseren. Het onderzoek naar de regulatie van TRPV5 heeft geleid tot belangrijke structurele kennis over de rol van het N-terminale domein in eiwitvouwing en nog belangrijker het ontdekken van de stimulerende effecten van zogenaamde stresshormonen op de kanaalactiviteit. De farmacologische stof dobutamine, uit de familie van stresshormonen zoals epinefrine, is voor het eerst in verband gebracht met de regulatie van het TRPV5 kanaal en het transcellulaire Ca^{2+} transport. *In vivo* studies zijn nodig om de calciotrope rol van dobutamine en andere β-adrenerge agonisten en ook antagonisten (β-blokkers) op renale Ca^{2+} resorptie en de totale Ca^{2+} balans te onderzoeken.

Vervolgens is met dit proefschrift een belangrijk begin gemaakt in het bestuderen van de regulatie van de renale NCX1 variant. Dit eiwit, NCX1.3, is gekloneerd uit de nier, om het daarna te karakteriseren. Hierbij is voor het eerst een interactie tussen NCX1.3 en calmoduline aangetoond. Calmoduline speelt een onmisbare rol in het functioneren van TRPV5 en PMCA. Het is interessant om te onderzoeken of de vele andere factoren die een effect hebben op TRPV5, ook een rol spelen in de regulatie van NCX1.3. Een goed voorbeeld is het effect van β-adrenerge signalering, omdat regulatie van NCX1 in het hart (splice variant NCX1.1) al in eerdere studies is beschreven. Hiervoor is het van groot belang eerst het nog controversiële onderwerp van fosforylatie van NCX1 helder te krijgen. Het onderzoek naar de rol en regulatie van NCX1.3 zal een grote bijdrage leveren aan het begrip van de functie van deze Ca^{2+} transporteur in het transcellulaire Ca^{2+} transport in de nier.

Naast het individueel bestuderen van TRPV5, NCX1.3 en in de toekomst ook PMCA, is het van essentieel belang om het samenspel van de verschillende componenten te onderzoeken. Met de ontwikkeling van een celmodel voor het distaal convoluut/verbindingsbuis, is hiervoor een cruciale basis gelegd. Door het kruisen met verschillende muismodellen zullen de verschillende componenten tot in detail kunnen worden bestudeerd. Daarnaast biedt het celmodel de kans om voor allerlei bekende en onbekende factoren hun rol op de Ca^{2+} resorptie *"ex vivo"* te kunnen bestuderen. Dit tezamen zal leiden tot een compleet beeld van de rol en het samenspel van de verschillende elementen, TRPV5, calbindine-D_{28k}, PMCA4 en NCX1, en regulatie door verschillende bekende en nog onbekende calciotrope factoren. Inzicht in de precieze regulatie van het transcellulair Ca^{2+} transport in de nier zal helpen in het begrijpen en oplossen van verschillende ziektes waar de Ca^{2+} balans is verstoord, zoals met name bij idiopathische hypercalciurie, waar de oorzaak van het renale Ca^{2+} verlies nog onbekend is.

7

Chapter 8

List of abbreviations
Curriculum Vitae
List of publications
Dankwoord

List of abbreviations

1,25-(OH)$_2$D$_3$	1,25-dihydroxyvitamin D$_3$
1α-OHase	25-hydroxyvitamin-D$_3$-1α-hydroxylase
[Ca^{2+}]$_i$	intracellular Ca^{2+} concentration
β-AR	β-adrenergic receptor
AQP2	aquaporin 2
ARD	ankyrin repeat domain
ATP	adenosine 5'-triphosphate
a.u.	arbitrary unit
B2R	bradykinin receptor subtype 2
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxy-methyl ester)
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaCl$_2$	calcium chloride
CaM	calmodulin
cAMP	3'- 5'-cyclic adenosine monophosphate
CaSR	Ca^{2+}-sensing receptor
CBD	Ca^{2+} binding domain
CB28k	calbindin-D$_{28k}$
cDNA	complement DNA
CHF	congestive heart failure
CNT	connecting tubule
COPAS	complex object parameter analyzer and sorter
C-terminus	carboxy terminus
DAG	diacylglycerol
DCT	distal convoluted tubule
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulphoxide
EDTA	ethylenediamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid
ENaC	epithelial sodium channel
Epi	epinephrine
ER	endoplasmic reticulum
fors	forskolin

8

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GSDB	goat serum dilution buffer
HEK293	human embryonic kidney 293 cells
HRP	horseradish peroxidase
HCl	hydrochloric acid
IC_{50}	50% inhibitory concentration
IP	immunoprecipitation
IP_3	inositol trisphosphate
IRES	internal ribosome entry site
K_D	dissociation constant
K^+	potassium ion
KCl	potassium chloride
kDa	kilo dalton
KHB	KREBS-Henseleit
$LaCl_3$	lanthanum chloride
MDCK	Madin-Darby canine kidney
mpkDCT	murine renal distal convoluted tubule
mesna	2-mercaptoethanesulfonic acid sodium salt
$MgCl_2$	magnesium chloride
Mg^{2+}	magnesium ion
mRNA	messenger RNA
n	sample size
NaCl	sodium chloride
NaOH	sodium hydroxide
Na^+	sodium ion
NCC	Na^+-Cl^- cotransporter
NCX	Na^+/Ca^{2+} exchanger
NE	norepinephrine
NHERF	Na^+/H^+ exchanger regulatory factor
NKCC2	Na-K-2Cl cotransporter
NMDG	N-methyl-D-glucamine
N-terminus	amino terminus
PAGE	polyacrylamide gel electrophoresis
PAR-1	protease-activated receptor-1
PBS	phosphate-buffered saline

8

PEI	polyethylenimine
PFO	perfluorooctanoic acid
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PMCA	plasma membrane Ca^{2+} ATPase
PMSF	phenylmethanesulfonylfluoride
PSS	physiological salt solution
PT	proximal tubule
PTH	parathyroid hormone
PTH1R	parahyroid hormone receptor type 1
PV	parvalbumin
RR	ruthenium red
SE	standard error
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase
SGK1	serum- and glucocorticoid-induced kinase 1
TAL	thick ascending loop of Henle
TEER	transepithelial electrical resistance
TM	transmembrane
TPA	12-*O*-tetradecanoylphorbol-13-acetaat
TRPV	transient receptor potential vanilloid
$TRPV5^{-/-}$	TRPV5 knockout
VDR	vitamin D receptor
VDRE	vitamin D-responsive element
WNK	with no lysine (K)
WT	wild-type
XIP	exchanger inhibitor peptide

8

Curriculum Vitae

Eline van der Hagen werd geboren op 26 maart 1986 te Tilburg. Daar behaalde zij in 2004 haar Gymnasium diploma aan het Koning Willem II College. Vervolgens studeerde zij Moleculaire (Levens) Wetenschappen aan de Wageningen Universiteit. Tijdens deze studie voltooide zij twee afstudeervakken en een wetenschappelijke stage van elk 6 maanden. Het eerste afstudeervak vond plaats bij de afdeling Voeding en Farmacologie onder leiding van dr. W.T. Steegenga. Daarna werkte zij aan haar tweede afstudeervak in de groep van prof. dr. D. Weijers van de afdeling Biochemie. Zij rondde haar studie af met een stage aan het Institute for Cell and Molecular Biosciences, Newcastle University (UK), in de groep van prof. dr. D. Ford. In november 2009 behaalde zij haar MSc diploma, met de specialisaties "Biomedisch Onderzoek" en "Biologische Chemie". Aansluitend is zij gestart als promovendus op de afdeling Fysiologie onder leiding van prof. dr. R.J.M. Bindels en prof. dr. J.G.J. Hoenderop, binnen het Radboud Institute for Molecular Life Sciences (RIMLS) onderdeel van het Radboudumc, wat heeft geleid tot het in dit proefschrift beschreven onderzoek. Tijdens haar promotieonderzoek heeft zij verschillende BSc en MSc studenten Biomedische Wetenschappen begeleid tijdens hun stage bij de afdeling Fysiologie.

8

List of publications

van der Hagen EA, van Loon EP, Verkaart S, Latta F, Bindels RJ, Hoenderop JG: Regulation of the renal Na^+/Ca^{2+} exchanger 1 (NCX1) variant by calmodulin. *Submitted, 2014*

van der Hagen EA*, Tudpor K*, Verkaart S, Lavrijsen M, van der Kemp AW, van Zeeland F, Bindels RJ, Hoenderop JG: β1-Adrenergic receptor signaling activates the epithelial calcium channel, transient receptor potential vanilloid type 5 (TRPV5), via the protein kinase A pathway. *J Biol Chem.* 289: 18489-18496, 2014

van der Hagen EA, Lavrijsen M, van Zeeland F, Praetorius J, Bonny O, Bindels RJ, Hoenderop JG: Coordinated regulation of TRPV5 mediated Ca^{2+} transport in primary distal convolution cultures. *Pflügers Arch. In Press, 2014*

de Groot T*, **van der Hagen EA***, Verkaart S, te Boekhorst VA, Bindels RJ, Hoenderop JG: Role of the transient receptor potential vanilloid 5 (TRPV5) protein N-terminus in channel activity, tetramerization, and trafficking. *J Biol Chem.* 286: 32132-32139, 2011

de Groot T*, Kovalevskaya NV*, Verkaart S, Schilderink N, Felici M, **van der Hagen EA**, Bindels RJ, Vuister GW, Hoenderop JG: Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. *Mol Cell Biol.* 31: 2845-2853, 2011

Coneyworth LJ*, Jackson KA*, Tyson J, Bosomworth HJ, **van der Hagen E**, Hann GM, Ogo OA, Swann DC, Mathers JC, Valentine RA, Ford D: Identification of the human zinc transcriptional regulatory element (ZTRE): a palindromic protein-binding DNA sequence responsible for zinc-induced transcriptional repression. *J Biol Chem.* 287: 36567-36581, 2012

*authors contributed equally

8

Dankwoord

Na bijna 5 jaar bloed, zweet en tranen, ik vrees zowel letterlijk als figuurlijk, is het na het schrijven van dit stukje toch echt bijna af! Het proefschrift waar het de afgelopen 5 jaar allemaal om draaide. Ondanks dat niet alles helemaal liep zoals gepland of gehoopt, ben ik heel blij en trots op het uiteindelijke resultaat! Dit heb ik natuurlijk niet alleen gedaan, en daarom wil ik hier heel graag iedereen bedanken die direct of meer indirect aan dit boekje hebben bijgedragen.

Daarbij wil ik natuurlijk beginnen met mijn promotoren prof. dr. **Joost Hoenderop** en prof. dr. **René Bindels**, zonder de kans die jullie me hebben gegeven om als PhD student 4 jaar op jullie succesvolle en goed georganiseerde afdeling te werken, was dit boekje er natuurlijk nooit geweest. Ik heb ontzettend veel geleerd van jullie manier van schrijven en presenteren. Bedankt voor alle mogelijkheden die ik heb gekregen.

Daarna wil ik heel graag **Sjoerd** bedanken, vanaf dag 1 was je betrokken bij mijn onderzoek, super fijn om iemand te hebben om mee te kunnen discussiëren over resultaten, technieken en nieuwe mogelijkheden. Je had altijd wel weer een geweldig (of soms wat minder geweldig) nieuw idee om te gaan onderzoeken. Zonder jou was dit boekje zeker niet zo mooi geweest, jouw invloeden en experimenten zijn in ieder hoofdstuk zichtbaar! Ik ben dan ook heel blij dat je me bij wilt staan als paranimf.

Ook wil ik **Theun** bedanken, ontzettend fijn dat ik met jou mijn eerste publicatie kon verwerven. De kritische discussies met jou en Sjoerd waren ontzettend leerzaam en productief, precies zoals het in mijn ogen zou moeten gaan. Ook na je "verhuizing" naar osmoregulatie heb ik altijd nog veel aan je adviezen gehad.

Kukiat, great that we succeeded to get the β-adrenergic story to the publication that it is now, the last requirement for us both to deliver a PhD thesis. Hopelijk ben je al je Nederlands nog niet verleerd als je terugkomt om te verdedigen.

Dan wil ik heel graag het "COPAS team" bedanken: **AnneMiete** voor alle essentiële muizen kennis en kunde, en **Hans**, **Marla** en **Femke**, als ik al die tubules zelf had moeten sorteren was het allemaal nooit gelukt. Ik weet hoe ontzettend geestdodend en frustrerend het werk is en ben jullie ontzettend dankbaar dat jullie hierin zoveel hebben kunnen helpen. Daarbij wil ik in het speciaal **Femke** bedanken, jij was vanaf het begin betrokken bij de COPAS en hebt daardoor het langst en het meest afgezien, gelukkig was het allemaal niet voor niets en hebben we een mooi verhaal af kunnen leveren en gaat het sorteren steeds ietsje makkelijker en beter! **Marla**, ook al kwam je later bij het "COPAS team", je kritische blik en onnavolgbare handigheid hebben het leven met de COPAS ontzettend verbeterd! En natuurlijk heel erg bedankt voor alle mooie stainings en de laatste transport assay resultaten.

Irene, onmisbaar in het lab, jouw "magische handen" die apparaten als vanzelf weer laten draaien, maar het meest natuurlijk om de geweldige organisatie die ervoor zorgt dat alles blijft werken en er nooit iets mist. En natuurlijk heel erg bedankt met de hulp bij het verkrijgen van al die lastige stofjes en niet te vergeten voor de gezelligheid bij de lunch.

Jeroen, na ¾ jaar afzien aan de "koude kant" van het lab, kwam er een zonnetje binnengelopen.

Al was je vrolijke geklets niet altijd even handig, het was ontzettend fijn in zo'n plezierige sfeer te mogen werken, dit kwam de productiviteit zeker ten goede! En ondanks dat we nooit hebben samengewerkt, was je altijd bereid mee te denken over wetenschappelijke en meer diplomatieke oplossingen, in beiden ben je onzettend goed, ik ben heel erg benieuwd waar dat toe gaat leiden.

Anke, raar eigenlijk dat we elkaar (voor mijn gevoel) pas zo laat echt tegen kwamen, toen ik verhuisde naar de "warme kant" van het lab. Ik gun niemand wetenschappelijke tegenslag en jij met al je kennis, kunde en enthousiasme verdient dat al helemaal niet, maar stiekem was het voor mij een hoopgevend voorbeeld om te zien hoe je uiteindelijk toch zo'n mooi resultaat hebt weten te behalen. Het is ons gelukt!

Liz en **Ellen**, ik kan er niet omheen om jullie samen te noemen, als doldwaze duo met jullie (zelfverzonnen) liedjes en ik moet toegeven dat ondanks dat het niet echt om aan te horen is, misschien juist daarom toveren jullie een lach op ieders gezicht. Maar jullie zijn vooral ook ontzettend verschillend. **Liz**, ik heb ontzettend veel bewondering voor je efficiënte manier van werken en hoe je op je doel af gaat, je bent er bijna! **Ellen**, als student bij Sjoerd hebben we al veel en fijn samengewerkt aan het FGF23/Klotho verhaal, helemaal leuk toen je besloot als PhD student verder te gaan. Voor mij super dat juist jij voortzet wat ik heb achtergelaten, vooral het NCX1 stuk maar ook de PMCAs. Hopelijk gaat het jou ook een mooi proefschrift brengen, ik heb er het volste vertrouwen in!

Lauriane, after moving around in the lab, I ended up in the "NCX unit" with you, my only fellow NCX colleague. I much enjoyed our discussions, you were of great help. I also want to thank you, together with **Maxime**, for critically reading the last parts of this thesis.

Mijn studenten: **Sanne**, **Marga**, **Jali**, **Britta**. Heel erg bedankt voor alle mooie resultaten en leuke discussies, hopelijk hebben jullie veel geleerd, ik zeker ook van jullie!

Annelies, onze treinreizen terug naar Arnhem en avonden bij de PhD retreat waren altijd gezellig, en fijn ook dat ik bij late feestjes mee terug kon met de auto!

Ofcourse, also, I would like to thank all other colleagues that made the work so much fun! Thanks for the nice chats during breaks, visits to the aesculaaf, helpful discussions, etc, etc.

Dan wil ik nu graag diegenen bedanken die buiten het lab hebben bijgedragen aan dit boekje door morele steun, het tonen van interesse en het bieden van de nodige afleiding.

Jaarclubgenootjes, de gezellige BBQs, weekeindjes naar Cochem en Norwich en andere geslaagde uitjes waren een zeer welkome afwisseling! En ja, ik weet het, nu ben ik aan de beurt om iets leuks te regelen, RAM!

Lieve **Ruud**, **Dinie**, **Thomas**, **Laura**, **David** en **Pauline**, bedankt voor alle gezellige BBQs, etentjes, bezoekjes en het tonen van jullie interesse, ontzettend fijn!

Papa, **Mama**, **Rob**, **Marloes**, het was niet altijd makkelijk als enige "beta" in het gezin, misschien juist dankzij jullie vaak skeptische opmerkingen ben ik stug doorgegaan met het bestuderen van die "molecuultjes", hopelijk zijn jullie ook trots op het resultaat! En natuurlijk heel erg

8

bedankt voor alle gezelligheid bij de verschillende etentjes, uitjes, feestjes etc. Daarbij wil ik ook zeker **Helle** en **Aerent** niet vergeten! Bedankt ook voor de interesse in het wel en wee van het onderzoek gedurende al die jaren. **Papa**, **Mama**, jullie staan altijd klaar met raad en daad, niets is te gek om het ons zo goed mogelijk te maken. Zonder alle kansen en mogelijkheden die jullie mij hebben gegeven had ik dit nooit kunnen bereiken, bedankt!

Lieve **Remco**, jij hebt deze tijd met al zijn ups en downs natuurlijk van het dichtstbij meegemaakt. Bij jou kan ik altijd tot rust komen na een dag hard werken, eentje vol frustraties of juist de succesjes vieren. Helemaal geweldig dat we dit zo samen hebben kunnen doen. Nu samen op naar een nieuw avontuur!