TRPV4 channels in the human urogenital tract play a role in cell junction formation and epithelial barrier

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Abstract
Aim: The molecular interactions between transient receptor potential vanilloid subtype 4 channels (TRPV4) and cell junction formation were investigated in the human and mouse urogenital tract.

Materials and Methods: A qualitative study was performed to investigate TRPV4 channels, adherence junctions (AJs) and tight junctions (TJs) in kidney, ureter and bladder tissues from humans and wild-type and transgenic TRPV4 knockout (−/−) mice with immunohistochemistry, Western blotting, immunoprecipitation and reverse transcription-PCR. Cell junction formation in the wild-type and TRPV4 knockout (−/−) mouse was evaluated with immunohistochemistry and transmission electron microscope (TEM) techniques.

Results: TRPV4 channels are predominantly located in membranes of epithelial cells of the bladder, ureter and the collecting ducts of the kidney. There is a molecular interaction between the TRPV4 channel and the AJ. TEM evaluation showed that AJ formation is disrupted in the TRPV4 −/− mouse resulting in deficient intercellular connections and integrity of the epithelium.

Conclusions: TRPV4 is believed to be a mechanoreceptor in the bladder. This study demonstrates that TRPV4 is also involved in intercellular connectivity and structural integrity of the epithelium.

Keywords: adherence junction, barrier, transient receptor potential vanilloid subtype 4, urinary bladder, urothelium.

This study investigates the interactions between the cation channel transient receptor potential vanilloid subtype 4 (TRPV4) and cell junctions in urinary epithelia.

TRPV4 is a non-selective Ca²⁺-permeable cation channel that is part of the TRP-channel family that is involved in diverse physiological processes like heat–cold detection, vision, mechanosensation, pain and hearing (Nilius et al. 2003, 2004, Nilius & Voets 2005, Everaerts et al. 2008). The TRPV4 channels are formed by six membrane spanning helices and a cytoplasmic located N- and C-terminus and are expressed predominantly in epithelial cells (Nilius et al. 2003, Everaerts et al. 2008, Mochizuki et al. 2009). Besides this, there is evidence that TRPV4 channels are present in specific neurones (Vriens et al. 2004, Cao et al. 2009). The TRPV4 channel can be activated by mechanical stretch, shear stress (flow), temperature differences and specific chemical stimuli (Vriens et al. 2004, Cao et al. 2009). The modes of activation occur through different pathways (Vriens et al. 2004). Their gating function is dependent on intra- and extracellular Ca²⁺ concentrations (Nilius et al. 2003).

In the urogenital tract, TRPV4 channels are located in the epithelial lining of the connecting tube/collecting duct cells of the kidney, and the epithelia of the...
TRPV4 channels are investigated for mechanosensory properties and its involvement in bladder filling sensations (Mochizuki et al. 2009, Gevaert et al. 2007). Reduced mechanosensation was observed in the transgenic TRPV4 knockout (−/−) mouse (Gevaert et al. 2007). The phenotype is characterized by bladder dysfunction with an increased bladder capacity and altered voiding behaviour (Gevaert et al. 2007). A lack of TRPV4 channels inhibits afferent signalling by reducing ATP excretion in the urothelium (Mochizuki et al. 2009, Gevaert et al. 2007). There are also reports that showed TRPV4 localization on nerves in dorsal root ganglia and dorsal horns, which might also influence bladder function (Cao et al. 2009).

According to the location of TRPV4 channels in the kidney, it is believed that TRPV4 either is involved in regulation of osmosis, or as a flow receptor in the distal tubuli or collecting ducts (Berrout et al. 2012). TRPV4 is expressed in aldosterone-sensitive cells containing aquaporin-2 receptors in the kidney (Galizia et al. 2012). Both flow and water uptake are regulated in these nephron segments. The exact function of TRPV4 channels in the kidney is however still not fully clarified.

Mechanoactivation of TRPV4 channels is thought to occur via a distinct pathway in which cell swelling activates phospholipase A2 which induces P450 epoxygenase-dependent arachidonic acid metabolite formation (Vriens et al. 2004), the latter being the endogenous agonist that activates the channel (Vriens et al. 2004). There is evidence that TRPV4 channels are connected to adherens junctions (AJs) and the actin cytoskeleton (Becker et al. 2009, Goswami et al. 2010, Sokabe et al. 2010, Janssen et al. 2011). The AJ is a rigid cell junction that plays a vital role in maintenance of the epithelial barrier and helps to provide structure and organization in epithelial and endothelial tissues (Goodwin & Yap 2007, van Roy & Berx 2008). The extracellular component of the AJ consists of E-cadherin dimers are essential for the intercellular connections. The intracellular component consist of β-3, γ- and α-catenins of which the latter is connected to the actin cytoskeleton (Goodwin & Yap 2007, van Roy & Berx 2008). This rigid intercellular network is capable of transporting mechanical forces across neighbouring cells and may be enable the activation of TRPV4 channels. Previous research in bladder urothelial cells demonstrated a distinct molecular connection between TRPV4 channels and the intracellular α-catenin complex of the adherence junction (Janssen et al. 2011). Besides this, there is evidence from different organ systems that TRPV4 deficiency impairs barrier and cell junction formation (Willette et al. 2008, Sokabe et al. 2010).

The objective of this study was to explore the location and molecular interaction of TRPV4 channels and barrier forming cell junctions in the human and mouse urogenital tract.

Materials and methods

Tissue and cells

The local human and animal ethical committees of the Radboud University Nijmegen Medical Center approved the use of patient and animal materials. We obtained pathology verified, non-tumorous tissue from human bladders (n = 6), kidneys (n = 2) and ureters (n = 1). For RNA isolations from human bladders, we used cold cup biopsies (n = 3) that were taken from patients treated for pure stress incontinence. Mouse bladders and kidneys were harvested from C57BI 6J mice (TRPV4+/+)(eight bladders, five kidneys from individual mice) and transgenic TRPV4 knockout mice (TRPV4 −/−) with a C57BI 6J background (eight bladders, five kidneys from individual mice). TRPV4 −/− mice were generously provided by Suzuki et al. and housed and bred in our central animal facility (Suzuki et al. 2003a,b).

Immunofluorescence

Immunofluorescence (IF) assays were performed to investigate the location of TRPV4 channels and the relation with AJs in the urogenital tract of humans and mice. A table of all antibodies used is included in the Data S1. Tissues were embedded in Tissue-Tek® O.C.T. (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and snap frozen in isopentane. From frozen tissue, 4-μm sections were cut in a cryostat microtome (Thermo Fisher Scientific, Waltham, MA, USA) and air-dried. Sections were fixed with 3% v/v paraformaldehyde (10 min), following rinsing (three times) with Tris-buffered saline with 0,05% v/v Tween solution (TBS-T) and blocking with 20% v/v goat-serum in TBS-T. After this, sections were incubated overnight (4 °C) with primary antibodies against TRPV4 (polyclonal, donated by Department of Otolaryngology, Stanford University School of Medicine; Nilius et al. 2003), two against E-cadherin (Takara bio inc., Otsu, Japan & Sigma Aldrich, Steinheim, Germany) and an occludin (Invitrogen, Camarillo, CA, USA) antibody for tight junction evaluation. Alexa’s 488 & 594 (Invitrogen) labelled antibodies were used as secondary antibodies, and DAPI (Merck) was used for visualizing nuclei. Omission of the first antibody was taken as a control. Sections were analysed using
Imaging Densitometer (Hercules, CA, USA). Western blots were acquired using the Bio-Rad GS-690 Imaging Densitometer (Hercules, CA, USA). Images of the IF assays. Differences were that Poly-HRP-α-RA/Rb/Mo IgG (Immunologic, Duiven, The Netherlands) was used as secondary antibody and bound antibodies were visualized by DAB (Immunologic). No DAPI was used. Sections were analysed using normal binocular microscope (Leica DMR®).

**Immunohistochemistry**

Tight junction expression in the wild-type and TRPV4+/− mouse were also investigated with immunohistochemistry. For this, the occludin antibody (Invitrogen) was used. Procedures were comparable to the IF assays. Differences were that Poly-HRP-α-RA/Rb/Mo IgG (Immunologic, Duiven, The Netherlands) was used as secondary antibody and bound antibodies were visualized by DAB (Immunologic). No DAPI was used. Sections were analysed using normal binocular microscope (Leica DMR®).

**Western blotting**

Kidney samples from humans, wild-type and TRPV4−/− mice were used to investigate the expression of TRPV4 in the kidney tissue and to assess the specificity of the TRPV4 antibody. Tissues were rinsed twice with saline and mechanically homogenized with a polytron (IKA analyse, Staufen, Germany). After this, samples were transferred to an extraction buffer containing 300 mM sucrose, 1 mM EGTA, 5 mM EDTA, 25 mM imidazole and protease inhibitors. The samples were centrifuged at 100 g (4 °C) for 2 min. Protein concentrations of the supernatants of the specimens were determined with the Bio-Rad protein assay. After this, 10 μg of each samples was loaded on a 10% w/v SDS gel and transferred to a PVDF membrane (GE healthcare). Precipitates were analysed with the previously described antibodies for TRPV4, E-cadherin, α- and β-catenin to determine whether these proteins could be detected in the immunocomplexes. Images of the immunoprecipitates were acquired using the Bio-Rad GS-690 Imaging Densitometer.

**Real-time RT-PCR**

Real-time RT-PCR was performed to investigate the presence and location of TRPV4 channels in the human bladder. Cold cup biopsies were snap frozen in liquid nitrogen and processed by step sectioning. Bladder biopsies were immunohistochemically evaluated for the presence of urothelial layer with a standard haematoxylin–eosin staining. We included three histological normal looking biopsies of which two had a normal urothelial layer present and one without any urothelium present (Fig. 1a). Total RNA from 6 × 20 μm cold cup biopsy sections per sample was isolated using TRIzol reagent, according to the manufacturer’s instructions (Invitrogen). Concentration and purity of the RNA was determined on a Nanodrop-1000 spectrophotometer (Thermo Scientific Waltham, MA USA).

Total RNA (400 ng) was DNase-I-treated (Invitrogen), and cDNA was synthesized using random hexamer primers and SuperScript II-MMLV reverse transcriptase (Invitrogen). The RT reaction was diluted 3 times in PCR grade H2O. Gene expression was determined by SYBR Green qPCR, using SYBR Green PCR mix (Roche) and 2 μL cDNA as a template. RNA not subjected to reverse transcriptase was used as a negative control. Gene-specific primers, including urothelium-specific and smooth muscle-specific primers, are listed in the Table S1. qPCR was performed on a LightCycler480 instrument (Roche), using the following amplification conditions: 5 min. 95 °C, followed by cycles of 10 s. 95 °C, 20 s. 60 °C, 20 s. 72 °C. Cp values were determined using the LC480 SW1.5 software (Roche).

**Transmission electron microscopy (TEM)**

TEM was used to investigate cell junction formation in the TRPV4+/− mouse on an ultrastructural level. For this, bladders from wild-type (n = 2) and
TRPV4+/− mice (n = 2) were removed. Tissues were fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4°C, and post-fixed with 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h. After a rinsing period of 3 h with 0.1 M phosphate buffer (pH 7.4), the samples were dehydrated in an ascending series of ethanols and embedded in Epon 812. Ultrathin sections (60 nm) were picked up on formvar-coated grids, post-stained with lead citrate and uranyl acetate and examined in a JEOL 1010 electron microscope (Peabody, MA, USA).

Results
The light micrographs and electron micrographs shown should be considered as typical examples of the evaluated sectioned material.

Location of TRPV4 channels in the urogenital tract
Specificity of the antibody for TRPV4 was confirmed by comparing kidneys of wild-type (TRPV4+/+) and TRPV4+/− mice. No signal was detected in TRPV4+/− mouse tissues in both immunofluorescence assays.
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(AJ formation in the TRPV4/C0 mouse urothelium, while they were clearly distinguishable in both wild-type and TRPV4/C0 mice. However, no adherence junctions were detected in the TRPV4/C0 mouse urothelium, while they were clearly distinguishable in the wild type.

Besides these abnormalities, we also detected abnormal vesicular exocytosis in the TRPV4/C0 mouse bladder umbrella cells. Intracellular vesicles in the umbrella cell type were much larger compared to wild types and also contained large amounts of clearly visible material that was expelled into the bladder lumen after fusion of the vesicles with the outer cell membrane (Fig. 4).

Interaction between TRPV4 channels and AJs. Disturbed AJ formation in TRPV4/C0 mice

To study the interaction of TRPV4 channels and AJs in the urogenital tract, cell junction formation was investigated in the urogenital tract of the TRPV4/C0 mouse using IF and immunohistochemistry experiments and also evaluated on an ultrastructural level with TEM. The results from the IF experiments confirmed a similar co-localization between TRPV4 channels and AJs in mice bladders and kidneys (Fig. 3). AJ formation in the wild-type mouse bladders resembled the human bladder with clearly visible narrow cell junction ridges between neighbouring urothelial cells. AJ formation in the TRPV4/C0 mouse bladders differed from this with less pronounced intercellular ridges that appeared interrupted. Similar observations were carried out in the collecting ducts of TRPV4/C0 mice kidneys. Tight junction formation was investigated with occludin immunostainings. The results showed that tight junctions were still formed in both bladders and kidneys from TRPV4/C0 mice (Fig. 4).

On an ultrastructural level, evaluation of wild-type and TRPV4/C0 bladders with TEM revealed clear urothelial abnormalities in the TRPV4/C0 mice (Fig. 5). This included enlarged intercellular spaces between urothelial cells, which were hardly detectable in the wild-type mouse. When looking at cell junction formation in the urothelium in higher detail, tight junctions and desmosomes were clearly detectable in both wild-type and TRPV4/C0 mice. However, no adherence junctions were detected in the TRPV4/C0 mouse urothelium, while they were clearly distinguishable in the wild type.

Molecular connection between TRPV4 channels and AJs

With immunoprecipitation (IP) experiments on human kidney tissue, we investigated potential molecular interactions between TRPV4 and AJ proteins (Fig. 1d). The TRPV4 antibody was used for the pull-down experiment, and the immunoprecipitates were investigated for the presence of E-cadherin, α- and β-catenin proteins. The negative control α-tubulin was not present in the TRPV4 precipitate (Data S1). Results confirmed that TRPV4 has a molecular connection to α-catenin, the intracellular AJ protein that is connected to the actin cytoskeleton. Lower concentrations of β-catenin and E-cadherin were also detected in the precipitate in, respectively, decreasing concentrations, confirming that TRPV4 channels are connected to AJs.

TRPV4 mRNA expression in bladder urothelial cells

We analysed TRPV4 mRNA expression in cold cup bladder biopsy specimens by real-time RT-PCR. The sample that had the urothelium was absent (Fig. 1a), and only the HPRT housekeeping gene and the smooth muscle-specific transcript ACTB were expressed (Fig. 1b). In the two specimens containing a healthy urothelial layer on standard HE staining, the urothelium-specific transcripts, CDH1, FGFR3(IIIb) and KRT20 were detected. In the latter samples, ACTB was also expressed, as these specimens all contained a smooth muscle cell component. TRPV4 expression was found in the CDH1/FGFR3/KRT20 positive samples and hardly in the sample without urothelium (approximately a 100-fold higher expression in urothelium-enriched samples), indicating that TRPV4 expression is predominantly restricted to urothelial cells (Fig. 1b).

Expression of TRPV4 in the human kidney was confirmed with Western blotting experiments and immunofluorescence assays (Fig. 1c & 2). The immunofluorescent signal for TRPV4 was stronger in the kidneys compared to the human bladder and ureter. TRPV4 was clearly visible on the epithelial cell membranes of the collecting tubules, with also a co-localization with AJs (Fig. 2). AJs were clearly visible as dot-like structures between the cell membranes of two adjacent umbrella cells. Simultaneous incubation with the TRPV4 antibody revealed that these ‘dots’ or ‘puncta’ showed distinct co-localization with TRPV4 (Fig. 2). TRPV4 expression in lower urothelial layers was observed in the human and ureter, but the signal was less pronounced (Fig. 2). In the mice bladders, the TRPV4 signal was seen more equally distributed throughout the urothelium (Fig. 3). With IF, no signal of TRPV4 expression was seen in the deeper layers of the bladders of human and mice specimens (lamina propria and detrusor layers) (Data in Figures S1–S6).

Results confirmed that TPRV4 has a molecular connection to α-catenin, the intracellular AJ protein that is connected to the actin cytoskeleton. Lower concentrations of β-catenin and E-cadherin were also detected in the precipitate in, respectively, decreasing concentrations, confirming that TRPV4 channels are connected to AJs.

Interaction between TRPV4 channels and AJs. Disturbed AJ formation in TRPV4–/– mice

To study the interaction of TRPV4 channels and AJs in the urogenital tract, cell junction formation was investigated in the urogenital tract of the TRPV4–/– mouse using IF and immunohistochemistry experiments and also evaluated on an ultrastructural level with TEM. The results from the IF experiments confirmed a similar co-localization between TRPV4 channels and AJs in mice bladders and kidneys (Fig. 3). AJ formation in the wild-type mouse bladders resembled the human bladder with clearly visible narrow cell junction ridges between neighbouring urothelial cells. AJ formation in the TRPV4–/– mouse bladders differed from this with less pronounced intercellular ridges that appeared interrupted. Similar observations were carried out in the collecting ducts of TRPV4–/– mice kidneys. Tight junction formation was investigated with occludin immunostainings. The results showed that tight junctions were still formed in both bladders and kidneys from TRPV4–/– mice (Fig. 4).

On an ultrastructural level, evaluation of wild-type and TRPV4–/– bladders with TEM revealed clear urothelial abnormalities in the TRPV4–/– mice (Fig. 5). This included enlarged intercellular spaces between urothelial cells, which were hardly detectable in the wild-type mouse. When looking at cell junction formation in the urothelium in higher detail, tight junctions and desmosomes were clearly detectable in both wild-type and TRPV4–/– mice. However, no adherence junctions were detected in the TRPV4–/– mouse urothelium, while they were clearly distinguishable in the wild type.

Besides these abnormalities, we also detected abnormal vesicular exocytosis in the TRPV4–/– mouse bladder umbrella cells. Intracellular vesicles in the umbrella cell type were much larger compared to wild types and also contained large amounts of clearly visible material that was expelled into the bladder lumen after fusion of the vesicles with the outer cell membrane (Fig. 4).
Figure 2 Immunofluorescence experiments on presence and co-localization of TRPV4 channels and AJs in the urogenital tract epithelium. TRPV4 channels and the AJ-specific molecule E-cadherin were investigated in human bladder (n = 6) (a–c), ureter (n = 1) (d–f) and kidney (n = 2) (g–l). AJs were clearly visible as dot-like structures between the cell membranes of two adjacent umbrella cells. Simultaneous incubation with the TRPV4 antibody revealed that these ‘dots’ or ‘puncta’ (white arrows) had a distinct immunoreactivity for TRPV4. TRPV4 was clearly visible on the epithelial cell membranes of the collecting tubules, with also a distinct co-localization with AJs. Besides these areas, there were also sections of the kidney tubuli that either solitary expressed AJs (orange arrows) or TRPV4 (red arrows).
A qualitative evaluation was performed to localize TRPV4 channels in the urogenital tract and investigate the interaction between TRPV4 and epithelial cell junctions in humans and mice. Our results demonstrate that TRPV4 is predominantly located on epithelial cells of the bladder, ureters and in the collecting ducts of the kidney. There is a distinct co-localization between TRPV4 channels and AJs in the investigated epithelial cell layers. Subsequent immunoprecipitation experiments demonstrated that there is a molecular interaction between TRPV4 and α-catenin, which is the intracellular complex of the AJ that connects to the actin cytoskeleton. We used transgenic TRPV4−/− mouse to study the interaction between TRPV4 channels and AJs. Immunofluorescence assays and TEM evaluation on bladder tissue showed a disruption in AJ formation in the TRPV4−/− phenotype, resulting in a decrease in epithelial integrity.

The TRPV4−/− mice that was used is a well-investigated strain (Suzuki et al. 2003a, b, Janssen et al. 2011). There are other transgenic TRPV4−/− mouse strains we did not have access to for confirmation of our experiments (Gevaert et al. 2007). For the TEM experiments, only bladder tissue was chosen, because not all epithelial linings in kidney tissues express TRPV4 channels, making it far less suitable for comparative evaluation.

The function of TRPV4 in the kidney is still unclear. The TRPV4−/− mouse phenotype has distinct abnormalities in osmolar balance (Everaerts et al. 2010a, b). It has therefore been suggested that the lack of TRPV4 channels in the kidney may be responsible for this, as TRPV4 channels are located in the aldosterone-sensitive cells of the distal nephron (Berrout et al. 2012, Galizia et al. 2012). There is however convincing evidence that shows that the osmolar disturbance in the TRPV4−/− is actually caused by a lack of TRPV4 channels in the brain ventricular (Mamenko et al. 2011). There is evidence that water absorption in the collecting ducts occurs via transmembrane transport via activated aquaporin-2 channels in the principal cells and not through

Figure 3 Immunofluorescence experiments on TRPV4 and adherence junction formation in wild-type and transgenic TRPV4−/− mice in the urogenital tract epithelium. TRPV4 channels were located in bladder urothelium in wild type (WT; n = 3) (a–d) and co localized with the AJ-specific protein E-cadherin (c). In the transgenic TRPV4−/− mouse bladder (n = 3) (e–h), no signal for TRPV4 was detected. AJs were continuously formed at the epithelial cell borders in wild-type mouse bladders (b and enlarged in d) and kidneys (i) (white arrows). In contrast, in the TRPV4−/− bladder (e and enlarged in g) and kidney (j), AJs in the epithelial cell borders had notable interrupted areas between adjacent cells (white arrows).
extracellular transport (Galizia et al. 2012). There are some indications that kidney TRPV4 channels regulate water absorption indirectly by regulating K⁺ and Na⁺ uptake in the collecting ducts (Mamenko et al. 2011). A regulatory function in cell junction formation by TRPV4 channels would be interesting for kidney function.

The results from our RT-PCR and immunofluorescence experiments demonstrate that TRPV4 channels are predominantly located in epithelial layers. No study was performed on TRPV4 channels in other urogenital organs like the prostate or urethra. The latter region is of interest, as the urethra is also exposed to flow and stretch and humans are able to sense urethral flow. Different groups have investigated the location of TRPV4 channels in the urogenital tract with comparable results (Birder et al. 2007, Gevaert et al. 2007, Everaert et al. 2008, 2010a, b, Berrout et al. 2012, Galizia et al. 2012). Besides in the epithelium, Gevaert et al. and other groups also reported on TRPV4 expression in the walls of blood vessels (Wissenbach et al. 2000, Gevaert et al. 2007). Thorneloe et al. (2008) described TRPV4 expression in spindle like cells in the detrusor layer. The results from our RT-PCR experiments and immunofluorescence stainings (details shown in Data S1) did not match these results.

Cystometry experiments by other groups demonstrated that mechanical activation of TRPV4 channels is important for bladder afferent signalling (Birder et al. 2007, Gevaert et al. 2007, Mochizuki et al. 2009). Stretch activates TRPV4 channels independently from heat or agonists like 4α-phorbol 12,13-didecanoate (Vriens et al. 2004). The interaction between TRPV4 channels and the tensile network that consist of AJs and the actin cytoskeleton appears to be important for TRPV4 functioning. This is supported by investigations by Becker et al., who demonstrated that TRPV4 is connected to actin-filaments (Becker et al. 2009, Goswami et al. 2010). Additionally, Suzuki et al. (2003a, b) reported that stretch activation of TRPV4 was abolished by the application of actin-freezing agents.

The results from the ultrastructural investigation of the TRPV4+/− mouse bladder imply that a lack of TRPV4 channels is responsible for the formation of enlarged vesicles with the congestion of cell material and membrane proteins in the umbrella cells. Vesicular endo- and exocytosis is an adaptation in bladder umbrella cells that minimizes the exposure of the

**Figure 4** Tight junction formation in the mouse urogenital tract epithelium. Immunohistochemical (a, b) and immunofluorescence (IF) (c-f) investigations on wild-type (WT)(a, c, e) and transgenic TRPV4+/− mouse (b, d, f) bladders. Tight junctions were visualized with an occludin immunostaining and are highlighted in the images with black and white arrows. Tight junctions in the WT bladders (n = 3) are formed in the luminal umbrella cell type urothelial cells. In the TRPV4+/− mouse bladder (n = 3), adjacent urothelial cells have enlarged intercellular spaces (b), compared to the WT mouse (a). Tight junctions also appear to be less frequently visible in the TRPV4+/− mouse bladder compared to the WT on immunohistochemistry experiments. However, this difference could not be confirmed with IF (d). In the kidney, there is a similar expression of tight junctions between WT (n = 2) and TRPV4+/− mice (n = 2) in the luminal epithelial cells.
Figure 5 Transmission Electron Microscopy (TEM) evaluation of cell junction formation of the wild-type (WT) and transgenic TRPV4−/− mouse bladder. WT (n = 2) (a–c, g–i) and transgenic TRPV4−/− mouse (n = 2) (d–f, j–l) bladders were investigated on ultrastructural level with TEM. In the WT urothelium, adjacent cells were tightly packed with hardly any distinguishable cell borders (a and i; turquoise arrow). Tight junctions (red arrows) were visible between the most luminal cell borders of neighbouring umbrella cells (b). AJs (orange arrows) and desmosomes (blue arrows) were detectable between adjacent urothelial cell membranes (c). In the TRPV4−/− bladder, adjacent urothelial cells have enlarged intercellular spaces (turquoise arrows) (d and l), and absent AJ formation (f). Tight junctions (red arrow) (e) and desmosomes (blue arrow) (f) were still present. Vesicular exocytosis was detected in both WT (g and h) and TRPV4−/− mice (j and k) umbrella cells and seen as the fusion of vesicles with the luminal membrane (pink arrows). The intracellular vesicles in the TRPV4−/− umbrella cells were enlarged (purple arrows) and contained more proteins compared to the WT (j and k).
outer cell membranes to urine (Hudolkin et al. 2012). Vesicles with membrane proteins are stored intracellular and merge with the outer membrane during stretch of the bladder wall and a reverse process takes place after bladder emptying. Transport of these vesicles is dependent on the actin cytoskeleton and is directly influenced by mechanical strain. This process could also be important for secretion of ATP for afferent signalling, although there is contrasting evidence that urothelial ATP excretion does not occur through vesical excretion (Ferguson et al. 1997, Lewis & Lewis 2006). The abnormal vesicle formation could be caused by the absent interaction between TRPV4 channels and the actin cytoskeleton. Additionally, Cuajungco et al. demonstrated that TRPV4 interacts with PACSIN 3 (Cuajungco et al. 2006). The latter is a cytoskeleton protein that is involved in vesicular membrane transport and endocytosis. To our knowledge, there are no reports on PACSIN 3 proteins in the bladder urothelium (Modregger et al. 2000). Our results are however indicative that TRPV4 has a role in vesicular endo- and exocytosis in the bladder urothelium, which could influence bladder sensory pathways. The content of the cell material in the vesicles needs further study.

The relation between TRPV4 and barrier forming cell junctions has been investigated in other organs like skin and long tissue. In lung, applying a TRPV4 antagonist in high doses induces severe barrier failure in pulmonal microvessels (Willette et al. 2008). In skin, TRPV4 reduces tight junction formation and causes an impairment in epithelial barrier (Sokabe et al. 2010). Reduced tight junction formation was not observed in our study, and we have not performed functional assays to measure epithelial resistance. New studies also show that TRPV4 influences both cellular migration and fixation in tissues (Mrikonjić et al. 2015). How TRPV4 exactly mediates cell junction formation in epithelia and the degree it contributes to epithelial resistance still requires further study. Even so, there appears to be a mutual dependence, which suggests that there is a bridge between the two classical functions in epithelia being 1) barrier formation and 2) sensory function.

In summary, we can say that although the interaction between TRPV4 channels and cell junction formation in the epithelia of the urogenital tract generates plausible hypothesis. Further study is mandated to clarify the physiological implications for this in different organ systems.

**Conclusion**

This study has demonstrated an important role for TRPV4 channels in cell junction formation in the urogenital tract. A lack of TRPV4 impairs the ability to construct a sufficient coherent epithelial barrier, implying that in the epithelium, there is an association between barrier function and afferent signalling.

**Conflict of interest**

There are no conflict of interests to be reported for me and my co-authors concerning this manuscript and its publication.

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Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Antibody specifications.
Figure S1. Immunoprecipitation experiment interaction of TRPV4 and Aβ related proteins on kidney tissues. Results from negative control αf-1 tubulin.
Figure S2. Additional immunofluorescence imaging at higher magnification of TRPV4 expression in the luminal umbrella cell layer and in the detrusor layer in the human bladder.
Figure S3. Additional IF imaging at higher magnification of TRPV4 and E-cadherin (Adherence junctions) expression in the human kidney.
Figure S4. Additional IF images of adherence junction formation in the wild type and TRPV4–/– mice kidneys.
Figure S5. Additional immunochemistry images of urothelial barrier related structures uroplakin and tight junctions in the TRPV4–/– and wild type mice bladders.
Figure S6. Additional TEM and immunohistochemical (IH) staining to demonstrate increased intercellular spaces between adjacent urothelial cells in the bladders of TRPV4–/– mice.