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Molecular structure and regulation of the epithelial calcium channels TRPV5 and TRPV6

Qing Chang
I want to bring out the secrets of nature and apply them for the happiness of man. I don't know of any better service to offer for the short time we are in the world. (Thomas Edison)

我想揭示大自然的秘密，用来造福人类。我认为，在我们的短暂一生中，最好的贡献莫过于此了。（爱迪生．T.）

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Molecular structure and regulation of the epithelial calcium channels TRPV5 and TRPV6

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op maandag 14 mei 2007
om 13.30 uur precies

door

Qing Chang

geboren op 14 November 1976
te Xi’an, China
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Molecular structure and regulation of the epithelial calcium channels TRPV5 and TRPV6

An academic essay in the Medical Sciences

Doctoral thesis

to obtain the degree of doctor from Radboud University Nijmegen
on the authority of Rector Magnificus, prof. dr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans
to be defended in public on Monday, 14 May 2007
at 13.30 hours

by

Qing Chang

born in Xi'an, China
on the 14th of November 1976
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Dr. T. van Kuppevelt
All I am, or can be, I owe to my family. (Abraham Lincoln)
我之所有,我之所能,都归功于我的家庭。(林肯)

To my deeply-loved parents
and Dong
仅以此书献给我挚爱的父母和牛栋
Home is the place where, when you have to go there, it has to take you in.
(Frost Robert)
无论何时何地家永远是向游子敞开大门的地方。(罗伯特. F.)
**Table of Contents**

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P. 1</td>
</tr>
<tr>
<td>General introduction</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>P. 35</td>
</tr>
<tr>
<td>Molecular determinants in TRPV5 channel assembly</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>P. 57</td>
</tr>
<tr>
<td>Direct interaction with Rab11a targets the epithelial Ca(^{2+}) channels TRPV5 and TRPV6 towards the plasma membrane</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P. 81</td>
</tr>
<tr>
<td>The β-glucuronidase klotho hydrolyzes and activates the TRPV5 channel</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>P. 99</td>
</tr>
<tr>
<td>Tissue kallikrein stimulates Ca(^{2+}) reabsorption <em>via</em> PKC-dependent plasma membrane accumulation of TRPV5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>P. 125</td>
</tr>
<tr>
<td>N-glycosylation of TRPV5 determines channel stability at the plasma membrane</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>P. 143</td>
</tr>
<tr>
<td>Klotho primarily affects the epithelial Ca(^{2+}) channels TRPV5 and TRPV6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>P. 159</td>
</tr>
<tr>
<td>General discussion</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>P. 189</td>
</tr>
</tbody>
</table>
| Summary  
Samenvatting (Summary in Dutch)  
中文简介 (Summary in Chinese) |
| 10      | P. 209|
| Acknowledgement                        
Curriculum vitae                        
List of publications                    
List of abbreviations                   |
If winter comes, can spring be far behind? (P. B. Shelley)

冬天来了，春天还会远吗？(雪莱．P．B．)
Chapter 1

General introduction

Department of Physiology
Nijmegen Centre for Molecular Life Sciences
Radboud University Nijmegen Medical Centre
The Netherlands
The only limit to our realization of tomorrow will be our doubts of today.
(Franklin Roosevelt)

实现明天理想的唯一障碍是今天的疑虑。(罗斯福．F．)
Introduction

The TRP superfamily

The Transient Receptor Potential (TRP) superfamily of proteins consists of cation-selective ion channels with molecular structure similarities but diverse functions [1]. The first member of TRP family was discovered in studies that examined Drosophila phototransduction pathway [2], in which the photoreceptor cells of Drosophila exhibit sustained receptor potentials in response to continuous light exposure. Cosens and Manning [2-4] reported in 1969 that a group of mutant flies exhibited a transient receptor potential upon continuous light exposure and named it TRP. The TRP gene was cloned in 1989 [3] and subsequently shown to encode a Ca\(^{2+}\)-permeable cation channel [4]. Since then, several channels that have sequence and structural similarities to the Drosophila TRP have been cloned from flies, worms, and mammals. TRP channels are widely expressed and have diverse functions, ranging from thermal, tactile, taste, osmolar and fluid flow sensing to transepithelial Ca\(^{2+}\) and Mg\(^{2+}\) transport [5-13]. Mutations of TRP proteins result in kidney-related diseases, including hereditary hypomagnesemia with secondary hypocalcemia (HSH) caused by mutations of TRPM6, autosomal dominant polycystic kidney disease caused by mutations of TRPP2, and focal segmental glomerulosclerosis caused by mutations of TRPC6 [5, 14-21].

A previous consensus report proposed a unified nomenclature for the TRP superfamily [22]. It classified TRP channels into TRPC, TRPV, and TRPM subfamilies on the basis of amino acid sequence and structural similarity. More recent classification has expanded TRP superfamily to include three other subfamilies, TRPP, TRPML, and TRPN [1, 22, 23]. Unlike most of ion channel families, the TRP superfamily is identified on the basis of homology only [1, 23-25]. The mode of activation and selectivity for TRP channels are diverse. Some TRP channels are activated by ligands, whereas others are regulated by physical stimuli (for instance, heat) or yet-unknown mechanisms [1, 23, 25-31]. All TRP channels are cation selective, but the selectivity ratio for Ca\(^{2+}\) versus the monovalent cation Na\(^{+}\) (\(P_{Ca}/P_{Na}\)) varies widely [1, 23, 25-34]. Structurally, TRP channels have six transmembrane (TM) segments and intracellularly localized amino (N-tail) and carboxyl (C-tail) termini, similar to the topology of voltage-gated K\(^{+}\), Na\(^{+}\) and Ca\(^{2+}\) channels, cyclic nucleotide-gated channels and hyperpolarization-activated channels [1] (Figure 1A). The four subunits of TRP channels assemble as tetramers to form cation-permeable pores. In
the cytosolic tails, a TRP domain of a conserved 25 amino acids stretch is located in the C-tail, and potential regulatory sites including several ankyrin repeats and enzyme domains are localized in the N- and C-tails. For instance, TRPV5 and TRPV6 contain PDZ motifs and protein kinase C (PKC) phosphorylation sites in both N- and C-tails, TRPM6 and TRPM7 contain an α-kinase domain in the C-tail, and TRPM2 contains a phosphatase domain in its C-tail. Furthermore, between TM segment 5 and 6 there is a short hydrophobic stretch that forms the pore forming region of these channels. Some TRP channels contain potential N-linked glycosylation sites, for instance, TRPV5 and TRPV6 have one at the asparagine residue N358 or N357 between TM segment 1 and 2, respectively [1] (Figure 1B).

**TRPV5 and TRPV6: unique members of the TRP superfamily**

The epithelial Ca\(^{2+}\) channels, TRPV5 and TRPV6, represent two highly homologous members within the TRP superfamily, which are mainly expressed in Ca\(^{2+}\)-transporting epithelia [25, 35]. The genes of TRPV5 and TRPV6 are juxtaposed on human chromosome 7q35 [36-38]. The two distinct genes comprise 15 exons, encoding proteins of approximately 730 amino acids [28, 29, 37, 39]. TRPV5 and TRPV6 display 30–40% homology with other members of the TRP family, and possess unique properties that distinguish them from other TRP channels [9, 40]. For instance, TRPV5 and TRPV6 retain a Ca\(^{2+}\) over Na\(^{+}\) selectivity, which is much larger (\(P_{\text{Ca}}/P_{\text{Na}} > 100\)) than observed for any other member of the TRP superfamily (\(P_{\text{Ca}}/P_{\text{Na}} \leq 9\)) [3, 25, 32-34]. TRPV5 and TRPV6 share 75% homology at the amino acid level, and the main sequence differences are located in the N- and C-tails. Electrophysiological studies using human embryonic kidney cells (HEK293) heterologously expressing TRPV5 or TRPV6, show that these channels permeate Ca\(^{2+}\) [25, 40, 41] and exhibit a similar ion permeation sequence for divalent cations (Ca\(^{2+}\) > Sr\(^{2+}\) ≈ Ba\(^{2+}\) > Mn\(^{2+}\)). However, the relative permeability for Ba\(^{2+}\) in relation to Ca\(^{2+}\) is significantly less for TRPV6 compared to TRPV5. Furthermore, TRPV5 has a 100-fold higher affinity for the blocker ruthenium red than TRPV6 [41].

The tissue distribution of TRPV5 and TRPV6 has been studied extensively by Northernblot, RT-PCR analysis and immunohistochemistry [28, 32, 33, 36, 38, 41-46]. In humans, both channels are co-expressed in the organs that mediate transcellular Ca\(^{2+}\) transport, such as small intestine, colon, kidney, bone and placenta but also in exocrine
Chapter 1

A

TRPC | 3-4 | Yes | No
TRPM | 0   | Yes | Yes*
TRPML| 0   | No  | No
TRPN | 29  | No  | No
TRPP | 0   | No  | No
TRPV | 3-6 | Yes | No

* ADP ribose pyrophosphatase domain for TRPM2, and kinase domain for TRPM6 and TRPM7

TRP box

EWKFARTKLWMSYFEEGTLPPPFN

TK L DD X X

TRP domain

... The number of ankyrin repeats

B

Ca²⁺

pore

NH₂

COOH

Potential PKC phosphorylation site
Potential PKA/cGK phosphorylation site
Potential CaMKII phosphorylation site
Potential N-linked glycan
Ankyrin repeat
TRP domain
α-kinase domain

The different numbers of ankyrin repeats
tissues such as pancreas, prostate, mammary gland, sweat gland and salivary gland. The relative mRNA levels of TRPV5 and TRPV6 in tissues co-expressing these channels are different. In general, TRPV5 seems to be the major isoform in kidney, which is localized along the apical membrane of the epithelial cells lining the late part of the distal convoluted tubule (DCT2) and the connecting tubule (CNT) [45, 47]. In these nephron segments TRPV5 co-localizes with other proteins involved in Ca\textsuperscript{2+} transport, such as calbindin-D\textsubscript{28K} (CaBP\textsubscript{28K}), Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger type 1 (NCX1) and plasma membrane Ca\textsuperscript{2+}-ATPase 1b (PMCA1b) [47]. In contrast to TRPV5, TRPV6 is abundantly expressed in small intestine. In the duodenum, TRPV6 protein is present in the luminal membrane along the villus tips [46, 47]. In addition, TRPV6 mRNA is located in the exocrine acinar cell of the pancreas. The expression of TRPV6 in prostate is increased in prostate cancer and correlates with the tumor grade [48].

The structure of TRPV5 and TRPV6 shows the typical topology features shared by all members of the TRP family [35] (Figure 1B). The N- and C-tails of TRPV5 and TRPV6 contain several conserved putative regulatory sites that might be involved in regulation of channel activity and trafficking. For instance, the N-tails of these channels contain ankyrin repeats, which play a crucial role in protein–protein interactions [49]. In addition, the N- and C-tails of TRPV5 and TRPV6 each contain potential internal PDZ motifs, which can function as part of a molecular scaffold via interaction with PDZ-domain containing proteins, involved in membrane protein routing, trafficking and regulating (Figure 1B) [50, 51]. Particularly, TRPV5 and TRPV6 possess several potential phosphorylation sites for PKC, cAMP- and cGMP-dependent protein kinase (PKA and cGK) and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) in their N- and C-tails. Many of these putative phosphorylation sites are conserved among species, which
suggest an important role in TRPV5 and TRPV6 function (Table 1). The characteristic pore region of TRPV5 and TRPV6 is unique for its high Ca\(^{2+}\) selectivity [34, 40, 52]. A single aspartic residue in the pore region at position number 542 (D542) of TRPV5 is crucial for Ca\(^{2+}\) permeation, which is completely conserved in TRPV6 (D541). Mutation of D542 into an alanine abolishes Ca\(^{2+}\) permeation, but does not affect the permeation of monovalent cations [52].

**Table 1. Number of putative phosphorylation sites in the N- and C-tails of TRPV5 and TRPV6**

<table>
<thead>
<tr>
<th></th>
<th>TRPV5</th>
<th></th>
<th>TRPV6</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Rabbit</td>
<td>Rat</td>
<td>Mouse</td>
<td>Human</td>
</tr>
<tr>
<td>PKC</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>PKA/cGK</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CaMKII</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Sequence alignment was performed with the following GenBank sequences: TRPV5: rabbit (*Oryctolagus cuniculus* AJ133128), rat (*Rattus norvegicus* AB032019), mouse (*Mus musculus* AF336378), human (*Homo sapiens* AJ271207). TRPV6: rat (*R. norvegicus* AF160798), mouse (*M. musculus* AB037373), human (*H. sapiens* AJ487964). PKC: protein kinase C; PKA/cGK: cAMP- and cGMP-dependent protein kinase; CaMKII: Ca\(^{2+}\)/calmodulin-dependent protein kinase II; ND: not determined; Conserved: the number of conserved phosphorylation sites among all species.

**N-glycosylation of TRPV5 and TRPV6**

Protein glycosylation is the process or result of addition of saccharides to proteins. Two types of glycosylation exist: N-linked glycosylation to the amide nitrogen (NH\(_2\)) of asparagine side chains and O-linked glycosylation to the hydroxyl oxygen (OH) of serine and threonine side chains. It has been reported that most membrane proteins of mammalian cells are glycoproteins (proteins containing glycans). Such glycans have two major functions. Inside the cell, they help proteins fold and assemble correctly in the endoplasmic reticulum (ER), and they might also act as a signal for the trafficking of glycoproteins. Outside the cell, they provide specific recognition structures for interaction with a variety of external ligands [53]. Protein N-glycosylation is a metabolic process that has been highly conserved in evolution. In all eukaryotes, N-glycosylation is obligatory for viability [54]. It functions by modifying appropriate asparagine residues of proteins with oligosaccharide structures, thus influencing their properties and bioactivities. N-glycoprotein biosynthesis involves a multitude of enzymes, including glycosyltransferases and glycosidases [54-56].
Introduction

It has been well known that N-linked oligosaccharides arise when blocks of 14 sugars are added cotranslationally to newly synthesized polypeptides in the ER [57]. These glycans are then subjected to extensive modification as the glycoproteins mature and move through the ER via the Golgi complex to their final destination inside and outside the cell. In ER, it has been reported that N-glycans play a pivotal role in protein folding, oligomerization, quality control, sorting, and transport, in addition, they are used as universal tags that allow specific lectins and modifying enzymes to establish order [58]. In the Golgi complex, the glycans acquire more complex structures and new functions. The difference of glycan synthesis and processing between the ER and the Golgi complex represents an evolutionary adaptation that allows efficient adding of the potential oligosaccharides [57-60]. N-glycosylation of proteins is highly conserved from yeast to human and has a significant effect on modulating protein structure and localization to facilitate proper folding and trafficking of membrane proteins [59]. The glycosylation of proteins can be modulated by adaptations in the biosynthetic pathway. For instance, dietary variations and also hormones like estradiol can have a significant effect on the display of cell-surface carbohydrate epitopes [61]. The effects of cotranslational protein modification on the process of protein folding were initially poorly understood. Time-resolved fluorescence energy transfer has been used to assess the impact of glycosylation on the conformational dynamics of flexible oligopeptides [62]. The results showed that glycosylation causes the modified peptides to adopt different conformations, and for some peptides this change may lead to more compact conformations that better approximate the conformation of these peptides in the final folded protein. This result further implied that glycosylation can trigger the timely formation of structural elements and thus assist in the complex process of protein folding [62].

The role of N-glycosylation of transmembrane proteins has received great attention, and it appears that in some cases it is involved in membrane targeting. To date, several studies have focused on the role of N-glycosylation in ion channel activity. It has been demonstrated that the gating and trafficking of the voltage gated K⁺ (Kᵥ) channel is regulated by N-glycosylation. Kᵥ1.1 and Kᵥ1.4 channels are plasma membrane glycoproteins involved in action potential repolarization. It has been shown previously that N-glycosylation affects the gating function of Kᵥ1.1, in addition, the cell-surface trafficking of Kᵥ1.1 and Kᵥ1.4 is influenced by glycosylation as well [63-69]. It shows that
prevention of N-glycosylation of K\textsubscript{v1.4} decreased its protein stability, induced its high intracellular retention, and decreased its cell-surface protein levels [70]. Understanding the different trafficking programs of K\textsubscript{v1} channels, and whether they are altered by N-glycosylation, will highlight the different posttranslational mechanisms available to cells to modify their cell-surface ion channel levels and possibly their signaling characteristics. Furthermore, Freeman and co-workers elucidated that N-glycosylation influences gating and pH sensitivity of the K\textsubscript{v} channel subunit I(sK) [71]. Pabon et. al demonstrate that N-glycosylation of G-protein-activated inwardly rectifying K\textsuperscript{+} channel (GIRK1) at asparagine residue N119 and the renal outer-medullary K\textsuperscript{+} channel (ROMK1) at asparagine residue N117 has different consequences in K\textsuperscript{+} channel function [72]. Similarly, previous studies indicate that inactivation of protein N-glycosylation reduces Na\textsuperscript{+}-pump activity [73]. The role of N-glycosylation in epithelial Na\textsuperscript{+} channel (ENaC) trafficking was previously obscure. All three ENaC subunits possess numerous potential N-glycosylation sites [74], and it is clear that all subunits are glycosylated in cells. All the glycosylation sites are utilized in \(\alpha\)-ENaC, and mutating some or all of these sites does not seem to affect channel activity [74, 75]. It suggests that at least sugar modification of \(\alpha\)-subunit does not affect proper trafficking to and insertion of the ENaC channel at the plasma membrane. In addition, the role of N-glycosylation in Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter (NKCC2) functional properties was assayed in one (N442Q or N452Q) or both (N442, N452Q) N-glycosylation mutants by Western blotting and confocal microscopy. The study suggested that prevention of N-glycosylation reduces NKCC2 functional expression by affecting insertion into the plasma membrane and the intrinsic activity of the cotransporter [76]. It has been thoroughly studied that the mutation of N-glycosylation sites influences other protein activities including the gamma-aminobutyric acid (GABA) transporter (GAT1) [77], the voltage-gated Ca\textsuperscript{2+} channels (Ca\textsubscript{v}) [78] and cyclic nucleotide-gated ion channel (CNG) [79]. For instance, N-glycosylation of the asparagine residues N136 and N184 is necessary for subunit-mediated regulation of Ca\textsubscript{v} [78]. In addition, the mutation of the well-conserved N-glycosylation site in bovine retinal CNG channel affects the channel function by changing the electrostatic energy profile of the external vestibule near the pore [79].

Within the TRP superfamily, it has been demonstrated that N-glycosylation regulates TRPV1 channel activity [80]. However, in addition to the well-known fact that deglycosylation negatively regulates protein trafficking, Xu and co-workers showed that
deglycosylation activates TRPV4 channel in response to hypotonic stress, which is associated with an increase in plasma membrane targeting of TRPV4 [81]. Like other known TRP channels, both TRPV5 and TRPV6 channels contain a single conserved N-glycosylation site, which is localized in the extracellular loop between TM region 1 and 2. This N-glycosylation site locates at the asparagine residue N358 or N357 in TRPV5 and TRPV6, respectively. The studies have demonstrated that these channels are not only translated as core proteins, but are also complex-glycosylated resulting in higher molecular weight proteins ranging from 85–100 kDa [82]. The intensity of glycosylated TRPV5 and TRPV6 bands is reduced after incubation with endoglycosidase H (endoH), which hydrolyzes high mannoses. In addition, the bands completely disappear after treatment with peptide N-glycosidase F (endoF), which removes all types of sugars. This observation indicates that both epithelial channels are highly mannose- and complex-glycosylated [82]. N-glycosylation of TRPV5 and TRPV6 may play an important role in protein folding, intracellular trafficking and channel regulation.

**Architecture of TRPV5 and TRPV6**

The assembly of ion channels has received a lot of attention. For instance, ENaC is composed of three structurally related subunits (α, β, γ) that form a tetrameric channel. It has been indicated that multiple domains throughout αENaC, when co-expressed with αβγENaC, confer a dominant negative phenotype [83]. Thus, these domains may play an important role in facilitating and stabilizing subunit-subunit interactions that allow for the proper assembly and functional expression of the heterooligomeric ENaC [83]. In addition, the tetrameric ROMK provides an important pathway for K⁺ secretion by the mammalian kidney. The X-ray crystal structure of KirBac1.1, a prokaryotic ortholog of ROMK, has suggested that channel gating involves intermolecular interactions of the N- and C-tail domains of adjacent subunits [84]. A considerable amount of information about channel subunit assembly has been accumulated by studies on Kᵥ channels that are structurally related to TRP channels. Previous studies indicated that the subfamily of Shaker-like Kᵥ channels possesses a T1 domain in their N-tail, which confers subfamily specificity and inter-subunit assembly [85]. The designated T1 domain, a highly conserved N-tail cytoplasmic domain (around 130 amino acids) immediately preceding the first putative TM helix S1, was found to form a tetrameric ring with a narrow, positively charged central pore [86]. This highly conserved T1 domain has been shown to spontaneously form tetramers in the absence of TM sequences [86, 87]. TM domain
(TM1-3) has been implicated to be involved in $K_v$ channel assembly [88]. Crystallization of T1 domain further verified the reported biochemical and functional data and implicated that T1 is also involved in channel gating [86, 89].

Similar to $K_v^+$ channels, it has been shown that heteromeric complex formation can modify the activity of the TRP channels. The *Drosophila* TRP and TRPL members were identified first to form channel hetero-multimers, which are associated with receptors and regulators in a signaling complex [90, 91]. Moreover, it has been shown for TRPC1 and TRPC3 that hetero-oligomers of these channels possess distinctive properties compared to the channel alone [92]. In addition, Strubing et al. [93] demonstrated that TRPC1 and TRPC5 are subunits of a heteromeric neuronal channel, and co-expression of these channels in HEK293 cells results in a novel non-selective cation channel, unlike that of any reported TRPC channel. Other TRPCs also exclusively assemble into homo- or hetero-tetramers within the confines of TRPC subfamilies, for instance, TRPC4 and TRPC5, TRPC3, TRPC6 and TRPC7 [94]. Recently, the oligomeric structure of another TRP member, TRPV1, was studied by biochemical cross-linking. The findings suggested the predominant existence of tetramers within the TRPV1 family [95].

Previously, Hoenderop and coworkers unraveled that TRPV5 and TRPV6 form homo- and hetero-tetrameric channel complexes with four subunits configured in a head-to-tail fashion [82] (Figure 2). Since TRPV5 and TRPV6 are co-expressed in some tissues, it allows oligomerization of these channels *in vivo* [36, 41, 43]. This study has combined several independent methods to demonstrate that TRPV5 and TRPV6 are functional as homo- and heterotetrameric $Ca^{2+}$ channels with novel properties [82]. First, chemical cross-linking experiments revealed protein band shifts from monomeric TRPV5 and TRPV6 to multimeric compositions. Secondly, sucrose gradient centrifugation confirmed
that TRPV5 and TRPV6 channel complexes have a molecular weight in line with a tetrameric configuration. Thirdly, co-immunoprecipitations demonstrated that TRPV5 and TRPV6 subunits are physically linked to each other. Fourthly, electrophysiological analyses of concatemeric polypeptides revealed that all (hetero)tetrameric TRPV5 and TRPV6 channels are functional with differences in transport kinetics. Finally, the tetrameric structure was investigated in a functional assay, following a similar approach to that previously used to prove the tetrameric stoichiometry of the structurally related Shaker-like K\textsuperscript{+} channel [96] and cyclic nucleotide-gated channel (CNG) [97]. Detailed information concerning protein structure and assembly of ion channels containing six TM domains is available for Shaker-like K\textsuperscript{+} and CNG channels. The selective assembly of heteromeric CNG channels requires a trimer-forming C-tail leucine zipper (CLZ) domain [98]. The clustering of four subunits in six TM domain channels is assumed to create an aqueous pore centered around the 4-fold symmetry axis [86]. It has been previously demonstrated that a single aspartic residue in the aqueous pore region of TRPV5 (D542) determines the Ca\textsuperscript{2+} permeation of the channel [52], which is completely conserved in TRPV6 (D541). The tetrameric architecture of TRPV5 and TRPV6 implies that four aspartates (TRPV5-D542 and TRPV6-D541) contribute to the selectivity filter for Ca\textsuperscript{2+}, by analogy with the four negatively charged glutamates and/or aspartates that determine the Ca\textsuperscript{2+} selectivity in Ca\textsubscript{v} channels [99, 100]. Although the overall structure of TRPV5 and TRPV6 is similar to that of Ca\textsubscript{v} channels, the mode of subunit assembly appears to be different for TRPV5 and TRPV6, since four individual TRPV5 and/or TRPV6 subunits have to assemble to form a functional channel, whereas functional Ca\textsubscript{v} channels are monomeric proteins containing four homologous internal repeats. It has been shown that Ca\textsubscript{v} channels assemble through α-interaction domain (AID), β-interaction domain (BID) and an extensive, conserved hydrophobic cleft (named the a-binding pocket, ABP) [101, 102]. As TRPV5 and TRPV6 exhibit different channel kinetics, heterotetrameric TRPV5 and TRPV6 proteins displayed properties that, depending on the subunit configuration, are intermediate between TRPV5 and TRPV6. An increased number of TRPV5 subunits in such a concatemer expresses more TRPV5-like properties, indicating that the stoichiometry of TRPV5 and TRPV6 heterotetramers influences the channel properties [82]. In addition, replacing individual TRPV5 subunits by TRPV6 subunits in a TRPV5 tetramer has major effects on Ba\textsuperscript{2+} permeability, Ca\textsuperscript{2+}-dependent inactivation and the block by ruthenium red. In this way, Ca\textsuperscript{2+}-transporting epithelia co-expressing TRPV5 and TRPV6 may be able to generate a pleiotropic set of functional heterotetrameric
Chapter 1

channels. Variation in the individual subunits of this tetramer could provide a mechanism for fine tuning the Ca\(^{2+}\) transport kinetics in Ca\(^{2+}\)-transporting epithelia. Consequently, it would be interesting to determine which molecular structures in TRPV5 and TRPV6 are involved in tetramerization. Hypothetically, assembly of TRPV5 and TRPV6 channels might involve the ankyrin repeats and the PDZ motif in the N- and C-tail [103]. The different characteristics of TRP channels compared to other ion channels are shown in Table 2, including their selectivity, localization, type of glycosylation and assembly domains, and so on.

Table 2. The comparison of TRP channels with the other ion channels

<table>
<thead>
<tr>
<th>Channels</th>
<th>Selectivity</th>
<th>Renal localization</th>
<th>N-gly. Tetra. Assembly domain</th>
<th>Topology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP</td>
<td>Ca(^{2+}), Na(^{+}), Mg(^{2+})</td>
<td>DCT, CNT, TAL</td>
<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
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<td>Yes</td>
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<td>ENaC</td>
<td>Na(^{+})</td>
<td>DCT, CNT, CD</td>
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<tr>
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<td>K(^{+})</td>
<td>TAL, CD</td>
<td>Yes</td>
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</tbody>
</table>

TRP: transient receptor potential channel; Ca\(_v\): voltage-gated Ca\(^{2+}\) channel; CNG: cyclic nucleotide-gated channel; K\(_v\): voltage-gated K\(^{+}\) channel; ENaC: epithelial Na\(^{+}\) channel; ROMK: renal outer-medullary K\(^{+}\) channel; N-gly.: N-glycosylation; tetra.: tetramerization; AID: \(\alpha\)-interaction domain; BID: \(\beta\)-interaction domain; ABP: \(\alpha\)-binding pocket; CLZ: C-tail leucine zipper domain. DCT: distal convoluted tubule; CNT: connecting tubule; TAL: thick ascending limb of Henle’s loop; IMCD: inter modular collecting duct; PT: proximal tubule; CD: collecting duct; TM: transmembrane domain.

Ankyrin repeats of TRPV5 and TRPV6

In general, ankyrin repeats can interconnect integral membrane proteins such as ion channels with the spectrin-actin based cytoskeletal elements in specialized membrane domains, enabling protein trafficking to the plasma membrane and channel regulation via actin filaments [104, 105]. Ankyrin-binding proteins include the voltage-dependent
Na⁺ channel, Na⁺-K⁺-ATPase, Na⁺-Ca²⁺-exchanger, IP₃ receptor and ryanodine receptor Ca²⁺ release channels. The membrane-binding domain of ankyrins is comprised of one or more copies of a 33-residue repeat known as the ankyrin repeat. This protein-protein interaction module is involved in a diverse set of cellular functions, and consequently, defects in ankyrin repeat proteins result in a number of human diseases [106, 107]. TRPV5 and TRPV6 contain several ankyrin repeat domains in their N-tails, which could be involved in the maintenance and targeting of these channels to specific membrane regions, as has been demonstrated for a neural-specific isoform of ankyrin in the localization of Na⁺ channels [105]. Interestingly, Erler et al. [108] identified a specific N-tail domain encompassing the third ankyrin repeat being a stringent requirement for physical assembly of TRPV6 subunits. Deletion of this repeat or mutation of critical residues within this repeat renders non-functional channels that do not co-immunoprecipitate or form tetramers. It was proposed that the third ankyrin repeat initiates a molecular zipper process that proceeds past the fifth ankyrin repeat and creates an intracellular anchor that is necessary for functional subunit assembly [108]. These studies provide evidence that ankyrin repeats in the N-tail of TRPV6 are essential for subunit assembly [108]. Importantly, it is likely that this assembly domain also exists in the N-tail of TRPV5, because these two channels share 75% homology at the amino acid level, raising the possibility that both N-tail or C-tail assemble together in order to form functional heterotetrameric channel complexes of TRPV5 and TRPV6 [82]. Detailed sequence comparison of the N- and C-tails of the TRPV5 and TRPV6 channels reveals significant differences, which may account for the unique electrophysiological properties [41, 82]. In addition, the assembly domains could differ between the two channels in order to specifically allow self-assembly or the formation of heterotetrameric channels [82]. Deleting or mutating assembly domains could cause a change in tertiary structure and/or prevent the interaction with auxiliary proteins, thereby affecting channel trafficking and activity of TRPV5 and TRPV6.

**The role of TRPV5 and TRPV6 in Ca²⁺ homeostasis**

Maintenance of body Ca²⁺ homeostasis is of vital importance for many physiological functions including intracellular signaling processes, synaptic transmission in neurons, neuronal excitability, muscle contraction, blood clotting, fertilization and bone formation. The extracellular Ca²⁺ concentration should, therefore, be tightly regulated. Ca²⁺ homeostasis in humans is achieved by a mechanism tightly controlling the concerted
actions of intestinal Ca\(^{2+}\) absorption, exchange the Ca\(^{2+}\) from the bone mass and renal Ca\(^{2+}\) reabsorption [109, 110]. There are two pathways for Ca\(^{2+}\) to pass through renal epithelium and reach the blood compartment. The major one is by passive paracellular transport together with Na\(^{+}\) in the proximal tubules and the second is by active transcellular Ca\(^{2+}\) transport in DCT and CNT tubules (Figure 3) [111]. Even though the distal part of the nephron is responsible for only ~15% of total renal Ca\(^{2+}\) reabsorption, it is generally regarded as the site for fine-tuning of the urinary Ca\(^{2+}\) reabsorption independently of the Na\(^{+}\) balance [45, 112, 113]. This transport is mediated by three discrete steps: (i) Ca\(^{2+}\) entry from the pro-urine across the apical membrane via TRPV5; (ii) Ca\(^{2+}\) buffering and intracellular diffusion from apical to basolateral membrane by calbindins; (iii) Ca\(^{2+}\) extrusion across the basolateral membrane via NCX1 and/or PMCA1b into the blood (Figure 3) [28, 29, 109].

**Figure 3. Localization and mechanism of epithelial Ca\(^{2+}\) transport.** (A) Model of the nephron, the functional unit of the kidney, depicting the TRPV5 expressing sections in the DCT and CNT tubule. (B) Epithelia can absorb Ca\(^{2+}\) by paracellular and transcellular transport. Passive and paracellular Ca\(^{2+}\) transport takes place across the tight junctions and is driven by the electrochemical gradient for Ca\(^{2+}\). The active form of vitamin D, 1,25-dihydroxy-vitamin D\(_3\) (1,25-(OH)\(_2\)D\(_3\)), stimulates the individual steps of transcellular Ca\(^{2+}\) transport by increasing the expression levels of the luminal Ca\(^{2+}\) channels, calbindins, and the extrusion systems. Active and transcellular Ca\(^{2+}\) transport is carried out as a three-step process. Following entry of Ca\(^{2+}\) through the (hetero)tetrameric epithelial Ca\(^{2+}\) channels, TRPV5 and TRPV6, Ca\(^{2+}\) bound to calbindin diffuses to the basolateral membrane. At the basolateral membrane, Ca\(^{2+}\) is extruded via a ATP-dependent Ca\(^{2+}\)-ATPase (PMCA1b) and a Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX1). In this way, there is net Ca\(^{2+}\) absorption from the luminal space to the extracellular compartment. DCT: distal convoluted tubule; CNT: connecting tubule; PMCA1b: plasma membrane Ca\(^{2+}\)-ATPase 1b; NCX1: Na\(^{+}\)-Ca\(^{2+}\) exchanger 1.
TRPV5 and TRPV6 are generally considered as the gatekeepers of transcellular Ca\(^{2+}\) transport, and therefore the regulation of Ca\(^{2+}\) influx across the luminal membrane through these channels is particularly crucial. To unravel the role of TRPV5 and TRPV6 in maintaining the Ca\(^{2+}\) balance, the characterization of TRPV5 and TRPV6 knockout mice is instrumental. Hoenderop et al. [114] generated TRPV5 null (TRPV5\(^{-/-}\)) mice by genetic ablation of TRPV5 to investigate the requirement of TRPV5 functioning in renal and intestinal Ca\(^{2+}\) (re)absorption. Interestingly, metabolic studies demonstrated that TRPV5\(^{-/-}\) mice display a significant calciuresis compared to wild-type littermates (TRPV5\(^{+/+}\)) while remaining normocalcemia. However, the active metabolite vitamin D (1,25(OH)\(_2\)D\(_3\)) levels in serum are significantly elevated in TRPV5\(^{-/-}\) mice compared with TRPV5\(^{+/+}\) mice. In addition, polyuria and urine acidification were consistently observed in TRPV5\(^{-/-}\) mice, which have been elucidated to reduce the potential risk of renal stone formation due to urine acidification [113, 115]. Micropuncture studies indicated that the renal Ca\(^{2+}\) transport defect is localized in the DCT/CNT. Furthermore, ablation of TRPV5 in the DCT is accompanied by a concomitant decrease in calbindin-D\(_{28k}\) and NCX1 mRNA levels. The excessive renal Ca\(^{2+}\) wasting is compensated by an enhanced intestinal Ca\(^{2+}\) absorption as a result of increased TRPV6 and calbindin-D\(_{9k}\) expression, explaining the normocalcemia. Moreover, the TRPV5\(^{-/-}\) mice exhibit significant disturbances in bone structure, including reduced trabecular and cortical bone thickness compared with TRPV5\(^{+/+}\) mice [109]. All together, ablation of the TRPV5 gene seriously disturbs renal Ca\(^{2+}\) handling, resulting in compensatory intestinal hyperabsorption and bone abnormalities. Hediger and co-workers [116] addressed the functional role of TRPV6 in Ca\(^{2+}\) absorption by inactivation of the mouse TRPV6 gene. These TRPV6 null (TRPV6\(^{-/-}\)) mice were placed on a Ca\(^{2+}\)-deficient diet and subsequently applied in a 45Ca\(^{2+}\) absorption assay. TRPV6\(^{-/-}\) mice show a consistent decrease in Ca\(^{2+}\) absorption over time. From their preliminary data it was concluded that TRPV6\(^{-/-}\) mice show a significant Ca\(^{2+}\) malabsorption, suggesting that TRPV6 is indeed the rate-limiting step in 1,25-OH\(_2\)D\(_3\)-dependent Ca\(^{2+}\) absorption.

**Regulation of TRPV5 and TRPV6**

TRPV5 and TRPV6 are synthesized in the endoplasmic reticulum (ER) and are subsequently modified by oligosaccharide structures at appropriate asparagine residues through the ER to the Golgi complex, and finally transported towards their destination: plasma membrane. Regulation of TRPV5 and TRPV6 can, hypothetically, occur at the
level of (i) transcriptional and translational regulation by 1,25-dihydroxy-vitamin D$_3$ (1,25-(OH)$_2$D$_3$), dietary Ca$^{2+}$, estrogen and parathyroid hormone (PTH); (ii) intracellular trafficking and routing regulation to the plasma membrane by S100A10/annexin 2; (iii) direct (in)activation of channel activity at the plasma membrane by pH, intracellular Ca$^{2+}$, and Ca$^{2+}$ sensors (calmodulin and 80K-H) (Figure 4).

Figure 4. Integrated model of TRPV5 and TRPV6 regulation. TRPV5 and TRPV6 is synthesized in the ER and is subsequently transported towards the plasma membrane. Regulation of TRPV5 and TRPV6 can, hypothetically, occur at the level of (1) transcriptional and translational regulation by 1,25-(OH)$_2$D$_3$, dietary Ca$^{2+}$, estrogen and PTH; (2) intracellular trafficking and routing of channels to the plasma membrane by S100A10/annexin 2; (3) direct (in)activation of channel activity at the plasma membrane by pH, intracellular Ca$^{2+}$, and (4) Ca$^{2+}$ sensors: 80K-H and calmodulin. [Ca$^{2+}$]: Ca$^{2+}$ concentration; PTH: parathyroid hormone; 1,25-(OH)$_2$D$_3$: 1,25-dihydroxy-vitamin D$_3$, CaM: calmodulin.

A. TRPV5 and TRPV6 regulation at the transcriptional and translational level

Parathyroid hormone

Parathyroid hormone (PTH) is the calcitropic hormone controlling the Ca$^{2+}$ balance [109, 117, 118]. Early studies using micropuncture and cell preparations demonstrated that PTH directly stimulates active Ca$^{2+}$ reabsorption in the distal part of the nephron [119, 120]. In addition, PTH stimulates the activity of 1$\alpha$-OHase, a crucial enzyme in the biosynthesis of 1,25-(OH)$_2$D$_3$ and, thereby, increases the 1,25-(OH)$_2$D$_3$-dependent absorption of Ca$^{2+}$ in the small intestine [121]. Van Abel et al. [122] recently
demonstrated that parathyroidectomy (PTX) in rats results in decreased serum PTH levels and hypocalcaemia, which is accompanied by decreased TRPV5 and calbindinD28k mRNA levels and protein abundance [122]. Supplementation with PTH restores serum Ca2+ concentrations, TRPV5 and calbindinD28k abundance in kidney, suggesting that PTH affects renal Ca2+ handling through the regulation of TRPV5.

**1,25-dihydroxy-vitamin D3**

Various studies have provided evidence that the expression of TRPV5 and TRPV6 is tightly controlled by 1,25-dihydroxy-vitamin D3 (1,25-(OH)2D3) [42, 123-133]. In mice, a single dose of 1,25-(OH)2D3 up-regulates renal TRPV5 and duodenal TRPV6 mRNA levels, illustrating the genomic vitamin D response [126, 133]. In vitamin D receptor knockout (VDR−/−) mice, duodenal TRPV6 mRNA levels are significantly down-regulated and associated with decreased intestinal Ca2+ absorption and hypocalcemia [38, 126]. Targeted ablation of 1α-OHase in mice, leading to an impairment of 1,25-(OH)2D3 biosynthesis, was shown to result in severe hypocalcemia and down-regulation of both renal TRPV5 and intestinal TRPV6 expression. Repletion with 1,25-(OH)2D3 restores Ca2+ channel expression levels and normalizes serum Ca2+ levels. Taken together, the transcriptional and translational levels of TRPV5 and TRPV6 is regulated by 1,25-(OH)2D3.

**Estrogen and androgen**

Given the fact that the postmenopausal estrogen deficiency results in a negative Ca2+ balance associated with osteoporosis [134, 135] and this disturbed Ca2+ balance can be corrected by estrogen replacement therapy [136-139]. The effect of estrogen on the proteins involved in active Ca2+ (re)absorption was investigated in ovariectomized (OVX) 1α-OHase knockout (1α-OHase−/−) mice. 17β-estradiol replacement therapy results in upregulation of renal TRPV5, which is accompanied by a normalization of serum Ca2+ levels. Furthermore, in duodenum both TRPV5 and TRPV6 mRNA levels are upregulated in these supplemented mice [125, 140]. Thus, TRPV5 and TRPV6 expression is transcriptionally controlled by estrogen in a vitamin D-independent manner. Van Cromphaut and co-workers [141] reported that renal TRPV5 and duodenal TRPV6 expression is reduced in estrogen receptor knockout mice and subsequently upregulated by estrogen treatment. In addition, Peng and co-workers [48] showed that TRPV6 expression is particularly increased in androgen-sensitive prostate adenocarcinoma cell
lines as compared to androgen-insensitive cells. Dihydrotestosterone and androgen-receptor (AR) antagonists were shown to regulate TRPV6 in these cell lines. Thus, besides estrogens, androgens also appear to regulate TRPV6 expression.

**Dietary Ca\(^{2+}\)**

In addition to the calciotropic hormones, there are indications that Ca\(^{2+}\) itself can affect TRPV5 and TRPV6 expression. To further address this question, studies were performed in VDR\(^{-/-}\) and 1α-OHase\(^{-/-}\) mice fed with normal and high Ca\(^{2+}\) diets [124, 127]. Importantly, high dietary Ca\(^{2+}\) intake restores the reduced renal TRPV5 and intestinal TRPV6 expression in 1α-OHase\(^{-/-}\) mice, which is accompanied by normalization of the serum Ca\(^{2+}\) concentration. In contrast, the Ca\(^{2+}\)-enriched rescue diet increases the serum Ca\(^{2+}\) concentration, and subsequently reduces the expression of renal TRPV5 and calbindin-D\(_{28K}\) in wild-type mice [124, 126, 127]. Similarly, dietary rescue of the hypocalcemic state in VDR\(^{-/-}\) mice amends the significantly decreased duodenal TRPV5 and TRPV6 mRNA expression, whereas in wild-type mice high dietary Ca\(^{2+}\) content decreases TRPV5 and TRPV6 expression due to a decrease of serum 1,25-(OH)\(_{2}\)D\(_{3}\) level [126].

**Table 3. Hormonal regulation of TRPV5 and TRPV6 in the target organs**

<table>
<thead>
<tr>
<th>Kidney</th>
<th>Duodenum</th>
<th>Reference</th>
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<tr>
<td></td>
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<tr>
<td>High Ca(^{2+}) diet</td>
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<td>↑</td>
</tr>
<tr>
<td>1α-OHase(^{-/-})</td>
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<td>ND</td>
</tr>
<tr>
<td>1α-OHase(^{-/-})+dietary Ca(^{2+})</td>
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<td>ND</td>
</tr>
<tr>
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<td>↓</td>
<td>↓</td>
</tr>
<tr>
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<td>ND</td>
</tr>
<tr>
<td>Androgen</td>
<td>ND</td>
<td>↓</td>
</tr>
<tr>
<td>AR+antagonist</td>
<td>ND</td>
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</tr>
<tr>
<td>PTX</td>
<td>↓</td>
<td>=</td>
</tr>
<tr>
<td>PTX+PTH</td>
<td>↑</td>
<td>=</td>
</tr>
</tbody>
</table>

↑: upregulation; ↓: downregulation; UN: undetectable; =: not changed; ND: not determined; Wild-type: wild-type mice; VitD: 1,25(OH)\(_{2}\)D\(_{3}\); 1α-OHase\(^{-/-}\): 1α-OHase knockout mice; VDR\(^{-/-}\): vitamin D receptor knockout mice; O VX: ovarioectomized; AR: androgen receptor; PTX: parathyroidectomized; PTH: parathyroid hormone.
Therefore, these studies showed that Ca\(^{2+}\) supplementation can up-regulate TRPV5 and TRPV6 gene transcription in the absence of circulating 1,25-(OH)\(_2\)D\(_3\). Hormonal regulation of TRPV5 and TRPV6 is summarized in Table 3.

**B. Regulation of TRPV5 and TRPV6 activity at the plasma membrane**

**pH**

Studies using primary cultures of rabbit CNT and CCD cells indicated that acidification of the apical medium inhibits transcellular Ca\(^{2+}\) reabsorption [29, 142, 143]. In addition, pH directly influences TRPV5 and TRPV6 channel activity at the apical plasma membrane. TRPV5 expressing *Xenopus laevis* oocytes showed decreased \(^{45}\)Ca\(^{2+}\) uptake when the incubation medium is acidified and extracellular acidification significantly reduces Ca\(^{2+}\) influx through TRPV5 [144]. Extracellular pH also affects current kinetics including extracellular Mg\(^{2+}\) blockade and Ca\(^{2+}\) affinity. The mean current density decreases at acidic pH and increases at alkaline pH. Recently, Yeh and co-workers [144] showed that mutation of the glutamic acid (Glu) residue at position 522 to glutamine (Gln) near the pore helix decreases the inhibition of TRPV5 by extracellular acidification. Taken together, these data suggest that the luminal pH directly regulates Ca\(^{2+}\) entry through the epithelial Ca\(^{2+}\) channels *in vivo*.

**Ca\(^{2+}\)-dependent feedback inhibition**

In mammalian cells, TRPV5 and TRPV6 are constitutively open at a low-intracellular Ca\(^{2+}\) concentration and a negative membrane potential [32, 145, 146]. These findings suggest that intracellular Ca\(^{2+}\) exerts a negative feedback mechanism on TRPV5 and TRPV6 activity [41]. Both Ca\(^{2+}\) channels inactivate during hyperpolarizing voltage-steps and this inhibition is reduced when Ca\(^{2+}\) is substituted for Ba\(^{2+}\) or Sr\(^{2+}\) as charge carriers. However, the inactivation of TRPV6 is characterized by a slow decline after an initial fast inactivation phase, whereas TRPV5 only shows the slow inactivation phase [145, 147]. In addition, the difference in Ca\(^{2+}\)-dependent inactivation between TRPV5 and TRPV6 is restricted to the first intracellular loop between TM domain 2 and 3 [147]. These data suggested that TRPV5 and TRPV6 activity is inhibited by Ca\(^{2+}\) influx through the channel, which could be a crucial mechanism for the regulation of TRPV5 and TRPV6 at the plasma membrane under physiological conditions [35, 148, 149].
Ca\textsuperscript{2+} sensors

1. Calmodulin

Calmodulin (CaM) is a ubiquitous cytosolic protein known to play a pivotal role in Ca\textsuperscript{2+}-dependent inactivation, acting as a Ca\textsuperscript{2+} sensor, thereby facilitating both activation and inactivation of different ion channels, including voltage- and ligand-gated Ca\textsuperscript{2+} channels [150], Ca\textsuperscript{2+} pumps, and other proteins in a Ca\textsuperscript{2+}-dependent manner [151]. CaM consists of four Ca\textsuperscript{2+}-binding EF-hand structures, which are localized in the N- and C-tails. Ca\textsuperscript{2+} binding to CaM is highly cooperative with Ca\textsuperscript{2+} binding first to the C-tail EF-hands, which have the highest affinity for Ca\textsuperscript{2+}, followed by Ca\textsuperscript{2+} binding to lower affinity sites located in the N-tail [152]. CaM has been shown to modulate several other members from the TRP superfamily [153-163], for instance, modulation of TRPV1 and TRPC1. TRPV5 and TRPV6 activity is negatively regulated by the intracellular Ca\textsuperscript{2+} concentration and CaM could mediate the regulation of the activity of TRPV6. Niemeyer et al. showed for the first time that CaM binds to the C-tail of human TRPV6 in a Ca\textsuperscript{2+}-dependent manner [151, 164]. The regulation of TRPV6 by CaM was recently confirmed by Lambers et al. [165]. By combination of pull-down assays and co-immunoprecipitations, it was demonstrated that CaM binds to both TRPV5 and TRPV6 in a Ca\textsuperscript{2+}-dependent fashion. The binding of CaM to mouse TRPV6 is localized to the TM domain and consensus CaM binding motifs locate in the N-tail [1–5-10 motif, TRPV6-(88-97)] and C-tail [1–8-14 motif, TRPV6-(643-656)], suggesting a mechanism of regulation involving multiple interaction sites [165]. Electrophysiological measurements of HEK293 cells heterologously co-expressing Ca\textsuperscript{2+}-insensitive CaM mutants and TRPV6 revealed a significantly reduced Ca\textsuperscript{2+} inward current, whereas, no effect was demonstrated on currents of TRPV5-expressing cells [165]. This finding is remarkable given the high homology between both channels, similar Ca\textsuperscript{2+}-dependent regulation of channel activity, and binding of CaM to both channels [41, 165, 166].

2. 80K-H

In an effort to identify novel regulators of Ca\textsuperscript{2+} reabsorption by TRPV5, Gkika et al. used cDNA microarray analysis, which identified PKC substrate 80K-H as a potential associated protein involved in the Ca\textsuperscript{2+}-dependent control of TRPV5 [167]. This study reported a specific interaction between 80K-H and TRPV5 and showed that a highly conserved short peptide sequence (MLERK) in the TRPV5 C-tail is necessary for 80K-H binding [167]. Furthermore, 80K-H was shown to bind Ca\textsuperscript{2+} directly and inactivation of its
two EF-hand structures totally abolishes Ca\(^{2+}\) binding. Electrophysiological studies using 80K-H mutants showed that three domains of 80K-H (a pair of EF-hand structures, a highly acidic glutamic stretch and a His–Asp–Glu–Leu sequence) are critical determinants for TRPV5 activity. Importantly, inactivation of the EF-hand pair reduces the TRPV5-mediated Ca\(^{2+}\) current and increases the TRPV5 sensitivity for intracellular Ca\(^{2+}\), accelerating the feedback inhibition of the channel. None of the 80K-H mutants alters the TRPV5 plasma membrane localization or the association of 80K-H with TRPV5, suggesting that 80K-H has a direct effect on TRPV5 activity at the plasma membrane. It was shown that both proteins co-localize in the distal part of the nephron, indicating that regulation of TRPV5 by 80K-H could occur \textit{in vivo}. Furthermore, parallel transcriptional regulation of both proteins by 1,25-(OH)\(_2\)D\(_3\) and dietary Ca\(^{2+}\) was demonstrated [168]. Taken together, 80K-H acts as novel Ca\(^{2+}\) sensor controlling TRPV5 channel activity at the plasma membrane.

C. Regulation of trafficking of TRPV5 and TRPV6

\textit{S100A10}

Directed trafficking of channels and transporters to the plasma membrane is essential for transcellular ion transport. Previously, an auxiliary protein of TRPV5 and TRPV6 was identified by screening a mouse kidney cDNA library with a yeast two-hybrid system. A bait was constructed with the cytoplasmic C-tail of TRPV5. This study described the identification of the first auxiliary protein for both TRPV5 and TRPV6, named S100A10, which specifically associates with the C-tail of these epithelial Ca\(^{2+}\) channels [169]. Van de Graaf et al. [169] provided the first evidence of a regulatory role for the S100A10-annexin 2 heterotetramer in the trafficking of TRPV5 and TRPV6. S100A10 is predominantly present as a heterotetrameric complex with annexin 2, which has been implicated in numerous biological processes including endocytosis, exocytosis, and membrane-cytoskeleton interactions [170]. The association of S100A10 with TRPV5 and TRPV6 is restricted to a short conserved peptide sequence VATTV located in the C-tail of these channels. This stretch is conserved among all identified species of TRPV5 and TRPV6 [169]. The first threonine (T) of this sequence was identified as the crucial amino acid for binding and channel function. When this particular threonine is mutated, the activity of TRPV5 and TRPV6 is abolished accompanied by a major disturbance in their subcellular localization. This suggests that the S100A10-annexin 2 heterotetramer
facilitates the translocation of TRPV5 and TRPV6 channels towards the plasma membrane. The importance of annexin 2 in this process was demonstrated by showing that small interfering RNA (siRNA)-based downregulation of annexin 2 significantly inhibits TRPV5 and TRPV6 currents. The latter demonstrated that annexin 2 in conjunction with S100A10 is crucial for TRPV5 and TRPV6 activity. Taken together, these findings showed that the S100A10-annexin 2 complex is a significant component for the trafficking of ion channels to the plasma membrane in general and in particular a major regulator of TRPV5 and TRPV6 function and, therefore, maintenance of the Ca^{2+} balance.

**Goal of this thesis**

Ca^{2+} homeostasis is of utmost importance for the normal development and function of the body. Active transcellular Ca^{2+} transport involves a chain of Ca^{2+} transport proteins mediating apical Ca^{2+} influx, transport to the basolateral membrane and extrusion into the bloodstream. Regulation of the two epithelial Ca^{2+} channels, TRPV5 and TRPV6 that mediate the rate-limiting cellular Ca^{2+} entry steps, is pivotal to control the transcellular Ca^{2+} transport rate. In this respect, elucidation of the mechanisms underlying the molecular structure and regulation of TRPV5 and TRPV6 activity is instrumental to better comprehend the molecular nature of Ca^{2+} homeostasis. Although the understanding of function, regulation and structure assembly of the TRP family is developing rapidly, many aspects of TRPV5 and TRPV6 regulation remain elusive. The goal of this thesis was, therefore, to unravel the molecular structure and regulation mechanisms of TRPV5 and TRPV6.

In *chapter 2*, the assembly motif of TRPV5 forming tetrameric complexes was investigated. To this aim, different experimental approaches, including GST pull-down, co-immunoprecipitation assays were applied, by which two critical assembly domains in the N- and C-tail of TRPV5 were identified. At the functional level, electrophysiological analysis, $^{45}$Ca$^{2+}$ uptake and immunocytochemical assays elucidated the pivotal role of TRPV5 assembly in the channel trafficking to the plasma membrane. The aim of *chapter 3* was to identify new TRPV5 or TRPV6 interacting proteins. To this end, we employed a yeast two-hybrid approach using the C-tail of either TRPV5 or TRPV6. The identified interacting protein Rab11a was subsequently characterized using biochemical,
histological, and functional analyses to elucidate a novel operation mode for Rab11a in the regulation of TRPV5 and TRPV6 trafficking to the plasma membrane. In chapter 4, we performed microarray analysis in TRPV5−/− mice in order to screen for genes encoding candidate proteins involved in Ca2+ reabsorption. Via this method, the anti-aging hormone klotho was identified as a putative regulatory protein of TRPV5. Using electrophysiological analysis, 45Ca2+ uptake and cell-surface biotinylation assays to characterize the klotho function, this study showed for the first time a novel mechanism through which a glycosylated cell-surface protein, TRPV5, is regulated by the extracellular β-glucuronidase klotho. In chapter 5, the aim was to investigate the molecular mechanism of hypercalciuria observed in tissue kallikrein knockout (TK−/−) mice. To this end, the relation between TK expression and hypercalciuria was investigated in vivo using Tk−/− and TRPV5−/− mice. Subsequently, the effect of TK on transcellular Ca2+ transport was examined in primary cultures of renal CNT and CCT cells. The signaling pathway through which TK acts on Ca2+ reabsorption as well as its effect on TRPV5 surface expression were delineated in TRPV5-expressing cells. Chapter 6 demonstrated the effect of different glycosidase proteins in addition to β-glucuronidase and klotho on TRPV5 activity. Using 45Ca2+ uptake assay, our study identified another two TRPV5 stimulatory glycosidases: endoF and sialidase. Further, by performing cell-surface biotinylation assay, the mechanism of this glycosidase-mediated TRPV5 activation and the role of N-glycosylation in TRPV5 regulation have been revealed. Moreover, to examine whether the klotho effect is restricted to TRPV5 or more generally applicable to other renal apically localized channels or transporters in DCT cells, we selected the candidates within the TRP channels including TRPV6, TRPV4 and TRPM6. Additionally, a Na+-Cl− cotransporter (NCC) was picked up as a candidate in this study. By applying 45Ca2+ and 22Na+ uptake analyses, we demonstrated a predominantly stimulatory effect of klotho on the epithelial Ca2+ channels TRPV5 and TRPV6. Finally, the findings in this thesis are generally discussed and a summary is presented in chapter 8.

Reference


Introduction


119. P.A. Friedman, Coutermarch B.A., Kennedy S.M., et al. Parathyroid hormone stimulation of calcium transport is mediated by dual signaling mechanisms...


Chapter 2

Molecular determinants in TRPV5 channel assembly

Q. Chang, E. Gyftogianni, S.F.J. van de Graaf, S. Hoefs,
F.A. Weidema, R.J.M. Bindels & J.G.J. Hoenderop

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The Netherlands

The most glorious moment in your life are not the so-called days of success, but rather those days when out of dejection and despair you feel rise in you a challenge to life, and the promise of future accomplishment. (Gustave Flaubert)

你的一生中，最为辉煌的一天并不是功成名就的那些天，而是从悲叹与绝望中产生对人生的挑战和对未来辉煌的期盼的那些日子。（福楼拜 G）
Abstract

The epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6 mediate the Ca\(^{2+}\) influx in 1,25-dihydroxy-vitamin D\(_3\)-responsive epithelia, and are, therefore, essential in the maintenance of the body Ca\(^{2+}\) balance. These Ca\(^{2+}\) channels assemble in (hetero)tetrameric channel complexes with different functional characteristics regarding Ca\(^{2+}\)-dependent inactivation, ion selectivity and pharmacological block. GST pull-downs and co-immunoprecipitations demonstrated an essential role of the intracellular N- and C-tails in TRPV5 channel assembly by physical interactions between N-N tails, C-C tails and N-C-tails. Patch-clamp analysis in human embryonic kidney (HEK293) cells and \(^{45}\)Ca\(^{2+}\) uptake experiments in *Xenopus laevis* oocytes co-expressing TRPV5 wild-type and truncates indicated that TRPV5\(_{\Delta N}\) (deleted N-tail) and TRPV5\(_{\Delta C}\) (deleted C-tail) decreased channel activity of wild-type TRPV5 in a dominant-negative manner, whereas TRPV5\(_{\Delta N\Delta C}\) (deleted N-tail/C-tail) did not affect TRPV5 activity. Oocytes co-expressing wild-type TRPV5 and TRPV5\(_{\Delta N}\) or TRPV5\(_{\Delta C}\) showed virtually no wild-type TRPV5 expression at the plasma membrane, whereas co-expression of wild-type TRPV5 and TRPV5\(_{\Delta N\Delta C}\) displayed normal channel surface expression. This indicates that TRPV5 trafficking towards the plasma membrane was disturbed by assembly with TRPV5\(_{\Delta N}\) or TRPV5\(_{\Delta C}\), but not with TRPV5\(_{\Delta N\Delta C}\). TRPV5 channel assembly signals were refined between amino acid position 64-77 and 596-601 in the N-tail and C-tail, respectively. Pull-down assays and co-immunoprecipitations demonstrated that N- or C-tail mutants lacking these critical assembly domains were unable to interact with tails of TRPV5. In conclusion, two domains in the N-tail (64-77) and C-tail (596-601) of TRPV5 are important for channel subunit assembly, subsequent trafficking of the TRPV5 channel complex to the plasma membrane and channel activity.

Introduction

TRPV5 and TRPV6 constitute the Ca\(^{2+}\) influx pathway in 1,25-dihydroxy-vitamin D\(_3\)-responsive epithelia, including small intestine, kidney and placenta, and play a vital role in the process of Ca\(^{2+}\) (re)absorption \([1-4]\). Both channels belong to a distinct subfamily (TRPV) within the superfamily of Transient Receptor Potential channels (TRP). The TRP family consists of a diverse group of non-voltage-gated cation channels, including TRPC (canonical), TRPM (melastatin) and TRPV (vanilloid) subfamilies, which varies
Molecular determinants in TRPV5 channel assembly

significantly in their selectivity and mode of activation [5]. The understanding of the function, gating, regulation and structure assembly of the TRP family is developing rapidly. Initially, it was demonstrated that the *Drosophila* TRP and TRPL members form heteromultimeric channels associated in a supramolecular signaling complex with specific receptors and regulators [6]. Moreover, it has been identified that there are many channel compositions within the TRPC family, for instance, TRPC1 & 3, TRPC1 & 5, TRPC4 & 5, TRPC3 & 6 & 7 [7-9]. Within the TRPV family, the oligomeric structure of TRPV1 was studied by biochemical cross-linking and the predominant existence of tetramers was suggested [10]. More recently, it has been reported that TRPV5 and TRPV6 form homo- or hetero-tetramers in order to generate a pleiotropic set of functional channels with different Ca²⁺ transport [11-13]. TRPV5 and TRPV6 share 75% sequence homology at the amino acid level [11-13] and display several similar functional properties, including the permeation profile for monovalent and divalent cations [14], and regulation by calcitropic hormones [15-21]. However, detailed sequence comparison of the N- and C-tails of the TRPV5 and TRPV6 channels reveals significant differences, which may account for the unique electrophysiological properties including differences of inactivation, kinetic properties, and affinity for the blocker ruthenium red between these two homologous channels [22].

A considerable amount of information in channel subunit assembly has been accumulated by studies on voltage-gated K⁺ (Kᵥ) channels that are structurally related to the TRP channels. Previous studies indicated that the subfamily of Shaker-related Kᵥ channels possesses a T1 domain in the N-tail, which confers subfamily specificity and inter-subunit assembly [23]. This highly conserved T1 domain has been shown to spontaneously form tetramers in the absence of transmembrane sequences [24, 25]. Crystallization of this domain further verified the reported biochemical and functional data and implicated that T1 is also involved in channel gating [25, 26]. To date, no information is available about assembly domains in TRP proteins. Because TRPV5 shares common structural features with Kᵥ channels, we hypothesized that there is at least one assembly signal within this channel.

The aim of the present study was, therefore, to identify the regions in TRPV5 involved in channel assembly. Using different experimental approaches, including pull-down, co-immunoprecipitation, patch-clamp and immunocytochemical analysis, two critical
domains in the N-tail and C-tail, were identified to be involved in TRPV5 channel assembly and subsequent trafficking to the plasma membrane.

**Material and methods**

**DNA constructs and cRNA synthesis**

The N- and C-tail of TRPV5 were amplified and tagged with a HA or Flag tag, respectively, by the use of PCR on the full-length cDNA of TRPV5, and subsequently cloned into the pGEX 6p-2 (Amersham Pharmacia biotech) vector and the pT7Ts *Xenopus laevis* oocytes expression vector [27]. (For HA-tagged TRPV5 N-tail, forward primer 5’-ATGTACCCATACGACGTGCCAGACTACGCAGGGGCCTGTCCACCCAAGG-3’, and reverse primer 5’-TTAAGGCGGCCGTATTTCCTTC-3’. For Flag-tagged TRPV5 N-tail, forward primer 5’-ATGGACTACAAGGATGACGATGACAAGGGGGCCTGTCCACCCAAGG-3’, and reverse primer 5’-TTAAGGCGGCCGTATTTCCTTC-3’. For HA-tagged TRPV5 C-tail, forward primer 5’-ATGTACCCATACGACGTGCCAGACTACGCAGGCGACACTCACTGGCGGG-3’, and reverse primer 5’-TCAGAAATGGTAGACTTCC-3’. For Flag-tagged TRPV5 C-tail, forward primer 5’-ATGGACTACAAGGATGACGATGACAAGGGCGACACTCACTGGCGGG-3’, and reverse primer 5’-TCAGAAATGGTAGACTTCC-3’). TRPV5 truncates in which the N-tail (TRPV5ΔN), C-tail (TRPV5ΔC) or both tails (TRPV5ΔNΔC) were deleted, were obtained by PCR and cloned into the pCINeo/IRES-GFP vector and subcloned into the pT7Ts vector [21]. Deletion mutants of the assembly domain in the N-tail (deleted amino acids 64-76) or the C-tail (deleted amino acids 595-600) were obtained by *in vitro* mutagenesis and subcloned into the PT7Ts vector. (For N-tail deletion, forward primer 5’-CTGCGTCTCCTTAAGATAGCTGTGGGGGAGACGGCG-3’, and reverse primer 5’-CCACAGGAAGCGGCCGTGCTGCTCCGCTGGG-3’. For C-tail deletion, forward primer 5’-GTTGTGCCACACCACCTGATGCTGGCTCTCCGCTGG-3’, and reverse primer 5’-CTGCGTCTCCTTAAGATAGCTGTGGGGGAGACGGCG-3’, and reverse primer 5’-TCAGAAATGGTAGACTTCC-3’). TRPV5 truncates in which the N-tail (TRPV5ΔN), C-tail (TRPV5ΔC) or both tails (TRPV5ΔNΔC) were deleted, were obtained by PCR and cloned into the pCINeo/IRES-GFP vector and subcloned into the pT7Ts vector [21]. Deletion mutants of the assembly domain in the N-tail (deleted amino acids 64-76) or the C-tail (deleted amino acids 595-600) were obtained by *in vitro* mutagenesis and subcloned into the PT7Ts vector. (For N-tail deletion, forward primer 5’-CTGCGTCTCCTTAAGATAGCTGTGGGGGAGACGGCG-3’, and reverse primer 5’-CCACAGGAAGCGGCCGTGCTGCTCCGCTGGG-3’. For C-tail deletion, forward primer 5’-GTTGTGCCACACCACCTGATGCTGGCTCTCCGCTGG-3’, and reverse primer 5’-CTGCGTCTCCTTAAGATAGCTGTGGGGGAGACGGCG-3’, and reverse primer 5’-TCAGAAATGGTAGACTTCC-3’). pT7Ts constructs were linearized and cRNA was synthesized *in vitro* as described previously [21]. All constructs were verified by sequence analysis.
Electrophysiology
The full-length cDNA encoding wild-type TRPV5, TRPV5ΔN, TRPV5ΔC and TRPV5ΔNΔC were transfected in HEK293 cells as described previously [22, 28]. Currents using the whole-cell configuration were measured with an EPC-9 (HEKA Elektronik, Lambrecht, Germany; 8-Pole Bessel filter 10 kHz). Electrode resistances were between 2 and 5 MΩ, capacitance and series resistance were compensated, and access resistance was monitored continuously. The step protocol consisted of 3 s voltage steps to –100 mV from a holding potential of +70 mV. The standard extracellular solution contained 150 mM NaCl, 1 mM CaCl₂, 6 mM CsCl, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES/CsOH (pH 7.4). Monovalent cation currents were measured in nominally Ca²⁺- and Mg²⁺- free solution (free Ca²⁺ concentration is 10 nM), and Ca²⁺ currents in 1 mM CaCl₂ but Mg²⁺- free solutions. Monovalent cation currents were inhibited by replacing 150 mM NaCl with an equimolar amount of N-methyl-D-glucamine NMDG-Cl. The standard internal (pipette) solution contained: 20 mM CsCl, 100 mM Cs aspartate, 1 mM MgCl₂, 10 mM BAPTA, 4 mM Na-2ATP and 10 mM HEPES / CsOH (pH 7.2). Cells were kept in a nominally Ca²⁺-free medium to prevent Ca²⁺ overload, and exposed for a maximum of 5 min to a Krebs solution containing 1.5 mM Ca²⁺ before sealing the patch pipette to the cell. All experiments were performed at room temperature (RT; 20–22°C).

GST fusion proteins and pull-down assay
PGEX6p-2 constructs were transformed in E.coli BL21, and GST-fused proteins were expressed and purified as described previously [21]. Proteins of full-length TRPV5, TRPV5 N-tail, C-tail or N- and C-tail deletion mutants were translated in vitro with [³⁵S]methionine (Dupont/NEN Research Products, Boston, MA) for 120 min at 30°C using rabbit reticulocyte lysates (Promega, Madison, WI). In vitro translated proteins were added to purified GST-fused truncants, immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech AB). After 2 h incubation at RT, the beads were washed extensively with pull-down buffer [20 mM Tris-HCl PH 7.4, 140 mM NaCl, 1 mM CaCl₂, 0.2% (v/v) Triton-X-100, 0.2% (v/v) NP-40], and bound proteins were eluted with SDS-PAGE loading buffer and separated on SDS-polyacrylamide gels. Following electrophoresis, gels were analyzed by autoradiography [21].
Injection of oocytes and total membrane isolation

*Xenopus laevis* oocytes were prepared and injected as described previously [21]. To isolate total lysates, 20 oocytes were homogenized in 200 μl homogenization (HBA) buffer [20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 5 mM NaH₂PO₄, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin] and centrifuged twice at 100 g for 10 min at 4°C to remove yolk proteins. Next total lysates were taken up in Laemmli buffer (2 μl/oocyte) at 37°C for 30 minutes [2, 22, 29].

Immunoblot analysis

Total lysates were subjected to SDS-PAGE electrophoresis (12-16% w/v) and blotted onto PVDF membranes (Millipore Corp. Bedford, MA, USA). Blots were incubated with 5% (w/v) non-fat dried milk (NFDM) in TBS-T [Tris-buffered saline (pH 7.4) containing 0.2% (v/v) Tween-20]. Immunoblots were incubated overnight at 4°C with mouse anti-HA (1:4000) or mouse anti-Flag (1:8000) antibodies (Sigma chemical Co., St. Louis, MO, USA), in 1% or 5% (w/v) NFDM in TBS-T, respectively. After washing, immunoblots were incubated at RT with the corresponding secondary antibody sheep anti-mouse IgG peroxidase (Sigma), (1:2000) in TBS-T. For co-immunoprecipitation assays, immunoblots were incubated overnight at 4°C with mouse anti-Flag-peroxidase coupled antibody (1:2000) (Sigma), 5% (w/v) NFDM in TBS-T. Immunopositive bands were visualized using an enhanced chemo-luminescence system (Pierce, Rockford, IL, USA).

Co-immunoprecipitation

Twenty microliter equivalents of protein A-coupled agarose beads (Pharmacia, Uppsala, Sweden) were pre-incubated for 3 h at RT with 2 μl of monoclonal anti-HA antibody (Sigma) in 0.7 ml of IPP500 [500 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) NP-40, 0.1% (v/v) Tween-20, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin] and 0.1% (w/v) bovine serum albumin. The beads were washed three times with IPP100 [100 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) NP-40, 0.1% (v/v) Tween-20, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin]. Thirty oocytes were co-injected (each 12.5 ng) with HA- and Flag-tagged TRPV5 N-tail or C-tail cRNA or cRNA transcribed form the deletion mutants. Oocytes were subsequently homogenized with 150 μl of solubilization buffer [20 mM Tris pH 8.0, 10% (v/v) glycerol, 5 mM EDTA, 0.5% (v/v) NP-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin] and incubated on ice for 1 h and centrifuged at 16,000 g for 1 h at 4°C. The solubilized proteins were added to
antibody bound beads in sucrose buffer [100 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 0.1% (v/v) Triton X-100, 10% (w/v) sucrose, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin] and incubated o/n at 4°C. Subsequently, the beads were washed with IPP100 and proteins were eluted in Laemmli as described previously [29].

**Immunocytochemistry**

*Xenopus laevis* oocytes were injected with 5 ng cRNA of HA-tagged TRPV5, or co-injected with 5 ng cRNA of HA-tagged TRPV5 and 10 ng non-tagged TRPV5 mutants (TRPV5ΔN, TRPV5ΔC, TRPV5ΔNΔC), respectively. Immunocytochemistry was performed as described previously [30, 31].

**45Ca2+ uptake assay**

*Xenopus laevis* oocytes were injected with 5 ng HA-TRPV5 cRNA only or co-injected with 10 ng TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC cRNA. Ca2+ uptake was determined 2 days after injection as described previously [2].

**Plasma membrane isolation**

*Xenopus laevis* oocytes were injected with 5 ng HA-TRPV5 cRNA only or co-injected with 5 ng HA-TRPV5 and 10 ng TRPV5 truncates cRNA (TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC). After 48 h follicle membranes were manually removed and 12 oocytes were incubated in MBSS [20 mM 2-N-morpholino ethanesulfonic acid, 80 mM NaCl, pH 6.0, containing 1 % (v/v) positively charged colloidal silica (Ludox Cl, Aldrich, Bornem, Belgium)] at 4°C for 30 min [29]. Subsequently, oocytes were washed twice in MBSS and incubated with 0.1 % (v/v) sodium polyacrylicacid (Aldrich) in MBSS at 4°C for 30 min. Oocytes were homogenized in HBA buffer and plasma membranes were isolated by serial centrifugation at 4x 13.5g, 2x 24g and 2x 38g. One ml supernatant was exchanged with HBA buffer after each centrifugation step. Finally, membranes were pelleted at 16,000 g for 30 min, dissolved in SDS sample buffer and analyzed by immunoblotting.

**Statistical analysis**

In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). P values below 0.05 were considered significant.
Results

Pull-down assay of N-tail and C-tail of TRPV5

Figure 1. Pull-down analysis of N-tail and C-tail of TRPV5. GST fusion proteins containing N-tail and C-tail of TRPV5 were immobilized on glutathione-Sepharose 4B beads and incubated with \textit{in vitro} translated [\textsuperscript{35}S]methionine full-length TRPV5, TRPV5 N-tail or C-tail. The assembly was detected between \textit{in vitro} translated full-length of TRPV5 and GST-fused TRPV5 N-tail or C-tail \textbf{(A)}. Furthermore, an interaction of the N-tail with the N-tail \textbf{(B)} or the C-tail with C-tail \textbf{(C)} was observed. Incubation of the GST-N-tail with both \textit{in vitro} translated N-tail and C-tail indicated a preference for N-N interaction rather than N-C interaction \textbf{(D)}. No binding to GST alone was detected.

The role of TRPV5 N-tail and C-tail in the channel assembly was initially examined using pull-down assays. TRPV5 N-tail and C-tail were expressed as GST-fusion proteins and subsequently tested for their interaction with \textit{in vitro} translated full-length of TRPV5 or the TRPV5 N-tail and C-tail. As depicted in \textbf{Figure 1A}, the GST-fused N-tail and C-tail associated with \textit{in vitro} translated [\textsuperscript{35}S]methionine-TRPV5. Moreover, pull-down analysis demonstrated N-tail-N-tail \textbf{(Figure 1B)} and C-tail-C-tail interactions \textbf{(Figure 1C)}. To investigate whether the N-tail preferentially interacts with the N- or C-tail, the GST-N-tail was incubated with \textit{in vitro} translated N- and C-tail simultaneously. This pull-down experiment indicated that the GST-N-tail preferentially binds the \textit{in vitro} translated N-tail \textbf{(Figure 1D)}. All interactions were specific, as no interaction with GST alone was observed.
Molecular determinants in TRPV5 channel assembly

Co-immunoprecipitation of N-tail and C-tail of TRPV5

The findings from the pull-down experiments suggested that the N-tail and C-tail of TRPV5 contributed to channel assembly. We tested, therefore, whether the N-tail and C-tail of TRPV5 can be co-immunoprecipitated. First, Immunoblot analysis confirmed expression of both tails that were specifically detected by the applied anti-HA or anti-Flag antibody, respectively. The anti-HA antibody specifically detected the HA-N-tail in total lysates from oocytes co-expressing HA-N-tail and Flag-C-tail, whereas anti-Flag antibody only recognized the Flag-C-tail (Figure 2A). The HA- and Flag-tagged N-tails (Figure 2B), HA- and Flag-tagged C-tails (Figure 2C), or HA-N-tail with Flag-C-tail of TRPV5 (Figure 2D) were co-expressed and subsequently immunoprecipitated using the anti-HA antibody. Immunoblots containing the immune complexes were probed with the anti-Flag antibody. Interestingly, Figure 2 showed that Flag-tagged N-tail or C-tail was co-immunoprecipitated with the HA-tagged N-tail or C-tail of TRPV5, demonstrating N-N, C-C and N-C interactions in a TRPV5 channel complex.

Figure 2. Co-immunoprecipitation of N-tail with C-tail of TRPV5. *Xenopus laevis* oocytes were co-injected with cRNA of HA- and Flag-tagged N-tails, C-tails or HA-tagged N-tail and Flag-tagged C-tail of TRPV5, respectively. The cell lysates were processed for immunoprecipitation and immunoblot analysis. Anti-HA antibody specifically recognized HA-N-tail in total lysates from oocytes co-expressing HA-N-tail and Flag-C-tail, whereas anti-Flag antibody only detected the Flag-C-tail (A). Subsequently, Oocyte lysates were subjected to immunoprecipitation using the anti-HA antibody, and immunoblots containing protein complexes were probed with the anti-Flag antibody. Co-immunoprecipitations were observed between HA- and Flag-tagged N-tails (B), HA- and Flag-tagged C-tails (C), and HA-tagged N-tail and Flag-tagged C-tail of TRPV5 (D).
Functional analysis of TRPV5 channel assembly

(A) 

(B) 

(C) 

(D) 

Figure 3. Functional analysis of TRPV5 channel assembly. (A) $^{45}$Ca$^{2+}$ uptake was measured in oocytes injected with TRPV5 cRNA only or co-injected with TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC cRNA. (B) HEK293 cells were transfected with wild-type TRPV5 or TRPV5 truncates including TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC, or co-transfected with wild-type TRPV5 and TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC, and subsequently analyzed by patch-clamp analysis. Asterisk indicates significance compared to TRPV5 transfected cells, p<0.05). The Na$^{+}$ currents in HEK293 cells transfected with TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC only were not significant different from non-transfected cells (data not shown). HA-TRPV5 and truncates were co-injected in Xenopus laevis oocytes. To isolate plasma membrane fractions follicle membranes were removed and plasma membranes were coated with silica. Plasma membrane fractions (C) or total membranes (D) were isolated, blotted and analyzed for TRPV5 expression using monoclonal anti-HA.

To further elucidate the functional consequences of the observed interactions between N-N tail, N-C tail and C-C tail, the effect of co-expressing TRPV5 wild-type and truncates (TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC) on TRPV5 activity at the plasma membrane was determined in Xenopus laevis oocytes and HEK293 cells. Firstly, oocytes were injected with HA-TRPV5 only or co-injected with TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC cRNA. Expression of TRPV5 resulted in ~3.5-fold increase of $^{45}$Ca$^{2+}$ influx compared to non-injected oocytes (Figure 3A). Co-expression of TRPV5ΔN significantly decreased the TRPV5-mediated Ca$^{2+}$ influx compared to TRPV5 alone, while co-expression of TRPV5ΔC completely inhibited Ca$^{2+}$ influx to a level that was indistinguishable from non-
injected oocytes. In contrast, co-expression of TRPV5ΔNΔC had no significant effect on TRPV5 channel activity (Figure 3A). 45Ca2+ uptake in oocytes expressing only TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC was not different from non-injected oocytes (data not shown). Secondly, patch-clamp analysis was performed to investigate the electrophysiological properties of channel complexes consisting of wild-type and TRPV5 truncates. Truncated channels, including TRPV5ΔN, TRPV5ΔC and TRPV5ΔNΔC, were (co)expressed with wild-type TRPV5 in HEK293 cells. As shown in Figure 3B, HEK293 cells expressing TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC did not yield any Na+ currents and were not different from non-transfected cells (data not shown). Remarkably, HEK293 cells co-expressing wild-type TRPV5 and TRPV5ΔN or TRPV5ΔC displayed significantly reduced Na+ currents compared to wild-type TRPV5 alone. Inversely, Na+ currents measured in HEK293 cells co-expressing wild-type TRPV5 and TRPV5ΔNΔC were not significantly affected, indicating that it is unlikely that the transmembrane domains of TRPV5 are critical determinants for subunit assembly.

**TRPV5 routing is disturbed by mutant channel assembly**

In order to understand the molecular mechanism underlying the dominant-negative effect of TRPV5 truncates (TRPV5ΔN and TRPV5ΔC) on wild-type TRPV5 channel activity, a biochemical and immunocytochemical approach was combined. The expression of HA-TRPV5 was determined by analyzing plasma membrane and total membrane fractions of oocytes expressing TRPV5 alone or in combination with the truncates. A distinct band at a molecular weight of ~85 kDa corresponding to the glycosylated form of TRPV5 was observed in plasma membrane fractions of TRPV5-expressing oocytes (Figure 3C). Importantly, wild-type TRPV5 expression was significantly reduced or absent in plasma membrane fractions of oocytes co-expressing TRPV5ΔN or TRPV5ΔC, respectively. However, co-injection of TRPV5ΔNΔC had no effect on the plasma membrane localization of TRPV5 (Figure 3C). Importantly, total membrane fractions showed that TRPV5 was expressed to a similar extent in the absence and presence of the TRPV5 truncates (Figure 3D). These results were verified by immunocytochemistry to detect TRPV5 localization at the plasma membrane. In contrast to non-injected oocytes (Figure 4A), wild-type TRPV5 cRNA-injected oocytes displayed a strong immunopositive labeling along the plasma membrane, whereas the cytoplasm was only faintly stained (Figure 4B). Importantly, plasma membrane staining...
of TRPV5 was significantly decreased in oocytes co-expressing HA-TRPV5 and TRPV5ΔN and to a lesser extent HA-TRPV5 and TRPV5ΔC (Figure 4D, E). A strong immunopositive staining was, however, present along the plasma membrane of oocytes co-injected with HA-TRPV5 and TRPV5ΔNΔC (Figure 4C). The localization of TRPV5 in the presence of TRPV5ΔN or TRPV5ΔC was predominantly intracellular. Together, these results indicated that the trafficking of HA-TRPV5 from cytosol towards the plasma membrane was disturbed by assembly with TRPV5ΔN or TRPV5ΔC, but not with TRPV5ΔNΔC, explaining the reduced channel activity. Importantly, immunoblot analysis demonstrated that the total expression of TRPV5 was equal under all conditions (Figure 3D).

Identification of the assembly signal in the N- and C-tail of TRPV5

To gather more detailed information regarding structural requirements for channel subunit assembly of TRPV5, a series of deletion mutants of the N- and C-tails were constructed (Figure 5). Truncated forms of TRPV5 N-tail and C-tail were expressed as
GST-fusion proteins and subsequently tested for their interaction with in vitro translated TRPV5 N-tail and C-tail using pull-down assays. As depicted in Figure 5 (left part), the interaction between GST-fused N-tail truncations and the in vitro translated N-tail was abolished at amino acid position 64 and 55, whereas truncations at the positions 240 up to 77 associated with in vitro translated N-tail. In addition, pull-down analysis demonstrated that the association of the C-tail and [35S]methionine-TRPV5 C-tail disappeared when the tail was truncated at position 596, whereas truncations at position 704 up to 601 did not affect the interaction (Figure 5, right part). Therefore, two critical regions for TRPV5 channel assembly were located between amino acid position 64-77 (Figure 5, left part) and 596-601 (Figure 5, right part) in the N-tail and C-tail, respectively.

**Figure 5.** Mapping of assembly domains in TRPV5. GST fusion proteins containing different portions of the N-tail (left) and C-tail (right) of TRPV5 were constructed according to the schematic drawing. These proteins were immobilized on glutathione-Sepharose 4B beads and then incubated with in vitro translated [35S]methionine TRPV5 N-tail (left) or C-tail (right). Interaction of the N-tail or C-tail with the GST fusion proteins was determined by immunoblotting. The assembly domain in the N-tail was localized between 64 and 77 (left), whereas the interaction region in the C-tail (right) was observed between 596 and 601. GST was used as a negative control in the pull-down experiments. These assembly domains identified in the N- and C-tail are functionally conserved within TRPV5 and TRPV6 members as indicated by the alignments.
To further elucidate the role of the assembly domains these regions were deleted in the N-tail (N-tail\(\Delta64-76\)) and the C-tail (C-tail\(\Delta596-600\)) and subsequently analyzed by pull-downs and co-immunoprecipitations as described above. First, the N-tail and the C-tail of TRPV5 were expressed as GST-fusion proteins and subsequently analyzed for their interaction with in vitro \[^{35}S\]methionine translated N-tail, C-tail and the N-tail and C-tail deletion mutants. In contrast to wild-type N-tail and C-tail, N-tail\(\Delta64-76\) and C-tail\(\Delta596-600\) did not interact with the N- and C-tail (Figure 6A). Second, *Xenopus laevis* oocytes were co-injected with wild-type and mutant HA-N-tail and Flag-N-tail or HA-C-tail and Flag-C-tail. Co-immunoprecipitations demonstrated that assembly between HA-N-tail/Flag-N-tail\(\Delta64-76\), HA-N-tail/Flag-C-tail\(\Delta596-600\) and HA-C-tail/Flag-C-tail\(\Delta596-600\) was abolished, whereas binding between wild-type N- and C-tails was observed (Figure 6B, left). Importantly, both wild-type and mutant tails were equally expressed (Figure 6B, right).

![Figure 6. Pull-downs and co-immunoprecipitation assays of N- and C-tail deletion mutants of TRPV5. (A)](image) GST fusion proteins containing the N-tail and C-tail of TRPV5 were immobilized on glutathione-Sepharose 4B beads and incubated with in vitro translated \[^{35}S\]methionine TRPV5 N-tail and C-tail or N-tail mutant lacking amino acid residues 64-76 (N-tail\(\Delta64-76\)) and C-tail mutant lacking amino acid residues 596-600 (C-tail\(\Delta596-600\)). (B) *Xenopus laevis* oocytes were co-injected with HA-N-tail and Flag-N-tail or Flag-N-tail\(\Delta64-76\), HA-N-tail and Flag-C-tail or Flag-C-tail\(\Delta596-600\), HA-C-tail and Flag-C-tail or Flag-C-tail\(\Delta596-600\), respectively. Cell lysates were processed for immunoprecipitation, immunoblotting and subsequently protein complexes were probed with the anti-Flag antibody. Both wild-type and mutant tails were equally expressed (right).
Discussion

In the present study molecular determinants were identified in TRPV5 that play a key role in the formation of the functional channel complex. Using a combined approach of several independent methods we conclude that at least two regions in the cytosolic tails of TRPV5 are important for channel assembly and subsequent routing to the plasma membrane. This conclusion is based on (i) pull-down assays and co-immunoprecipitations that demonstrated physical interactions between the N-tail/N-tail, N-tail/C-tail and C-tail/C-tail of TRPV5; (ii) $^{45}\text{Ca}^2+$ uptake measurements in oocytes and patch-clamp analysis in HEK293 cells that showed the dominant-negative nature of TRPV5 truncated channel subunits, lacking the N-tail or C-tail, on TRPV5 wild-type channel activity; (iii) plasma membrane fractions and immunocytochemical analysis that demonstrated a disturbed trafficking of TRPV5 in oocytes co-expressing TRPV5$_\Delta$C and TRPV5$_\Delta$N towards the plasma membrane explaining the molecular mechanism of the reduced channel activity; (iv) mapping assays that revealed two critical assembly domains located at positions 64-77 in the N-tail and 596-601 in the C-tail of TRPV5; (v) deletion of these assembly domains in the N-tail and C-tail that abolished the interaction between the tails pointing to a important role of these regions in channel assembly.

By analogy with other cation channel subunits consisting of six transmembrane-spanning domains, TRP members are believed to assemble into homo- or heterotetrameric complexes. These complexes have been verified by classical methods such as co-immunoprecipitation, crosslinking analysis or functional assays applying dominant negative pore mutants [7-10, 32]. Recently, we demonstrated a (hetero)tetrameric stoichiometry for TRPV5 and TRPV6 [32]. Heterotetrameric TRPV5 and TRPV6 proteins displayed properties that, depending on the subunit configuration, are intermediate between TRPV5 and TRPV6. Exchanging TRPV5 for TRPV6 subunits in a channel tetramer has major effects on Ba$^{2+}$ permeability, Ca$^{2+}$-dependent inactivation and the block by ruthenium red [32]. In this way, Ca$^{2+}$ transporting epithelia co-expressing TRPV5 and TRPV6 may be able to generate a pleiotropic set of functional heterotetrameric channels. To date, little information is available about assembly domains in TRP proteins.
Our understanding of the architecture of functional TRP channels reflects only the endpoint of a complex and poorly understood assembly pathway. Current research is beginning to elucidate the intermediate steps between the synthesis and insertion of individual subunits into the membrane. Our data indicated N-tail-N-tail and C-tail-C-tail interactions within TRPV5 channel complexes. In addition, co-immunoprecipitations demonstrated that assembly of TRPV5 subunits occurred not only within N-tails or C-tails, but also between the N-tail and C-tail. In order to further explore the crucial domains in the N-tail and C-tail for TRPV5 channel assembly, we constructed a series of truncated deletion mutants. Pull-down analysis indicated that a short peptide stretch at 64-77 is necessary for N-tail/N-tail interaction, whereas the critical region for C-tail/C-tail interaction is located between amino acids 596 and 601. Furthermore, we demonstrated by pull-down assays and co-immunoprecipitations that N- and C-tail mutants lacking these assembly domains are not able to interact with the TRPV5 tails. These findings indicated that the interaction between TRPV5 channel subunits depends on assembly signals present in the N-tail and C-tail.

It is likely that assembly also occurs within the N-tail and C-tail of TRPV6 because TRPV5 and TRPV6 share more than 75% homology at the amino level, raising the possibility that both N-tail and C-tail assembled together in order to form functional heterotetrameric channel complexes of TRPV5 and TRPV6 [22]. Recently, Niemeyer and co-workers demonstrated that the third ankyrin repeat within the N-tail of TRPV6 is critical for TRPV6 subunit assembly. This repeat initiates a molecular zippering process that proceeds past the fifth ankyrin repeat and, thereby, creates an intracellular anchor necessary for functional subunit assembly. In addition, deletion of this region prevented TRPV6-TRPV6 self association and generation of functional channels [33]. Pull-down mapping experiments and co-immunoprecipitations in our study identified an assembly domain in the N-tail of TRPV5 which is located more upstream, overlapping with the first ankyrin repeat. The most significant binding was found between the N-terminal regions 64 to 77. However, a slightly stronger binding signal was observed with the truncant at position 162 that contains the first three ankyrin repeats [33]. These findings may suggest that the ankyrin repeat identified in TRPV6 could also be involved in TRPV5 channel assembly. Ankyrin repeats have been frequently implicated in protein-protein interactions. In addition, structure prediction programs indicated that both assembly
domains identified in TRPV5 participate in a predicted α-helical structure that is often engaged in protein-protein interactions to form higher order structures [34].

Detailed information about the domains involved in the process of channel subunit assembly of TRPV5 is a prerequisite for obtaining further insight into the function and properties of this channel. $^{45}\text{Ca}^{2+}$ uptake measurements demonstrated that the $\text{Ca}^{2+}$ influx is significantly decreased or abolished in oocytes co-injected with wild-type TRPV5 and TRPV5ΔN or TRPV5ΔC, respectively. These findings are consistent with the patch-clamp analysis in which the Na$^+$ currents were significantly reduced in HEK293 cells co-expressing wild-type TRPV5 with TRPV5ΔN or TRPV5ΔC. Plasma membrane fractions and immunocytochemistry in oocytes demonstrated the dominant negative effect of TRPV5ΔN or TRPV5ΔC on trafficking of the wild-type TRPV5 channel to the plasma membrane. Intriguingly, TRPV5 surface expression and activity was significantly more reduced in cells co-expressing TRPV5ΔC compared to TRPV5ΔN. This suggests a major role of the N-tail domain rather than the C-tail domain in channel assembly, which was further substantiated by pull-down experiments in which the GST-N-tail preferentially binds the N-tail compared to the C-tail. Importantly, we demonstrated that the TRPV5 channel activity was not significantly affected in cells co-expressing wild-type TRPV5 with TRPV5ΔNΔC, which was further substantiated by a normal channel distribution at the plasma membrane of the oocyte underlining the specific involvement of the cytoplasmic tails.

Our observations are in line with the structurally related K$^+$ channels. The role of the N-tail in the oligomerization of Shaker K$^+$ subunits was first recognized by the laboratory of Jan and co-workers [35]. They identified a cytoplasmic sequence preceding the first transmembrane segment that was conserved among Kv1 subfamily members. In Shaker channels, the conserved region is found between residues 97 and 196 and named T1 [36]. Subfamily-specific assembly is provided primarily by polar interactions encoded in a conserved set of amino acids at its tetramerization interface [25]. Despite the overall structural similarity between the various K$^+$ channel families, there appears to be little or no mixing of K$^+$ channel subunits between channel families [26, 35, 37-39]. In the Shaker Kv1 subfamily, the N-tail domain mediates oligomerization and prevents heteromultimer formation with members of other Kv subfamilies [35]. Structures of the T1 domains of Shaker K$^+$ channel subfamilies provided valuable structural insights into understanding
both channel assembly and functional regulation of the entire channel molecule through conformational changes [25]. Interestingly, previous studies suggested that T1 plays a role not only in channel assembly, but also in channel gating, and that conformational changes across the buried polar interface between subunits are a crucial part of the gating process [40]. Further studies will address the question whether the identified assembly signals in TRPV5 play also a role in channel gating.

In summary, both the N-tail (64-77) and C-tail (596-601) are critical for TRPV5 channel assembly. Truncated TRPV5 channels at the N- or C-tail oligomerize with wild-type TRPV5 channels leading to the formation of mutant channel complexes that do not reach the plasma membrane. Therefore, assembly of channel subunits is essential for routing of the TRPV5 channel complex and subsequent activity at the plasma membrane.

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References
Molecular determinants in TRPV5 channel assembly


Direct interaction with Rab11a targets the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6 towards the plasma membrane


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As fruit needs not only sunshine but cold nights and chilling showers to ripen it, so character needs not only joy but trial and difficulty to mellow it. (Hugh Black)

水果不仅需要阳光，也需要凉夜，寒冷的雨水能使其成熟。人的性格陶冶不仅需要欢乐，也需要考验和困难。 (布莱克 H)
Abstract

TRPV5 and TRPV6 are the most Ca\textsuperscript{2+}-selective members of the Transient Receptor Potential (TRP) family of cation channels and play a pivotal role in the maintenance of body Ca\textsuperscript{2+} balance. However, little is known about the mechanisms controlling the plasma membrane abundance of these channels to regulate epithelial Ca\textsuperscript{2+} transport. In this study, we demonstrated the direct and specific interaction of GDP-bound Rab11a with TRPV5 and TRPV6. Rab11a co-localized with TRPV5 and TRPV6 in vesicular structures underlying the apical plasma membrane of Ca\textsuperscript{2+}-transporting epithelial cells. This GTPase recognized a conserved stretch in the C-tail of TRPV5 that is essential for channel trafficking. Furthermore, co-expression of GDP-locked Rab11a with TRPV5 or TRPV6 resulted in significantly decreased Ca\textsuperscript{2+} uptake, caused by diminished channel cell-surface expression. Together, our data demonstrated the important role of Rab11a in the trafficking of TRPV5 and TRPV6. Rab11a exerts this function in a novel fashion since it operates via direct cargo interaction while in the GDP-bound configuration.

Introduction

TRPV5 and TRPV6 form a distinct group of highly Ca\textsuperscript{2+}-selective channels belonging to the Transient Receptor Potential (TRP) channel superfamily. This family fulfills a plurality of physiological functions, which vary from photo transduction, nociception, olfaction, heat and cold sensation to epithelial Ca\textsuperscript{2+} transport [1]. TRPV5 and TRPV6 mediate the rate-limiting luminal influx step of transcellular Ca\textsuperscript{2+} transport [2, 3]. Therefore, understanding the regulation of these channels is of utmost importance for our insight in the Ca\textsuperscript{2+} homeostasis. TRPV5 and TRPV6 display constitutive activity [4], suggesting that channel abundance at the cell surface is of crucial importance for regulation of the Ca\textsuperscript{2+} influx via these channels. It is becoming increasingly evident that channel trafficking is essential in determining the activity of TRP proteins [5-9]. For instance, inducible vesicular translocation and plasma membrane insertion has been described for several TRP channels, for instance, TRPV2, TRPC3, TRPC5 and TRPL. This trafficking process stimulated the Ca\textsuperscript{2+} influx from the extracellular compartment essential to growth factor- [6, 8], carbachol- [5] or light-induced signaling [7]. However, little is known about the molecular mechanisms involved in trafficking towards the plasma membrane of TRP channels in general and in particular of TRPV5 and TRPV6.
Several protein families have been described to play key roles in cargo trafficking to and from specific cellular compartments, including the plasma membrane. One of these families consists of the Rab GTPases. The ability to act as molecular switches that cycle between GTP- and GDP-bound states, underlies the functionality of this family. Many Rab proteins show a distinct subcellular localization, making them ideal candidates to govern the specificity of vesicle trafficking, most likely cooperatively operating with other proteins [10, 11]. Further characterization of the largely elusive mechanism underlying the function of Rab proteins in ion channel trafficking could further contribute to the already enigmatic Rab-dependent regulation of cargo trafficking in the highly organized cellular transport machinery.

The aim of this study was to identify regulatory proteins directly interacting with TRPV5 or TRPV6. Using biochemical, histological and functional analyses we demonstrate a novel operation mode for Rab11a in the regulation of TRPV5 and TRPV6 trafficking to the plasma membrane requiring direct interaction with these cargo molecules.

**Materials and methods**

**DNA constructs and cRNA synthesis**

The C-tail of mouse TRPV5 and TRPV6 and deletion mutants of TRPV5 in pGEX6p-2 were obtained as described previously [12]. A VSV tag-encoding oligo duplex (sense 5’-CATGGCATACTGATATCGAAATGAACCGCCTGGGTAAGGCCGCCTTT-3’ and antisense 5’-CTAGAAGGGCGCGTTACCCAGGCCTTTCA-3’) was inserted into the Ncol/XbaI restriction sites of the pTLN oocyte expression vector. Rab11a was cloned into this construct by PCR (forward primer 5’-GGCGCGCTCGATATCGAAATGAACCGCCTGGGTAAGGCCGCCTTT-3’ and reverse primer 5’-GTGAACTTGCGGGGTTTTTCAGTATCTACGA-3’) using the pACT2 construct as a template and subsequently subcloned into pGEX6p2 (Amersham Biosciences, Uppsala, Sweden) and pCB7 [13]. MluI and SalI sites were introduced into the lentivirus transfer vector pLV-CMV-GFP [14] by site-directed mutagenesis replacing the GFP stop codon. Rab11aS25N was subsequently cloned into these sites by PCR using the forward primer 5’-GGCGCGCTCGATATCGAAATGAACCGCCTGGGTAAGGCCGCCTTT-3’ and the reverse primer 5’-ATGAACTTGCGGGGTTTTTCAGTATCTACGA-3’. TRPV5 was cloned into eGFP-C1 (Clontech Palo Alto, CA) by PCR (forward primer 5’-TCCGGACGGGGGGGATGGGGGTCGCTGTCCACC-3’ and reverse primer 5’-
CCGGTGATCTGATCAG-3'). The construct encoding DsRed-fused Rab11a was generously provided by Dr. U. Rescher (University of Muenster, Germany) and S20V and S25N mutants were subsequently obtained by site-directed mutagenesis. All constructs were verified by sequence analysis. Oocyte expression constructs were linearized and TRPV5, TRPV6 and Rab11a cRNA was synthesized \emph{in vitro} using SP6 RNA polymerase as described previously [15].

**Yeast two-hybrid system**

Yeast was subsequently transformed with pAS-1 containing the TRPV6 C-tail, and a mouse kidney cDNA library (Clontech, Palo Alto, CA) present in the pACT2 vector. Screening of the library was performed as described previously [12]. Yeast two-hybrid results were confirmed using purified library plasmids and negative controls were performed by replacing a binding partner with either a pAS-1 construct containing the N-tail (amino acids 1-53) of rat γENaC or the empty pACT2 vector.

**GST-TRPV5 and TRPV6 fusion protein and interaction assays**

pGEX6p-2 constructs were transformed in \textit{Escherichia coli} BL21 and GST fusion proteins were expressed and purified according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ). GST-Rab proteins were prepared in GTP or GDP bound conformation as described [16]. Rab11a S25N was cleaved from GST using Precission Protease (Amersham Biosciences), concentrated using Centriprep YM10 (Millipore, Amsterdam, The Netherlands) and checked by SDS-PAGE. \textsuperscript{[35]S]Methionine-labeled full-length Rab11a or TRPV5/TRPV6 protein was prepared using a reticulocyte lysate system (Promega, Madison, WI). HEK293 cells were transfected with Rab11a, Rab22b or Rab7 constructs and lysed in pull-down buffer [20 mM HEPES, 100 mM NaCl, 5 mM MgCl\textsubscript{2}, 1 mM DTT, 100 μM GTP or GDP and 0.4% (v/v) Triton X-100 (pH 7.5)]. Rab proteins, TRPV5 or TRPV6 were added to GST or GST-fusion proteins immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) in pull-down buffer and incubated for 2 h at room temperature. After extensive washings, bound proteins were eluted with SDS-PAGE loading buffer and visualized by autoradiography or immunoblotting using rabbit anti-Rab11 (Zymed, San Fransisco, CA), monoclonal anti-VSV (Sigma, St Louis, MO, for Rab11 and Rab22b) or anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA, for Rab7). The quality and quantity of GST or GST-fused proteins was routinely analyzed by Coomassie staining.
Interaction with Rab11a regulates TRPV5 and TRPV6

Co-immunoprecipitation

*Xenopus laevis* oocytes were co-injected with 10 ng TRPV5 and 10 ng Rab11a S25N cRNA as described [15]. After 48 h injected oocytes were lysed in sucrose buffer containing 20 mM Tris pH 7.4, 5 mM EDTA, 135 mM NaCl, 0.1 % (v/v) NP-40, 0.5 % (w/v) sodium deoxycholate and 10 % (w/v) sucrose, centrifuged at 100 g for 5 min and subsequently incubated on ice for 60 min. The lysates were centrifuged for 30 min at 16,000 g and supernatants were incubated with monoclonal anti-HA antibodies (Sigma, St Louis, MO) immobilized on protein A-agarose beads (Kem-En-Tec A/S, Kopenhagen, Denmark) for 16 h at 4 °C. Immunoprecipitated proteins were analyzed by immunoblot analysis using rabbit anti-Rab11a antibodies.

CNT and CCD primary cell culture and transepithelial Ca\(^{2+}\) transport

Connecting tubules (CNT) and cortical collecting ducts (CCD) were immunodissected from the kidney cortex of New Zealand White rabbits (± 0.5 kg), placed in primary culture on permeable filters (0.33 cm\(^2\); Costar) and transepithelial Ca\(^{2+}\) transport was measured as described previously [17] in the presence or absence of 1 μM ruthenium red (Sigma, St Louis, MO). Cells were subsequently fixed in 3% (w/v) paraformaldehyde in PBS for 30 min at 4 °C.

Lentiviral infection of primary CNT/CCD cells

Third generation lentiviruses were produced by co-transfection of the packaging vectors pRSV-Rev, pMDL g/p RRE, and pMD2G from Tronolab (Lausanne, Switzerland) and the transfer vector into Human Embryonic Kidney 293T cells as described before [18]. The titer was determined by p24 HIV ELISA (Murex Diagnostics, Dartford, UK). Primary rabbit CNT/CCD cells were infected with lentivirus immediately before plating, in the presence of polybrene (8 μg/ml) using 1 or 10 virus particles per cell (1 or 10 MOI). Virus was removed after 24 h. Transepithelial Ca\(^{2+}\) transport was measured 6 days post-infection in the presence of 10 μM forskolin as described before [17].

Immunofluorescence microscopy

Immunohistochemistry on mouse kidney sections was performed as described previously [19] using affinity purified guinea pig antiserum against TRPV5 and rabbit anti-Rab11 antibodies. Serial sections were incubated with rabbit anti-TRPV6 or rabbit anti-Rab11a antibodies. *Xenopus laevis* oocytes were injected with 5 ng HA-tagged TRPV5 cRNA or 5 ng Flag-tagged TRPV6 cRNA with or without 10 ng
Rab11a cRNA. Two days after injection immunocytochemistry was performed as described [15] using anti-HA or rabbit anti-TRPV6 (dilution 1: 400). HeLa cells were grown on coverslips and transfected with Effectene (QIAGEN, Valenica, CA) according to the manufacturer's protocol. Sixteen hours after transfection, cells were fixed with 4% (w/v) PFA in PBS for 10 minutes at RT, incubated with 50 mM NH$_4$Cl in PBS for 5 min, washed with PBS and mounted in Mowiol containing 4% (w/v) n-propyl-gallate as antifade agent. Cells fixed on permeable filter supports were stained with guinea pig anti-TRPV5 and rabbit anti-Rab11 and appropriate secondary antibodies coupled to Alexa 488 or 596 and mounted in Mowiol. The cell surface was visualized by biotinylation from the apical and basolateral compartment, followed by incubation with streptavidin-Oregon green 488, essentially as described [20]. This procedure predominantly stained the basolateral cell surface. Images were taken sequentially with a Biorad MRC 1024 confocal microscope or using a Zeiss LSM510 Meta confocal microscope, emitting at 488 nm and at 543 nm using a 30 mW argon laser and a 1 mW helium/neon laser. Emissions were collected using a 505-530 nm band-pass filter or a 560 nm long-pass filter. XZ scans were constructed from 35 confocal optical sections (0.2 μm apart). All negative controls, including non-injected oocytes or sections incubated with preimmune serum or conjugated antibodies alone, were devoid of staining.

$^{45}$Ca$^{2+}$ uptake assay

Xenopus laevis oocytes were co-injected with TRPV5 or TRPV6 and Rab11a cRNA. Ca$^{2+}$ uptake was determined 2 days after injection as described previously [21].

Plasma membrane isolation

Xenopus laevis oocytes were injected with 5 ng TRPV5 cRNA only or co-injected with 5 ng TRPV5 and 10 ng Rab11a cRNA. After 48 h follicle membranes were manually removed and plasma membranes were isolated from 12 oocytes as described before [22].

Statistical analysis

In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). P values below 0.05 were considered significant.
Interaction with Rab11a regulates TRPV5 and TRPV6

Results

Identification of Rab11a as a TRPV5- and TRPV6-associated protein

Using the C-tail of TRPV6 as bait to screen a mouse kidney cDNA library with the yeast two-hybrid technique, we isolated full length Rab11a, a member of the Rab family of small GTPases, as a TRPV6-interacting protein. Rab11a strongly interacted with TRPV6 (Figure 1A), whereas β-galactosidase activity was not observed with the negative control, in the absence of prey or after co-transformation of the bait with the empty pACT2 (prey) vector.
The interaction between TRPV6 and Rab11a was further substantiated using GST pull-down assays. *In vitro* translated $[^{35}\text{S}]$methionine-labeled TRPV6 strongly interacted with Rab11a S25N, a mutant deficient in GTP binding. Binding to wild-type (WT) Rab11a was moderate and only minor binding to Rab11a S20V, a GTPase-deficient Rab11a mutant [23], was observed (Figure 1B). Identical results were obtained with $[^{35}\text{S}]$methionine-labeled TRPV5 (Figure 1B). The reverse binding reaction, using GST, GST-TRPV5 C-tail or GST-TRPV6 C-tail incubated with *in vitro* translated $[^{35}\text{S}]$methionine-labeled Rab11a S20V or Rab11a S25N mutants, confirmed the TRPV5- and TRPV6-binding specificity for Rab11a S25N (Figure 1D).

![Figure 2](image)

*Figure 2.* (A) VSV tagged Rab11a S25N or Rab22b S19N and myc-tagged Rab7 T22N were transiently expressed in HEK293 cells and incubated with GST or GST fused to the C-tail of TRPV5 or TRPV6 and interactions were analyzed by immunoblot analysis using monoclonal anti-VSV or anti-Myc. (B) Coomassie staining of the precipitated GST fusion proteins demonstrating equal amounts of GST or GST fused to the C-tail of TRPV5 or TRPV6. (C) Rab11a S25N was expressed in bacteria as a GST fusion protein, GST was cleaved of using Precision protease and purified Rab11a S25N was incubated with immobilized and purified GST-TRPV5 and bound Rab11a detected by immunoblot analyses using anti-Rab11 antibodies (left panel). The integrity and purity of Rab11a S25N was verified by Coomassie staining (right panel).
GST alone failed to show any binding in all conditions. The integrity and quantity of the GST-fusion proteins was analyzed by Coomassie staining and shows that equivalent amounts of protein were used in these assays (Figure 1C, E). In order to further assess the Rab11a interaction, HA-tagged TRPV5 and Rab11a S25N were co-expressed in *Xenopus laevis* oocytes and subjected to immunoprecipitation using monoclonal anti-HA antibodies. HA-tagged TRPV5 was specifically precipitated using these monoclonal antibodies, as indicated by the specific bands at ~70 kDa for the core TRPV5 protein and ~90 kDa for the complex-glycosylated TRPV5 (Figure 1F). Rab11a S25N was co-precipitated with TRPV5 from these oocytes, as represented by an immunopositive band of ~25 kDa (Figure 1G).

Subsequently, the specificity for Rab11a was determined using distant members of the Rab family of GTPases. Rab22b S19N and Rab7 T22N, both GTP binding-deficient Rab proteins, did not bind TRPV5 and TRPV6, indicating the specificity of the interaction of TRPV5 and TRPV6 with Rab11a (Figure 2A). GST, GST-fused TRPV5 and GST-fused TRPV6 were present in equal amounts, as demonstrated by Coomassie staining (Figure 2B). GST alone did not show any binding in these conditions. To investigate whether the association between Rab11a and the epithelial Ca²⁺ channels is direct, recombinant Rab11a S25N was purified as GST-fusion protein and subsequently cleaved from GST using Precision protease. The purity of recombinant Rab11a was analyzed by Coomassie staining (Figure 2C, right panel). Purified Rab11a S25N displayed similar TRPV5-binding efficiencies (Figure 2C), demonstrating that the interaction is direct and does not require additional proteins.

**Co-localization of Rab11a with TRPV5 and TRPV6**

Subsequently, we examined whether TRPV5 colocalized with wild-type Rab11a and the GTP- and GDP-locked Rab11a mutants. To this end, HeLa cells were transiently transfected with constructs encoding EGFP-tagged TRPV5 and DsRed-tagged Rab11a (WT, S20V or S25N) and the subcellular localization of TRPV5 and Rab11a was visualized by confocal laser scanning microscopy. TRPV5 was clearly present in many vesicles distributed throughout the cytoplasm where it showed prominent colocalization with wild-type Rab11a (Figure 3A) and to a lesser extend in the ER. Similarly, TRPV5 colocalized also with Rab11a S20V, the GTP-locked form of Rab11a, in vesicular structures, strongly resembling the pattern observed with wild-type Rab11a (Figure 3B). Interestingly, a significant vesicular colocalization of TRPV5 was observed with GDP-locked Rab11a S25N (Figure 3C), opposed to the dispersed localization of Rab11a S25N observed previously [24, 25]. These findings
are in line with our GST pull-down and co-immunoprecipitation results and suggest that GDP-bound Rab11a is at least in part vesicle-associated in TRPV5-expressing cells. DsRed-fused wild-type Rab4 showed little or no colocalization with TRPV5, indicating that this channel is particularly enriched in the Rab11a-positive compartment (Figure 3D). Furthermore, YFP-fused TRPV2 did not colocalize with Rab11a, but showed a strong plasma membrane staining (Figure 3E).

A clear plasma membrane localization of TRPV5 was not observed in either of the conditions, although a significant TRPV5-mediated Ca\(^{2+}\) influx was measured in TRPV5-transfected HeLa cells compared to none-transfected cells (data not shown). This suggests that a low number of TRPV5 channels are present at the plasma membrane to facilitate Ca\(^{2+}\) influx but prevent cytosolic overload with Ca\(^{2+}\). To
determine whether the interaction can also occur under physiological conditions the cellular colocalization of endogenous TRPV5 and TRPV6 with endogenous Rab11a was investigated. Immunopositive staining for Rab11a colocalized with TRPV5 in specific kidney tubules, previously identified as distal convoluted (DCT) and connecting tubules (CNT) [19] (Figure 4A). Furthermore, serial sections were stained for the presence of Rab11a and TRPV6, revealing co-expression of both proteins in the luminal domain of CNT and cortical collecting ducts (CCD) [26] (Figure 4B).

Figure 4. Co-localization of endogenous TRPV5 and TRPV6 with Rab11a. (A) Mouse kidney sections were co-stained with antibodies against TRPV5 and Rab11a. (B) Serial kidney sections were stained with antibodies against TRPV6 or Rab11a. (C, D) Immunodissected CNT and CCD cells were grown on permeable filter supports and double-labeled with anti-TRPV5 and anti-Rab11 or (E) steptavidin-Oregon green to visualize the cell surface. Arrowheads indicate the positions utilized to render XZ projections from 35 confocal optical sections (0.2 μm apart). (F) Transcellular Ca\(^{2+}\) transport in control situations and in the presence of 1 μM ruthenium red was measured prior to fixation to ensure monolayer integrity and cell viability. The robust transcellular Ca\(^{2+}\) transport demonstrates the quality of the preparation. Bar denotes 100 μm (A, B) or 5 μm (* P<0.05) (C-E).
detailed localization studies were subsequently performed on primary cultures of CNT and CCD cells, immunodissected from rabbit kidney. These cells exhibited net apical-to-basolateral Ca\(^{2+}\) transport, indicating the integrity and functionality of the preparation. Ca\(^{2+}\) transport was blocked (from 63 ± 3 to 5 ± 2 nmol/cm\(^2\)/h) by ruthenium red, a polycationic dye that blocks TRPV5 activity with an IC\(_{50}\) of 111 nM [27] (Figure 4F).

![Figure 5. The Rab11a binding site in TRPV5.](image)

**Figure 5.** The Rab11a binding site in TRPV5. (A) GST fusion proteins containing different portions of the C-tail of mouse TRPV5 were constructed according to the schematic drawing. (B) These proteins were immobilized on glutathione-Sepharose 4B beads and then incubated with in vitro translated Rab11a S25N. Interaction of Rab11a with the GST fusion proteins was determined by autoradiography. The region between amino acids 595 and 601 was essential for binding. Mutation of this region (595-5G-601) diminished the interaction with Rab11a (right panel). (C) GST or GST-fused to the C-tail of TRPV5, immobilized on glutathione Sepharose 4B beads were incubated with in vitro translated Rab22b and precipitated proteins were analyzed by autoradiography. Rab22b did not associate with TRPV5, demonstrating the specificity of the Rab11a binding. (D) TRPV5 and TRPV6 sequences, varying from human to zebrafish, were aligned using the Clustal method, demonstrating complete conservation of the Rab11a binding-site during evolution. (E) cRNA encoding full-length TRPV5 or TRPV6 was injected in \textit{Xenopus laevis} oocytes and the localization of the channel was investigated by immunocytochemistry. Mutation of 5 amino acids in the Rab11a-binding region resulted in significantly disturbed TRPV5 and TRPV6 trafficking, resulting in a low number of channels at the cell surface. (F) Accordingly, TRPV5-mediated \(^{45}\)Ca\(^{2+}\) uptake was significantly decreased in \textit{Xenopus laevis} oocytes, injected with TRPV5 595-5G-601 compared to oocytes injected with wild-type TRPV5. Similar functional results were obtained between wild-type and 601-5G-607 mutant TRPV6. Bar denotes 25 μm (* P<0.05).
TRPV5-staining localized predominantly to the apical side of the cell and was absent from the basolateral membrane that is in line with its physiological function as apical Ca\textsuperscript{2+} influx channel (Figure 4E). Importantly, TRPV5 immunopositive staining overlapped with that of Rab11a in vesicular structures (Figure 4C). Analysis of z-stacks of these cells (Figure 4D), combined with plasma membrane staining (Figure 4E) confirmed the colocalization of Rab11a and TRPV5 in a subapical region. Thus, these findings further substantiate the physiological relevance of the interaction between the epithelial Ca\textsuperscript{2+} channels and Rab11a.

**Characterization of the Rab11a binding site in TRPV5**

To map the Rab11a binding site, a series of deletion mutants of the C-tail of TRPV5 was constructed as depicted in Figure 5A. These mutants were expressed and purified as GST-fusion proteins and incubated with in vitro translated Rab11a S25N. Rab11a interacted with TRPV5 truncates up to position 601, narrowing the binding site to a helical region of 29 amino acids. Two truncated TRPV5 mutants, containing stop codons at position 596 or 591, failed to co-precipitate Rab11a (Figure 5B). Therefore, the region between amino acids 595 and 601 of TRPV5 is essential for Rab11a binding. Subsequently, this region, corresponding to the amino acid sequence MLERK, was mutated into glycines (595-5G-601) and the binding of Rab11a was re-analyzed by GST pull-down. The interaction between Rab11a and the mutant 595-5G-601 TRPV5 was significantly decreased, further indicating the relevance of this MLERK domain for Rab11a interaction (Figure 5B).

Of note, the binding region for the previously identified TRPV5-interacting protein S100A10 is located upstream of this Rab11a binding region [12] and the T593A (amino acid numbering according to the mouse sequence, accession number NP001007573) mutation in TRPV5, which is crucial for S100A10 binding, had no effect on binding of Rab11a (data not shown), indicating the specificity of the identified region. GST alone did not bind Rab11a and in vitro translated Rab22b did not show any affinity for TRPV5 under these conditions (Figure 5C). Importantly, the diminished binding of Rab11a to the TRPV5 595-5G-601 mutant was accompanied by an impaired trafficking of TRPV5, as was demonstrated by immunocytochemical analysis of *Xenopus laevis* oocytes injected with TRPV5 wild-type or 595-5G-601 mutant cRNA. Wild-type channels showed robust plasma membrane localization, whereas the binding-deficient mutant is mainly localized to the cytoplasm (Figure 5E). We also mutated this region in TRPV6 (601-5G-607) and investigated whether similar trafficking defects were displayed. In line with our results with TRPV5, wild-
type TRPV6 showed significant plasma membrane staining, whereas TRPV6 601-5G-607 was absent from the plasma membrane but accumulated intracellularly. (Figure 5E). The functional role of Rab11a binding was subsequently investigated by $^{45}\text{Ca}^{2+}$ uptake measurements in oocytes. Expression of wild-type TRPV5 resulted in a ~5-fold increase in the $\text{Ca}^{2+}$ uptake compared to non-injected oocytes, whereas expression of the TRPV5 595-5G-601 mutant resulted in a $\text{Ca}^{2+}$ uptake that was indistinguishable from non-injected oocytes (Figure 5F).

Figure 6. Role of Rab11a in TRPV5 and TRPV6 trafficking towards the plasma membrane. $^{45}\text{Ca}^{2+}$ uptake was measured in oocytes injected with TRPV5 cRNA (A) or TRPV6 cRNA (B) only or co-injected with Rab11a S20V, Rab11a S25N, or Rab22b S19N cRNA. Asterisk denotes significantly (P<0.05) different from non-injected oocytes. HA-TRPV5 and Rab11a cRNA was co-injected in Xenopus laevis oocytes. Plasma membranes (C) or total membranes (D) were isolated and blotted for TRPV5 using monoclonal anti-HA. Oocytes were injected with HA-tagged TRPV5 cRNA with or without Rab11a cRNA and TRPV5 localization was determined by immunocytochemistry using monoclonal anti-HA (E). Rab11a S25N expression decreased the TRPV5 plasma membrane localization. Representative images of three independent experiments are shown. Bar denotes 10 μm.
Similar results were obtained with TRPV6 and the TRPV6 601-5G-607 mutant (Figure 5F). The functional significance of the identified Rab11a-binding site is further underscored by sequence conservation among all members of the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6 identified so far (Figure 5D), ranging from human to zebrafish.

**Rab11a S25N inhibited TRPV5- and TRPV6-mediated Ca\(^{2+}\) influx**

The effect of Rab11a expression on TRPV5 and TRPV6 activity at the plasma membrane was determined in *Xenopus laevis* oocytes, using a \(^{45}\)Ca\(^{2+}\) uptake assay. Expression of TRPV5 and TRPV6 resulted in a ~5-fold increase of Ca\(^{2+}\) influx compared to non-injected oocytes (Figure 6A, B). Co-expression of Rab11a S25N significantly decreased the TRPV5- and TRPV6-mediated Ca\(^{2+}\) influx to a level that was indistinguishable from non-injected oocytes (Figure 6A, B). Co-expression of Rab11a S20V or Rab22b S19N had no significant effect on TRPV5 and TRPV6 activity. Ca\(^{2+}\) uptake in oocytes expressing only Rab11a S20V or Rab11a S25N was not different from non-injected oocytes (0.49 ± 0.07, 0.42 ± 0.05 versus 0.44 ± 0.04 pmol Ca\(^{2+}\)/oocyte/h, respectively). The effect of Rab11a S25N is consistent with a role for Rab11a in TRPV5- and TRPV6- targeting to the plasma membrane. Therefore, we investigated trafficking of TRPV5 in *Xenopus laevis* oocytes. A distinct band at the size of complex glycosylated TRPV5 [28] was observed by immunoblot analysis in plasma membrane preparations of TRPV5-injected oocytes (Figure 6C). The core TRPV5 protein was not detectable, indicating the purity of the plasma membrane preparation obtained by using this method [22]. Importantly, TRPV5 could not be detected in the plasma membrane of oocytes co-expressing TRPV5 and Rab11a S25N, whereas co-injection of Rab11a S20V resulted in normal plasma membrane localization of TRPV5 (Figure 6C). In all conditions, TRPV5 was produced and glycosylated to a similar extent as in the absence of mutant Rab11a protein (Figure 6D). These results were verified by immunocytochemical analysis of TRPV5- and Rab11a-expressing oocytes. Immunopositive staining for TRPV5 was predominantly localized along the plasma membrane (Figure 6E). Co-injection of TRPV5 with Rab11a S25N resulted in largely dispersed intracellular immunopositive staining with virtually no staining of TRPV5 at the plasma membrane, while expression of Rab11 S20V had no effect (Figure 6E).

The role of Rab11a in the regulation of TRPV5 was further investigated using primary cultures of CNT/CCD cells grown to confluence on permeable filter supports.
Lentivirus-mediated expression of GFP-fused Rab11a S25N or GFP only was verified by confocal laser scanning microscopy (Figure 7A).

GFP-Rab11a S25N displayed a tubulovesicular localisation, while GFP showed a predominant diffuse cytosolic pattern. Rab11a S25N partially co-localized with endogenous TRPV5, whereas GFP alone showed no co-localization (Figure 7A), in line with the colocalization results obtained in HeLa cells (Figure 3). The expression of GFP and GFP-fused Rab11a S25N was further confirmed by immunoblot analysis.

Figure 7. Rab11a S25N inhibits transcellular Ca\textsuperscript{2+} transport in primary cultures of CNT/CCD cells. CNT/CCD cells were immunodissected from rabbit kidney, infected with lentivirus and plated on permeable filter supports. Confluent monolayers of CNT/CCD cells were analysed six days post-infection. (A) Expression of GFP and GFP-fused Rab11a S25N was investigated by confocal laser scanning microscopy and localization of the GFP signal was compared with endogenous TRPV5. (B) Lentiviral expression of GFP or GFP-fused Rab11a S25N was quantified by immunoblot analysis using anti-GFP antibodies confirming the size and integrity of the proteins. (C) Net apical to basolateral Ca\textsuperscript{2+} transport was measured in forskolin-stimulated (10 μM) CNT/CCD monolayers infected with lentivirus expressing either GFP alone or GFP-fused Rab11a S25N. Infections were performed at densities of 1 or 10 lentivirus particles per cell (1 or 10 MOI), showing a concentration-dependent inhibition of Ca\textsuperscript{2+} transport by Rab11a S25N (* p<0.05). Bar denotes 10 μm, NI; not infected.
using anti-GFP antibodies showing single bands of comparable intensity at the expected height for GFP and GFP-fused Rab11a S25N (Figure 7B). Importantly, transcellular Ca\(^{2+}\) transport showed a significant and dose-dependent inhibition in Rab11a S25N-expressing primary CNT/CCD monolayers compared to monolayers expressing GFP only. Viral infections did not affect the transepithelial resistance in either condition confirming the integrity of the monolayer (data not shown). Furthermore, no differences in TRPV5 expression were observed in infected versus non-infected cells. Therefore, the results obtained with the renal primary cultures are consistent with our observations using TRPV5-expressing *Xenopus* oocytes and further substantiate the role of Rab11a in the targeting of TRPV5 to the plasma membrane.

**Discussion**

The present study demonstrates a novel function of Rab11a mediating trafficking of TRPV5 and TRPV6 towards the plasma membrane by direct cargo interaction. First, GDP-bound Rab11a directly and specifically binds to a conserved stretch in the C-tail of TRPV5 and TRPV6, demonstrating a unique interaction between a TRP channel and a GDP-bound Rab. Second, the epithelial Ca\(^{2+}\) channels colocalize with Rab11a in subapical vesicles present in Ca\(^{2+}\)-transporting cells of the kidney. Third, functional data suggests that cargo interaction (in the GDP status) and subsequent GTP binding are required for Rab11a-mediated TRPV5 and TRPV6 targeting towards the plasma membrane.

**GDP-bound Rab11a as a novel TRPV5- and TRPV6-interacting protein**

Our study describes for the first time cargo proteins that interact directly with Rab11a. Although Rab proteins are known to interact with a large variety of effectors [10, 29, 30], only a few studies have demonstrated direct interaction between a Rab GTPase and a cargo molecule [31]. Recently, it was reported that the polymeric IgA receptor (pIgR) interacts directly with Rab3b, controlling IgA-stimulated transcytosis [32]. The second example is the interaction between Rab5a and the angiotensin II type 1A receptor [33]. Finally, Pfeffer and coworkers were the first to demonstrate a mechanism in which Rab proteins interact indirectly with cargo. They showed that a ternary complex of Rab9, mannose-6-phosphate receptor and the adaptor protein TIP47 plays a role in the vesicular transport of the mannose-6-phosphate receptor [34]. Direct interaction of Rabs with cargo could provide further insight into the mechanisms of Rab localization and function. The recruitment of Rabs to specific
membranes is mediated by the Rab C-tail [35]. This suggests the involvement of Rab-interacting proteins in the membrane localization of Rab proteins. An intriguing possibility is that certain Rab-accessory proteins are cargo themselves, contributing to the membrane localization of specific Rab proteins. As TRPV5 and TRPV6 preferentially interacted with Rab11a in its inactive configuration, TRPV5 and TRPV6 may support the recruitment of Rab11a to specific membrane compartments in Ca$^{2+}$-transporting epithelia. Similarly, it was suggested that the direct interaction between Rab3b and plgR provides a partial explanation for the specificity of binding of Rab3b to plgR-containing vesicles [32]. Furthermore, direct interaction of Rabs with cargo could contribute to the targeting of proteins to their proper destination. Direct association of TRPV5 and TRPV6 with Rab11a could support the translocation of these channels into recycling endosomes and thereby constitute the “delivery machinery” destined to transport these channels to the apical plasma membrane. Identification of additional novel cargo that interacts with Rab proteins could provide means to further dissect the mechanism of Rab activity and thus a novel insight into the molecular machinery of membrane traffic.

Co-localization of Rab11a and TRPV5 and TRPV6

Further evidence for a role of Rab11a in TRPV5 and TRPV6 regulation is the predominant co-localization of Rab11a with TRPV5 or TRPV6 along the apical domain of DCT, CNT and CCD, corroborating previous Rab11 localization data from Goldenring et al. [36]. Furthermore, Rab11a is present along the luminal membrane of enterocytes, where TRPV6 expression is prominent [36, 37]. The specific expression pattern in kidney and intestine supports a role for Rab11a in the regulation of TRPV5 and TRPV6. Also at the subcellular level, Rab11a shows significant co-localization with TRPV5 in subapical vesicular structures. Previous functional and histological studies have identified Rab11-positive structures as (apical) recycling endosomes, which are specialized compartments involved in (polar) sorting of endocytosed membrane proteins [23, 38-41]. Furthermore, Rab11 has been demonstrated to play a role in transport from the trans-Golgi network to the plasma membrane [42, 43]. In addition, Ang and coworkers have recently demonstrated a role for recycling endosomes as intermediates in the transport from Golgi to the plasma membrane [44]. Our study is the first to indicate that cargo molecules that travel via these Rab11-enriched structures can be directly bound to Rab11a.
Rab11a binding targets TRPV5 and TRPV6 towards the plasma membrane

The unique role of Rab11a in targeting of TRPV5 and TRPV6 towards the plasma membrane by direct interaction to the channel was further established using combined biochemical, functional and immunocytochemical analyses. In order to identify the direct consequences of Rab11a association with the epithelial Ca\(^{2+}\) channels, we mutated the Rab11a binding site in TRPV5 and TRPV6. The interaction between Rab11a and TRPV5 and TRPV6 is localized to a helical stretch in the C-tail. A stretch of five amino acids at position 595-601 within the Rab11a binding site of TRPV5 was demonstrated to be required for Rab11a binding. Moreover, this region is conserved among all identified species of TRPV5 and TRPV6. Mutations in this stretch resulted in a lack of TRPV5- and TRPV6-mediated Ca\(^{2+}\) uptake, based on defective trafficking of TRPV5 and TRPV6, in line with an essential role for Rab11a in targeting TRPV5 and TRPV6 towards the plasma membrane. However, as this stretch has been implicated in the interaction of TRPV5 with 80K-H, a Ca\(^{2+}\)-binding protein [45] and in TRPV5 channel assembly [46], Rab11a-independent factors can not be excluded to explain the impaired trafficking of this mutant. Therefore, we further substantiated the functional role of Rab11a in the trafficking of TRPV5 and TRPV6 using Rab11a mutants in Xenopus oocytes and primary cultures of Ca\(^{2+}\)-transporting cells from rabbit kidney. GDP-locked Rab11a expression strongly reduced the TRPV5- and TRPV6-mediated Ca\(^{2+}\) influx, resulting from a significantly decreased number of Ca\(^{2+}\) channels at the plasma membrane. This effect can be explained in two ways. First, in analogy with dominant negative Ras mutants [47], Rab11a S25N could sequester a Rab11a guanine nucleotide exchange factor (GEF) and thereby block the activation of Rabs. However, expression of another dominant negative Rab protein (Rab22b S19N) did not inhibit TRPV5 and TRPV6 activity. This either suggests that Rab GEF inhibition is not essential to block TRPV5 and TRPV6 trafficking, or that Rab11a S25N associates with a specific GEF that does not bind Rab22b S19N. It is currently unknown which protein operates as a GEF for Rab11a and how promiscuous this GEF is. Rab11a S20V has a lower affinity for Rab GEFs [23, 48], explaining the lack of functional consequences of expression of this mutant.

A second explanation for the dominant negative effect of Rab11a S25N is a competition with endogenous Rab11a for the Rab11a binding site in TRPV5 and TRPV6. Because Rab11a S25N can not bind GTP, it will not be activated and this impairs TRPV5 and TRPV6 trafficking towards the plasma membrane. Rab11a S20V cannot compete with endogenous Rab11a for TRPV5 and TRPV6 binding, explaining the lack of function of this mutant. Together, these data indicate that cargo interaction (in the GDP status) as well as subsequent GTP binding is required for Rab11a-
mediated TRPV5 and TRPV6 trafficking. Our findings show a novel interaction between a Rab GTPase and TRP channels and point to a unique role for Rab11a in the regulation of TRPV5 and TRPV6 channel trafficking. We propose the following model for the role of Rab11a in trafficking of TRPV5 and TRPV6 (Figure 8). Initially, cytosolic GDP-bound Rab11a specifically interacts with TRPV5 and TRPV6 in a cytoplasmic compartment. Subsequently, Rab11a is docked to this compartment, while GDP is exchanged with GTP. At this state, Rab11a effectors will associate and Rab11a does no longer interact with TRPV5 and TRPV6. Finally, active GTP-bound Rab11a mediates transport of TRPV5 and TRPV6 containing structures to the plasma membrane, where membrane fusion allows Ca\textsuperscript{2+} influx.

In conclusion, our data provide new insight into the molecular machinery of TRP channel trafficking via direct interaction between a Rab GTPase and apically targeted cargo. This association is involved in the physiological regulation of TRPV5 and TRPV6 cell-surface abundance, a critical component in Ca\textsuperscript{2+} homeostasis. The future challenge is to find the signals that modulate the interaction of TRPV5 and TRPV6 and Rab11a to fine-tune cell-surface expression of these epithelial channels.
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References


Interaction with Rab11a regulates TRPV5 and TRPV6

Chapter 4

The β-glucuronidase klotho hydrolyzes and activates the TRPV5 channel

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Why should work be such a significant source of human satisfaction. A good share of the answer rests in the kind of pride that is stimulated by the job, by the activity of accomplishing. (Leonard R. Sayles)

为什么工作竟然是人们获得满足和快乐如此重要的源泉呢？最主要的答案就在于，工作和通过工作所取得的成就，能极大的激发我们的自豪感。（塞尔斯. L. R.）
Abstract

Blood Ca$^{2+}$ concentration is maintained within a narrow range despite large variations in dietary input and body demand. The unique Transient Receptor Potential ion channel, TRPV5, has been implicated in this process. We found that TRPV5 is stimulated by the anti-aging hormone klotho via a novel extracellular activation mechanism. Klotho, secreted in the pro-urine, hydrolyzes by its β-glucuronidase activity the extracellular TRPV5 sugar residues, entrapping the channel protein at the plasma membrane to maintain durable Ca$^{2+}$ channel activity and membrane Ca$^{2+}$ permeability in kidney. Thus, klotho provides a novel concept of activation of a cell-surface TRP protein by extracellular hydrolysis of its N-linked oligosaccharides.

Materials and methods

DNA constructs
pCINeo/IRES-GFP-TRPV5, pCINeo/IRES-GFP-HA-TRPV5, pCINeo/IRES-GFP-TRPV6 constructs were generated as described previously [1, 2]. The coding sequence of wild-type klotho was amplified from mouse (strain C57BL/6) kidney cDNA, cloned into the pGEX6p-2 vector and tagged at the N-tail with a HA-tag and subsequently subcloned into the pCINeo/IRES-GFP. The TRPV5 (TRPV5-N358Q) and TRPV6 (TRPV6-N357Q) mutants were obtained by in vitro mutagenesis in pCINeo/IRES-GFP-TRPV5 and pCINeo/IRES-GFP-TRPV6 cDNA, respectively. All constructs were verified by DNA sequence analysis.

Quantitative real-time PCR analysis
Total RNA was isolated from mouse kidney, brain, lung, muscle, heart, liver, spleen, duodenum, ileum and bone using TRIzol and subjected to reverse transcription as described [4]. The primers and probes for the real-time PCR analysis were 5’-GCCGACCATTTCAGGGATTA-3’ and 5’-TGATCCAGTACTTGACCTGACCA-3’ and the probe 5’-CGAGCTCTGCTTCCGCCACTTCG-3’. Klotho expression levels were presented as a ratio to the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) as described previously [4].

Immunohistochemistry
Immunohistochemistry was performed as described [5]. Mouse kidney sections were
Klotho hydrolyzes and activates the TRPV5 channel

incubated for 16 hours at 4 °C with affinity purified mouse anti-calbindin-D<sub>28K</sub> (1:100), rabbit antiserum against klotho (1:500) and guinea pig anti TRPV5 (1:200). To visualize the proteins corresponding secondary Alexa 488/594 conjugated antibodies (1:300) (Molecular Probes, Eugene, USA) were used.

**Collection and concentration of conditioned culture media**

HEK293 cells were transfected with 15 μg pCINeo/IRES-GFP-HA-klotho or pCINeo/IRES-GFP empty-vector cDNA in 85 mm petri-dishes. After 3 days of transfection, the conditioned culture media was collected and concentrated 10 times using the centrprep columns ultracel YM-30 (Millipore corporation, Bedford, MA, U.S.A.).

**Transfection of HEK293 cells and preparation of cell lysates**

HEK293 cells were transfected with HA-TRPV5, TRPV6, TRPV5-N358Q, TRPV6-N357Q and/or HA-klotho pCINeo/IRES-GFP constructs. Two days after transfection, cells were homogenated, centrifuged at 200 g for 10 min and subsequently eluted in Laemmlli buffer followed by an incubated of 30 min at 37°C. For total membrane isolation of HEK293 cells, the cells were homogenized in HBA [20 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA pH 8.0, 80 mM sucrose, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml pepstatin], and then centrifuged for 15 min, at 4,000 g at 4°C, in addition 30 min at 13,000 rpm at 4°C. The pellet was dissolved in 30 μl of laemmli buffer and incubated at 37°C for 30 min. The proteins were detected by SDS-PAGE gel electrophoresis followed by autoradiography.

**Protein and immunoblot analysis**

Total protein cell lysates or total membranes were isolated from HEK293 cells as described above or from C57BL6 mouse kidney as described previously [6]. The samples were subjected to SDS-PAGE electrophoresis (6-8% w/v). Immunoblots were incubated overnight at 4°C with primary antibodies including mouse anti-HA (1:4000) and guinea pig anti-TRPV5 (1:4000), rabbit MO3 anti-TRPV6 (1:5000) and rat anti-klotho (bio-connect, Huissen, the Netherlands) (1:1000), in 1% (w/v) NFDM in TBS-T, respectively. After washing, immunoblots were incubated at room temperature with the corresponding secondary antibody goat anti-mouse IgG peroxidase (1:10000) (Sigma, St Louis, MO, USA), goat anti-guinea pig IgG peroxidase (1:10000) (Sigma), goat anti-rabbit IgG peroxidase (1:10000) (Sigma) or goat anti-rat IgG peroxidase (1:5000) (Sigma) in TBS-T.
Immunoprecipitation of klotho
Protein A-coupled agarose beads were pre-incubated for 3 h at RT with monoclonal anti-HA antibody (Sigma) in PBS and subsequently washed three times with PBS. Culture media collected from klotho-expressing HEK293 cells (klotho-containing supernatant) was then added to the washed antibody-bound protein A beads (Pharmacia, Uppsala, Sweden) and incubated for 16 hours at 4°C. After incubation, the remaining supernatant was subsequently analyzed for klotho expression using the anti-klotho antibody and $^{45}$Ca$^{2+}$ uptake in TRPV5 expressing HEK293 cells.

Urine TCA precipitation
24 hour urine was collected from mice housed in metabolic cages and centrifuged at 200 g for 10 min at 4°C to remove debris. Proteins in the urine were precipitated using ice-cold trichloroacetic acid (TCA) (Ocr os organics, New Jersey, USA). The urine TCA mixture was centrifuged at 13,000 rpm for 10 min at 4°C, and the pellet was subsequently washed with 300 μl acetone. Next, the pellet was air-dried (3 min), dissolved in laemmli buffer containing 0.1 M DTT and 150 mM Tris (pH 8.8) and analyzed by immunoblot analysis for the expression of klotho.

Biotinylation
HEK293 cells were transfected with 15 μg pCINEO/IRES-GFP-HA-TRPV5 or pCINEO/IRES-GFP empty-vector cDNA in 85 mm petri-dishes, which were coated with poly-L-lysine (0.1% v/v). One day after transfection, cells were incubated with conditioned media for 16 hours. Dishes with confluent transfected HEK293 cells were placed on ice and washed twice with ice-cold PBS pH 8.0 / NaOH containing 1 mM MgCl$_2$ and 0.5 mM CaCl$_2$ (PBSB). Cells were then incubated for 30 min at 4°C with NHS-LC-LC-biotin (final concentration 0.5 mg/ml, Pierce, Ettenleur, the Netherlands), freshly diluted in PBSB. Biotinylation was terminated by rinsing the dishes twice with PBSB containing 0.1% (w/v) bovine serum albumin and once with PBS pH 7.4 without Ca$^{2+}$ or Mg$^{2+}$. Cells were collected from plates and lysed with 1 ml lysis buffer, incubated for 60 min at 4°C. Following measurement of the protein content of the cell extract, samples (900 μg of protein) were added to 150 μl of neutravidin beads, pre-equilibrated in the lysis buffer and rotated overnight at 4°C. The biotin-avidin agarose complexes were then harvested by centrifugation at 14,000 g for 10 min and washed four times with the lysis buffer. Finally, the beads were resuspended in 60 μl of 2x Laemmli buffer and incubated at 37°C for 30
Klotho hydrolyzes and activates the TRPV5 channel

min prior to SDS-PAGE.

**Electrophysiology**
To investigate the effect of klotho, HEK293 cells were transfected with HA-TRPV5 and incubated with culture media collected from HEK293 cells transfected with pClNeo/IRES-GFP (empty-vector supernatant) or pClNeo/IRES-GFP-HA-klotho (klotho-containing supernatant) for 16 hours. Macroscopic currents were measured in the whole-cell patch-clamp configuration using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany; 8-Pole Bessel filter 10 kHz) as described previously in detail [7].

**45Ca²⁺ uptake assay**
HEK293 cells were transfected with HA-TRPV5 or co-transfected with HA-TRPV5 and HA-klotho pClNeo/IRES-GFP constructs. In order to investigate the effect of conditioned media HEK293 cells expressing TRPV5, TRPV6 or TRPV5-N358Q, TRPV6-N357Q were incubated for 16 hours or for the indicated time points with conditioned media. Subsequently, 45Ca²⁺ uptake was determined as described previously [2]. β-Glucuronidase from bovine liver and D-saccharic acid-1,4-lacton (Sigma) were used.

**Measurement of transcellular Ca²⁺ transport in primary culture of rabbit kidney connecting tubule and cortical collecting duct**
Rabbit kidney connecting tubules and cortical collecting ducts were immunodissected from kidney cortex of New Zealand White rabbits (~0.5 kg) using antibody R2G9 and then placed in primary culture on permeable filters (0.33 cm²; Costar, Cambridge, MA, USA) as described in detail previously [8]. At confluency, monolayers of rabbit connecting tubular and cortical collecting duct cells growing on permeable filters were washed twice and preincubated in PSS containing 140 mM NaCl, 2 mM KCl, 1 mM K₂HPO₄, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 5 mM L-alanine, 5 µM indomethacin, 10 mM Hepes/Tris, pH 7.4, for 15 min at 37°C. Subsequently, the monolayers were incubated in PSS (100 µl to apical and 600 µl to basolateral compartment), in the presence of empty-vector supernatant (apical), klotho-containing supernatant (apical), β-glucuronidase (apical) or 10 nM PTH (basolateral, Sigma) as indicated in the text. After 90 min transepithelial Ca²⁺ transport was measured by using a colorimetric assay kit as described [8].
Glucose labeling
HEK293 cells were transfected with 2.5 μg pCINEO/IRES-GFP-HA-TRPV5 or pCINEO/IRES-GFP empty-vector cDNA. Cells were incubated in the presence of 4.5 μCi/well of D-[U-14C]glucose (Amersham, Buckinghamshire, UK). 32 hour after transfection the cells were incubated for 16 hours with empty-vector supernatant, klotho-containing supernatant or β-glucuronidase. Subsequently, cells were washed and homogenized with 300 μl of solubilization buffer [0.5% (v/v) NP-40, 20 mM Tris pH 8.0, 10% (v/v) glycerol, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin] and incubated on ice for 1 hours, and subsequently centrifuged at 16,000 g for 1 hour at 4°C to pellet undissolved proteins. The solubilized proteins were diluted with 0.7 ml of sucrose buffer [100 mM NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 0.1% (v/v) Triton X-100, 10% (w/v) sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin]. In the mean time, 20 μl equivalents of protein A-coupled agarose beads were pre-incubated for 3 hours at room temperature with 2 μl of monoclonal anti-HA antibody (Sigma) in 0.7 ml of IPP500 [500 mM NaCl, 10 mM Tris pH 8.0, 0.1% (v/v) NP-40, 0.1% (v/v) Tween-20, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin] and 0.1% (w/v) bovine serum albumin. The beads were washed three times with IPP100 [100 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) NP-40, 0.1% (v/v) Tween-20]. Furthermore, the cell lysates were added to the washed antibody-bound protein A beads and incubated for 16 hours at 4°C. After incubation, the beads were washed three times with IPP100, incubated in 25 μl of Laemmli buffer for 30 min at 37°C. The eluted proteins were subsequently subjected to immunoblotting and liquid scintillation counting.

Statistical analysis
In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). P values below 0.05 were considered significant.

Result and Discussion

Ca²⁺ is an essential ion in all organisms, where it plays a crucial role in processes ranging from formation and maintenance of the skeleton to temporal and spatial regulation of neuronal function. Concentration of blood Ca²⁺ decreases with age in both men and women, and several studies provided evidence that this negative Ca²⁺ balance is linked
Klotho hydrolyzes and activates the TRPV5 channel

to aging-associated disorders. The Ca^{2+} balance is maintained by the concerted action of three organ systems including the gastrointestinal tract, bone and kidney. Until recently, the mechanism by which Ca^{2+} ions enter the absorptive epithelia was unknown. A major breakthrough in completing the molecular details of this influx pathway was the identification of the epithelial Ca^{2+} channel family consisting of two members of the Transient Receptor Potential (TRP) superfamily, namely TRPV5 and TRPV6 [9, 10]. TRPV5 comprises the Ca^{2+} channel predominantly involved in renal Ca^{2+} handling, whereas TRPV6 was postulated to mediate intestinal Ca^{2+} absorption. TRPV5 and TRPV6 are the most Ca^{2+}-selective channels in the TRP superfamily.

Figure 1. Localization and tissue distribution of klotho. (A) Immunohistochemical analysis of klotho (green), TRPV5 (red) and calbindin-D_{28K} (red) in mouse kidney sections. (B) RNA was extracted from several mouse tissues including kidney, brain, lung, muscle, heart, liver, spleen, duodenum (duod.), ileum and bone and subsequently klotho expression was determined by quantitative real-time PCR analysis (n=3 mice per condition). Klotho expression levels were presented as a ratio to the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT). (C) Protein lysates were prepared from mouse kidney and 24 hour urine was collected from mice (n=3). Both samples were analyzed for the expression of klotho proteins by immunoblotting. Klotho-containing supernatant (klotho super) from HEK293 cells expressing klotho was included as a positive control.
Mice lacking TRPV5 display diminished renal Ca\(^{2+}\) reabsorption despite enhanced vitamin D levels, causing severe hypercalciuria [11]. In addition, compensatory hyperabsorption of dietary Ca\(^{2+}\) was measured in intestine of TRPV5 knockout mice. Furthermore, these mice exhibit significant abnormalities in bone structure, including reduced trabecular and cortical bone thickness [11]. In the process of identifying regulatory proteins in the maintenance of the Ca\(^{2+}\) balance by oligonucleotide-based microarray chip analysis, we identified klotho as a significantly down-regulated gene in kidneys of TRPV5 knockout mice. Klotho, which is a type I membrane glycoprotein of 130 kD [12], is indeed abundantly expressed in mouse kidney as determined by quantitative real-time PCR analysis (Figure 1B). In all other analyzed tissues including brain, lung, muscle, heart, liver, spleen, duodenum, ileum and bone at least 500 times lower mRNA expression levels of klotho were estimated. Immunoblotting showed a clear band at \(~\)130 kD in mouse kidney lysates corresponding to a significant renal klotho abundance and accordingly klotho protein was detected in mouse urine (Figure 1C). Nabeshima and co-workers generated a mice strain in which the klotho gene was inactivated [12]. Klotho gene ablation results in a syndrome resembling human aging, including short life span, bone aberrations, infertility, skin atrophy and hypercalcemia together with an increased serum vitamin D level. Klotho exhibits a considerable homology to \(\beta\)-glucosidase enzymes of bacteria, plants and eukaryotes [13]. In general \(\beta\)-glucosidases participate in essential steps of synthesis and degradation of polysaccharides, which are involved in processes such as pathogen defense [14], control of signal transduction [15] and modification of hormones [16]. In humans, inheritable deficiencies of glycosidases are known to induce a large variety of impairments as lysosomal storage diseases, Gaucher’s and Krabbe’s disease and lactose intolerance [17]. In addition to the aging-associated disorders observed in the klotho null mice polymorphisms in the klotho gene have been linked to reduced bone mineral density in humans [18, 19]. However, the molecular function of klotho and the down-stream targets of this anti-aging hormone remain to be identified.

Previous studies have indicated that the expression of both klotho and TRPV5 is tightly controlled by the calcitropic hormone vitamin D, which may suggest a functional link between these proteins in the maintenance of the Ca\(^{2+}\) balance [9, 20]. Based on this correlation and the conspicuous overlap in the pathophysiology of TRPV5 and klotho knockout mice, for instance, disturbed Ca\(^{2+}\) homeostasis and vitamin D metabolism, we
Klotho hydrolyzes and activates the TRPV5 channel

hypothesized that TRPV5 is a down-stream target of the anti-aging hormone klotho. To test this theory, we first performed detailed immunohistochemical analysis on mouse kidney, which demonstrated a complete co-localization of TRPV5, the vitamin D-sensitive Ca\(^{2+}\)-transporting protein calbindin-D\(_{28K}\) and the klotho protein in the distal convoluted (DCT) and connecting tubule (CNT), nephron segments particularly known to be responsible for active transepithelial Ca\(^{2+}\) reabsorption (Figure 1A). Despite the fact that klotho mutant mice show systemic aging phenotypes, only limited organs express the klotho gene [12].
Subsequently, we co-expressed klotho with TRPV5 in human embryonic kidney (HEK293) cells. Functional studies using these cells showed a TRPV5-mediated $^{45}\text{Ca}^{2+}$ influx that was significantly enhanced by co-expression of klotho (Figure 2A). Our demonstration that klotho is detectable in urine together with previous studies reporting that klotho is present in serum and cerebrospinal fluid [21], suggests that klotho operates from the extracellular site to regulate TRPV5 activity. To investigate this mechanism, HEK293-TRPV5 cells were incubated overnight (16 hours) with the culture media collected from klotho-expressing HEK293 cells (klotho-containing supernatant) or empty-vector-expressing HEK293 cells (empty-vector supernatant). Intriguingly, TRPV5 activity was significantly increased by klotho-containing supernatant, whereas no effect was observed with empty-vector control supernatant (Figure 2B). Klotho protein with a molecular weight of 130 kDa was clearly detectable in supernatant obtained from HEK293-klotho cells, whereas no specific band was noticeable in the empty-vector control supernatant (Figure 2B). Klotho did not alter TRPV5 expression (data not shown) suggesting a specific effect on channel kinetics or trafficking towards the plasma membrane. HEK293-TRPV5 cells were treated overnight (16 hours) with klotho-containing supernatant or empty-vector supernatant, and subsequently analyzed by whole cell patch-clamp analysis. Both the Na$^+$ and Ca$^{2+}$ currents were significantly stimulated by klotho treatment (Figure 2C). Measurements in divalent free solution (EDTA 0.1 mM) revealed a significant increase in channel activity (Figure 2C, left). Application of a hyperpolarizing voltage step from a holding potential of +70 to -100 mV...
Klotho hydrolyzes and activates the TRPV5 channel showed an increased Ca\(^{2+}\) peak current, whereas no significant differences on the Ca\(^{2+}\)-dependent inactivation behavior of the channel was observed (Figure 2C, middle). By applying a voltage ramp to cells perfused with a solution containing 10 mM Ca\(^{2+}\) a significant rise in current amplitude was obtained (Figure 2C, right). Together with the fact that TRPV5 is a constitutive active Ca\(^{2+}\) channel with an open probability close to 100% [22], these findings imply an increased channel abundance at the plasma membrane due to the incubation with extracellular klotho. The stimulatory effect of klotho on TRPV5 activity was maximal at 16 hours of incubation, but a significant increase in channel activity was already apparent after 2 hours (Figure 2D). Since it has been demonstrated that klotho exhibits β-glucuronidase activity [3], we examined whether purified β-glucuronidase from bovine liver (EC 3.2.1.31) could mimic the stimulatory effect of klotho on TRPV5 channel activity. To this end, HEK293-TRPV5 cells were incubated for 16 hours with increasing concentrations of β-glucuronidase. Remarkably, β-glucuronidase mimicked the effect of the klotho-containing supernatant on TRPV5 channel activity with a maximal stimulation at 250 μg/ml (Figure 2E). To further characterize whether the β-glucuronidase activity of klotho is responsible for the stimulatory action, we studied the effect of the potent klotho inhibitor D-saccharic acid 1,4-lactone [3]. As shown in Figure 2F, the klotho-mediated increase of TRPV5 activity was dose-dependently inhibited by this blocker with an apparent IC\(_{50}\) of 0.5 μM demonstrating that the stimulatory action of klotho can be attributed to its β-glucuronidase activity. Subsequently, klotho was immunoprecipitated from the klotho-containing supernatant and the remaining fluid was indeed depleted of klotho and importantly failed to increase TRPV5 channel activity (Figure 2G). Together, these findings demonstrated that klotho via its β-glucuronidase activity directly activates TRPV5.
Next, the effect of klotho and β-glucuronidase was evaluated in kidney primary cultures. These primary cultures of rabbit distal convoluted and connecting tubules form confluent monolayers that exhibit many characteristics of the original polarized epithelium, including PTH- and vitamin D-stimulated transepithelial Ca\textsuperscript{2+} transport [8]. Apical addition of klotho-containing supernatant or β-glucuronidase significantly stimulated transcellular Ca\textsuperscript{2+} transport in these primary kidney cells, whereas the control supernatant did not affect the transport process (Figure 3).

To elucidate the molecular mechanism underlying the stimulatory effect of extracellular klotho on TRPV5 channel activity, we studied the role of glycosylation in this process. Heterologous expression of TRPV5 in HEK293 cells and subsequent immunoblot analysis of cell lysates revealed specific bands of TRPV5 with a molecular size ranging from 75 to 90-100 kDa (Figure 4A). These bands were not detected in empty-vector-transfected cells. The immunoreactive protein bands at 75 kDa reflect the core TRPV5 protein, while the 90-100 kDa band represents the complex glycosylated TRPV5 product [23]. Sequence analysis predicts a N-glycosylation site (N358) positioned between transmembrane segment 1 and 2. The functional role of glycosylation in...
klotho-mediated TRPV5 stimulation was investigated by mutating the asparagine residue in this putative glycosylation site into a glutamine (TRPV5-N358Q), which prevented TRPV5 glycosylation upon expression in HEK293 cells. These cells showed normal Ca\(^{2+}\) influx that was not significantly different from cells expressing wild-type TRPV5 channels (Figure 4A). Interestingly, the klotho and β-glucuronidase-stimulatory effect on TRPV5 was abolished in the HEK293 cells expressing this TRPV5-N358Q mutant, whereas HEK293-TRPV5 cells showed an increased Ca\(^{2+}\) influx activity (Figure 4A). These results indicated that abolishment of the glycosylation by mutagenesis of the N-glycosylation site prevents accumulation of TRPV5 at the plasma membrane. This suggests that the klotho-mediated TRPV5 accumulation is due to the specific hydrolysis of extracellular sugar residues from the channel protein. Next, we showed that klotho also augments the activity the highly homologues TRPV6 channel, while the glycosylation-deficient TRPV6 (N357Q) mutant failed to respond to klotho and the β-glucuronidase (Figure 4B).
Thus, klotho stimulates TRPV channel activity by sugar hydrolysis at the conserved glycosylation site between transmembrane segment 1 and 2. To investigate klotho-mediated hydrolysis of the glycosylated TRPV5 protein, HEK293 cells transiently expressing the channel were labeled for 48 hours with D-[U-14C]glucose.

Figure 4. Molecular mechanism of klotho-stimulated TRPV5 channel activity. HEK293 cells expressing TRPV5 or TRPV5-N358Q (A), TRPV6 or TRPV6-N357Q (B) were exposed to klotho-containing supernatant (klotho-super) or 250 μg/ml β-glucuronidase (Glu) for 16 hours and subsequently assayed for 45Ca2+ uptake. Asterisks indicate significant difference from 45Ca2+ influx in HEK293-TRPV5 cells or in HEK293-TRPV6 cells, respectively (p<0.05, n=4). TRPV5 and TRPV6 expression was analyzed in the cell lysates and total membrane fractions indicating that glycosylation was impaired in the mutant TRPV5-N358Q and TRPV6-N357Q, respectively.

HEK293-TRPV5 cells were labeled for 48 hours with D-[U-14C]glucose. After 32 hours cells were treated with klotho-containing supernatant (klotho-super), empty-vector supernatant (empty-super) or 250 μg/ml β-glucuronidase (Glu) and subjected to immunoprecipitation using the guinea pig anti-TRPV5 antibody. Precipitated D-[U-14C]glucose-TRPV5 was eluted from the beads and processed for liquid scintillation counting. Asterisk indicates significant difference from HEK293-TRPV5 cells treated with empty-vector supernatant (p<0.05, n=6).

HEK293 cells were transfected with TRPV5 and incubated for 16 hours with klotho-containing supernatant (klotho-super), empty-vector supernatant (empty-super) or 250 μg/ml β-glucuronidase (Glu). TRPV5 expression was determined by immunoblotting in total cell lysates to demonstrate equal expression (left). Cell-surface proteins were biotinylated extracellularly, precipitated with neutravidin-agarose beads and immunoblotted for TRPV5 (middle: low exposure, right: high exposure). As a negative control biotine was omitted in the procedure.
After 32 hours of D-[U-14C]glucose labeling the cells were incubated for 16 hours with klotho-containing supernatant, empty-vector control supernatant or β-glucuronidase and subsequently TRPV5 was immunoprecipitated. Incubation of the HEK293-TRPV5 cells with klotho-containing supernatant or β-glucuronidase showed a decrease in D-[U-14C]glucose-labeled TRPV5 precipitate indicating extracellularly cleaved 14C-sugars compared to the empty-vector control supernatant (Figure 4C). These data demonstrate that klotho exhibits β-glucuronidase activity by hydrolysing sugars from glycosylated TRPV5 channels. To obtain insight into the functional consequences of the klotho action on TRPV5, the plasma membrane expression of TRPV5 was examined in klotho- and control-treated HEK293-TRPV5 cells by plasma membrane surface biotinylation which demonstrated that the klotho-containing supernatant enhanced the plasma membrane TRPV5 expression explaining the observed increase in channel activity (Figure 4D, middle: low exposure, right: high exposure). Treatment of the cells with empty-vector supernatant had no effect on cell-surface expression indicating the specificity of the klotho action. Total expression of TRPV5 in cell lysates was equal in all conditions (Figure 4D, left). The klotho-mediated increase in channel abundance was fully mimicked by the addition of 250 μg/ml β-glucuronidase indicating that extracellular hydrolysis of TRPV5 associated carbohydrates and consequent plasma membrane gathering depends on the glucuronidase activity of the klotho protein.

N-glycosylation of proteins is highly conserved from yeast to human and has a significant stabilizing effect on large regions of the backbone protein structure to facilitate proper folding and trafficking of membrane proteins. Protein glycosylation has been implicated in cellular functions such as modulating protein structure and localization, cell-cell recognition, and signaling in multicellular systems. The glycosylation of proteins can be modulated by adaptations in the biosynthetic pathway. For instance, dietary variations and also hormones like estradiol can have a profound effect on the display of cell-surface carbohydrate epitopes [24]. The present study now demonstrates that glycosylated cell-surface proteins are extracellularly controlled by glucuronidases to regulate their biological function. Thus, we provide a new concept of a regulatory mechanism by glycosylation in which the secreted urinary β-glucuronidase klotho hydrolyzes the N-linked oligosaccharides of TRPV5 to entrap channels at the plasma membrane. Since klotho and TRPV5 co-localize in the distal part of the nephron, klotho could exert its stimulatory effect via an auto- and/or paracrine mechanism. Furthermore, TRPV5 and
klotho are both positively regulated by the biosynthetic vitamin D pathway. This concomitant control will ensure high Ca\textsuperscript{2+} transport capacity and reduce Ca\textsuperscript{2+} excretion to preserve normal blood Ca\textsuperscript{2+} levels during periods of dietary Ca\textsuperscript{2+} insufficiency.

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References
Klotho hydrolyzes and activates the TRPV5 channel

Chapter 5

Tissue kallikrein stimulates Ca\textsuperscript{2+} reabsorption via PKC-dependent plasma membrane accumulation of TRPV5

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The ideals that have lighted my way, and time after time have given me new courage to face life cheerfully, have been kindness, beauty and truth. (Albert Einstein)

有些理想曾为我们引过道路，并不断给我新的勇气以欣然面对人生，那些理想就是一真、善、美。（爱因斯坦．A．）
Abstract

The Transient Receptor Potential Vanilloid 5 (TRPV5) channel determines urinary Ca^{2+} excretion and is therefore critical for Ca^{2+} homeostasis. Interestingly, mice lacking the serine protease tissue kallikrein (TK) exhibit robust hypercalciuria comparable to the Ca^{2+} leak in TRPV5 knockout mice. Here, we delineated the molecular mechanism through which TK stimulates Ca^{2+} reabsorption. Using TRPV5-expressing primary cultures of renal Ca^{2+}-transporting epithelial cells, we showed that TK activates Ca^{2+} reabsorption. The stimulatory effect of TK was mimicked by bradykinin (BK) and could be reversed by application of JE049, a BK receptor type 2 (B2R) antagonist. A cell permeable analog of diacylglycerol (DAG) increased TRPV5 activity within 30 minutes via protein kinase C activation of the channel, since mutation of TRPV5 at the putative PKC phosphorylation sites S299 and S654 prevented the stimulatory effect of TK. Cell-surface labeling revealed that TK enhanced the amount of wild-type TRPV5 channels, but not of the TRPV5 S299A and S654A mutants, at the plasma membrane by delaying its retrieval. In conclusion, TK stimulates Ca^{2+} reabsorption via the BK-activated PLC/DAG/PKC pathway and the subsequent stabilization of the TRPV5 channel at the plasma membrane.

Introduction

The overall Ca^{2+} balance is regulated by a homeostatic mechanism tightly controlling the concerted actions of intestinal Ca^{2+} absorption, exchange of Ca^{2+} from bone and renal Ca^{2+} reabsorption. In kidney, Ca^{2+} can cross the epithelial cells and reach the blood compartment via two pathways: passive (paracellular) and active (transcellular) Ca^{2+} reabsorption. Active Ca^{2+} reabsorption takes place in the distal convoluted (DCT) and the connecting (CNT) tubules [1]. Although it accounts only for ~15% of total renal Ca^{2+} reabsorption, it is generally considered the site for fine-tuning of urinary Ca^{2+} excretion. Furthermore, active Ca^{2+} reabsorption is the primary target for regulation by calcitropic hormones, including 1,25-dihydroxyvitamin D_{3} and parathyroid hormone (PTH), enabling the organism to regulate Ca^{2+} reabsorption to the body’s demand.

The Transient Receptor Potential Vanilloid 5 (TRPV5) channel is expressed along the apical membrane of DCT and CNT and represents the rate-limiting step in renal
Transcellular Ca\(^{2+}\) reabsorption [1]. Inactivation of TRPV5 in mice (TRPV5\(^{-/-}\)) abolishes active Ca\(^{2+}\) reabsorption in kidney resulting in severe hypercalciuria [2]. To compensate this renal Ca\(^{2+}\) leak, TRPV5\(^{-/-}\) mice exhibit intestinal Ca\(^{2+}\) hyperabsorption. In addition, the bone structure of these mice is significantly disturbed as illustrated by a reduced trabecular and cortical bone thickness [2]. Hence, these data demonstrate the key function of TRPV5 in active Ca\(^{2+}\) reabsorption and its essential role in the body Ca\(^{2+}\) homeostasis.

Interestingly, hypercalciuria was recently observed in tissue kallikrein-deficient (TK\(^{-/-}\)) mice [3]. TK is a serine protease produced in CNT [4], where it co-localizes with TRPV5 [2]. Proteolytic enzymes such as TK are synthesized as inactive precursors or zymogens, to prevent protein degradation and to enable spatial and temporal regulation of enzymatic activity. The precursor of TK is converted to the mature active form before entering the luminal tubular compartment. Trypsin, plasma kallikrein, plasmin and thermolysin can cleave in vitro the TK precursor, but the endogenous activator is still unknown [5]. Once activated, TK is excreted and can process low molecular weight kininogen to release kinin which acts through kinin receptors such as the bradykinin (BK) 2 receptor (B2R) [6]. Remarkably, recent studies show that TK can directly activate the B2R independently of BK release [7]. However, the molecular events that link TK to Ca\(^{2+}\) balance are at present unknown.

The aim of this study was, therefore, to elucidate the molecular mechanism of hypercalciuria observed in TK\(^{-/-}\) mice. To this end, the relation between TK expression and hypercalciuria was investigated in vivo using TK\(^{-/-}\) and TRPV5\(^{-/-}\) mice. Subsequently, the effect of TK on transcellular Ca\(^{2+}\) transport was examined in primary cultures of renal CNT/cortical collecting duct (CCD) cells. Finally, the signaling pathway through which TK acts on Ca\(^{2+}\) reabsorption and its effect on TRPV5 surface expression were delineated in TRPV5-expressing cells.

**Material and methods**

**Animal experiments**

TK\(^{-/-}\) mice were produced as previously described [3, 8] and fed ad libitum two Na\(^{+}\) diets containing 0.3% w/w or 3% w/w for 14 days. Subsequently, 24 hour urine was
collected from TK\(^{-}\) and TK\(^{+/+}\) mice housed in metabolic cages and blood was obtained by orbital puncture. Measurements of biological parameters in the animals' plasma and urine were performed as previously described [3]. Urinary Na\(^{+}\), Ca\(^{2+}\) and Mg\(^{2+}\) excretion was expressed as ratios to urinary creatinine excretion to take into account the variations in urine collection. TRPV5\(^{-}\) mice were generated as described previously [2]. At the age of 4 weeks, mice were fed ad libitum two diets containing 0.02% w/w or 2% w/w Ca\(^{2+}\) for 5 weeks and were subsequently placed in metabolic cages. 24 hour urine samples were collected and subjected to TCA precipitation. The animal ethics board of the Radboud University Nijmegen approved all animal experimental procedures.

**Urine protein precipitation by TCA**

10% v/v of the 24 hour urine volume collected of TRPV5\(^{-}\) mice was centrifuged at 200 g for 10 minutes at 4°C to remove cell debris. Proteins in the urine were precipitated using ice-cold TCA (Acros organics, New Jersey, USA). The TCA mixture was centrifuged at 13,000 g for 10 minutes at 4°C, and the pellet was subsequently washed with 300 \(\mu\)l acetone. Next, the pellet was air-dried for approximately 3 minutes, dissolved in Laemmli buffer containing 0.1 M DTT and 150 mM Tris (pH 8.8) and analyzed by immunoblot analysis for the expression of TK using a rabbit anti-TK antibody (Calbiochem, San Diego, CA, USA). Immunopositive bands were scanned using an imaging densitometer to determine pixel density (Molecular Analyst Software, BioRad Laboratories, Hercules, CA).

**DNA constructs and cell culture**

The pCINeo/IRES-GFP-TRPV5 and pCINeo/IRES-GFP-HA-TRPV5 constructs were generated as described previously [9]. Single and combined PKC mutants were generated by alanine substitution of the six putative phosphorylation sites of TRPV5 (S144A, S299A, S316A, S654A, S664A, S698A) using \textit{in vitro} mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA, USA). The B2R pcDNA3 was a kind gift from Prof G. Erdös, MD (Department of Pharmacology, University of Illinois, Chicago, USA). HEK293 cells were transfected at 70% of confluency using polyethylenimine (Polysciences, Inc., Warrington, USA) or Lipofectamin 2000 (Invitrogen Life Technologies, Breda, The Netherlands). After 48-60 hours, cells were used for \(^{45}\)Ca\(^{2+}\) uptake assays, patch-clamp and/or biotinylation.
Tissue kallikrein stimulates TRPV5 activity

experiments. Prior to the assays, cells were incubated for 1 hour in serum-free medium containing the particular compound.

Transcellular Ca\(^{2+}\) transport in renal primary cultures
CNT/CCD tubules were immunodissected from kidney cortex of New Zealand White rabbits (~0.5 kg) using antibody R2G9 and then placed in primary culture on permeable filters (0.33 cm\(^2\); Costar, Cambridge, MA, USA) as described before [10]. At confluence, monolayers were used for the transepithelial Ca\(^{2+}\) transport assay as previously described [10]. Transepithelial potential difference and resistance were checked before and after transport measurement to confirm the integrity of the monolayer.

\(^{45}\text{Ca}^{2+}\) uptake assay
HEK293 cells were transfected with pClNeo/IRES-GFP-HA-TRPV5 or pClNeo/IRES-GFP empty-vector cDNA. Ca\(^{2+}\) uptake was determined in uptake medium (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 10 mM Na-acetate, 2 mM NaH\(_2\)PO\(_4\), 20 mM HEPES-Tris, pH 7.4 supplemented with 10 \(\mu\)M felodipine, 10 \(\mu\)M methoxyverapamil, 1 mM BaCl\(_2\) and 1 \(\mu\)Ci/ml \(^{45}\text{CaCl}_2\)) for 10 minutes at room temperature (20–25°C). Each well was washed extensively with stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 1.5 mM LaCl\(_3\), 10 mM Na-acetate, 20 mM HEPES-Tris, pH 7.4) at 4 °C, incubated with 0.05 % w/v SDS and the lysates were counted for radioactivity using liquid scintillation.

Electrophysiology and solutions
Patch-clamp experiments were performed as described previously [11] 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). Two voltage protocols were used: a ramp, to establish the I-V relation in nominally DVF or in 10 mM Ca\(^{2+}\)-containing extracellular solutions and a hyperpolarizing step protocol to measure the Ca\(^{2+}\)-dependent inactivation. Na\(^{+}\) current densities were calculated from the current at -80 mV during the ramp protocols, while the Ca\(^{2+}\) currents were calculated from the current at -80 mV during the ramp protocols or from Ca\(^{2+}\) peak values were extracted from the current at -100 mV during the step protocol.
The analysis and display of patch-clamp data were performed using Igor Pro software (WaveMetrics, Lake Oswego, USA).

**Cell-surface labeling with biotin**

Human Embryonic Kidney (HEK293) cells were transfected with 15 µg pCINeo/IRES GFP-HA-TRPV5, pCINeo/IRES-GFP-TRPV5-S299A, pCINeo/IRES-GFP-TRPV5-S654A or pCINeo/IRES-GFP empty-vector cDNA in poly-L-lysine (Sigma, St Louis, MO, USA) coated 88 mm dishes. 48 hours after transfection, cells were incubated for 1 hour with 100 nM TK and 1 µM JE049. The biotinylation assay was performed, cells were homogenized in 1 ml lysis buffer as described previously [12] using the NHS-LC-LC-biotin (Pierce, Etten-Leur, The Netherlands). Finally, biotinylated proteins were precipitated using neutravidin-agarose beads (Pierce). TRPV5 expression was analyzed by immunoblot for the precipitates (plasma membrane fraction) and for the total cell lysates using the guinea pig anti-TRPV5 antibody [13]. For the biotinylation assay determining TRPV5 cell-surface turnover, the biotinylation experiment was pursued as described above. For time point 0 hour, cells were collected from plates and lysed, immediately after biotinylation. Other plates of cells were further cultured after biotinylation for 1, 3, 6 or 12 hours, then washed once with ice-cold PBS (pH 7.4), and subsequently homogenized in lysis buffer. All samples were processed as described above.

**Compounds**

TK, BK, PMA and OAG were purchased from (Sigma, St Louis, MO, USA). U73122 and U73343 were purchased from Upjohn Laboratories (Kalamazoo, MI, USA). JE049, formerly known as icatibant or HOE140, which was a kind gift of Dr. J. Pünter (Aventis Pharma Deutschland GmbH, Frankfurt, Germany).

**Statistical analysis**

In all experiments, the data is expressed as mean ± S.E.M. Overall statistical significance was determined by analysis of variance (ANOVA). In case of significance, differences between the means of two groups were analyzed by unpaired t-test, while multiple comparisons between groups were performed by Bonferroni post-hoc tests. \( p<0.05 \) were considered significant. The statistical analyses were performed using the SPSS software (SPSS Inc, Chicago, Illinois, USA).
Result

**TK expression levels inversely correlate with urinary Ca\(^{2+}\) excretion**

The relation between urinary TK expression and urinary Ca\(^{2+}\) excretion was investigated by using wild-type (TK\(^{+/+}\)) and TK\(^{-/-}\) mice fed a 0.3 % w/w (normal) and 3 % w/w (high) Na\(^{+}\) diet. Urinary Ca\(^{2+}\) excretion was significantly increased in TK\(^{+/+}\) mice on high Na\(^{+}\) diet compared to mice on normal diet (\(p<0.05\), \(n=12\) mice) (Figure 1A). Conversely, Ca\(^{2+}\) excretion in TK\(^{-/-}\) mice on normal Na\(^{+}\) diet was significantly higher (140 ± 13 %) than in TK\(^{+/+}\) mice (\(p<0.05\), \(n=12\) mice), but remained constant on a high Na\(^{+}\) diet (\(p>0.2\), \(n=12\) mice) (Figure 1A). On the high Na\(^{+}\) diet there was no significant difference in urinary Ca\(^{2+}\) excretion between TK\(^{+/+}\) and TK\(^{-/-}\) mice. Body weight, food intake, urinary creatinine as well as plasma values of Ca\(^{2+}\), creatinine and protein concentrations did not differ between mice genotypes on either diet (Table 1).

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\(^{*}p<0.05\) versus same genotype on 0.3 % w/w Na\(^{+}\) diet (\(n=12\) mice).

**TK is up-regulated in TRPV5\(^{-/-}\) mice**

The amount of TK excreted in the urine was determined by trichloroacetic acid (TCA) protein precipitation of 24 hour urine collected from wild-type (TRPV5\(^{+/+}\)) and TRPV5\(^{-/-}\) mice fed either a 0.02 % w/w (low) or a 2 % w/w (high) Ca\(^{2+}\) diet. Immunoblot analysis
of the TCA-precipitate showed the specific TK band migrating at ~40 kDa (Figure 1B). Semi-quantitative densitometry of the TK corresponding protein band demonstrated that urinary TK excretion increased in TRPV5-/- mice compared to TRPV5+/+ littermates on low Ca²⁺ diet, whereas the high Ca²⁺ diet did not affect TK excretion between TRPV5-/- and TRPV5+/+ genotypes (Figure 1C).

TK stimulates Ca²⁺ transport in renal primary cell cultures

The effect of TK on transcellular Ca²⁺ transport was evaluated in primary cultures of renal CNT/CCD cells. Application of 100 nM TK to the apical side of the monolayer significantly stimulated Ca²⁺ transport compared to non-treated monolayers, while no effect was observed when TK was added to the basolateral side only (Figure 2A). Importantly, apical addition of TRPV5 channel blocker ruthenium red (10 µM) completely inhibited baseline transepithelial Ca²⁺ transport and abolished the stimulatory effect of TK (Figure 2A). TK action was mimicked by apical addition of 100 nM trypsin, which like TK displays serine protease activity (Figure 2B), as well as 1 µM BK (Figure 2C). Interestingly, the stimulatory effect on Ca²⁺ reabsorption of both BK and TK was inhibited by apical application of 1 µM JE049, a B2R antagonist (Figure 2C). Basolateral treatment of trypsin, BK or JE049 did not affect transcellular Ca²⁺ transport across the monolayer (data not shown).
TK enhances TRPV5 channel activity

The stimulatory effect of TK on Ca\(^{2+}\) transport was further examined in TRPV5-transfected HEK293 cells. Incubation of these cells with TK concentrations ranging from 0.05 to 5,000 nM enhanced \(^{45}\)Ca\(^{2+}\) influx in a dose-dependent manner with a maximal stimulation at 500 nM and an EC\(_{50}\) of 6.00 ± 0.04 nM (Figure 3A), whereas no effect was observed in mock-transfected cells (data not shown).

![Graph A](image1)

**Figure 2. Stimulatory effect of TK on active Ca\(^{2+}\) reabsorption.** (A) Monolayers of rabbit primary cultures of CNT/CCD cells were incubated with 100 nM TK for 90 minutes at the apical, basolateral or both sides. In one condition cell monolayers were incubated for 90 minutes in the presence of the TRPV5 blocker ruthenium red (RR, 10 µM, apical side) in the presence or absence of TK (100 nM, apical side). Vehicle-treated cells were used as control (CTRL) and net apical to basolateral Ca\(^{2+}\) transport was measured. Data are expressed as means ± S.E.M. (n=6 wells). *p<0.05 versus CTRL, #p<0.05 versus TK-treated. (B) Primary cells were treated apically with 100 nM trypsin or 100 nM TK. (C) 1 µM BK and 100 nM TK were applied at the apical side of the monolayers in the presence (open bars) or absence (closed bars) of 1 µM JE049, a B2R antagonist. In all experiments vehicle-treated cells were used as control (CTRL) and net apical to basolateral Ca\(^{2+}\) transport was measured. Data are expressed as means ± S.E.M. (n=4 wells). *p<0.05 versus CTRL.

Further, TK action on TRPV5 channel activity was investigated by the whole-cell patch-clamp technique in TRPV5-expressing HEK293 cells. Pre-incubation with 100 nM TK increased the inward Ca\(^{2+}\) current in response to the step protocol, but the Ca\(^{2+}\)-dependent inactivation of the TRPV5 currents remained unchanged (Figure 3B).

The current-voltage (I-V) relation of the currents in response to a voltage ramp did not alter after pre-incubation with 100 nM TK (Figure 3C), but the inward Ca\(^{2+}\) current at -80 mV increased by 167 ± 14 % compared to non-treated, control (CTRL) cells (p<0.05, n=15 cells) as depicted in Figure 3D. Likewise, in divalent free (DVF) solution...
a significant increase of 149 ± 17 % in Na⁺ inward currents at -80 mV (p<0.05, n=15 cells) was observed, while the I-V relation remained unchanged (Figure 3E-F).

**Figure 3. Effect of TK on TRPV5-transfected HEK293 cells.** (A) HEK293 cells were transfected with TRPV5 and treated for 1 hour with TK in concentrations ranging from 0.05 to 5,000 nM and finally ⁴⁵Ca²⁺ uptake was measured. Values are expressed as means ± S.E.M. (n=6 wells). (B) Averaged Ca²⁺ currents measured with 10 mM Ca²⁺ in the extracellular solution during a 3 seconds step to -100 mV from a holding potential of +70 mV in TRPV5-transfected HEK293 cells treated (dotted trace) or non-treated (solid trace) with 100 nM TK. (C) Current-voltage relations measured from 450 milliseconds voltage ramps in 10 mM Ca²⁺-containing extracellular solution from non-treated control (CTRL) (solid trace) or TK treated (dotted trace) TRPV5-expressing HEK293 cells. (D) Average density of the Ca²⁺ current measured as in (C), was 400 ± 56 pA/pF (n=15 cells) for TK treated cells compared to 240 ± 33 pA/pF (n=15 cells) for control (CTRL) cells. (E) Current-voltage relations measured from 450 milliseconds voltage ramps in nominally DVF solution in TRPV5-transfected HEK293 cells treated (dotted trace) or non-treated (solid trace) with TK. (F) Average Na⁺ current density at -80 mV in nominally DVF solution were 745 ± 85 pA/pF (n=15 cells) for TK treated cells compared to 500 ± 55 pA/pF for control (CTRL) cells (n=15 cells). *p<0.05 versus CTRL.

**TK increases TRPV5-mediated currents via the B2R**

Since BK mimicked the TK action on Ca²⁺ transport in primary cultures of CNT/CCD cells, the effect of BK on TRPV5-expressing HEK293 cells was further studied. First, the expression of the B2R in HEK293 cells was confirmed by immunoblot analysis. B2R-transfected HEK293 cells were used as a positive control. In both mock and B2R-transfected HEK293 cells, the receptor was detected as a single immunopositive band of ~69 kDa (Figure 4A), confirming the presence of the endogenous B2R in this cell line. Subsequently, TRPV5-transfected HEK293 cells were incubated for 1 hour with 1 µM BK and functionally characterized by patch-clamp measurements in comparison to
non-treated (CTRL) cells. Interestingly, BK treatment resulted in a significant increase of in both Ca$^{2+}$ (Figure 4D) and Na$^+$ (Figure 4F) currents ($p<0.05$, $n=12$ cells) compared to CTRL cells, without affecting the Ca$^{2+}$-dependent inactivation (Figure 4B) or the I-V relation of the currents (Figure 4C and 4E).

Because the different treatments of TRPV5-expressing HEK293 cells affected both Ca$^{2+}$ and Na$^+$ current amplitudes leaving unaffected the other current properties, only the Ca$^{2+}$ currents were depicted to demonstrate the effect of particular compounds.
Incubation of TRPV5-transfected cells with 1 μM JE049 reversed the stimulatory effect of TK on TRPV5-mediated currents. The Ca\textsuperscript{2+}-dependent inactivation of Ca\textsuperscript{2+} currents remained unchanged for cells treated with TK or TK and JE049 (Figure 4G). Ca\textsuperscript{2+} current amplitudes measured from the step protocol were significantly lower, 285 ± 50 pA/pF, for the JE049-treated cells ($p<0.05$, n=11 cells) compared to TK-treated cells (Figure 4H).

**TK stimulates TRPV5-mediated currents via the PLC-dependent PKC pathway**

**Figure 5.** TK action is mediated by the DAG/PKC-dependent PLC signalling pathway. TRPV5 expressing cells were incubated for 1 hour with 100 nM TK and subsequently treated as described below. (A) 10 minutes incubation with 10 μM of the PLC inhibitor U73122 reverted the average Ca\textsuperscript{2+} currents amplitude of the TK treated cells to values similar to control (CTRL) cells, contrary to its inactive form U73343 which let the TK effect unaltered (n=10 cells). (B) Ca\textsuperscript{2+} current of cells pre-treated for 1 hour with 10 μM OAG Ca\textsuperscript{2+} currents was 355 ± 30 pA/pF and for control (CTRL) cells 191 ± 25 pA/pF. (C) Time-course of TRPV5 current stimulation by OAG. Points represent Ca\textsuperscript{2+} peak current measured at -100 mV using the step protocol from cells pre-treated with 10 μM OAG for 15, 30 and 60 minutes or non-treated cells. *$p<0.05$ versus non-treated cells at 0 minutes. (D) Average Ca\textsuperscript{2+} currents for cells expressing TRPV5 mutated for all its six PKC phosphorylation sites were 123 ± 36 pA/pF and TK treated cells were 113 ± 25 pA/pF (n=10 cells for each condition). (E) Point mutation of the PKC phosphorylation sites S299 and S654 did not affect Ca\textsuperscript{2+} currents in comparison with the other four PKC mutants upon TK treatment. Data are expressed as percentage of Ca\textsuperscript{2+} currents measured of cells expressing PKC-TRPV5 point mutants treated with 100 nM TK normalized to non-treated control (CTRL) cells (n=12 cells for each mutant).
Tissue kallikrein stimulates TRPV5 activity

To elucidate in detail the mechanism through which TK enhances TRPV5 activity, the following strategies were followed. First, pre-treatment with 10 μM of the PLC inhibitor U73122 for 10 minutes abolished the TK stimulatory effect on TRPV5-mediated Ca²⁺ currents, whereas 10 μM of its inactive analogue U73343 had no effect (p<0.05, n=10 cells) (Figure 5A). Then, TRPV5-transfected cells were incubated for 1 hour with 10 μM 1-oleoyl-acetyl-sn-glycerol (OAG), a synthetic diacylglycerol (DAG) analogue. OAG mimicked the stimulatory effect of TK on TRPV5-mediated Ca²⁺ currents (p<0.05, n=10 cells) (Figure 5B). The stimulatory effect of OAG was observed within 30 minutes after addition (Figure 5C). Next, the effect of TK on the sextuple PKC phosphorylation-deficient mutant was tested. In HEK293 cells expressing this mutant, the stimulatory effect of TK was abolished (Figure 5D). Subsequently, the PKC phosphorylation sites were individually mutated into an alanine residue. TK increased Ca²⁺ currents of all single PKC mutants, except of S299A and S654A (p<0.05, n=12 cells) (Figure 5E).

Figure 5. (F) Incubation with 1 μM PMA for 24 hours was used to down-regulate the PMA-sensitive PKC isoforms in TRPV5-transfected cells. In both cases, non-treated (NT), or incubation with PMA for 24 hours, TK (black bars) was still able to significantly increase TRPV5-mediated Ca²⁺ currents compared to control cells (white bars) (n=14 cells for each condition) *p<0.05 versus CTRL cells.

Figure 6. B2R confers specificity to TK-dependent stimulation of TRPV5 activity. (A) Averaged Ca²⁺ currents measured with 10 mM Ca²⁺ in the extracellular solution during a 3 seconds step to -100 mV from a holding potential of +70 mV in TRPV5-transfected HEK293 cells treated with 1 mM ATP (dotted trace), treated with 1 mM ATP and 1 μM of the B2R antagonist JE049 (dashed line) or non-treated control (solid trace). (B) Current-voltage relationships measured from 450 milliseconds voltage ramps in 10 mM Ca²⁺-containing solution in TRPV5-transfected HEK293 cells treated with ATP (dotted trace), treated with ATP and JE049 (dashed trace) or non-treated (solid trace) with ATP. (C) Average density of the Ca²⁺ peak current measured as in (B), was 300 ± 54 pA/pF for ATP-treated cells and 322 ± 65 pA/pF for ATP and JE049, compared to 190 ± 23 pA/pF (n=15 cells) for control (CTRL) cells. *p<0.05 versus CTRL.
Furthermore, to determine which PKC isoforms are involved in this process, cells were incubated for 24 hours with 1 μM of phorbol ester (phorbol 12-myristate 13-acetate-PMA) to down-regulate the PMA-sensitive PKC isoforms expressed in HEK293 cells as previously described [14]. Then, these PMA-pretreated cells were treated as aforementioned with 100 nM TK for 1 hour. TK was still able to significantly increase (164 ± 14% of control, p<0.05, n=14 cells) the TRPV5-mediated Ca\(^{2+}\) currents (Figure 5F). Vehicle-incubated (NT) cells were used as control for the effect of PMA on TRPV5 currents and PMA incubation left TRPV5-mediated currents unaffected.

Subsequently, the possible involvement of other G-protein coupled receptors in addition to the B2R was investigated. To this end, purinergic receptors were activated by the addition of extracellular ATP (1 mM for 1 hour) to TRPV5-expressing HEK293 cells. Indeed, as shown in the Figure 6, ATP treatment enhanced the inward Ca\(^{2+}\) currents in these cells, while leaving the I-V relationship and Ca\(^{2+}\)-dependent inactivation unchanged. Moreover, after pre-treatment with the B2R antagonist JE049, ATP was still able to stimulate the TRPV5 currents, indirectly demonstrating the specificity of TK stimulation via the B2R.

**TK increases cell-surface expression of TRPV5 by delaying channel retrieval**

![Figure 7. TK affects the plasma membrane expression of TRPV5.](image)

The effect of TK on the amount of TRPV5 channel expressed at the plasma membrane was investigated in HEK293 cells. TRPV5-transfected cells were incubated with 100 nM TK alone or in combination with 1 μM JE049 for 1 hour and then subjected to cell-surface biotinylation. Biotinylated cell lysates were precipitated with neutravidin-
Tissue kallikrein stimulates TRPV5 activity

agarose beads and immunoblotted for TRPV5. Mock-transfected and non-biotin-treated cells were used as negative controls. TK treatment enhanced TRPV5 expression in the biotinylated fraction, whereas JE049 abolished this effect (Figure 7A, left panel). Importantly, TRPV5 was equally expressed in total cell lysates of all tested conditions (Figure 7A, right panel). The observed increase in cell-surface expression of TRPV5 by TK could be due to either an enhanced trafficking from the Golgi apparatus to the cell surface or a reduction in channel retrieval from the plasma membrane. The kinetics of cell-surface retrieval was measured in HEK293 cells using cell-surface biotinylation. After a 1 hour TK treatment, the retrieval of TRPV5 channel from the plasma membrane was decreased by 71, 46, and 17 % ($p<0.05$, n=3 blots) at the time points of 1, 3 and 6 hours, respectively (Figure 7B). TRPV5 expression in cell lysates was identical in all tested conditions (Figure 7C). These results suggested that TK increases cell-surface expression of TRPV5 by delaying channel retrieval from the plasma membrane.

TRPV5 PKC mutants S299A and S654A are insensitive to TK treatment

The effect of TK on the TRPV5 PKC mutants S299A and S654A was investigated in HEK293 cells by biotinylation analysis. TRPV5 wild-type (WT), S299A and S654A transfected cells were incubated with or without 100 nM TK for 1 hour and then the kinetics of cell-surface retrieval was measured in HEK293 cells using cell-surface biotinylation. Without TK treatment (Figure 8A), the plasma membrane expression of TRPV5 WT, S299A and S654A was decreased at the similar extent at the time points
of 1, 3, 6 and 12 hours. Interestingly, with TK treatment (Figure 8B), the retrieval of TRPV5 WT channel from the plasma membrane was decreased, but retrieval of S299A and S654A was not altered. TRPV5 WT, S299A and S654A expression in cell lysates was identical in all tested conditions (data not shown). These results indicated that the TK effect of delaying channel retrieval from the plasma membrane was abolished in TRPV5 PKC mutants S299A and S654A.

Discussion

The present study showed that extracellular TK stimulates TRPV5-mediated Ca\(^{2+}\) reabsorption by activating the B2R and subsequently the DAG/PKC pathway resulting in accumulation of TRPV5 channels at the cell surface. This conclusion is based on the following experimental observations: (i) Urinary Ca\(^{2+}\) excretion in mice is inversely related to the expression of TK; (ii) TK stimulates Ca\(^{2+}\) reabsorption in primary cultures of renal CNT/CCD cells, which can be blocked by the B2R antagonist JE049; (iii) the stimulatory effect of TK is mediated by the PLC/DAG/PKC-pathway and requires the
Tissue kallikrein stimulates TRPV5 activity

two conserved PKC sites S299 and S654 in TRPV5; (iv) TK enhances Ca\(^{2+}\) transport by increasing TRPV5 abundance at the plasma membrane.

TK stimulated TRPV5-mediated Ca\(^{2+}\) transport via the B2R receptor. The physiological relevance of this latter finding is substantiated by the co-localization of B2R with TK [15] and TRPV5 [2] in DCT and CNT. B2R belongs to the seven-transmembrane domain G protein-coupled receptor superfamily [16] and signals via G\(_{\alpha}\) protein with consequent activation of PLC. PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP\(_{2}\)) in inositol trisphosphate (IP\(_{3}\)) and DAG [17]. Commonly, IP\(_{3}\) releases Ca\(^{2+}\) from the endoplasmic reticulum stores, while DAG activates PKC [17]. Certainly, our results show that TRPV5 activation requires PIP\(_{2}\) breakdown following B2R stimulation and relies on DAG as a down-stream effector. Subsequently, DAG increases TRPV5 activity via PKC phosphorylation of the channel since potential PKC phosphorylation deficient mutants of TRPV5 at positions S299 and S654 lack the TK effect. This is the first study demonstrating that TRPV5 is activated through its PKC phosphorylation sites. Among the six predicted phosphorylation sites in the TRPV5 sequence, two serines, S299 and S654 are critical for TRPV5 activation by TK. Importantly, TRPV5 closest homologue, TRPV6, did not respond to TK (data not shown). Since the second serine (S654) is not conserved in TRPV6, both serines are apparently critical for stimulation of TRPV5 by TK. In addition, pre-incubation with PMA for 24 hours down-regulates \(\alpha\), \(\beta\), and \(\epsilon\) isoforms of PKC, but not PKC [18]. This latter PKC isoform is presumably involved in TK increase of TRPV5 currents since the stimulatory effect of PKC was similar in PMA-pretreated and control cells. Interestingly, stimulation of active Ca\(^{2+}\) transport in primary cultures of renal CNT/CCD also involves PMA-insensitive PKC isotypes [19] among which the isotype PKC can be activated by B2R [20]. However, B2R knockout mice (B2R\(^{-/-}\)) mice showed no change in urine Ca\(^{2+}\) excretion [3], in contrast with TK\(^{-/-}\) mice, suggesting that compensation mechanisms could mask the TK effect on the Ca\(^{2+}\) balance in these knockout mice. For instance, it has been shown that in these knockout mice a compensatory induction of the B1 receptor occurs that could theoretically be a new target for TK (Duka et al., 2001). Alternatively, TK exerts its in vivo effect via a mechanism independent of the B2R.

Previous studies showed that TRP channels can be activated by different signaling molecules of the PLC pathway. For example, the three canonical TRP members
TRPC3, 6 and 7 are characterized by their sensitivity to DAG [21]. Alternatively, the vanilloid and melastatine members of TRP family, TRPV1 [22], TRPV5 [23], TRPM4 [24], TRPM5 [25], TRPM7 [26] and TRPM8 [27] can be directly regulated by PIP2. Thus, in vivo TRPV5 activation by PIP2 [23] or DAG/PKC could adjust TRPV5 activity in response to physiological fluctuations. In addition, Ca2+ entering the cell through TRPV5 could prevent the electrostatic interaction between the negatively charged PIP2 and the channel by screening the negative charge on the lipid head group, as proposed for Mg2+ and TRPM7 [28].

Stimulation of Ca2+ transport through TRPV5 upon TK action could be the result of an increase in either open probability of the channel or in expression of TRPV5 at the plasma membrane. Surface biotinylation analysis showed that TK increases the amount of TRPV5 channels at the plasma membrane. Thus, PKC activation of TRPV5 following the TK application would regulate the balance between constitutive exocytosis and endocytosis in favor of the former leading to the accumulation of TRPV5 at the cell surface. Similarly, the epidermal growth factor (EGF) prevents the internalization of plasma membrane TRPC3 [29]. The role of the cytoskeleton in this translocation process is presently unknown. It is possible that PKC-dependent phosphorylation of TRPV5 leads to activation of motor proteins that transport the channels towards the plasma membrane. This process could involve the FKBP52 protein, characterized previously as a TRPV5 regulatory protein [30], since FKBP52 is known to interact with the motor protein dynein [31, 32]. Interestingly, TRPM7 associates to the actomyosin cytoskeleton upon BK stimulation regulating cell adhesion [33]. Remarkably, the protein synaptotagmin has been proposed to regulate the exocytosis of TRPC5 [34]. However, the cytoskeletal elements and the motor proteins participating in the incorporation of TRPV5 at the plasma membrane remain to be identified. Furthermore, accumulation of channels at the cell surface can also occur by increased incorporation into the plasma membrane. Indeed, PKC potentiation of TRPV1 promotes the recruitment of a channel vesicular pool to the cell surface [35]. This exocytosis process of TRPV1 is dependent on the soluble N-ethylmaleimide-sensitive-factor attachment proteins receptor (SNARE) [35], known to act as membrane recognition molecules and acceptors for vesicle trafficking, docking and fusion [36]. It would be interesting to investigate the role of similar scaffold proteins in the assembly of TRPV5 and PKC upon TK treatment, considering that such signaling
pathways are currently indicated to function in spatially distinct microdomains [37]. For instance, the A-kinase-anchoring protein, AKAP, has been described to coordinate the subcellular localization of second messenger-regulated enzymes, such as PKC in order to modulate the activity of K+ channel, KCNQ/M, upon agonist stimulation via Gq-coupled pathway [38].

The aforementioned data is in support of plasma membrane recycling of TRPV5 protein as mechanism of channel regulation. A tight control of TRPV5 activity is of primordial importance for body Ca2+ homeostasis, since TRPV5 constitutes the fine-tuned Ca2+ entry step in active Ca2+ reabsorption [1]. Besides the hormonal regulation of TRPV5, accessory proteins play a role in modulating channel trafficking and activity [9, 39]. Together with a recent report on the β-glucuronidase klotho [12], our study introduces a new mechanism of TRPV5 regulation which is based on extracellular enzymatic activation. TK and klotho co-localize with TRPV5 in the distal part of the nephron where they activate the channel from the luminal side. A comparable enzymatic regulation is described for the epithelial Na+ channel, ENaC, present in CNT, by the channel activating proteases, CAP-1, CAP-2 and CAP-3 [40]. Unlike the protease CAP and the β-glucuronidase klotho, TK stimulates TRPV5 indirectly via activation of the B2R that then induces a redistribution of TRPV5 channels towards the plasma membrane. On the contrary, CAPs which are membrane-bound serine proteases, act directly on the channel gating by enhancing the open probability of ENaC [41] to stimulate epithelial Na+ absorption. Likewise, klotho directly activates TRPV5 by enzymatic modification of the N-glycan to stabilize TRPV5 channels at the plasma membrane [12].

Interestingly, we showed that an increase in Na+ supply induced a Ca2+ wasting in WT mice. It is well know that a high Na+ diet increases the plasma volume that triggers the renin-angiotensin-aldosterone system (Nijenhuis et al., 2005). These hormones will reduce the reabsorption of Na+ and consequently Ca2+ in the proximal tubules as a negative feedback response. Ultimately, this will increase the urinary excretion of Ca2+. However, the anticipated calciuria was not observed in TK-/- mice, possibly these mice can not further increase their urinary Ca2+ excretion. In addition, the rates of urinary Ca2+ excretion in WT and TK-/- mice were similar on a high Na+ diet, whereas they markedly differ on a normal Na+ diet. This strongly supports the fact that TK
participates in the renal response to a high Na$^+$ diet and to the attendant decrease in urinary Ca$^{2+}$ reabsorption.

![Figure 9. Schematic diagram of TK stimulatory effect on TRPV5.](image)

TK activates the B2R and subsequently PLC, leading to the hydrolysis of PIP$_2$ in IP$_3$ and DAG. DAG induces subsequently the phosphorylation of TRPV5 through PKC-dependent mechanism with consequent stabilization of TRPV5 channel at the plasma membrane. The increased number of channels at the plasma membrane is the result of delayed channel retrieval ensuing enhanced Ca$^{2+}$ transport through TRPV5.

Here, we demonstrated that TK displays autocrine or paracrine stimulation of active Ca$^{2+}$ reabsorption in a dose-dependent manner. As shown in the mice models, reduction in TK expression increases urinary Ca$^{2+}$ excretion due to impaired tubular Ca$^{2+}$ reabsorption. Conversely, genetic ablation of TRPV5 doubled the renal expression of TK possibly due to counterbalance the significant Ca$^{2+}$ wasting observed in the TRPV5$^{-/-}$ mice by maximally stimulating the process of Ca$^{2+}$ reabsorption. Indeed, an increase in dietary Ca$^{2+}$ intake as an alternative manoeuvre to offset the renal Ca$^{2+}$ loss in TRPV5$^{-/-}$ mice prevented the compensatory increase in TK expression. These results underline the role of TK during Ca$^{2+}$ restriction. It is, therefore, interesting to study the Ca$^{2+}$ excretion in humans with a genetically reduced TK activity. In this respect, a loss-of-function polymorphism of the TK gene in which the active-site arginine at position 53 is changed into a histidine (R53H), reduces by 50-60% TK activity is interesting [42]. Partial genetic deficiency in TK activity of the R53H subjects is associated with a form of arterial dysfunction [43]. TK is well known as a vasodilator factor of renal cortical blood vessels through BK production, while abnormalities of TK levels has long been documented in the pathogenesis of hypertension [44]. It is
Tissue kallikrein stimulates TRPV5 activity

Therefore tempting to speculate that TK stimulation of TRPV5-mediated Ca\(^{2+}\) reabsorption plays a role in TK hypotensive action, since urinary Ca\(^{2+}\) leak has been correlated with higher baseline systolic and diastolic blood pressures [45].

In conclusion, our data demonstrated that TK reduces urinary Ca\(^{2+}\) excretion by an autocrine and paracrine stimulation of TRPV5-mediated Ca\(^{2+}\) reabsorption explaining the hypercalciuria in TK\(^{-}\) mice. **Figure 9** illustrates the molecular pathway linking TK to stimulation of TRPV5, as elucidated in the present study. TK activates the B2R and through the PLC derived-messenger DAG initiates the phosphorylation of the TRPV5 PKC sites S299 and S654. Subsequently, Ca\(^{2+}\) influx through TRPV5 is enhanced by the accumulation and stabilization of the channel at the plasma membrane. Thus, TK-directed translocation of TRPV5 channels constitutes a mechanism by which renal cells can fine-tune the Ca\(^{2+}\) reabsorption.

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Tissue kallikrein stimulates TRPV5 activity


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Tissue kallikrein stimulates TRPV5 activity
Chapter 6

N-glycosylation of TRPV5 determines channel stability at the plasma membrane

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In preparation
Happiness, I have discovered, is nearly always a rebound from hard work.
(David Grayson)
我发现，辛勤工作的报酬几乎总是幸福。(格雷森. D.)
Abstract
Transient Receptor Potential Vanilloid 5 (TRPV5) constitutes the gatekeeper of transepithelial Ca\(^{2+}\) reabsorption and plays, therefore, an essential role in the maintenance of the Ca\(^{2+}\) balance. TRPV5 is complex glycosylated containing a N-linked glycan located at the asparagine residue N358. Here, we report that TRPV5 channel activity is stimulated by the glycosidases: sialidase that hydrolyzes sialic acid residues and peptide N-glycosidase F (endoF) that removes the entire N-glycan. These two glycosidases activate TRPV5 by enhancing channel expression at the plasma membrane via hydrolysis of the TRPV5 extracellular N-glycan, similar to the recently reported glycosidases \(\beta\)-glucuronidase and klotho. In contrast, \(\beta\)-glucosidase, \(\beta\)-galactosidase and endoglycosidase H (endoH) did not exhibit this stimulatory effect on TRPV5 activity. Furthermore, cell-surface biotinylation was employed to determine the channel turnover at the plasma membrane. It showed that \(\beta\)-glucuronidase delays TRPV5 retrieval at the cell surface. Finally, we demonstrated that the TRPV5 glycosylation-deficient mutant TRPV5-N358Q has a reduced retrieval rate at the plasma membrane compared to the wild-type channel. These data suggest that N-glycosylation plays a crucial role in TRPV5 retrieval and glycosidase-mediated channel activation.

Introduction
Ca\(^{2+}\) is an essential ion in all organisms, where it plays a pivotal role in processes ranging from formation and maintenance of the skeleton to temporal and spatial regulation of neuronal function. The Ca\(^{2+}\) balance is tightly controlled by the concerted action of three organ systems - the gastrointestinal tract, bone and kidney. A major breakthrough in the mechanism by which Ca\(^{2+}\) ions enter the absorptive epithelia was the identification of two epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6 that are the most Ca\(^{2+}\)-selective channels within the Transient Receptor Potential (TRP) superfamily [1, 2]. TRPV5 is essential for renal Ca\(^{2+}\) handling, whereas TRPV6 is postulated to mediate intestinal Ca\(^{2+}\) absorption.

In the process of identifying regulatory proteins of TRPV5 and TRPV6 by oligonucleotide-based microarray chip analysis, we previously discovered klotho as a significantly down-regulated gene in kidneys of the mice lacking TRPV5 (TRPV5\(^{-/-}\) mice) [3]. Klotho, which is a type I membrane glycoprotein of 130 kD, exhibits a considerable homology to \(\beta\)-glucosidase enzymes of bacteria, plants and eukaryotes [4]. Klotho gene
N-glycosylation determines TRPV5 channel stability

ablation results in a syndrome resembling human aging, including short life span, bone aberrations, infertility, skin atrophy and hypercalcemia together with increased vitamin D serum levels [4]. In contrast, klotho extends the life span when overexpressed in mice [5], by regulating the insulin / insulin-like growth factor 1 (IGF1) signaling pathway. To date, the elucidation of the physiological functions of klotho is developing rapidly. Previous studies indicated that klotho may function as ligand, co-receptor, and enzyme, and in addition the protein is secreted in serum, cerebrospinal fluid and urine [3, 6]. Klotho has been implicated in the maintenance of the Ca\(^{2+}\) and phosphate balance [7].

The molecular action of klotho in maintaining the Ca\(^{2+}\) balance was observed in a recent study showing that klotho enhances TRPV5 activity [3]. We demonstrated by immunohistochemistry on kidney sections a co-localization of klotho with TRPV5 in the renal distal convoluted (DCT) and connecting tubules (CNT) [3]. Functional analysis using human embryonic kidney (HEK293) cells showed that TRPV5 activity is significantly enhanced by extracellularly secreted klotho, which can be mimicked by \(\beta\)-glucuronidase. Intriguingly, the stimulatory effect of klotho or \(\beta\)-glucuronidase on TRPV5 was abolished in HEK293 cells expressing the TRPV5 glycosylation-deficient mutant TRPV5-N358Q, indicating that N-glycosylation plays a crucial role in the TRPV5 activation. N-glycosylation of proteins is highly conserved from yeast to human and has a significant effect on modulating protein structure and localization to facilitate appropriate folding and trafficking of membrane proteins [8]. To date, considerable studies have demonstrated that prevention of N-glycosylation negatively influences trafficking and activity of many transporters or channels [9-13]. It has been thoroughly studied that gating, cell-surface trafficking and pH sensitivity of the voltage gated K\(^+\) (K\(_v\)) channels are regulated by N-glycosylation [14-16]. Furthermore, within the TRP superfamily, N-glycosylation influences also TRPV1 and TRPV4 channels [17, 18]. These data point to a potential function of N-glycosylation influencing trafficking, gating and activity across diverse members of membrane proteins.

However, up to date, the exact role of N-glycosylation in the klotho-mediated TRPV5 regulation is elusive. Based on a previous study, we hypothesized that other glycosidases may mimic the stimulatory effect of klotho. Therefore, the current study aims to investigate the molecular mechanism underlying the klotho-mediated TRPV5 activation as well as the specificity of this effect. To this end, we investigated the effect of
diverse members of the glycosidase superfamily, to which β-glucuronidase and klotho belong, namely β-glucosidase, β-galactosidase, sialidase, endoglycosidase H (endoH), and peptide N-glycosidase F (endoF). These glycosidases hydrolyze various sugar residues including glucuronic acid, glucose, galactose, sialic acid, high mannoses and the entire N-glycan. New enzymes in the glycosidase family that activate TRPV5 activity have been discovered using $^{45}$Ca$^{2+}$ uptake analysis in HEK293 cells expressing the channel. Subsequently, the molecular mechanism through which klotho stimulates TRPV5 was delineated by cell-surface biotinylation experiments that address channel turnover at the plasma membrane.

**Material and methods**

**DNA constructs**
pCINeo/IRES-GFP-TRPV5, pCINeo/IRES-GFP-HA-TRPV5 [19], and pCINeo/IRES-GFP-HA-klotho [3] constructs were generated as described previously. The TRPV5 mutant (TRPV5-N358Q) was obtained by *in vitro* mutagenesis in pCINeo/IRES-GFP-TRPV5 cDNA. All constructs were verified by DNA sequence analysis.

**Collection and concentration of conditioned culture media**
HEK293 cells were transfected with 15 μg pCINeo/IRES-GFP-HA-klotho or pCINeo/IRES-GFP empty-vector cDNA in a 85 mm petri-dish. After 3 days of transfection, the conditioned culture media was collected and concentrated 10 times using the centriprep columns ultracel YM-30 (Millipore corporation, Bedford, MA, U.S.A.).

**Transfection of HEK293 cells and preparation of total cell membrane**
HEK293 cells were transfected with 2 μg pCINeo/IRES-GFP-HA-TRPV5 or pCINeo/IRES-GFP empty-vector cDNA in a 6-well plate. Two days after transfection, the cells were homogenized in HBA buffer [20 mM Tris (pH 7.4 / HCl), 5 mM MgCl$_2$, 5 mM NaH$_2$PO$_4$, 1 mM EDTA (pH 8.0 / NaOH), 80 mM sucrose, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml pepstatin], and then centrifuged for 15 min at 4,000 g at 4°C, followed by 30 min spin down at 16,000 g at 4°C. The pellet was dissolved in 30 μl of laemmli buffer and incubated at 37°C for 30 min. The proteins were detected by SDS-PAGE gel electrophoresis followed by autoradiography.
Enzymatic treatment of HEK293 cells

HEK293 cells were transfected with 2 μg pCINeo/IRES-GFP-TRPV5-N358Q or pCINeo/IRES-GFP-HA-TRPV5 or pCINeo/IRES-GFP empty-vector cDNA in a 6-well plate. Two days after transfection, cells were homogenized for total membrane isolation as described above. Subsequently, the total membrane fraction was dissolved in DMEM medium (Cambrex Bio Science, Verviers, Belgium). Culture media collected from klotho-expressing HEK293 cells (klotho-containing supernatant), β-glucuronidase from bovine liver (Sigma, St. Louis, MO, U.S.A.), β-glucosidase (Sigma), β-galactosidase (Promega, Madison, WI, U.S.A.), endoH, endoF (New England Biolabs, Ipswich, MA, U.S.A), or sialidase (Calbiochem, San Diego, CA, U.S.A.) was added to the medium at 37°C for 1 hour. The final concentration of β-glucuronidase is 310 U/ml, β-glucosidase or β-galactosidase is 27 U/ml, endoH or endoF is 10,000 U/ml, and sialidase is 27 μU/ml, respectively. In addition, laemmli buffer was added to the medium after glycosidase treatment, and incubated for 30 min at 37°C. The protein was detected by SDS-PAGE.

45Ca²⁺ uptake assay

HEK293 cells were transfected with 2 μg pCINeo/IRES-GFP-TRPV5-N358Q, pCINeo/IRES-GFP-HA-TRPV5 or pCINeo/IRES-GFP empty-vector cDNA in a 6-well plate. One day after transfection, HEK293 cells expressing HA-TRPV5 or TRPV5-N358Q were incubated for 16 hours with klotho-containing supernatant, β-glucuronidase, β-glucosidase, β-galactosidase, endoH, endoF or sialidase in 300 μl DMEM medium at 37°C with 5% (v/v) CO₂. The final concentration of β-glucuronidase is 310 U/ml, β-glucosidase or β-galactosidase is 27 U/ml, endoH or endoF is 10,000 U/ml, and sialidase is 27 mU/ml, respectively. Subsequently, 45Ca²⁺ uptake was determined as described previously [3, 19].

Biotinylation

HEK293 cells were transfected with 15 μg pCINeo/IRES-GFP-TRPV5-N358Q, pCINeo/IRES-GFP-HA-TRPV5 or pCINeo/IRES-GFP empty-vector cDNA in a 85 mm petri-dish, which were coated with 0.1% (v/v) poly-L-lysine. One day after transfection, cells were incubated with klotho-containing supernatant or the investigated glycosidases at 37°C with 5% (v/v) CO₂ for 16 hours. Dishes with transfected confluent HEK293 cells were subsequently processed for the biotinylation assays as described previously [3].
Biotinylation assay to determine TRPV5 cell-surface turnover
HEK293 cells were transfected with 15 μg pCINeo/IRES-GFP-HA-TRPV5 or pCINeo/IRES-GFP-TRPV5-N358Q in a 85 mm petri-dish, which were coated with poly-L-lysine (0.1% v/v). One day after transfection, cells were incubated with 310 U/ml β-glucuronidase for 16 hours at 37°C with 5% (v/v) CO₂. The day after incubation, the biotinylation assay was pursued as described previously [3]. For time point 0 hour, cells were collected from plates and lysed with 1 ml lysis buffer immediately after biotinylation. The cells were further cultured in DMEM medium for 1, 3, 6 and 12 hours at 37°C with 5% (v/v) CO₂, subsequently washed with ice-cold PBS, and homogenized in 1 ml lysis buffer, followed by immunoprecipitation using neutravidin beads.

Immunoblot analysis
Total membrane isolation or biotinylation assay was performed in HEK293 cells as described above. The samples were subjected to SDS-PAGE electrophoresis (8% w/v). Immunoblots were incubated overnight at 4°C with guinea pig anti-TRPV5 (1:4,000) in 1% (w/v) non-fat dried milk (NFDM) in TBS-T [Tris-buffered saline (TBS) buffer (150 mM NaCl, 10 mM Tris pH 7.4 / HCl) containing 0.2% (v/v) Tween-20]. After washing, immunoblots were incubated at room temperature with goat anti-guinea pig IgG peroxidase (1:10,000) (Sigma, St. Louis, MO, U.S.A.) in TBS-T.

Statistical analysis
In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). P values below 0.05 were considered significant.

Results
EndoF and sialidase stimulate TRPV5 activity
The effect of different glycosidases on TRPV5 activity was determined in HEK293 cells expressing TRPV5 using a ⁴⁵Ca²⁺ uptake assay. These cells were incubated for 16 hours with various members of the glycosidase superfamily, including β-glucosidase, β-galactosidase, sialidase, endoH and endoF. Klotho-containing supernatant and β-glucuronidase were used as positive controls. HEK293 cells expressing TRPV5 showed an increase in Ca²⁺ influx (~2 fold) compared to empty-vector (mock)-transfected HEK
N-glycosylation determines TRPV5 channel stability

cells (Figure 1A). Remarkably, incubation with sialidase or endoF significantly enhanced the TRPV5-mediated Ca\(^{2+}\) influx, same as the klotho-containing supernatant or β-glucuronidase incubation. However, cells incubated with β-glucosidase, β-galactosidase or endoH showed a Ca\(^{2+}\) influx indistinguishable from vehicle treated TRPV5-expressing cells. Importantly, sialidase and endoF did not stimulate Ca\(^{2+}\) influx in mock-transfected HEK293 cells (Figure 1A).

![Figure 1. Stimulation of TRPV5 activity by endoF and sialidase. (A) \(^{45}\)Ca\(^{2+}\) uptake was measured in HEK293 cells transfected with cDNA of TRPV5 or mock. Subsequently, the cells were exposed for 16 hours to different members of the glycosidase superfamily including β-glucuronidase, klotho-containing supernatant, sialidase, endoF, endoH, β-glucosidase and β-galactosidase. After 16 hours of incubation, the cells were analyzed for \(^{45}\)Ca\(^{2+}\) influx analysis. Sialidase and endoF incubated mock-transfected HEK293 cells were used as control. Asterisk indicates significant difference from HEK293-TRPV5 cells. #, indicates significant difference from mock-transfected HEK293 cells (p<0.05, n=6). (B) HEK293-TRPV5 cells were treated with different glycosidases as described above and analyzed by immunoblotting analysis.]

**Effect of glycosidases on TRPV5 N-glycosylation**

Heterologous expression of TRPV5 in HEK293 cells and subsequent immunoblot analysis of total cell membrane isolation revealed two specific bands of TRPV5 with molecular sizes of 70-75 and 85-100 kDa. The band of 70-75 kD reflected the core TRPV5 protein, whereas the band of 85-100 kD represented the complex glycosylated
form (Figure 1B). The complex-glycosylated TRPV5 band disappeared after endoF treatment, but was not altered by the other investigated glycosidases (Figure 1B). Importantly, total expression of TRPV5 was identical in all conditions.

![Image](image.png)

Figure 2. Enhancement of TRPV5 expression at the cell surface by klotho, β-glucuronidase, sialidase and endoF. HEK293 cells were transfected with TRPV5 and incubated for 16 hours with β-glucuronidase, klotho-containing supernatant, sialidase and endoF. (A) Cell-surface proteins were biotinylated, precipitated with neutravidin-agarose beads and immunoblotted for TRPV5. (B) TRPV5 expression was determined by immunoblotting in total cell lysates to demonstrate equal expression. As a negative control biotin was omitted in the procedure.

Klotho, β-glucuronidase, sialidase and endoF enhance TRPV5 expression at the plasma membrane

To address the molecular mechanism of glycosidase-mediated TRPV5 activation, cell-surface protein biotinylation assays were performed with TRPV5-expressing HEK293 cells treated with the various glycosidases. Cell-surface protein labeling indicated that channel expression at the plasma membrane is significantly increased by enzymatic treatment with endoF, sialidase, klotho or β-glucuronidase (Figure 2A). Importantly, immunoblot analysis showed that the total membrane expression of TRPV5 was equal in all conditions (Figure 2B). The intensity of complex-glycosylated band of TRPV5 reduced upon the endoF treatment in both plasma membrane and total membrane fractions.

β-glucuronidase increases channel activity by delaying TRPV5 retrieval at the plasma membrane

The retrieval of TRPV5 from the plasma membrane to the cytosol was measured using metabolic labeling in combination with cell-surface biotinylation. Channel abundance of wild-type TRPV5 at the plasma membrane decreased by 41% (p<0.05, n=3) within 1
N-glycosylation determines TRPV5 channel stability

hour due to protein retrieval (Figure 3A). However, this apparent retrieval process was delayed by 98%, 85% and 77% (p<0.05, n=3) after the exposure to β-glucuronidase at 1, 3 and 6 hours, respectively (Figure 3B). Importantly, total expression of TRPV5 was not altered by β-glucuronidase treatment (data not shown). Semi-quantification of the immunoblot demonstrated significantly delayed retrieval kinetics of TRPV5 in HEK293 cells treated with β-glucuronidase, compared to non-treated cells (Figure 3C).

Retrieval of the TRPV5 glycosylation-deficient mutant is delayed at the plasma membrane

To elucidate the molecular mechanism responsible for the delay in klotho-mediated TRPV5 retrieval, we studied the role of N-glycosylation in this process. Sequence analysis predicted a conserved N-glycosylation site (N358) positioned between TM segment 1 and 2 of TRPV5. The functional role of N-glycosylation in klotho-mediated TRPV5 stimulation was investigated by mutating the asparagine residue in this putative

Figure 3. TRPV5 retrieval at the plasma membrane is delayed by β-glucuronidase. HEK293 cells were transfected with TRPV5 and incubated for 16 hours with β-glucuronidase. TRPV5 cell-surface expression was determined by biotinylation for 30 min, and the amount of TRPV5 channels at the plasma membrane with (B) or without (A) β-glucuronidase treatment was analyzed by immunoblotting analysis at the different time points as depicted. (C) Semi-quantification of the immunoblot indicated the significantly different retrieval process with or without β-glucuronidase treatment.
glycosylation site into a glutamine (TRPV5-N358Q) [3]. Immunoblot analysis showed that N-glycosylation is impaired in TRPV5-N358Q, since the upper complex-glycosylated TRPV5 band of this mutant was not detectable (Figure 4C) [3]. HEK293 cells expressing TRPV5-N358Q exhibited wild-type TRPV5 Ca\(^{2+}\) influx (Figure 4A) [3]. The apparent retrieval process of TRPV5 was decreased in the TRPV5-N358Q mutant by 100%, 60%, 53% and 45% (n=3) at 1, 3, 6 and 12 hours, respectively (Figure 4B). Total expression of TRPV5 and TRPV5-N358Q was similar in all conditions (Figure 4C).

β-glucuronidase does not change the retrieval kinetics of TRPV5-N358Q

To address the functional consequences of β-glucuronidase action on TRPV5-N358Q, HEK293 cells expressing TRPV5 or TRPV5-N358Q were treated for 16 hours with β-
N-glycosylation determines TRPV5 channel stability

glucuronidase. $^{45}\text{Ca}^2+$ uptake and cell-surface biotinylation assays were subsequently performed. $^{45}\text{Ca}^2+$ uptake analysis demonstrated that TRPV5-N358Q expressed a comparable Ca$^2+$ influx to wild-type TRPV5, which was not enhanced by β-glucuronidase treatment (Figure 4A). Furthermore, cell-surface biotinylation showed that β-glucuronidase is not able to change the retrieval kinetics of TRPV5-N358Q at the plasma membrane (Figure 5B), whereas wild-type TRPV5 retrieval was significantly delayed by this enzyme (Figure 5A). Importantly, total expression of TRPV5 and TRPV5-N358Q was equal in all conditions (Figure 5C, D).

![Figure 5](image)

Figure 5. Retrieval kinetics of TRPV5-N358Q are not affected by β-glucuronidase. HEK293 cells were transfected with TRPV5 or TRPV5-N358Q, and subsequently exposed to β-glucuronidase for 16 hours. TRPV5 cell-surface expression was determined by biotinylation for 30 min, and the amount of TRPV5 channels at the plasma membrane with or without β-glucuronidase treatment was analyzed by immunoblotting analysis at the different time points as depicted (A, B). Total TRPV5 expression was determined by immunoblotting to demonstrate equal expression (C, D).

**Discussion**

Here we identified, in addition to klotho and β-glucuronidase, sialidase and endoF as novel glycosidases stimulating TRPV5 activity by hydrolyzing the N-oligosaccharides of the channel, resulting in stabilization of TRPV5 at the plasma membrane. This conclusion is based on the following experimental observations: (i) sialidase, endoF,
klotho and β-glucuronidase enhanced TRPV5 channel abundance at the plasma membrane, resulting in increased TRPV5-mediated Ca\(^{2+}\) influx. In contrast, β-glucosidase, β-galactosidase and endoH did not exhibit this stimulatory effect on TRPV5 activity. (ii) β-glucuronidase stabilized TRPV5 at the cell surface by delaying channel protein retrieval. (iii) the glycosylation-deficient mutant TRPV5-N358Q showed delayed retrieval kinetics compared to the wild-type channel. (iv) β-glucuronidase did not affect TRPV5-N358Q retrieval due to absence of the entire N-glycan.

In general glycosidases participate in essential steps of synthesis and degradation of polysaccharides, which are involved in processes such as pathogen defense [21], control of signal transduction [22] and modification of hormones [23]. At present, the biological function of glycosidases remains scarce. So far, some studies suggested that sialidase and endoF can regulate ion channel activity [24]. In support of the previous findings, our data show that sialidase and endoF activate TRPV5, substantially extending the members of glycosidases extracellularly modulating TRPV5 activity besides klotho and β-glucuronidase [3]. Sialidase is known to hydrolyze sialic acid residues from glycosylated proteins [25]. A novel mechanism has been described through which hydrolysis of surface sialic acids by sialidase or removal of the complete surface N-glycan of voltage-gated Na\(^{+}\) channel (Na\(_v\)) can directly modulate channel gating, and therefore affect cardiac, skeletal muscle, and neuronal excitability [24]. Moreover, it has been described that K\(_{v1.1}\) channels contain significant amounts of sialic acids, which appear to be the negative charges at the channel surface sensitive to Ca\(^{2+}\). The data suggested that sialidase treatment modifies K\(_{v1.1}\) function by influencing the channel voltage dependence of activation [26]. EndoF is a well-known glycosidase, which removes the entire N-glycan of proteins. Glycans participate in many key biological processes including cell adhesion, molecular trafficking, receptor activation, signal transduction, and endocytosis [27]. It has been reported that N-glycans could serve as a signal for degradation by the SCF(Fbx2) ubiquitin ligase complex [28]. The F-box protein Fbx2 binds specifically to proteins attached to N-linked high-mannose oligosaccharides and subsequently contributes to ubiquitination of N-glycosylated proteins. These studies highlight the role of N-glycans in protein degradation besides its typical role in protein folding, quality control, sorting, transport and channel activity [29]. Interestingly, the complex-glycosylated TRPV5 band of 85-100 kDa disappeared after endoF treatment, but was not changed by the other investigated glycosidases. It
N-glycosylation determines TRPV5 channel stability

indicated that klotho and sialidase only hydrolyze specific N-oligosaccharides of TRPV5, not detectable as a shift in molecular weight of the channel by SDS-PAGE. However, this hydrolysis can already activate TRPV5. In addition, based on the result that β-glucosidase, β-galactosidase and endoH did not increase TRPV5-mediated Ca\(^{2+}\) influx, our data revealed that these specific N-oligosaccharides, responsible for the channel activation, are β-glucuronic acid and sialidic acid, hydrolyzed by β-glucuronidase and sialidase, respectively.

Stimulation of Ca\(^{2+}\) influx through TRPV5 upon glycosidase treatment could be the result of an increase in either open probability of the channel or in expression of TRPV5 at the plasma membrane. Cell-surface biotinylation analysis showed that sialidase, endoF, klotho and β-glucuronidase increase TRPV5 channel abundance at the plasma membrane. Thereby, TRPV5 activation following the glycosidase application might be due to either an enhanced trafficking from the Golgi apparatus to the cell surface or a reduction in channel retrieval from the plasma membrane. Metabolic labeling in combination with cell-surface biotinylation subsequently revealed that the apparent retrieval process of TRPV5 is significantly delayed upon exposure to β-glucuronidase, indicating that β-glucuronidase increases TRPV5 activity by stabilizing the channel at the plasma membrane. Most likely, this also applies to sialidase and endoF-mediated increase in TRPV5 activity.

To further clarify the molecular mechanism underlying the N-glycan-mediated TRPV5 retrieval, we investigated the retrieval kinetics of the TRPV5 glycosylation-deficient mutant (TRPV5-N358Q). Our result corroborates the importance of N-glycosylation in TRPV5 stability at the cell surface, since TRPV5-N358Q showed a low turnover compared to wild-type channels. In addition, β-glucuronidase did not further delay the TRPV5-N358Q retrieval due to absence of the entire N-glycan of this mutant, confirming that N-glycosylation plays a key role in the retrieval process of TRPV5, and therefore glycosidase-mediated channel activation. N-glycosylation of proteins is highly conserved from yeast to human and has a significant effect on protein structure and localization to facilitate proper folding and trafficking of membrane proteins [8]. To date, considerable studies have conclusively demonstrated that prevention of N-glycosylation negatively influences gating, cell-surface trafficking and activity of many channels or transporters,
for example, the γ-aminobutyric acid (GABA) transporter (GAT1) [9], voltage-gated Ca\(^{2+}\) channels (Ca\(_v\)) [10], cyclic nucleotide-gated ion channel (CNG) [11], Na\(^+-\)K\(^+-\)2Cl\(^-\) cotransporter (NKCC2) [13], G-protein-activated inwardly rectifying K\(^+\) channel (GIRK1), renal outer-medullary K\(^+\) channel (ROMK1) [12], and the K\(_v\) channels [14-16]. In addition, the human ether-à-go-go-related gene (HERG) channel without N-glycosylation degrades more rapidly than the glycosylated channel, resulting in a reduced cell-surface stability [30]. However, in addition to the well-known fact that deglycosylation negatively influences protein trafficking, stability and activity, Xu and co-workers showed that deglycosylation activates TRPV4 channel [18]. They demonstrated that the TRPV4 mutant (TRPV4-N651Q) is not glycosylated. HEK293 cells expressing TRPV4-N651Q display an enhanced Ca\(^{2+}\) entry resulted from an increased plasma membrane abundance of TRPV4-N651Q compared to the wild-type channel. This finding supports the present study showing that deglycosylation of TRPV5 increases channel expression at the cell surface. Furthermore, our data add a new dimension to the traditional effect of N-glycosylation on ion channel trafficking and degradation by showing that deglycosylation delays TRPV5 retrieval to increase channel stability. Future studies should investigate whether the same mechanism applies to other TRP channels.

Together with a recent report on a serine protease, tissue kallikrein (TK) [31], our study introduces a new mechanism through which Ca\(^{2+}\) reabsorption can be stimulated via extracellular urinary enzymes. To date, there is only limited information about extracellular regulation of ion channels and transporters. It has been previously described that epithelial Na\(^+\) channel (ENaC) is extracellularly regulated by the channel activating proteases CAP-1, CAP-2 and CAP-3 [32]. CAPs, which are membrane-bound serine proteases, act directly on channel gating by enhancing the open probability of ENaC to stimulate epithelial Na\(^+\) absorption [33]. Unlike CAPs, TK stimulates TRPV5 indirectly via activation of the bradykinin (BK) 2 receptor (B2R), which then induces a redistribution of TRPV5 channels towards the plasma membrane [31]. Sialidase, endoF, klotho and β-glucuronidase, directly activate TRPV5 by enzymatic modification of the N-glycan to stabilize TRPV5 channels at the plasma membrane. Klotho and TK co-localize with TRPV5 at the apical membrane of the distal part of the nephron. Of particular interest is that these two proteins are secreted into the pro-urine from which they activate the TRPV5 channel, and therefore Ca\(^{2+}\) reabsorption. Thus, it would be tempting to explore additional urinary proteins extracellularly regulating TRPV5. Urinary secreted
N-glycosylation determines TRPV5 channel stability

glycosidases could be potential candidates crucial in maintaining the Ca^{2+} balance. Unraveling the molecular mechanisms by which extracellular calciotropic factors control the activity of TRPV5 is essential in keeping Ca^{2+} levels within a healthy range.

In conclusion, our data demonstrated that sialidase and endoF together with klotho and β-glucuronidase stimulate TRPV5-mediated Ca^{2+} influx by the hydrolysis of the N-glycan of the channel, resulting in an increased channel abundance at the plasma membrane by a reduced retrieval rate.

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Reference


Klotho primarily affects the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6

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In preparation
The important thing in life is to have a great aim, and the determination to attain it. (Johan Wolfgang von Goethe)

人生重要的事情就是确定一个伟大的目标，并决心实现它。（歌德. J. M.）
Abstract
The klotho gene encodes a single-pass transmembrane protein and functions as an aging-suppressing hormone. It was recently demonstrated that klotho plays a pivotal role in the maintenance of Ca\(^{2+}\) homeostasis via regulation of the Transient Receptor Potential (TRP) ion channel TRPV5, the gatekeeper of renal active Ca\(^{2+}\) reabsorption. Klotho stimulates TRPV5 activity by hydrolysis of N-oligosaccharides, resulting in stabilization of the channel at the plasma membrane. To further investigate whether the action of klotho can be extended to other renal apically localized channels or transporters in distal convoluted tubules (DCT), four different proteins were selected including TRPV4, TRPV6, TRPM6 and the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) co-transporter (NCC). \(^{45}\)Ca\(^{2+}\) uptake assays in human embryonic kidney (HEK293) cells expressing the proteins of interest demonstrated that TRPV6-mediated Ca\(^{2+}\) influx is significantly increased by klotho or \(\beta\)-glucuronidase. However, \(\beta\)-glucuronidase did not enhance the Ca\(^{2+}\) influx mediated by TRPV4 and TRPM6. In addition, \(^{22}\)Na\(^{+}\) uptake analysis in mammalian kidney cortical collecting duct (mpkCCD) cells showed that thiazide-sensitive Na\(^{+}\) uptake mediated by NCC is not influenced by \(\beta\)-glucuronidase. Thereby, our data revealed that the stimulatory effect of klotho is predominantly applicable to the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6, but not to TRPV4, TRPM6 and NCC.

Introduction
The klotho gene was originally identified as a gene mutated in a mouse strain that develops multiple aging-like phenotypes [1]. Mice defective in klotho expression (klotho\(^{-/-}\) mice) exhibit a syndrome resembling human aging, including short life span, hypoactivity, muscle atrophy, skin atrophy, osteopenia, vascular calcification, abnormality in phosphate and Ca\(^{2+}\) metabolism [1]. Conversely, overexpression of the klotho gene extends the life span in mice, by regulating the insulin / insulin-like growth factor 1 (IGF1) signaling pathway [2]. These observations suggest that klotho may function as an aging-suppressor and be involved in the regulation of aging and age-related diseases. The klotho gene encodes a single-pass transmembrane protein. The extracellular region of the protein is composed of two domains, each having a homology to \(\beta\)-glucosidase of bacteria and plants. In addition, \(\beta\)-glucuronidase activity is detected when artificial substrates are added to the extracellular domain of klotho [3]. The extracellular domain of klotho is secreted into serum, urine and cerebrospinal fluid [4, 5]. Intriguingly, klotho is only mainly expressed in the renal DCT cells and the choroid plexus in the brain, but
Klotho primarily affects TRPV5 and TRPV6

multiple aging-like phenotypes of klotho−/− mice are involving almost all organ systems. Therefore, klotho itself may function as a humoral factor.

To date, the identification of the physiological functions of klotho is developing rapidly. It has been demonstrated that klotho regulates the insulin / IGF1 [2] and fibroblast growth factor (FGF23) signaling pathway [6]. More recently, it was shown that klotho plays a crucial role in the maintenance of Ca2+ and phosphate balance. Appealing evidence of the involvement of klotho in Ca2+ homeostasis came from the study showing that klotho activates the epithelial Ca2+ channel TRPV5 [4]. TRPV5 functions as the gatekeeper of renal transcellular Ca2+ reabsorption, and is regulated at multiple levels including transcription, translation, intracellular trafficking and channel activity at the plasma membrane [7]. We demonstrated [4] by functional analysis using HEK293 cells that TRPV5 activity is significantly enhanced by secreted klotho, which can be mimicked by β-glucuronidase. Intriguingly, the stimulatory effect of klotho or β-glucuronidase on TRPV5 was abolished in HEK293 cells expressing the TRPV5 glycosylation-deficient mutant TRPV5-N358Q, indicating a pivotal role of N-glycosylation in the TRPV5 activation. Since klotho co-localizes with TRPV5 in renal DCT cells where it activates the channel from the pro-urine [4], it is tempting to explore whether the activities of other renal apically localized channels or transporters could be influenced by klotho.

The aim of the present study was, therefore, to examine whether the klotho effect is restricted to TRPV5 or more generally applicable to other renal apically localized proteins in DCT cells. To this end, we selected candidates within the TRP channels including TRPV4, TRPV6 and TRPM6. These channels have structural similarities with TRPV5 and are permeable to Ca2+. Additionally, the thiazide-sensitive Na+-Cl− cotransporter (NCC) was selected as a candidate in this study. Peptide N-glycosidase F (endoF) that removes the entire N-glycan was applied to elucidate the role of N-glycosylation in the regulation of the investigated proteins. By using 45Ca2+ and 22Na+ uptake analyses, we demonstrated that β-glucuronidase does not activate TRPV4, TRPM6 and NCC, but only stimulates TRPV5 and TRPV6. Thereby, a unique stimulatory effect of klotho on the epithelial Ca2+ channels TRPV5 and TRPV6 was discovered.
Material and methods

DNA constructs
pCINeo/IRES-GFP-HA-TRPV5, pCINeo/IRES-GFP-TRPV6 [8], pCINeo/IRES-GFP-TRPV4 [9], pCINeo/IRES-GFP-HA-TRPM6 [10], pCB6-Flag-NCC [11] and pCINeo/IRES-GFP-HA-klotho [4] constructs were generated as described previously. All constructs were verified by DNA sequence analysis.

Collection and concentration of conditioned culture media
HEK293 cells were transfected with 15 μg pCINeo/IRES-GFP-HA-klotho or pCINeo/IRES-GFP empty-vector cDNA in a 85 mm petri-dish. Three days after transfection, the conditioned culture media was collected and concentrated 10 times using the centriprep columns ultrace1 YM-30 (Millipore corporation, Bedford, MA, U.S.A).

Transfection of HEK293 cells and preparation of total cell membrane
HEK293 cells were transfected with 2 μg pCINeo/IRES-GFP-HA-TRPV5, pCINeo/IRES-GFP-TRPV6, pCINeo/IRES-GFP-HA-TRPM6, pCINeo/IRES-GFP empty vector, or 0.5 μg pCINeo/IRES-GFP-TRPV4 cDNA in a 6-well plate. MpkCCD cells were transfected with 2.5 μg pCB6-Flag-NCC or pCB6 empty vector cDNA in a 6-well plate [11]. Two (TRPV4, TRPV5 and TRPV6) or three days (TRPM6 and NCC) after transfection, the cells were homogenized in HBA buffer [20 mM Tris (pH 7.4 / HCl), 5 mM MgCl2, 5 mM NaH2PO4, 1 mM EDTA (pH 8.0 / NaOH), 80 mM sucrose, 1 mM phenyl-methylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml pepstatin], and then centrifuged for 15 min, at 4,000 g at 4°C, in addition to 30 min at 16,000 g at 4°C. The pellet was dissolved in 30 μl of laemmli buffer and incubated at 37°C for 30 min. The proteins were detected by SDS-PAGE gel electrophoresis followed by autoradiography.

45Ca2+ uptake assay
HEK293 cells were transfected with 2 μg pCINeo/IRES-GFP-HA-TRPV5, pCINeo/IRES-GFP-TRPV6, pCINeo/IRES-GFP-HA-TRPM6, pCINeo/IRES-GFP empty-vector, or 0.5 μg pCINeo/IRES-GFP-TRPV4 cDNA in a 6-well plate. HEK293 cells expressing TRPV4, TRPV5, TRPV6, or TRPM6 were incubated for 16 hours with klotho-containing supernatant, 310 U/ml β-glucuronidase (Sigma, St. Louis, MO, U.S.A), or 10,000 U/ml endoF (New England Biolabs, Ipswich, MA, U.S.A) in 300 μl DMEM medium (Cambrex...
Bio Science, Verviers, Belgium) at 37°C with 5% (v/v) CO2, respectively. Subsequently, 

45Ca2+ uptake was determined as described previously [4]. For the 45Ca2+ uptake in TRPV4, TRPV5 and TRPV6, the depletion buffer was KHB buffer (pH 7.4 / 1 M Tris) containing 110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 10 mM Na-acetate, 2 mM NaH2PO4, 20 mM HEPES, 4 mM L-lactate, 10 mM D-glucose and 1 mM L-alanine. The uptake buffer was 45CaCl2 (1 μCi/ml) in KHB buffer with 0.1 mM Ca2+, 4 mM L-lactate, 10 mM D-glucose, 1 mM L-alanine and voltage-gated Ca2+ channel inhibitors (10 μM felodipine, 10 μM verapamil, and 1 mM BaCl2). The stop buffer contained 110 mM NaCl, 5 mM KCl, 1.2 mM MgCl2, 10 mM Na-acetate, 20 mM HEPES, 0.5 mM CaCl2 and 1.5 mM LaCl3. For 45Ca2+ uptake in TRPM6, the depletion buffer (pH 7.4 / 1 M Tris) contained 110 mM NaCl, 5 mM KCl, 10 mM Na-acetate, 20 mM HEPES, 4 mM L-lactate, 10 mM D-glucose and 1 mM L-alanine. The uptake buffer was 45CaCl2 (1 μCi/ml) in depletion buffer with 0.1 mM CaCl2, 4 mM L-lactate, 10 mM D-glucose, 1 mM L-alanine, 10 μM felodipine and 10 μM verapamil. The stop buffer was depletion buffer with 1.5 mM LaCl3 and 0.5 mM CaCl2.

22Na+ uptake
For 22Na+ uptake experiments, mpkCCD cells were transfected with 2.5 μg pCB6-Flag-NCC or pCB6 empty-vector cDNA in a 6-well plate as described previously [11]. MpkCCD cells expressing Flag-NCC were incubated for 16 hours with 310 U/ml β-glucuronidase or 10,000 U/ml endoF at 37°C with 5% (v/v) CO2 after two days of transfection. The cells were subsequently washed and performed with 22Na+ uptake measurement as previously described [11].

Cell culture
MpkCCD cells were derived from a 1-month-old SV-PK/Tag transgenic mouse [11]. Cells were cultured in defined medium consisting of a 1:1 mixture of DMEM:Ham's F-12 (Invitrogen, Breda, the Netherlands), supplemented with 2% (v/v) heat-inactivated fetal calf serum, 5 μg/ml insulin (Sigma, St. Louis, MO, U.S.A), 1 nM dexamethasone (Sigma), 90 nM sodium selenate (Sigma), 5 μg/ml transferrin (Sigma), 10 ng/ml EGF (Sigma), 4.5 mM glutamine (Gibco-BRL, Breda, the Netherlands), 0.2% (w/v) D-glucose (Sigma), 30 μg/ml gentamicin and 20 mM HEPES (Gibco-BRL), equilibrated with 5% CO2–95% air at 37°C.
Protein and immunoblot analysis

Total membranes were isolated from HEK293 or mpkCCD cells. The samples were subjected to SDS-PAGE electrophoresis (6% w/v or 8% w/v). Immunoblots were incubated overnight at 4°C with primary antibodies including guinea pig anti-TRPV5 (1:4,000), mouse anti-HA (1:4,000), rabbit anti-TRPV4 (1:5,000), rabbit anti-TRPV6 (1:2,000) in 1% (w/v) non-fat dried milk (NFDM), or rabbit anti-NCC A857 (1:10,000) [11] in 5% (w/v) NFDM in TBS-T [Tris-buffered saline (TBS) buffer (150 mM NaCl, 10 mM Tris pH 7.4 / HCl) containing 0.2% (v/v) Tween-20]. After washing, immunoblots were incubated at RT with the corresponding secondary antibody including goat anti-guinea pig IgG peroxidase (1:10,000) (Sigma, St. Louis, MO, U.S.A), sheep anti-mouse IgG peroxidase (1:10,000) (Sigma), or goat anti-rabbit IgG peroxidase (1:5,000) (Sigma) in TBS-T.

Statistical analysis

In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). P values below 0.05 were considered significant.

Results

The β-glucuronidase klotho or endoF stimulates TRPV5 activity
The effect of klotho, β-glucuronidase or endoF on TRPV5 activity was determined in HEK293 cells expressing the channel using a $^{45}$Ca$^{2+}$ uptake assay. The TRPV5-transfected HEK293 cells were incubated for 16 hours with culture media collected from klotho-expressing HEK293 cells (klotho-containing supernatant), β-glucuronidase or endoF. $^{45}$Ca$^{2+}$ uptake assay demonstrated that the expression of TRPV5 resulted in a ~2 fold increase of Ca$^{2+}$ influx compared to cells transfected with empty-vector (mock) cDNA (Figure 1A). Pre-incubation with klotho-containing supernatant, β-glucuronidase, or endoF significantly enhanced the TRPV5-mediated Ca$^{2+}$ influx. The complex-glycosylated TRPV5 band of 85-100 kDa disappeared after endoF treatment, but was not noticeably altered by klotho-containing supernatant or β-glucuronidase (Figure 1B). Importantly, total expression of TRPV5 was equal in all conditions.

The β-glucuronidase klotho enhances TRPV6-mediated Ca$^{2+}$ influx

Because TRPV6 is a close homologue of TRPV5, we subsequently tested the klotho effect on TRPV6. TRPV6-transfected HEK293 cells were incubated for 16 hours with klotho-containing supernatant or β-glucuronidase. $^{45}$Ca$^{2+}$ uptake analysis showed that TRPV6 expressing cells display a ~1.5 fold increase of Ca$^{2+}$ influx compared to mock transfected cells (Figure 2A). Incubation with klotho-containing supernatant or β-glucuronidase for 16 hours resulted in a significantly enhanced Ca$^{2+}$ influx mediated by TRPV6. Total expression of TRPV6 in all conditions was similar. In addition, endoF
inhibited complex glycosylation of TRPV6 as indicated by immunoblot showing that the upper complex-glycosylated band of TRPV6 disappeared after endoF treatment. However, the glycosylation was not changed by incubation with klotho-containing supernatant or β-glucuronidase (Figure 2B).

![Graph A](image)

![Graph B](image)

**Figure 2. Activation of TRPV6 channel by β-glucuronidase or klotho.** (A) HEK293 cells were transfected with TRPV6 and incubated for 16 hours with β-glucuronidase (β-Glu) and klotho-containing supernatant (klo-super). The cells were subsequently analyzed for 45Ca\(^{2+}\) influx. Asterisk indicates significant difference from HEK293-TRPV6 cells. #, indicates significant difference from mock-transfected HEK293 cells (p<0.05, n=6). (B) HEK293-TRPV6 cells were treated with klotho-containing supernatant, β-glucuronidase or endoF, and subsequently analyzed by immunobloting analysis.

**Regulation of TRPV4 activity by β-glucuronidase or endoF**

To test whether TRPV4 activity is regulated by β-glucuronidase or endoF, 45Ca\(^{2+}\) uptake assays were performed. TRPV4-transfected HEK293 cells displayed a ~2-3 fold increased Ca\(^{2+}\) influx compared to mock transfected cells. TRPV4-mediated Ca\(^{2+}\) influx was not influenced by β-glucuronidase, but significantly increased by endoF (Figure 3A). The intensity of the complex-glycosylated band of TRPV4 was reduced by endoF treatment (Figure 3B), whereas the total expression of TRPV4 was the same in all conditions.
Klotho primarily affects TRPV5 and TRPV6

Figure 3. Regulation of TRPV4 by endoF or β-glucuronidase. (A) HEK293 cells were transfected with TRPV4 and incubated for 16 hours with β-glucuronidase (β-Glu) or endoF. TRPV4 activity was determined by $^{45}$Ca$^{2+}$ uptake assay. (B) The total expression of TRPV4 channels with or without β-glucuronidase and endoF treatment was analyzed by immunoblotting. Asterisk indicates significant difference from HEK293-TRPV4 cells. #, indicates significant difference from mock-transfected HEK293 cells (p<0.05, n=6).

β-glucuronidase treatment does not stimulate TRPM6 activity

The effect of β-glucuronidase on TRPM6 channel was examined by $^{45}$Ca$^{2+}$ uptake experiments. Our functional analyses demonstrated that TRPM6 transfected cells exhibit a ~1.5 fold increase of Ca$^{2+}$ influx compared to mock-transfected cells. However, β-glucuronidase incubation for 16 hours did not enhance the Ca$^{2+}$ influx mediated by TRPM6.

Figure 4. TRPM6 channel is insensitive to β-glucuronidase treatment. HEK293 cells expressing TRPM6 were exposed to β-glucuronidase (β-Glu) for 16 hours and subsequently assayed for $^{45}$Ca$^{2+}$ uptake. #, indicates significant difference from mock-transfected HEK293 cells (p<0.05, n=6).
NCC activity is insensitive to β-glucuronidase or endoF treatment

Figure 5. β-glucuronidase or endoF is unable to stimulate NCC cotransporter. (A) mpkCCD cells were transfected with Na+-Cl cotransporter (NCC) cDNA. Two days after transfection, the cells were exposed to β-glucuronidase (β-Glu) or endoF for 16 hours. Subsequently, NCC activity was determined by 22Na+ uptake assay. 100 μM hydrochlorothiazide (thiazide) was added to the cells to examine the specificity of Na+ transport by NCC. #, indicates significant difference from mock-transfected HEK293 cells (p<0.05, n=6). (B) The effect of endoF or β-glucuronidase on N-glycosylation of NCC was analyzed by immunoblotting.

22Na+ uptake experiments demonstrated that the Na+ influx in NCC transfected mpkCCD cells was significantly increased as compared to mock transfected cells (Figure 5A). Hydrochlorothiazide (thiazide) reduced the 22Na+ influx in NCC expressing mpkCCD cells, indicating that the NCC-expressing cells displayed a thiazide-sensitive Na+ uptake. Furthermore, the thiazide-sensitive Na+ uptake was not influenced by β-glucuronidase or endoF treatment. The complex-glycosylated band of NCC of 115 kDa completely disappeared after endoF treatment (Figure 5B). Importantly, total expression of NCC in all conditions was similar.

Discussion

Based on the fact that klotho co-localizes with TRPV5 in the renal DCT cells where it is secreted and activates the channel from the pro-urine [4], we explored whether the activities of other renal apically localized channels or transporters in DCT cells (including
Klotho primarily affects TRPV5 and TRPV6

TRPV4, TRPV6, TRPM6 and NCC) can also be extracellularly influenced by klotho. Our data discovered a predominant effect of klotho on the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6. This conclusion is substantiated by the following experimental observations: (i) klotho and \(\beta\)-glucuronidase stimulated TRPV5- or TRPV6-mediated Ca\(^{2+}\) influx. (ii) TRPV4- or TRPM6-mediated Ca\(^{2+}\) influx was not enhanced by \(\beta\)-glucuronidase. (iii) \(\beta\)-glucuronidase did not increase the thiazide-sensitive Na\(^{+}\) influx displayed in NCC transfected mpkCCD cells.

TRPV5 and TRPV6 represent two highly homologous members within the TRP superfamily [12, 13] constituting a distinct class of highly Ca\(^{2+}\)-selective channels [14, 15], which are mainly expressed in Ca\(^{2+}\)-transporting epithelia. TRPV5 shares 75\% homology with TRPV6 at the amino acid level [16], and these two channels have similar molecular structures, electrophysiological features, and the unique pore characteristics [17]. By using \(^{45}\)Ca\(^{2+}\) uptake experiments, we were able to demonstrate that incubation with klotho-containing supernatant or \(\beta\)-glucuronidase for 16 hours results in a significantly enhanced Ca\(^{2+}\) influx mediated by TRPV6. The immunoblot showed identical total expression of TRPV6 in all conditions, indicating that klotho or \(\beta\)-glucuronidase increases either open probability of the channel or TRPV6 abundance at the plasma membrane. The latter is most likely, since a cell-surface biotinylation assay has shown a significantly increased TRPV5 expression at the plasma membrane by klotho [4]. The present study further substantiates klotho as a regulator of both TRPV5 and TRPV6 channels. The tissue distribution of TRPV5 and TRPV6 in humans indicates that both channels are co-expressed in several organs that mediate transcellular Ca\(^{2+}\) transport [18], such as duodenum, colon, and kidney, but also in pancreas, prostate, mammary, sweat and salivary glands. Klotho is predominantly expressed in the renal DCT cells and the choroid plexus in the brain, and the extracellular domain of klotho is secreted into serum, urine and cerebrospinal fluid [4, 5]. Therefore, the secreted klotho may function as a humoral factor extracellularly modulating TRPV5 and TRPV6 activity in various target organs where these two epithelial Ca\(^{2+}\) channels are expressed. Both TRPV5 and TRPV6 channels contain a conserved N-glycosylation site at the asparagine residue N358 (TRPV5) or N357 (TRPV6) in the extracellular loop between TM region 1 and 2, respectively [4]. Immunoblot analysis showed that the intensity of the complex-glycosylated TRPV5 and TRPV6 bands is reduced after incubation with endoglycosidase H (endoH) hydrolyzing N-linked high mannoses [19]. These bands disappear after
treatment with endoF removing the entire N-glycan, indicating that both epithelial
channels are similarly complex glycosylated [19]. N-glycosylation plays a crucial role in
TRPV5 regulation by klotho [4]. Likely, a comparable function applies to N-glycosylation
of TRPV6 in the klotho-mediated channel stimulation

Furthermore, we investigated whether β-glucuronidase activates two other members of
TRP superfamily, TRPV4 and TRPM6, which share similar molecular structures with
TRPV5 and TRPV6. TRPV4 and TRPM6 are Ca^{2+}-permeable cation channels [20, 21],
localized in the renal DCT cells. In general, TRPV4 plays a key role in cell volume
regulation [22], and is widely expressed including in lung, spleen, kidney, testis, fat, brain,
cochlea, skin, smooth muscle, liver, and vascular endothelium [23]. TRPM6 has a more
restricted expression pattern being predominantly present in absorbing epithelia [24],
and plays a potential role in the maintenance of Mg^{2+} balance [25]. Our results showed
that β-glucuronidase incubation does not simulate TRPV4 and TRPM6 channel activity.
Presumably, the N-oligosaccharides pivotal for TRPV5 and TRPV6 activation, which are
hydrolyzed by β-glucuronidase, are not present in TRPV4 and TRPM6. Alternatively,
hydrolysis of the N-oligosaccharides is not involved in the regulation of TRPV4 and
TRPM6. A consensus N-glycosylation motif was identified at the asparagine residue
N651 within the pore-forming loop between the fifth and sixth TM segments of TRPV4 [26,
27]. Mutation of this residue results in a glycosylation-deficient mutant TRPV4-N358Q,
which displays an increased Ca^{2+} entry resulted from the enhancement of TRPV4
expression at the plasma membrane [26]. Since deglycosylation increases TRPV4
channel activity [26], we applied endoF to remove TRPV4 N-glycosylation in order to
study the role of N-glycosylation in the channel regulation. The results showed that
endoF treatment enhances TRPV4-mediated Ca^{2+} influx. It supports the previous study
that deglycosylation activates TRPV4 channel, indicating a key role of N-glycosylation in
TRPV4 regulation. Up to date, detailed information regarding TRPM6 N-glycosylation
remains elusive. Therefore, comprehensive studies about N-glycosylation and N-glycan
structure of TRPM6 are a prerequisite to further understand TRPM6 regulation.

To substantiate the applicability of the effect of β-glucuronidase on other transporters
besides TRP channels, we investigated whether the thiazide-sensitive NCC can be
activated by β-glucuronidase. NCC is mainly expressed in the apical membrane of DCT,
where it is responsible for the reabsorption of ~10% of the filtered NaCl load [28, 29].
Interestingly, mutations in NCC have been implicated in the autosomal recessive renal tubular disorder Gitelman syndrome, characterized by hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria [30]. Our results demonstrated that the thiazide-sensitive Na\(^+\) influx mediated by NCC is not influenced by β-glucuronidase or endoF treatment. Detailed sequence analysis showed that rat NCC contains two potential N-glycosylation consensus sites, at the asparagine residue N404 and N424. Functional expression of NCC in *Xenopus laevis* oocytes revealed that the glycosylation-deficient mutants NCC-N404Q and NCC-N424Q display drastically decreased thiazide-sensitive \(^{22}\)Na\(^+\) uptake, resulting from reduced plasma membrane expression [31]. This observation suggests that N-glycosylation is essential for optimal function and cell-surface expression of NCC [31]. It may suggest a different role of N-glycosylation in NCC regulation compared to TRPV5 and TRPV6, explaining why NCC is insensitive to β-glucuronidase or endoF treatment.

In conclusion, a predominantly stimulatory effect of klotho has been discovered in the present study, which is only applicable to the epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6, but not to TRPV4, TRPM6 and NCC. This may result from diverse functions of N-glycosylation or various N-glycan structures of the investigated proteins. Therefore, detailed studies concerning the N-glycosylation and N-glycan structures of TRPV4, TRPV5, TRPV6, TRPM6 and NCC are warranted in the near future.

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**Reference**


General discussion

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The Netherlands
Almost any situation---good or bad ---is affected by the attitude we bring to.

(Lucius Annaus Seneca)

差不多任何一种处境——无论是好是坏——都受到我们对待处境的态度的影响。（西尼加 L A）
Introduction

The Transient Receptor Potential (TRP) superfamily of proteins consists of ion channels with molecular structure similarities [2]. TRP channels are extensively expressed and have diverse functions [3-11]. They are all cation selective, but the selectivity ratio for Ca$^{2+}$ and the monovalent cation Na$^+$ varies widely [2, 12-22]. The TRP superfamily is identified on the basis of homology only [2, 12, 13, 23], since the mode of activation and ion selectivity are diverse. Mutations of TRP proteins result in kidney-related diseases, including hereditary hypomagnesemia with secondary hypocalcemia (HSH) caused by mutations of TRPM6, autosomal dominant polycystic kidney disease caused by mutations of TRPP2, and focal segmental glomerulosclerosis caused by mutations of TRPC6 [3, 24-31]. The epithelial Ca$^{2+}$ channels, TRPV5 and TRPV6 uniquely represent two highly homologous members within the TRP superfamily, which are mainly expressed in Ca$^{2+}$-transporting epithelia [13, 32]. The molecular identification of TRPV5 [17] and TRPV6 [16, 33], boosted the research addressing the molecular mechanism of transepithelial Ca$^{2+}$ transport. TRPV5 primarily fulfills the role as a gatekeeper of epithelial Ca$^{2+}$ transport in the kidney [32], whereas TRPV6 forms the main Ca$^{2+}$ influx pathway in small intestine [34]. These channels convey the rate-limiting step in active Ca$^{2+}$ transport and play, therefore, a pivotal role in Ca$^{2+}$ homeostasis. The structure of TRPV5 and TRPV6 shows the typical topology features shared by all members of the TRP family [32]. The amino (N-tail) and carboxyl (C-tail) termini of TRPV5 and TRPV6 contain several conserved putative regulatory sites (for instance, ankyrin repeats [35] and potential internal PDZ motifs [36]) that might be involved in regulation of channel trafficking and activity. Particularly, TRPV5 and TRPV6 possess several potential phosphorylation sites in their N- and C-tails. Many of these putative phosphorylation sites are conserved among species, which suggest an important role in TRPV5 and TRPV6 functioning. TRPV5 and TRPV6 form homo- or hetero-tetrameric channel complexes with four subunits in a head-to-tail fashion [37]. These two channels contain a conserved N-glycosylation site in the extracellular loop between transmembrane (TM) region 1 and 2, which is localized at the asparagine residue N358 or N357 in TRPV5 and TRPV6, respectively. It has been reported that these channels are complexly glycosylated resulting in higher molecular weight proteins with corresponding molecular weight of about 85-100 kDa [37]. N-glycosylation of TRPV5 and TRPV6 may play an important role in protein folding, intracellular trafficking and channel regulation.
Detailed study about the molecular structure and regulation of these epithelial Ca\textsuperscript{2+} channels is crucial for a comprehensive understanding of Ca\textsuperscript{2+} homeostasis. The activity of TRPV5 and TRPV6 can be controlled by different regulatory mechanisms [38], for instance, hormone-mediated TRPV5 and TRPV6 expression, pH, intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+} sensors (80K-H and calmodulin)-controlled channel activity and S100A10-mediated trafficking regulation. To get deeper insight into the molecular structure and regulation of TRPV5 and TRPV6, the studies described in this thesis have been performed. This chapter will discuss and summarize the findings of these studies.

**Molecular structure of TRPV5 and TRPV6**

*Heterotetrameric TRPV5 and TRPV6 channels and their assembly domains*

By co-immunoprecipitations and molecular mass determination of TRPV5 and TRPV6 complexes using sucrose gradient sedimentation it was demonstrated that these two channels form homo- and heterotetrameric channel complexes. When concatemeric channels are constructed in a head-to-tail fashion consisting of four different TRPV5 and/or TRPV6 subunits, they display intermediate properties between TRPV5 and TRPV6 channels depending on the subunit configuration [37]. Previously, Hellwig and co-workers showed by fluorescence resonance energy transfer and co-immunoprecipitation experiments that TRPV5 and TRPV6 seem to be unique in forming heterotetramers [39], whereas the other TRPV channels preferentially form homotetramers. Thus, regulation of the relative expression levels of TRPV5 and TRPV6 may be a mechanism to fine-tune the Ca\textsuperscript{2+} transport kinetics in TRPV5 and TRPV6 co-expressing tissues. Indeed, several studies indicated that certain tissues co-expressing TRPV5 and TRPV6 would allow oligomerization of these channels *in vivo* [34, 38, 40]. An extensive analysis of TRP channel tissue distribution shows that TRPV5 and TRPV6 are co-expressed in brain, kidney, pancreas, small intestine, colon, prostate and testis [40].

Detailed information concerning protein structure and assembly of ion channels containing six TM domains is available for Shaker-like voltage-gated K\textsuperscript{+} (K\textsubscript{V})\textsuperscript{+} and cyclic nucleotide-gated channels (CNG). It has been demonstrated that CNG channel assembly requires a trimer-forming C-tail leucine zipper (CLZ) domain [41]. K\textsubscript{V} channels process a highly conserved N-tail cytoplasmic T1 domain (around 130 amino acids),
found to form a tetrameric ring with a narrow, positively charged central pore [42]. This designated \( \text{T1} \) domain has been shown to spontaneously form tetramers in the absence of TM sequences [42, 43]. Crystallization of the \( \text{T1} \) domain further verifies the reported biochemical and functional properties [42, 44]. The overall structure of \( \text{TRPV5} \) and \( \text{TRPV6} \) is similar to that of voltage-gated \( \text{Ca}^{2+} \) (\( \text{Ca}_v \)) channels. It has been investigated that functional \( \text{Ca}_v \) channels are monomeric proteins containing four homologous internal repeats, which assemble via an \( \alpha \)-interaction domain (AID), \( \beta \)-interaction domain (BID) and an extensive, conserved hydrophobic cleft (named the \( \alpha \)-binding pocket, ABP) [45, 46]. Similarly, \( \text{TRPV5} \) and \( \text{TRPV6} \) are shown to assemble into homo- and/or heterotetrameric complexes. However, the crucial domains involved in the assembly of these two channels were until recently poorly understood. Therefore, the identification of the precise assembly domains is a prerequisite to better understand \( \text{TRPV5} \) and \( \text{TRPV6} \) tetramerization. In chapter 2, the molecular determinants in \( \text{TRPV5} \) that play a key role in the formation of the functional channel complex were identified. Both the N-tail and C-tail are critical for \( \text{TRPV5} \) channel assembly. Furthermore, functional \( ^{45}\text{Ca}^{2+} \) influx study in \textit{Xenopus laevis} oocytes and patch-clamp analysis in HEK293 cells co-expressing \( \text{TRPV5} \) wild-type and truncated channels lacking either the N-tail or C-tail indicated that these N- or C-tail lacking truncants exert dominant-negative effects on channel activity, resulted from a disturbed trafficking of \( \text{TRPV5} \) to the plasma membrane. Therefore, assembly of channel subunits is essential for trafficking of the \( \text{TRPV5} \) channel complex and subsequent activity at the plasma membrane. Niemeyer et al. [47] identified the third ankyrin repeat being a stringent requirement for physical assembly of \( \text{TRPV6} \) subunits. Our studies in chapter 2 showed that at least two regions in the cytosolic tails (64-77 in the N-tail and 596-601 in the C-tail) are involved in channel assembly [48]. Physical interactions between the N-tail & N-tail, N-tail & C-tail and C-tail & C-tail of \( \text{TRPV5} \) were demonstrated by pull-down and co-immunoprecipitations in \textit{Xenopus laevis} oocytes. When these two critical regions in the N-tail (64-77) and C-tail (596-601) involved in channel assembly are deleted, the interactions between the tails are abolished. In addition, the identified N-tail assembly domain includes an ankyrin repeat, substantiating the involvement of these protein–protein binding modules in \( \text{TRPV5} \) assembly. The importance of both the N- and C-tail in \( \text{TRPV} \) channel oligomerization, including \( \text{TRPV5} \) and \( \text{TRPV6} \), was recently confirmed by Hellwig et al. [39]. Their study provided evidence that ankyrin repeats in the N-tail of \( \text{TRPV5} \) and \( \text{TRPV6} \) are essential for subunit assembly [39, 48], which is in line with our results in chapter 2. Deleting or mutating
assembly domains could cause a change in the tertiary structure and/or prevent the interaction with auxiliary proteins, thereby affecting channel trafficking and activity. For instance, the assembly domain in the C-tail (596-601) of TRPV5 is a helical stretch containing five amino acids (MLERK), also known to bind the auxiliary proteins Rab11a and 80K-H [49-51] (Figure 1). Impaired trafficking observed in the TRPV5 mutant lacking the MLERK motif could be explained by the inability to interact with the necessary auxiliary proteins that play an essential role in TRPV5 trafficking and functioning [50]. Taken together, both N- and C-tail are crucial for TRPV5 assembly. Assembly of channel subunits is essential for trafficking of the TRPV5 channel complex and subsequent activity at the plasma membrane. In order to get more insight into the TRPV5 and TRPV6 assembly, protein crystallography of these channels would be the most challenging approach to determine the molecular structure in the near future.

**Molecular regulation of TRPV5 and TRPV6 channel**

The detailed studies in the molecular regulation of TRPV5 and TRPV6 further extended to proteins that act as the putative regulators of these two channels. Our recent findings demonstrated that TRPV5 and TRPV6 can be regulated at the trafficking level by Rab11a, at the channel activity level at the plasma membrane by NHERF2 / NHERF4, FKBP52, BSPRY, calbindin-D$_{28k}$ and RGS2. In addition, these channels could also be regulated by extracellular proteins including klotho, β-glucuronidase, sialidase, peptide N-glycosidase F (endoF) and tissue kallikrein (TK). An integrated model of TRPV5 and TRPV6 regulation by different proteins and binding sites of channel-associated proteins is depicted in Figure 1.

**FKBP52, NHERF2/4, BSPRY, calbindin-D$_{28k}$, and RGS2-mediated channel activity regulation**

It has been recently reported [52] that expression of the FKBP52 protein reduces TRPV5-mediated Ca$^{2+}$ and Na$^+$ current, suggesting that FKBP52 decreases the number of functional channels at the plasma membrane. Van de Graaf et al. [53] showed the first physiology role of novel protein BSPRY in the directly negative regulation of TRPV5, which is involved in inhibitory signaling cascades that control the activity of the epithelial Ca$^{2+}$ channels at the cell surface. In addition, it has been demonstrated that NHERF2 and NHERF4 hypothetically increase cell-surface TRPV5 abundance and activity in a
Figure 1. Integrated model of TRPV5 and TRPV6 regulation by different proteins and binding sites of channel-associated proteins. TRPV5 and TRPV6 are synthesized in the endoplasmic reticulum (ER) and subsequently transported towards the plasma membrane. Rab11a mediates the trafficking of TRPV5 and TRPV6 to the plasma membrane, and it binds to MLERK domain in the C-tails of TRPV5 and TRPV6. FKBP52 decreases the number of functional channels at the plasma membrane. NHERF2 and NHERF4 are hypothesized to enhance the stability of TRPV5 and TRPV6 at the plasma membrane. NHERF2 binds to the last amino acids YHE and NHERF4 binds to amino acid region 596-617 of TRPV5. In addition, calbindin-D28k and RGS2 regulate TRPV5 or TRPV6 by direct association with the channel. Calbindin-D28k binds to both N- and C-tails of TRPV5 in a Ca^{2+} dependent manner, and RGS2 binds to N-tail of TRPV6, however, the exact binding domains of these two proteins were not identified. Furthermore, BSPRY is involved in inhibitory signaling cascades that control the activity of the epithelial Ca^{2+} channels at the cell surface, and binds to C-tail of TRPV5 without identification of binding domains. TRPV5 and TRPV6 have one N-linked glycan at the asparagine residue N358 or N357 between transmembrane (TM) segment 1 and 2, respectively. Glycosidases - klotho, sialidase and endoF activate TRPV5 from outside by hydrolyzing N-glycan of the channel at the site N358. Tissue kallikrein (TK) extracellularly stimulates TRPV5 by activating the bradykinin 2 receptor (B2R), which requires the two conserved PKC sites – serine residue S299 and S654 in the channel. In total, there are six predicted PKC phosphorylation sites as shown in the figure. The tetramerization domains of TRPV5 locate between 64-77 region of N-tail and MLERK region in C-tail. 64-77 region in the N-tail of TRPV5 located more upstream, overlaps with the first ankyrin repeat.
direct fashion via protein-protein interaction [54-56]. Furthermore, Lambers et al. [57] showed that calbindin-D₂₈k acts in Ca²⁺-transporting epithelia as a dynamic Ca²⁺ buffer, regulating Ca²⁺ concentration in close vicinity to the TRPV5 pore by direct association with the channel. More recently, RGS was identified as a protein directly interacting with TRPV6 to negatively affect the channel gating properties [58].

Rab11a-mediated trafficking regulation

A novel function of Rab11a as an associated protein mediating trafficking of TRPV5 and TRPV6 to the plasma membrane by direct cargo protein interaction has been demonstrated in chapter 3. Rab11a is a small GTPase involved in trafficking via recycling endosomes [59, 60]. Rab proteins are known to interact with a large variety of effectors [61-63], but only a few studies have demonstrated direct interactions between a Rab GTPase and a cargo molecule [64]. The ability to act as molecular switches that cycle between GTP- and GDP-bound states, underlies the functionality of the Rab GTPase family. Although the role of Rab GTPase in protein trafficking has long been recognized, the underlying mechanisms are far from understood. Our study in chapter 3 describes for the first time cargo proteins that interact directly with Rab11a. As TRPV5 and TRPV6 preferentially interact with GTP binding-deficient (GDP-locked) mutant Rab11a S25N in its GDP-bound configuration, this direct association of TRPV5 and TRPV6 with Rab11a could support the translocation of these channels into recycling endosomes and thereby constitute the “delivery machinery” destined to transport these channels to the apical plasma membrane. Further evidence for a role of Rab11a in TRPV5 and TRPV6 regulation is the predominant colocalization of Rab11a with TRPV5 or TRPV6 along the apical domain of the DCT and CNT cells. At the subcellular level, Rab11a shows a significant colocalization with TRPV5 in subapical vesicular structures. Previous functional and histological studies have identified Rab11-positive structures as (apical) recycling endosomes, which are specialized compartments involved in (polar) sorting of endocytosed membrane proteins [59, 60, 65-67]. Furthermore, Rab11 has been demonstrated to play a role in transport from the trans-Golgi network to the plasma membrane [68, 69]. The unique role of Rab11a in targeting of TRPV5 and TRPV6 to the plasma membrane by direct interaction to the channel was further established by combined biochemical, functional, and immunocytochemical analyses. The MLERK domain in TRPV5 and TRPV6, conserved among all identified species of these two
channels, was demonstrated to be required for Rab11a binding. Mutations in this stretch induce a significantly diminished TRPV5- and TRPV6-mediated Ca\textsuperscript{2+} influx, resulted from impaired trafficking of the channels. It indicates an essential role for Rab11a in targeting TRPV5 and TRPV6 to the plasma membrane. Moreover, this study further substantiates the functional role of Rab11a in the trafficking of TRPV5 and TRPV6 using Rab11a mutants by the result that GTP binding-deficient (GDP-locked) Rab11a mutant Rab11a S25N expression strongly reduces the TRPV5- and TRPV6-mediated Ca\textsuperscript{2+} influx. The expression of another dominant negative Rab protein (Rab22b S19N) does not inhibit TRPV5 and TRPV6 activity indicating the specificity of the Rab11a effect. These data indicate that cargo interaction (in the GDP status), as well as subsequent GTP binding, is required for Rab11a-mediated TRPV5 and TRPV6 trafficking. Taken together, our data provide new insight into the molecular machinery of TRP channel trafficking via direct interaction between a Rab GTPase and apically targeted cargo. This association is involved in the physiological regulation of TRPV5 and TRPV6 cell-surface abundance, a critical component in Ca\textsuperscript{2+} homeostasis.

**Klotho, β-glucuronidase, sialidase, endoF and TK–mediated extracellular regulation**

**Klotho**

The klotho gene was originally identified as a gene mutated in a mouse strain that developed multiple aging-like phenotypes [70]. Mice defective in klotho gene expression (klotho\textsuperscript{-/-} mice) exhibit a syndrome resembling human aging, including short life span, hypoactivity, muscle atrophy, skin atrophy, osteopenia, vascular calcification, abnormality in phosphate and Ca\textsuperscript{2+} metabolism [70]. In contrast, overexpression of the klotho gene extends life span in mice, by regulating the insulin / insulin-like growth factor 1 (IGF1) signaling pathway [71]. These observations suggest that the klotho gene may function as an aging-suppressor, and klotho could be involved in the regulation of human aging and related diseases [72-77]. The klotho gene encodes a single-pass transmembrane protein. The extracellular domain of the protein is composed of two homologous domains, each having a homology to β-glucosidase of bacteria and plants. In addition, β-glucuronidase activity is detected when artificial substrates are added to the extracellular domain of klotho [78, 79]. The klotho protein is secreted into serum,
urine and cerebrospinal fluid [71, 78, 79]. Intriguingly, the klotho gene is only notably expressed in the renal DCT cells and the choroid plexus in the brain, but multiple aging-like phenotypes of klotho<sup>−/−</sup> mice are involving almost all organ systems. Therefore, klotho itself may function as a humoral factor.

To date, the identification of klotho molecular functions is developing rapidly. Klotho appears to regulate the insulin / IGF1 signaling pathway via binding of klotho to the putative klotho receptor [71]. This is a mechanism for life span extension, evolutionarily conserved from worms to mammals [80]. Further analysis demonstrates that klotho, both the full length and secreted forms bind to multiple fibroblast growth factor (FGF23) receptors [81], and that FGF23 requires klotho to activate FGF signaling pathway. FGF23 was identified as a gene mutated in patients with autosomal dominant hypophosphatemic rickets [82], and inhibits phosphate transport in renal proximal tubules. This indicates that klotho plays an important role in phosphate metabolism [83, 84]. It has been recently investigated that klotho also plays a crucial role in the maintenance of Ca<sup>2+</sup> homeostasis. One of the first signs that klotho regulates Ca<sup>2+</sup> metabolism is the observation that klotho<sup>−/−</sup> mice have slightly increased serum levels of Ca<sup>2+</sup> and develop bone abnormalities including osteoporosis [70]. In addition, klotho may play as a negative regulator of active vitamin D synthesis [70]. Appealing evidences of the involvement of klotho in Ca<sup>2+</sup> homeostasis came with the investigation shown in chapter 4 that klotho activates the epithelial Ca<sup>2+</sup> channel TRPV5 [85]. Previous studies have indicated that the expression of both klotho and TRPV5 is tightly controlled by the calcitropic hormone vitamin D, which may suggest a functional link between these proteins in the maintenance of the Ca<sup>2+</sup> balance [38, 86]. Based on this correlation, the significant downregulation of klotho expression in TRPV5<sup>−/−</sup> mice shown by real-time PCR [85], and the conspicuous overlap in the pathophysiology of TRPV5<sup>−/−</sup> and klotho<sup>−/−</sup> mice (for instance, disturbed Ca<sup>2+</sup> and vitamin D metabolism), we hypothesized that TRPV5 is a down-stream target of the anti-aging hormone klotho.

The study in chapter 4 further uncovers the relationship between TRPV5 and klotho. Co-expression of klotho with TRPV5 in HEK293 cells, or application of culture media collected from klotho-expressing HEK293 cells (klotho-containing supernatant) to TRPV5-transfected HEK293 cells, significantly stimulates TRPV5-mediated <sup>45</sup>Ca<sup>2+</sup> uptake in these cells. Cell-surface biotinylation experiments revealed a significant
increase in plasma membrane localization of TRPV5 whereas total expression is not affected after klotho treatment. These effects were mimicked by the purified β-glucuronidase indicating that this enzymatic activity of klotho is responsible for the increased TRPV5 activity. Interestingly, the klotho or β-glucuronidase-mediated stimulatory effect on TRPV5 was abolished in HEK293 cells expressing the TRPV5 glycosylation-deficient mutant TRPV5-N358Q without the entire N-glycan. Further, D-[U-\(^{14}\)C]glucose labeling assay demonstrated that klotho exhibits β-glucuronidase activity by hydrolyzing the N-glycan of glycosylated TRPV5 channels. Our results indicate that N-glycosylation plays a crucial role in TRPV5 activation by β-glucuronidase klotho, in addition, the N-glycan acts as the target in this stimulation event. We provide a novel concept of a regulatory mechanism through which the secreted β-glucuronidase klotho hydrolyzes the extracellularly exposed N-glycan of TRPV5 to entrap channels at the plasma membrane. The physiological relevance of the aforementioned findings is substantiated by the co-localization of klotho with TRPV5 in the renal DCT and CNT cells, where klotho activates the channel from the pro-urine side. Klotho is the first example of an extracellular enzyme modifying the sugar chains of an ion channel to initiate a signaling cascade controlling transport activity. During aging, Ca\(^{2+}\) loss predominates Ca\(^{2+}\) intake [64]. As klotho deficiency is associated with a phenotype resembling aging, it may well be that impaired klotho activity in the elderly is responsible for reduced Ca\(^{2+}\) reabsorption via TRPV5. Future studies will have to corroborate this notion. The discovery of klotho-mediated extracellular TRPV5 activation has greatly enhanced our comprehension of TRPV5 regulation and klotho functioning, which may prove to be the model in unraveling the anti-aging effect of this novel hormone.

Since klotho co-localizes with TRPV5 in renal DCT cells where it activates the channel from the pro-urine, it is tempting to explore whether the activities of other renal apically localized channels or transporters could be influenced by klotho. The aim of the study in chapter 7 was, therefore, to examine whether the klotho effect is restricted to TRPV5 or more generally applicable to other renal apically localized proteins in DCT cells. To this end, we selected candidates within the TRP channels including TRPV4, TRPV6 and TRPM6. These channels have structural similarities with TRPV5 and are permeable to Ca\(^{2+}\). Additionally, the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC) was selected as a candidate in this study. Using \(^{45}\)Ca\(^{2+}\) uptake experiments, we were able to demonstrate that incubation with klotho-containing supernatant or β-glucuronidase for 16 hours
results in a significantly enhanced Ca\(^{2+}\) influx mediated by TRPV6. The immunoblot showed identical total expression of TRPV6 in all conditions, indicating that klotho or β-glucuronidase increases either open probability of the channel or TRPV6 abundance at the plasma membrane. The latter is most likely, since a cell-surface biotinylation assay has shown a significantly increased TRPV5 expression at the plasma membrane by klotho [85]. The study in chapter 7 further substantiates klotho as a regulator of both TRPV5 and TRPV6 channels. The tissue distribution of TRPV5 and TRPV6 in humans indicates that both channels are co-expressed in several organs that mediate transcellular Ca\(^{2+}\) transport [87], such as duodenum, colon, and kidney, but also in pancreas, prostate, mammary, sweat and salivary glands. Klotho is predominantly expressed in the renal DCT cells and the choroid plexus in the brain, and the extracellular domain of klotho is secreted into serum, urine and cerebrospinal fluid [78, 85]. Therefore, the secreted klotho may function as a humoral factor extracellularly modulating TRPV5 and TRPV6 activity in various target organs where these two epithelial Ca\(^{2+}\) channels are expressed. Both TRPV5 and TRPV6 channels contain a conserved N-glycosylation site at the asparagine residue N358 (TRPV5) or N357 (TRPV6) in the extracellular loop between TM region 1 and 2, respectively [85]. Immunoblot analysis showed that the intensity of the complex-glycosylated TRPV5 and TRPV6 bands is reduced after incubation with endoglycosidase H (endoH) hydrolyzing N-linked high mannoses [37]. These bands disappear after treatment with endoF removing the entire N-glycan, indicating that both epithelial channels are similarly complex glycosylated [37]. N-glycosylation plays a crucial role in TRPV5 regulation by klotho [85]. Likely, a comparable function applies to N-glycosylation of TRPV6 in the klotho-mediated channel stimulation.

Furthermore, we investigated whether β-glucuronidase activates two other members of TRP superfamily, TRPV4 and TRPM6, which share similar molecular structures with TRPV5 and TRPV6. TRPV4 and TRPM6 are Ca\(^{2+}\)-permeable cation channels [88, 89], localized in the renal DCT cells. In general, TRPV4 plays a key role in cell volume regulation [90], and is widely expressed including in lung, spleen, kidney, testis, fat, brain, cochlea, skin, smooth muscle, liver, and vascular endothelium [91]. TRPM6 has a more restricted expression pattern being predominantly present in absorbing epithelia [24], and plays a potential role in the maintenance of Mg\(^{2+}\) balance [25]. Our results in chapter 7 showed that β-glucuronidase incubation does not simulate TRPV4 and TRPM6
channel activity. Presumably, the N-oligosaccharides pivotal for TRPV5 and TRPV6 activation, which are hydrolyzed by β-glucuronidase, are not present in TRPV4 and TRPM6. Alternatively, hydrolysis of the N-oligosaccharides is not involved in the regulation of TRPV4 and TRPM6. Up to date, detailed information regarding TRPM6 N-glycosylation remains elusive. Therefore, comprehensive studies about N-glycosylation and N-glycan structures of TRPV4 and TRPM6 are a prerequisite to further understand regulation of these two channels.

To substantiate the applicability of the effect of β-glucuronidase on other transporters besides TRP channels, we investigated whether the thiazide-sensitive NCC can be activated by β-glucuronidase. NCC is mainly expressed in the apical membrane of DCT, where it is responsible for the reabsorption of ∼10% of the filtered NaCl load [92, 93]. Interestingly, mutations in NCC have been implicated in the autosomal recessive renal tubular disorder Gitelman syndrome, characterized by hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria [94]. Our results in chapter 7 demonstrated that the thiazide-sensitive Na⁺ influx mediated by NCC is not influenced by β-glucuronidase treatment. Detailed sequence analysis showed that rat NCC contains two potential N-glycosylation consensus sites, at the asparagine residue N404 and N424. Functional expression of NCC in *Xenopus laevis* oocytes revealed that the glycosylation-deficient mutants NCC-N404Q and NCC-N424Q display drastically decreased thiazide-sensitive $^{22}$Na⁺ uptake, resulting from reduced plasma membrane expression [95]. This observation suggests that N-glycosylation is essential for optimal function and cell-surface expression of NCC [95]. It may suggest a different role of N-glycosylation in NCC regulation compared to TRPV5 and TRPV6, explaining why NCC is insensitive to β-glucuronidase treatment.

Taken together, klotho hydrolyzes N-oligosaccharides of TRPV5 to entrap the channel at the plasma membrane leading to an increased channel activity. This stimulatory effect of klotho is only predominantly applicable to the epithelial Ca²⁺ channels TRPV5 and TRPV6, but not to TRPV4, TRPM6 and NCC. This may result from diverse functions of N-glycosylation or various N-glycan structures of the investigated proteins. Therefore, detailed studies concerning the N-glycosylation and N-glycan structures of TRPV4, TRPV5, TRPV6, TRPM6 and NCC are warranted in the near future.
**EndoF and sialidase**

The study in chapter 4 about klotho-mediated TRPV5 activation highlights the crucial role of the N-glycan in TRPV5 regulation. Thus, we hypothesized that other glycosidases that hydrolyze N-glycans may mimic the stimulatory effect of klotho. Therefore, the study in chapter 6 aimed to investigate the molecular mechanism underlying the klotho-mediated TRPV5 activation as well as the specificity of this effect. To this end, we investigated the effect of diverse members of the glycosidase superfamily, to which β-glucuronidase and klotho belong, namely β-glucosidase, β-galactosidase, sialidase, endoH, and endoF. These glycosidases hydrolyze various sugar residues including glucuronic acid, glucoses, galactoses, sialic acid, high mannoses and the entire N-glycan. In general, glycosidases participate in essential steps of synthesis and degradation of polysaccharides, which are involved in processes such as pathogen defense [96], control of signal transduction [97] and modification of hormones [98]. In humans, inheritable deficiencies of glycosidases are known to induce a large variety of impairments such as lysosomal storage diseases, Gaucher’s and Krabbe’s disease and lactose intolerance [99]. At present, the biological function of glycosidases remains scarce. So far, some studies suggested that sialidase and endoF can regulate ion channel activity [100]. Sialidase is known to hydrolyze sialic acid residues from glycosylated proteins [101]. A novel mechanism has been described through which hydrolysis of surface sialic acids by sialidase or removal of the complete surface N-glycan of voltage-gated Na⁺ channel (Naᵥ) can directly modulate channel gating, and therefore affect cardiac, skeletal muscle, and neuronal excitability [100]. Moreover, it has been described that Kᵥ1.1 channels contain significant amounts of sialic acids, which appear to be the negative charges at the channel surface sensitive to Ca²⁺. The data suggested that sialidase treatment modifies Kᵥ1.1 function by influencing the channel voltage dependence of activation [102]. EndoF is a well-known glycosidase, which removes the entire N-glycan of proteins. Glycans participate in many key biological processes including cell adhesion, molecular trafficking, receptor activation, signal transduction, and endocytosis [103]. It has been reported that N-glycans could serve as a signal for degradation by the SCF(Fbx2) ubiquitin ligase complex [104]. The F-box protein Fbx2 binds specifically to proteins attached to N-linked high-mannose oligosaccharides and subsequently contributes to ubiquitination of N-glycosylated proteins. These studies highlight the role of N-glycans in protein degradation besides its
typical role in protein folding, quality control, sorting, transport and channel activity [1]. In support of the previous findings, our data in chapter 6 showed by $^{45}\text{Ca}^{2+}$ uptake measurements that sialidase or endoF stimulates TRPV5-mediated Ca$^{2+}$ influx [85]. Thereby, we substantially extend the members of glycosidases extracellularly modulating TRPV5 activity besides klotho and $\beta$-glucuronidase [85]. Interestingly, the complex-glycosylated TRPV5 band of 85-100 kDa disappeared after endoF treatment, but was not changed by the other investigated glycosidases. It indicated that klotho and sialidase only hydrolyze specific N-oligosaccharides of TRPV5, not detectable as a shift in molecular weight of the channel by SDS-PAGE. However, this hydrolysis can already activate TRPV5. In addition, based on the result that $\beta$-glucosidase, $\beta$-galactosidase and endoH do not increase TRPV5-mediated Ca$^{2+}$ influx, our data revealed that these specific N-oligosaccharides, responsible for the channel activation, are $\beta$-glucuronic acid and sialic acid, hydrolyzed by $\beta$-glucuronidase and sialidase, respectively.

Stimulation of Ca$^{2+}$ influx through TRPV5 upon glycosidase treatment could be the result of an increase in either open probability of the channel or in expression of TRPV5 at the plasma membrane. Cell-surface biotinylation analysis showed that sialidase, endoF, klotho and $\beta$-glucuronidase increase TRPV5 channel abundance at the plasma membrane. Thereby, TRPV5 activation following the glycosidase application might be due to either an enhanced trafficking from the Golgi apparatus to the cell surface or a reduction in channel retrieval from the plasma membrane. Metabolic labeling in combination with cell-surface biotinylation subsequently revealed that the apparent retrieval process of TRPV5 is significantly delayed upon exposure to $\beta$-glucuronidase, indicating that $\beta$-glucuronidase increases TRPV5 activity by stabilizing the channel at the plasma membrane. Most likely, this also applies to sialidase and endoF-mediated increase in TRPV5 activity. Hereby, our data indicated a novel retrieval pathway controlled by the N-glycan of TRPV5, in which N-glycosylation plays a pivotal role.

N-glycosylation
N-glycosylation of proteins is highly conserved from yeast to human and has a significant effect on modulating protein structure and localization to facilitate proper folding and trafficking of membrane proteins [105]. The role of N-glycosylation of transmembrane proteins has received great attention, and it appears that in some cases
General discussion

it is involved in membrane targeting. To date, several studies have focused on the role of N-glycosylation in the variety of ion channels or transporters. It has been conclusively demonstrated that prevention of N-glycosylation negatively influences proteins activity including the γ-aminobutyric acid (GABA) transporter (GAT1) [106], the voltage-gated Ca\(^{2+}\) channels (Ca\(_v\)) [107], cyclic nucleotide-gated ion channel (CNG) [108] and Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC2) [109]. Further, Pabon et al. demonstrate that N-glycosylation of G-protein-activated inwardly rectifying K\(^+\) channel (GIRK1) at the asparagine residue N119 and the renal outer-medullary K\(^+\) channel (ROMK1) at the asparagine residue N117 has different consequences in K\(^+\) channel function [110]. In addition, it has been demonstrated that the gating, cell-surface trafficking and pH sensitivity of K\(_v\) channels are regulated by N-glycosylation [111-113]. Moreover, the human ether-à-go-go-related gene (HERG) channel without N-glycosylation degrades more rapidly than glycosylated channel, resulting in a reduced cell-surface stability [114]. Within the TRP superfamily, N-glycosylation regulates also TRPV1 channel activity [115]. Understanding the role of N-glycosylation in protein trafficking, degradation and activity will highlight the different posttranslational mechanisms modifying the cell-surface ion channel expression levels and possibly their signaling characteristics. However, in addition to the well-known fact that deglycosylation negatively influences protein trafficking, stability and activity, Xu and co-workers showed that deglycosylation activates TRPV4 channel [116]. They identified a consensus N-glycosylation motif at the asparagine residue N651 within the pore-forming loop between the fifth and sixth TM segments of TRPV4. Mutation of this residue results in a glycosylation-deficient mutant TRPV4-N358Q. HEK293 cells expressing TRPV4-N651Q display an enhanced Ca\(^{2+}\) entry resulted from an increased plasma membrane abundance of TRPV4-N651Q compared to the wild-type channel. The structurally related TRP channels share a nearly identically situated and experimentally confirmed N-glycosylation site, which promotes rather than limits channel insertion into the plasma membrane. Since deglycosylation increases TRPV4 channel activity [116], we applied endoF to remove TRPV4 N-glycosylation in order to study the role of N-glycosylation in the channel regulation. As revealed in chapter 7, \(^{45}\)Ca\(^{2+}\) uptake measurements showed that endoF increases TRPV4-mediated Ca\(^{2+}\) influx, indicating an enhanced TRPV4 channel activity via deglycosylation. The immunoblotting subsequently demonstrated that the complex-glycosylated band of TRPV4, which is around 85-100 KDa, is diminished after endoF treatment. It supports the previous study that
deglycosylation activates TRPV4 channel, indicating a key role of N-glycosylation in TRPV4 regulation.

To further clarify the molecular mechanism underlying the N-glycosylation-mediated TRPV5 retrieval shown in chapter 6, we investigated the retrieval kinetics of the TRPV5 glycosylation-deficient mutant (TRPV5-N358Q). Our result corroborates the importance of N-glycosylation in TRPV5 stability at the cell surface, since TRPV5-N358Q showed a low turnover compared to wild-type channels. In addition, β-glucuronidase did not further delay the TRPV5-N358Q retrieval due to absence of the entire N-glycan of this mutant, confirming that N-glycosylation plays a key role in the retrieval process of TRPV5, and therefore glycosidase-mediated channel activation. Taken together, our findings emphasize that deglycosylation increases cell-surface expression of TRPV4 and TRPV5 channels. Moreover, it adds a new dimension to the traditional effect of N-glycosylation on ion channel trafficking and degradation by showing that deglycosylation delays TRPV5 retrieval to increase channel stability. Future studies should investigate whether the same mechanism applies to other TRP channels.

*Tissue kallikrein (TK)*

TK is a serine protease produced in CNT [117], where it co-localizes with TRPV5 [118]. Proteolytic enzymes such as TK are synthesized as inactive precursors or zymogens, to prevent protein degradation and to enable spatial and temporal regulation of enzymatic activity. The precursor of TK is converted to the mature active form before entering the luminal tubular compartment [119]. Once activated, TK is excreted and can process low molecular weight kininogen to release kinin, which acts through kinin receptors such as the bradykinin (BK) 2 receptor (B2R) [120]. Remarkably, recent studies showed that TK can directly activate the B2R independently of BK release [121]. Interestingly, mice lacking TK (TK−/− mice) exhibit robust hypercalciuria comparable to the Ca^{2+} leak in TRPV5−/− mice. [122]. However, the molecular events that link TK to Ca^{2+} balance were until recently unknown. The study in chapter 5 delineates the molecular mechanism through which TK stimulates Ca^{2+} reabsorption via TRPV5, which explains hypercalciuria observed in TK−/− mice. Using TRPV5-expressing primary cultures of rabbit CNT and CCD cells, we showed that TK stimulates Ca^{2+} reabsorption. This stimulatory effect of TK is mimicked by BK and could be reversed by application of JE049, a B2R antagonist. The effect of these compounds on TRPV5 activity is validated in TRPV5-transfected
HEK293 cells by electrophysiological measurement. Furthermore, using a phospholipase C (PLC) inhibitor and a cell permeable analog of diacylglycerol (DAG) it was demonstrated that TK activates PLC/DAG pathway. The analog of DAG increases TRPV5 activity via PKC activation of the channel, since mutation of TRPV5 at the putative PKC phosphorylation sites S299 and S654 prevents the stimulatory effect of TK. Cell-surface biotinylation revealed that TK enhances the amount of TRPV5 channels at the plasma membrane by delaying its retrieval. Therefore, we conclude that TK stimulates Ca$^{2+}$ reabsorption via the BK-activated PLC/DAG/PKC pathway and induces the subsequent stabilization of the TRPV5 channel at the plasma membrane. The physiological relevance of this finding is substantiated by the co-localization of B2R with TK [123] and TRPV5 [118] in DCT and CNT cells. This is the first study demonstrating that TRPV5 is activated through PKC phosphorylation sites. Among the six predicted phosphorylation sites in the TRPV5 sequence, two serines, S299 and S654 are apparently critical for TRPV5 activation by TK. Importantly, TRPV5 closest homologue, TRPV6, does not respond to TK. Since the second serine (S654) is not conserved in TRPV6, both serines are all critical for stimulation of TRPV5 by TK. Previous studies showed that TRP channels can be activated by different signaling molecules of the PLC pathway. For example, TRPC3, TRPC6 and TRPC7 are characterized by their sensitivity to DAG [124]. Alternatively, TRPV1 [125], TRPV5 [126], TRPM4 [127], TRPM5 [128], TRPM7 [129] and TRPM8 [130] can be directly regulated by PIP2. Thus, in vivo TRPV5 activation by PIP2 [131] or DAG/PKC could adjust TRPV5 activity in response to physiological fluctuations. In addition, Ca$^{2+}$ entering the cell through TRPV5 could prevent the electrostatic interaction between the negatively charged PIP2 and the channel by screening the negative charge on the lipid head group, as proposed for Mg$^{2+}$ and TRPM7 [132].

Since cell-surface biotinylation analysis showed that TK increases the amount of TRPV5 channels at the plasma membrane by delaying channel retrieval. Thus, PKC activation of TRPV5 following the TK application would regulate the balance between constitutive exocytosis and endocytosis in favor of the former leading to the accumulation of TRPV5 at the cell surface. The role of the cytoskeleton in this translocation process is presently unknown. It is possible that PKC-dependent phosphorylation of TRPV5 leads to activation of motor proteins that transport the channels towards the plasma membrane. Interestingly, TRPM7 associates to the actomyosin cytoskeleton upon BK stimulation.
regulating cell adhesion [133]. Remarkably, the protein synaptotagmin has been proposed to regulate the exocytosis of TRPC5 [134]. Furthermore, accumulation of channels at the cell surface can also occur by increased incorporation into the plasma membrane. Indeed, PKC potentiation of TRPV1 promotes the recruitment of a channel vesicular pool to the cell surface [135]. This exocytosis process of TRPV1 is dependent on the soluble N-ethylmaleimide-sensitive-factor attachment proteins receptor (SNARE) [135], known to act as membrane recognition molecules and acceptors for vesicle trafficking, docking and fusion [136]. However, the cytoskeletal elements and the motor proteins participating in the incorporation of TRPV5 at the plasma membrane remain to be identified. It would be intriguing to investigate the role of similar scaffold proteins in the assembly of TRPV5 and PKC upon TK treatment, considering that such signaling pathways are currently indicated to function in spatially distinct microdomains [137]. Taken together, TK-directed translocation of TRPV5 channels constitutes a mechanism by which renal cells can fine-tune the Ca\textsuperscript{2+} reabsorption.

**Extracellular regulation of TRPV5 and TRPV6**

Together with the results in chapter 4 on the β-glucuronidase klotho, chapter 5 on the TK, and chapter 6 on the endoF and sialidase, our study introduces a new mechanism through which Ca\textsuperscript{2+} reabsorption can be stimulated via extracellular urinary enzymes. To date, there is only limited information about extracellular regulation of ion channels and transporters. It has been previously described that epithelial Na\textsuperscript{+} channel (ENaC) is extracellularly regulated by the channel activating proteases CAP-1, CAP-2 and CAP-3 [138-140]. CAPs, which are membrane-bound serine proteases, act directly on the channel gating by enhancing the open probability of ENaC to stimulate epithelial Na\textsuperscript{+} absorption [141]. Subsequently, other serine protease - trypsin, prostatin and TMPRSS2 were subsequently identified to regulate ENaC activity [139, 140, 142]. Similarly, the activity of acid-sensing ion channels (ASICs), neuronal Na\textsuperscript{+} channels, were also found to be influenced by serine protease - trypsin [143, 144]. However, unlike the protease CAP, TK stimulates TRPV5 indirectly via activation of the B2R, which then induces a redistribution of TRPV5 channels towards the plasma membrane. Klotho, β-glucuronidase, sialidase and endoF directly activate TRPV5 by enzymatic modification of the N-glycan to stabilize TRPV5 channels at the plasma membrane resulted from the channel retrieval delaying. In future studies, the effect of serine or other proteases on epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6 should be investigated. Klotho and TK co-
General discussion

localize with TRPV5 at the apical membrane of the distal part of the nephron. Of particular interest is that these two proteins are secreted into the pro-urine from which they activate the TRPV5 channel, and therefore Ca\(^{2+}\) reabsorption. Thus, it would be tempting to explore additional urinary proteins extracellularly influencing TRPV5 activity. Urinary secreted glycosidases could be good candidates in maintaining the Ca\(^{2+}\) balance. In order to determine potential urinary regulators, urinary proteomics will be a powerful tool, which has been used for many years for the diagnosis and monitoring of renal disease [145]. Together, unraveling the molecular mechanisms by which extracellular calcitropic factors control the activity of TRPV5 involved in hormonal regulation of Ca\(^{2+}\) balance is essential in keeping Ca\(^{2+}\) levels within a healthy range.

The N-glycan structure of TRPV5

In mature glycoproteins, N-glycans are structurally diverse [1]. The sugar composition and the number and size of branches in the sugar tree vary among glycans, among glycoproteins, and among cell types, tissues, and species [146, 147]. However, when initially added in the ER to grow new polypeptides, the glycans do not display such diversity. The so-called “core glycans” are homogeneous and relatively simple [1, 146, 147] (Figure 2).

![Figure 2. The N-linked core oligosaccharide. N-glycans are added to proteins in the endoplasmic reticulum (ER) as “core oligosaccharides” that have the depicted structure. These are bound to the polypeptide chain through an N-glycosidic bond with the side chain of an asparagine (Asn) residue that is part of the Asn-X-Ser/Thr consensus sequence. Terminal glucose and mannose residues are removed in the ER by glucosidases and mannosidases [1]. Asn: asparagine; Ser: serine; Thr: threonine; X: any amino acid.](image-url)
Such N-glycan structures remain uniform until the glycoproteins reach the Golgi apparatus, where structural diversity is introduced through a series of nonuniform modifications [1]. When the glycoprotein moves to the Golgi complex, the glycan chains undergo further trimming. In many cases, new sugars are added during terminal glycosylation to produce complex N-glycans [146-151]. However, the number of branches generated is variable, as are the number and identity of sugars added. This biosynthesis pathway is still in puzzle in several respects, for instance, which sugars will be added during the final glycosylation pathway, and what are the determinants for the different sugar addition?

By the present study in chapter 6, it was shown that hydrolysis of the N-glycan stabilizes the channel at the plasma membrane, leading to the enhancement of TRPV5 activity. In addition, our data elucidated that β-glucuronic acid hydrolyzed by β-glucuronidase and sialic acid removed by sialidase are responsible for TRPV5 activity. Thus, based on the above findings and the theory of N-glycan biosynthesis [146-151], the potential TRPV5 N-glycan structure is predicted. In ER, the TRPV5 N-glycan will be the homogeneous
and relatively simple “core oligosaccharides” (Figure 2) [146, 147], which is shared by nearly all N-glycoproteins. In Golgi complex, high mannoses and other sugars are added during terminal glycosylation to produce the complex N-glycan [1, 152]. The N-glycan of mature TRPV5 could be composed of part of “core oligosaccharides” (three mannoses and two N-acetylglucosamines) [1, 146-151]. Sialic acid and glucuronic acid are present at the end of the sugar tree, since these two sugars are responsible for TRPV5 activity. Therefore, the speculated N-glycan structure of mature TRPV5 is shown in Figure 3.

Conclusion and future direction

In this thesis, the studies unraveling the molecular structure and regulation mechanisms of TRPV5 and TRPV6 were described. The molecular determinants in TRPV5 were identified that play a key role in the formation of the functional channel complex, and substantiated that assembly of channel subunits is essential for routing to and subsequent activity of TRPV5 at the plasma membrane. In addition, our data provided new insight into the molecular machinery of TRPV5 and TRPV6 channel trafficking via direct interaction between a Rab GTPase (Rab11a) and apically targeted cargo. Of particular interest is that our study introduces a new mechanism through which Ca\textsuperscript{2+} reabsorption can be stimulated via extracellular activation of TRPV5 by urinary enzymes, including TK, sialidase, endoF, klotho and β-glucuronidase. TK stimulates TRPV5 indirectly via activation of the B2R, which then induces a redistribution of TRPV5 channels towards the plasma membrane. Sialidase, endoF, klotho and β-glucuronidase directly activate TRPV5 by enzymatic modification of the N-glycan to stabilize TRPV5 channels at the plasma membrane. This extracellular control will ensure high Ca\textsuperscript{2+} transport capacity and reduce Ca\textsuperscript{2+} excretion to preserve normal plasma Ca\textsuperscript{2+} levels during periods of dietary Ca\textsuperscript{2+} insufficiency and aging. The identification and elucidation of TRPV5 and TRPV6 regulation by extracellular proteins have greatly contributed to our understanding of the regulation of apical Ca\textsuperscript{2+} influx in epithelial cells. However, this is only the first step in a long endeavor. The major challenges in the near future will be to explore additional urinary proteins extracellularly regulating TRPV5 and TRPV6. Urinary secreted glycosidases could be potential candidates in this case, and urinary proteomics would be a powerful tool, which has been used for many years to diagnose and monitor renal disease. We have to obtain further insight into the N-glycosylation process of TRPV5, TRPV6 and other TRP channels, in addition, establish a comprehensive view of N-glycan structures of TRPV5 and TRPV6, and their contribution to channel regulation.
To unravel the molecular mechanisms by which extracellular factors control the activity of TRPV5 and TRPV6 is essential in enduring Ca\(^{2+}\) levels within a healthy range. This will complete our picture of Ca\(^{2+}\) homeostasis.

**Reference**


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Summary

Samenvatting (Dutch summary)

中文简介 (Chinese summary)
It is no use doing what you like; you have got to like what you do. (Winston Churchill)

不能爱哪行才干哪行，要干哪行爱哪行。（丘吉尔·W.）
Summary

Chapter 1. Introduction

The Transient Receptor Potential (TRP) superfamily of proteins consists of ion channels with structural similarities. TRP channels are widely expressed and have diverse functions in our body. They are all cation selective, but the selectivity ratio for Ca\(^{2+}\) and the monovalent cation Na\(^{+}\) varies extensively. The TRP superfamily is identified on basis of homology only, since the mode of activation and ion selectivity is quite different. Some TRP channels are activated by ligands, whereas others are regulated by physical stimuli (for instance, heat) or yet-unknown mechanisms. Mutations of TRP channels result in kidney-related diseases, including hereditary hypomagnesemia with secondary hypocalcemia (HSH) caused by mutations of TRPM6, autosomal dominant polycystic kidney disease caused by mutations of TRPP2, and focal segmental glomerulosclerosis caused by mutations of TRPC6. The epithelial Ca\(^{2+}\) channels, TRPV5 and TRPV6, uniquely represent two highly homologous members within the TRP superfamily, which are mainly expressed in Ca\(^{2+}\)-transporting epithelia. The molecular identification of TRPV5 and TRPV6 boosted the research addressing the molecular mechanism of transepithelial Ca\(^{2+}\) transport. TRPV5 primarily fulfills the role as a gatekeeper of epithelial Ca\(^{2+}\) transport in the kidney, whereas TRPV6 forms the main Ca\(^{2+}\) influx pathway in small intestine. These channels convey the rate-limiting step in active Ca\(^{2+}\) transport and play, therefore, a pivotal role in Ca\(^{2+}\) homeostasis. The structure of TRPV5 and TRPV6 shows the typical topology shared by all members of the TRP family, which have six transmembrane (TM) segments, intracellularly localized amino (N-tail) and carboxyl (C-tail) termini. The N- and C-tail of TRPV5 and TRPV6 contain several conserved putative regulatory sites that are involved in regulation of channel trafficking and activity, for instance, ankyrin repeats, potential internal PDZ motifs and protein kinase C (PKC) phosphorylation sites. TRPV5 and TRPV6 form homo- and/or heterotetrameric channel complexes. When concatemeric channels are constructed in a head-to-tail fashion consisting of four different TRPV5 and/or TRPV6 subunits, they display intermediate properties between TRPV5 and TRPV6 channels depending on the subunit configuration. These two channels contain a conserved N-glycosylation site in the extracellular loop between TM region 1 and 2, which is localized at the asparagine residue N358 (TRPV5) or N357 (TRPV6). It has been reported that these two channels are complex glycosylated resulting in higher molecular weight proteins ranging from 85–
100 kDa. N-glycosylation of TRPV5 and TRPV6 may play an important role in protein folding, intracellular trafficking and channel regulation. TRPV5 and TRPV6 are synthesized in the endoplasmic reticulum (ER) and are subsequently modified at appropriate asparagine residues through the ER to the Golgi complex, and finally transported towards the plasma membrane as their final destination. The activity of TRPV5 and TRPV6 can be controlled by different regulatory mechanisms [37], for instance, hormone-mediated TRPV5 and TRPV6 expression, pH, intracellular Ca\(^{2+}\) and Ca\(^{2+}\) sensors (80K-H and calmodulin)-controlled channel activity and S100A10-mediated trafficking regulation. Our recent findings demonstrated that NHERF2 / NHERF4, FKBP52, BSPRY, calbindin-D\(_{28k}\) and RGS2 could also regulate TRPV5 and TRPV6 channel activity at the plasma membrane. However, up to date, many aspects of TRPV5 and TRPV6 regulation remain elusive. The aim of this thesis was, therefore, to unravel the molecular structure and regulation mechanisms of TRPV5 and TRPV6.

Chapter 2. Molecular structure of TRPV5

TRPV5 and TRPV6 were shown to assemble into homo- and/or heterotetrameric complexes. However, the crucial domains involved in the assembly of these two channels were until recently poorly understood. Here, the molecular determinants in TRPV5 that play a key role in the formation of the functional channel complex were identified. Both the N-tail and C-tail are critical for TRPV5 channel assembly. Functional \(^{45}\)Ca\(^{2+}\) influx study in *Xenopus laevis* oocytes and patch-clamp analysis in human embryonic kidney (HEK293) cells co-expressing TRPV5 wild-type and truncated channels lacking either the N-tail or C-tail indicated that these N- or C-tail deletion truncants exert dominant-negative effects on channel activity, resulted from a disturbed trafficking of TRPV5 to the plasma membrane. At least two regions in the cytosolic tails (64-77 in the N-tail and 596-601 in the C-tail) are involved in channel assembly. When these two critical regions are deleted, physical interactions between the N-tail & N-tail, N-tail & C-tail and C-tail & C-tail of TRPV5 are abolished. In addition, the identified N-tail assembly domain includes an ankyrin repeat, substantiating the involvement of these protein–protein binding modules in TRPV5 assembly. Taken together, both N- and C-tail are crucial for TRPV5 assembly. Assembly of channel subunits is essential for trafficking of the TRPV5 channel complex and subsequent activity at the plasma membrane.
Chapter 3. Rab11a as a novel regulator of TRPV5 and TRPV6 trafficking

Rab11a is a small GTPase involved in protein trafficking via recycling endosomes. A novel function of Rab11a as an associated protein mediating trafficking of TRPV5 and TRPV6 to the plasma membrane by direct cargo protein interaction was demonstrated. Rab11a predominantly colocalizes with TRPV5 or TRPV6 along the apical domain of the renal distal convoluted (DCT) and connecting tubules (CNT). TRPV5 and TRPV6 preferentially interact with the GTP binding-deficient (GDP-locked) mutant Rab11a S25N in its GDP-bound configuration. This direct association could support the translocation of these channels into recycling endosomes and thereby constitute the “delivery machinery” destined to transport these channels to the apical plasma membrane. The amino-acid domain 596-601 (MLERK) in C-tail of TRPV5 or TRPV6, conserved among all identified species of these two channels, is required for Rab11a binding. Mutations in this stretch induce a significantly diminished TRPV5- and TRPV6-mediated Ca\(^{2+}\) influx, resulted from impaired trafficking of the channels. GTP binding-deficient (GDP-locked) mutant Rab11a S25N expression strongly reduces the TRPV5- and TRPV6-mediated Ca\(^{2+}\) influx. These results indicate an essential role for Rab11a in targeting TRPV5 and TRPV6 to the plasma membrane, in which cargo interaction (in the GDP status), as well as subsequent GTP binding is required. Taken together, our data provide new insight into the molecular machinery of TRPV5 and TRPV6 channel trafficking via direct interaction between a Rab GTPase and apically targeted cargo. This association is involved in the physiological regulation of TRPV5 and TRPV6 cell-surface abundance, a critical component in Ca\(^{2+}\) homeostasis.

Chapter 4 and 7. Klotho as a unique extracellular stimulator of TRPV5 and TRPV6

The klotho gene was originally identified as a gene mutated in a mouse strain that develops multiple aging-like phenotypes. Mice defective in klotho expression (klotho\(^{-/-}\) mice) exhibit a syndrome resembling human aging. Klotho gene encodes a single-pass transmembrane protein, whose extracellular domain is secreted into serum, urine and cerebrospinal fluid. The extracellular domain of klotho has a homology to β-glucosidase. The recent study suggested that klotho may play a crucial role in the maintenance of Ca\(^{2+}\) and phosphate homeostasis. Appealing evidences of the involvement of klotho in Ca\(^{2+}\) homeostasis came from the studies described in chapter 4 showing that klotho activates the epithelial Ca\(^{2+}\) channel TRPV5. Real-time PCR analysis indicated that klotho expression is downregulated in kidneys of TRPV5 knockout (TRPV5\(^{-/-}\)) mice. Co-
expression of klotho with TRPV5 in HEK293 cells, or application of klotho-containing supernatant (culture media from klotho-expressing HEK293 cells) to TRPV5-transfected HEK293 cells, significantly stimulates TRPV5-mediated $^{45}\text{Ca}^{2+}$ influx. Cell-surface biotinylation experiments revealed a significant increase in TRPV5 channel abundance at the plasma membrane. These effects could be mimicked by the purified $\beta$-glucuronidase indicating that the enzymatic activity of klotho is responsible for the increased TRPV5 activity. Interestingly, the klotho and $\beta$-glucuronidase-mediated stimulatory effect on TRPV5 is abolished in HEK293 cells expressing the TRPV5 glycosylation-deficient mutant TRPV5-N358Q without the entire N-glycan. It indicates that N-glycosylation plays a crucial role in TRPV5 activation by $\beta$-glucuronidase klotho, in addition, the N-glycan acts as the target in this stimulatory event. The physiological relevance of the aforementioned findings is substantiated by the co-localization of klotho with TRPV5 in the renal DCT and CNT cells, where klotho activates the channel from the pro-urine side. Hereby, we provide a new concept of a regulatory mechanism by glycosylation in which the secreted urinary $\beta$-glucuronidase klotho hydrolyzes the extracellular exposed N-linked oligosaccharides of TRPV5 to entrap channels at the plasma membrane. Furthermore, it raises the intriguing question concerning the specificity of klotho stimulatory effect on TRPV5. To answer this question, we performed the studies described in chapter 7. Here, we selected different ion channels within the TRP superfamily, including TRPV4, TRPV6, and TRPM6, in order to examine whether the klotho effect is restricted to TRPV5 or may be more generally applicable to other renal apically localized channels in DCT cells. These selected channels have structural similarities with TRPV5 and are all permeable to Ca$^{2+}$. Additionally, the Na$^+$-Cl$^-$ cotransporter (NCC) was chosen as a candidate in this study since it is also expressed in DCT. By using $^{45}\text{Ca}^{2+}$ influx analysis, we showed that klotho-containing supernatant or $\beta$-glucuronidase incubation results in a significantly enhanced Ca$^{2+}$ influx mediated by TRPV6, further substantiating klotho as a regulator of both TRPV5 and TRPV6 channels. However, $\beta$-glucuronidase treatment for 16 hours does not increase the Ca$^{2+}$ influx mediated by TRPV4 or TRPM6. In addition, the thiazide-sensitive Na$^+$ influx mediated by NCC was not influenced by $\beta$-glucuronidase as shown by $^{22}\text{Na}^+$ uptake experiments. Therefore, a unique stimulatory effect of klotho was disclosed that is only applicable to the epithelial Ca$^{2+}$ channels TRPV5 and TRPV6, but not to TRPV4, TRPM6 and NCC.
Chapter 5. Tissue kallikrein as a modulator of TRPV5

Tissue kallikrein (TK) is a serine protease produced in CNT, where it co-localizes with TRPV5. Mice lacking TK (TK-/- mice) exhibit robust hypercalciuria, which was also observed in TRPV5-/- mice. Our study delineates the molecular mechanism through which TK stimulates renal Ca\(^{2+}\) reabsorption via TRPV5. It explains the hypercalciuria observed in TK-/- mice. Using TRPV5-expressing primary cultures of rabbit renal CNT and cortical collecting duct (CCD) cells, we showed that TK stimulates Ca\(^{2+}\) reabsorption in these cells. This stimulatory effect of TK is mimicked by bradykinin (BK) and could be reversed by application of JE049, a bradykinin 2 receptor (B2R) antagonist. The effect of these compounds on TRPV5 activity is validated in TRPV5-transfected HEK293 cells by electrophysiological measurement. Using a phospholipase C (PLC) inhibitor and a cell permeable analog of diacylglycerol (DAG) it was demonstrated that TK activates PLC/DAG pathway. The analog of DAG increases TRPV5 activity via PKC activation of the channel, since mutation of TRPV5 at the putative PKC phosphorylation sites of S299 and S654 prevents the stimulatory effect of TK. Cell-surface biotinylation revealed that TK enhances the amount of TRPV5 channels at the plasma membrane by delaying channel retrieval. Taken together, TK stimulates Ca\(^{2+}\) reabsorption via the BK-activated PLC/DAG/PKC pathway and induces the subsequent stabilization of the TRPV5 channel at the plasma membrane.

Chapter 6. EndoF and sialidase as another two extracellular activators of TRPV5

Based on the forementioned study on klotho, we hypothesized that other glycosidases that hydrolyze N-oligosaccharides of TRPV5 may mimic the stimulatory effect of klotho. Therefore, we investigated the effect of diverse members of the glycosidase superfamily, to which \(\beta\)-glucuronidase and klotho belong, namely \(\beta\)-glucosidase, \(\beta\)-galactosidase, sialidase, endoglycosidase H (endoH) and peptide N-glycosidase F (endoF). \(^{45}\)Ca\(^{2+}\) uptake analysis showed that sialidase and endoF increase TRPV5-mediated Ca\(^{2+}\) influx, resulted from enhanced TRPV5 channel abundance at the plasma membrane. In contrast, \(\beta\)-glucosidase, \(\beta\)-galactosidase and endoH do not stimulate TRPV5 activity. Cell-surface biotinylation experiments demonstrated that \(\beta\)-glucuronidase stabilizes TRPV5 at the cell surface by significantly delaying channel retrieval. In addition, the glycosylation-deficient mutant TRPV5-N358Q shows reduced retrieval kinetics compared to wild-type channels. Here, we identified in addition to klotho and \(\beta\)-glucuronidase, sialidase and endoF as novel glycosidases extracellularly modulating TRPV5 activity.
Summary

These glycosidases activate TRPV5 by hydrolyzing the N-oligosaccharides of the channel, resulting in the stabilization of the TRPV5 channel units at the plasma membrane, in which N-glycosylation plays a crucial role and controls TRPV5 stability at the cell surface.

Chapter 8. Conclusion and future perspectives

In this thesis, the studies unraveling the molecular structure and regulation mechanisms of TRPV5 and TRPV6 were described. The molecular determinants in TRPV5 were identified that play a key role in the formation of the functional channel complex, and substantiated that assembly of channel subunits is essential for routing to and subsequent activity of TRPV5 at the plasma membrane. In addition, our data provided new insight into the molecular machinery of TRPV5 and TRPV6 channel trafficking via direct interaction between a Rab GTPase (Rab11a) and apically targeted cargo. Of particular interest is that our study introduces a new mechanism through which Ca\textsuperscript{2+} reabsorption can be stimulated via extracellular activation of TRPV5 by urinary enzymes, including TK, sialidase, endoF, klotho and β-glucoronidase. TK stimulates TRPV5 indirectly via activation of the B2R, which then induces a redistribution of TRPV5 channels towards the plasma membrane. Sialidase, endoF, klotho and β-glucoronidase directly activate TRPV5 by enzymatic modification of the N-glycan to stabilize TRPV5 channels at the plasma membrane. This extracellular control will ensure high Ca\textsuperscript{2+} transport capacity and reduce Ca\textsuperscript{2+} excretion to preserve normal plasma Ca\textsuperscript{2+} levels during periods of dietary Ca\textsuperscript{2+} insufficiency and aging. The identification and elucidation of TRPV5 and TRPV6 regulation by extracellular proteins have greatly contributed to our understanding of the regulation of apical Ca\textsuperscript{2+} influx in epithelial cells. However, this is only the first step in a long endeavor. The major challenges in the near future will be to explore additional urinary proteins extracellularly regulating TRPV5 and TRPV6. Urinary secreted glycosidases could be potential candidates in this case, and urinary proteomics would be a powerful tool, which has been used for many years to diagnose and monitor renal disease. We have to obtain further insight into the N-glycosylation process of TRPV5, TRPV6 and other TRP channels, in addition, establish a comprehensive view of N-glycan structures of TRPV5 and TRPV6, and their contribution to channel regulation. To unravel the molecular mechanisms by which extracellular factors control the activity of TRPV5 and TRPV6 is essential in enduring Ca\textsuperscript{2+} levels within a healthy range. This will complete our picture of Ca\textsuperscript{2+} homeostasis.
Samenvatting

Hoofdstuk 1. Introductie
De eiwitten van de Transient Receptor Potential (TRP) superfamilie vormen ionkanalen die voorkomen in het hele lichaam. TRP-ionkanalen hebben overeenkomsten in structuur, maar onderling verschillende functies. Terwijl deze ionkanalen selectief zijn voor cationen, varieert de selectiviteitsratio voor calcium en natrium sterk. Bovendien wordt een deel van de TRP-kanalen geactiveerd door middel van interactie met een ligand, terwijl anderen worden gereguleerd door stimuli zoals temperatuur. Mutaties in TRP-kanalen kunnen resulteren in niergerelateerde ziekten, zoals erfelijke hypomagnesiëmie met secundaire hypocaliëmie (HSH) dat veroorzaakt wordt door mutaties in TRPM6 en focale segmentale glomerulosclerose ten gevolge van mutaties in TRPC6. Twee leden van de TRP-superfamilie (TRPV5 en TRPV6) zijn homologe calciumkanalen, welke voornamelijk tot expressie komen in calciumtransporterende epitheelcellen. De identificatie van TRPV5 en TRPV6 heeft het inzicht in de moleculaire mechanismen die betrokken zijn bij het transepitheliaal calciumtransport enorm vergroot. TRPV5 vervult de rol van poortwachter bij de epitheliale calciumresorptie in de nier, terwijl TRPV6 voornamelijk verantwoordelijk is voor de opname van calcium in de dunne darm. De structuur van beide kanalen bestaat uit 6 transmembraanregio’s en intracellulair gelegen N- en C-termini, eigenschappen die gedeeld worden met alle leden van de TRP-superfamilie. Zowel de N- als de C-terminus van TRPV5 en TRPV6 bevatten geconserveerde domeinen welke betrokken zijn bij de regulatie van deze kanalen. Beide kanalen bevatten bovendien geconserveerde glycosyleringsdomeinen in de extracellulaire lus tussen transmembraanregio 1 en 2, welke geïdentificeerd zijn als asparagine-residuen N$_{358}$ in TRPV5 en N$_{357}$ in TRPV6. N-glycosylering is belangrijk voor de vouwing, het transport naar de plasmamembraan en de regulatie van eiwitten. De synthese van TRPV5 en TRPV6 vindt plaats in het endoplasmatisch reticulum, waarna een modificatie plaatsvindt aan bovengenoemde asparagineresiduen. Via het Golgicomplex worden de calciumkanalen naar de plasmamembraan getransporteerd. Er zijn diverse belangrijke mechanismen die bepalen hoeveel calcium daadwerkelijk via TRPV5 of TRPV6 een cel instroomt, zoals regulatie door hormonen, transportprocessen in de cel, de locale zuurgraad, intracellulaire calciumconcentraties en regulatie via geassocieerde eiwitten. Uit recent onderzoek is gebleken dat S100A10, NHERF2/NHERF4, FKBP52, BSPRY, calbindin-D$_{28K}$ en RGS2 als geassocieerde
eiwitten betrokken zijn bij de regulatie van TRPV5 en TRPV6. Vele moleculaire details van de mechanismen die betrokken zijn bij TRPV5- en TRPV6-afhankelijk calciumtransport zijn echter nog onbekend. Dit proefschrift beschrijft de moleculaire structuur en regulatiemechanismen van TRPV5- en TRPV6-afhankelijk calciumtransport.

Hoofdstuk 2. De moleculaire structuur van TRPV5

Hoofdstuk 3. Rab11a, een nieuwe regulator van TRPV5 en TRPV6
Rab11a is een GTPase eiwit dat aanwezig is in recycling-endosomen, celorganellen die betrokken zijn bij het transport van eiwitten naar de plasmamembraan. Rab11a colokaliseert met TRPV5 en TRPV6 in cellen van het distaal convoluut en de verbindingsbuis. Aminozuurdomein 596-601 (MLERK) in de C-termini van beide kanalen is geconserveerd in diverse species en is verantwoordelijk voor binding aan Rab11a. Mutaties in dit domein verlagen de TRPV5- en TRPV6-afhankelijke calciumstroom en dit is het resultaat van een verstoord transport van TRPV5- en TRPV6-calciumkanalen naar de plasmamembraan. TRPV5 en TRPV6 binden direct aan GDP-gebonden Rab11a, terwijl GTP-gebonden Rab11a weinig tot geen associatie met deze kanalen vertoont. Expressie van een Rab11a-mutant, welke geblokkeerd is in de GDP-status leidt tot een afname van de hoeveelheid TRPV5 op de plasmamembraan. Deze bevindingen suggereren dat Rab11a betrokken is bij het transport van TRPV5 en TRPV6 naar de plasmamembraan.
Hoofdstuk 4 en 7. Klotho, een extracellulaire stimulator van TRPV5 en TRPV6

Klotho is een transmembraane eiwit dat met één domein de plasmamembraan passeert. Het extracellulaire domein van klotho wordt uitgescheiden in bloed, urine en hersenvocht en vertoont een functionele overeenkomst met het enzym β-glucosidase. Muizen zonder klotho-expressie (klotho knock-out muizen) tonen symptomen die geassocieerd worden met een verstoorde calciumbalans en versnelde veroudering. In hoofdstuk 4 staat de onderzoeksvraag centraal wat de rol is van klotho bij het behoud van een normale calciumhuishouding. Expressie van TRPV5 samen met klotho in HEK293 cellen of toediening van klotho-bevattend kweekmedium aan HEK293 cellen waarin TRPV5 tot expressie is gebracht, stimuleert het TRPV5-afhankelijk calciumtransport. Een toename van het aantal TRPV5-kanalen op de plasmamembraan leidt tot een vergrote calciuminflux. Bovengenoemde effecten van klotho kunnen worden nagebootst door toediening van β-glucuronidase. Dit suggereert dat de enzymatische activiteit van klotho verantwoordelijk is voor het stimulerende effect van klotho op TRPV5. Wanneer de TRPV5 glycosyleringsmutant TRPV5-N\textsuperscript{358}Q (zonder N-gebonden suikergroepen) in HEK293 cellen tot expressie wordt gebracht, verdwijnen de stimulerende effecten van zowel klotho als β-glucuronidase, waaruit blijkt dat N-glycosylering een cruciale rol speelt in de activatie van TRPV5 door klotho. De fysiologische relevantie van de voorgenoemde bevindingen wordt bevestigd door de apicale colocalisatie van TRPV5 en klotho in cellen van het distaal convoluut en de verbindingsbuis van de muizennier. In deze nefronsegmenten kan klotho TRPV5 stimuleren vanuit de voorurine. Hiermee is een nieuw regulatiemechanisme blootgelegd waarbij klotho extracellulaire N-gebonden suikergroepen in TRPV5 hydrolyseert hetgeen leidt tot een verhoogde TRPV5-expressie in de plasmamembraan en daardoor stimulatie van het transepitheliaal calciumtransport.

In hoofdstuk 7 is het onderzoek beschreven waarin de specificiteit van het klotho effect wordt aangetoond. Dit heeft geleid tot de identificatie van TRPV6 als tweede TRP-kanaal dat gereguleerd wordt door klotho en β-glucuronidase. Er is geen verandering in de activiteit van de TRP-kanalen TRPV4, TRPM6 en de thiazide-gevoelige Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporteur (NCC) aangetoond na toediening van klotho of β-glucuronidase. Het effect van klotho lijkt dus specifiek voor TRPV5 en TRPV6 en speelt geen rol bij de regulatie van TRPV4, TRPM6 en NCC.
Hoofdstuk 5. Kallikreine, een modulator van TRPV5
Kallikreine (TK) is een serineprotease die geproduceerd wordt in de verbindingsbuiscellen van de nier en hier colocaliseert met TRPV5. Muizen zonder TK (TK knock-out muizen) vertonen hypercalciurie, een kenmerk ook aanwezig in TRPV5 knock-out muizen. Hoofdstuk 5 beschrijft het moleculaire mechanisme waarmee TK de TRPV5-afhankelijke calciumresorptie stimuleert. Dit effect is aangetoond in primaire cellen van verbindings- en corticale verzamelbuizen, geïsoleerd uit konijnen en vervolgens gevalideerd in TRPV5-getransfecteerde HEK293 cellen. Analyse van de eiwitexpressie laat zien dat het aantal TRPV5-kanalen op de plasmamembraan verhoogd wordt door TK. Dit stimulerende effect van TK kan worden nagebootst met bradykinine en wordt daarnaast geblokkeerd met JE049, een antagonist van de bradykininereceptor. Door gebruik te maken van een fosfolipase C (PLC) remmer en een celperemeabele analoog van diacylglycerol (DAG) is aangetoond dat TK de PLC/DAG route activeert. DAG stimuleert het TRPV5-kanaal via PKC-activatie. Een TRPV5-kanaal waarin de PKC fosforyleringsplaatsen S299 en S654 zijn gemuteerd is ongevoelig geworden voor TK. Samengevat kan gesteld worden dat TK via activering van de bradykininereceptor het aantal TRPV5-kanalen dat tot expressie komt op de plasmamembraan verhoogt via de PLC/DAG/PKC route.

Hoofdstuk 6. EndoF en sialidase, twee activatoren van TRPV5
Gebaseerd op het onderzoek beschreven in hoofdstuk 5, is de hypothese ontstaan dat behalve klotho ook andere glycosidases de hydrolyse van N-gebonden oligosacchariden in TRPV5 kunnen bewerkstelligen. Hoofdstuk 6 beschrijft de effecten van β-glucosidase, β-galactosidase, sialidase, endoglycosidase H (endoH) en peptide N-glycosidase F (endoF) op de activiteit van TRPV5. Analyse van de calciumopname in HEK293 cellen na toediening van deze enzymen heeft sialidase en endoF geïdentificeerd als stimulatoren van het TRPV5-afhankelijk calciumtransport. Behalve klotho en β-glucuronidase moduleren ook sialidase en endoF de TRPV5-activiteit extracellulair via de hydrolyse van de N-gebonden suikergroepen van het kanaal. Dit resulteert in de stabilisatie van TRPV5 in de plasmamembraan en een toename van het TRPV5-afhankelijk calciumtransport.
Hoofdstuk 8. Conclusies en toekomstperspectieven

In dit proefschrift zijn de determinanten geïdentificeerd die een rol spelen bij de formatie en regulatie van functionele TRPV5- en TRPV6-kanalen. Hiermee zijn nieuwe inzichten verkregen in de moleculaire werkingsmechanismen betrokken bij het transport van TRPV5 en TRPV6 naar de plasmamembraan zoals de functie van het TRPV5-geassocieerde eiwit Rab11a. Een nieuw mechanisme is ontdekt waarbij TRPV5 wordt gereguleerd door de extracellulaire enzymen TK en glycosidases als klotho, β-glucuronidase en sialidase. Deze mechanismen verhogen de capaciteit van de calciumresorptie en verlagen derhalve de calciumexcretie via de urine. Dit resulteert in het behoud van een normale calciumconcentratie in het bloed gedurende perioden van een verstoorde calciumbalans door bijvoorbeeld een lage calciuminname of een verminderde calciumabsorptie in de darm tijdens veroudering. In de toekomst zal het van belang zijn andere extracellulaire eiwitten te identificeren die betrokken zijn bij de regulatie van het TRPV5- en TRPV6-afhankelijk calciumtransport. Glycosidases, welke uitgescheiden worden in de urine, zijn hiervoor potentiële kandidaten. Toepassing van nieuwe eiwitanalysetechnieken in urinemonsters zal hierbij een krachtig hulpmiddel kunnen zijn. Inzicht in de N-glycosylering van TRP-kanalen in het algemeen en de suikergroepen aanwezig op de TRPV5- en TRPV6-kanalen in het bijzonder zal verdere opheldering geven over de betrokkenheid van deze domeinen in de regulatie van ionkanalen. Het vergaren van kennis over de extracellulaire factoren die de activiteit van ionkanalen kunnen beïnvloeden is essentieel voor het behoud van een goede calciumbalans in het lichaam.
中文简介

第一章   导言

TRP（Transient Receptor Potential）家族是一类分子结构具有相似性的离子通道蛋白。TRP家族通道蛋白的表达十分广泛，并且功能各异。所有TRP家族的通道蛋白都具有阳离子通透性，但是对于二价钙离子与单价钠离子的选择通透性大相径庭，而且具有多种激活模式。正是如此，所以我们只能从蛋白分子结构的同源性来判断某一蛋白是否属于TRP家族。某些TRP通道蛋白的突变可导致多种与肾脏有关的疾病，如伴有继发性低钙血症的遗传性低镁血症【hereditary hypomagnesemia with secondary hypocalcemia（HSH）】，多囊肾（polycystic kidney disease），以及中心肾小球硬化（focal segmental glomerulosclerosis）等。在众多的TRP家族蛋白中，上皮钙离子通道TRPV5和TRPV6是具有高度结构同源性的典型代表，它们主要表达在与钙离子运输相关的上皮细胞中。TRPV5和TRPV6通道的发现使得对上皮钙离子转运分子机制的研究得到了极大关注。这两个蛋白有效地控制着上皮钙离子的转运传输，从而在调节钙离子血液平衡方面扮演着举足轻重的角色。但是TRPV5和TRPV6在调节钙离子重吸收方面的分工略有不同，TRPV5主要在肾脏上皮钙离子运输中起关键作用，而TRPV6主要形成小肠里的钙离子流入通道。TRPV5和TRPV6的分子结构显示出典型的所有TRP家族成员共有的拓扑结构特征——具有六个跨膜区和胞内的氨基端和羧基端。在TRPV5和TRPV6的氨基端和羧基端上还包含着一些特异的高度保守的调节位点，例如ankyrin重复片断、内部潜在的PDZ序列模式和磷酸化位点，这些位点可能对于通道活性的调节至关重要。TRPV5和TRPV6都是由四个亚单位构成的同源或异源性通道复合四聚体，此四聚体以头尾相接的方式结合在一起。当四个TRPV5和TRPV6亚单位组成异源通道复合四聚体时，此四聚体显示处介于TRPV5和TRPV6的特性，而此特性由四聚体中配置的TRPV5和TRPV6亚单位数量来决定。TRPV5和TRPV6通道在位于第一和第二跨膜区之间的细胞外环上包含一个高度保守的N糖基化位点。这个N糖基化位点在TRPV5和TRPV6上分别位于第358或第357个氨基酸残基——天冬氨酸。我们已经清楚，这两个通道不仅被翻译为核心蛋白，并且通过高度复杂的糖基化形成分子量约为85-100kDa的大分子蛋白质。TRPV5和TRPV6的N糖基化在蛋白质折叠、转运以及通道活性调节方面扮演着重要角色。TRPV5和TRPV6在内质网内合成，随后在由内质网向高尔基体装配的过程中被位于天冬氨酸的单糖所调节，并且最终运往它们的目的地——血浆膜。TRPV5和TRPV6可被多种机制所调控，包括由各类激素介导的在蛋白转录
和翻译方面的调控，由 pH 值、细胞内钙离子浓度和钙离子传感蛋白（calmodulin 和 80K-H）控制的在血浆膜活性方面的调控，由 S100A10/annexin 2 调节的在通道向血浆膜转运过程中的调控等。我们的最新研究进一步表明 NHERF2/NHERF4、FKBP52、BSPRY、calbindin-D28k 和 RGS2 等蛋白也可调控 TRPV5 或 TRPV6 在血浆膜上的活性。尽管近年来在 TRPV5 和 TRPV6 功能、调控和通道装配等方面的研究已经有了迅速发展，但目前的研究成果还不能完全解释有关这两个蛋白调控以及分子结构方面的所有机理。因此本论文旨在解开 TRPV5 和 TRPV6 的分子结构和调控之谜。

第二章 TRPV5 的分子结构

早先研究已经表明，TRPV5和TRPV6可以装配形成同源或异源四聚体通道，但是此四聚体的装配位点还不明晰。因此，我们做了大量的研究试图找寻在 TRPV5复合四聚体通道形成过程中起着重要作用的分子。本章的研究表明，氨基端和羧基端在 TRPV5通道装配方面的作用具有同等的重要性。我们在 Xenopus laevis 的卵母细胞和人类肾脏胚胎细胞【human embryonic kidney（HEK293）cells】中共表达 TRPV5通道与缺失氨基端或羧基端的通道突变体。功能试验分析表明这些缺失了氨基端、羧基端的突变体表现了对通道活性的显性抑制作用，此抑制作用的产生是由于此突变体不能与 TRPV5 通道装配成四聚体，从而干扰了TRPV5蛋白向血浆膜的转运。我们的研究还进一步表明，TRPV5蛋白中至少有二个区域（位于氨基端第64至77位的氨基酸残基序列和位于羧基端第596至601位的氨基酸残基序列）在通道装配的过程起决定作用。当缺失这两个重要的区域时，TRPV5通道氨基端与氨基端，氨基端与羧基端，羧基端与羧基端的物理交互作用将被彻底消除。另外，位于氨基端第64至77位的氨基酸残基序列与所谓的 ankyrin第一重覆片断重叠，证实了这些“蛋白质——蛋白质”互作模块在 TRPV5 装配中具有重要作用。总结以上观点，通道亚单位的装配是决定 TRPV5 蛋白能否运送到血浆膜以及其在血浆膜上活性大小的根本条件。

第三章 Rab11a——全新的TRPV5和TRPV6转运调节蛋白

Rab11a 是一个小型的 GTP 酶，它主要作用于由循环内涵体控制的蛋白转运过程。本章的研究阐明了 Rab11a 的作用，即可以作为 TRPV5 和 TRPV6 的直接互作蛋白从而调节这两个通道到达血浆膜的过程，继而充当了全新的 TRPV5 和 TRPV6 的转运调控器。Rab11a 与 TRPV5 或 TRPV6 在肾脏远曲小管【distal convoluted tubule（DCT）】、连接管【connecting tubule（CNT）】细胞的顶膜上共同表达，并且 TRPV5 和 TRPV6 优先地与 Rab11a 的突变体
Rab11a S25N互作。Rab11a S25N缺少了与GTP互作的特性。Rab11a与TRPV5和TRPV6的直接互作作用能够支持这些通道迁移到循环内涵体，从而构成“传递器”最终运输这些通道到达血浆膜。Rab11a与TRPV5和TRPV6的互作区域位于通道的羧基端，并且由在所有物种中全部保守的五个氨基酸残基（595到601：MLERK）所组成。位于这一互作区域的突变体将阻碍TRPV5和TRPV6到达血浆膜地转运，从而进一步导致由TRPV5和TRPV6介导的钙离子流入的显著减少。另外，我们的研究发现GDP锁定的Rab11a突变体Rab11a S25N与TRPV5和TRPV6的共表达也会导致由TRPV5和TRPV6介导的钙离子流入的衰减，这充分证明了Rab11a在TRPV5和TRPV6转运至血浆膜过程中的重要作用。综上所述，我们的研究提供了在TRPV5和TRPV6通道分子转运方面的新模式，进而证明了与Rab11a GTP酶的直接互作将有效地调节TRPV5和TRPV6通道的转运，此调节机制在控制TRPV5与TRPV6的细胞表面丰盈度及钙离子血液平衡方面起着至关重要的作用。

Klotho——TRPV5和TRPV6特异的细胞外激活因子

Klotho基因最早被发现是由于此基因的突变引起了小鼠的衰老化。大量的研究数据进而证明缺失Klotho蛋白表达的小鼠的确表现出一系列与人类衰老极为类似的症状。Klotho基因编码一个穿膜蛋白质，此蛋白质位于细胞膜外端的部分经证实将会分泌到血液、尿液以及脑脊液中，这个被分泌的外端部分与β—葡萄糖苷酶（β—glucosidase）具有同源性。最新研究揭示Klotho在维持钙离子血液平衡方面起着相当重要的作用。第四章中的研究数据极为有力地证明了Klotho在维持钙离子血液平衡方面的重要作用。RT-PCR分析表明Klotho在TRPV5敲除小鼠肾脏中的表达明显降低。TRPV5与Klotho在HEK293细胞内的共表达或用含有Klotho的细胞培养液处理TRPV5表达的HEK293细胞都会明显地刺激由TRPV5介导的钙离子吸收。细胞表面生物素标记实验证实了在血浆膜上TRPV5的大幅度增加是产生这一现象的原因。β—葡萄糖醛酸酶（β—glucuronidase）可以模拟该刺激作用，表明Klotho所含的葡萄糖醛酸酶的活性对刺激增加TRPV5的活性起着决定作用。有趣的是，由Klotho以及葡萄糖醛酸酶对TRPV5的刺激作用在表达了TRPV5糖基化缺失突变体TRPV5-N358Q的HEK293细胞里被彻底消除，这个糖基化缺失突变体被证实没有N糖链。通过D-[U-14C]葡萄糖标志试验我们又进一步阐明了Klotho和β—葡萄糖醛酸酶能够水解位于已经糖基化了的TRPV5蛋白表面的糖链。这些数据表明，N糖基化在由葡萄糖醛酸酶和Klotho介导的对TRPV5通道的激活中扮演了关键的角色，而且N糖链在这一过程中起到了靶子的作用。Klotho和TRPV5在肾脏远曲小管（DCT）以及连接管
（CNT）中的共表达为此研究发现提供了有力的生理依据，在正常的生理状况下，Klotho 从肾脏与 TRPV5 共表达的细胞内被分泌到原尿中进而调节 TRPV5 的活性。我们第四章的研究提供了一个有关 TRPV5 调控机制的新概念。在此调控机制中，被分泌的 Klotho 蛋白水解 TRPV5 通道的细胞外 N 糖链引起此通道在血浆膜上表达的增加，从而加大由 TRPV5 引起的钙离子吸收。与此同时，这个研究也提出了一些有关 Klotho 蛋白在 TRPV5 刺激作用中特异性的问题，我们因此开始了第七章的研究。在第七章中，我们选择了一些具有代表性的蛋白质来进一步研究 Klotho 的刺激作用是否仅限于 TRPV5 或者对其它蛋白亦有活性，例如，我们选择了位于 TRP 家族之内的 TRPV6、TRPV4 和 TRPM6 通道。这些通道与 TRPV5 具有结构相似性并且对钙离子有选择通透性。另外，对钠离子有选择通透性的钠离子氯离子运输蛋白 【Na⁺-Cl⁻ cotransporter (NCC)】也被用到了这项研究中。钙离子吸收试验数据表明，葡萄糖醛酸酶和 Klotho 显著增加了由 TRPV6 介导的钙离子流入，从而证明了 Klotho 能够作为 TRPV5 和 TRPV6 共同的调节因子来维持钙离子的血液平衡。但是，葡萄糖醛酸酶并不能刺激 TRPV4 和 TRPM6 的通道活性。而且，钠离子吸收试验还进一步阐明了由 NCC 介导的对噻嗪类药物敏感的钠离子流入不会受到葡萄糖醛酸酶的影响。因而，我们证实了 Klotho 对上皮钙离子通道 TRPV5 和 TRPV6 激活作用的特异性，此作用并不适用于 TRPV4, TRPM6 和 NCC。此特异作用的产生也许来源于这些蛋白不同的糖基化方式和它们各自的 N 多聚糖结构。

第五章 组织血管激肽释放酶——TRPV5通道活性的调节器

组织血管激肽释放酶【Tissue kallikrein (TK)】是在肾脏连接管 (CNT) 中产生的丝氨酸蛋白酶。这一蛋白酶在肾脏集合管与 TRPV5 通道共同表达。TK 敲除小鼠表现出明显的高钙尿症，此症状与在 TRPV5 敲除小鼠中表现的钙离子流失现象高度相似，我们为解释这一生理现象展开了大量的研究工作，本章对这些研究做了详尽的描述。通过对野生鼠肾脏连接管（CNT）以及皮质集合管（CCD）细胞的培养，我们发现 TK 能够刺激这些细胞中的钙离子吸收。TK 的这一刺激作用可被血管缓激肽 【bradykinin (BK)】 所模拟，但是被血管缓激肽第二受体 【bradykinin 2 receptor (B2R)】 拮抗剂 JE049 所阻抗。这些化合物的活性都能够由在 HEK293 细胞中进行的电生理试验所证实。此外，试验中应用磷脂酶 C 【phospholipase C (PLC)】 抑制剂以及具有细胞通透性的甘油二酯 【diacylglycerol (DAG)】 类似物检测到 TK 激活了 PLC/DAG 路径。位于 TRPV5 假定磷酸化位点 S299 和 S654 的突变体阻碍了 TK 对此通道的刺激作用，此结果表明了甘油二酯类似物可通过激活 TRPV5 磷酸
化来增加通道的活性。细胞表面半衰期生物素标记试验揭示了TK凭借延迟TRPV5的回收降解而提高TRPV5在血浆膜的表达数量。因此，我们得出结论，即TK通过BK激活的PLC/DAG/PKC通路来增加TRPV5通道在血浆膜的稳定性，进而刺激钙离子的重吸收。

第六章 缩氨酸N端切割糖苷酶F和唾液酸酶——两个新型的TRPV5细胞外激活酶

基于上述对于Klotho蛋白功能的一系列深入研究，我们假定具有N端糖链切割特性的其它糖苷酶成员能够模拟Klotho的活性。因此我们进一步展开了对Klotho和葡萄糖醛酸酶所属糖苷酶家族中其它成员的研究，这些糖苷酶包括β－葡萄糖苷酶（β-glucosidase），β-半乳糖苷酶（β-galactosidase），唾液酸酶（sialidase），内切糖苷酶H【endoglycosidase H (endoH)】以及缩氨酸N端切割糖苷酶F【peptide N-glycosidase F (endoF)】。钙离子吸收试验表明唾液酸酶和endoF明显增加了TRPV5通道在细胞表面的表达，从而刺激了由TRPV5介导的钙离子吸收。但是，其它糖苷酶成员β－葡萄糖苷酶，β-半乳糖苷酶和endoH并没有显示出对TRPV5的刺激作用。通过细胞表面半衰期生物素标记试验，我们证实了β－葡萄糖醛酸酶显著延迟了TRPV5的回收降解，从而导致了TRPV5通道在血浆膜上表达数量的增加。此外，我们的研究还发现TRPV5糖基化缺失突变体TRPV5-N358Q的回收降解明显慢于TRPV5通道，从而导致此突变体在细胞表面的稳定性大大提高，β－葡萄糖醛酸酶也因此不能够再刺激TRPV5-N358Q的活性。我们在本章的研究进一步证实除Klotho和β－葡萄糖醛酸酶之外，唾液酸酶和endoF也可作为新的糖苷酶于细胞外调节TRPV5的活性。这四个糖苷酶由于切割TRPV5的N糖链而引起TRPV5降解的延迟，这一过程将导致TRPV5通道在细胞表面稳定性的提高，从而大幅度的刺激由TRPV5介导的钙离子吸收。N糖基化控制着TRPV5在细胞表面的稳定性，因而在由糖苷酶介导的TRPV5激活作用中起着极为重要的作用。

第八章 结论及未来展望

在本论文中，我们对有关TRPV5和TRPV6分子结构和调节机制方面的研究做了详细的阐述和全面的总结。我们的研究表明，位于氨基端第64至77位以及羧基端第596至601位的氨基酸残基序列对TRPV5通道的装配的作用同等重要，通道亚单位的装配是TRPV5转运至血浆膜及随后在血浆膜上活性程度的根本决定因素。此外，我们的研究提出了在TRP通道分子转运方面的新模式，进而证明了与Rab11a GTP酶的直接互作可以有效地调节TRP通道的转运。此调节机制在控制TRPV5与TRPV6的细胞表面丰盈度及血液钙离子平衡方面起着十分关
键的作用。我们的研究还阐明了组织血管激肽释放酶（TK）通过血管缓激肽（BK）激活的PLC/DAG/PKC通路来增加TRPV5通道在血浆膜的稳定性，进而刺激钙离子的重吸收。我们的研究进一步表明，TRPV5和TRPV6的活性可由细胞外糖苷酶控制。这些糖苷酶包括β－葡萄糖醛酸酶、Klotho和endoF。最终我们证实了Klotho的激活作用对上皮钙离子通道TRPV5和TRPV6具有明显的特异性。这里，我们提供了一个N糖基化调控TRPV5活性的新概念，这一概念揭示上述糖苷酶由于切割TRPV5 N糖链而引起TRPV5降解的延迟，该过程将导致TRPV5通道在细胞表面稳定性的提高，从而大幅度的刺激由TRPV5介导的钙离子吸收。这一调节机制将保持高钙运输容量并减少钙离子在尿中的排泄，从而保证在饮食钙离子不足和衰老过程中具有正常的血液钙离子平衡。我们的研究结果提出了关于TRPV5和TRPV6生理功能的新概念，并且基于对细胞外蛋白调节TRPV5和TRPV6过程的进一步阐述，我们指出了全新的关于上皮细胞顶膜钙离子流入的调节机制，但这只是窥豹一斑。未来工作应主要着眼于对TRPV5、TRPV6和其它TRP通道N糖基化过程的进一步研究。我们必须建立一个详细的TRPV5和TRPV6N糖链的结构图，并且深入研究这些糖链对通道的调控作用。搞清细胞外调钙因子控制TRPV5和TRPV6活性的分子机制是保持机体在一个健康范围内维持正常的钙离子水平的根本保证，这同时也有助于我们了解血液钙离子平衡调控的完整机制。
Chapter 10

Acknowledgement
Curriculum vitae
List of publications
List of abbreviations
No pain, no palm; no thorns, no throne; no gall, no glory; no cross, no crown. (William Penn)

没有播种，何来收获；没有辛劳，何来成功；没有磨难，何来荣耀；没有挫折，何来辉煌。（佩恩 W）
Acknowledgement

A journey is always easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of four years of hard working whereby I have been accompanied and supported by kind people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them. In fact, I have never thought that this is going to be the most difficult article ever written by me because of too many people involved.

I would like to express my deep and sincere gratitude to my supervisors, Prof. Dr. Rene Bindels and Dr. Joost Hoenderop. I could not have imagined having better supervisors for my Ph.D., and without their supervision, wide knowledge, perceptiveness and logical way of thinking that have been of great value for me I would never have finished. THANKS, Rene and Joost, for offering me such a precious opportunity to be a Ph.D. student in your group, supplying me a nice lab condition to work, your guidance and intuition have provided a wonderful basis for the present thesis.

It is impossible to overstate my gratitude to Prof. Dr. Carel van Os. Five years ago, with his enthusiasm, his inspiration, and his great efforts to explain things about Nijmegen clearly and simply during our first meeting in China, I decided to initiate my journey to Holland. He opened the door of science to me, in this fantastic new world of mine I enjoyed myself so much, got more matured and made a great achievement, which I could never think of before my Ph.D. journey started. THANKS, Carel, for all the encouragement, support and understanding.

I am deeply grateful for Eugenie, who has put so much effort to help my husband and me with applying visas to come over, showing me around, giving me a bicycle, finding me the first Chinese shop in Nijmegen… I could take an extra paper to list all the things you have done for me, which made my first winter in Nijmegen warm! THANK
YOU, for all the encouragement and understanding! I like very much pleasant talks and lovely dinners we have had!

**Wouter and Kirsten**, my dearest paranimfens, there are no one else who deserve this more than you. You were the closest colleagues, and now are good friends of mine. I do appreciate more than words to say that all your effort, friendship, trust, accompany and humor made my life in the lab full of fun and stimulation! I enjoyed our days in U4 that much, still laughing while thinking of the jokes you have ever made. THANKS, for keeping me happy, for making my life more colorful.

I would like to say a big 'THANK-YOU' to **Rob and Bob**. You let me be more cheerful and stronger whenever and where ever I have been with all of your support, understanding and encouragement. Hi, guys, wish you a great achievement in your career, hope to see you around!

Now I warmly thank **Stan, Susan** and **Annemiete** for your valuable advices and friendly helps. Our extensive discussions around my work and interesting explorations in experiments have been very helpful and supportive for this study. Dank u veel!

I am indebted to all colleagues of mine for providing an inspiring, cooperative and pleasant lab environment in which I learn and grow. I am especially grateful to **Arjen, Joost. S, Sylvie, Dennis, Tom, Els, Monique. VA and Monique. G**. Your overly enthusiasm and integral view on research have made a deep impression on me. I owe you lots of gratitude for having me shown this way of research. Thanks a lot, all of my colleagues in Physiology Department!

Of course, my Chinese club in NCMLS, **Gang, Yuedan, Peng** and **Liyan**, many many thanks for giving me the feeling of being at home at work. I enjoyed a lot our lunch meetings and dinner parties. I am so lucky to have you, my friends in the lab to make me laugh, share laughter and even tears what ever I have. 非常感谢你们，亲爱的朋友们，祝愿你们天马行空，前程似锦! And then, my gratitude will be always with another two members of our Mandarine-speaking community, **Ted** and **Chuanhui**. Your
kindness, friendship and dignity make my life a bit lighter, THANKS for all the cute presents you have given to us, hopefully, see you soon in Taiwan!

On my opinion, doing a Ph.D. is a sacred task and this was definitely one of the best decisions of my life. Additional energy and vitality for my Ph.D. were provided externally through my involvement in several social activities. Thereby, thanks, my dear international friends in or out of the lab, Ester, Mirto, Stephanie, Catalin, Celine, Vassilis, Artur and Nazad. I can NOT imagine how miserable my life here would have been without your genuine presence. I love all the drinks, dinners, parties and dancing what ever we have done together, and I do believe that we will be whole-life friends no matter whether we are physically apart or not. Ester, my first and best Dutch friend ever, I miss our beautiful house very much, it gives me numerous happy memories! Being your roommate and friend is my great fortune. Wish you healthy and happy forever!!! Mirto, my dear girl, I owe you a lot for getting incredible supports, helps, understanding, trust and comfort from you. Thank you so much for me wonderful meals, giving me warm words, and sharing my stories.

Αγαπητη Μυρτα, σε ευχαριστω πολυ για την υποστηριξη, το κουραγιο και την παρουσια α σου μεσα στο εργαστηριο. Nazad, I am so grateful and happy to have you come along in my journey, thanks for all encouraging talks, mails and phone calls, your great accompany makes my sky much brighter, very good luck with your research in the lab! Steph, have fun in Mexico! Here, dear friends of mine, I wish you all the best with what ever you are doing and going to do!

I feel the deepest sense of gratitude for my Chinese friends in Nijmegen as well, Zhihai, Ying, Jinxing, Dongyu, Lao Nie, Xiaoling, Jidong, Xiaogang, Weibo, Han yan, Huijuan, Hu Lin, Gao Jian, Cao Xin, Jingze, Zheng hang, Xie Rui, Wang Qin, Dajin, Yu Bin and Zhang Zheng … The happy memories of all of you provide me a persistent inspiration for the journey of my Ph.D. You make Nijmegen my second home-town, and get my life much easier and more fulfilled! Dear Zhihai and Ying, wish you both all success in Shang Hai!
Chapter 10 Acknowledgement

The chain of my gratitude would be definitely incomplete if I would forget to thank my parents, Dong, Chun and Hongtao, who form part of my vision and told me the good things that really matter in life. Dad and Mom, you born me, raised me, support me, teach me, and love me. To you I dedicate this thesis. My deepest and sincere love is given to my husband, Dong. You came to Nijmegen only for me with sacrificing all you had in China, career, family and fortune, your unconditional love, support and patience make my life in Holland brighten, and relieve me from all I have been through. Thanks for your love!

亲爱的爸爸，妈妈，感谢你们赐给予我所有美好的一切：善良，坚强和开朗达观的个性。感谢你们在我成长的历程中给予我的无微不至的关怀和谆谆教诲。这些永远是我生命中最宝贵的财富，有你们这样的父母，是我的福气。我爱你们！亲爱的妹妹，妹夫，谢谢你们的大力支持与不断鼓励，谢谢你们让我在异国他乡也常常感受到家庭的温暖。

亲爱的老公，你一定是上苍派驻到我身边的守护者，我常常怀着感恩的心感谢你的付出和我们的爱，它是我能够拿到博士学位的动力和力量源泉，它让我相信真爱可以永恒！

Qing
Curriculum vitae

Qing Chang was born in Xi’an of China on November 14th, 1976. In 2000, she accomplished her medicine study, and received the M.D. (medical doctor) Degree at Xi’an Jiaotong University, China. She obtained one of the highest scores in the end of her M.D. study, and received more than 10 different prizes during the study, including “the Best Medical Graduate Award”, Shann’Xi Province, China in 2000; five times “the Annual Student Prize of Xi’an JiaoTong University”; twice “the Federal Award of Best Medical Student of China”. After Qing Chang obtained her M.D. degree, she started to work at Xi’an Jiaotong University as an assistant professor and medical doctor in the Department of Pathology in 2000. Early interests in scientific research encouraged her to start as a Ph.D. student in the Department of Physiology, Radboud University Nijmegen Medical Centre, the Netherlands, in 2002. Her Ph.D. project was founded by Dutch “ZonMW Vernieuwingsimpuls” grant. Her supervisors were Prof. Dr. R.J.M. Bindels and Dr. J.G.J. Hoenderop. She enjoyed her scientific work, and devoted herself utmost into the challenging Ph.D. project named “Molecular Structure and Regulation of the Epithelial Calcium Channels TRPV5 and TRPV6”. During her Ph.D. study, she obtained “the Best Poster Presentation Award” in the annual Ph.D. conference of Nijmegen Centre for Molecular Life Sciences (NCMLS) in 2005, and “Chinese Government Award for Outstanding Ph.D. Students Abroad” in March of 2006. From November of 2006 to March of 2007, she was employed by Dr. Reuven Agami as a postdoc in the Department of Tumor Biology of the Netherlands Cancer Institute (NKI). Since March of 2007 until today, she has been working as a clinical research scientist and pharmacogeneticist in the Department of Global Clinical Development, NV Organon.
List of publications


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>1,25(OH)₂D₃</td>
<td>1,25-dihydroxy-vitamin D₃</td>
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<td>[Ca²⁺]ᵢ</td>
<td>intracellular Ca²⁺ concentration</td>
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<td>ADPLD</td>
<td>autosomal dominant polycystic liver disease</td>
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<td>AGE</td>
<td>advanced glycosylation end products</td>
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<td>AID</td>
<td>α-interaction domain</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>ATL</td>
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<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<td>CaSR</td>
<td>Ca²⁺-sensing receptor</td>
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<td>CCD</td>
<td>cortical collecting duct</td>
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<td>collecting duct</td>
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<td>CFTR</td>
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<tr>
<td>CIP</td>
<td>channel-interacting PDZ domain protein</td>
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<td>fibroblast growth factor</td>
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<td>green fluorescent protein</td>
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<td>GIRK</td>
<td>G-protein-activated inwardly rectifying K⁺ channel</td>
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<td>Description</td>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>G protein-coupled receptor kinase</td>
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<td>HPRT</td>
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<td>i</td>
<td>unitary channel current</td>
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<td>I</td>
<td>whole cell current</td>
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<td>inter modular collecting duct</td>
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<td>NHERF</td>
<td>NHE regulating factor</td>
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<td>PAGE</td>
<td>polyacrylamide electrophoresis gel</td>
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<td>phospholipase C</td>
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<td>Pₒ</td>
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<td>parathyroid hormone</td>
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<td>sodium dodecyl sulphate</td>
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<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive-factor attachment proteins receptor</td>
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<td>tissue kallikrein knock-out mice</td>
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<td>Tris</td>
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<td>TRP melastatin</td>
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<td>TRPV</td>
<td>TRP vanilloid</td>
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<tr>
<td>TRPV5&lt;sup&gt;+&lt;/sup&gt;</td>
<td>TRPV5 knock-out mice</td>
</tr>
<tr>
<td>TRPV5&lt;sup&gt;+&lt;/sup&gt;/calbindin-D&lt;sub&gt;28k&lt;/sub&gt; &lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>TRPV5/calbindin-D&lt;sub&gt;28k&lt;/sub&gt; double knock-out mice</td>
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<tr>
<td>VDDR-1</td>
<td>vitamin D-dependent rickets type I</td>
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<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
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<tr>
<td>VDR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>vitamin D receptor knock-out mice</td>
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<tr>
<td>Y2H</td>
<td>yeast two-hybrid</td>
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