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## REGULATION OF FUNCTIONAL EXPRESSION OF MECHANOSENSITIVE TRPV4 CHANNEL IN THE DISTAL NEPHRON BY DIETARY POTASSIUM AND SODIUM INTAKE

Nabila Boukelmoune

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REGULATION OF FUNCTIONAL EXPRESSION OF MECHANOSENSITIVE  
TRPV4 CHANNEL IN THE DISTAL NEPHRON BY DIETARY POTASSIUM AND  
SODIUM INTAKE

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REGULATION OF FUNCTIONAL EXPRESSION OF MECHANOSENSITIVE  
TRPV4 CHANNEL IN THE DISTAL NEPHRON BY DIETARY POTASSIUM AND  
SODIUM INTAKE

A  
THESIS

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M.D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment  
of the Requirements  
for the Degree of

MASTER OF SCIENCE

By  
Nabila Boukelmoune, BS., MS  
Houston, Texas  
December, 2014

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# **REGULATION OF FUNCTIONAL EXPRESSION OF MECHANOSENSITIVE TRPV4 CHANNEL IN THE DISTAL NEPHRON BY DIETARY POTASSIUM AND SODIUM INTAKE**

Nabila Boukelmoune, MS.

Advisory professor: Oleh. M. Pochynyuk, Ph.D.

The  $\text{Ca}^{2+}$ -permeable TRPV4 channel is predominantly expressed in the distal nephron (DN) and its activity is essential for  $[\text{Ca}^{2+}]_i$  elevations in response to increased tubular flow. Here, I probed the physiological mechanisms controlling TRPV4 function and expression in the DN. I found that renal TRPV4 expression and mRNA levels were significantly increased by high  $\text{K}^+$  diet (5%) and decreased by dietary  $\text{K}^+$  restriction (0.003%). In contrast, variations in  $\text{Na}^+$  regimen had no apparent effect on TRPV4 expression and mRNA levels. Regulation of TRPV4 protein expression by  $\text{K}^+$  diet was independent of aldosterone action, since saturation of systemic mineralocorticoid signaling with DOCA, a precursor of aldosterone, had little effect on TRPV4 protein abundance in the kidney. Confocal immunofluorescence in split-opened DNs showed that high  $\text{K}^+$  and  $\text{Na}^+$  intake resulted in redistribution of the channel towards the apical plasma membrane of DN cells, while  $\text{K}^+$  and  $\text{Na}^+$  restrictions caused cytosolic distribution of TRPV4. Augmented TRPV4 expression and localization to the apical plasma membrane during high  $\text{K}^+$  and  $\text{Na}^+$  intake were associated with significantly augmented flow-induced  $[\text{Ca}^{2+}]_i$  responses in DN cells. In summary, my findings demonstrate that high  $\text{K}^+$  and  $\text{Na}^+$  intake regulate TRPV4 status to properly respond to elevated tubular flow during

these physiological stimuli. I also propose that impaired regulation of TRPV4 in the DN during variations in dietary intake may result in systemic defects in  $K^+$  and  $Na^+$  balance contributing to cardiovascular abnormalities.

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## ABBREVIATIONS

AA	Arachidonic Acid
BK	Big Potassium (large conductance $K^+$ channel)
$Ca^{2+}$	Calcium
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaM	Calmodulin (Calcium-Modulated protein)
CNT	Connecting tubule
CCD	Cortical Collecting Duct
CD	Collecting Duct
$Cl^-$	Chloride
DN	Distal Nephron
DOCA	Deoxycorticosterone acetate
$EC_{50}$	Half maximum Effective Concentration
EETs	Epoxyeicosatrienoic acids
ENaC	Epithelial $Na^+$ Channel
IC	Intercalated Cell
$Mg^{2+}$	Magnesium
mRNA	Messenger RNA
$K^+$	Potassium
PC	Principal Cell
$4\alpha$ -PDD	$4\alpha$ -phorbol 12,13-didecanoate
PIP2	Phosphatidylinositol 4,5-bisphosphates
PKA	Protein Kinase A
PKC	Protein Kinase C

PMA	Phorbol Myristate Acetate
ROMK	Renal Outer Medullary K <sup>+</sup> channel
SK3	Small-conductance calcium-activated K <sup>+</sup> channel
Na <sup>+</sup>	Sodium
TRP	Transient Receptor Potential
TRPA1	Transient Receptor Potential ANKTM1
TRPC	Transient Receptor Potential Canonical
TRPL	TRP-like channel
TRPM	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPN	Transient Receptor Potential NOMPC
TRPP	Transient Receptor Potential Polycystin
TRPV	Transient Receptor Potential Vanilloid

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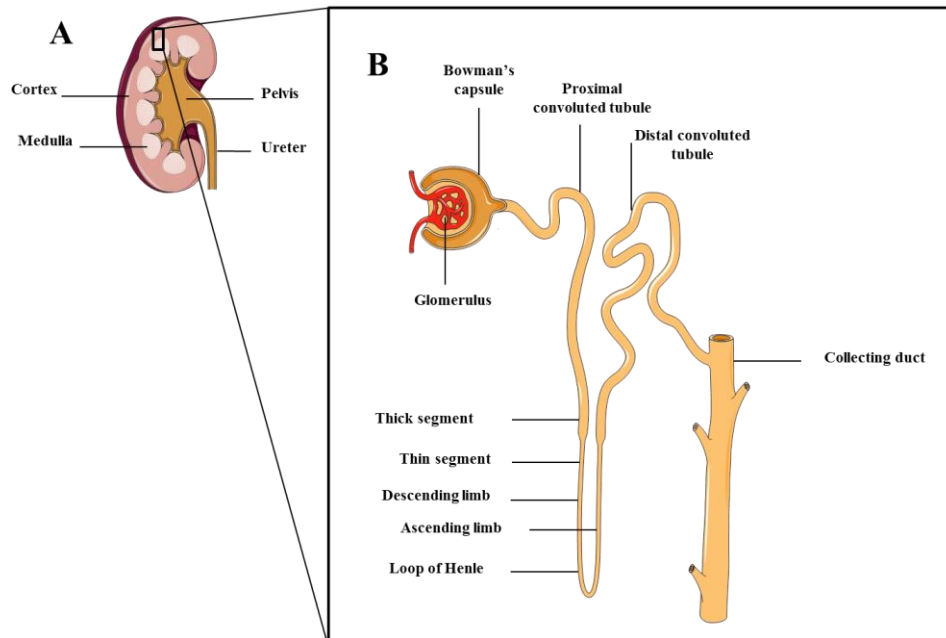
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## **INTRODUCTION**

## **1. Overview of kidneys function**

The kidneys are paired retroperitoneal bean-shaped organs situated in the posterior side of the abdomen, on each side of the vertebral column. A bisected kidney consists of two distinct regions: an outer region, the cortex, and an inner region, the medulla as illustrated in figure 1A. The functional unit of the kidney is the nephron. In humans, there are 300,000 to 1 million nephrons per kidney and in mouse there are ~ 11,000 (1-4). Structurally, each nephron consists of a glomerulus (site for ultrafiltration), located in the cortex and one double hairpin-shaped tubule that extends into the medulla and connects to the collecting duct system, which merges to join the ureter (Figure 1B). Physiologically, the nephrons play an important role in the filtration of blood by removing the excess of electrolytes and water, as well as eliminating waste products such as urea and creatinine by producing and excreting urine. This is crucial for maintaining whole body fluid homeostasis. Small molecules such as ions, glucose and amino acids, are also freely filtered and reabsorbed.

The main function of the glomerulus is to produce an ultrafiltrate of plasma which is further processed by the tubule via reabsorptive and secretory mechanisms. The renal tubule is a hollow structure consisting of a monolayer of epithelial cells. The tubule is formed of distinct segments, the proximal tubule, the thick and thin descending limbs, the thin and thick ascending limbs and the distal convoluted tubule (Figure 1). The fine tuning of water and electrolytes occurs in the late parts of the renal tubule and specifically in the collecting duct (CD). All that is not reabsorbed is excreted in the urine. Ultimately, the urine is emptied in the urinary bladder through the renal pelvis and ureters.



**Figure 1.** Kidney and renal tubule structure. (A) Kidney structure showing the cortex, medulla, pelvis and ureter. (B) Structure of the different regions of the renal tubule. Adapted from Servier medical art <http://www.servier.com/Powerpoint-image-bank>

## 2. Electrolytes transport in the distal nephron

The distal nephron (DN), which includes the connecting tubule (CNT) and the collecting duct (CD) system, serves to fine-tune water and electrolytes transport to match dietary intake with urinary excretion (5,6). The DN is composed of two distinct cell populations, morphologically and functionally different. Principal cells (PCs) are the most abundant cell type, representing 70-75% of the total cell population (5). PCs mediate  $\text{Na}^+$  absorption (mainly via the epithelial  $\text{Na}^+$  channel ENaC), water reabsorption (via aquaporin type 2 channels) and  $\text{K}^+$  secretion (through the Renal Outer Medullary  $\text{K}^+$  channel (ROMK)-dependent pathways. The remaining cells in the distal nephron are intercalated cells (ICs). ICs are responsible for acid/base transport (7). ICs also contribute to the large conductance  $\text{K}^+$  channel (BK)-dependent



$K^+$  secretion and probably the small-conductance calcium-activated potassium channel SK3-dependent potassium secretion, in response to elevated  $K^+$  intake (8,9).

### **3. Hormonal regulation in the distal nephron**

Transport in the DN segment is highly regulated. Indeed, this segment is very sensitive to hormones including aldosterone, angiotensin II, and vasopressin. The central hormonal regulator of  $Na^+$  and  $K^+$  transport is aldosterone. Increased plasma  $[K^+]$  after a dietary  $K^+$  load triggers adrenal aldosterone secretion which in turn stimulates  $K^+$  secretion. Furthermore, activation of the Renin-Angiotensin-Aldosterone system (RAAS) in response to dietary  $Na^+$  restriction increases ENaC expression via the aldosterone–mineralocorticoid receptor (MR) pathway.

### **4. Mechanical stress in the distal nephron**

Renal tubular cells face a constant exposure to a variety of mechanical forces such as circumferential stretch, osmotic pressure gradients, hydrostatic pressure and fluid shear stress (10-19). The ultrafiltrate delivery through tubules provokes a frictional force which, in turn regulates vectorial transport of  $Cl^-$ ,  $Mg^{2+}$ , glucose,  $K^+$  and  $Na^+$  (10,19-22) and a variety of other processes such as cytoskeleton organization and activity of transcription factors (12). The greatest values of a mechanical stress occur in the distal nephron and the collecting duct, where it is viewed as an important stimulus for the regulation of water and electrolytes transport (23). Specifically, elevations in tubular fluid flow increase  $Na^+$  reabsorption and  $K^+$  secretion in the distal nephron through several mechanisms. One of which involves the activation of ion channels, such as the apically localized epithelial  $Na^+$  channel (ENaC). ENaC determines electrogenic  $Na^+$  reabsorption within the DN, and was shown to be stimulated in response to elevations in tubular flow rates within physiological range in isolated rabbit cortical collecting

ducts (CCDs), possibly by alterations of the cytoskeleton, leading to an increase in  $\text{Na}^+$  reabsorption (6,24,25). Another mechanism is the crucial regulation of  $\text{K}^+$  balance, since plasma  $\text{K}^+$  concentration influences cellular electrical excitability, and is important for proper functioning of neurons, skeletal and cardiac muscles.  $\text{K}^+$  homeostasis and  $\text{K}^+$  secretion in the distal nephron depend on ENaC- mediated  $\text{Na}^+$  absorption. The high flow rate directly activates ENaC, thereby increasing the driving force for  $\text{K}^+$  secretion (21,26-28).  $\text{K}^+$  transport is complex, under normal physiological conditions; renal  $\text{K}^+$  secretion is mediated through the highly selective apical  $\text{K}^+$  channel ROMK (Kir1.1) (21,29,30). In contrast, under high  $\text{K}^+$  dietary intake other  $\text{K}^+$  channels contribute to its secretion. Indeed, flow-dependent  $\text{K}^+$  secretion occurs through activation of the luminal  $\text{Ca}^{2+}$  activated  $\text{K}^+$  channel (BK) in response to flow elevations in the distal tubule and CD (14,23,31-34). This process is  $\text{Ca}^{2+}$ -dependent, it parallels with flow induced  $\text{Ca}^{2+}$  influx and is mediated through transducers such as TRP channels. Recent experimental evidence strongly suggests that the activity of  $\text{Ca}^{2+}$ -permeable TRPV4 channel, highly expressed on the apical plasma membrane of the distal nephron cells, mediates elevations in  $[\text{Ca}^{2+}]_i$  caused by fluid flow. In cultured and native distal nephron cells, inhibition and genetic interference with TRPV4 function abrogate elevations in  $[\text{Ca}^{2+}]_i$  in response to shear stress caused by fluid flow (23,35,36).

In addition to BK,  $\text{Ca}^{2+}$ -dependent small conductance SK3 (KCa2.3) channel, expressed predominantly at the apical border of the CNT and CD, is activated by mechanical stimuli. SK3 has a higher affinity for  $\text{Ca}^{2+}$  over that observed for BK channel and can potentially play a role in  $\text{K}^+$  secretion, as observed in TRPV4 positive cells of the CNT and CCD (8,37).

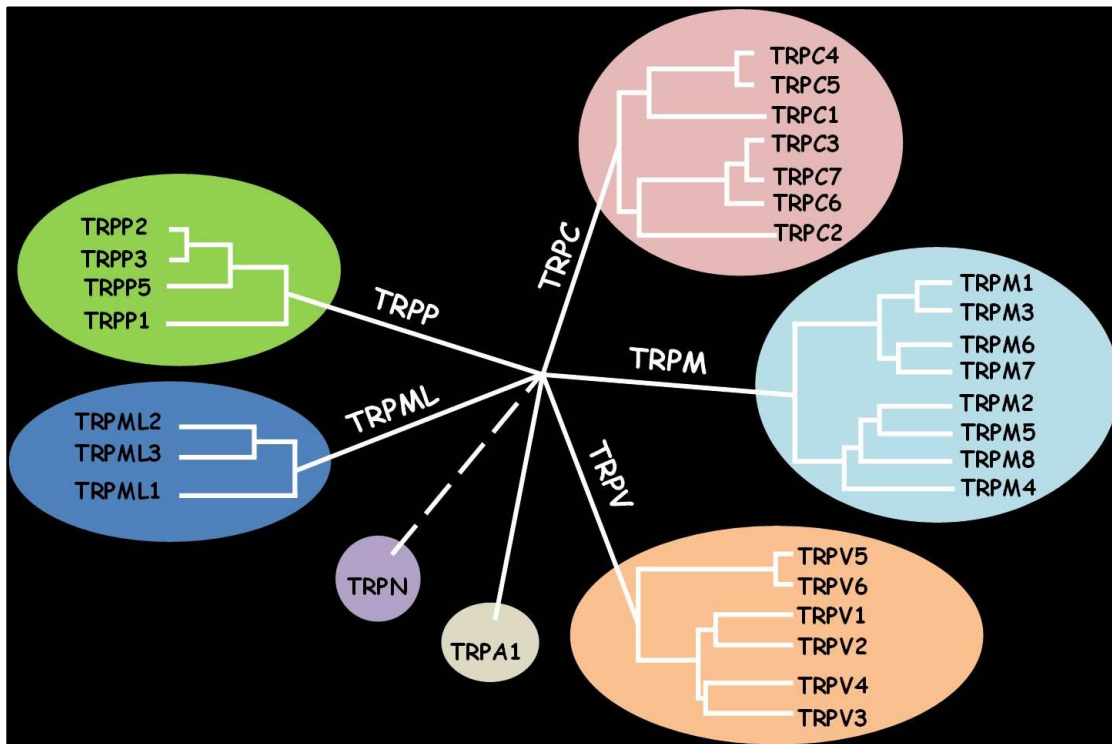
## 5. The Transient Receptor Potential family of ion channels

### i. Description:

Transient receptor potential (TRP) genes were first described in the fruit fly *Drosophila melanogaster* (38). Studies in the fly visual system identified a mutant presenting a transient response to steady light as compared to a sustained response in the wild-type, as recorded by electro-retinograms (39). The mutant form was named transient receptor potential. Later, the *trp* gene was identified by Montell and Rubin in 1989 (38). Because of its structural homology with other cation channels and permeation properties, the product of the *trp* gene was proposed to be, a 6-transmembrane segment protein functioning as a  $\text{Ca}^{2+}$  permeable cation channel (40). The length of Human TRP genes is about 911 kb with 11 to 39 exons (41). Members of the TRP channel family form seven subfamilies, and the number of channels within each subfamily varies across species. Within each subfamily, the transmembrane domain shares the highest homology. Based on sequence homology, the 7 TRP channels subfamilies are classified as follows and represented in figure 2:

1. TRPCs for canonical (7 members), the closest homolog to *Drosophila* Trp channels (42).
2. TRPVs for Vanilloid (6 members), named in reference to the first identified channel, TRPV1. This channel is activated by Capsaicin, member of vanilloids. In this study, we focus on the fourth member of TRPV sub-family (TRPV4) (42).
3. TRPM subfamily (8 members) for Melastatin (TRPM1), first cloned from a murine melanoma cell line (42).
4. TRPMLs for MucoLipins (3 members) (42).
5. TRPPs for Polycystins (5 members), issued originally from 2 genes, Polycystic Kidney Diseases 1 and 2 (PKD1 for TRPP1, and PKD2 for TRPP2) (42).

6. TRPN (1 member), named after the NO-mechano-potential C (NOMP-C) channel of *Caenorhabditis elegans*. The channel is distinguishable by a succession of ankyrin repeats in its amino-terminal region (42,43). And to date, the only TRPN family member identified is from Zebrafish.
7. TRPA1 (or ANKTM1) a distant member of TRP channels family (44).



**Figure 2.** TRP channels family tree in mammals.

## ii. General structure of TRP channels

Based on sequence analysis, in analogy with voltage gated potassium channels, TRP channels superfamily are organized in tetramers (45). TRP channels exist as homotetramers and

can also form heterotetramers with other members of TRP family. An example of heteromeric interactions is the direct association between TRPP2 and TRPV4 channels to form mechanosensitive heteromeric complexes (46-48).

Despite different subfamily groups, monomers have the same architecture, composed of 6 hydrophobic transmembrane domains (S1-S6) separated by hydrophilic segments (Figure 3). Between segments 5 and 6 is a P loop. The association of P-loops forms the channel pore. The S5-S6 loop also forms the selectivity filter of the channel pore (49). The amino and carboxy termini are cytoplasmic and include specific domains which differ between subfamilies:

- a. Ankyrin repeats with a structural homology and regularity at the N-terminus of TRPV (6 repeats) and TRPC subfamilies (4-5 repeats). Another category of channels have numerous repeats: TRPA1, TRPN subfamily with 17-29 repeats. These repeats allow interactions with the cytoskeleton and protein-protein interactions (50).
- b. A conserved TRP box on the 6<sup>th</sup> transmembrane domain. This sequence is believed to be a site of regulation of TRP channels by lipids such as phosphatidylinositol 4,5-bisphosphates (PIP2) (51-53).
- c. Enzymatic domains (chanzymes) (54). For example, TRPM2 has an ADP-ribose pyrophosphatase “NUDIX” domain (55), TRPM6 and TRPM7 have an alpha kinase domain with a phosphotransferase activity (56).

### **iii. Transient Receptor Potential Vanilloid family**

The TRPV family comprises 6 members which form 3 subfamilies: TRPV1/TRPV2/TRPV4, and TRPV5/TRPV6 ( $\text{Ca}^{2+}$  selective channels), and TRPV3 (Figure 2) (57). TRPV1- 4 are thermosensitive, polymodal, non-selective cation channels, conducting  $\text{Ca}^{2+}$

and  $\text{Na}^+$ , with a permeability ratio  $P_{\text{Ca}}/P_{\text{Na}}$  between 1 and 10 (49,58,59). TRPV4, also known as OTRPC4, VRL-2, VR-OAC, and TRP12, was first cloned based on homology with *C. elegans* OSM-9 (60). It is a  $\text{Ca}^{2+}$ - permeable channel expressed in a wide variety of tissues and organs such as the kidneys, lungs, heart, brain, dorsal root and trigeminal ganglia, and endothelial cells. TRPV4 was first identified as a channel activated by hypotonicity-induced cell swelling (59-64). TRPV4 has been demonstrated to be sensitive to a wide variety of physical and chemical stimuli. Thus, it is considered as a polymodal channel. The channel is activated by shear stress, hypoosmotic cell swelling, non-noxious temperatures, acidity, phorbol esters (PKC activating and non-activating phorbol esters) and downstream metabolites of arachidonic acid (epoxyeicosatrienoic acids) (65-67). My research project aims at understanding the regulation and function of TRPV4 in the kidney.

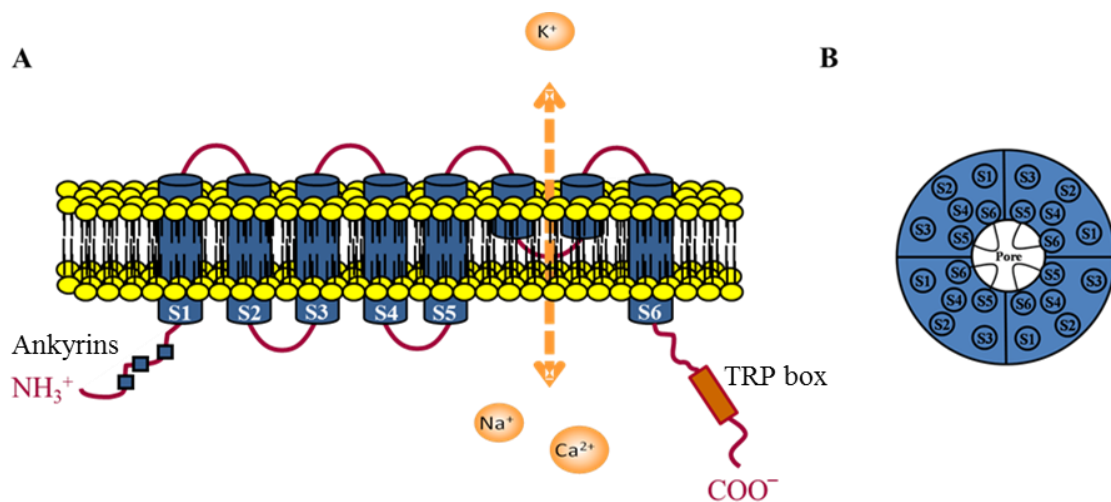
## **6. TRPV4**

### **i. Structure**

Mammalian TRPV4 ortholog contains approximately 871 amino acids. The channel permeates calcium and sodium ions, with a relatively high calcium permeability (permeability ratio  $P_{\text{Ca}}^{2+}/P_{\text{Na}}^+ = 6$ ) (68). The amino terminus side of TRPV4 has at least three ankyrin repeat domains (60), four protein kinase phosphorylation sites, and a cAMP- dependent Protein kinase A phosphorylation site upstream of the ankyrin repeats. In addition, the presence of a Src family dependent tyrosine phosphorylation site has been localized within the first ankyrin domain. TRPV4 has been demonstrated to be activated downstream of PKC activation in response to mechanical stress. In addition, at 37° C, PKC activation by phorbol esters has been shown to lead to the channel opening and to rise of intracellular  $[\text{Ca}^{2+}]$ . In response to hypotonic stress, a Src-mediated phosphorylation of a tyrosine residue within the first ankyrin repeat has been

reported (69). The pore loop is composed of hydrophobic pore helix and an ion selectivity segment which constitute the channel selectivity filter. The carboxy terminal tail of the channel is the docking site for at least two interacting proteins. One of these two sites has been reported to bind calmodulin (70) and a mutation in this region results in a loss of  $\text{Ca}^{2+}$  dependent calmodulin binding and a decrease in TRPV4 currents (60,71). In addition, a microfilament associated protein 7 (MAP7) interacts with the channel upstream of the calmodulin binding site (72).

Activation of PKC by PMA, a potent and specific activator, substantially enhanced both the gating and the phosphorylation of TRPV4. Bradykinin, which activates  $G_q$  and hence PKC, also potentiated activation of TRPV4 by phosphorylation of 3 potential sites close to the N-terminal domain: Ser<sup>162</sup>, Thr<sup>175</sup>, and Ser<sup>189</sup> (73). In addition to PKC activation of PKA by forskolin, an adenylyl cyclase activator enhanced TRPV4 gating. An effect reversed by the specific PKA inhibitor, H89 (73). The effect of PKA and PKC on TRPV4 was facilitated by the scaffolding protein AKAP79, which tethers both kinases to TRPV4, thus enhancing the channel function and phosphorylation (73).



**Figure 3.** TRP channels general structure. **(A)** TRP channel structure showing the 6 transmembrane segments (S1-S6), a p-loop between S5 and S6, the ankyrins repeats, and the TRP box at the N- and C-termini respectively. **(B)** Upper view of a TRP channel, with the different segments organized to form the pore region.

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## ii. Channel activation

TRPV4 has been reported to be activated by a wide variety of stimuli including:

### 1. Physical stimuli

- *TRPV4 and osmolarity changes*

TRPV4 is activated at physiological osmolarities (270-300 mOsmol/l) and modulated by changes in extracellular osmolarity. Increases in osmolarity above 300 mOsmol/l have been reported to reduce the channel activity (61,74). In contrast, a hypotonic medium increases TRPV4 activity (60,61,74). In response to hypotonicity, tyrosine phosphorylation by members of the Src family of tyrosine kinases was suggested, and mutation of Tyr 253 residue has been shown to abolish TRPV4 response (69). In addition to tyrosine phosphorylation, TRPV4-mediated responses to changes in osmolarity result from activation of endogenous signaling pathways. Cell swelling induces the activation of phospholipase A2 (PLA2), release of arachidonic acid (AA) from membrane phospholipids, and cytochrome P450 (CYP450) epoxygenase dependent metabolism of AA to epoxyeicosatrienoic acids (EETs) activating the channel.

TRPV4 channel regulates volume change, causing regulatory volume decrease (RVD) and increase in response to hypo and hypertonicity, respectively. As an example, TRPV4 elicits  $\text{Ca}^{2+}$



influx into bronchial epithelial cells that triggers regulatory volume decrease via  $\text{Ca}^{2+}$  dependent potassium channels (75). Furthermore, in salivary gland epithelium, TRPV4 interacts with Aquaporin 5 (AQP5), a channel which plays a role in the regulation of water permeability and cell volume (75). Indeed, acinar cells lacking TRPV4 or AQP5 displayed reduced  $\text{Ca}^{2+}$  entry and loss of RVD (75). Recent studies proposed an association of AQP2 and TRPV4 in the renal cortical collecting duct (76). This association is suggested to be required for calcium entry followed by the efflux of ions and osmotically active organic solutes (76).

- *TRPV4 and tubular fluid flow*

Distal nephron cells are permanently exposed to variations in protourine flow. These changes in flow can be modulated by the rate of glomerular filtration, tubuloglomerular feedback, diuretics, chronic diabetes, hypertension or variations of dietary sodium, potassium and protein intake (77-85). Changes in fluid hemodynamics produces shear stress, transmural pressure and stretch which affects epithelial cells of the tubule. Several studies demonstrated that cellular responses to this stimuli are mediated through elevations in  $[\text{Ca}^{2+}]_i$  (10,13-19). This suggests that  $\text{Ca}^{2+}$  permeable TRPV4 channel, abundantly expressed in the distal nephron, plays a role in sensing flow in this region.

- *Temperature*

TRPV4 can be activated by heat. The temperature threshold for the channel activation is between 27°-35°C. The physiological function of TRPV4 as a temperature sensor is supported by its expression in the thermosensory regions of the hypothalamus (86) and the vascular endothelium, where it plays a role in temperature-dependent  $\text{Ca}^{2+}$  homeostasis (87). TRPV4

temperature sensitivity was also observed in heterologous expression system, in human embryonic kidney HEK293 cells (36,86).

## 2. Chemical stimuli

TRPV4 is activated by synthetic compounds such as: 4 $\alpha$ -PDD and the specific channel agonist GSK1016790A. TRPV4 is also activated by phorbol ester derivatives and arachidonic acid metabolites (65-67). Moreover, the channel can be blocked using Ruthenium Red, RN1734 and the specific antagonist HC 067047.

- *Activation by 4 $\alpha$ -phorbol ester derivatives*

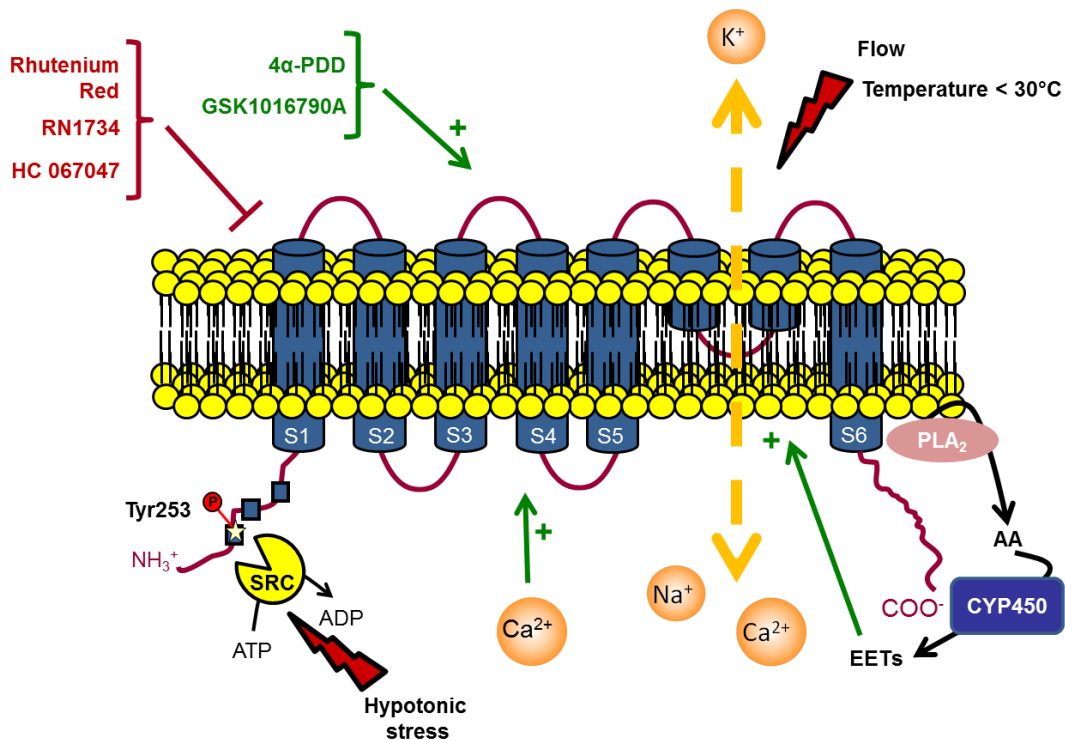
4 $\alpha$ -phorbol ester derivatives (4 $\alpha$ -PDD) are exogenous agonists of TRPV4. 4 $\alpha$ -PDD has been shown to activate the channel with an EC<sub>50</sub> of 0.2  $\mu$ M (88). They are considered to be specific activators of TRPV4, since they weakly activate TRPV1 and other channels. In the mechanism of TRPV4 activation by 4 $\alpha$ -phorbols (89), several mutations in the S3–S4 region have been reported to alter the channel sensitivity to 4  $\alpha$ -PDD and heat, suggesting the important role of this region in the channel activation.

- *Activation by arachidonic acid metabolites*

TRPV4 can be activated by products of metabolism of arachidonic acid and cannabinoid anandamide, such as epoxyeicosatrienoic acids (5, 6) and (8, 9)-EETs produced by CYP450 epoxygenase (90).

- *Regulation of TRPV4 by  $\text{Ca}^{2+}$*

Activation and inactivation of TRPV4 is regulated by  $\text{Ca}^{2+}$  flux. TRPV4 activity is reduced in the absence of extracellular  $\text{Ca}^{2+}$  (70). Activation of the channel by 4- $\alpha$  phorbol ester or hypotonicity is reduced in the absence of  $\text{Ca}^{2+}$  (70). TRPV4 is activated by small elevations in  $[\text{Ca}^{2+}]_i$  via mechanisms involving direct binding of calmodulin (CaM) to the channel. Mutations of the CaM-binding site, preventing  $\text{Ca}^{2+}$ -CaM binding has been shown to decrease the rate and extent of channel activation. In contrast to modest  $[\text{Ca}^{2+}]_i$  elevations, high increase in  $[\text{Ca}^{2+}]_i$  lead to TRPV4 inhibition and desensitization via a negative feedback mechanism protecting cells from the cytotoxic effect of a high intracellular  $[\text{Ca}^{2+}]_i$  (70).



**Figure 4.** TRPV4 regulatory pathways. AA:Arachidonic acid, EETs: poxyeicosatrienoic

acids, PLA2:phospholipase A2, Src: src family of tyrosine kinases, CYP450: cytochrome P450 epoxidase.

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### **iii. TRPV4 channelopathies:**

Channelopathies are disorders resulting from the dysfunction of ion channels. Mutations in TRPV4 channel genes cause alterations of TRPV4 structure and function which has been linked to several pathologies such as skeletal diseases (skeletal dysplasias) and neuropathies (distal hereditary spinal muscle atrophies, congenital distal hereditary motor neuropathies) (91).

## **7. Significance of proposed research**

The distal nephron is under continuous exposure to mechanical stimuli, such as tubular flow. This physical stress is a critical regulator of distal nephron transport rate. Variations in dietary  $K^+$  and  $Na^+$  intake produce changes in tubular flow and osmotic gradients, creating a mechanical stress perceived by epithelial cells in the renal tubule. These cells respond to mechanical stress by increasing intracellular calcium levels. These responses are critical regulators of a variety of processes ranging from transport of water and solutes to cellular growth and differentiation. Inability to perceive mechanical stimuli has been linked to numerous pathologies associated with systemic imbalance of electrolytes and to development of polycystic kidney disease (PKD). TRPV4 has been identified as a mechanosensitive channel (23). We have recently identified a pivotal role of TRPV4 in cellular calcium response to elevated tubular flow in distal nephron cells (92). Indeed, this flow mediated  $Ca^{2+}$ -response was abolished in TRPV4 knockout mice (35).

While TRPV4 activity is essential for  $[Ca^{2+}]_i$  elevations in response to increased tubular flow, the underlying physiological mechanisms governing the channel function remain unclear and require further investigation.

In this study, I aim to investigate the impact of sodium and potassium diets on TRPV4 function, expression and subcellular localization in mouse distal nephron cells. I hypothesize that increased flow to the distal nephron in response to variations in  $Na^+$  and  $K^+$  intake will impact TRPV4 channel activity and possibly its expression and subcellular localization.

## **MATERIALS AND METHODS**

## **MATERIALS AND METHODS**

For all experiments, 6 to 8 weeks old wild type, male C57BL/6 mice (Charles rivers laboratories, Wilmington, MA) were separated into 5 groups and were given a high sodium (2% Na<sup>+</sup>, TD.92034), low sodium (< 0.01% Na<sup>+</sup>, TD.90228), high potassium (5%), low potassium (0.01% K<sup>+</sup>) or regular (0.32% Na<sup>+</sup>, 0.9% K<sup>+</sup>, TD.7912) diets respectively, for 7 days. All diets were purchased from Harlan Teklad (Madison, WI, USA). Animals had free access to tap water. In some experiments, animals were subcutaneously injected with Deoxycorticosterone acetate (DOCA, 2.4 mg/injection/animal) for 3 consecutive days. Animal use and welfare adhered to the National Institutes of Health Guide for the Care and use of Laboratory Animals following a protocol reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The University of Texas Health Science Center at Houston.

### **1. Tissue isolation**

Mice were sacrificed by CO<sub>2</sub> administration and cervical dislocation. Kidneys were immediately isolated and stored on ice. Kidneys were subsequently decapsulated and homogenized followed by either analyses by Western blotting, RNA isolation (see below), or cut into thin slices (<1mm) for isolation of individual nephrons for Ca<sup>2+</sup> imaging and Immunohistochemistry using a well-established protocol in our lab (93).

### **2. Western blot analyses**

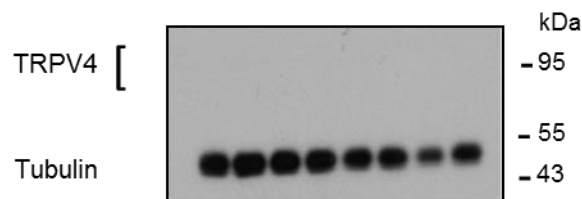
Kidneys were dissected and placed on ice, decapsulated and homogenized in a dounce glass homogenizer in 1 ml of ice-cold hypotonic lysis Buffer (Table 1). 1mM PMSF and 2 mg/ml EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) were added to the lysates to prevent protein degradation. Homogenates were centrifuged at 1,000 g for 15 minutes

at 4 °C. The supernatants were removed and stored at – 80°C for later use. Protein concentration was determined with Bradford assay using Immunoglobulin G (IgG, Abcam, Cambridge, UK) as a standard. Each standard (10 µl/well of the following concentrations of IgG: 0.5 µg/µl, 0.3 µg/µl, 0.15 µg/µl, 0.1 µg/µl and 0.05 µg/µl) and samples (10 µl/well) were placed into a 96 well plate in triplicates. 200 µl of Coomassie dye (Bio-rad, Hercules, CA, USA) was added for the colorimetric reaction. The absorbance shift of the Coomassie dye in each sample was measured at a wavelength of 595 nm using a microplate reader (Molecular devices spectro). Protein concentration was determined for each sample using the IgG standard curve. Samples were further diluted to a final concentration of 1 µg/µl using hypotonic lysis buffer (Table 1), reduced in 5X SDS buffer (Table 1) with 20 mM Dithiothreitol and denatured at 75°C for 10 minutes. 15 µg of each samples were loaded per well of a 5% stacking gel followed by a separation on a 9% polyacrylamide gels (Table 1) at 150 volts during 1 hour 40 minutes. Proteins were then transferred to a nitrocellulose membrane using wet transfer in towbin buffer (Table 1) at 100 volts for 1 hour 30 minutes at 4 °C. The nitrocellulose membrane was blocked for 1 hour in 5 % filtered nonfat milk (LabScientific Inc, New Jersey, USA) in TBS-Tween wash buffer (Table 1) and subsequently incubated with anti-actin (1:2000 dilution, Abcam, Cambridge, UK) or anti-tubulin (1:2000 dilution, Abcam, Cambridge, UK) and either with anti-TRPV4 (1:1000 dilution, Alomone Labs, Jerusalem, Israel) or anti-TRPC3 (1:500 dilution, Alomone Labs, Jerusalem, Israel) primary antibodies for 2 hours at room temperature. Membranes were incubated with TRPV4 or TRPC3 control antigens provided by vendor (Alomone Labs, Jerusalem, Israel) to verify for antibody specificity. Membranes were washed 3 times for 5 minutes in TBS-Tween wash buffer and then incubated with peroxidase-conjugated goat anti-rabbit secondary antibodies (1:30,000 dilution, Bio-Rad, Hercules, CA) for 1 hour at room temperature. Membranes were washed 3 times for 5 minutes in wash buffer and incubated in a mixture of



ECL reagents (Thermo Scientific, Waltham, MA, USA) following manufacturer's protocol. Membranes were attached to a cassette and subsequently exposed to an X-Ray film (Phoenix Research Products, Candler, NC, USA) at different exposure. Blots were analyzed using Image Studio Lite version 3.1 software (LI-COR, Lincoln, Nebraska, USA). TRPV4 and TRPC3 protein upper bands (~ 95 kDa) were selected and their intensities were normalized to the corresponding actin bands (~ 45 kDa) used as a loading control.

<b>Table 1: chemicals and solutions</b>	
Hypotonic lysis buffer	50 mM Tris base, 1% Triton X-100, 5 mM EDTA, 7<pH<8
5 X SDS reducing buffer	10 % SDS, 50 % Glycerol, 1M Tris pH 6.8, 0.5 % Bromophenol blue
9 % resolving gel	6.6 ml H <sub>2</sub> O, 4.5 ml 30 % acrylamide, 3.75 ml 1.5 M Tris pH 8.8, 150 µl 10 % SDS, 150 µl 10 % APS, 15 µl TEMED
5 % stacking gel	3.42 ml H <sub>2</sub> O, 1.02 ml 30% acrylamide, 1.5 ml 0.5 M Tris pH 6.8, 60 µl 10 % SDS, 60 µl 10 % APS, 6 µl TEMED
10 X electrode buffer, 1L	144.2 g Glycine, 30.3 g Tris base, 10 g SDS
Towbin buffer, 1L	200 ml Methanol, 14.4 g Glycine, 3 g Tris base
Wash buffer, 1L	150 mM NaCl, 50 mM Tris-HCL, pH 7.5, 0.1 % Tween 20
Physiological buffer	150 mM NaCl, 5mM KCl, 5 mM Glucose, 10 mM HEPES, 1 Mm CaCl <sub>2</sub> , 2mM MgCl <sub>2</sub>



**Figure 5.** Absence of TRPV4 bands following incubation with control antigen.

Representative Western Blot of TRPV4 expression in whole kidney lysates of C57BL/6 mice. Lysates were run on a polyacrylamide gel and probed with TRPV4 blocking peptide, anti-TRPV4 and anti-tubulin antibodies.

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### **3. RNA extraction and RT- quantitative PCR (RT-qPCR)**

Kidneys were isolated, decapsulated and homogenized in TRIzol Reagent, 1 ml per 50-100 mg of tissue (Ambion, Life Technologies), using a glass teflon homogenizer and then incubated 5 minutes at room temperature to dissociate the nucleoprotein complex. 0.2 ml chloroform (Fisher Scientific) was added to the homogenate and mixed for 15 seconds. Samples were incubated for 3 minutes at room temperature followed by a centrifugation at 12,000 x g for 15 minutes at 4°C. This step allowed a phase separation where the mixture separates into three distinct phases. RNA was in the transparent upper aqueous phase. Subsequently, the supernatant was removed and RNA was precipitated by adding 0.5 ml of 100 % Isopropanol (Sigma). Samples were incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4°C. Finally, supernatants were removed and RNA pellets were washed with 75% ethanol per 1 ml TRIzol reagent used initially. Samples were then quickly vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. Supernatant was discarded and RNA pellets were air-dried until becoming transparent, RNA was then resuspended in 30 µl RNase-free water (Gibco, Life Technologies). RNA concentrations and quality were determined spectrophotometrically at 260 and 280 nm. To determine RNA quality, Agilent 2100 Bioanalyzer (Agilent Technologies) was used for electrophoretic separation of very small amounts of RNA that can be detected by laser induced fluorescence. cDNA was synthesized in

270  $\mu$ l total volume by addition of 3  $\mu$ l well<sup>-1</sup> RT master mix consisting of : 45  $\mu$ l RT 10X buffer, 90  $\mu$ l deoxynucleotides mix, 54.6  $\mu$ l random primer, 62.4  $\mu$ l H<sub>2</sub>O and 18  $\mu$ l Affinity Script enzyme to a 96 well plate followed by a 2  $\mu$ l volume of sample (20 ng/  $\mu$ l). Each sample was analyzed in triplicate plus a control without reverse transcriptase. Plates were covered with Biofilm A (MJR) and incubated in a thermocycler (MJR, Waltham, MA, USA) for 30 minutes at 50 °C followed by 5 minutes at 72°C. Subsequently 3  $\mu$ l of PCR master mix (52.5  $\mu$ l Jumpstart 10X, 70  $\mu$ l primer/probe mix (Table 2), 59.5  $\mu$ l MgCl<sub>2</sub>, 21  $\mu$ l deoxynucleotides mix, 909.5  $\mu$ l H<sub>2</sub>O and 5.3  $\mu$ l Taq Polymerase (Sigma) were added to each well of the cDNA plate. Plate was then placed in a light cycler 480 II and Quantitative RT-PCR was performed following cycling conditions: denaturation at 95 °C for 2 minutes, amplification at 95 °C for 12 seconds and amplification at 60 °C for 30 seconds for a total of 40 cycles.

**Table 2: Probes and primer sequences**

TRPV4	Probe	5'-/56-FAM/CCTGCTTGT/ZEN/GTACCTGCTCTTCATGA/31ABkFQ/-3'
	Primer 1	5'-TCGTCACAGACCTTCATGTTG-3'
	Primer 2	5'-CCTCTTCAAAGACCTCTTCCG-3'
HPRT	Probe	5'-/56-FAM/CTTGCTGGT/ZEN/GAAAAGGACCTCTCGAA/31ABkFQ/-3'
	Primer 1	5'-AACAAAGTCTGGCCTGTATTC-3'
	Primer 2	5'-CCCCAAAATGGTTAAGGTTGC-3'

#### 4. [Ca<sup>2+</sup>]<sub>i</sub> measurements

Kidney slices were placed in an ice cold physiological solution buffered with HEPES pH 7.4 (Table 1). Distal nephrons were identified by their typical morphology and color, they were mechanically isolated from kidney slices by micro-dissection using watchmaker forceps under a stereomicroscope. Isolated distal nephrons were attached to poly-L-lysine coated coverslips (5\*5 mm). Coverslips containing distal nephrons were placed in a perfusion chamber mounted

on a Nikon Eclipse Ti inverted microscope and perfused with bath solution at room temperature. In order to access the apical membrane, distal nephrons were split opened with two sharpened micropipettes controlled with micromanipulators. Intracellular calcium levels were measured in individual cells within the split opened area of distal nephrons from mice kept on high sodium, high potassium and control diets using Fura-2 fluorescence radiometric imaging. Split opened distal nephrons were loaded with 2  $\mu\text{mol/L}$  of Fura -2/acetoxymethyl ester (EMD biosciences, USA) in a bath solution at room temperature for 40 minutes. Then, they were placed in an open-top imaging chamber (Warner RC-10) with a bottom coverslip viewing window and the chamber attached to the microscope stage of an inCa imaging workstation (Intracellular Imaging, Inc). Cells were imaged using a 20x Nikon super fluor objective and regions of interest were selected for individual cells. Typically 10-30 cells were simultaneously monitored per coverslip and the results were averaged for each experiment. Intracellular calcium was estimated from the Fura-2 fluorescence by repetitive brief excitation at 340 nm and 380 nm and by calculating the ratio of the emission intensities ( $R = F_{340}/F_{380}$ ) at 511 nm every 5 seconds in order to avoid decay in Fura-2 signals due to photobleaching (92,94). This ratio represents an index of relative changes in  $[\text{Ca}^{2+}]_i$ . The ratio can be converted to actual  $[\text{Ca}^{2+}]_i$  values after calibration of fluorescence intensities in permeabilized cells (with ionomycin) in solutions with known  $[\text{Ca}^{2+}]_i$  (94,95). InCytIm2 -version 5.29c software (Intracellular Imaging, OH, USA) was used to visualize and quantify Fura-2 fluorescence in split-opened distal nephrons.

## **5. Immunofluorescence microscopy**

Freshly isolated split opened distal nephrons from mice fed with low  $\text{Na}^+$ , low  $\text{K}^+$ , high  $\text{Na}^+$  and high  $\text{K}^+$  diets were fixed in 4% paraformaldehyde in Phosphate buffered saline PBS (pH 7.4) for 15 minutes. After fixation, samples were washed 3 times for 5 minutes in PBS and

permeabilized in 0.1% Triton X-100 for 5 minutes then washed 3 times for 5 minutes in PBS. To prevent non-specific staining, samples were treated with 10 % normal goat serum (Jackson ImmunoResearch laboratories) in PBS for 30 minutes at room temperature. Following washing in PBS, split opened distal nephrons were incubated in a mixture of TRPV4 primary antibody diluted 1:1000, Alomone labs), 1% normal goat serum and 0.1% Triton X-100 in PBS during 3 hours. Subsequently, samples were washed 3 times for 5 minutes in PBS and then incubated with goat anti-rabbit IgG labeled with Alexa Fluor 488 (1:2000 dilution, Invitrogen) in the presence of 1% normal goat serum and 0.1% Triton X-100 in PBS for 90 minutes at room temperature, in the dark; followed by three washes in PBS. Nuclei were stained with Dapi (1.5  $\mu$ M, Calbiochem) for 1 minute in the dark. Samples were attached to glass slides using mounting medium (Thermo Scientific). Samples were examined using a Nikon Eclipse confocal fluorescence microscope with a 40x Plan fluor oil immersion objective. Samples were excited with 405 and 488 nm lasers and emission captured with a 16-bit CoolSNAP HQ<sup>2</sup> camera (photometrics) interfaced to a computer running NIS-Elements version 4.00 software. 3-D stacks of split opened distal nephrons were generated from a series of confocal plane images with 0.25  $\mu$ m steps on Z- axis (93).

## **6. Statistical methods**

Summary data were given as mean values  $\pm$  SEM as indicated in the figures. Differences among the groups were analyzed with a one-way ANOVA. The significance level was defined as  $p < 0.05$ .

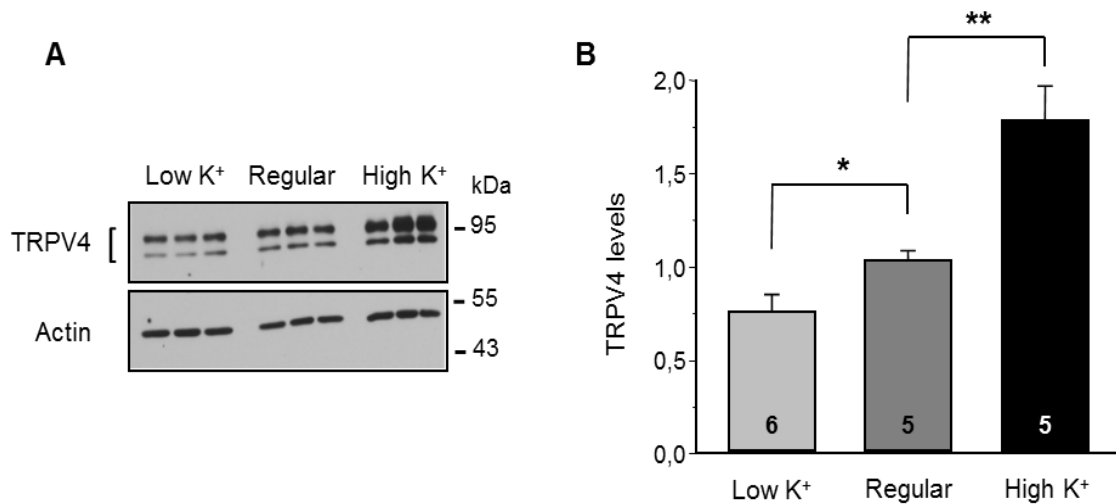
## **RESULTS**

## RESULTS

We have recently demonstrated that  $\text{Ca}^{2+}$  permeable TRPV4 channel is essential for flow-dependent  $[\text{Ca}^{2+}]_i$  elevations (35,92,93). Interestingly, it has been shown that variations in dietary potassium and to a lesser extent, sodium intake affect tubular fluid delivery leading to an increase of the flow rate in the distal nephron, thereby augmenting mechanical stress. Thus, we hypothesized that increased  $\text{Na}^+$  and  $\text{K}^+$  intakes, will lead to respective increase in TRPV4 expression and function. To address this hypothesis, I employed a variety of experimental approaches including western blotting; real time quantitative PCR, Fura-2  $\text{Ca}^{2+}$  imaging and immunofluorescence microscopy.

### **1. Changes in dietary potassium intake regulate renal TRPV4 expression**

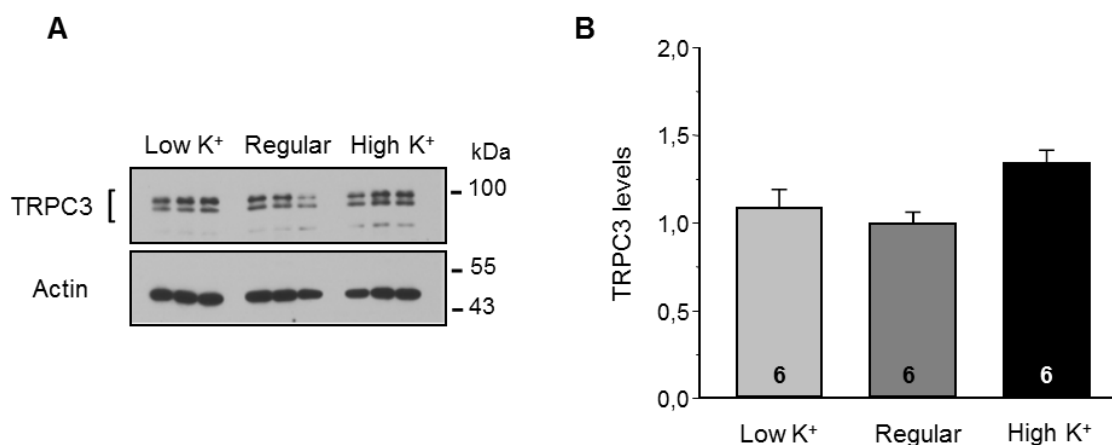
To assess the effects of variations in the  $\text{K}^+$  diet on renal TRPV4 expression, we performed western blotting as described in Materials and Methods. Figure 6A shows a representative western blot monitoring TRPV4 expression in whole kidney homogenates from C57BL/6 mice fed low, regular, and high  $\text{K}^+$  diets for 7 days. Since TRPV4 expression in the kidney is restricted to the distal tubule, I used whole kidney homogenates, as a reliable indicator of changes in the distal nephron. TRPV4 protein expression appeared as a duplet around ~ 95 kDa of a glycosylated (upper) and non-glycosylated (lower) bands. Renal TRPV4 levels were significantly increased in high  $\text{K}^+$  diet samples and significantly reduced in low  $\text{K}^+$  diet samples as compared to the control conditions (Figure 6B).



**Figure 6.** Effect of high K<sup>+</sup> diet on TRPV4 expression in the kidney. (A) Representative Western Blot of TRPV4 expression in whole kidney lysates of C57BL/6 mice fed with low (0.01%), regular (0.9%) and high (5%) K<sup>+</sup> diet for 7 days. Lysates were run on a polyacrylamide gel and probed with anti-TRPV4 antibody. (B) Summary graph comparing TRPV4 expression levels from whole kidney homogenates similar to that shown in (A). Intensities of TRPV4 bands were normalized to intensities of the respective actin bands. \* Significant decrease versus regular diet (P ≤ 0.05 by One-way ANOVA). \*\* Significant increase versus regular diet (P ≤ 0.01 by One-way ANOVA). The data are represented as means ± SEM with numbers of individual experiments embedded in the graph.

To examine if this regulation was specific to TRPV4, I next probed the levels of TRPC3, another mechanosensitive, Ca<sup>2+</sup> permeable TRP channel, expressed in the distal nephron. As opposed to TRPV4, TRPC3 protein levels remained unchanged in high, low and regular K<sup>+</sup> diet conditions (Figure 7A and B). Overall, these results suggest that TRPV4 is specifically regulated by variations in K<sup>+</sup> regimen.



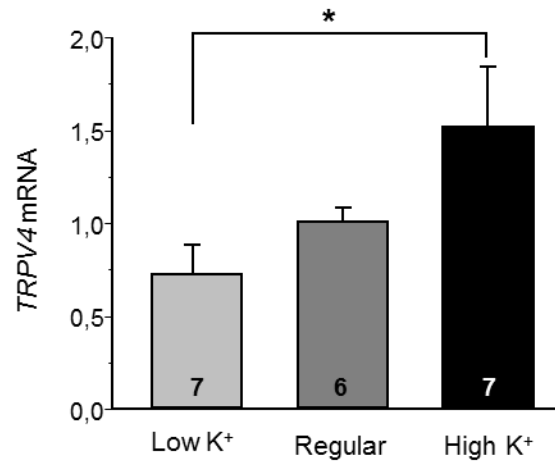


**Figure 7.** Effect of High K<sup>+</sup> diet on renal TRPC3 protein levels. **(A)** Representative Western Blot of TRPC3 expression in whole kidney lysates of C57BL/6 mice fed a low (0.01%), regular (0.9%) and high (5%) K<sup>+</sup> diets. Lysates were run on a polyacrylamide gel and probed with anti-TRPC3 or actin antibodies. **(B)** Summary graph comparing renal TRPC3 expression levels from western blots similar to that shown in **(A)**. Intensities of TRPC3 bands were normalized to the intensities of the respective actin bands. The data are represented as means ± SEM with numbers of individual experiments embedded in the graph.

## 2. Regulation of *TRPV4* mRNA levels by variations in K<sup>+</sup> diet

My results in Figure 6 demonstrated that TRPV4 levels are enhanced by a high potassium diet. I next asked whether TRPV4 regulation also occurs at the mRNA level. To test for this, I performed a real-time quantitative PCR to monitor changes in *TRPV4* mRNA levels. Consistent with our hypothesis, I observed a significant augmentation of relative *TRPV4* mRNA levels in high K<sup>+</sup> diet and a reduction in low K<sup>+</sup> diet as compared to control conditions (Figure 8).

Therefore, I concluded that TRPV4 is significantly modulated by potassium diet at both the mRNA and the protein levels.

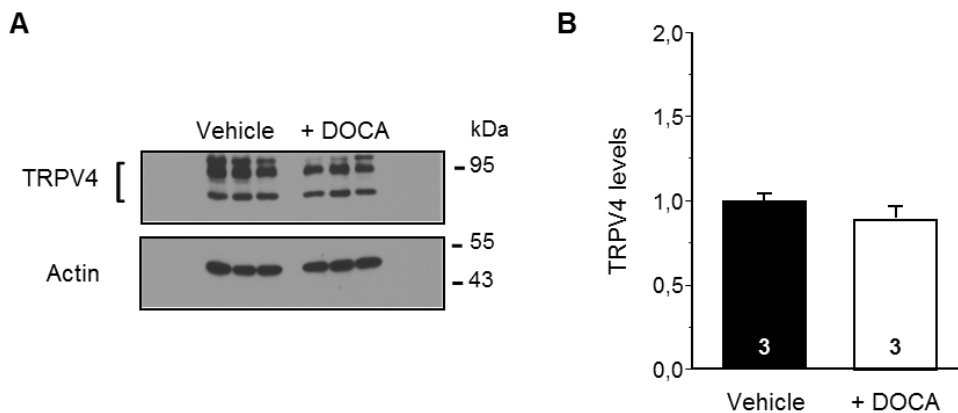


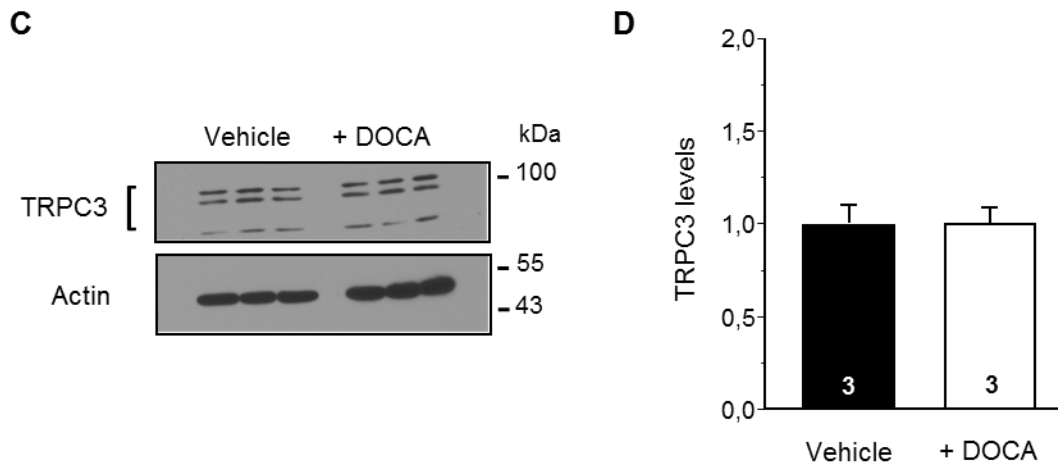
**Figure 8.** Effect of high and low K<sup>+</sup> diet on *TRPV4* mRNA levels. (A) Relative expression of *TRPV4* mRNA detected by qRT-PCR in kidneys of C57BL/6 mice fed with low (0.01%), regular (0.9%) and high (5%) K<sup>+</sup> diets for 7 days. Total RNA was reverse transcribed into cDNA using random primers and real time q-PCR was performed using *TRPV4* primers covering exons 12-13, to monitor changes in mRNA levels. Mean *TRPV4* cycle threshold values were normalized to the respective HPRT cycle threshold values. \* Significant increase versus low K<sup>+</sup> diet ( $P \leq 0.05$  by One-way ANOVA). The data are represented as means  $\pm$  SEM with numbers of individual experiments embedded in the graph.

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### 3. Renal TRPV4 expression is not regulated by aldosterone

Elevated  $K^+$  intake leads to increase in plasma  $K^+$  levels and this, in turn, stimulates aldosterone release by the adrenal cortex to promote renal  $K^+$  excretion (21,26-28). To test whether the increase in TRPV4 protein levels during systemic administration of a high potassium regimen is due to increased plasma aldosterone concentrations, I injected mice with Deoxycosterone Acetate (DOCA), a precursor of aldosterone, at saturating concentrations (2.4 mg), during 3 consecutive days in order to maximally stimulate aldosterone cascade. DOCA treatment had no effect on kidney TRPV4 protein levels, as shown in figure 9A and the summary graph 9B. In addition to TRPV4, I observed no changes in TRPC3 protein abundance after DOCA administration as represented in figure 9C and the summary graph 9D. The absence of changes in TRPV4 and TRPC3 protein abundance points to an aldosterone independent regulation of the channels in the distal part of the renal nephron. The absence of aldosterone action suggests that the increase in tubular flow per se may account for the increase in TRPV4 expression in response to a high  $K^+$  diet.



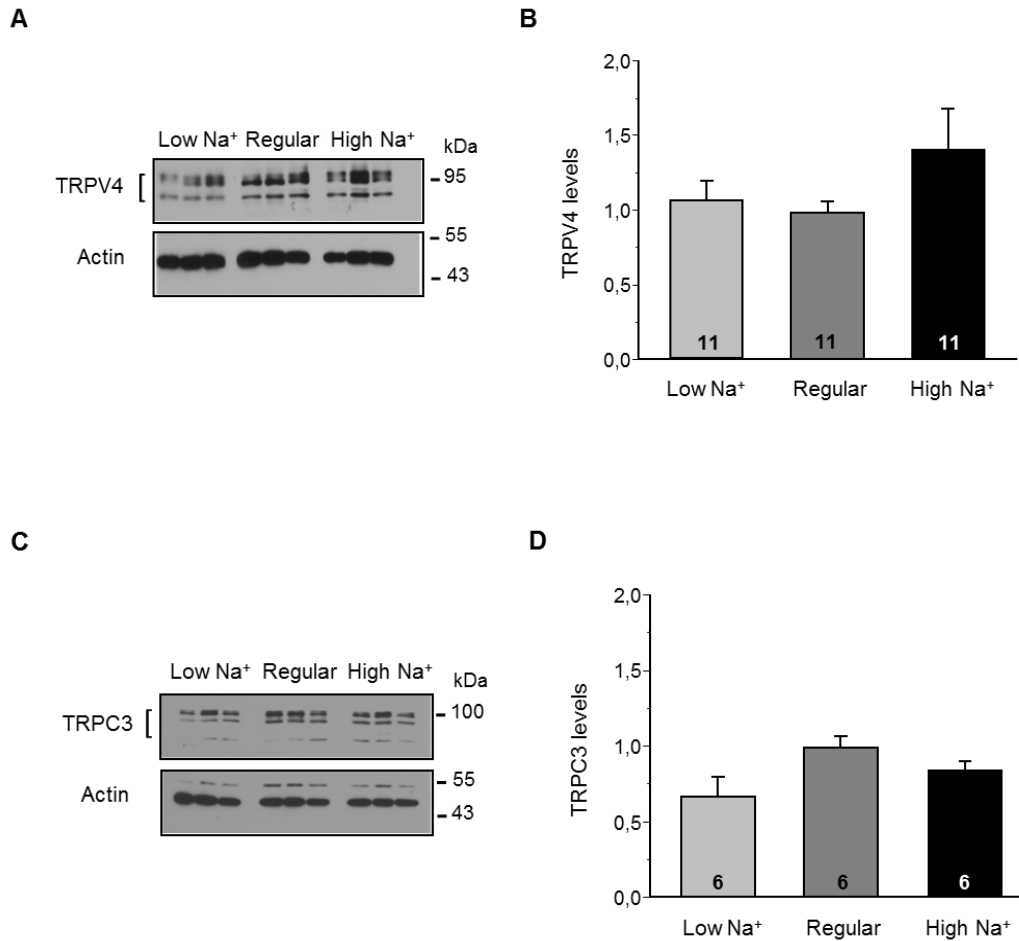


**Figure 9. Effect of saturation of mineralocorticoid status with DOCA on TRPV4 and TRPC3 expression.** (A) and (C) Representative Western Blot of TRPV4 (A) or TRPC3 (C) expression in whole kidney lysates of C57BL/6 mice injected with 2.4 mg DOCA for 3 consecutive days before experimentation. Lysates were run on a polyacrylamide gel and probed with either anti-TRPV4 or anti-TRPC3 antibodies. (B) and (D) Summary graphs comparing renal TRPV4 (B) or TRPC3 (D) expression levels from western blots similar to that shown in (A) and (B). Intensities of TRPV4 and TRPC3 bands were normalized to the intensities of the respective actin bands. The data are represented as means  $\pm$  SEM with numbers of individual experiments embedded in the graph.

#### 4. High and low Na<sup>+</sup> diet have little effect on total TRPV4 expression in the kidney and no effect on TRPC3 protein abundance

Similarly, high sodium intake also produces a rise in sodium delivery to the distal nephron to increase urinary sodium excretion. This leads to a higher tubular flow in the DN. To assess the effect of variations in Na<sup>+</sup> diet on TRPV4, I probed the channel expression in whole kidney homogenates from mice fed low, high and regular Na<sup>+</sup> regimens for 7 days (Figure 10A).

Surprisingly, a high sodium intake produced only a modest insignificant raise of TRPV4 protein levels as compared to low and regular diets (figure 10B). Similarly, TRPC3 levels were unchanged by variations in dietary  $\text{Na}^+$  intake (figure 10C and D).



**Figure 10.** Effect of high and low  $\text{Na}^+$  diet on TRPV4 and TRPC3 expression in the kidney. (A) and (C) Representative Western Blot of TRPV4 and TRPC3 expression in whole kidney lysates of C57BL/6 mice fed with low (<0.01%), regular (0.32%) and high (2%)  $\text{Na}^+$  diets for 7 days. Lysates were run on a polyacrylamide gel and probed with either anti-TRPV4 or anti-TRPC3 antibodies. (B) and (D) Summary graphs comparing renal TRPV4 or TRPC3 expression levels from western blots similar to that shown in (A)

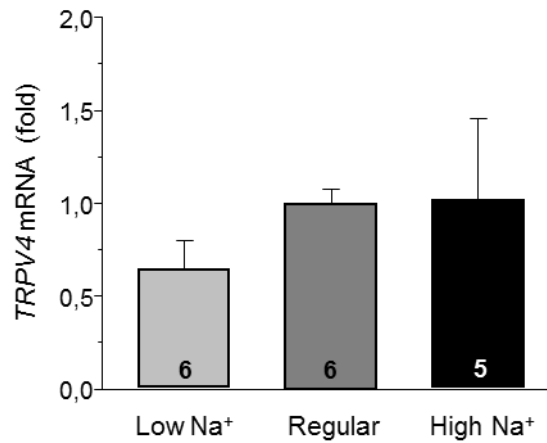
and (C). Intensities of TRPV4 and TRPC3 bands were normalized to the intensities of the respective actin bands. The data are represented as means  $\pm$  SEM with numbers of individual experiments embedded in the graph.

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## **5. Regulation of *TRPV4* mRNA levels by variations in Na<sup>+</sup> diet**

I have shown that variations in Na<sup>+</sup> diet, and particularly high Na<sup>+</sup> diet had little effect on TRPV4 protein abundance, as represented in figure 10. I next tested if these changes in Na<sup>+</sup> intake affect *TRPV4* mRNA, by performing a real time q-PCR. As summarized in figure 11, high Na<sup>+</sup> diet does not affect TRPV4 mRNA levels as compared to regular diet. Furthermore, low Na<sup>+</sup> diet elicits a small reduction in TRPV4 mRNA levels as compared to control conditions.

The absence of stimulatory effect of a high Na<sup>+</sup> diet on TRPV4 suggest that either an increase in flow does not regulate TRPV4 expression, or different modes of adaptation exist in the distal nephron in response to elevated K<sup>+</sup> and Na<sup>+</sup> intake. It needs to be noticed, though, that the lack of increase in TRPV4 expression by high Na<sup>+</sup> intake does not exclude other regulations of the channel at the post-translational level, such as trafficking and gating.

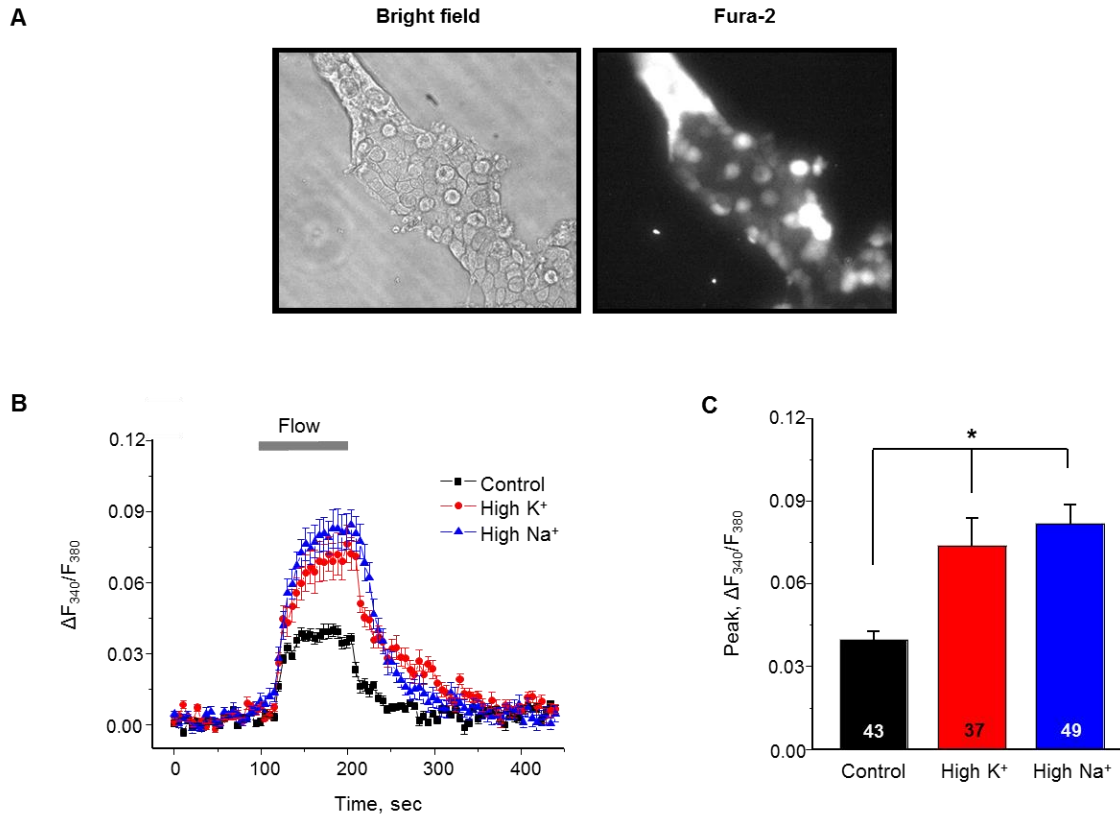


**Figure 11.** Effect of high and low Na<sup>+</sup> diets on *TRPV4* mRNA levels. **(A)** Relative expression of *TRPV4* mRNA detected by qRT-PCR in kidneys of C57BL/6 mice fed with low (<0.01%), regular (0.32%) and high (2%) Na<sup>+</sup> diets for 7 days. Total RNA was reverse transcribed into cDNA using random primers and real time q-PCR was performed using *TRPV4* primers covering exons 12-13, to monitor changes in mRNA levels. Mean *TRPV4* cycle threshold values were normalized to the respective *HPRT* cycle threshold values. The data are represented as means  $\pm$  SEM with numbers of individual experiments embedded in the graph.

## 6. TRPV4-mediated responses to elevated flow are augmented by high K<sup>+</sup> and Na<sup>+</sup> diets

We next probed how an increase in K<sup>+</sup> and Na<sup>+</sup> diet affect TRPV4-dependent [Ca<sup>2+</sup>]<sub>i</sub> elevations in response to flow in distal nephron cells. Individual DNs were mechanically isolated from kidney sections and split-opened with sharp micropipettes to get a monolayer of cells. This approach allows access to the apical plasma membrane, to monitor in real-time changes in [Ca<sup>2+</sup>]<sub>i</sub> at the cellular level, in response to mechanical stimulation (Figure 12A). Changes in Ca<sup>2+</sup> concentrations in response to flow are a reliable readout of TRPV4 activity, as

previously demonstrated by our group (92). Freshly isolated split opened distal tubules were subjected to an abrupt 10 X elevation in flow, producing a shear stress of approximately 3 dyn/cm<sup>3</sup> (92,93). This flow elevation caused a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> in high potassium and sodium as compared to control conditions (Figure 12B and C). These results show that TRPV4- mediated [Ca<sup>2+</sup>]<sub>i</sub> elevations are regulated by both high Na<sup>+</sup> and K<sup>+</sup> diets.



**Figure 12: Regulation of TRPV4- mediated mechanosensitive [Ca<sup>2+</sup>]<sub>i</sub> responses by high K<sup>+</sup> and Na<sup>+</sup> diets.** (A) Representative micrographs of a split-opened murine distal nephron taken under bright-field illumination (left) and fluorescent emission of FURA-2 with 380 nm excitation (right). This technique enables unequivocal separation of a fluorescent signal from individual cells within a split opened area. It also allows direct mechanical manipulations with the apical surface of cells. (B) The average time course of



$[Ca^{2+}]_i$  responses to 10x increases in flow (1.5 ml/min to 15 ml/min) over the apical surface (shown with the gray bar on top) recorded from distal nephrons from mice put on either regular, high  $K^+$ , or  $Na^+$  diets. (C) Summary graph comparing the average responses to flow in control and high  $K^+$  and  $Na^+$  diets. \* - Significant increase versus control ( $P \leq 0.05$  by One-way ANOVA). The data are represented as means  $\pm$  SEM with number of individual experiments embedded in the graph.

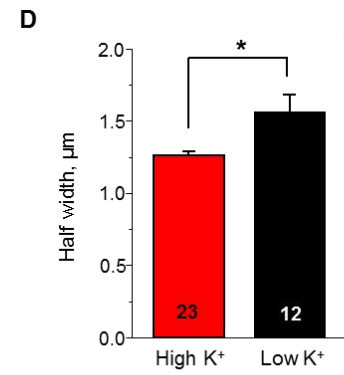
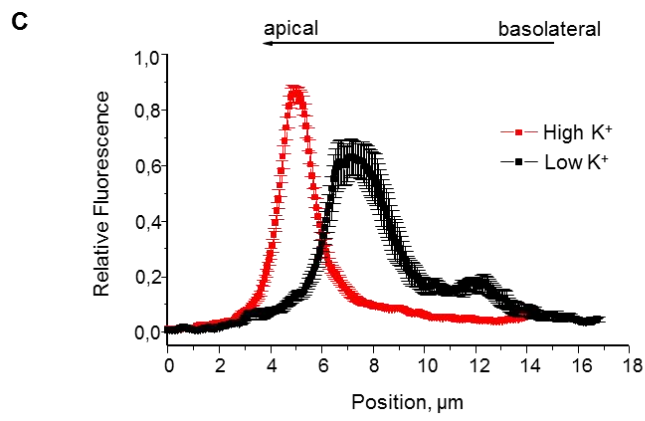
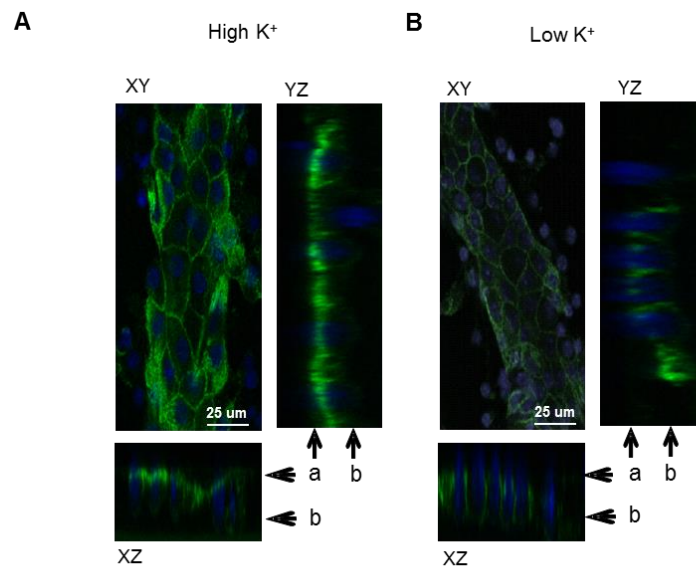
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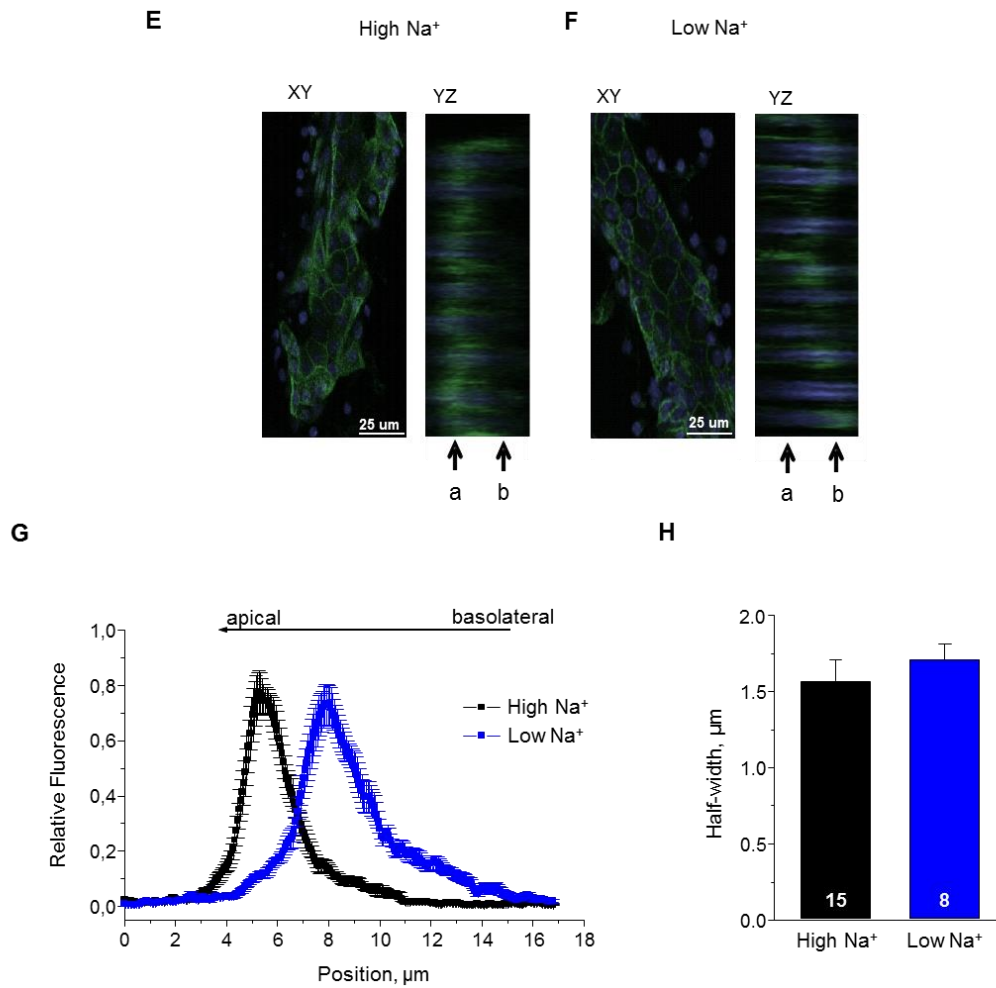
## **7. TRPV4 subcellular localization in the distal nephron is regulated by dietary $K^+$ and $Na^+$**

I next examined whether TRPV4 localization in distal nephron cells is controlled by variations in potassium and sodium diets. I monitored intracellular channel distribution using immunofluorescence confocal microscopy in split-opened distal nephrons (as described in methods). As shown in Figure 13, TRPV4 expression was predominantly cytosolic under low potassium and sodium conditions, as apparent in a representative confocal fluorescent image in figures 13B and F. In contrast, TRPV4 fluorescent signal was shifted to the apical membrane under high potassium and sodium conditions, with a more prominent effect produced by high  $K^+$  diet (Figure 13A versus 13E). In order to perform a quantitative analysis of the observed channel subcellular localization, I used line scan analysis of the fluorescent signal distribution along the z-axis in cross-sections of three dimensional stacks similar to those shown in figure 13. Figures 13C and G represent the average distribution of fluorescence intensity reporting TRPV4 expression in low and high potassium and sodium diets, respectively. As illustrated in figure 13, high potassium and sodium diets shifted the fluorescent signal toward the apical area. In addition, high potassium diet caused the sharpening of the fluorescence distribution.

Summarized in figures 13D and H, the half-width of the fluorescence intensity was significantly reduced from  $\sim 1.6 \pm 0.12 \mu\text{m}$  ( $n = 12$ ) in low  $\text{K}^+$  to  $\sim 1.3 \pm 0.03 \mu\text{m}$  ( $n = 23$ ) in high  $\text{K}^+$  diet. Whereas, for  $\text{Na}^+$  diet, the half-width was  $\sim 1.7 \pm 0.1 \mu\text{m}$  ( $n = 15$ ) in low  $\text{Na}^+$  diet and  $\sim 1.6 \pm 0.14 \mu\text{m}$  ( $n = 8$ ) in high  $\text{Na}^+$  diet. These values were not significantly different. These results suggest that TRPV4 subcellular localization and trafficking to the apical membrane is regulated by high  $\text{K}^+$  and  $\text{Na}^+$  diets.

In summary, our data suggest a differential regulation of mechanosensitive TRPV4 channel by high  $\text{K}^+$  and  $\text{Na}^+$  diets. While high  $\text{K}^+$  diet elicits increase in TRPV4 protein, mRNA, channel activity and translocation to the apical membrane, a high  $\text{Na}^+$  diet does not affect TRPV4 protein, but causes an increase in the channel activity and its translocation to the apical membrane.





**Figure 13:** Variations in dietary  $\text{K}^+$  and  $\text{Na}^+$  intakes control TRPV4 subcellular localization in distal nephron cells. (A), (B), (E) and (H) Representative confocal plane micrographs (axes are shown) and respective cross-sections showing TRPV4 subcellular localization (anti-TRPV4, pseudocolor green) in split-opened distal nephrons from mice kept on high and low  $\text{K}^+$  diets, as shown in (A) and (B) and high and low  $\text{Na}^+$  diets, as shown in (E) and (F). Nuclear DAPI staining is shown by pseudocolor blue. “a” and “b” indicate apical and basolateral sides, respectively. (C) and (G) The distribution of intensities of averaged fluorescent signals representing TRPV4 localization along a line on Z-axis in individual cells from distal nephrons

similar to that shown in **(A)**, **(B)**, **(E)** and **(F)**. For each individual cell, the fluorescent signals were normalized to their respective maximal values. The position of the apical and basolateral sides is shown with arrow on the top. **(D)** and **(H)** Summary graph of half-width means for distributions of fluorescence signals shown in **(C)** and **(G)**. \* Significant increase in high versus low  $K^+$  diet ( $P \leq 0.05$  by One-way ANOVA). The data are represented as means  $\pm$  SEM with number of individual experiments embedded in the graph.

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## **DISCUSSION**

## DISCUSSION

The present study provides important novel insights into the mechanisms regulating TRPV4-based mechanosensitivity in the distal nephron. It is well established that regulation of electrolytes transport (i.e.  $\text{Na}^+$  and  $\text{K}^+$ ) to match dietary intake occurs in the distal nephron. There,  $\text{Ca}^{2+}$  permeable TRPV4 channel, in addition to being abundantly expressed, plays a central role in  $\text{Ca}^{2+}$  elevations in response to changes in tubular fluid dynamics. Mechanosensitive  $[\text{Ca}^{2+}]_i$  responses are important determinants of many physiological processes in late nephron segments, including flow-dependent  $\text{Na}^+$  and  $\text{K}^+$  secretion (14). Here, I identified physiological determinants controlling total renal TRPV4 abundance, in addition to functional activity and subcellular localization in murine distal nephrons. I show that both protein abundance and messenger RNA of TRPV4 in the kidney are up regulated by dietary  $\text{K}^+$  load. In contrast, increased dietary  $\text{Na}^+$  has a limited to no effect on the channel protein and mRNA levels. Furthermore, I found that high  $\text{K}^+$  and  $\text{Na}^+$  loads are responsible for augmented TRPV4 activation in response to elevated flow over the apical plasma membrane, in addition to TRPV4 translocation to the apical membrane. This regulation of TRPV4 is essential for balancing ions excretion and reabsorption.

It has been previously established that both high  $\text{K}^+$  and  $\text{Na}^+$  diets affect tubular fluid delivery, leading to an augmented flow rate to the distal nephron, thereby augmenting mechanical stress (11,12). Our group has provided experimental evidence that TRPV4 mediates  $\text{Ca}^{2+}$  influx in distal nephron epithelial cells in response to elevated luminal flow. First, we and others have confirmed that TRPV4 is abundantly expressed in the distal nephron (35,92,93,96). Second, we demonstrated that genetic interference with the channel disrupts  $\text{Ca}^{2+}$  responses to shear stress in cultured collecting duct cells (35). Furthermore, we showed that TRPV4

knockdown in mice abolishes flow induced  $[Ca^{2+}]_i$  elevations in the connecting tubule and the cortical collecting duct (35). In addition, we demonstrated that pharmacological inhibition of the channel, using a highly selective antagonist, HC-067047 prevents changes in  $[Ca^{2+}]_i$  during increased flow (92,93). These observations led us to hypothesize that this channel plays a pivotal role in regulating electrolytes transport in the distal nephron.

The kidney is the primary site for regulation of  $K^+$  homeostasis. When plasma  $K^+$  levels increase in response to a high  $K^+$  intake, a state known as hyperkalemia, aldosterone secretion by the adrenal cortex increases. This increase affects the activity and abundance of ion channels, such as those responsible for  $K^+$  secretion (6,97-100). Our findings that TRPV4 protein levels are not altered in response to the aldosterone precursor deoxycorticosterone acetate (DOCA) injections, suggests that the channel is probably independently regulated of aldosterone release. The question then arises as to which hormones could regulate the channel in the distal nephron. I envision this as a possible direction of my future research.

Gao F *et al.* have shown that high sodium intake (4%) didn't increase TRPV4 expression in the renal cortex and medulla of Wistar rats (101). This result is consistent with my observations that renal TRPV4 protein expression and mRNA levels are not enhanced in response to dietary  $Na^+$  load. The lack of regulation of TRPV4 expression by high  $Na^+$  diet is likely due to the fact that there is little need to increase  $K^+$  excretion under these conditions.

In this study, I demonstrate that TRPV4 activity is controlled by high  $K^+$  and  $Na^+$  regimens. The combined immunohistochemical and functional analysis point to an expression of functional TRPV4 along the apical /subapical region of the cells. Our group has shown that, in the kidney, TRPV4 channel activity and trafficking are controlled by PKC- and PKA-dependent pathways, respectively. While PKA-dependent pathway promotes TRPV4 trafficking



and translocation to the apical membrane; PKC-dependent pathway increases the channel activity at the plasma membrane (93). However, on the mechanism of PKA-mediated regulation of channel translocation from the basolateral to the apical membrane, we propose that TRPV4 trafficking in distal nephron cells could be under control of antidiuretic hormone (vasopressin). This might be tested in my future work.

As stated before, to ensure  $K^+$  homeostasis in response to  $K^+$  load, its secretion is increased in distal nephron cells through regulation of locally expressed ion channels. The renal outer medullary potassium (ROMK) channels are responsible of basal  $K^+$  secretion. BK channels, expressed in PCs and ICs of the CNT and CD are important for flow-dependent  $K^+$  secretion, a process dependent on the presence of  $Ca^{2+}$  (6). Several studies have shown that pharmacological blockade or genetic ablation of BK channel markedly blunts flow-induced  $K^+$  secretion (102-104). In addition to ROMK and BK channels, Berrouit J *et al* have shown that TRPV4 mediated  $Ca^{2+}$  influx, leading to increase in  $[Ca^{2+}]_i$ , activates SK3 channel, a high  $Ca^{2+}$  affinity-activated  $K^+$  channel (8), abundantly expressed in the distal nephron. This is believed to be pivotal for the regulation of  $K^+$  excretion. My assessment of renal TRPV4 protein abundance during a high dietary  $K^+$  intake is in agreement with my quantitative analysis of the channel mRNA levels and by functional studies in split-opened murine distal nephrons, where I demonstrate the increase in TRPV4 mediated  $Ca^{2+}$  responses to elevated flow. In summary, high  $K^+$  diet maximally stimulates TRPV4 protein expression, mRNA and translocation to the apical membrane. While,  $Na^+$  diet has a different effect, since  $Na^+$  reabsorption does not require  $Ca^{2+}$ .

It would be of great interest to test for possible interactions between TRPV4 and  $Ca^{2+}$ -activated  $K^+$  channels, such as BK channels, to determine whether such regulation is due to their

association or indirectly via independent signaling entities with a coordinated function in distal nephron cells.

To summarize my studies, first, I have identified novel physiological stimuli controlling TRPV4 mechanosensitivity and intracellular  $\text{Ca}^{2+}$  responses, to regulate transport rates in the distal nephron. Second, I have demonstrated that  $\text{Na}^+$  and  $\text{K}^+$  loads control TRPV4 channel function and/or expression as well as its translocation to the apical membrane of distal nephron cells. This regulation is envisioned to be an adaptive response to elevated tubular flow in the course of these physiological stimuli, but it is still unclear whether diets exert a direct action on TRPV4 or may involve activation of a mediator or hormone, and this would be the subject of future studies in the field.

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## **VITA**

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