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François Seghers, août 2017

LIST OF ABBREVIATIONS

4αPDD	4 α -Phorbol 12,13-didecanoate
5',6'-EET	5',6'-epoxyeicosatrienoic acid
AA	arachidonic acid
AC	adenylate cyclase
ADPKD	autosomal dominant polycystic kidney disease
AJ	<i>adherens</i> junction
AQP1	aquaporin 1
AQP2	aquaporin 2
ARD	ankyrin repeat domain
AT1	angiotensin I
AT2	angiotensin II
AVPR2	vasopressin receptor type 2
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
[Ca²⁺]_i	intracellular Ca ²⁺ concentration
cAMP	cyclic adenosine monophosphate
CD	collecting duct
CN	connecting tubule
CYP	cytochromes P450
DCT	distal convoluted tubule
DRG	dorsal root ganglion
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
ECM	extracellular matrix
FA	focal adhesion
FK	forskolin
Gd³⁺	gadolinium
GFR	glomerular filtration rate
GsMTx4	<i>Grammostola spatulata</i> spider toxin
GTB	glomerulotubular balance
HEK cell	human embryonic kidney cell
IBMX	3-isobutyl-1-methylxanthine
IP	intraperitoneal

JG	juxtaglomerular
LMWP	low molecular weight proteins
MNC	magnocellular neurosecretory cell
mPTC	mouse proximal tubule cell
NO	nitric oxide
NOS	nitric oxide synthase
OBAA	3-(4-octadecyl)-benzoylacrylic acid
OVLT	organum vasculosum laminae terminalis
PLA₂	phospholipase A2
PVN	paraventricular nucleus
PT	proximal tubule
RVD	regulatory volume decrease
RAS	renin-angiotensin system
RR	ruthenium red
RT-qPCR	reverse transcription and quantitative polymerase chain reaction
Ser 824	serine 824 (TRPV4)
SON	supraoptic nucleus
TAL	thick ascending limb of the Henle loop
TRP	transient receptor potential (channel)
VP	vasopressin

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INTRODUCTION

Detection and transduction of osmomechanical stimuli

Detection of environmental mechanical forces and its transduction in a biological response is a fundamental property of all living organisms ranging from simple bacteria to complex multicellular organisms. Deformation upon mechanical stress is an inherent property of every biological compound and, as expected, living organisms have developed and conserved structures that detect and translate mechanical stimuli into biological signals in order to adapt to changing environments or to maintain homeostasis. Cell membrane, consisting of a lipid bilayer containing various membrane proteins, is the major interface where this transduction is at play [1].

The first mechanical stress that life experienced was the osmotic force. Bacterial cells were exposed to osmotic challenges, facing hypoosmotic shocks as in rain or coping with the accumulation of metabolism-derived intracellular osmolytes. In order to maintain the cell integrity, they have developed stretched open channel proteins that sense membrane tension and allow an osmolytes efflux, relieving cell pressure. These prototypical mechanosensitive ion channels have been extensively studied [1, 2].

Eukaryotic cells are also facing mechanical stimuli. Unlike bacterial cells, they lack a rigid cell wall and possess a cytoskeleton, which implies a different repartition of the forces applied on the cells, and a different panel of force sensors compared to prokaryotic organisms.

At the level of individual cells, migration, adhesion, proliferation or differentiation are processes that integrate mechanical stimuli from subcellular structures. In complex multicellular organisms, it has become crucial for every type of differentiated cell to detect and interact with adjacent cells, as well as with the extracellular matrix (ECM) and with the external environment. Eventually, in cells that are located at physical transduction interfaces, complex organisms have developed specialized mechanosensitive systems. These are clearly illustrated in highly specific sensorial capacities such as touch or hearing. Though, other vital, unconscious homeostatic processes also rely on mechanotransduction. The blood pressure regulation feedback is based on the so called baroreceptors; arterial smooth muscle adapts to blood pressure according to the myogenic reflex; osmoreceptors detect and tightly regulate

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systemic osmolality; intestinal peristalsis requires coordination; bone mineralization is determined by its loading...

In eukaryotes, what does convert a physical force into a cellular response? Molecular components of mechanotransduction systems are proteins that are specialized in linking external physical stimuli to intracellular effectors. Even if virtually all proteins are sensitive to mechanical stresses, some are particularly designed to detect physical forces, due to their structure and localization. They are mainly the membrane ion channels on one hand, and cytoskeleton and adhesion proteins on the other hand.

The core elements of the mechanosensitive cytoskeleton consist in actin and actomyosin fibers, called stress fibers. They participate in passive and active detection of the cell mechanical environment. They are anchored to specific membrane complexes exposed to external forces. These membrane complexes are of two major types: the *adherens* junctions (AJs) that form cell-to-cell adhesion, and the focal adhesions (FAs) that are linked to ECM proteins like collagen. These adhesion sites work as important mechanotransducers, as they transduce to the cell external forces from ECM or neighbouring cells. They consist in large multiproteic complexes organized around transmembrane adhesion receptors (cadherins for AJs and integrins for FAs), which are linked to the actin cytoskeleton through a variety of linker proteins [3, 4] [5]. These complexes comprise also other proteins such as enzymes and ion channels [6]. The mechanotransduction mechanism at play relies on the modification of the physical interactions between the binding partners within the complexes as force is applied. The multiple actin fibers connecting the adhesion sites create a network capable of detecting not only the amplitude but also the direction of the forces applied on the cell [5]. In addition to passively sensing external forces, these mechanosensitive systems can also be dynamically loaded as contractile actomyosin stress fibers can apply tension on FAs and AJs, allowing the cell to probe its mechanical environment. Mechanically induced processes involving adhesion structures and cytoskeleton are relatively slow, consisting of cytoskeleton modification (within minutes) and transcriptional effects (within hours). They are mainly involved in migration, adhesion, proliferation or differentiation.

In the FAs, integrins are heterodimeric transmembrane receptors composed of α and β subunits. Their activation by the modification of the extracellular domain shifts integrins from a low- to high-affinity state for ligands binding to their cytoplasmic tail, such as kindlin and talin. Mechanical stretch on integrins activates the focal adhesion kinase FAK and Src family

kinases that trigger cytoskeleton rearrangement. Integrins mediate information about ECM stiffness, which impact on cell proliferation and differentiation via the action of transcriptional activators. Integrin-mediated mechanotransduction plays thus an important role in development and tissue homeostasis [7].

In the AJs, cadherins are Ca^{2+} -dependent cell-cell adhesion molecules composed of an extracellular domain, a transmembrane domain and a C-terminal cytoplasmic domain. The extracellular domain is responsible for homophilic interactions with cadherin expressed at the surface of neighboring cells, which trigger interactions with actin filaments via the cytoplasmic proteins α - and β -catenin. Mechanical forces therefore propagate across cells in tissues through AJs and drive coordinated tissue remodeling such as wound healing, apical cell constriction, cell intercalation and cell migration [8].

Faster events in mechanotransduction are thought to be mediated by the opening of mechanosensitive ion channels at the cell membrane, allowing the detection of acute physical stimulation of the cell [9]. Indeed, due to the asymmetric concentrations of ions on both sides of the resting cell membrane, opening of a transmembrane ion channel results in a sudden change in membrane potential and/or in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), depending on the channel selectivity, that may rapidly trigger a cascade of cellular response.

Mechanosensitive ion channels

The first direct observation of eukaryotic mechanosensitive ion channels was obtained in electrophysiological studies, where suction in patch-clamp experiments increased channel activity, suggesting the existence of an ion channel whose gating was dependent on membrane stretch [10]. Later on, many candidate mechanosensitive channels were identified. They appear to vary greatly in sequence and function and do not belong to one single structural family. Mechanosensitivity is a general property, like voltage sensitivity. It requires that the channel changes shape enough during stress to switch from closed to open state (or open to closed). All protein channels are basically mechanically deformable, but whether this mechanical sensitivity is physiologically relevant has then to be determined [11].

Gating mechanisms

Different gating mechanisms may lead to the mechanical opening of an ion channel. The simplest gating mechanism consists in the opening of the channel directly due to the deformation of the surrounding lipid bilayer. The lipid bilayer forming the cell membrane experiences surface tension that creates lateral tension in the bilayer. All proteins embedded in this layer are subject to these forces. Added stretch on the membrane will affect magnitude and direction of these forces, resulting in conformational changes in the membrane proteins (**FIGURE 1A**). As the lipid composition of the membrane is not homogenous, this model is also proposed to explain the tendency of many mechanosensitive ion channels to be modulated by exogenous or endogenous lipids, that would modify the physicochemical composition of the membrane in the surroundings of the channel [12].

In a second gating mechanism, the tethered model, the channel is linked to elements of the cytoskeleton, the ECM or others membrane proteins that would activate it by transmitting the stress (**FIGURE 1B**). In this line, it has been proposed that some mechanosensitive ion channels could be directly linked to the actin fibers [6].

The lipid bilayer stretch model is derived from studies on the bacterial mechanosensitive channels, where membrane tension alone has been demonstrated to recapitulate the mechanosensitive channels activation. The physical properties of the eukaryotic cell membrane are complex. It is heterogenous in lipid composition, it is surrounded and anchored in an ECM, and its tension is passively or actively regulated by the cytoskeleton. Activation of mechanosensitive channels was successfully achieved either by

selectively twisting integrins (the major components of the AJs anchoring the ECM to the cytoskeleton)[13] or by pulling on an actin stress fiber (the core element of the cytoskeletal network) [6]. These experiments validated the tethered model as a mechanosensitive ion channel gating mechanism. Beside their role as tethers, the constraints caused by the ECM and the cytoskeleton scaffolds can render the cell membrane somewhat loose, in excess [1]. Its tension is low when it is locally constrained by dense cytoskeleton attachment and rises when cytoskeleton is disrupted or in artificial bilayers. It is reasonable to imagine that in some case the cytoskeleton would prevent channels from opening rather than pulling them open [11]. This can explain the apparently different properties of the same channel observed in various expression models.

It has long been thought that eukaryotic mechanosensitive ion channels were rather activated according to the tethered model, where channels are attached to cytoskeleton and ECM elements. But it has recently been demonstrated that the eukaryotic two-pore-domain K^+ channels and the Piezo channels reconstituted in liposomes can be activated due to the sole lipid bilayer distension, and it could surely apply also to other mechanosensitive ion channels [14]. It seems that the lipid bilayer and the tethered models coexist in eukaryotic cell physiology.

Beside direct mechanical gating, indirect mechanotransduction mechanisms have also been described, where the ion channel is not the force-sensing element of the process, but rather the signal transducer. It involves the enzymatic production of soluble messengers that would eventually activate the channel downstream a signaling cascade (**FIGURE 1C**). In that case, the opening of the channel would be somehow delayed from the mechanical stimulus, and the channel would not be directly mechanically gated but would act as a second messenger operated channel [15]. Similarly, the channel gating could occur upon phosphorylation by a mechanically activated kinase.

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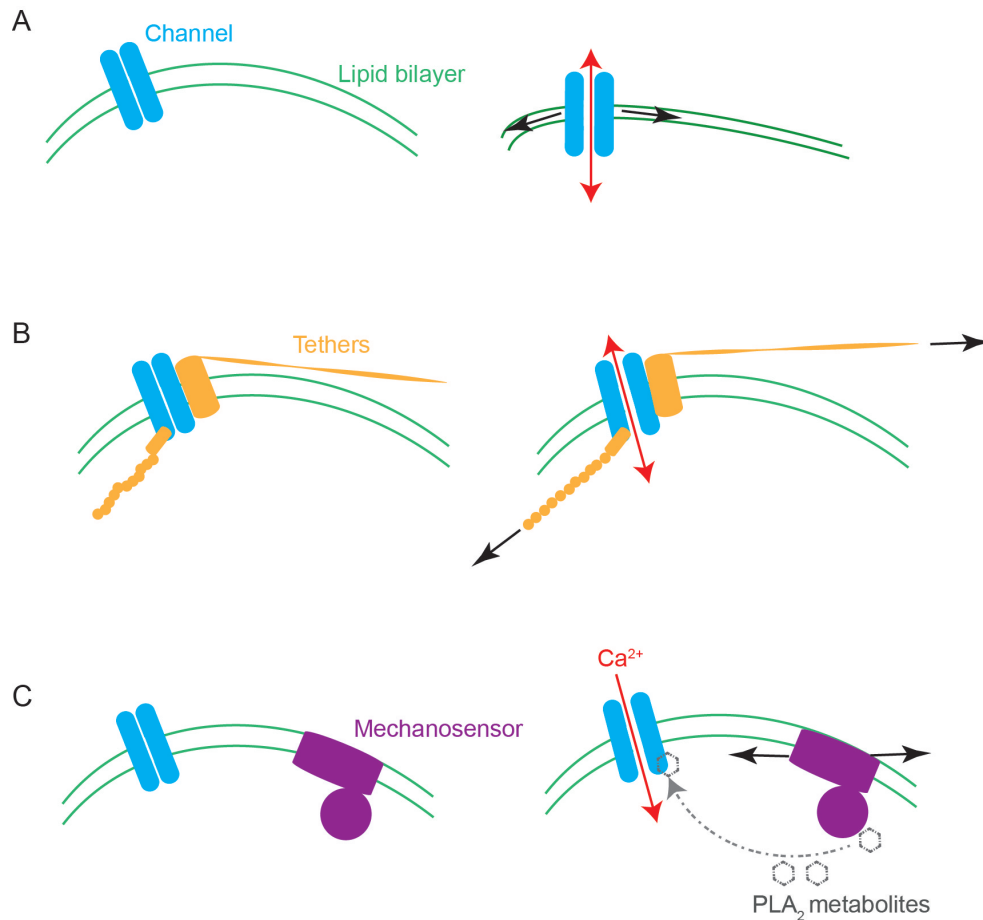


FIGURE 1. Different gating mechanisms of mechanosensitive ion channels. A. Opening of the channel by direct transmission of the tension of the surrounding lipid bilayer. B. Transmission of the stresses via the link of the channel to tethers (cytoskeleton, ECM or others membrane proteins). C. Indirect mechanotransduction involving the enzymatic production of soluble messengers eventually activating the channel.

Classification

The ion channels that have been involved in mechanotransduction processes or that have shown mechanosensitive properties belong to different families. There are no defined “force-sensing” domains [12]. Several criteria have been proposed for an ion channel to qualify as a mechanosensitive ion channel, although they are very restrictive and based on the well-characterized bacterial mechanosensitive ion channels [2, 16]. The candidate must be expressed in a mechanosensitive cell and its deletion should lead to a deficit in the mechanically induced response, without preventing the normal development of that cell. The structure of the channel should be characterized and should account for the mechanotransduction process so that alteration of the physical properties of the channel should alter the properties of the response. Furthermore, mechanical gating of the channel should be achievable in a heterologous expression system or when the channel is reconstituted

in an artificial lipid bilayer [2]. The channel is considered as directly mechanically gated if the latency between the stimulus and the current elicited is below 5 milliseconds (otherwise it is rather considered as mechanically sensitive *lato sensu*) [17].

Researchers have set to work and tested each new candidate, using diverse *in vitro* and *in vivo* approaches. Mechanically activated ion channels respond to diverse physical forces; these forces can be classified as osmotically driven volume changes (*e. g.* in epithelia), active cell deformation via the actomyosin activity (muscle, cell migration), cell stretch or compression (osteoarticular system, blood vessels), fluid shear stress on cell surfaces, or deformation of a specialized subcellular element (cilium in auditory hair cells) [18].

Various *in vitro* assays have been designed to apply a force on isolated cells or tissue preparations [16]. It is a challenge to design the *in vitro* mechanical stimuli protocols for studying mechanosensitive ion channels. Variable results can arise from different cellular models (endogenous or heterogenous ion channel expression), different physical stimuli (type, amplitude, velocity, frequency...) and different biophysical environment (glass, plastic, ECM coating...). Moreover, the channel of interest may be associated in complexes with auxiliary proteins that would be required to express its mechanosensitive properties, including other membrane proteins or channels, cytoskeleton or ECM elements.

In the field of electrophysiological techniques, patch-clamp experiments can classically detect ion channel activity during mechanical stimulation (during suction within the patch pipette, cell poking, substrate deformation or osmotic shock), or neuronal firing upon mechanical stimulation can be measured on nervous tissue preparations. $[Ca^{2+}]_i$ imaging is also used to assess the activation of the ion channels upon shear stress, cell stretching or cell deformation (**FIGURE 2**). The widely used osmotic shock can stretch the cell membrane, but it is slower and less uniform than other mechanical stimulations, because the cell membrane curvature and compliance are not constant, and it involves compensatory cytoskeleton changes [11]. Besides, cells also respond to osmotic stimulation with activation of intracellular signaling cascades that could also activate channels indirectly [19]. One should then extrapolate osmosensitive to mechanosensitive properties with caution.

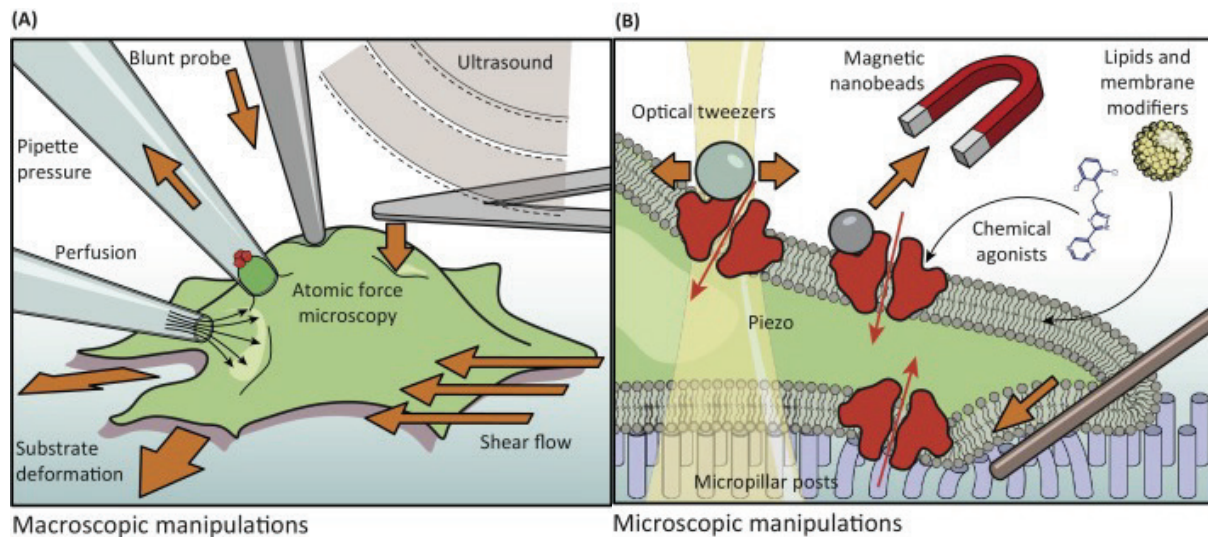


FIGURE 2. A. Macroscopic methods for stimulating populations of mechanosensitive ion channels, whose activity can be measured with electrophysiological methods or through $[Ca^{2+}]$ imaging. Direct deformation of the cell can be achieved with a blunt probe, with atomic force microscopy or with a high-pressure perfusion; by contrast, shear flow applies a parallel stress to the substrate surface. Membrane lipid bilayer stretch assays are typically performed through modulation of the pressure in a patch pipette, while stretching of a flexible substrate integrates the stimulation of cell adhesion structures. Vibration can be applied through ultrasound stimulation.

B. Microscopic modes of mechanosensitive ion channel stimulation. Deflection of micropillars stimulates a small population of ion channels through membrane deformation. Direct activation of a channel can be achieved through magnetic or optical control of nanoparticles bound to specific channel domains. Lipids that modify the membrane structure such as cholesterol can modulate mechanosensitive ion channels. Reproduced from [20]

Beyond the *in vitro* observation of candidate mechanosensitive ion channels, researchers also focus on their involvement in physiological processes. Results from cell or tissue culture experiments and *in vivo* testing are supported by the development of specific inhibitors, and gene targeting technologies that have allowed the production of mutant models to question the physiological role of the mechanosensitive ion channels. Some issues yet limit their study. The mechanosensitive ion channels are usually expressed at low levels in endogenous cells [16]. Even in sensory cells, they are quantitatively not the major component of the cell receptors, as opposed to photoreceptors in retinal cells or olfactory chemoreceptors [2]. In some cases, developmental changes in whole constitutional knockout animals may disturb the physiological study by developmental defects [21, 22]. It is also frequently observed that the deletion or the blocking of a candidate mechanosensitive ion channel only partially impairs the biological process where it is involved in, suggesting redundancy and compensation among mechanosensors [23-26].

In general, the ion channels that are called mechanosensitive in the literature have been successfully mechanically activated in electrophysiological experiments and are expressed in mechanotransduction sites, and they have been variably involved in physiologically relevant processes.

The DEG/ENaC/ASIC ion channels

Within this family of two transmembrane domains Na^+ channels, the degenerin (DEG) channels are mechanosensitive channels that were characterized in somatosensory neurons of *Caenorhabditis elegans* (*C. elegans*), an invertebrate where they mediate touch sense [16]. Homologues in mammals include the epithelial Na^+ channel (ENaC), that is well-known for its role in apical sodium reabsorption in the distal nephron epithelium, which is shear stress activated [27], and the Acid Sensing Ion Channels (ASIC), that are expressed in the central and peripheral nervous systems. ASIC2 is expressed in the autonomic neurons innervating the aortic arch and is required for the baroreceptor and autonomic control of the circulation [28].

The mechanosensitive potassium channels

Three channels belonging to the “two pore domain” K^+ channels ($\text{K}_{2\text{P}}$) family have been deeply studied about their mechanosensitive properties: TREK-1 (TWIK-1 related K^+ channel), TREK-2 and TRAAK (TWIK-related arachidonic acid-stimulated K^+ channel). Several in depth electrophysiological, biochemical and structural studies have shown that these three channels are directly gated by the lipid membrane tension. Their physiological roles are not yet fully understood but they are expressed in the dorsal root ganglia (DRG) and would tune the activity of mechanosensitive neurons [16].

The transduction channels in hair cells

In the inner ear, hair cells convert mechanical forces from sound waves into electrical signals that are relayed to the brain. They have been widely studied as prototypical mechanotransducers. They contain stereocilia bundles on their apical surface that are connected to each other by small strands called tip links, in a typical tethered model. The physical strain induced by tip link movement is thought to gate a mechanically activated ion channel located at the lower end of each tip link. Four genes have been shown to be necessary components of the hair cell mechanotransducer complex: Transmembrane Channel Like proteins 1 and 2 (TMC1 and TMC2), Tetraspan Membrane Protein of Hair Cell Stereocilia (TMHS), and Transmembrane Inner Ear Expressed Gene (TMIE) but despite many years of research the complete identity of the mechanotransducer complex remains unknown [16].

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The Piezo ion channels

The recently discovered Piezo channels are very large cations permeable channels (2521 and 2752 amino acids for human Piezo1 and human Piezo2, respectively) with numerous transmembrane domains. They do not share homology with other known proteins [20]. Piezo channels are inherently mechanosensitive as mechanical perturbations of the lipid bilayer alone are sufficient to activate Piezo channels [14]. A recent study has however also shown that Piezo1 could be activated by pulling on extracellular collagen IV [29]. In the presence of a constant stimulus, Piezo-mediated currents decay rapidly due to the channel inactivation.

In vertebrates, Piezo channels are required for mechanotransduction in a number of biological processes [14]. No homologs have been identified in bacteria or yeast, suggesting an evolutionary need for a novel mechanosensor in higher organisms [20]. They are abundantly expressed in a wide range of cells. Piezo1 is mainly expressed in nonsensory tissues exposed to fluid pressure and flow such as endothelium or kidney and bladder epithelia where it senses shear stress, or red blood cells where it participates to cell volume regulation. Piezo2 is predominantly found in sensory nervous tissues and is strongly involved in light touch sensation and proprioception [30]. The two channels differ on their electrophysiological properties, Piezo1 appearing as a polymodal sensor of diverse mechanical forces whereas Piezo2 activation seems rather limited to fine mechanical touch [20]. Human mutations in the genes encoding the Piezo channels have been identified in hereditary spherocytosis, congenital lymphatic dysplasia (Piezo1) and arthrogryposis (Piezo2) [20].

The TRP ion channels

The Transient Receptor Potential (TRP) channels were first described in *Drosophila melanogaster* (*D. melanogaster*), where photoreceptors carrying *trp* gene mutations exhibited a transient voltage response to continuous light. Despite large variety in functions this ion channel family, the more recently discovered channels were all named after these three letters. TRP channels are identified by sequence homology rather than gating mechanism, because their functions are disparate and still under study [31]. They have been implicated in the normal and pathologic physiology of many mammalian processes due to their ability to coordinate and integrate a spectrum of physical and chemical stimuli [31, 32]. Among them, several have been described in mechanotransduction mechanisms [17].

TRP channels subunits possess six transmembrane domains with a pore region between the fifth and the sixth transmembrane loops. They form cationic channels with special ion selectivity for each isoform [32]. Mammalian TRP channels subunits are structurally divided into six subfamilies: TRPC (7 members), TRPV (6), TRPM (8), TRPA (1), TRPP (3) and TRPML (3) (**FIGURE 3**). Subunits assemble as homo- or heterotetrameric polypeptides. TRP channels are almost ubiquitously expressed and most isoforms have splice variants, so that every cell has a panel of TRP channel proteins.

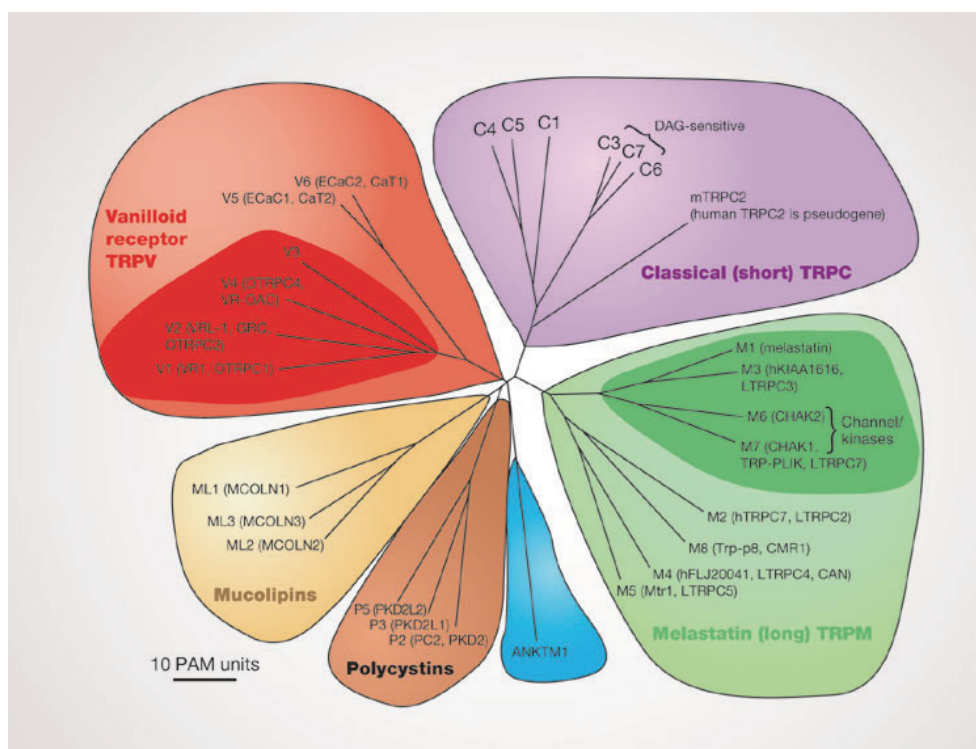


FIGURE 3. Mammalian TRP family tree. Reproduced from [31]

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Their known functions are diverse. They are the interface between a wide range of physical and chemical stimuli and the cell physiology. Some are activated by diverse botanical or inflammatory compounds, some are sensitive to intracellular messengers such as lipids or cytosolic calcium. In mammals, TRP channels allow for example to appreciate sweet, bitter and umami tastes, or to discriminate warmth, heat and cold. There is also evidence for a role of mechano- or osmosensitive TRP isoforms in the osmosensing, the regulation of cell volume and in other mechanical stimuli transduction. In many cases, the involvement of the TRP channels isoforms needs to be confirmed, and their exact role in the signaling process determined, whether it would be stimulus detection or transmission, or response amplification [15, 33].

Several TRP channels have been described as mechanosensitive. Electrophysiological studies have succeeded in activating TRP channels by stretching the cell membrane or by hypotonic shock. One important issue is that several of these data relies on models of heterologous overexpression. Indeed, it is difficult to study the electrophysiology of endogenous TRP channels due to their relatively low level of expression. At the level of the cell and organ physiology, deciphering the role of TRP channels is also complicated by their interaction with other proteins. It is also thought that redundancy and compensation between the different isoforms of TRP channels can occur in KO models. However, the finding of so many mechanosensory candidates in this channel superfamily suggests that they are critical for mechanical signaling [2].

The classical TRPC channels are the most closely related to the *D. melanogaster* TRP. They are mainly involved in store-operated calcium entry, and can be activated by intracellular messengers, G-protein-coupled receptors or tyrosine kinase receptors. Some TRPC channel isoforms have been suggested to present mechanosensitive properties [31]. TRPC1 has been shown to form a stretch-activated ion channel in *Xenopus* oocytes, but this could not be reproduced in mammalian CHO cells [34, 35]. TRPC3 and TRPC6 as homo- or heteromultimers can confer mechanosensitivity, with variable results and gating mechanisms depending on the cellular model. When co-expressed in a neuron cell line they were mechanosensitive. Mice deficient in TRPC3 and TRPC6 exhibited impaired non-nociceptive mechanosensation [25]. Physiologically, TRPC6 is also involved in arterial myogenic tone [36]. In kidneys, TRPC6 is expressed in podocytes where it is activated by mechanical stimuli. Interestingly, gain of function mutations in TRPC6 have been implicated in hereditary nephrotic syndrome [37]. Hypoosmotic- and pressure-induced membrane stretch

activate TRPC5 channels in HEK cells [38], and stretch activation of TRPC5 has been described on rat aortic baroreceptor neurons [39].

The TRPV channels were named after their first member TRPV1 showed activation by vanilloid compounds such as capsaicin, the active compound of pepper. TRPV1-4 channels share the common property of being heat sensitive. TRPV1 is mainly expressed in the nervous system, where it plays a major role as a nociceptor in nerve endings. While mechanosensitivity of the channel has not really been studied, it might be osmosensitive [40]. In the central nervous system, it has been shown to regulate the activity of osmosensory neurons upon osmotical stimulation [41]. The activation of TRPV2 by hypotonic shock or membrane stretch is more documented [15]. Very fast (<30 ms) TRPV2 activation was achieved in alveolar cells [42]. It is also expressed in smooth, cardiac and skeletal muscle. TRPV2 participates to the toxic Ca^{2+} overload of skeletal muscle cells in Duchenne's dystrophy [43]. TRPV4 will be extensively discussed below.

There are only few reports on the sensitivity of TRPM channels to mechanical stimuli or changes in cell volume [15]. TRPM3, which is expressed in brain, pancreas and kidney, displays cell volume-regulated activity when overexpressed in HEK cells [44]. TRPM7, that conducts preferentially Mg^{2+} over Ca^{2+} , is ubiquitously expressed. It has been activated in HEK cells by hypotonicity, shear stress and membrane stretch [15].

TRPA1, the only mammalian member of the TRPA subfamily, contains 14 N-terminal ankyrin repeat domains (ARDs), *i. e.* peptidic sequences that mediate protein to protein interaction, which possibly could serve as anchoring points for mechanical gating of the channel. It is activated by cold and by several botanical compounds such as mustard, cinnamon or garlic extracts [45]. TRPA1 is also activated by hypertonic solutions. Its main site of expression is sensory neurons, and it is mainly involved in nociception. TRPA1 is also expressed and could participate in mechanotransduction in the inner ear hair cells [15].

Among the TRPP subfamily, TRPP1 and 2 (better known as polycystins 1 and 2) are the products of the genes responsible for autosomal dominant polycystic kidney disease (ADPKD). The presence of TRPP proteins on renal epithelial primary cilia could render them capable of sensing fluid flow, osmolarity, and mechanical stretch [33]. TRPP2 is a 6 transmembrane domains ion channel whereas TRPP1 is less related to the classical TRP

Introduction

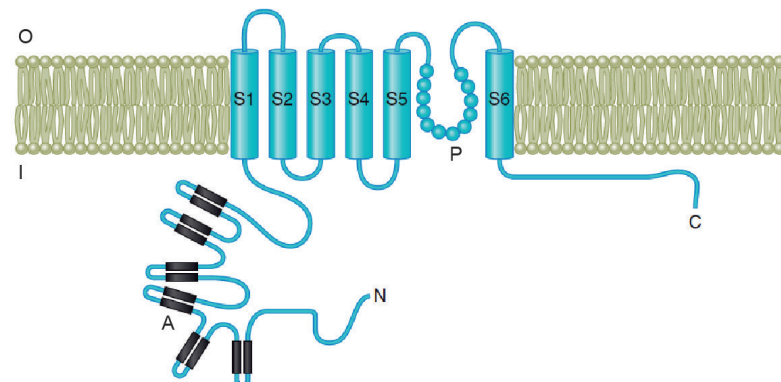
structure. Renal epithelial cells lacking functional TRPP1 or 2 form cilia but have an impaired fluid-flow sensation. It is suggested that the impaired mechanical stress sensing would impact the tissue morphogenesis in ADPKD [46]. However, evidence for direct mechanical activation of polycystins is still lacking. Instead, it has been shown that TRPP2 heteromerizes with TRPV4 to gain mechanosensitive properties. There would be a multiprotein cilium-associated mechanosensitive complex including at least TRPP1-2 and TRPV4 [47]. TRPP2 is also widely expressed in vascular endothelial and smooth muscle cell membrane, and flow-sensitive TRPP2-V4-C1 heterotetramers have been described in endothelium [48].

The osmo- and mechanosensitive TRPV4 ion channel

TRPV4 was first described in the early 2000's through homology screening as the mammalian homologue of OSM-9, a protein responsible for osmosensitivity and touch in *C. elegans*. [49-51]. It was found to be structurally related to the members of the TRPV channels family and was later classified as the fourth of the TRPV channels subfamily [52, 53]. Following the initial experiments performed on this ion channel, TRPV4 was the first mammalian ion channel clearly involved in osmosensing. In several TRPV4 transfected cell lines, it was found to trigger a large Ca^{2+} entry upon hypoosmotic challenge [49, 50]. What is more, expression of TRPV4 in OSM-9-deficient *C. elegans* restored hypertonicity and mechanical avoidance that were markedly impaired in this mutant. This observation was strongly suggestive of mechanosensitive properties of the TRPV4 channel [54].

Human as well as mouse TRPV4 monomer consists of 871 amino acids. It is formed by six transmembrane α -helices (S1–S6) crossing the lipid bilayer, linked by connecting loops of which the fifth is the pore-forming loop, and N- and C-termini that are localized in the cytoplasm (**FIGURE 4**) [55, 56]. TRPV4 normally assembles as a homotetramer, but heterotetramers have been reported in association with TRPC1 and TRPP2 monomers. The human TRPV4 possesses 5 splice variants affecting the N-terminus. The N-terminus that harbors six ARDs and the C-terminus are critical for correct channel assembly [57]. TRPV4 possesses several phosphorylation sites. One of them, the serine 824 (Ser 824), is interestingly located within an actin/tubulin-binding domain on the C-terminus. It has been shown that phosphorylation on the Ser 824 residue activates TRPV4, promotes its binding to actin over tubulin and is associated with focal plasma membrane localization [58].

FIGURE 4.
Representation of a TRPV4 monomer at the cell membrane. O, outer side of the cell; I, inner side of the cell; N, N-terminus; A, ankyrin repeat domains; S1-6, transmembrane segments; P, pore forming loop; C, C-terminus. Modified from [55]



Introduction

Within the TRPV channels family, TRPV4 displays stronger homology with TRPV1-3 than with TRPV5-6 (**FIGURE 3**). Differences in the TRPV4 amino acids sequence between species are small (human/mouse 95.2% identity and 96.9% similarity)[55]. TRPV1-4 channels are detected in the evolutionary tree of life as from teleosts, although TRPV ancestors are found in *C. elegans* and in *D. melanogaster*. This relatively late onset argues for very specific functions requirement. The highest purifying evolutionary pressure has been exerted over TRPV4, whereas TRPV2 sequences are the more divergent between species. TRPV1, TRPV2 and TRPV3 are located on the same chromosome, suggesting that the three isoforms originate from gene duplications. TRPV4 is located in another chromosome, indicating an earlier or distinct genetic variation event. The highly conserved TRPV domains are the ARDs, the membrane proximal domain, the transmembrane domains and the TRP domain, whereas divergence between the isotypes relies mostly in the N- and C-termini and the fifth pore-forming loop [59].

The TRPV4 ion channel is called polymodal because it responds to different types of unrelated stimuli. Although TRPV4 was originally considered to be a channel activated upon hypotonic cell swelling, functional characterization of the channel was greatly advanced by the discovery that the synthetic phorbol ester 4 α -Phorbol 12,13-didecanoate (4 α PDD) acts as a robust and direct channel activator [60]. Build on the properties of 4 α PDD, the search for possible endogenous TRPV4 agonists focused on endocannabinoids, a class of endogenous lipids that includes amides and esters of long-chain polyunsaturated fatty [61]. TRPV4 was successfully activated by anandamide and arachidonic acid (AA), via their metabolism by cytochromes P450 (CYP) and production of 5',6'-epoxyeicosatrienoic acid (5',6'-EET) [56, 62]. Besides, TRPV4 can also be activated by modestly elevated temperatures, lower than those required to activate the TRPV1-3 channels. TRPV4 is thought to be constitutively active at normal body temperature, potentiating other activating stimuli [61]. More recently, following a small molecule screening assay, GSK1016790A was identified as a novel TRPV4 channel activator. This very potent agonist activates mouse TRPV4 channels at a half maximal effective concentration of 18 nM [63]. Pharmacological blockers of the channel comprise the non-specific blocker ruthenium red (RR), and the specific blockers RN-1734 and HC-067047 [55].

Numerous studies have revealed that the physiological functions of TRPV4 are diverse. They share as a common point that TRPV4 activation produces a Ca²⁺ influx in the cells that is necessary for the activation of intracellular effectors [55]. From a physiological

point of view, TRPV4 is thus considered as a Ca^{2+} channel, with Na^+ relative permeability values of 6–10 for Ca^{2+} and 2–3 for Mg^{2+} . The TRPV4 currents are greatly diminished in absence of extracellular Ca^{2+} . TRPV4 is expressed in a broad range of tissues, including lung, spleen, kidney, testis, fat, brain, cochlea, skin, smooth muscle, liver, and blood vessels [56].

Facing the first osmosensitive mammalian channel, several groups rushed into investigating the gating mechanism of TRPV4 during hypotonic shock. Different ways of activation were proposed and the debate is not yet over.

As a cell bathed in hypotonic solution swells, the shortest way would be a direct mechanical opening of the channel by the membrane stretch. TRPV4 activation in response to membrane stretch in patch clamp experiments was demonstrated on a large study (>200 patches) in a heterologous expression system in *Xenopus laevis* oocytes, although in pH and voltage conditions promoting TRPV4 gating [64]. A recent study tempers this result, demonstrating that it is not an efficient mechanical stimulus for gating TRPV4 in physiological conditions [23]. Besides, another recent study proposes that TRPV4 is a volume rather than an osmolarity sensing channel, as activation of TRPV4 could be achieved by changing cell volume without the use of osmotic gradients [65].

Other researchers plead for an indirect activation of TRPV4 upon hypoosmotic stimulation. As mentioned above, in addition to their effect on membrane tension, osmotic challenges are known to induce cytoskeletal and enzymatic activation, where TRPV4 could not be the primary mechanotransducer itself. It was proposed that hypotonic stimulation would activate TRPV4 through phosphorylation [66], but this observation was later contradicted [61]. Besides, it has been shown that TRPV4 is activated by phospholipase A_2 (PLA_2)- and CYP- dependent production of 5',6'-EET, an endogenous lipid that is produced during hypotonic shock [61].

Mammalian TRPV4 has been described as an osmosensor in several physiological processes. The role of TRPV4 as a sensor of extracellular hypotonicity is well-established, but its activation by extracellular hypertonicity has been reported by a few studies, although not at the cellular level [55]. Nociceptive C fibers from DRG neurons were stimutable by hypotonic, but also hypertonic stimulation in a TRPV4 dependent manner [67, 68]. TRPV4 was also described as an osmosensor in the airway smooth muscles [69] and in renal epithelial cells [70]. Its role in systemic osmoregulation will be detailed in the results and discussion sections.

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TRPV4 has been shown to be necessary for the regulatory volume decrease (RVD), a process that allows cell to recover from swelling in a hypotonic environment and that is Ca^{2+} -dependent. This process is of particular importance in epithelia subjected to hypoosmolar liquids like keratinocytes, intestinal and renal epithelial cells, and in glial cells that participate to brain volume balance [71, 72].

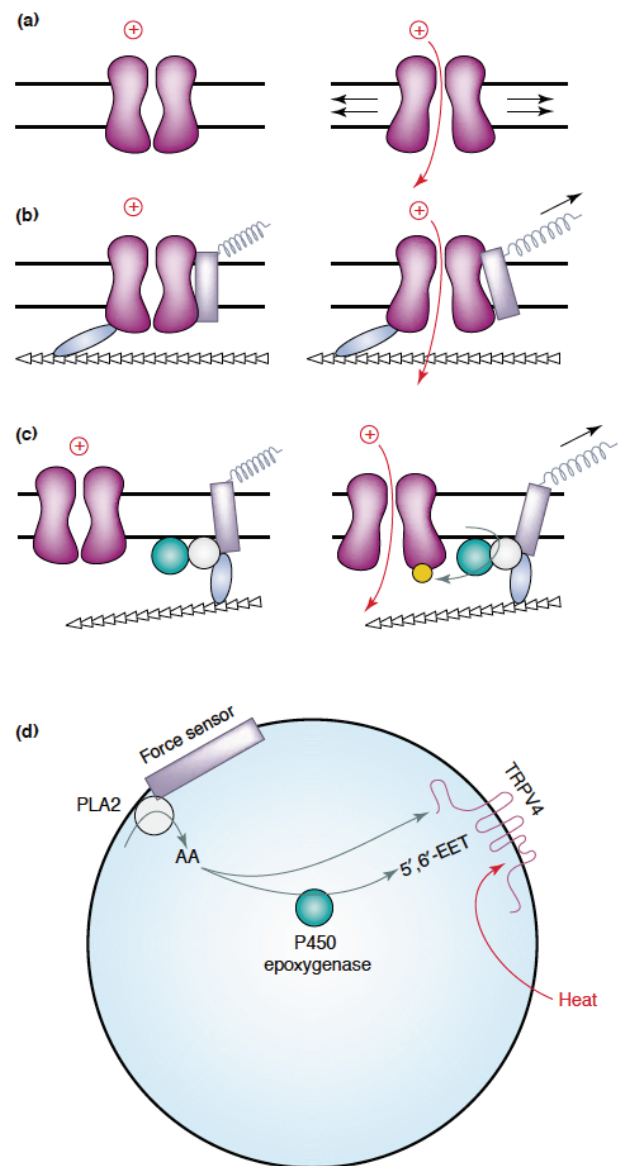
Beyond cell volume regulation and osmosensation, several mechanical stimuli deforming the cell membrane have clearly demonstrated a physiological role of TRPV4 in mechanosensation. As the exact TRPV4 gating mechanism even during the well-studied hypotonic shock is not certain, the mechanism with which TRPV4 responds to pure mechanical forces has still to be demonstrated (**FIGURE 5**). At the molecular level, the gating mechanisms could be the same as the ones involved in TRPV4 activation during hypoosmotic challenge. TRPV4 could be directly activated by membrane stretch [64]. The mechanical activation of TRPV4 could be downstream of a PLA_2 -dependent pathway, as observed after the osmotic stimulus. In this line, shear stress was shown to cause an endothelial dependent vasodilation through PLA_2 and TRPV4 activation [73]. Membrane stretch and endogenous lipids could actually be related stimuli, both altering the forces within the lipid membrane. In another model, ultra-rapid activation of TRPV4 (within milliseconds) was observed by magnetically twisting beta-integrin in AJs, but not by twisting other transmembrane proteins, pleading for a direct physical link between the channel and cytoskeleton proteins and not a deformation of the lipid membrane [13]. In line with this hypothesis, TRPV4 was found in the AJs of the urothelium [74] and in intercellular junctions between keratinocytes, and it was coimmunoprecipitated with catenin, a protein that links transmembrane cadherin to the actin cytoskeleton [75]. Recently, an electrophysiological study comparing the effectiveness of different *in vitro* mechanical stimuli on TRPV4 gating in chondrocytes [23] showed that the most effective stimulus was a membrane deformation at a cell-substrate contact point, most probably an AJ. The latency was $<2\text{ms}$ and the activation was independent of the PLA_2 activity, indicating a direct mechanical gating. Two other stimuli, pressure-induced membrane stretch in the patch pipette or cell indentation produced almost no TRPV4 gating. These results show how mechanosensitivity depends on the membrane domain properties and the type of stimulus, and highlight a role of TRPV4 in the intracellular transduction of stresses originating in the ECM. However, the identity and the functions of the putative tethers are still to be demonstrated. The mechanosensitive TRPV4 structure to function relationship is still unknown. N-terminal ARDs are potential interaction

sites with cytoskeleton and linking proteins. Although they were described later as critical for correct multimerization and trafficking of the channel [57], Liedtke *et al.* studied the function of a mutated TRPV4 lacking the whole N-terminus (half of the protein). The channel was surprisingly present at the plasma membrane and responded to hypotonic shock, although less robustly than the intact variant [50]. Interestingly, the C-terminus was shown to bind actin and tubulin [76], and this interaction is modulated by the phosphorylation of Ser 824 [58].

TRPV4 can form heteromultimers with TRPP2 and TRPC1. Such complexes are found in the primary cilium of distal renal epithelial cells (TRPV4-TRPP2) or in the vascular endothelial and smooth muscle cell membrane (TRPV4-TRPP2-TRPC1), where they show mechanosensitive properties [47, 48].

FIGURE 5. Various mechanisms proposed for the mechanical gating of TRPV4 ion channel.

a. Direct activation through modification of the lipid bilayer tension. b. Direct activation through deformation of intracellular, transmembrane or extracellular linking proteins. c. Indirect activation via mechanosensitive phosphorylation or second messenger. d. Description of the PLA₂-mediated activation of TRPV4, a mechanism proposed for TRPV4 osmosensing. TRPV4 is also activated by warm temperature. Reproduced from [77]



Introduction

At the physiological level, TRPV4 plays a role in several processes that involve cell stretch or compression, fluid shear stress on cell surfaces, or deformation of subcellular elements.

TRPV4 is expressed in small diameter neurons endings. *Trpv4*^{-/-} mice had a reduced sensitivity to tail compression and to chemical peritoneum irritation. TRPV4 thus seems to be involved in pressure and pain stimuli detection [78].

TRPV4 is expressed osteoblasts and osteoclasts and controls their differentiation and activity [79, 80]. Its role as a mechanosensor has been highlighted by the fact that mice lacking TRPV4 are protected against osteopenia upon unloading of mice hindlimbs [81]. The critical role of TRPV4 in skeletal development was demonstrated through the discovery of a TRPV4 gain-of-function mutation associated with a familial form of brachyolmia [82]. TRPV4 also plays a role of mechanosensor in the chondrocytes [23], where it regulates the metabolic response to mechanical loading [83].

TRPV4 is expressed in the endothelial and in the vascular smooth muscle cells. Its major role in vascular tone control and blood pressure regulation, observed in several studies on pressurized arteries, will be detailed in the results and discussion sections.

TRPV4 is expressed in the urothelium, where it is functionally connected to the AJs, and it is responsible for a calcium entry upon cell stretch [74, 84, 85]. Physiologically, TRPV4 participates in sensing modifications in bladder filling as mice lacking TRPV4 displayed impairment of bladder voiding [86, 87].

The renal tubular epithelium is a major site of TRPV4 expression. TRPV4 has been described as a flow sensor at the apical membrane of the distal tubule [70, 88]. Its role in the renal physiology will be detailed in the results and discussion sections.

Mutations in the human *TRPV4* gene are associated with hereditary diseases called *TRPV4* channelopathies. These affect the skeletal and the peripheral nervous systems with highly variable phenotypes. Skeletal affections consist in skeletal dysplasias mainly characterized by a short trunk with vertebral platyspondyly, scoliosis and defective ossification, or familial digital arthropathy brachydactyly that only affects fingers and toes [89]. Neurological affections comprise motor and sensory neuropathies, such as congenital distal spinal muscle atrophy, scapuloperoneal spinal muscle atrophy and Charcot - Marie - Tooth disease type 2C [90]. Some patients present mixed skeletal and neurological diseases. Why these two systems are specifically affected is unclear. The numerous reported mutations are spread over the whole *TRPV4* gene. In many cases, mutations associated with skeletal

dysplasias or neuropathies were reported to cause an apparent gain of channel function. However, loss-of-function mutations have also been reported. The relationship between the alteration of the channel function and the disease phenotype remains unclear [91].

Mechanotransduction in physiological systems: three examples

Mechanical forces at play in the kidney

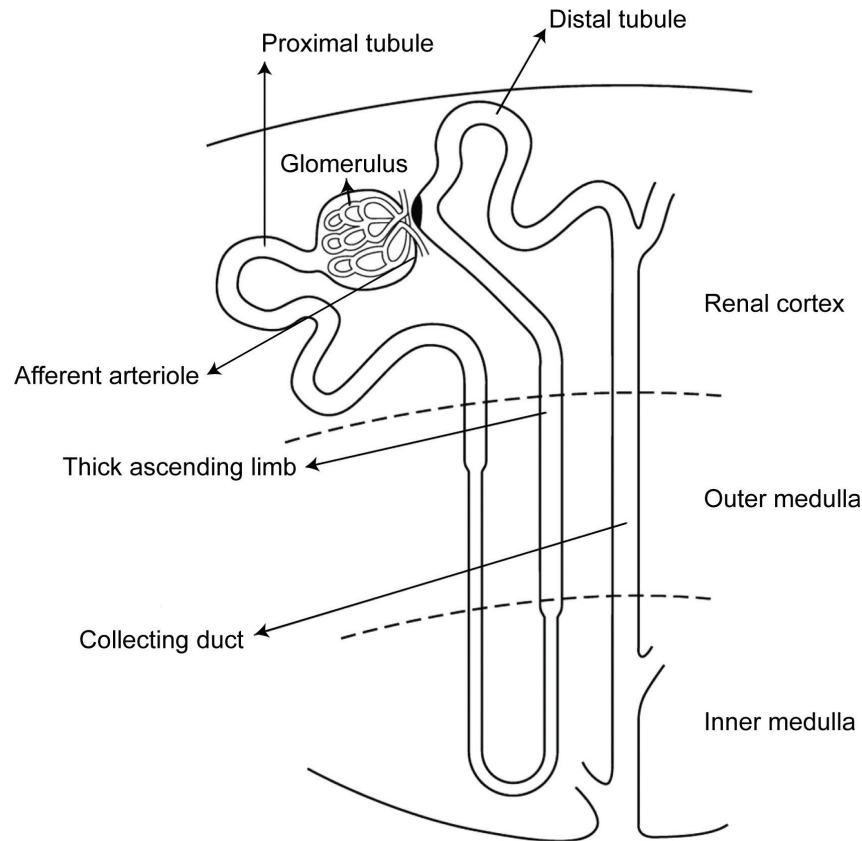


FIGURE 6. Anatomy of the renal nephron. Arterial blood carried by the afferent arteriole is filtered in the glomerulus through slit diaphragms formed by the podocytes. The glomerular ultrafiltrate is propelled through the PT and the final urine is formed by its modification along the successive nephron segments.

Kidneys are complex, highly organized organs. In humans, they are formed by nearly 1 million nephrons each (**FIGURE 6**) and they process about 180 liters of plasma daily to keep whole body homeostasis, under the dependence of external hormonal controls and internal autoregulatory reflexes. The latter rely on local chemical and physical signals among which mechanical forces such perfusion pressures, fluid shear stresses or osmotic gradients. To achieve their work, the nephron exploits mechanisms able to sense dynamic changes in composition and flow rates of protourine along the renal tubule.

Blood carried by the afferent arterioles enters the glomerular capillaries, and the plasma is filtered through slit diaphragms formed by specialized glomerular cells, the podocytes, to generate an ultrafiltrate that enters the lumen of the proximal tubule (PT).

Podocytes are exposed to tensile stress and to fluid shear stress arising from plasma filtration through the narrow slits. Several proteins could act as mechanosensors in podocytes converting mechanical force via a conformational change into a biochemical signal, such as the TRPC6 ion channel, and cytoskeleton, slit diaphragm and cell-matrix contact proteins. It was shown that podocytes respond to stretch as well as to fluid shear stress resulting from increasing renal arterial perfusion pressure by lengthening their foot processes [92].

The glomerular ultrafiltrate is propelled through the PT. This flow through the PT is variable according to the glomerular filtration rate (GFR). The role of flow in regulating reabsorption across the PT epithelium has been demonstrated that for Na^+ , HCO_3^- , K^+ , and water. The apical surface of PT cells senses changes in shear stress through their microvilli and primary cilia, and integrins and stretch-sensitive channels on the basolateral surface can transduce changes in radial stretch into physiological responses [93].

Distal segments of the nephron are also exposed to osmotic and mechanical stimuli. Depending on the hormonal status, they are partially or completely exposed to a hypotonic luminal milieu, to which they respond with elevations of $[\text{Ca}^{2+}]_i$. A proposed role for these Ca^{2+} signals is the onset of regulatory volume changes. Additionally, virtually all aspects of electrolyte transport, including K^+ secretion, Na^+ reabsorption and, Ca^{2+} influxes were known to be flow-sensitive in the distal nephron [94].

Blood pressure regulation

The cardiac and vascular smooth muscles produce forces through the circulatory system that allows correct systemic and local tissue perfusion. At the systemic level, two major systems control and coordinate these effectors: the autonomic nervous system and the hormonal renin-angiotensin system (RAS). The autonomic nervous system, whose central nuclei are located in the brain stem, controls blood pressure via heart, vessels and adrenal sympathetic and parasympathetic innervation. The RAS is a hormonal system where angiotensin II (AT2) is the main effector on the cardiovascular system and renin, that is secreted by the juxtaglomerular (JG) cells in the renal afferent arterioles, is the rate-limiting

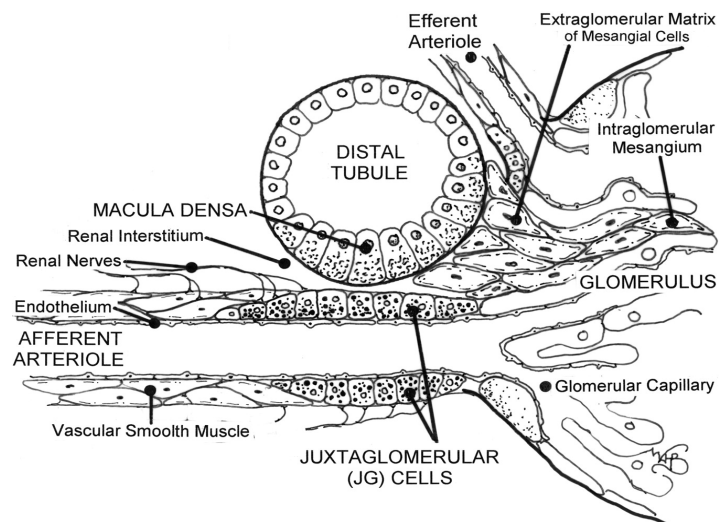
Introduction

enzyme. In order to maintain blood pressure homeostasis, a feedback on these systems is needed. The principal feedback mechanism takes place in mechanotransduction sites located within the circulatory system, where stretch of the elements of the circulatory system senses blood pressure. These sites are called the baroreceptors and are located in the carotid sinus and the aortic arch (the arterial baroreceptor) for the autonomic nervous system, and in the kidney vasculature (the renal baroreceptor) for the RAS.

The cells responsible for the arterial barosensitivity are the autonomic nerves efferences innervating the carotid sinus and the aortic arch, and the identification of molecular sensor has recently advanced. Several studies have explored the molecular nature of baroreceptor mechanosensors and suggested the participation of different ion channels, including acid-sensing ion channel 2 (ASIC2), epithelial sodium channel γ -subunit, TRPV1 and TRPC5 in baroreceptor functions [39, 95, 96].

However, much less is known about the molecular and even the cellular identity of the renal baroreceptor. The RAS is a fundamental blood pressure regulation system. It gained interest with the identification of patients suffering from hypertension related to renal artery partial obstruction, and the characterization of the various properties of the AT2 hormone [97]. Then it was established as a major physiological system. It relies on a hormonal cascade where the rate-limiting enzyme is renin. As a hormone as well as an enzyme at the same time, renin has its secretion regulated by several factors, and exerts its main physiological role as a protease that cleaves circulating angiotensinogen into angiotensin I (AT1). Renin is stored and secreted into circulation from renal JG cells, which are modified vascular smooth muscle cells of the renal afferent arteriole adjacent to the glomerulus (**FIGURE 7**). Although also stimulated by renal sympathetic nerve activity, circulating factors (AT2, endothelin) and distal urinary Na^+ concentration (via the tubuloglomerular feedback exerted by the *macula densa* in the distal tubule), its major secretion control mechanism is the renal baroreceptor.

FIGURE 7. Representation of the anatomic localization of the JG cells. JG cells are located in the wall of the renal afferent arterioles, where they are subjected to variations in blood pressure. They are also subjected to autonomous innervation and tubuloglomerular feedback via the *macula densa*. Reproduced from [98]



Based on experimental evidences, there is a consensus that the baroreceptor is located in the renal vasculature. In a drastic experiment, enhancement of renin secretion by lowering renal perfusion (by hemorrhage or suprarenal aortic obstruction) was still achievable in non-filtering denervated kidneys from adrenalectomized dogs [99]. The renal baroreceptor follows a pressure response relationship, with the renin secretion varying in reverse of the renal perfusion pressure [100]. However, it is not yet established whether the baroreceptor lies directly in the JG cells or in other elements of the renal vasculature, such as endothelial cells. Interestingly, the gap junction-forming protein connexin 40 was shown to be necessary to the pressure control on renin secretion and corrected positioning of the JG cells in the afferent arteriole wall [101].

Unlike most secretory cells, renin secretion from the JG cells is inversely related to $[Ca^{2+}]_i$, as illustrated by numerous experiments on isolated JG cells or perfused kidney [102]. Moreover it was shown that an increase in perfusion pressure was paralleled by a rise in $[Ca^{2+}]_i$ in JG cells, which was dependent on the presence of Ca^{2+} in the perfusion medium. The Ca^{2+} entry was blocked by gadolinium (Gd^{3+}), a mechanosensitive ion channels blocker [103]. It is then realistic to hypothesize that a mechanosensitive ion channel would mediate Ca^{2+} entry in JG cells, transducing the pressure level in an inhibitory Ca^{2+} -mediated feedback on renin release.

Introduction

Systemic osmoregulation

As early as in bacteria, metabolism was delimited by semi permeant membranes subjected to osmotic forces, resulting from the uncoupling of transmembrane osmolyte gradients from passive solvent fluxes. Detection and adaptation to osmotic stimuli was important as metabolism creates osmolytes that increase intracellular tonicity, and exposition to a hypotonic environment (like during rain) induces cell swelling and lysis. This explains why mechanosensitive ion channels are found in the membrane of bacteria.

In complex pluricellular organisms, cells are surrounded by an extracellular fluid with which they equilibrate their osmolality. In mammals, regulation of the extracellular medium osmolality is systematically regulated around 300 mOsm in a very conserved way, as it influences ionic strength and cell excitability, but also cell volume, a vital parameter in animals bearing a brain that is encased in a rigid cranium [104]. The stable systemic osmolality actually reflects a dynamic equilibrium. As in every homeostatic process, effectors lowering and rising extracellular osmolality are constantly active, ultimately orchestrated by central osmosensors. Fluctuations of the systemic osmolality result from intake or loss of water and sodium, which is the main extracellular osmolyte. Extracellular fluid hyperosmolality enhances thirst sensation to stimulate water intake, and raises vasopressin (VP) secretion, a hypothalamic antidiuretic hormone that promotes water reabsorption in the kidney. A hypoosmolar stimulus has the opposite effects [104].

The bases of central osmoregulation were established in the middle of the 20th century. Fundamental experiments showed that infusion of hypertonic saline in dog carotid artery was impacting their urinary volume. This effect was greatly diminished after posterior hypophysis removal, recognized as the site of antidiuretic hormone secretion, or internal carotid artery ligation, localizing the central osmosensing nuclei in the anterior hypothalamus [105].

The detailed anatomical localization of osmosensory neurons is complex (**FIGURE 8**). Such neurons have been identified in several hypothalamic regions: the organum vasculosum laminae terminalis (OVLT), the subfornical organ, and the magnocellular neurosecretory cells (MNCs) of the supraoptic (SON) and paraventricular (PVN) nuclei. The OVLT, an area comprised in the anterior wall of the third ventricle that lacks blood brain barrier, appears to be the most upstream osmosensitive nucleus as it projects to anterior cingulate and insular cortex, eliciting thirst sensation and behavioral responses, and to the

SON and the PVN [104]. VP is synthesized in the somata of MNCs in the SON and the PVN, where they coexist with oxytocin-releasing MNCs and parvocellular neurons. The MNCs project their axons to the posterior pituitary where. Their terminals, where VP is stored in vesicles, abut fenestrated capillaries. Osmotic changes are transduced at the MNCs somata, modifying the action potential discharge in their axon that eventually drives Ca^{2+} -dependent exocytosis of VP vesicles in the circulation [106].

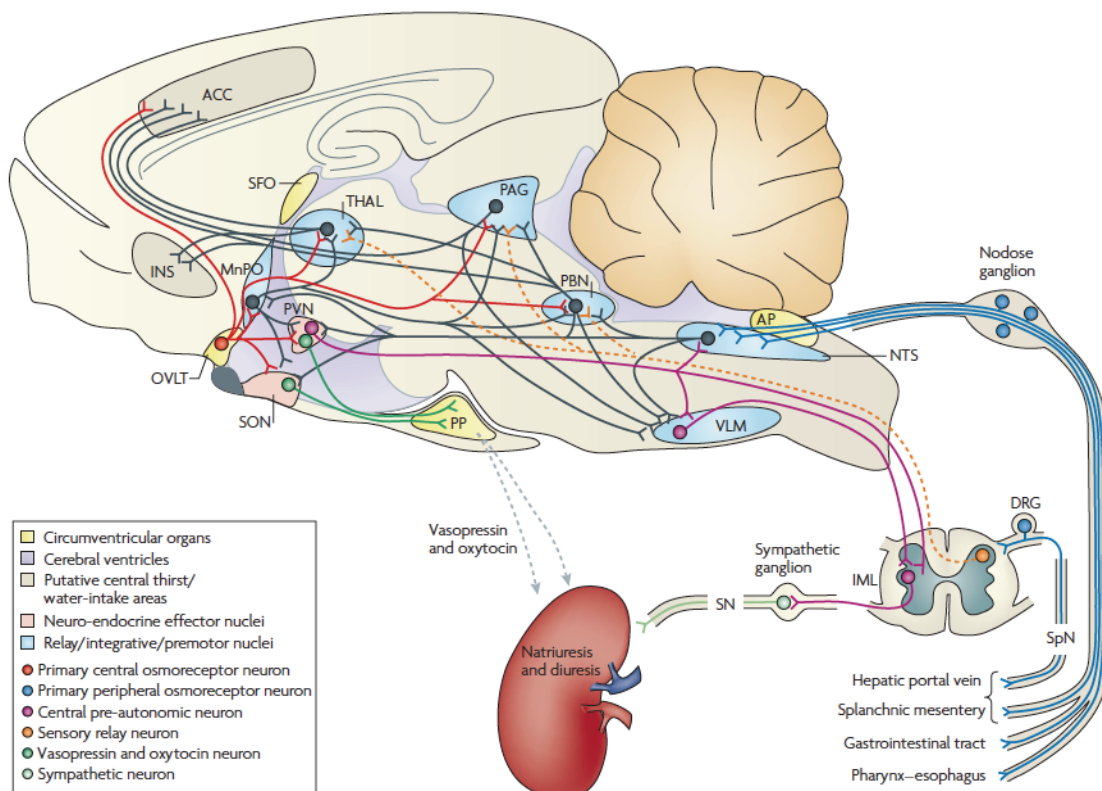


FIGURE 8. Osmoregulatory circuits in the mammalian brain and the periphery. ACC, anterior cingulate cortex; AP, area postrema; DRG, dorsal root ganglion; IML, intermediolateral nucleus; INS, insula; MnPO, median preoptic nucleus; NTS, nucleus tractus solitarius; OVLT, organum vasculosum laminae terminalis; PAG, periaqueductal grey; PBN, parabrachial nucleus; PP, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SN, sympathetic nerve; SON, supraoptic nucleus; SpN, splanchnic nerve; THAL, thalamus; VLM, ventrolateral medulla. Reproduced from [104]

Many subsets of neurons are osmosensitive in the central nervous system, but to qualify as osmosensors they must transduce the changes in extracellular osmolality into changes in action potential firing. Recordings on isolated neurons and hypothalamic slices have shown that the basal electrical activity of these cells effectively encodes the osmotic set point, and is proportionally excited by hypertonic stimuli and inhibited by hypotonic stimuli. These changes are encoded through proportional changes in the probability of opening of

Introduction

non-selective cation channels [104]. Although it is unknown whether all osmosensory neurons use the same type of transduction mechanism, the regulation of nonselective cation channel activity seems to encode increases and decreases in fluid osmolality and likely contributes to osmotic detection *in vivo* [106].

Electrophysiological recordings from acutely isolated OVLT and SON neurons were used to examine the effects of volume changes induced by osmotic stimuli and changes in patch pipette pressure. The modulation of the basal cation conductance upon osmotic stimulation could be reversed by an opposing pressure in the pipette, demonstrating that osmosensory transduction is essentially a mechanical process [107]. Indeed, unlike many cells that resist changes in volume through regulatory mechanisms, hypothalamic osmosensory neurons measure osmolality with their volume: they display changes in volume that are inversely proportional to extracellular osmolality and that can be sustained for many minutes, as opposed to neurons from other brain regions that neutralize tonicity-induced swelling or shrinkage by regulatory volume correction [108]. In addition to mechanosensitive cation channels, actin filaments are required for osmosensory transduction in MNCs. Their coupling to the osmosensitive ion channels is still unknown: scaffolds in specialized deformable membrane domains, tethers physically linked to the ion channel or to an intermediate mechanosensitive enzyme [104].

Beside the central, major osmosensors, peripheral osmosensitivite neurons have been described in the oropharyngeal cavity, the digestive tract, the splanchnic mesentery, the portal vein and the liver. They offer a buffering effect on thirst and VP secretion in anticipating the central response to ingestion of hypotonic or hypertonic food. The cellular identity of the peripheral osmosensors seems to consist in nerves endings innervating hepatic blood vessels neurons from the thoracic DRG [109].

AIM OF THE THESIS

The aim of this thesis is to better understand the involvement of the TRPV4 ion channel in osmoregulation and mechanotransduction in the fields of systemic osmoregulation and renal functions.

Osmosensitivity is a typical feature of the TRPV4 ion channel. It is expressed in the hypothalamic osmoregulatory nuclei and its participation in systemic osmoregulation has been suggested. However, TRPV4 is also expressed, and at a much higher level, in the kidney. In this organ, where water and ion flows are constantly modulated, the role of TRPV4 remains unclear. Its osmosensitive as well as mechanosensitive properties suggest that its activity could have an impact on several renal functions. We first aimed to characterize the localization of TRPV4 along the renal tubule. We then studied the regulation of water and sodium balance in *Trpv4*^{-/-} mice, investigating kidney and urine parameters, and central responses to hyperosmolar states. Following our observations on the *Trpv4*^{-/-} mice, we constructed a conditional neuronal *Trpv4* knockout mouse line in order to better understand the role of TRPV4 in the hypothalamic osmoregulatory nuclei.

We reported TRPV4 expression in the PT, which was so far unknown. We thus investigated further the phenotype of the *Trpv4*^{-/-} mouse in order to reveal its role in this part of the nephron, and we sought to understand the role of TRPV4 *in vitro* in PT cells cultures.

We also addressed the role of TRPV4 as a mechanosensor in JG cells, where we reported its expression. This particular renal cell population secretes renin, a blood pressure regulating hormone, whose secretion is known to be calcium- and mechanosensitive.

RESULTS

In the results section, which contains four chapters, we present experimental results obtained during the thesis.

Chapter I, entitled « **TRPV4 is associated with central rather than nephrogenic osmoregulation** », has been published as an article under the same name. It aims to describe the renal localization of TRPV4 and investigate the role of renal and central TRPV4 in systemic osmoregulation.

Chapter II, entitled « **Role of TRPV4 in the proximal tubule** », presents unpublished data exploring the role of TRPV4 in this nephron segment.

Chapter III, entitled « **TRPV4 participates in pressure-induced inhibition of renin secretion by juxtaglomerular cells** », has been published as an article under the same name. It focuses on the TRPV4-mediated mechanosensitivity of the JG cells and its impact on renin secretion. At the end of this chapter, we show interesting additional unpublished data that were not part of the published article due to length restriction.

Chapter IV, entitled « **Implication of TRPV4 in osmoregulation by hypothalamus neurons** », presents unpublished data describing the construction of a conditional TRPV4 knockout mouse line, and the first results obtained on a forebrain neuronal specific knockout of TRPV4.

CHAPTER I

TRPV4 is associated with central rather than nephrogenic osmoregulation

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Abstract

TRPV4 is a polymodal cation channel that is expressed in osmosensitive neurons of the hypothalamus as well as in tubular segments of the mammalian nephron. The segmental distribution and role(s) of TRPV4 in osmoregulation remain debated. To further analyze the physiological role of TRPV4, we investigated its renal distribution pattern and the functional consequences of its disruption in mouse models. Using reverse transcription and quantitative polymerase chain reaction (RT-qPCR) on microdissected segments, immunohistochemistry and a *LacZ* reporter mouse, we found that TRPV4 is abundantly expressed in the PT, the late distal convoluted tubule (DCT) and throughout the connecting tubule (CN) and collecting duct (CD), including principal and intercalated cells. TRPV4 was undetectable in glomeruli and of the thick ascending limb loop of Henle (TAL), and weakly abundant in the early DCT. Metabolic studies in *Trpv4*^{+/+} and *Trpv4*^{-/-} littermates revealed that the lack of TRPV4 did not influence activity, food and water intake, renal function and urinary concentration at baseline. Both *Trpv4*^{+/+} and *Trpv4*^{-/-} mice showed a similar response to furosemide, acute water loading, water deprivation, acid loading and dietary NaCl restriction. In the PT, specific activation of TRPV4 increased intracellular calcium concentration and modified endocytic function. No obvious malfunctions of the PT could be objectified at rest, *in vivo*. However, *Trpv4*^{-/-} mice showed a significantly lower VP synthesis and release after water deprivation, with a loss of the positive correlation between plasma osmolality and plasma VP levels, and a delayed water intake upon acute administration of hypertonic saline. This study reveals that, despite its abundant expression in proximal and distal nephron segments, TRPV4 does not play a major role in the kidney or its lack is efficiently compensated. Instead TRPV4 is critical for the release of VP, the sensation of thirst and the central osmoregulation.

Introduction

Osmoregulation is essential for every organism. In mammals, the homeostatic regulation of body water content involves a concerted action of the brain and the kidney. While the kidney is mainly an effector handling water, the control center is in the hypothalamus, where are located the most influential set of osmosensitive neurons [104, 110]. At the cellular level, ion channels are implicated in numerous osmotic and mechanical stimuli detection [9, 111]. Studies based on mouse models [54, 112] and human genetics [113] suggested that TRPV4 could be involved in systemic osmoregulation. However, although some evidence supported a physiological role of TRPV4 in the hypothalamic osmosensitive nuclei [54, 112, 114], others showed opposite results [41, 115]. Furthermore, the effect of TRPV4 gene disruption on NaCl and water handling by the nephron has not yet been studied.

TRPV4 is a cation channel that belongs to the vanilloid TRP subfamily [49]. TRPV4 responds to a large variety of stimuli, including osmotic cell swelling, mechanical stress, temperature, endogenous AA metabolites and phorbol esters [50, 56, 61, 62]. In view of its expression pattern in the brain, which includes neurons of the subfornical organ, the median preoptic area and the OVLT [50, 104, 114], TRPV4 is likely to play a role in osmoregulation. Indeed, these neurons are endogenously osmosensitive and project to MNCs in the SON and PVN of the hypothalamus [116, 117]. MNCs themselves possess intrinsic osmosensitivity [118, 119] and synthesize arginine VP, and they also express TRPV4 [114]. In response to increased plasma osmolality, VP is released from the posterior pituitary gland into the blood, and binds to the VP receptor type 2 (AVPR2) in the principal cells lining the CD of the kidney. This interaction leads to a signaling cascade causing phosphorylation and translocation of the water channels aquaporin 2 (AQP2) from intracellular vesicles to the apical plasma membrane, which facilitates osmotically-driven water reabsorption [120].

The role of TRPV4 in central osmosensors has been studied in two distinct *Trpv4* knockout mouse models [54, 112]. Under water deprivation, Mizuno *et al.* reported an exaggerated VP secretion in *Trpv4*^{-/-} mice while Liedtke *et al.* reported blunted VP secretion and thirst mechanism. Recently, using several hypertonic challenges, Kinsman *et al.* questioned TRPV4 implication in thirst sensation [115].

TRPV4 is abundantly expressed in the kidney [50, 121-123]. However, its localization along the nephron is controversial, possibly reflecting the lack of specificity of antibodies raised against TRP channels. A first study stated that TRPV4 was restricted to tubular segments constitutively impermeable to water: TAL, DCT and CD [121]. Along these

segments, TRPV4 was found in the basolateral side of the cells. Noteworthy, TRPV4 was absent from nephron segments exhibiting constitutive water permeability, *i.e.* the PT and descending thin limbs. A subsequent study showed that TRPV4 was restricted to CN and CD [122]. In view of these discrepant results, the physiological role of TRPV4 in the kidney remains unclear.

In order to clarify the role of TRPV4 in the kidney and in osmoregulation in general, we characterized the expression of TRPV4 in the mouse kidney by using RT-qPCR on isolated nephron segments, immunohistochemistry and a *Trpv4* reporter mouse model. We then evaluated the functional consequences of TRPV4 disruption in terms of renal function, general behavior and specific parameters related to the osmoregulation.

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Methods

Animals and sampling

Experiments were conducted on 12-week-old, male *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, generated by targeted deletion of exon 12 of the *Trpv4* gene [54]. The mice were backcrossed on a C57BL/6 background before starting the study. They were housed in light- and temperature-controlled room with *ad libitum* access to tap water and standard diet (AO3, SAFE, France). Basal activity was evaluated in individual ‘Physiocages’ (Panlab-Bioseb, Vitrolles, France) during 48 h after 24 h of habituation. Overnight urine collections were obtained in individual metabolic cages, after appropriate training. Blood was collected by venous puncture or decapitation (for VP measurement), plasma was stored at -20°C. Tissue samples were obtained after anesthesia with Sevoflurane (Abbott, Ottignies, Belgium) and were immediately processed for fixation and RNA/protein extraction. All protocols complied with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the local Ethics Committee.

Investigation of renal function

Mice were placed in metabolic cages to assess diuresis and renal handling of ions at baseline and during the following protocols. The urinary concentrating ability was tested after 24-h water deprivation. The capacity to excrete a water load was tested after intraperitoneal (IP) injection of sterile water (100μL/g) [124]. Parameters were also obtained after administration of a single dose of furosemide (10mg/kg; IP) or NaCl solution (0.5M; 0.8 mL/20 g; IP). The effect of Na⁺-depleted diet was investigated in *Trpv4*^{-/-} mice receiving deionized water *ad libitum* and a standard diet (1% NaCl) followed by a 0.01% NaCl diet for 17 days.

Analytic procedures

Sodium, potassium and chloride values were determined using a Kodak Ektachem DT60 II Analyzer (Johnson & Johnson, New Brunswick, NJ, USA). Other electrolytes, creatinine and urea were measured on a Synchron CX5 analyzer (Beckman Coulter, Fullerton,

CA, USA). Osmolality was measured on a Fiske osmometer (Needham Heights, MA, USA). Plasma VP levels were measured using EIA (Peninsula Laboratories, San Carlos, CA).

Microdissection of individual nephron segments and primary culture

Male *Trpv4*^{+/+} and *Trpv4*^{-/-} mice were decapitated, kidneys were removed and decapsulated. The dissection was done as described previously [125]. Mouse PT cells (mPTCs) culture and FITC-labeled albumin uptake assays were conducted as described previously [126].

Calcium measurement

mPTCs were incubated for 30 min at room temperature with 5 μ M Fura2-AM (Calbiochem, Camarillo, CA, USA) then washed for 1 hour in Krebs-HEPES buffer and mounted in the same medium on a Zeiss Axiovert 200 M inverted microscope (Zeiss Belgium, Zaventem, Belgium). Intracellular calcium ($[Ca^{2+}]_i$) in individual cells was measured using alternative excitation of Fura2-AM (0.5 Hz) at 340 and 380 nm using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument, Novato, CA, USA). Images were acquired with a Zeiss AxioCam camera coupled to a 510-nm emission filter and analyzed with Axiovision software.

Antibodies

Antibodies used for Western blotting and immunostaining are listed in **Supplementary TABLE 1**.

Western blotting

Extraction and immunoblotting of kidney samples were performed as described previously [127]. Normalization was obtained after membrane stripping and reprobing.

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Immunostaining

Mice were anesthetized with ketamine/xylazine (IP) and perfused through the left ventricle with PBS followed by paraformaldehyde-lysine-periodate fixative. 5µm thick cryosections (TRPV4) or sections from paraffin-embedded tissue blocks (AQP2, pAQP2 and NOS1) were incubated overnight at 4°C with primary antibodies. Binding sites were revealed by Cy3-conjugated goat anti-rabbit IgG antibodies. DAPI was used to counterstain nuclei. Sections were studied by epifluorescence on a Zeiss microscope.

Real-Time PCR

RNA extraction and RT-qPCR were performed as described previously [127]. For detailed conditions and primers, see **Supplementary Materials & Methods** and **Supplementary TABLE 2**.

Reporting of Trpv4 expression

A mouse strain was generated by homologous recombination inserting a *lacZ* reporter gene downstream the endogenous *Trpv4* promoter. Kidneys from 3-month-old heterozygous mice (*Trpv4*^{Wt/lacZ}) were isolated, cryoprotected and frozen sectioned (10µM). For β-galactosidase activity detection, sections were fixed in 2% glutaraldehyde, washed and stained overnight at 37°C in the PBS supplemented with 20mM K₃Fe(CN)₆, 20mM K₄Fe(CN)₆, 2mM MgCl₂ and 1mg/mL Xgal. They were then washed, and counterstained with Nuclear Fast Red. No staining was detected on sections from wild-type mice treated in the same conditions. For parallel immunostaining, frozen sections were fixed in methanol, then blocked and incubated with segment-specific antibodies. Binding sites were revealed using the AEC kit (Vector Laboratories).

Statistical analyses

Values are expressed as means ± standard error of the mean (SEM). Statistical comparisons were assessed by ANOVA or two-tailed Student's *t* test (GraphPad, San Diego, CA), as appropriate. *P* values of less than 0.05 were considered significant.

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Expression and distribution of TRPV4 in mouse kidney

We first investigated the segmental distribution of *Trpv4* mRNA in mouse kidney, using microdissected fractions characterized for segment-specific markers including podocin (*Nphs2*) for glomeruli, aquaporin 1 (*Aqp1*) for PT, $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (*Nkcc2*) for TAL, and *Aqp2* for CD (**FIGURE 1a**). The expression of *Trpv4* mRNA was detected in the convoluted (S1 to S2) and straight (S3) parts of the PT, as well as in the CD but neither in glomerulus and nor in TAL segments (**FIGURE 1b**).

The distribution pattern of TRPV4 was established by using a well-characterized antibodies that evidenced a single, specific 90-kDa band for TRPV4 by immunoblotting as well as a distinct tubular staining in wild-type *Trpv4*^{+/+} mouse kidney (**Supplementary FIGURE 1**). Immunofluorescence showed that TRPV4 was widely expressed in the mouse kidney (**FIGURE 2**). Based on co-distribution with segment-specific markers, TRPV4 was evidenced in the apical and basolateral membrane of PT cells (NaPi-2a; **FIGURE 2a**). In the distal nephron, a slight, basolateral staining for TRPV4 was detected in the first part of DCT, positive for the $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC), becoming more prominent in late DCT (**FIGURE 2b**) and continuing throughout the CN and the CD (AQP2; **FIGURE 2d**). Type A intercalated cells in the CD were clearly TRPV4 positive (H-ATPase; **FIGURE 2f**). However, intercalated cells in CN were weakly stained (**FIGURE 2e**). Importantly, TRPV4 immunoreactivity was absent from the TAL (NKCC2; **FIGURE 2c**).

These results were validated using a *LacZ* reporter mouse, allowing to assess the segment-specific expression of *Trpv4* by co-staining of β -galactosidase activity (**FIGURE 3a-e**). Confirming TRPV4 immunolocalization, the β -galactosidase activity detection reported *Trpv4* expression in the PT (AQP1), the DCT (NCC) and the CD (AQP2) but not in the glomerulus and the TAL (UMOD).

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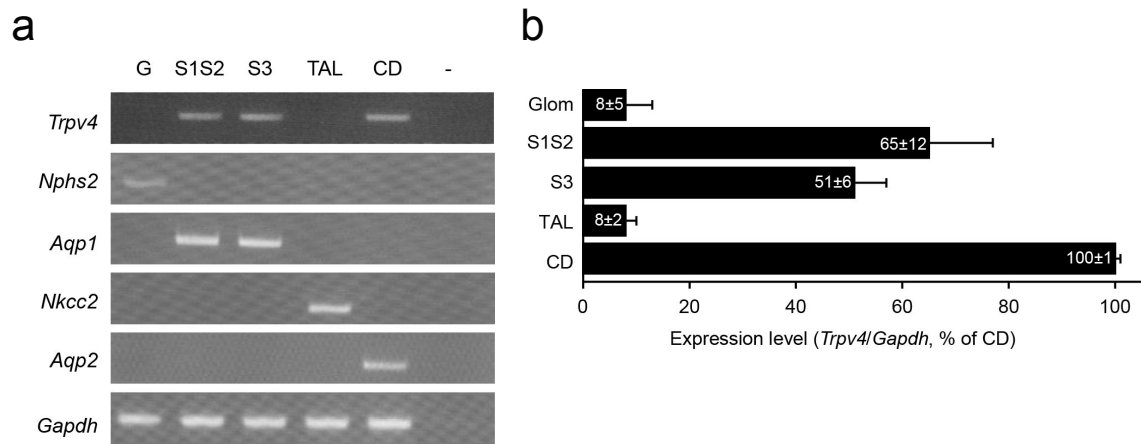


FIGURE 1. Differential expression of *Trpv4* in microdissected nephron segments. a. RT-qPCR of *Trpv4* mRNA with segment specific markers. *Nphs2* (Podocin) is expressed in glomeruli, *Aqp1* in PT, *Nkcc2* in TAL, and *Aqp2* in CD. RT-qPCR products were size-fractionated on 1.5% agarose gel. b. Semiquantitative RT-qPCR on microdissected segments demonstrates that *Trpv4* is expressed in the pars convoluta (S1S2) and the pars recta (S3) of the PT, and in the CD (n = 5 fractions)

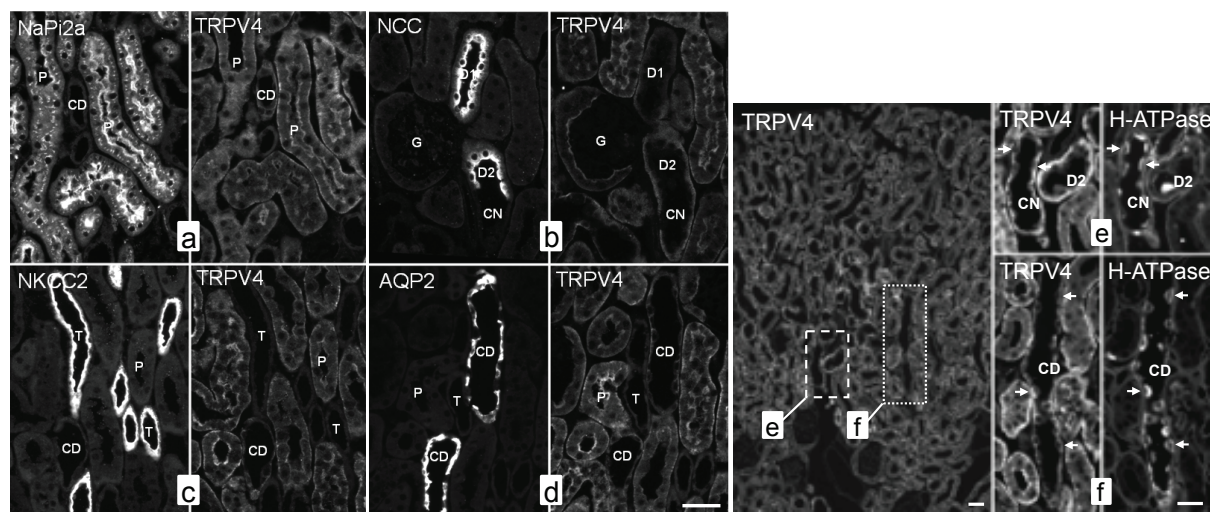


FIGURE 2. Parallel-staining of TRPV4 with different nephron segment-specific markers. a. Immunofluorescence staining of NaPi2a and TRPV4 showing coincident staining in PT (P). b. Immunostaining of NCC and TRPV4 depicting coexpression in early (D1) and late DCT (D2). TRPV4 immunoreactivity was absent in the glomerulus (G). c. Double staining of NKCC2 and TRPV4 showing absence of immunoreactivity in the TAL. d. CD show basolateral expression of TRPV4 and luminal AQP2 staining. e-f. Details of TRPV4 staining in the distal nephron. H-ATPase positive intercalated cells in CN are weakly stained (e). However, intercalated cells in CD are clearly TRPV4 positive (f). Scale bar = 50 μ m

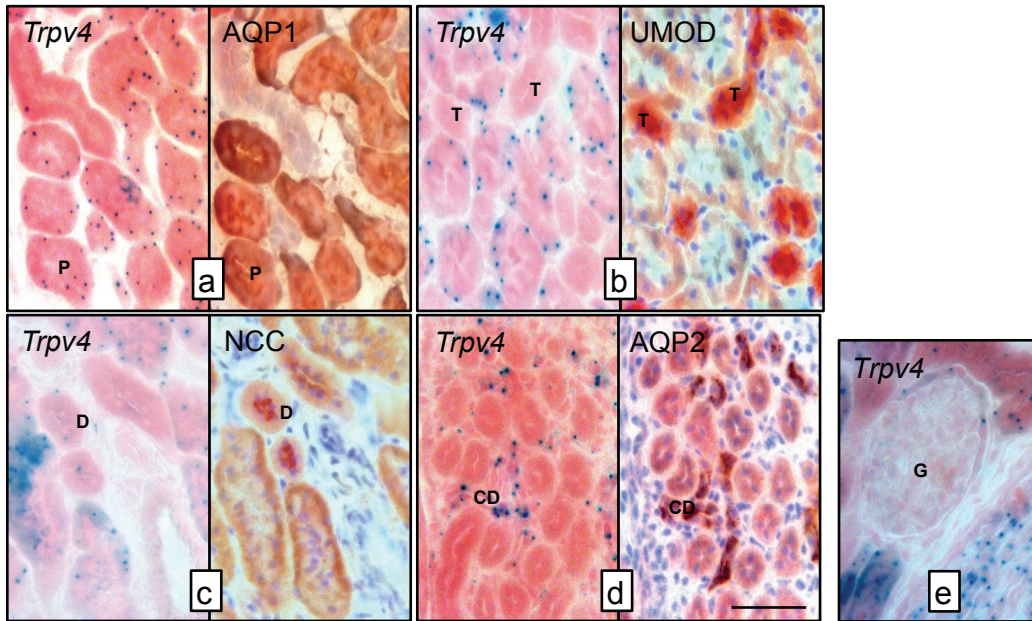


FIGURE 3. Segmental distribution of TRPV4 in the reporter mouse kidney. Serial sections of *Trpv4*^{Wt/lacZ} kidney (*Trpv4* expression is reported by beta-galactosidase staining) co-stained with segment-specific markers. a. Staining of *Trpv4* and AQP1 showing coexpression in PT (P). b. Staining of *Trpv4* and UMOD showing absence of expression in the TAL (T). c. Staining of *Trpv4* and NCC showing coexpression in the DCT (D). d. Staining of *Trpv4* and AQP2 showing coexpression in the CD. e. *Trpv4* is not expressed in the glomerulus (G). Scale bar = 50 μ m

Functional consequences of TRPV4 disruption

We next investigated whether the disruption of TRPV4 influences renal function in mouse. *Trpv4*^{-/-} mice had normal appearance, body weight, water intake and food intake, and showed no obvious behavioral abnormalities (testing in Physiocage, Panlab-Bioseb) as compared to *Trpv4*^{+/+} littermates (**TABLE 1**). Similarly, there were no significant alterations in the baseline biological parameters in plasma and urine (**TABLE 1**). Notably, *Trpv4*^{-/-} mice did not exhibit urinary loss of phosphate, glucose, calcium or low-molecular-weight protein (CC16), pleading against a major defect in PT function.

In order to test the physiological role of TRPV4 in the distal nephron, we administered a single dose of furosemide (10mg/kg; IP) to *Trpv4* mice (**FIGURE 4**). As expected, furosemide induced a strong diuretic response after 2 h, with decreased urine osmolality, and increased urinary excretion of Na⁺, K⁺ and Ca²⁺ (**FIGURE 4 a-e**). The response was similar in *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, suggesting functional integrity of the TAL and the CD.

We then evaluated whether the effect of sodium deprivation was affected by the deletion of TRPV4 (**FIGURE 4f-g**). *Trpv4* mice were fed a Na⁺-replete diet (1% NaCl) at baseline, followed by a Na⁺-depleted diet (0.01% NaCl) for 17 days. The dietary change resulted in

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very similar variations in the urinary excretion of Na^+ and K^+ in *Trpv4*^{+/+} and *Trpv4*^{-/-} mice both in terms of time-course and absolute levels during the sodium depletion test.

Because TRPV4 has also been detected in intercalated cells, we investigated whether the lack of TRPV4 could disrupt the ability to excrete ammonium during an oral acid load (**FIGURE 4h-i**). The response to both short-term (2 days) and long-term (6 days) NH_4Cl acid load showed that both the decrease in urine pH (**FIGURE 4h**) and the increase in urinary ammonium excretion (**Fig. 4i**) were similar in wild type and *Trpv4*^{-/-} mice. These data indicate that TRPV4 is not essential for the mineralocorticoid response or for the urinary acidification during metabolic acidosis.

Parameter	<i>Trpv4</i> ^{+/+}	<i>Trpv4</i> ^{-/-}	P
Body weight, g	21.81 ± 0.63	21.15 ± 0.49	0.42
Water intake, mL/g body weight	0.37 ± 0.01	0.39 ± 0.03	0.53
Food intake, g/g body weight	0.35 ± 0.04	0.38 ± 0.03	0.59
Activity, movements/48h	24177 ± 4988	27028 ± 3591	0.65
Rearing, standings/48h	6073 ± 741	6412 ± 831	0.77
Plasma			
Osmolality, mOsm/kg H ₂ O	321 ± 1.95	322 ± 2.26	0.68
Creatinine, mg/dL	0.11 ± 0.01	0.13 ± 0.01	0.08
Urea, mg/dL	22 ± 0.90	22 ± 1.56	0.78
Na^+ , mM	148 ± 0.65	150 ± 0.90	0.14
K^+ , mM	5.08 ± 0.15	4.84 ± 0.15	0.27
Cl^- , mM	121 ± 1.52	120 ± 1.41	0.80
VP, ng/mL	2.72 ± 0.42	3.57 ± 0.50	0.22
Urine			
Diuresis, $\mu\text{L}/\text{min} \cdot \text{g}$ body weight	0.036 ± 0.004	0.033 ± 0.004	0.57
Osmolality, mOsm/kg H ₂ O	2326.8 ± 133.21	2315.8 ± 88.73	0.94
pH	6.89 ± 0.13	6.59 ± 0.12	0.07
Na^+ /creatinine, nmol/ng creatinine	0.29 ± 0.02	0.31 ± 0.03	0.49
K^+ /creatinine, nmol/ng creatinine	0.60 ± 0.02	0.58 ± 0.03	0.67
Cl^- /creatinine, nmol/ng creatinine	0.37 ± 0.01	0.40 ± 0.03	0.34
Ca^{2+} /creatinine, ng/ng creatinine	0.11 ± 0.01	0.09 ± 0.01	0.31
Mg^{2+} /creatinine, ng/ng creatinine	1.20 ± 0.11	0.98 ± 0.08	0.12
Glucose, mg/dL	82.9 ± 2.60	86.6 ± 3.64	0.43
Phosphate, mg/dL	124 ± 24.1	153 ± 21.8	0.38
CC16, $\mu\text{g}/\text{L}$	9.44 ± 3.71	12.90 ± 3.98	0.54

TABLE 1. Physical activity and biological parameters in *Trpv4* mice at baseline. CC16, Clara cell 16-KD protein. Data are presented as means ± SEM; n = 10 mice per group

Effect of TRPV4 disruption on central osmoregulation

Previous studies have demonstrated that TRPV4 is located within the forebrain lamina terminalis, a structure that is essential for both thirst and VP secretion. To evaluate the central response to hyperosmotic stimuli, we challenged *Trpv4* mice with an IP injection of a hyperosmotic solution (0.5M NaCl, 0.04 mL/g body weight). Mice were single-housed, allowing the precise measurement of cumulative water intake (Physiocage testing). Under these conditions, water intake was significantly delayed in *Trpv4*^{-/-} mice ($T_{1/2}$ *Trpv4*^{-/-}: 1056 ± 245 sec versus $T_{1/2}$ *Trpv4*^{+/+} 672 ± 83 sec; **FIGURE 5a-b**), suggesting a central defect in osmoregulation.

In a second set of experiments, *Trpv4* mice were exposed to a 24 h water deprivation (**FIGURE 6**). The *Trpv4*^{-/-} mice and their control littermates had the same urine output and osmolality at baseline, with water deprivation resulting in a similar drop in urine volume (**FIGURE 6a**) and a similar increase in urine osmolality (U_{Osm}) in both groups (**FIGURE 6b**). Of interest, *Trpv4*^{-/-} mice exhibited significantly higher levels of plasma osmolality (P_{Osm}) after water deprivation (**FIGURE 6c**), as well as a lower increase in VP mRNA expression (**FIGURE 6d**) and plasmatic VP (**FIGURE 6e**) as compared to control littermates. Furthermore, the positive correlation between P_{Osm} and plasma VP levels, as observed in the *Trpv4*^{+/+} mice, was abolished in *Trpv4*^{-/-} mice (**FIGURE 6f**).

Results

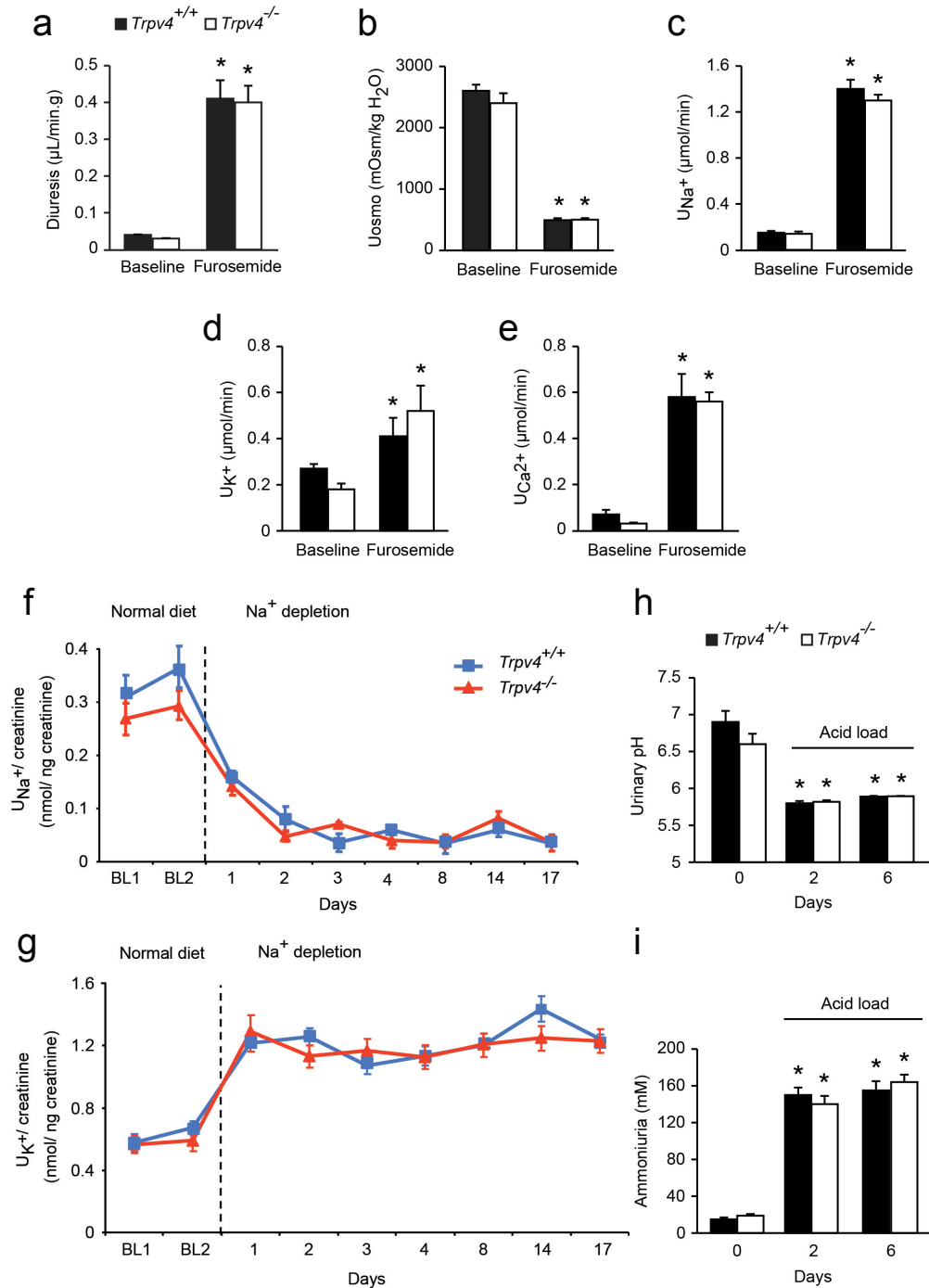
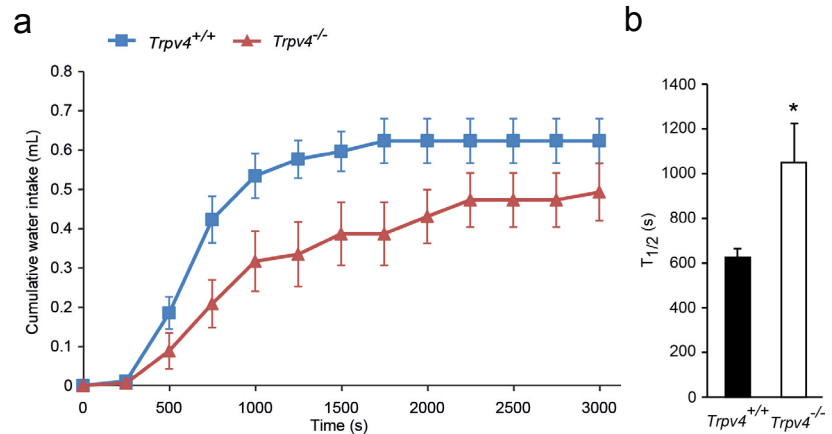


FIGURE 4. Renal function testing in *Trpv4*^{-/-} mice. a-e. Effects of furosemide on *Trpv4*^{+/+} and *Trpv4*^{-/-} mice. Furosemide injection induced an increase in diuresis (a) and a significant decrease in urine osmolality (b). Natriuresis (c), kaliuresis (d) and calciuresis (e) were increased as well. No difference was observed between *Trpv4*^{+/+} and *Trpv4*^{-/-} (n = 5 pairs). f-g. Urinary Na⁺ and K⁺ excretion obtained with normal and Na⁺-depleted diets. At baseline (BL1 and BL2), mice were fed a diet containing 1% NaCl. This baseline was followed by a Na⁺-depleted diet (0.01% NaCl) for 17 days (1-17). The amount of Na⁺ (f) and K⁺ (g) excreted in urine of *Trpv4*^{+/+} and *Trpv4*^{-/-} mice was determined. Results are expressed as nmol of Na⁺ and K⁺ excreted per ng of creatinine. No significant difference in Na⁺ and K⁺ excretion was observed between *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, at baseline and during sodium depletion (n = 5 pairs). h-i. Response to acid loading. Five pairs of mice were exposed to a NH₄Cl load for 0, 2 or 6 days. In *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, the acid load reduced the urinary pH (h) and induced an increase in urinary excretion of ammonium (i). The two groups showed a similar response to this challenge. * P < 0.05 vs baseline

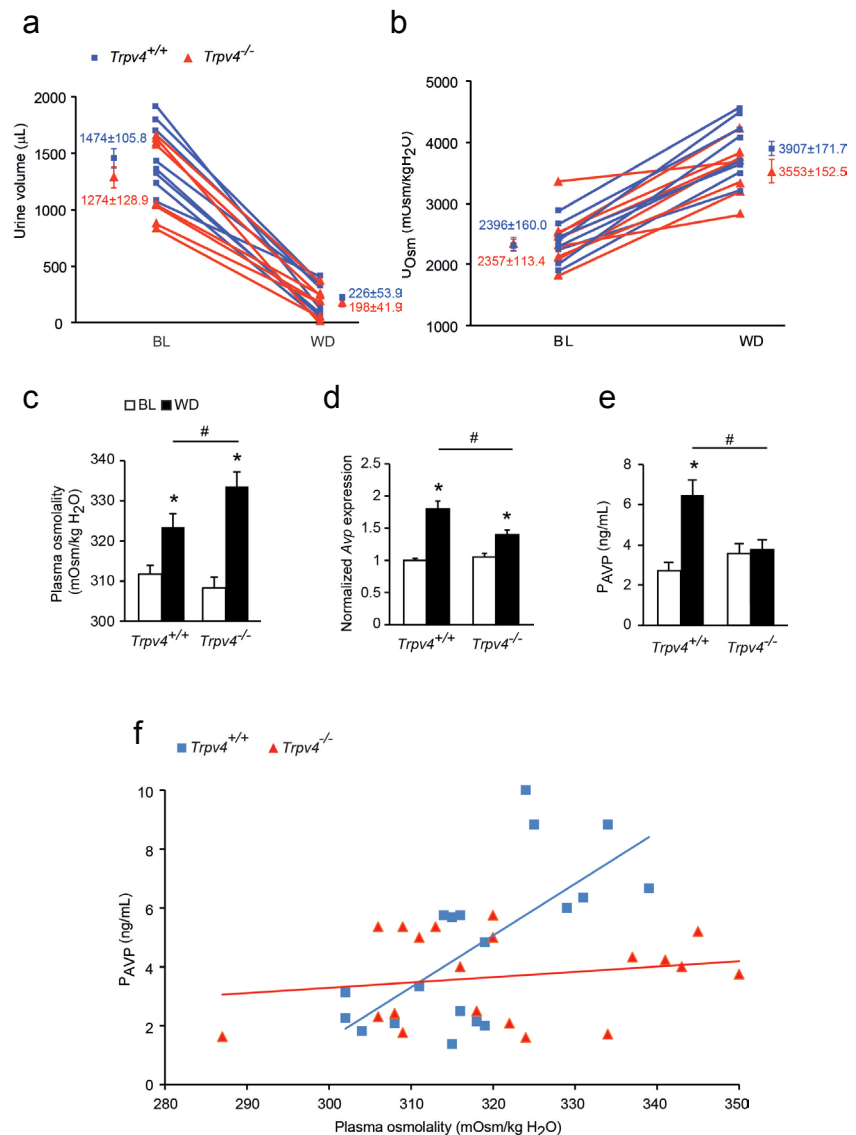
FIGURE 5. Hypertonic saline stimulation and water intake.

After adaptation in physiocage, mice were injected 0.5M NaCl i.p. and monitored for subsequent water intake. As compared to wild-type littermates, *Trpv4*^{-/-} mice show a decreased cumulative water intake (a, area under the curve: *Trpv4*^{-/-}, 953 ± 159 mL.s vs *Trpv4*^{+/+}, 1435 ± 143 mL.s; *P* = 0.04), and a significantly delayed water intake (b, *T*_{1/2}: KO 1056 ± 245 s vs WT 672 ± 83 s; *P* = 0.03) (*n* = 10 pairs). * *P* < 0.05 vs control

**FIGURE 6. Effects of water deprivation on water balance in *Trpv4*^{-/-} mice.**

Urine output (a) and urine osmolality (b) obtained at baseline (BL) and after 24h of water deprivation (WD) show the same variations in both strains. In comparison with *Trpv4*^{+/+} littermates, *Trpv4*^{-/-} mice exhibited a significantly higher rise in plasma osmolality (c) but a significantly lower upregulation of the levels of VP (*Avp*) mRNA in brain (d) and of the plasma levels of VP (*P*_{AVP}, e) after water deprivation.

f. Relationship between plasma osmolality (*P*_{Osm}) and VP (*P*_{AVP}). After 24h of water deprivation, in wild-type mice, the increase in *P*_{Osm} was reflected by an important increase in *P*_{AVP} (*R*²=0.47). This relationship was lost in *Trpv4*^{-/-} mice (*R*²=0.04). (*n* = 10 pairs, except for (f) *n* = 20 pairs). * *P* < 0.05 vs baseline. # *P* < 0.05 vs controls



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Effect of TRPV4 disruption on peripheral osmoregulation

The potential role of TRPV4 on the renal response to water deprivation was then investigated. Immunoblotting (whole kidney membrane fraction) showed similar membrane AQP2 and pS269 AQP2 levels in both genotypes at baseline and after water deprivation (**FIGURE 7a-f**). The levels of pS256 and pS261 AQP2 were also found to be similar between *Trpv4*^{-/-} mice and controls (**Supplementary FIGURE 2**). Immunostaining showed a similar increase in AQP2 signal intensity, membrane localization and phosphorylation in the CDs of *Trpv4*^{+/+} and *Trpv4*^{-/-} mice (**FIGURE 7g-h**). Of note, expression studies on whole kidney (RT-qPCR) showed that *Aqp2* mRNA expression levels, which were similar between both genotypes at baseline, showed a significantly lower rise after water deprivation in *Trpv4*^{-/-} mice. Expression of aquaporin 3 (*Aqp3*), *Avpr1* and *Avpr2* were similarly regulated in both genotypes (**FIGURE 8a-d**). Investigations of water handling were completed by injecting the V2R selective agonist dDAVP (1ng/g) (**FIGURE 9a**). This treatment resulted in a similar increase in U_{Osm} (after 5h) in both genotypes. In a reverse experiment, acute water loading (100μL/g; IP) demonstrated that *Trpv4*^{-/-} and *Trpv4*^{+/+} mice have a similar capacity to excrete water (**FIGURE 9b**).

These results, and the lack of reactivity of plasma VP under water deprivation, suggest the activation of a VP-independent pathway to increase membrane AQP2 in *Trpv4*^{-/-} mice [128-130]. In order to test whether the nitric oxide (NO) pathway could be involved, we compared the expression of nitric oxide synthases (NOS) in water-deprived *Trpv4*^{+/+} and *Trpv4*^{-/-} mice. There was a significant upregulation of NOS1 in *Trpv4*^{-/-} kidneys compared to controls, corresponding to enhanced NOS1 level in their inner medulla, with the highest signal in the vasculature (**FIGURE 10a-b**). Other isoforms were not upregulated (data not shown).

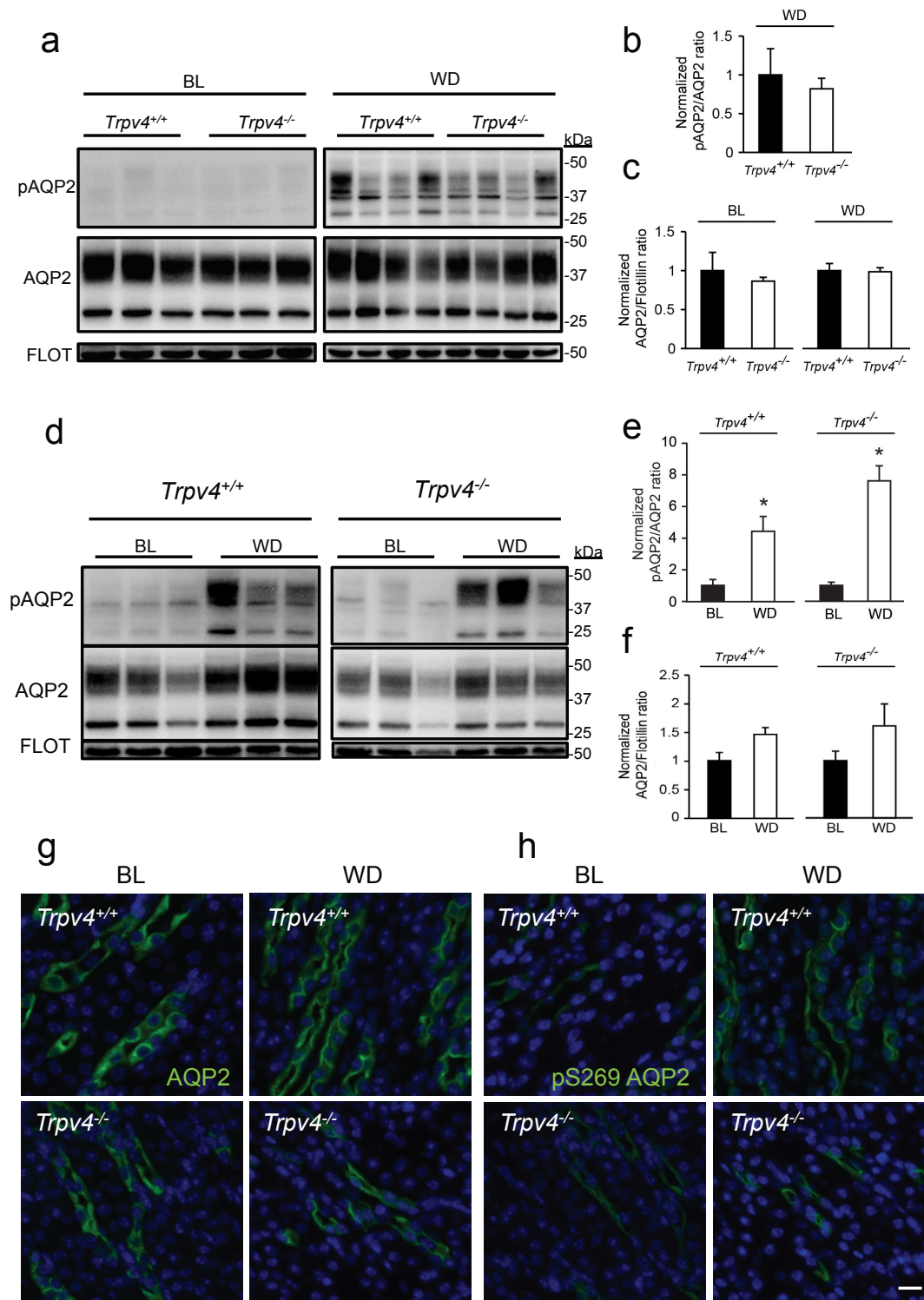


FIGURE 7. Effects of water deprivation on AQP2 protein in *Trpv4*^{-/-} whole kidney membrane extracts.

a. Immunoblotting of pS269 (pAQP2) and total AQP2 comparing *Trpv4*^{+/+} and *Trpv4*^{-/-} kidneys at baseline (BL) and after water deprivation (WD). b-c. Quantification of the immunoblotting signals. Flotillin (FLOT) is used as a membrane fraction loading control. No difference was found at baseline or after water deprivation.

d. Immunoblotting of pS269 and total AQP2 analyzing the response to water deprivation within each genotype.

e-f. Quantification of the immunoblotting signals. Like controls, *Trpv4*^{-/-} kidneys showed a significant rise in AQP2 phosphorylation after water deprivation. * $P < 0.05$ vs baseline. g-h. Immunostaining of AQP2 and pS269 AQP2 in CD under strictly controlled conditions showed a similar increase in signal and membrane localization in *Trpv4*^{+/+} and *Trpv4*^{-/-} CD after water deprivation. Scale bar = 50 μ m

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FIGURE 8. Effects of water deprivation on the expression of genes involved in osmoregulation in *Trpv4*^{-/-} kidneys. a. Expression studies on whole kidney (RT-qPCR) revealed similar levels in the mRNA expression of *Aqp2* between both genotypes at baseline, but a significantly lower rise after water deprivation in *Trpv4*^{-/-} mice. Levels of expression of *Aqp3* (b), *Avpr1a* (c) and *Avpr2* (d) mRNAs were similar between both genotypes at baseline and after water deprivation. * *P* < 0.05 vs baseline. #, *P* < 0.05 vs controls

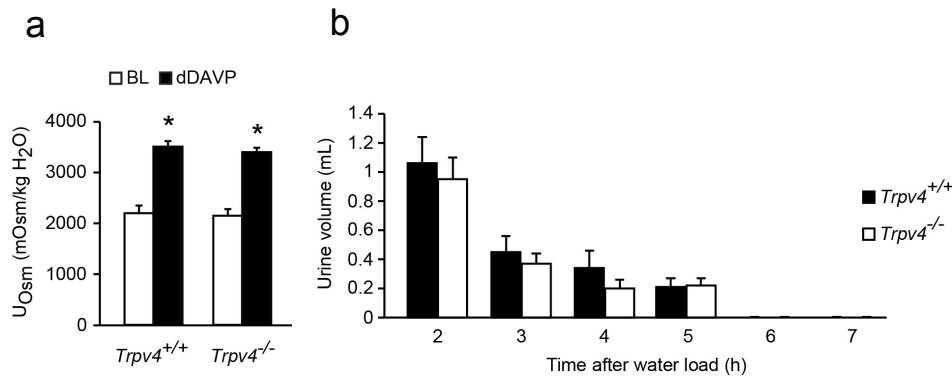
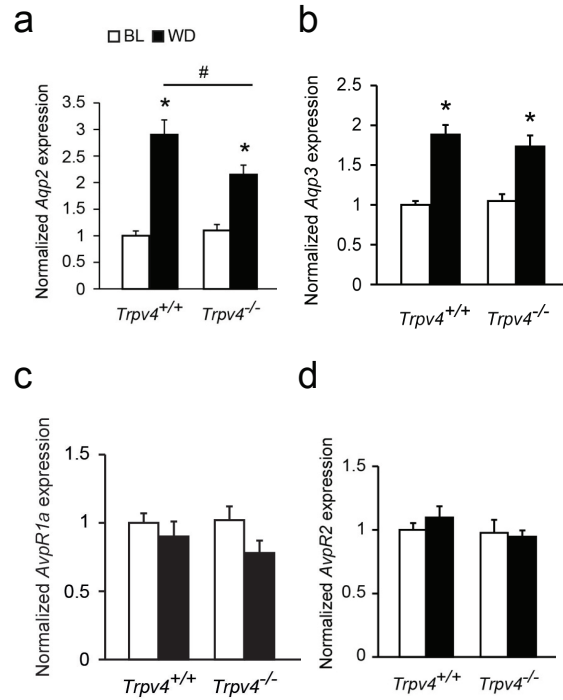
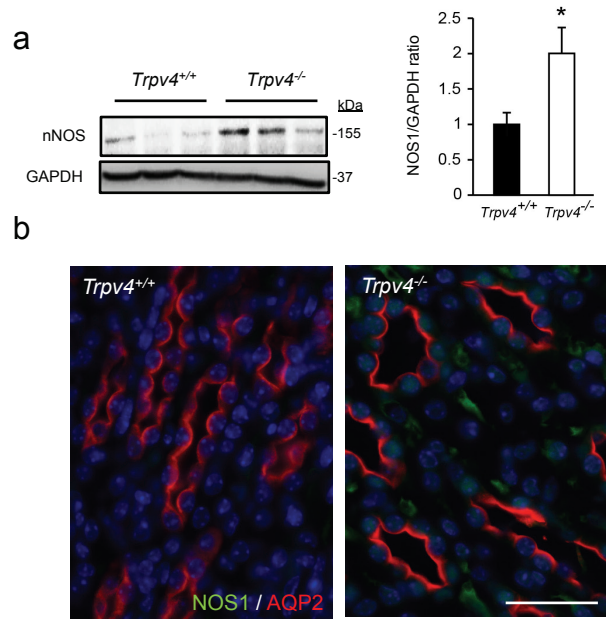


FIGURE 9. Investigations on water handling by the *Trpv4*^{-/-} kidney. a. Effect of dDAVP treatment on urine osmolality in *Trpv4*^{+/+} and *Trpv4*^{-/-} mice. Urine osmolality (U_{osm}) was measured at baseline and 5 h after injection of 1ng/g dDAVP (Minirin®, i.p.). The two groups of mice showed similar U_{osm} values at baseline (2238 ± 175 mOsm/kg H₂O vs. 2245 ± 144 mOsm/kg H₂O). dDAVP injection induced an increase of U_{osm}, however no significant difference was observed between *Trpv4*^{-/-} mice and wild-type littermates (3371 ± 78 mOsm/kg H₂O vs. 3534 ± 110 mOsm/kg H₂O; n = 6 pairs of mice). * *P* < 0.05 vs baseline. b. Response to water loading in *Trpv4*^{+/+} and *Trpv4*^{-/-} mice. Ten pairs of mice were administered an i.p. injection of water (100μL/g) and urine volume (mL) was measured every hour. Each group showed the same ability to excrete water after injection

FIGURE 10. Effects of water deprivation on NOS1 expression in *Trpv4*^{-/-} kidney.

a. Immunoblotting of NOS1 conducted on whole kidney extracts. Comparison of NOS1 levels in *Trpv4*^{+/+} and *Trpv4*^{-/-} kidneys after water deprivation revealed a significantly higher level in the *Trpv4*^{-/-} mice. GAPDH is used as a loading control. * $P < 0.05$ vs controls. b. Immunostaining on kidneys from water-deprived mice showed a higher NOS1 level in *Trpv4*^{-/-} inner medulla, particularly in the vessels, in comparison with controls. AQP2 is used to stain principal cells of the CD. Scale bar = 50 μ m



Effect of TRPV4 activation on PT cells primary culture

As often observed, the function of a protein can be masked in the KO model. For example, TRPV4 has been involved in osmosensation and shear stress sensing in the CD [131] while we did not find phenotypic differences in testing this segment in the *Trpv4*^{-/-} mice. As we show that TRPV4 is also expressed in the PT, we further analyzed its role on the endocytic function of primary cultures of PT epithelial cells. We first verified that we could activate TRPV4 in the mPTCs with the specific agonist GSK1016790A; we indeed observed that stimulation of wild type mPTCs with 100 nM GSK1016790A induced a large increase in $[Ca^{2+}]_i$ (FIGURE 11a). Then, we compared endocytosis of FITC-labeled albumin [126] between *Trpv4*^{+/+} and *Trpv4*^{-/-} mPTCs (FIGURE 11b). In standard conditions, the endocytosis of FITC-labeled albumin was similar between the two genotypes. However, activation of TRPV4 with GSK1016790A enhanced albumin endocytosis in *Trpv4*^{+/+} mPTCs. This effect was not observed in mPTCs isolated from *Trpv4*^{-/-} mice.

Results

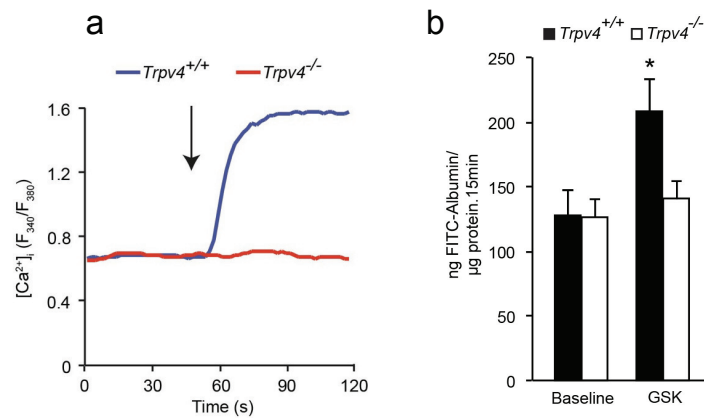


FIGURE 11. Role of TRPV4 on albumin endocytosis in mPTCs. a. Expression of a functional TRPV4 channel in the mPTCs was verified by monitoring $[Ca^{2+}]_i$ in the presence of the TRPV4 agonist GSK1016790A (100nM; arrow). Specificity of the agonist was verified on *Trpv4*^{-/-} mPTCs. The graph represents the average response of 10 cells for each condition and is representative of 5 different experiments. b. *Trpv4*^{+/+} and *Trpv4*^{-/-} mPTCs were exposed to 0.5 mg/mL FITC-labeled albumin for 15 minutes and the uptake was quantified. No significant difference was found at baseline, but in the presence of 100 nM GSK1016790A (GSK) albumin uptake was enhanced in *Trpv4*^{+/+} mPTCs and not in the *Trpv4*^{-/-} mPTCs (n=9 filters from 3 different mice for each condition). * $P < 0.05$ vs *Trpv4*^{+/+} baseline

Discussion

In this study, we detailed the expression profile of TRPV4 in mouse kidney and analyzed the consequences of the genetic deletion of TRPV4 on the central and nephrogenic components of osmoregulation. In contrast to previous studies, we demonstrate that TRPV4 is absent from the TAL but abundantly expressed in proximal and distal tubular segments. Despite the widespread expression pattern of TRPV4 in the kidney, its deletion has no detectable effect on a large range of renal function parameters. Conversely, the absence of TRPV4 is associated with abnormal thirst regulation and VP release, demonstrating a key role in central osmoregulation.

Knockout mice for TRPV4 express a relatively subtle phenotype [54, 112] [115]. Indeed, Mizuno *et al.* reported no perturbation under normal conditions, but an exaggerated secretion of VP in response to hyperosmolarity. Using another line, Liedtke *et al.* reported that *Trpv4*^{-/-} mice drank less and were slightly hyperosmolar. VP secretion in response to hyperosmotic stimulation was diminished, with lower fluid intake and increased latency of drinking. In a third study, Kinsman *et al.* found no difference in water intake following hyperosmotic challenge between this latter model and controls. Besides these KO models, other studies linked TRPV4 to VP secretion in the SON [114], modulated thirst with a TRPV4 agonist [132], or showed its role in osmosensitivity of the PVN [133, 134]. TRPV4 has also been described as a peripheral osmoreceptor in nerve endings surrounding hepatic blood vessels [109]. However, the fact that human patients with denervated liver (liver transplanted patients) have an elevated plasma osmolality in combination with elevated levels of the C-terminal pro-VP suggests an appropriate central response in absence of this peripheral osmoreceptor.

Our studies, based on the *Trpv4*^{-/-} mouse model developed by Liedtke *et al.* which was backcrossed on an inbred C57BL/6 background, reveal a significantly impaired ability of *Trpv4* KO mice to increase VP expression and secretion upon water deprivation. Furthermore, *Trpv4*^{-/-} mice had a significantly higher plasma osmolality and a reduced *Aqp2* mRNA upregulation after 24h water deprivation. The latter findings are probably related to defective VP secretion, as also evidenced by the loss of the typical, positive correlation between plasma osmolality and plasma VP levels. This correlation was also lost in the *Trpv1*^{-/-} mice (**FIGURE 6f** from this article can be paralleled to figure 6 from [135]), indicating that TRPV1 and TRPV4 are both needed in the mechanism of VP secretion. These findings indicate that the

Results

loss of TRPV4 alters the function of central osmoreceptors implicated in the regulation of VP secretion. The central defect is also reflected by an alteration of the mechanism of thirst, as evidenced by the significant delay in the water intake in the *Trpv4*^{-/-} mice after hypertonic saline injection.

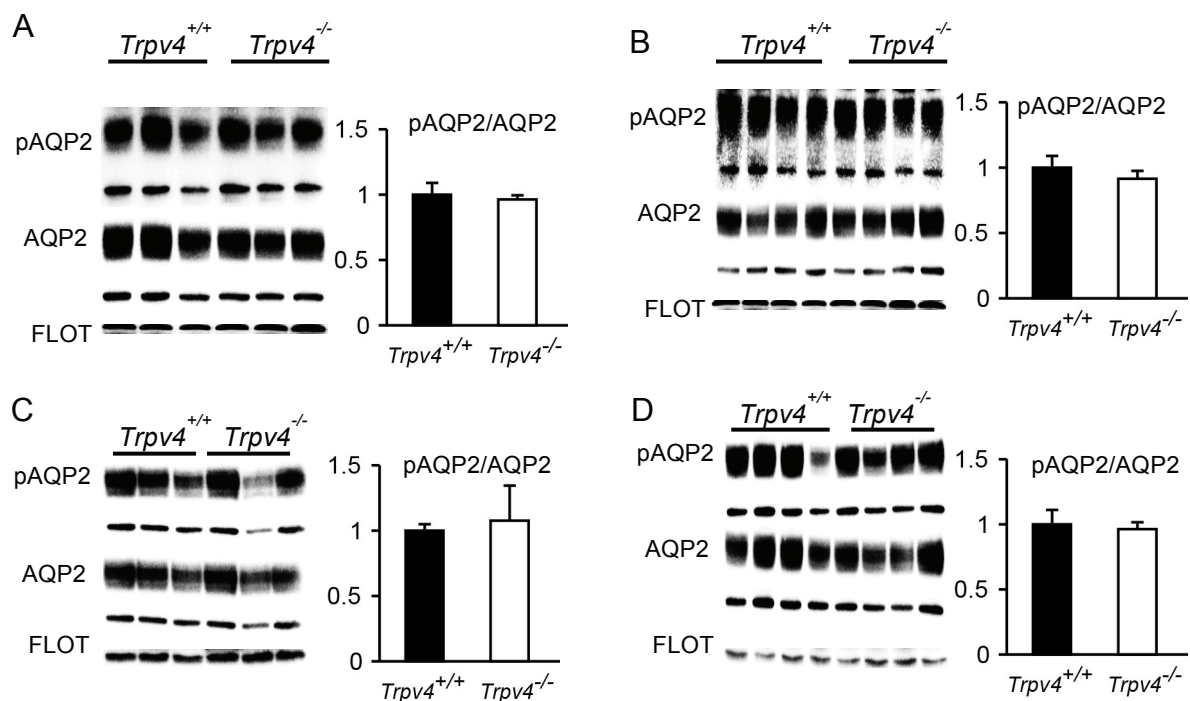
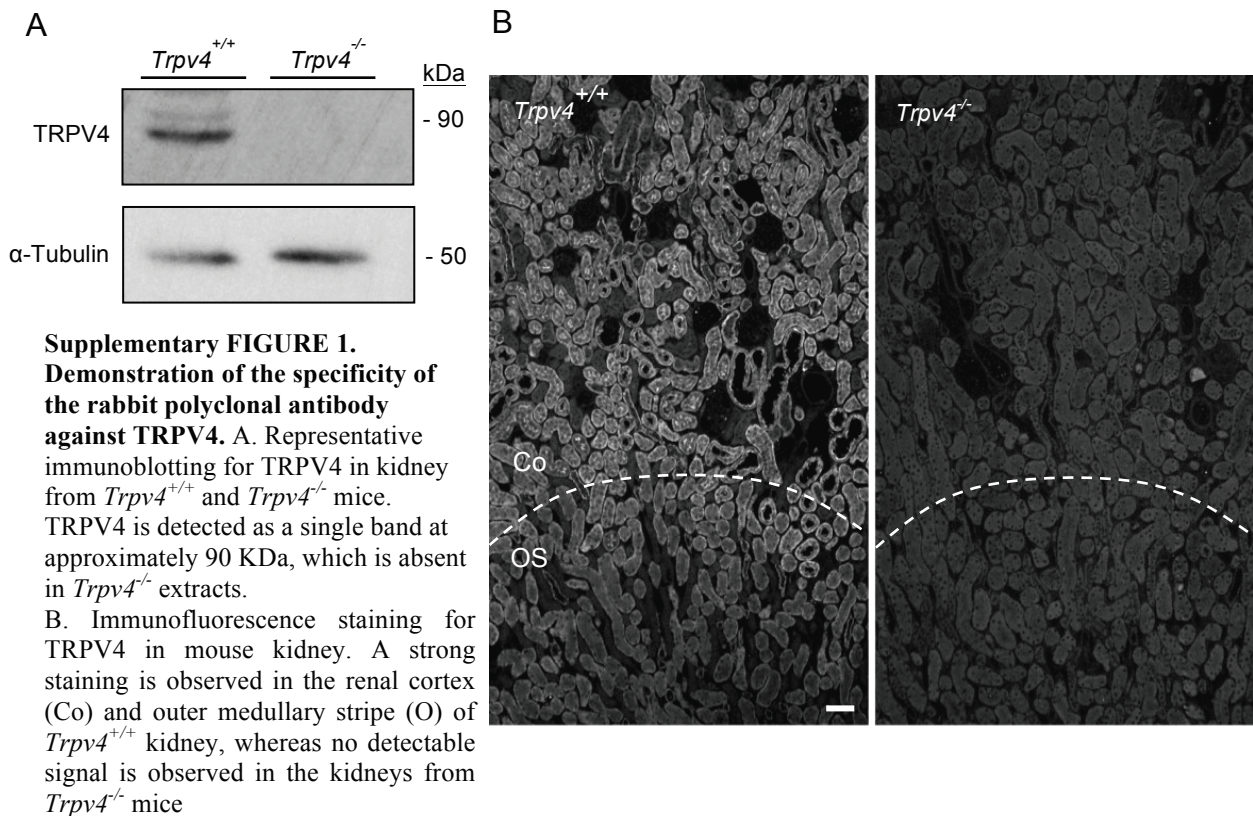
The distribution and physiological role of TRPV4 in the kidney remain debated [94, 131]. Our studies, based on RT-qPCR, immunostaining and gene expression reporting in mouse kidney, show consistently that TRPV4 is abundantly expressed in the apical and basolateral area of PT cells, and essentially at the basolateral membrane of the late DCT, the CN and the CD, including principal and intercalated cells. By contrast, TRPV4 is absent from the glomerulus and the TAL. This pattern of distribution differs from that published in two previous reports [121, 122]. Immunostaining for TRPV4 on rat and mouse kidneys performed by Tian *et al.* suggests that it is expressed at the basolateral membrane of the TAL and the downstream nephron segments, but was not validated on a KO model. On the other hand, Berrouit *et al.* showed expression (predominantly apical) from the CN through the papillary CD. While the antibody properties and the immunostaining conditions could explain the absence of positive PT staining in the latter study, our pattern is strongly supported by non-immunological evidences that are RNA expression and *lacZ* reporting studies.

Several lines of evidence were used to assess the potential role(s) of TRPV4 in these nephron segments. Studies performed at baseline failed to show any difference in behavior and plasma and urine parameters between *Trpv4*^{-/-} and *Trpv4*^{+/+} mice. An extensive set of functional tests was also used, failing to show significant differences in the renal response between *Trpv4*^{-/-} and *Trpv4*^{+/+} mice: furosemide treatment, NaCl depletion, acid loading, water deprivation, dDAVP treatment and water loading. These negative results have to be interpreted in view of *in vitro* studies that have demonstrated a physiological role of TRPV4 as a flow sensor, on mouse renal epithelial cell line [70] and isolated CD [122, 136, 137]. We now show that TRPV4 activation can enhance endocytosis in mPTCs, and further investigations will be needed to evaluate its participation to the mechanosensors regulating the PT functions [93, 138]. However, at rest, we could not detect any dysfunction of PT such as proteinuria, glycosuria or phosphaturia. The fact that the kidney is a site of high TRPV4 expression compared to other organs [50, 123], with TRPV4 being one of the most expressed TRP channels [123], may suggest that redundant mechanisms compensate for the lack of TRPV4 in *Trpv4*^{-/-} kidneys. Despite the defect in VP secretion, the renal response to 24h water deprivation was found to be functional in *Trpv4*^{-/-} mice, as urine concentrating ability and

AQP2 trafficking and phosphorylation were not significantly different between *Trpv4*^{-/-} mice and controls. Interestingly, we found an upregulation of NOS1 in the inner medulla of water deprived *Trpv4*^{-/-} mice, where NO signaling could be an alternative pathway to increase membrane AQP2 in absence of AVPR2 stimulation [128, 139].

In conclusion, despite abundant TRPV4 expression in proximal and distal nephron segments, the *Trpv4*^{-/-} mouse does not highlight a major renal dysfunction. Instead TRPV4 is critical for the release of VP, the thirst sensation and the central regulation of osmolality.

Supplementary Figures



Supplementary FIGURE 2. Immunoblotting of AQP2 conducted on whole kidney membrane extracts. Comparison and quantification of pS256 AQP2 (A-B) and pS261 AQP2 (C-D) levels in *Trpv4*^{+/+} and *Trpv4*^{-/-} kidneys at baseline and after water deprivation. Flotillin (FLOT) is used as a membrane fraction loading control. No difference was found between the two genotypes

Antigen	Host	Dilution		Source
		WB	IS	
TRPV4	Rabbit	1:1000	1:500	B. Nilius, Leuven
NaPi2a	Rabbit		1:500	H. Murer, Zurich
NCC	Rabbit		1:8000	J. Loffing, Zurich
NKCC2	Rabbit		1:20000	J. Loffing, Zurich
AQP2	Rabbit		1:40000	J. Loffing, Zurich
Flotillin	Mouse	1:1000		BD Biosciences
H-ATPase	Rabbit		1:500	C. Wagner, Zurich
α -Tubulin	Mouse	1:2000		Sigma
AQP1	Rabbit		1:200	Chemicon
Uromodulin	Sheep		1:1000	Meridian LS
NOS1	Mouse	1:1000	1:200	BD Biosciences

Supplementary TABLE 1. Antibodies used for immunoblotting and immunostaining. WB, Western blotting; IS, immunostaining

Gene	Forward primer	Reverse primer	PCR Product	Efficiency
<i>Gapdh</i>	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGGGGGAGA	176	1.04 ± 0.03
<i>Trpv4</i>	ACAAGAAGCGCCTGACTGAT	GATGAATTCACGCATGTTGC	155	1.02 ± 0.03
<i>Nphs2</i>	GTCTAGCCCATGTGTCCAAA	CCACTTTGATGCCCCAAATA	162	0.98 ± 0.03
<i>Aqp1</i>	GCTGTCATGTACATCATCGCCCAG	AGGTCATTGCGGCCAAGTGAAT	107	0.99 ± 0.02
<i>Slc12a1</i>	CCGTGGCCTACATAGGTGTT	GGCTCGTGTTGACATCTTGA	154	0.99 ± 0.04
<i>Aqp2</i>	TCACTGGGTCTTCTGGATCG	CGTTCCTCCCAGTCAGTGT	147	1.03 ± 0.04
<i>Avpr2</i>	GGAAATGGCAGTGGGGTATT	GGCACCAGACTGGCATGTAT	164	0.98 ± 0.04
<i>Actb</i>	TGCCCATCTATGAGGGCTAC	CCCGTTCAGTCAGGATCTTC	102	1.02 ± 0.02
<i>Arbp</i>	CTTCATTGTGGGAGCAGACA	TTCTCCAGAGCTGGGTTGTT	150	0.99 ± 0.01
<i>Ppia</i>	CGTCTCCTTCGAGCTGTTTG	CCACCCTGGCACATGAATC	139	1.02 ± 0.02
<i>Hprt1</i>	ACATTGTGGCCCTCTGTGTG	TTATGTCCCCCGTTGACTGA	162	0.99 ± 0.01
<i>Avp</i>	CAAGAGGGCCATCTCTGACA	GGGCAGGTAGTTCTCCTCC	160	1.01 ± 0.02

Supplementary TABLE 2. Primers used in RT-qPCR analyses

Results

Supplementary Methods

Real-Time PCR

Total RNA from whole kidney and brain was obtained by use of the AurumTM Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol. DNase I treatment was performed to remove genomic DNA. One microgram of RNA was reverse-transcribed with iScriptTM cDNA Synthesis Kit (Bio-Rad). Changes in target genes mRNA levels were determined by relative RT-qPCR with a CFX96TM Real-Time PCR Detection System (Bio-Rad) using iQTM SYBR Green Supermix (Bio-Rad) detection of single PCR product accumulation. Specific primers were designed using Primer3 [140] and the efficiency [141] of each set of primers was determined by dilution curves (see **Supplementary TABLE 1**). RT-qPCR analyses were performed in duplicate.

To characterize the microdissection, C_t differences between the reference (*Gapdh*) and target genes were calculated for each sample of each genotype. The formula used to quantify the relative changes in target gene over *Gapdh* mRNA is derived from the $2^{-\Delta C_t}$ formula as described by Pfaffl [141]. Real-time PCR results were confirmed using two reference genes, *Gapdh* and *Hprt1*. For brain and kidney, the expression stability of five possible reference genes (*Gapdh*, *Actb*, *Arbp*, *Ppia* and *Hprt1*) was verified using the GeNorm version 3.4 program [142, 143]. We selected the 5 reference genes for kidney and 3 reference genes for brain.

Reaction conditions for SYBR Green chemistry

RT-qPCR analyses were performed in duplicate with 100nM of both sense and anti-sense primers in a final volume of 20 μ L using iQTM SYBR Green Supermix (Bio-Rad). The PCR conditions were 95°C for 3 min followed by 40 cycles of 30 sec at 95°C, 1 min at 60°C. The PCR products were sequenced with the BigDye terminator kit (Perkin Elmer Applied Biosystems). The multiScreen SEQ₃₈₄ Filter Plate (Millipore, Billerica, USA) and Sephadex G-50 DNA Grade Fine (Amersham Biosciences, Piscataway, USA) dye terminator removal were used to purify sequences reactions before analysis on an ABI3100 capillary sequencer (Perkin Elmer Applied Biosystems).

CHAPTER II

Role of TRPV4 in the proximal tubule

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Introduction

In chapter I, we have aimed to characterize the TRPV4 renal expression pattern. While others find TRPV4 expression only in the CN and the CD [122], we clearly report for the first time expression of TRPV4 in the PT.

The PT is an epithelial organ that reabsorbs about 70% of the water, ions and solutes that are ultrafiltrated through the glomerulus, using transcellular and paracellular transports. It also expresses multiligand receptors, megalin and cubilin, that mediate the endocytosis of diverse organic molecules among which albumin and low molecular weight proteins (LMWP) that have passed through the glomerular filter [93, 144]. There is a linear relationship between PT reabsorption and GFR, which is known as the glomerulotubular balance (GTB). This local regulatory process ensures that the PT fractional reabsorption remains constant during variations of the GFR. It is independent of hormonal or neuronal control. The GTB is quantitatively the most important regulator of tubular reabsorption [145].

The modulation of PT functions by the amount of ultrafiltrate delivered to the PT suggests the presence of mechanotransduction structures. The mechanical stimulus itself originates in the filtration of arterial blood through slit diaphragms formed by podocytes in the renal glomerulus. The ultrafiltrated volume propelled into the PT creates a flow at the apical surface of the PT and raises the intraluminal pressure at 10-15 mm Hg [146]. This flow is pulsatile, with its amplitude and frequency varying according to filtration pressure and heart rate. Two types of force are thus developed by the entry of the ultrafiltrate in the PT lumen: a shear stress on the apical membrane, and a radial stretch as changes in intraluminal hydrostatic pressure modify the tubule diameter (**FIGURE 1**). The apical membrane of the PT cells, which is subjected to luminal flow, possesses a primary cilium and a brush border that maximizes the contact with the ultrafiltrate. Radial stretch for its part is sensed by cytoskeleton, adhesion proteins and stretch-sensitive ion channels at the basolateral or apical side of the epithelial cells [93].

Results

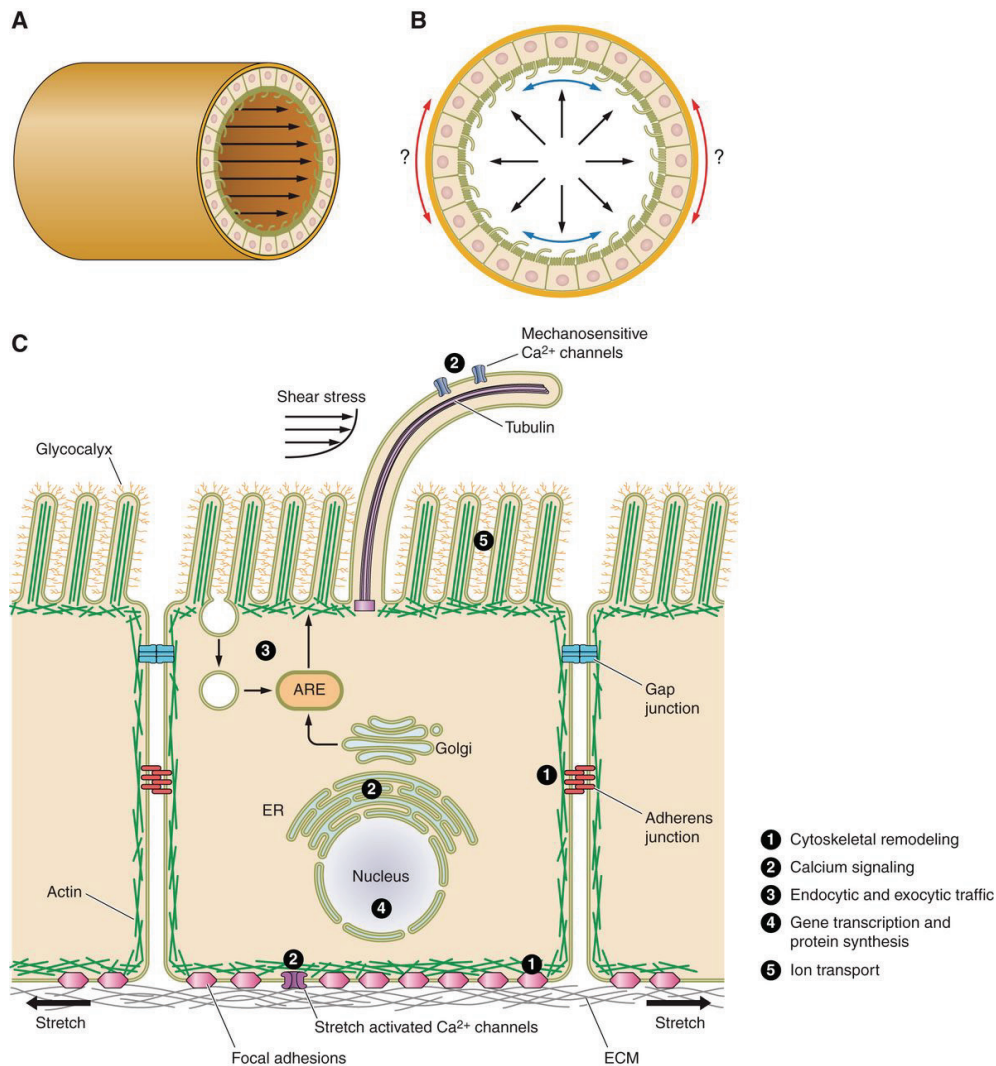


FIGURE 1. Mechanosensing in the PT. Illustration of the manifestation of flow as apical shear stress (A) or radial stretch (B) on the walls of the PT. C. PT functions initiated or modulated by shear stress and stretch. Reproduced from [93]

It has been shown that the presence of ions and water transporters at the apical membrane is enhanced upon mechanical stimulation of the PT cells by flow. This effect is attributed to trafficking of transporters containing vesicles to the apical membrane, as shown for the $\text{Na}^+\text{-H}^+$ -exchanger NHE3, which mediates PT Na^+ uptake, and the water channel AQP1 [147-149]. The cytoskeletal structures seem to play an important role in this mechanotransduction. In particular, the flow-induced transporter regulation was impaired upon disruption of the actin polymerization with cytochalasin, pointing the involvement of the acting brush-border in this mechanosensing [148, 150].

Elevations in GFR also increase the amount of filtered LMWP and other ligands of megalin and cubilin receptors. It seems that PT cells maintain a high constitutive endocytic capacity to efficiently retrieve these proteins, but whether PT cells modulate endocytosis in

response to mechanical stresses is not yet known [150]. A recent study suggests that shear stress enhances endocytosis in PT cells in a Ca^{2+} - and cilium-dependent way [138]. Besides, stretch is also known to modulate PT metabolism and functions. Cyclic stretch was shown to activate the PLA_2 , increasing cellular AA levels and activating extracellular signal-regulated kinases. This process was dependent on external Ca^{2+} and was blocked by Gd^{3+} , suggesting the involvement of stretch-activated ion channels [151].

Results

Methods

Immunostaining

Trpv4^{+/+} and *Trpv4*^{-/-} mice were deeply anesthetized by IP injection (10 ml/kg) of a solution containing 10 mg/mL ketamine and 1 mg/ml xylazine. After intracardiac perfusion with a 4°C buffered 4% paraformaldehyde PBS solution, the kidneys were removed and postfixed in the same solution for 2 hours, washed in PBS and cryoprotected overnight in 30% sucrose PBS. After embedding in Optimal Cutting Temperature compound (OCT, Tissue-Tek, VWR), 10µm thick cryosections were cut and stored at -80°C. Sections were blocked with a 3% BSA containing PBS solution and then incubated overnight with 1/200 anti-TRPV4 (Alomone) antibody. Binding sites were revealed with Alexa Fluor 488 (Thermofisher). Images were acquired with an Olympus FV1000 confocal microscope.

Cell culture

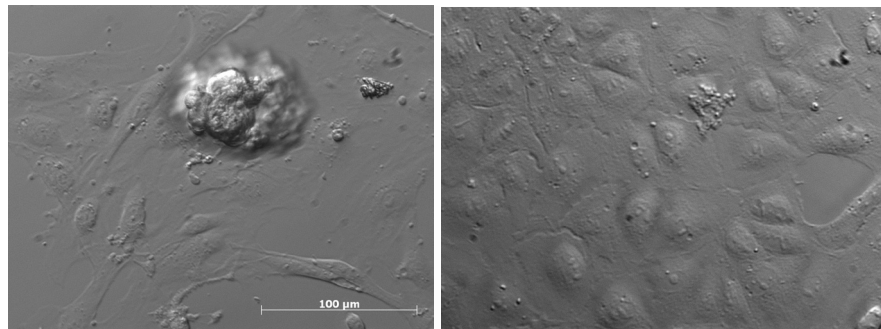
mPTCs from *Trpv4*^{-/-} and *Trpv4*^{+/+} mice were cultured as explained in chapter I (**FIGURE 2**). No differences in cell shape and size or in proliferation were observed between *Trpv4*^{-/-} and *Trpv4*^{+/+} mPTCs. *Trpv4* mRNA abundance was similar in freshly dissected PTs and mPTCs cultured for 7 days.

Ca²⁺ imaging

Loading of the mPTCs with Fura2-AM was achieved at room temperature in Krebs medium with 5µM Fura2-AM and 0.01% pluronic acid for 1h. Ca²⁺ imaging experiments were performed in Krebs-HEPES solution (NaCl 135 mM, KCl 5.9 mM, MgCl₂ 1.2 mM, CaCl₂ 1.8 mM, HEPES 11.6 mM, glucose 11.5 mM, pH 7.35). For hypotonic shock experiments, cells were bathed in a modified Krebs-HEPES solution where NaCl was partially omitted in the hypotonic solution (170m Osm), or replaced by mannitol in the isotonic solution (310 mOsm), in order to avoid changes in ionic gradients. For shear stress experiments, flow was applied on cells with an 18G needle connected to a peristaltic pump perfusion system and placed above the field. For measurement of [Ca²⁺]_i during cell stretching, cells were passaged on collagen I-coated flexible silicon chambers (ST-CH-04, B-

Bridge), placed on the microscope-mountable stretching instrument (ST-150, B-Bridge) and strained for 1s by 20% uniaxial stretching.

FIGURE 2. Phase contrast imaging of mPTCs culture. Two days after the dissection, development of mPTCs colonies out of PT fragments is visible (left panel). Seven days after culturing PT, mPTCs organize in a confluent epithelium (right panel)



Patch clamp experiments

Membrane currents were measured in the whole cell mode of the patch-clamp technique using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch electrodes had a resistance of $\sim 3 \text{ M}\Omega$ when filled with intracellular solution. An AgCl wire was used as a reference electrode. Currents were sampled at 20 kHz and digitally filtered at 2.9 kHz. A ramp protocol was applied consisting of a voltage step from the holding potential of 0 mV to -120 mV , followed by a 400-ms linear ramp to $+120 \text{ mV}$ applied every 2 s. The standard extracellular solution had the following composition (in mM): 150 NaCl, 6 KCl, 1 MgCl_2 , 1.5 CaCl_2 , 10 glucose, and 10 HEPES, buffered at pH 7.4 with NaOH. The osmolality of this solution was $320 \pm 5 \text{ mOsm}$. The pipette solution was composed of (in mM) 20 CsCl, 100 Cs-aspartate, 1 MgCl_2 , 10 HEPES, 4 Na_2ATP , 10 EGTA, and 1.93 CaCl_2 , adjusted to pH 7.2 with CsOH [85]. For hypotonic cell swelling, we superfused cells with a solution containing 105 mM NaCl, 6 mM CsCl, 1 mM MgCl_2 , 1.5 mM CaCl_2 , 10 mM glucose and 10 mM HEPES, buffered to pH 7.4 with NaOH. Isoosmolality was achieved by adding mannitol (80 mM) to this solution to reach 320 mOsm. Hypotonic cell swelling was induced by removing mannitol to lower the extracellular osmolality to 240 mOsm [152]. For hypotonic cell swelling in a low-chloride extracellular solution, 105 mM NaCl was replaced by 105 mM NaAsp. All measurements were carried out at room temperature.

Results

AT2 Infusion Studies

A 14-days infusion of AT2 (Tocris) at a dose of 1 ng/g/min dissolved in sterile saline was performed using osmotic mini-pumps (Alzet model 2004; Alza Corp., Mountain View, CA). The pumps were implanted subcutaneously in the dorsal region under ketamine-xylazine anesthesia (100-10µg/g). After a recovery period of 4 days, 24-hour urine was collected at 4, 7 and 14 days post-implantation in metabolic cages.

Urine analysis

After collection, 24-hour urine was centrifuged to remove debris, weighed and frozen at -80°C. Urinary albumin concentrations were determined using the ab108792 ELISA kit (Abcam), according to the manufacturer's protocol. Urinary glucose and phosphate concentrations were measured using a colorimetric reaction (Fuji Dri-Chem NX500 analyzer).

Results

Immunolocalization of TRPV4 demonstrated the presence of TRPV4 in the PT cells, mainly at the basolateral and slightly at the apical membranes (**FIGURE 3**), suggesting a possible involvement in both shear stress and stretch detection. We hypothesized that TRPV4 could act as a mechanosensor in the PT. The major mechanotransducer in modulation of water and Na^+ transport in the PT are the microvilli and this modulation is not considered as a Ca^{2+} -regulated mechanism [153]. However, the PT endocytic function could be modulated by Ca^{2+} -dependent processes [138]. In this line, we showed in chapter I that endocytosis was enhanced in a primary culture of PT cells upon TRPV4 activation.

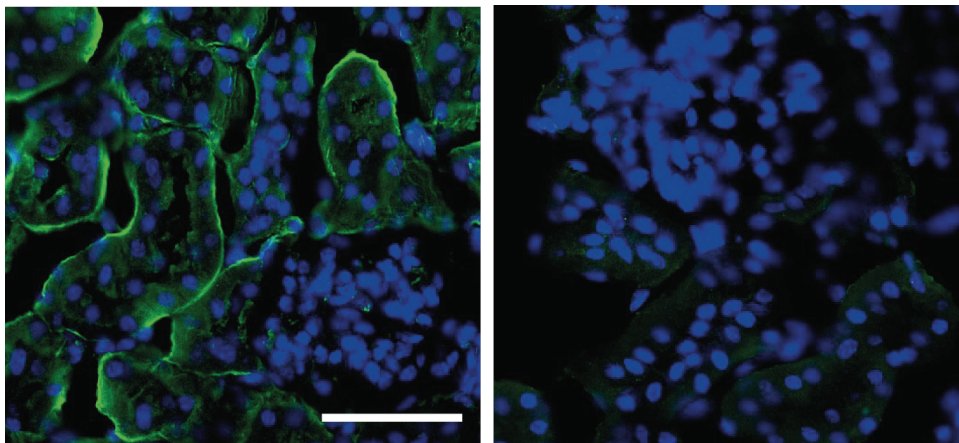


FIGURE 3. TRPV4 is expressed in PT. Immunolabelling of TRPV4 (in green) on renal cortex sections from *Trpv4*^{+/+} (left panel) and *Trpv4*^{-/-} mice (right panel). TRPV4 is mainly present at the basolateral side of PT epithelial cells. Scale bar: 50 μm

In chapter I, we also reported that, in basal conditions, no PT dysfunction was evidenced on the basis of urine analysis in the *Trpv4*^{-/-} mouse. Neither Na^+ -driven solute reabsorption (phosphate, glucose), nor LMWP uptake were affected by the deletion of TRPV4. We sought to increase the luminal protein delivery to the PT in order to challenge its endocytic capacity. To this purpose, we used a protocol of AT2 treatment in *Trpv4*^{-/-} and control mice in order to increase the permeability of the glomerular filter. Indeed, AT2 influences podocyte structure and function as it increases $[\text{Ca}^{2+}]_i$ and promotes apoptosis. Moreover, AT2 has a hypertensive effect that increases the filtration pressure. AT2 progressively contributes to the development of proteinuria and progressive kidney injury, as illustrated by the physiopathology of the diabetic nephropathy. Of note, the AT2 mouse model of renal injury strongly increases proteinuria but rarely produces substantial pathologic changes (such as global sclerosis and glomerulus collapse) [154].

Results

We collected urine from *Trpv4*^{-/-} and control mice before and during AT2 administration. The urinary output was significantly increased along AT2 infusion, but similarly between *Trpv4*^{-/-} and control mice (**FIGURE 4A**). AT2 increased albuminuria in control mice, but interestingly this increase was much larger in *Trpv4*^{-/-} mice (**FIGURE 4B**). As TRPV4 is not expressed in the glomerulus and as the diuresis increased to the same extent in AT2-treated *Trpv4*^{-/-} and control mice, the specific increase of proteinuria observed in *Trpv4*^{-/-} mice is suggestive of an endocytosis defect in *Trpv4*^{-/-} PT. Moreover, no significant differences in phosphate and glucose urinary levels were found between the two genotypes during the AT2 infusion, suggesting a specific effect on endocytosis (**FIGURE 4C-D**).

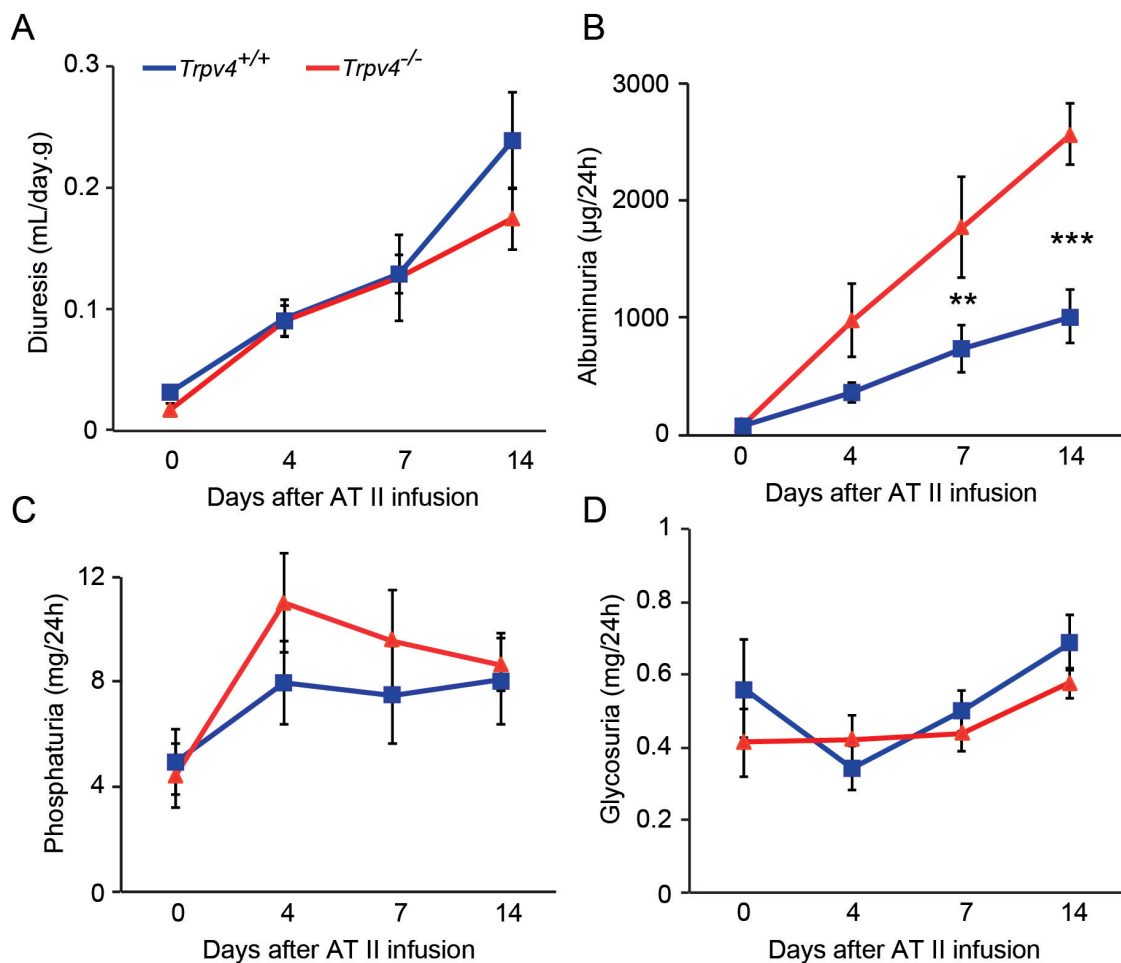


FIGURE 4. Urinary parameters of *Trpv4*^{-/-} and control mice before and during AT2 administration.

A. Evolution of the urine output during AT2 infusion. B. Evolution of the urinary loss of albumin during AT2 infusion. C. Evolution of the phosphaturia during AT2 infusion. D. Evolution of the glycosuria during AT2 infusion. ** $P < 0.01$ and *** $P < 0.001$, *Trpv4*^{-/-} vs *Trpv4*^{+/+}, two-way repeated measures ANOVA (n = 9 pairs)

In order to study the function of TRPV4 in the PT, we set up primary cultures of PT from *Trpv4*^{-/-} and control mice (mPTCs). We confirmed the expression of a functional TRPV4 channel in mPTCs using patch-clamp experiments. In mPTCs from *Trpv4*^{+/+} mice,

application of 100 nM GSK1016790A evoked an outwardly rectifying current, with an average reversal potential of +2 mV (**FIGURE 5A-B**). On the contrary, no current was observed in response to GSK1016790A in mPTCs from *Trpv4*^{-/-} mice (**FIGURE 5C-D**).

In *Trpv4*^{+/+} mPTCs, we also measured a robust current activation in response to hypotonic shock. This current was partially reversed by the specific TRPV4 antagonists RN1734 (**FIGURE 6A-B**) and HC067047 (data not shown). A current was also observed in mPTCs derived from *Trpv4*^{-/-} mice (**FIGURE 6C-D**), suggesting that the osmosensitivity of mPTCs is not exclusively due to TRPV4 channel gating. Interestingly, the reversal potential of the current activated by the hypotonic solution in mPTCs was about -20 mV (**FIGURE 6**). This value, near E_{Cl} , suggested that the current measured in response to the hypotonic shock was mainly carried by Cl^- ions (for a comprehensive review on Cl^- channels expressed in kidney see [155]).

To test this hypothesis, we blocked the Cl^- conductance in mPTCs using the well-known and aspecific Cl^- channels blocker DIDS (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid). **FIGURE 7A-B** shows that in *Trpv4*^{+/+} mPTCs the response to the hypotonic shock was partly blocked by DIDS. However, it was still possible to measure a robust current activation that was completely blocked by HC067047, with an average reversal potential of 0 mV (**FIGURE 7C-D**). Our results suggest that, under pharmacological blockade of Cl^- channels, the current observed in response to hypotonic shock in mPTCs is due to the opening of the TRPV4 channel. Similar results were obtained with the suppression of Cl^- conductance by replacement of Cl^- by aspartate. Hypotonic shock on *Trpv4*^{+/+} mPTCs using a 'low Cl^- ' solution (see Methods) evoked an outwardly rectifying current, with an average reversal potential of +8 mV that was completely blocked by HC067047 (**FIGURE 8A-B**). As expected, in these ionic conditions, the current recorded on *Trpv4*^{-/-} mPTCs in response to the hypotonic shock was significantly smaller compared to the current measured in *Trpv4*^{+/+} mPTCs (**FIGURE 8C-D**).

Results

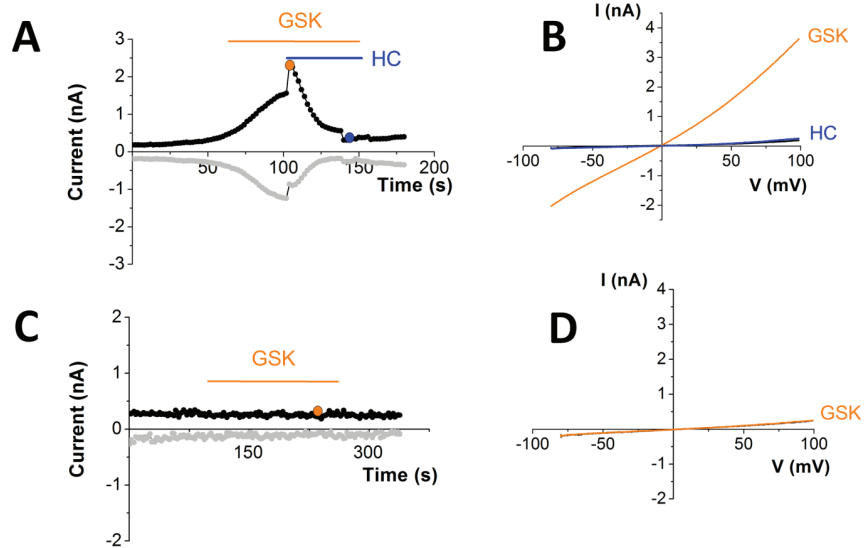


FIGURE 5. Effect of TRPV4 agonist/antagonist on mPTCs in patch-clamp experiments. Representative time-courses recorded at + 50 mV and -50 mV (black and grey curves, respectively; A) and I-V traces (B) of whole cell currents through *Trpv4*^{+/+} mPTCs, in the presence of GSK1016790A (100 nM) and HC067047 (1 μ M), at the indicated time intervals. Representative time-courses (C) and I-V traces (D) of whole cell currents through *Trpv4*^{-/-} mPTCs, during administration of GSK1016790A (100 nM). The black I-V traces showed in (B) and (D) are recorded in the absence of drugs at the beginning of the experiment

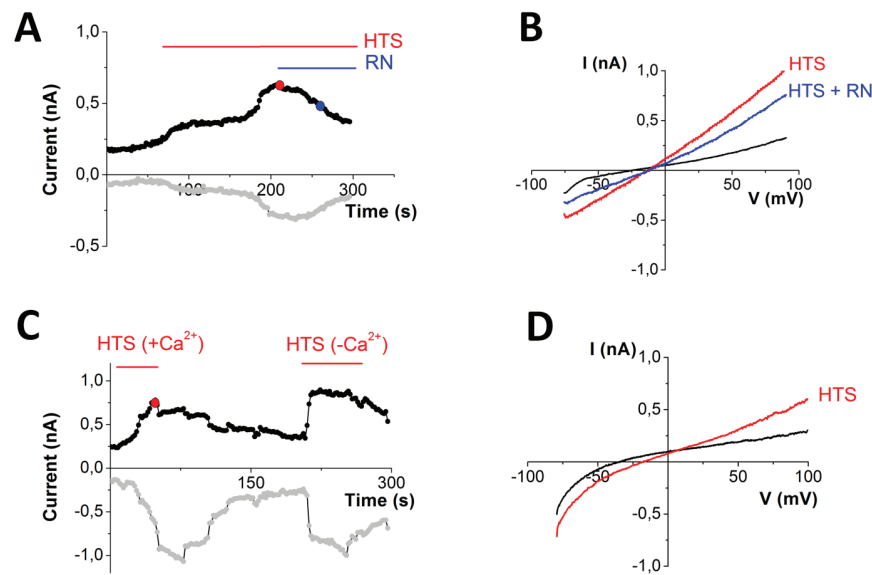


FIGURE 6. Effect of hypotonic solution stimulation on mPTCs in patch-clamp experiments. Time-course measured at membrane potential of +50 mV (black curve, A) and -50 mV (grey curve, A) and I-V trace (B) of whole cell currents through *Trpv4*^{+/+} mPTCs, during administration of hypotonic solution (HTS) and RN1734 (30 μ M), at the indicated time intervals. Representative time-courses recorded at + 50 mV (black curve, C) and -50 mV (grey curve, C) and I-V trace (D) of whole cell currents through *Trpv4*^{-/-} mPTCs, during administration of hypotonic solution with (+Ca²⁺) or without (-Ca²⁺), at the indicated time intervals. The black I-V traces showed in (B) and (D) are recorded before adding the drugs

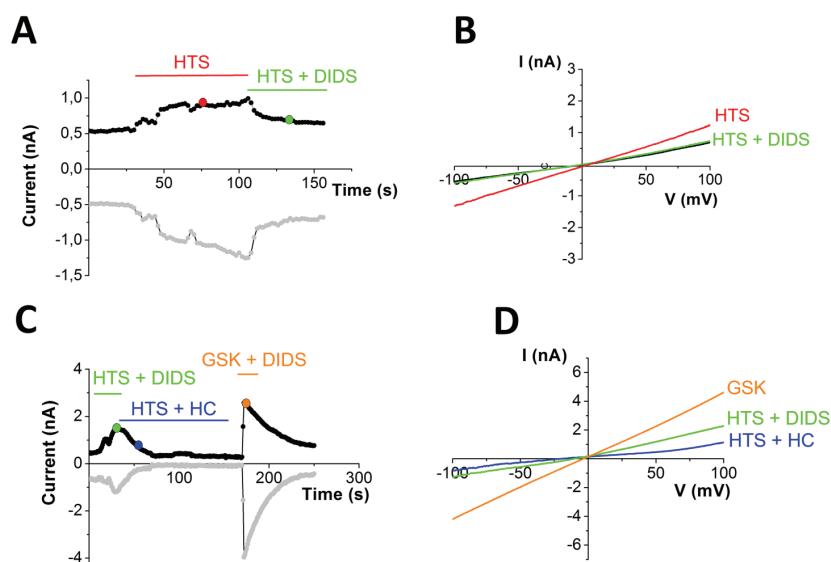


FIGURE 7. Effect of the chloride blocker DIDS on mPTCs in patch-clamp experiments. Time-course measured at membrane potential of +50 mV (black curve, A) and -50 mV (grey curve, A) and I-V trace (B) of whole cell currents through *Trpv4*^{+/+} mPTCs, during administration of hypotonic solution (HTS) and the chloride blocker DIDS (10 μ M), at the indicated time intervals. The black I-V trace is recorded in the absence of drugs at the beginning of the experiment. Representative time-courses recorded at +50 mV and -50 mV (black and grey curves, respectively; C) and I-V trace (D) of whole cell currents through *Trpv4*^{+/+} mPTCs, during administration of hypotonic solution, HC067047 (1 μ M) and GSK1016790A (100 nM), in the presence of DIDS (10 mM), at the indicated time intervals

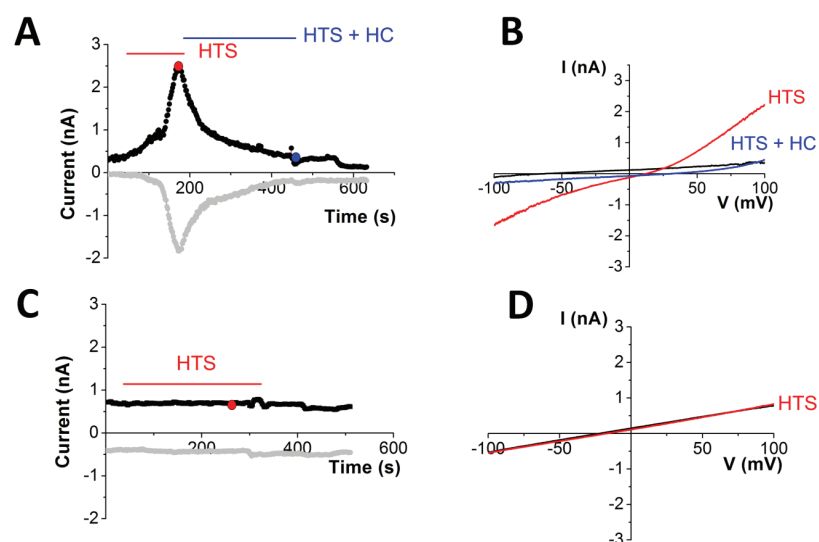
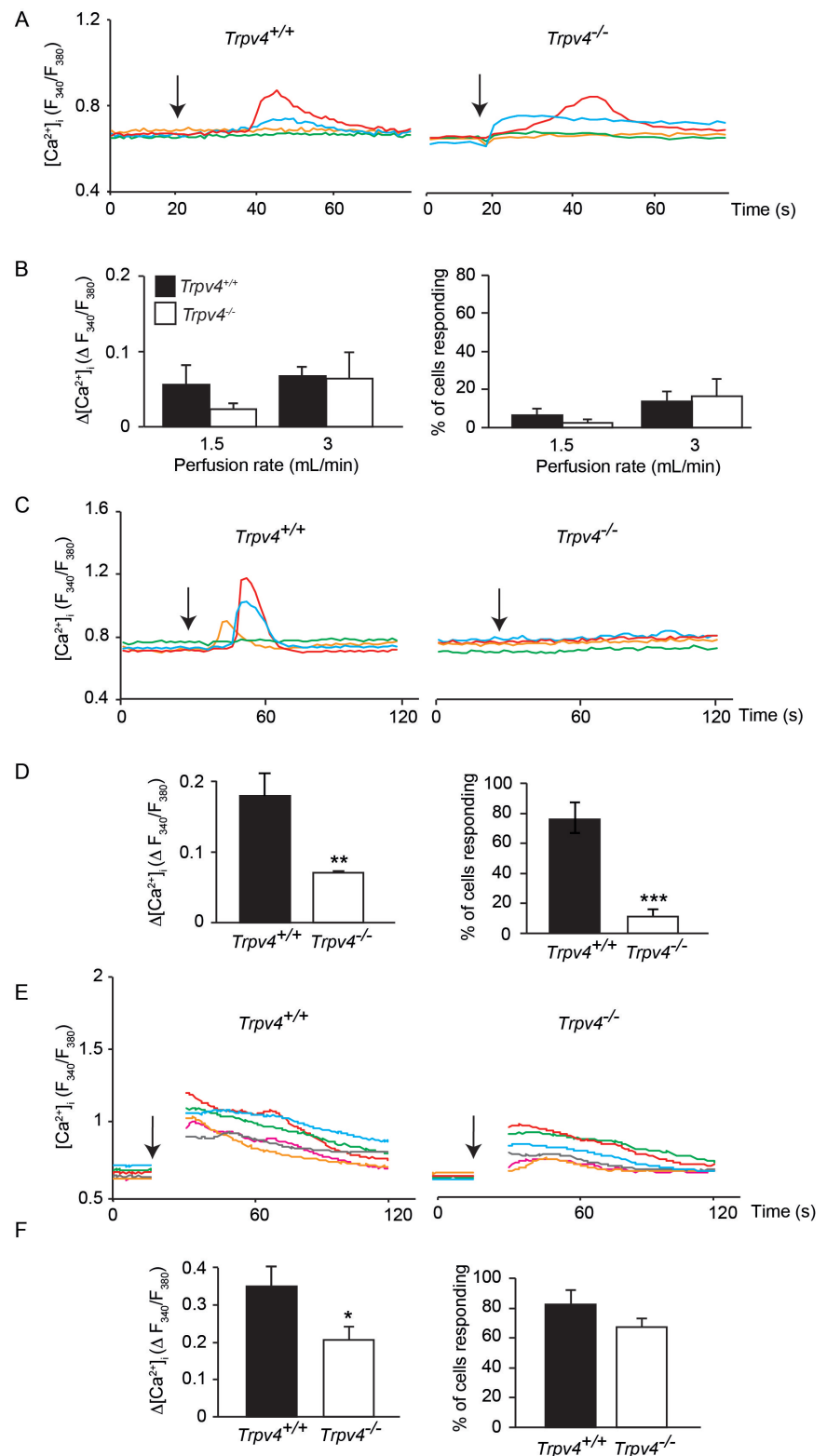


FIGURE 8. Effect of hypotonic 'low-chloride' solution stimulation on mPTCs in patch-clamp experiments. Representative time-course measured at membrane potential of +50 mV (black curve; A,C) and -50 mV (grey curve; A,C) and I-V trace (B,D) of whole cell currents through *Trpv4*^{+/+} and *Trpv4*^{-/-} mPTCs, respectively, during administration of a 'low-chloride' hypotonic solution (HTS, see Methods) and HC067047 (1 μ M), at the indicated time intervals. The black I-V traces showed in (B) and (D) correspond to our control conditions

Results

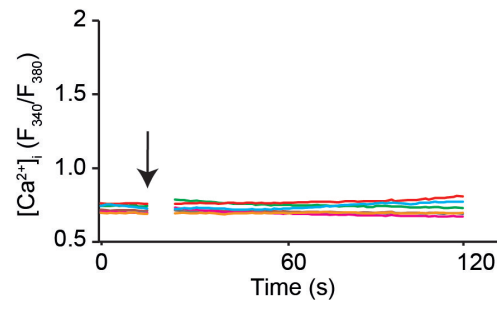
We then investigated whether we could mechanically activate TRPV4 in mPTCs, using Ca^{2+} measurement experiments. As TRPV4 channel has been described as a flow sensor in the CD [122], we first tested the response of mPTCs to shear stress. When a flow was applied on the surface of the cells, some sparse responses were observed among mPTCs. However, this phenomenon was independent of TRPV4 expression (**FIGURE 9A-B**). We then studied the response of mPTCs to the application of a hypotonic solution. The hypotonic cell swelling induced a $[\text{Ca}^{2+}]_i$ transient in mPTCs that was absent in *Trpv4*^{-/-} cells (**FIGURE 9C-D**). This could indicate that TRPV4 would act as a stretch sensor in the PT. In order to investigate the response of mPTCs to stretch, we grew them on stretchable silicon chambers. Stretch of the cells induced a rise in $[\text{Ca}^{2+}]_i$. We observed that this response depended on the presence of extracellular Ca^{2+} , indicating a Ca^{2+} entry through a mechanically activated channel (**FIGURE 10**). In *Trpv4*^{-/-} cells, the response to stretch was not abolished. However, there was a significant decrease in the amplitude of the response (**FIGURE 9E-F**).

FIGURE 9. Mechanical response of mPTCs in presence or absence of TRPV4. Changes in $[Ca^{2+}]_i$ were assessed during flow stimulation (A-B), hypotonic cell swelling (C-D) and cell stretching (E-F). For each stimulus, representative curves of individual mPTCs are shown for $Trpv4^{+/+}$ and $Trpv4^{-/-}$ cells (A, C, E). B, D, F. Average increase in $[Ca^{2+}]_i$ and fraction of the responding cells upon flow stimulation, hypotonic cell swelling and cell stretching (n=5 experiments with 15-20 cells). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to $Trpv4^{+/+}$ cells (t-test)



Results

FIGURE 10. Mechanical response of mPTCs in absence of extracellular Ca^{2+} . Representative curves of the $[\text{Ca}^{2+}]_i$ in individual mPTCs assessed during cell stretching



Perspective

In this chapter, we intended to study the role of TRPV4 in the PT, where we described its expression for the first time (chapter I). We also showed that its pharmacological activation enhanced endocytosis in a culture of PT cells. While *Trpv4*^{-/-} mice did not present urinary signs of PT dysfunction, we hypothesized that it could be activated during PT mechanical loading, as this triggers balancing processes increasing its reabsorptive activity [153]. In a mouse model of increased proteinuria, we showed that the lack of TRPV4 was associated with an exaggerated loss of albumin in the urine.

We then studied whether mechanical forces could trigger activation of TRPV4 in PT cells. We tested different mechanical stimuli on mPTCs, comparing their effect in presence or absence of TRPV4. Ca^{2+} transients were observed in some cells during shear stress, but independently of TRPV4 expression. The cells were however sensitive to hypotonic cell swelling in a TRPV4-dependent manner. Directly stretching mPTCs produced a robust $[\text{Ca}^{2+}]_i$ increase. Knockout of TRPV4 did not completely suppress the sensitivity of mPTCs to stretch, but reduced the amplitude of the transients. This result suggests that TRPV4 participates to the Ca^{2+} signaling upon mechanical stimulation of the mPTCs, probably in concert with other mechanosensitive channels. Interestingly, the mechanosensitive channel Piezo1 was shown to function as a stretch-activated channel in PT cells [156]. PT could be equipped with more than one mechanosensor, allowing diverse kinetics of transduction, and detection of stimuli varying in amplitude, frequency or rate of installation.

We plan to continue the study of TRPV4 in the PT by testing the effect of mechanical stimulation, in particular stretch, on the endocytic function of mPTCs *in vitro*. Taking advantage of patch-clamp techniques, we will compare the activation of TRPV4 on the apical and the basolateral membranes of freshly isolated PT. The possible involvement of Piezo1 in the mechanosensitive Ca^{2+} influxes in mPTCs will be assessed. Modulation of the intracellular effectors of endocytosis by a Ca^{2+} -dependent pathway is not classically described, but a recent study showed a Ca^{2+} -dependent modulation of endocytosis in the PT subjected to flow [138]. Further advance in the analysis of Ca^{2+} -sensitive processes in the endocytic function of the PT is still needed.

CHAPTER III

TRPV4 participates in pressure-induced inhibition of renin secretion by juxtaglomerular cells

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Abstract

The RAS is a crucial blood pressure regulation system. It consists of a hormonal cascade where the rate-limiting enzyme is renin, which is secreted in the blood flow by renal JG cells in response to low pressure in the renal afferent arteriole. In contrast, an increase in blood pressure in the afferent arteriole results in a decreased renin secretion. This is accompanied by a transitory increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) of JG cells. The inverse relationship between $[Ca^{2+}]_i$ and renin secretion has been called the “calcium paradox” of renin release. How increased pressure induces a $[Ca^{2+}]_i$ transient in the JG cells is however unknown. We observed that $[Ca^{2+}]_i$ transients induced by mechanical stimuli in JG As4.1 cells were almost completely abolished by HC067047 and RN1734, two inhibitors of TRPV4. They were also reduced by half by siRNA-mediated repression of TRPV4 expression but not after repression or inhibition of TRPV2 or Piezo1 ion channels. Interestingly, the stimulation of renin secretion by the adenylate cyclase (AC) activator forskolin (FK) was totally inhibited by cyclic stretching of the cells. This effect was mimicked by stimulation with GSK1016790A and 4αPDD, two activators of TRPV4 and inhibited in the presence of HC067047. Moreover, in isolated perfused kidneys from *Trpv4*^{-/-} mice, the pressure - renin relationship was significantly altered. Acute blocking of TRPV4 by adding HC067047 in perfusion in wild-type kidneys increased renin secretion by a factor of 2.5. *In vivo*, *Trpv4*^{-/-} mice presented increased plasma levels of renin and aldosterone compared to wild-type mice. Altogether, our results suggest that TRPV4 is involved in the pressure-induced entry of Ca^{2+} in JG cells, which inhibits renin release and allows the negative feedback regulation on blood pressure.

Introduction

The RAS controls internal salt and fluid balance and blood pressure. In the system, the aspartyl protease renin cleaves the angiotensinogen secreted by the liver at its N-terminus, transforming it in AT1. The angiotensin – converting enzyme then converts AT1 in AT2, which exerts different actions that control blood pressure: vasoconstriction, renal salt reabsorption, catecholamine and aldosterone release, stimulation of thirst and salt appetite.

The release of renin by JG cells constitutes the limiting step of the system and is therefore highly regulated. Indeed, JG cells are located in the media layer of the afferent arterioles of the kidney and respond to several hormonal factors such as β -adrenergic agonists that stimulate the release of renin or AT2, adenosine, VP, and atrial natriuretic peptide that inhibit its release.

Renin secretion is also controlled by the macula densa. There is an inverse relationship between luminal NaCl concentration at the macula densa and renin secretion, involving paracrine mediators such as prostaglandins, NO and adenosine [157].

The RAS is finally directly controlled by blood pressure [97, 158]. A decrease in blood pressure triggers renin secretion, which eventually leads to an AT2-induced increase in blood pressure. Inversely, an increase in blood pressure inhibits renin secretion. This inverse relationship between blood pressure in afferent arteriole and renin secretion constitutes a negative feedback allowing blood pressure stabilization. Several experiments show that this baroreflex feedback is distinct from the tubuloglomerular reflex [99, 159, 160], the most suggestive one demonstrating that pressure-dependent regulation of renin release is observed in isolated JG cells or in renin expressing cell lines [161-163]. In fact, in isolated JG cell lines, mechanical stretch prevents both agonists-stimulated renin release and renin mRNA accumulation [161]. Intracellular signaling pathways controlling renin secretion essentially involve cyclic adenosine monophosphate (cAMP) that stimulates secretion and Ca^{2+} that exerts the opposite effect [164-166]. Indeed, stimulation of β -adrenoreceptors or direct activation of ACs by FK increase cAMP concentration and cAMP activates protein kinase A (PKA) that triggers renin vesicles release by JG cells. VP, endothelin or AT2 increase $[\text{Ca}^{2+}]_i$ and inhibit renin release. In most secretory cells, $[\text{Ca}^{2+}]_i$ induces exocytosis. The inverse relationship between $[\text{Ca}^{2+}]_i$ and renin secretion has therefore been called the “calcium paradox” of renin release (reviewed in [167]). This effect is due, at least partially, to a direct inhibition of AC5 and AC6 by $[\text{Ca}^{2+}]_i$ [102], but also probably to the activation of a calcineurin-mediated control of renin exocytosis [168-170] or to the activation of a chloride

conductance [171]. Increased pressure in the afferent arteriole also leads to elevated $[Ca^{2+}]_i$ within JG cells in situ. Ca^{2+} transients seem to be due to an entry of Ca^{2+} from the extracellular medium with a possible reinforcement of the signal by a release of Ca^{2+} from the stores (endoplasmic reticulum) either via a Ca^{2+} -induced Ca^{2+} release process or via the activation of phospholipase C and the subsequent increase of inositol trisphosphate [163] (reviewed in [101]). Ion channels involved in pressure-induced entry of Ca^{2+} in JG cells have not been identified so far. They could involve mechanosensitive channels as well as voltage-dependent L-type Ca^{2+} channels but this is still a matter of debate [100, 103].

The present study was designed to investigate the possible involvement of mechanosensitive channels, in particular channels belonging to the TRP family or to the Piezo family [172-176]. We used the renin gene expressing and secreting cell line As4.1 to identify the mechanoreceptor involved and we validated our observations on a genetically modified mouse model. Our data suggest that TRPV4 ion channel participates in mechanosensitivity of JG cells, allowing pressure-induced calcium entry and subsequent inhibition of renin secretion.

Results

Methods

Cell culture and transfection

As4.1, a renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mouse, were purchased from ATCC. The cells were cultured in DMEM and supplemented with 10% fetal bovine serum (FBS). Cell cultures were maintained in humidified room air containing 5% CO₂ at 37°C. For [Ca²⁺]_i measurement experiments, As4.1 cells were seeded on 22-mm coverslips 48 h before the experiment. For transfection experiments, As4.1 cells were seeded in 6-wells culture dishes (Greiner) at a density of 5.10⁴ cells/well the day before transfection. Cells were transfected with Stealth RNAi siRNA (Negative Control, TRPV4, TRPV2 and Piezo1) and Lipofectamine RNAi MAX (Invitrogen) according to manufacturer's protocol. Cells were used at 72 h post-transfection.

Ethical approval

All animals were housed and handled according to the Belgian Council on Animal Care guidelines based on protocols approved by the Animal Ethics Committee of the Université Catholique de Louvain (2013/UCL/MD/017). Animals were given access to food and water *ad libitum* unless otherwise stated. At appropriate experimental time points, all animals were humanely killed by an overdose of anaesthetic followed by decapitation.

Animals

Experiments were conducted on 8 to 12-week-old, male *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, generated by targeted deletion of exon 12 of the *Trpv4* gene [177]. The mice were backcrossed on a C57BL/6 background before starting the study.

RT-qPCR

Total RNA was extracted from As4.1 using Trizol reagent (Invitrogen) according to the manufacturer's protocol, and reverse-transcribed using qScript Reverse Transcriptase (Quanta Biosciences, Gaithersburg, ME). cDNA-specific PCR primers were designed using Primer-BLAST and purchased from Eurogentec (Seraing, Belgium). Primer sequences are

given in **TABLE 1**. *Gapdh* was used as reference gene. Quantitative RT-qPCR was performed using SYBRGreen Mix (Bio-Rad, Hercules, CA, USA). The reaction was initiated at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, and extension at 72 °C for 10 s. Data were recorded on a MyiQ qPCR detection system (Bio-Rad), and cycle threshold (*Ct*) values for each reaction were determined using analytical software from the same manufacturer. Each cDNA was amplified in duplicate, and *Ct* values were averaged for each duplicate. The average *Ct* value for *Gapdh* was subtracted from the average *Ct* value for the gene of interest. The ΔC_t value obtained in siRNA condition was then subtracted from the ΔC_t value obtained in control condition, giving a $\Delta\Delta C_t$ value. As amplification efficiencies of primer pairs were comparable, the *Gapdh*-normalized expression of mRNAs was given by the relation $2^{-\Delta\Delta C_t}$.

Gene	Forward primer	Reverse primer	PCR Product (bp)
<i>Gapdh</i>	TGCACCACCAACTGCTTA	GGATGCAGGGATGATGTT	176
<i>Trpc1</i>	AACGATGGATGTGCGACCTG	GGCTTGGGCAAAGACACATC	95
<i>Trpc6</i>	CTTAGCAATGAGCTGGCAGTG	GCAGAGGTCCAAGAGACCAA	111
<i>Trpv1</i>	TGGGCATCATCAACGAGGAC	GGGAACCAGGGCAAAGTTCT	116
<i>Trpv2</i>	GCGAATGTTTACATCCGAGC	CTCCAGGAGGTAGGTCACCA	132
<i>Trpv4</i>	GGCTACTTCTACTTTGGGGAGC	TGTCGCCTCATGTCAGCTTT	113
<i>Trpa1</i>	CCTGACCTTTGGGCAGCTTA	ACCGCCAAGCCAATCAGTAA	78
<i>Trpm3</i>	ATGGAGTAAGCATGCACCGTTT	TGAGGGCCCATGTCTCGTAT	73
<i>Trpm7</i>	CACTGGCATGGGACAGAGTT	AAGGATCCAACCAGCCACTG	76
<i>Trpp2</i>	TGACCTACGGCATGATGAGC	GAGCCTTCGGTGAACCTCCA	148
<i>Piezo1</i>	CACCCGTATCTTGGGCAACT	ACTAGACGGAACCCCTGGAA	78
<i>Piezo2</i>	CTGCCCCTTGCCGAGATTAT	GTTGGGCCGCACAATGAAAT	101

TABLE 1. List of DNA primers used in quantitative RT-qPCR

Western blotting

Cells were collected in RIPA buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). 30 µg of protein for each sample was loaded on a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane and probed with a home-made anti-TRPV4 antibody described earlier [86] and with an anti-GAPDH antibody (Cell Signaling).

Results

Immunostaining

Trpv4^{+/+} and *Trpv4*^{-/-} mice were deeply anesthetized by IP injection (10 ml/kg) of a solution containing 10 mg/mL ketamine and 1 mg/ml xylazine. After intracardiac perfusion with a 4°C buffered 4% paraformaldehyde PBS solution, the kidneys were removed and postfixed in the same solution for 2 hours, washed in PBS and cryoprotected overnight in 30% sucrose PBS. After embedding in Optimal Cutting Temperature compound (OCT, Tissue-Tek, VWR), 10µm thick cryosections were cut and stored at -80°C. Sections were blocked with a 3% BSA containing PBS solution and then incubated overnight with 1/200 anti-TRPV4 (Alomone) and 1/200 anti-renin (R&D) antibodies. Binding sites were revealed with Alexa Fluor 488 and 594 (Thermofisher). Images were acquired with an Olympus FV1000 confocal microscope.

Calcium measurement and mechanical stimulation

As4.1 were incubated for 30 min at room temperature with 5 µM Fura2-AM (Calbiochem, Camarillo, CA, USA) in Krebs-HEPES buffer containing 11.5 mM HEPES, 135.5 mM NaCl, 5.9 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM D-glucose, pH 7.4. They were then washed for 1 hour in Krebs-HEPES buffer and mounted in the same medium on a Zeiss Axiovert 200 M inverted microscope (Zeiss Belgium, Zaventem, Belgium). [Ca²⁺]_i was measured in 15-20 individual As4.1 cells using alternative excitation of Fura2-AM (0.5 Hz) at 340 and 380 nm using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument, Novato, CA, USA). Images were acquired with a Zeiss AxioCam camera coupled to a 510-nm emission filter and analyzed with Axiovision software. Cytosolic free Ca²⁺ concentration was evaluated from the ratio of fluorescence emission intensities excited at the two wavelengths using the Grynkiewicz equation [178].

For measurement of [Ca²⁺]_i during mechanical stimulation, As4.1 cells were seeded on 22-mm coverslips 48 h before the experiment. A 18G needle, connected to a peristaltic pump perfusion system, was placed above the coverslip just above the field. Stimulation of the cells within the field was achieved by briefly activating the peristaltic pump. The flow of medium produced a deformation of the cell membrane visible in the video recording of the experiment. Mechanical stimulation was performed in Krebs-HEPES buffer on three separated spots of the coverslip in which 15 cells were analyzed. When an antagonist had to be tested, the medium was removed and replaced by fresh medium supplemented with the

antagonist. After 10 minutes, cells from three different spots were stimulated to test the effect of the antagonists on mechanical stimulation. This protocol prevented us from moving the stimulation system between control and treatment conditions. The video recording was systematically checked to ensure that the cells selected in the fields had their membrane deformed by the stimulus.

For measurement of $[Ca^{2+}]_i$ during cell stretching, As4.1 cells were cultured 24 h before the experiments in collagen I-coated flexible silicon chambers (ST-CH-04, B-Bridge) at a density of $2 \cdot 10^4$ cells/chamber. On the day of the experiment, the chambers were placed on the microscope-mountable stretching instrument (ST-150, B-Bridge). Cells were strained by a holded uniaxial stretching of 20%.

Renin secretion assay

As4.1 cells were seeded into 6-wells culture dishes 24 h before the experiment at a density of $2 \cdot 10^5$ cells/well. On the day of the experiment, the medium was removed and the cells were washed with DMEM. Then cells were incubated for 30 minutes at 37°C in DMEM or DMEM supplemented with either 5 μ M FK and 100 μ M 3-isobutyl-1-methylxanthine (IBMX) or FK, IBMX and 100 nM GSK1016790A, or FSK, IBMX and 1 μ M 4 α PDD. Experiments in Ca^{2+} free medium were performed in Ca^{2+} free DMEM (Invitrogen cat. 21068028) supplemented with 200 μ M EGTA). The supernatant was carefully collected and centrifuged at 5000 g for 5 minutes to eliminate possible floating cells, then stored at -80°C.

For measurement of renin secretion during cell stretching, As4.1 cells were seeded at confluence 24 h before the experiments in collagen I-coated flexible silicon chambers (ST-CH-10, B-Bridge), placed in the stretching instrument (ST-140, B-Bridge). FK treated cells were prestimulated for 6 hours to enhance the AC activity [102]. Cells were strained by a cyclic uniaxial stretching of 20% at 0.3Hz for 30 min.

Renin and aldosterone concentration measurement

Total renin concentration in cell culture supernatants and kidney perfusates was measured using the Mouse Renin 1 DuoSet ELISA (R&D Systems) according to manufacturer's protocol. Plasma aldosterone concentration was measured with an assay from Enzo Life Sciences (ADI-900-173) according to manufacturer's protocol.

Results

Plasma renin concentration measurement

To specifically measure active renin and avoid interference with pro-renin secreted by non-juxtaglomerular organs [179], we measured specific aspartyl protease activity of renin by measuring the fluorescence produced by cleavage into two separate fragments of a FRET peptide in which the fluorescence of 5-carboxyfluorescein (5-FAM) is quenched by the QXL 520 dye (Sensolyte® 520 Mouse Renin Assay Kit, Anaspec). Nine pairs of three months old males from *Trpv4*^{+/+} and *Trpv4*^{-/-} mice were single-housed during 3 days, then trunk blood was collected and centrifuged to collect plasma. The plasma was kept at -80°C until processing.

Isolated perfused kidney

Mice were injected intraperitoneally with a ketamine-xylazine mix for anaesthesia (see above) and with heparin (25U). On a heating plate (Vtrade, Fernelmont, Belgium), the abdomen was opened and the intestines were removed. Gonadal vessels were tied and loose ligations (FST 18020-60) were placed around the aorta and the vena cava, between the two renal pedicles and right under the left kidney pedicle. Iliac vessels were tied and aorta was clamped below the left renal artery. The aorta was then cannulated with a 24G catheter and the vena cava with a 22G catheter. The loose ligations were tightened and the clamp was removed. The left kidney was perfused with pre-warmed and gassed (95% O₂ - 5% CO₂) DMEM (Invitrogen 41966-029) at a pressure of 100mmHg and covered with wet compresses. Suprarenal aorta and spinal cord were sectioned to interrupt kidney innervation. The perfusion was continued for 10 minutes to allow complete washing out of the blood. The perfusate was collected from the venous catheter for 1 minute every 3 minutes, and during the time intervals, perfusion pressure could be changed. Perfusate was collected in duplicate at each pressure point, and two protocols were used: 100 – 60 – 140 mm Hg or 100 – 40 – 20 mm Hg. To ensure secretion stability, the maximal duration of an experiment was 30 minutes. At the end of the experiment, the kidney was weighed. The perfusate aliquots were weighed to determine the perfusate flow at each perfusion pressure, which was normalized on kidney weight, and stored at -80°C until determination of renin concentration. The renin secretion rate at each perfusion pressure (ng/min.g) was calculated as the product of normalized perfusate flow (mL/min.g) with perfusate renin concentration (ng/mL).

Reagents

The GsMTx4 toxin, isolated from *Grammostola spatulata* spider [180], and Yoda1 [181] were obtained from Tocris. Fura2-AM was purchased from Invitrogen (Molecular Probes); FK, IBMX, Gd^{3+} , GSK1016790A, HC067047, RN1734 and 3-(4-octadecyl)-benzoylacrylic acid (OBAA) from Sigma; 4 α PDD from Enzo Life Sciences; RR and endothelin from Merck Millipore.

Statistical analysis

Student's t test was used unless otherwise specified. Data are presented as means $\pm$ standard error of the mean (SEM). ANOVA or two-tailed unpaired Student's t tests were used to determine statistical significance except for reninemia measurements for which a non-parametric analysis was used (Mann-Whitney test).

Results

Results

As4.1 cells are mechanosensitive

We first verified the presence of a mechanosensitive calcium channel in As4.1 cells by monitoring changes in $[Ca^{2+}]_i$ during mechanical stimulation [163]. We tested a uniaxial strain in silicone stretchable chambers. A holded 20% elongation of the cells produced an elevation in $[Ca^{2+}]_i$ (**FIGURE 1A**), by comparison to unstretched cells. This elevation was absent when Ca^{2+} was removed from the extracellular medium (**FIGURE 1B-C**). Cells were also mechanically stimulated with a jet of external medium able to induce a deformation objectified by videomicroscopy. This mechanical stimulation also induced a Ca^{2+} transient in As4.1 cells that was dependent on extracellular Ca^{2+} (**FIGURE 1D-F**).

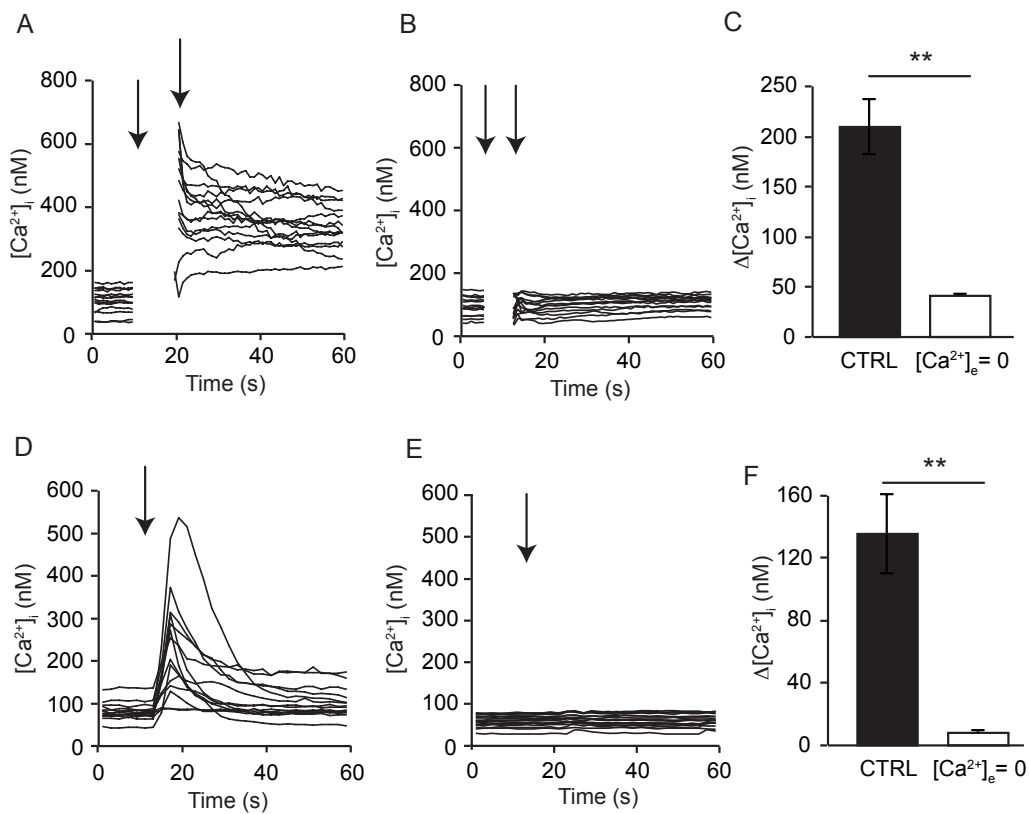


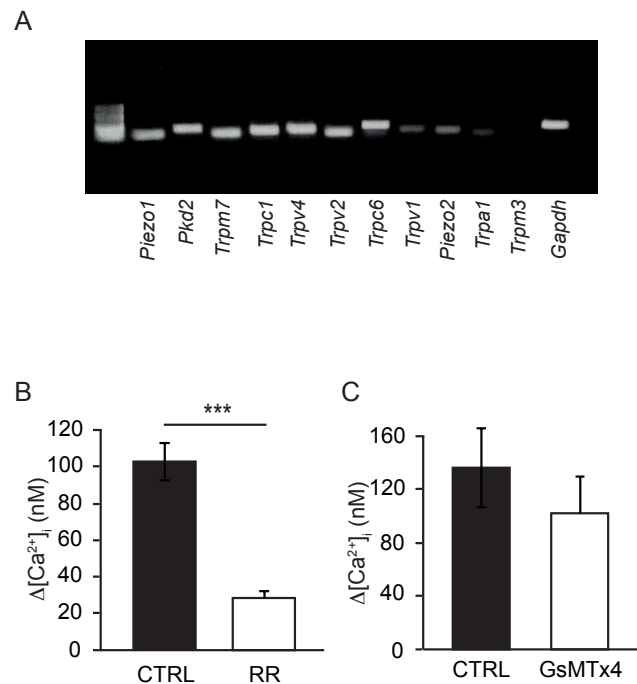
FIGURE 1. Mechanical stimulation of As4.1 cells elicits a rise in $[Ca^{2+}]_i$. A-B. $[Ca^{2+}]_i$ transients observed in As4.1 cells cultured on silicone chambers and stretched by 20% in the presence (A) or absence (B) of external Ca^{2+} . The first arrow indicates the initiation of the stretch and the second arrow the focus in the new observation field. C. Quantification ($n = 62$ vs 53). D-E. $[Ca^{2+}]_i$ transients observed in As4.1 cells mechanically stimulated (deformed) by a flow of medium (arrow) containing (D) or not (E) external Ca^{2+} . F. Quantification ($n = 50$ vs 51). ** $P < 0.01$

TRPV4 is needed for As4.1 mechanosensitivity

In order to identify the channel involved in the process, we performed a screening of putative mechanosensitive Ca^{2+} channels including members of the TRP and Piezo channels families [182]. At mRNA level, we found a significant expression of *Piezo1*, *Pkd2*, *Trpm7*, *Trpc1*, *Trpv4*, *Trpv2* and *Trpc6* (**FIGURE 2A**).

Using Ca^{2+} channels blockers, we narrowed the scope of investigation (**FIGURE 2B-C**). Application of 25 μM Gd^{3+} , a non-specific Ca^{2+} channel blocker, was found to inhibit mechanically induced Ca^{2+} entry (-74%, $P < 0.05$, data not shown), as well as RR (30 μM), an inhibitor of TRPV and Piezo channels. Finally, we used GsMTx4, a mechanosensitive channel blocker specific to Piezo and mechanosensitive TRPC channels (TRPC1 and TRPC6) [183]. Up to a concentration of 10 μM , GsMTx4 toxin failed to block the mechanically induced calcium entry. As the Ca^{2+} entry was blocked by RR but not GsMTx4, and as Piezo channel is known to be inhibited by GsMTx4 [24], TRPV ion channels were the more plausible mechano-activated channels in As4.1 cells.

FIGURE 2. Screening of putative mechanosensitive calcium channels in As4.1 cells. A. Expression of *Piezo1*, *Pkd2*, *Trpm7*, *Trpc1*, *Trpv4*, *Trpv2* and *Trpc6* measured by RT-qPCR performed on 2 μg of As4.1 total mRNA. B-C Sensitivity of mechanically (flow-induced cell deformation) induced Ca^{2+} response to Ca^{2+} channels blockers RR (n = 9) and GsMTx4 (n = 4). CTRL indicates the level of $[\text{Ca}^{2+}]_i$ transient observed in control conditions, before the addition of the blocker. *** $P < 0.001$



Using siRNAs we repressed the expression of *Trpv2*, *Trpv4* and *Piezo1* in these cells. The efficiency of repression was verified using quantitative RT-qPCR (**FIGURE 3A**) and immunoblotting for TRPV4 (**FIGURE 3B**). The response of As4.1 cells to TRPV2, TRPV4 and Piezo1 specific agonists was also tested. GSK1016790A (100nM), a TRPV4 specific

Results

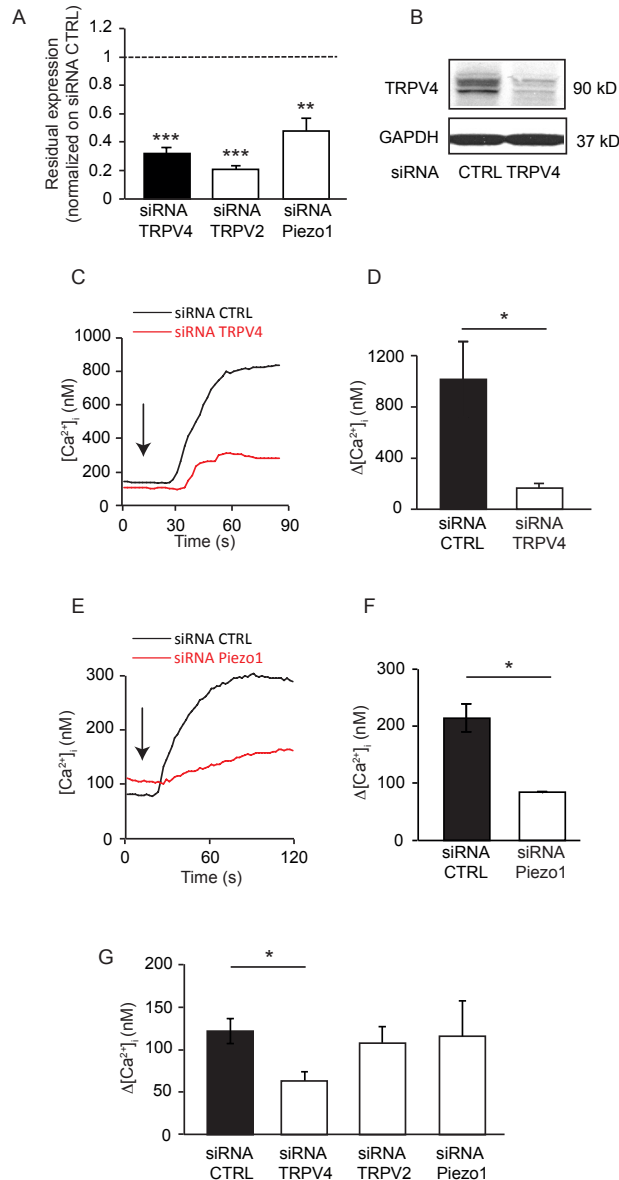
agonist [63], triggered a large increase in $[Ca^{2+}]_i$ that was diminished in the siRNA TRPV4 treated cells (**FIGURE 3C-D**). Yoda1 (15 μ M), a Piezo1 specific agonist [181], caused a smaller increase in $[Ca^{2+}]_i$ that was diminished in the siRNA Piezo1 condition (**FIGURE 3E-F**). No rise in $[Ca^{2+}]_i$ was observed upon addition of cannabidiol (100 μ M), a TRPV2 specific agonist [184] (**additional FIGURE 1A**). Interestingly, knocking-down *Trpv4* expression significantly reduced the Ca^{2+} response to mechanical stimuli, while knocking-down *Trpv2* or *Piezo1* expression did not alter it (Fig. 3G). The response was also not reduced by the presence of tranilast (100 μ M), an inhibitor of TRPV2 (**additional FIGURE 1**).

As expected, the response to GSK1016790A was clearly inhibited by RR and two specific blockers of TRPV4, HC067047 and RN1734 [185, 186], but insensitive to 10 μ M GsMTx4 toxin. As4.1 cells were also sensitive to another TRPV4 activator, 4 α PDD (**FIGURE 4A**).

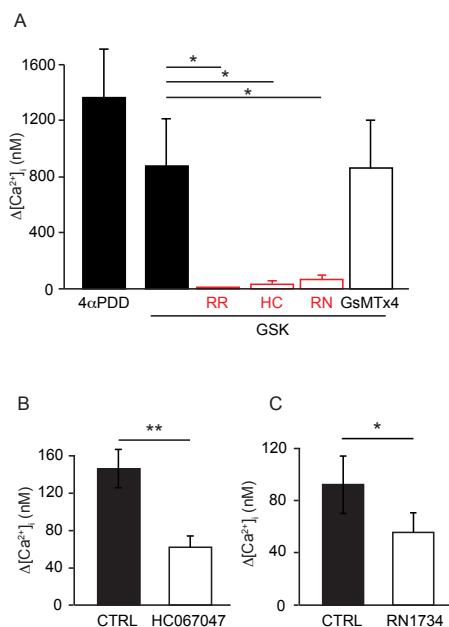
Finally, we tested the mechanical stimulation in the presence of HC067047 or RN1734. The rise of $[Ca^{2+}]_i$ was significantly reduced in the presence of these antagonists (Fig. 4B-C). Activation of TRPV4 by a mechanical stimulus is thought to be mediated by the PLA₂ pathway [61]. In our model, inhibition of PLA₂ with OBAA reduced the Ca^{2+} response to mechanical stimulus (-49%, $P < 0.01$, **additional FIGURE 2**). Altogether both pharmacological and siRNA strategies show that TRPV4 is involved in mechanical sensation of these cells.

FIGURE 3. Downregulation of *Trpv4* alters

As4.1 mechanosensitivity. A. Efficiency of downregulation of *Trpv4*, *Trpv2* or *Piezo1* verified by RT-qPCR. Residual expression is normalized on the respective controls (n=6 experiments for *Trpv4* and 5 for *Trpv2* and *Piezo1*). ** $P < 0.01$ and *** $P < 0.001$ comparing ΔCt values in each system with the respective controls. B. Western blotting comparing the expression of TRPV4 protein 72h after transfection with siRNA CTRL or siRNA TRPV4. C-D. Representative graph and quantification of the effect of GSK1016790A (100nM, arrow) on $[Ca^{2+}]_i$ in CTRL or TRPV4 siRNA-transfected cells (n=5 experiments). * $P < 0.05$. E-F. Representative graph and quantification of the effect of Yoda1 (15 μ M, arrow) on $[Ca^{2+}]_i$ in CTRL or Piezo1 siRNA-transfected cells (n=3 experiments). * $P < 0.05$. G. Effect of siRNA-induced repression of *Trpv4*, *Trpv2* or *Piezo1* expression on the Ca^{2+} response to mechanical stimulation (flow-induced cell deformation). $P = 0.03$ for one-way ANOVA and * $P < 0.05$ for multiple comparisons (Bonferroni)

**FIGURE 4. Pharmacological blockage of TRPV4 alters As4.1 mechanosensitivity.**

A. Quantification of the Ca^{2+} response of As4.1 cells to the addition of TRPV4 agonists 4 α PDD (1 μ M) or GSK1016790A (100nM). The Ca^{2+} response is abolished by TRPV4 blockers RR (30 μ M), RN1734 (20 μ M) and HC067047 (1 μ M), but not by GsMTx4 (10 μ M) (n = 12 to 23). $P < 0.001$ for one-way ANOVA and * $P < 0.05$ for multiple comparisons (Bonferroni). B-C. Effect of TRPV4 blockers HC067047 (n = 8 experiments) and RN1734 (n = 9) on Ca^{2+} response to mechanical stimulation (flow-induced cell deformation). CTRL indicates the level of $[Ca^{2+}]_i$ transient observed in control conditions, before the addition of the blocker. * $P < 0.05$ and ** $P < 0.01$



Results

TRPV4 regulates renin secretion in As4.1

We then investigated the “calcium paradox” of renin release. We first verified that we could stimulate renin secretion in As4.1 cells with the AC activator FK (5 μ M), in the presence of the phosphodiesterase blocker IBMX (100 μ M). We also verified that endothelin, known to inhibit renin secretion in a Ca²⁺-dependent fashion [102, 187], could block the stimulation of renin secretion in our model (-41%, $P<0.05$, **additional FIGURE 3**). Then, using GSK1016790A and 4 α PDD, two TRPV4 agonists that induce an entry of Ca²⁺ in As4.1 cells [188], we observed that activation of TRPV4 during FK-induced renin secretion stimulation significantly prevented the release of renin (**FIGURE 5A**). The same experiment performed in a Ca²⁺-free medium supplemented with 200 μ M EGTA showed that this effect was dependent on extracellular Ca²⁺ (**FIGURE 5B**). We next cultured As4.1 cells on stretchable silicone chambers in order to test the effect of mechanical stress on renin secretion. We observed that the FK-induced renin secretion stimulation was prevented by cycling stretching the cells (0.3 Hz, 30 min) and that this effect was reversed when TRPV4 was blocked with HC067047 (1 μ M) (**FIGURE 5C**). Using a Ca²⁺-free medium supplemented with 200 μ M EGTA, we verified that this process was also dependent on extracellular Ca²⁺ (**FIGURE 5D**).

Altogether, these results reveal that renin secretion is inhibited by mechanical stimulation via a Ca²⁺ entry through the TRPV4 channel.

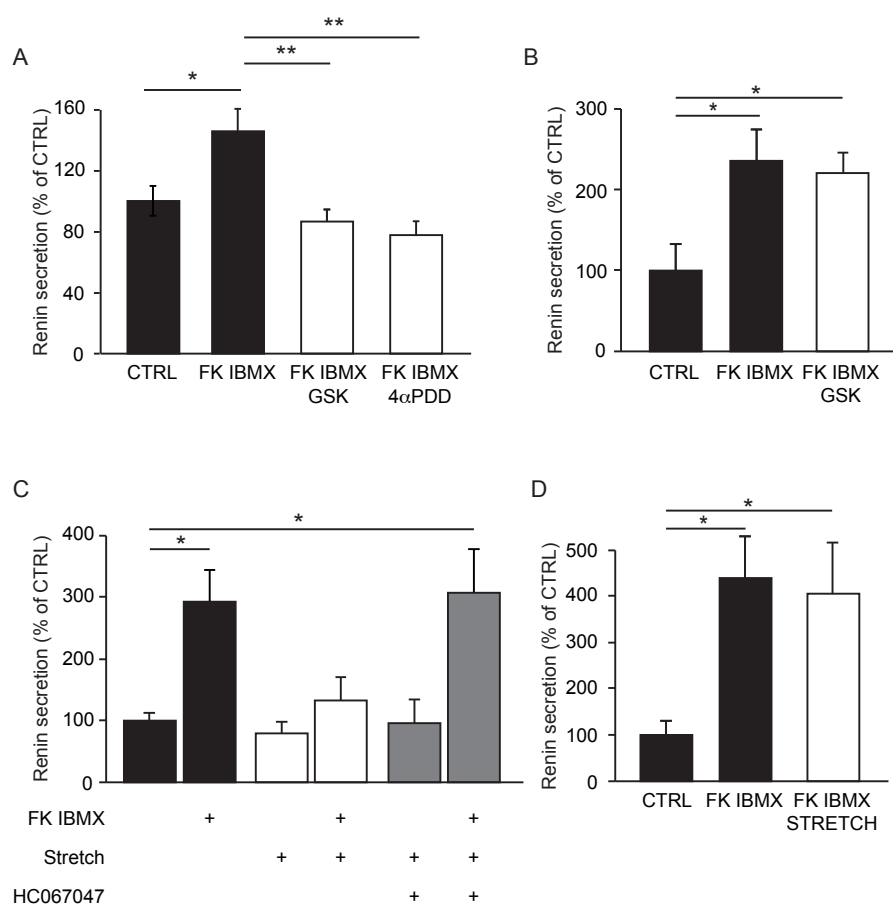


FIGURE 5. TRPV4 regulates renin secretion in As4.1 cells. A. Effects of TRPV4 activators GSK1016790A (100nM) or 4αPDD (1μM) on renin secretion in As4.1 cells in the presence of FK (5μM) and IBMX (100μM) (n = 9 wells for all conditions except for 4αPDD n = 6 wells). $P=0.001$ for one-way ANOVA. B. Effect of TRPV4 activator GSK1016790A on renin secretion stimulated by FK and IBMX in a Ca^{2+} free extracellular medium supplemented with 200 μM EGTA (n=4). $P< 0.05$ for one-way ANOVA. C. Effect of mechanical stress (stretch) on renin secretion stimulated by FK and IBMX, with or without addition of TRPV4 blocker HC067047 (HC) (n=6). $P< 0.001$, one-way ANOVA. D. Effect of mechanical stress (stretch) on renin secretion stimulated by FK and IBMX in a Ca^{2+} free extracellular medium supplemented with 200 μM EGTA (n=6). $P< 0.01$, one-way ANOVA. * $P< 0.05$ and ** $P< 0.01$ for multiple comparisons (Bonferroni)

Results

TRPV4 is expressed in mouse JG cells

In order to investigate whether TRPV4 could play a role in JG cells *in vivo*, we verified that TRPV4 was expressed in JG cells on sections from wild type mouse kidney, using a co-staining with renin. The anti-TRPV4 antibody was validated by probing sections from *Trpv4*^{-/-} kidney in parallel. As a positive control, PT cells were strongly stained at their basolateral membrane (**FIGURE 6**) [189].

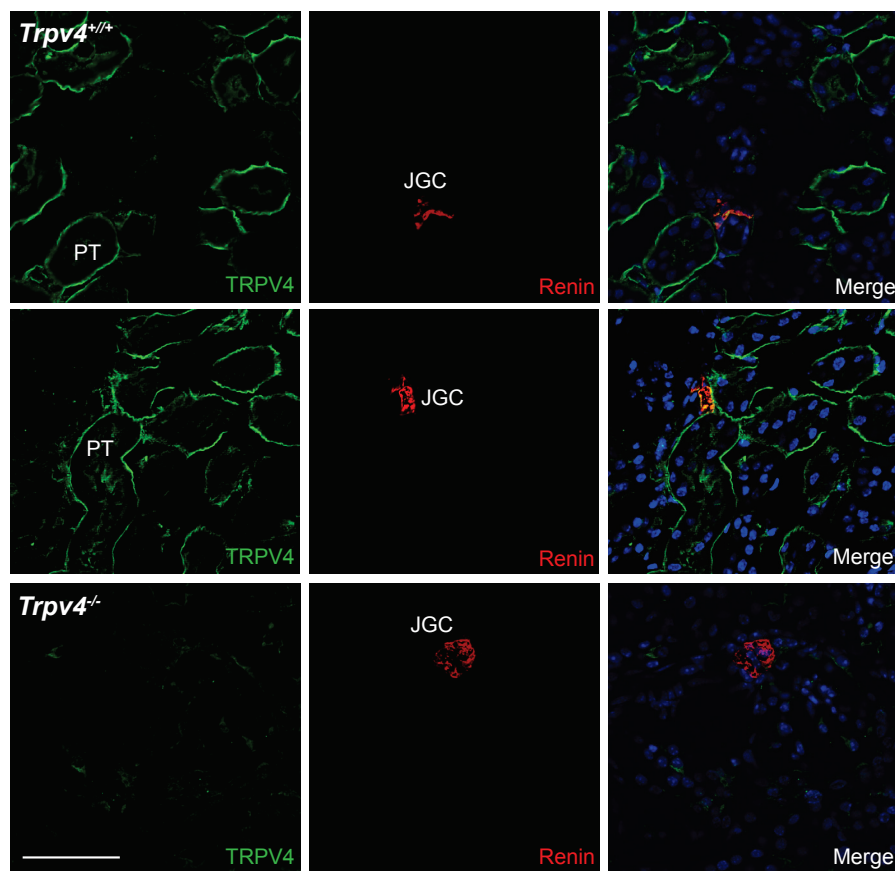


FIGURE 6. TRPV4 is expressed in the mouse JG cells. Co-immunolabelling of TRPV4 (in green) and renin (in red) on kidney sections from *Trpv4*^{+/+} (two upper rows) and *Trpv4*^{-/-} (last row) mice. Renin is used as a marker of the JG cells (JGC). TRPV4 is expressed in PT and JG cells, and is absent from *Trpv4*^{-/-} kidneys. Scale bar: 50 μ m

Lack of TRPV4 impairs pressure control on renin secretion in isolated perfused kidney

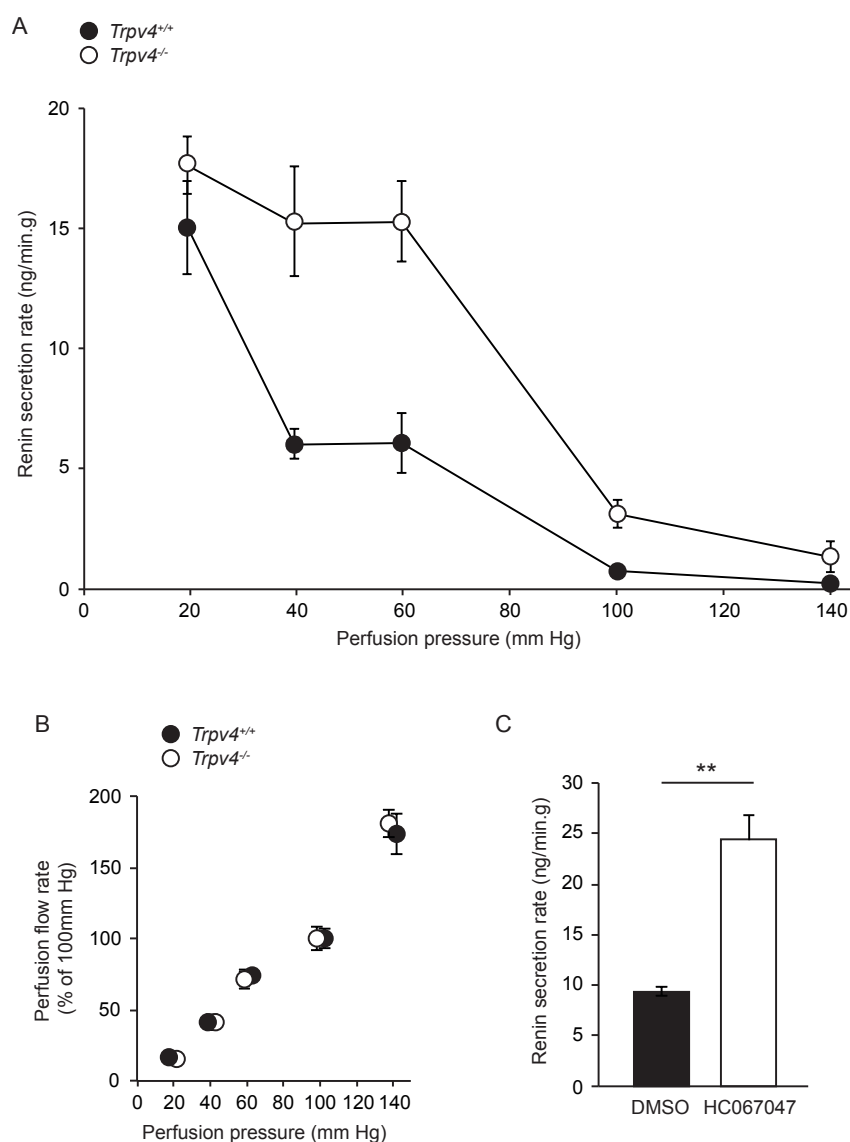
Isolated kidneys from *Trpv4*^{+/+} and *Trpv4*^{-/-} mice were perfused at different time-matched perfusion pressures. After collection of the venous effluent, the renin secretion rate was determined. We observed that, at a normal perfusion pressure (80 to 90 mmHg), renin secretion rate was higher in *Trpv4*^{-/-} kidneys and that the relationship between perfusion

pressure and renin release was shifted to the right in the kidneys from *Trpv4*^{-/-} mice compared to WT (**FIGURE 7A**). Perfusate flow (*Trpv4*^{+/+} 9.8 ± 0.8 versus *Trpv4*^{-/-} 11.1 ± 0.8 mL/min.g at 100 mm Hg) and variation in perfusate flow according to perfusion pressure were similar between the two genotypes (**FIGURE 7B**).

We next perfused isolated kidneys from wild-type mice with the TRPV4 inhibitor HC067047. According to the pressure–renin relationship observed in previous experiments, we chose to add the inhibitor at a perfusion pressure of 60 mm Hg. We observed a significantly higher renin secretion rate in the kidneys perfused with HC067047 than with the vehicle alone (DMSO) (**FIGURE 7C**). It is noteworthy that the perfusate flows remained similar in the different conditions (DMSO: 5.6 ± 1.2 and HC067047: 6.1 ± 0.6 mL/min.g).

FIGURE 7. Lack of TRPV4 impairs pressure control on renin secretion in mouse isolated perfused kidney.

A. Renin secretion rate determined on isolated kidneys from *Trpv4*^{+/+} and *Trpv4*^{-/-} mice at different perfusion pressures. * $P < 0.05$, two-way ANOVA revealing a significant difference in pressure-genotype interaction ($n = 7$ pairs). B. Perfusate flow measured at different perfusion pressures. No significant difference was found between the two genotypes. C. Effect of the TRPV4 inhibitor HC067047 on renin secretion rate measured on isolated kidneys from wild-type mice, compared to vehicle (DMSO). ** $P < 0.01$ ($n=3$)

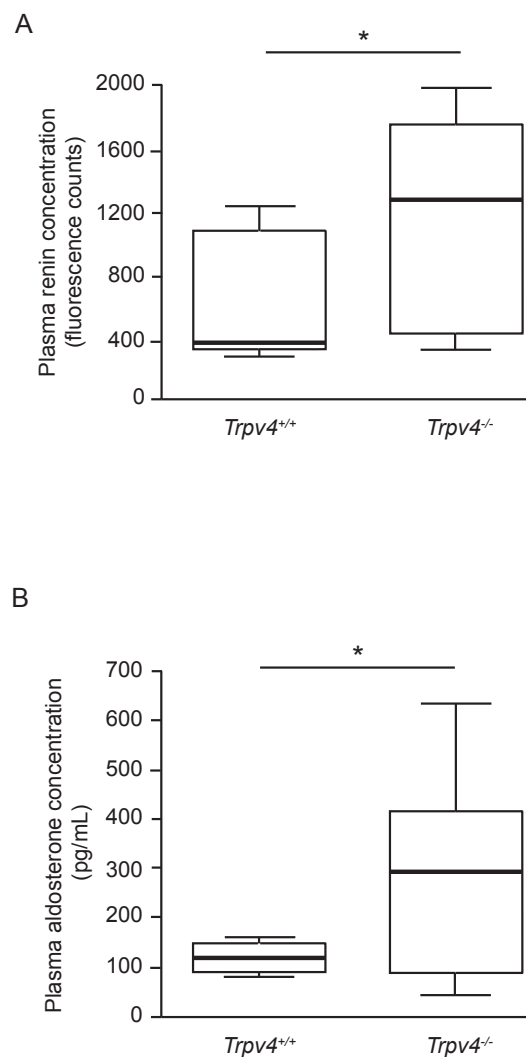


Results

Trpv4^{-/-} mice display a higher plasma renin concentration and a higher aldosterone concentration

We finally investigated plasma renin concentration in *Trpv4^{+/+}* and *Trpv4^{-/-}* mice. Trunk blood was collected in strictly similar conditions in the two genotypes. Renin concentration was measured by fluorescence after enzymatic cleavage of a quenched fluorescent substrate, and aldosterone concentration was measured by ELISA; Both renin and aldosterone concentration were found significantly elevated in *Trpv4^{-/-}* mice (**FIGURE 8A-B**).

FIGURE 8. *Trpv4^{-/-}* mice display a higher plasma renin concentration and an increased plasma concentration of aldosterone. A. Plasma renin concentration measured in *Trpv4^{+/+}* and *Trpv4^{-/-}* mice. **P*<0.05 (Mann-Whitney test, n = 9 vs 9). B. Plasma concentration of aldosterone measured in *Trpv4^{+/+}* and *Trpv4^{-/-}* mice. **P*<0.05 (Kolmogorov-Smirnov, n = 9 vs 9). Data are presented as box plots



Discussion

An increase in blood pressure in the afferent arteriole induces an increase of $[Ca^{2+}]_i$ in JG cells and results in a decrease in renin secretion. The present study was designed to decipher the mechanisms by which a mechanical stimulation of JG cells increases $[Ca^{2+}]_i$ and in particular to identify the mechanoreceptor involved.

We initially used a cellular model, the As4.1 cell line established from the ascites fluid of a six month old transgenic mouse harboring an intraparenchymal kidney tumor induced by transgene targeted tumorigenesis [190]. These cells express renin mRNA at a high level and secrete both renin and prorenin. Although the basal secretion is higher than in JG cells *in situ*, it is possible to further increase renin secretion by stimulation with β -adrenergic agonists or by directly activating AC by FK. In this cell line, AC modulation of renin secretion by $[Ca^{2+}]_i$ has been successfully achieved although to a lesser extent than in primary JG cells culture [102, 191]. Fig. 5 shows that in our conditions, *i.e.* after 6 h pre-stimulation of the cells with 5 μ M FK (see **Methods**), renin secretion could indeed be further stimulated by AC activators and inhibited by $[Ca^{2+}]_i$ increases (the calcium paradox).

To test whether these cells were mechanosensitive, we used two different protocols. First, we elongated by 20 % As4.1 cells kept in culture in silicone stretchable chambers. We observed an increase in $[Ca^{2+}]_i$ when cells were kept indefinitely stretched. However, this method did not allow us to monitor $[Ca^{2+}]_i$ within a specific cell during the stretch. Indeed, $[Ca^{2+}]_i$ was measured in a cell population before stretch and in another one just after the stretch. We therefore used another method to mechanically stimulate cells, namely a deformation of the cell by a flow of external medium. Both methods gave similar results, the mechanical stimulation induced an increase in $[Ca^{2+}]_i$ that depended on the presence of Ca^{2+} in the external medium. Thus the model seemed suitable to investigate the identity of the mechanoreceptor.

Several ion channels have been involved in mechanotransduction [9]. Candidates may belong, among others, to two protein families, the TRP channels and the Piezo channels families.

The TRP channel superfamily constitutes a large class of proteins expressed in many tissues and cell types. It is divided into six related subfamilies in mammals: the canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP) and mucolipin (TRPML) subfamilies [33]. All TRP channels are permeable to cations and most of

Results

them to calcium with a ratio PCa/PNa varying between 0.3 and 10 [192]. Several TRP channels present mechanosensitive properties, including TRPC1 and TRPC6, TRPV1, TRPV2 and TRPV4, TRPM3, TRPM7, TRPA1 and TRPP2 [2, 173]. Among these isoforms, we found in As4.1 cells a significant expression of *Trpc1* and *Trpc6*, *Trpv2* and *Trpv4*, *Pkd2* and *Trpm7*.

Piezo proteins constitute another family of potential mechanosensitive channels. The ion currents mediated by Piezo proteins are non-selective cation currents and have fast kinetics. Piezo1 is expressed especially in lungs, bladder and skin. Piezo2 is also highly expressed in neurons of the DRG [172, 173, 193]. Both Piezo1 and Piezo2 can be pharmacologically inhibited by Gd^{3+} and RR. Piezo1 is also more specifically inhibited by GsMTx4. Using quantitative RT-qPCR in As4.1 cells, we could detect the expression of *Piezo1*.

A first screening showed us that $[Ca^{2+}]_i$ transients induced by mechanical stimuli in JG As4.1 cells were inhibited by Gd^{3+} and RR, suggesting that the mechanosensitive channel involved might belong to the TRPV subfamily or to the Piezo family. We also tested the sensitivity of the response to GsMTx4. This toxin has been shown to efficiently inhibit Piezo1 (K_d near 200 nM) [172]. It also inhibits mechanosensitive channels of the TRPC subfamily (TRPC1 and TRPC6) [183, 194] and TRPA1 [195]. TRPV4 is reputed not to be sensitive to GsMTx4 [24] although this has been challenged recently [196]. In As4.1 cells, we observed that GsMTx4 (up to 10 μ M) did not inhibit $[Ca^{2+}]_i$ transients triggered by direct activation of TRPV4 by its specific agonist GSK1016790A. The deformation-induced response was also insensitive to GsMTx4, suggesting that the best candidate could belong to the TRPV subfamily. We refined our study by specifically repressing the expression of TRPV2, TRPV4 and Piezo1 and found that deformation-induced $[Ca^{2+}]_i$ transients were dependent on TRPV4. This finding was corroborated by the use of specific inhibitors of TRPV4 such as HC06704, RN1734 and PLA₂ inhibitor OBAA. As expected, application of the TRPV4 agonists GSK1016790A and 4 α PDD elicited $[Ca^{2+}]_i$ transients and inhibited the renin secretion obtained after stimulation of the cells with FK and IBMX. We also observed that the FK-induced renin secretion stimulation was prevented by cycling stretching the cells (0.3 Hz, 30 min) and that this effect was dependent on TRPV4.

We then investigated the role of TRPV4 in pressure-induced regulation of renin secretion *in vivo*. First, immunohistochemistry revealed that mouse JG cells indeed express

TRPV4. Then, studying isolated (denervated) perfused kidneys from *Trpv4*^{+/+} and *Trpv4*^{-/-} mice, we found that renin secretion was significantly increased at a normal basal pressure of 100 mmHg in *Trpv4*^{-/-} mice and that the pressure / renin secretion relationship was shifted to the right in the kidneys from *Trpv4*^{-/-} mice compared to wild-type, emphasizing the importance of TRPV4 in the process. Interestingly, the pressure / renin secretion relationship was not abolished, suggesting the involvement of another, less sensitive mechanosensitive process. As expected, pharmacological inhibition of TRPV4 with HC067047 also increased renin secretion without affecting the perfusate flow in *Trpv4*^{+/+} mice.

The next obvious question is whether these mice are hypertensive. This has been studied previously, and the answer is negative [112, 197-199]. Indeed, mean arterial blood pressure was around 88 mm Hg in both *Trpv4*^{+/+} and *Trpv4*^{-/-} mice under baseline conditions [199, 200]. Note that in our study, the difference in the relationship between blood pressure and renin secretion (isolated kidneys) was observed at physiological pressures (between 80 and 90 mm Hg). As a consequence, basal levels of renin and aldosterone were increased in *Trpv4*^{-/-} mice *in vivo*. In accordance with our hypothesis, it has been shown that acute systemic activation or inhibition of TRPV4 respectively lowers or increases blood pressure [197, 199, 201]. Moreover, it has been reported recently that mice deficient in serum paraoxonase 1, an HDL-associated lipolactonase, present a hypotension-related TRPV4 overactivity potentially due to an abnormally high renal level of 5',6'-EET, an AA derivative activating TRPV4 [197]. However, the observation that blood pressure was unchanged in resting conditions in *Trpv4*^{-/-} mice suggests that other mechanisms of blood pressure regulation counterbalance the effects of renin and aldosterone. Besides, TRPV4 is expressed in several organs involved in blood pressure regulation such as kidneys (proximal and distal tubules), blood vessels (endothelium and smooth muscle), peripheral nerves and hypothalamus, complicating the interpretation of its role *in vivo* [55, 104, 122, 123, 202].

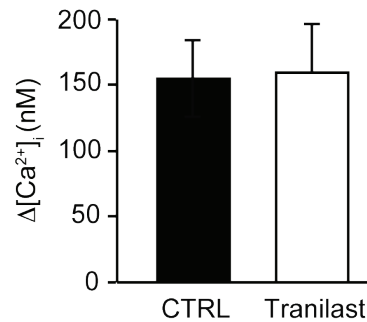
Taken together, our observations thus suggest that TRPV4 ion channel is involved in mechanotransduction in JG cells. When the pressure in the afferent arteriole increases, TRPV4 allows Ca²⁺ entry in JG cells, which inhibits renin secretion.

Additional unpublished data

In this section, we present additional physiological data obtained on the JG cell line As4.1 that were not included in the published paper due to length limitation.

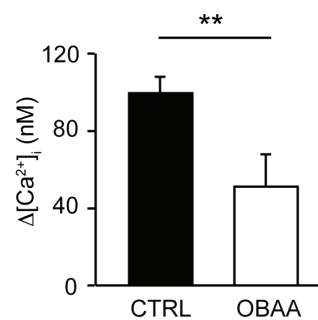
Because the TRPV channel blocker RR was efficiently blocking the mechanically induced Ca^{2+} entry in As4.1 cells, and because *Trpv2* mRNA was expressed in these cells, we sought to know if pharmacological TRPV2 blocking would affect the response to mechanical stimulation in As4.1 cells. As shown in **additional FIGURE 1**, the response to mechanical deformation was not affected by the presence of tranilast (100 μM), a poorly specific inhibitor of TRPV2. These data confirm what was observed in **FIGURE 3G** from the results section, where As4.1 cells transfection with *Trpv2* siRNA did not alter the response to mechanical stimulation.

Additional FIGURE 1. Quantification of the Ca^{2+} response to mechanical stimulation (flow-induced cell deformation) in presence of the TRPV2 blocker tranilast (100 μM). CTRL indicates the level of $[\text{Ca}^{2+}]_i$ transient observed in control conditions, before the addition of the blocker (n=4)



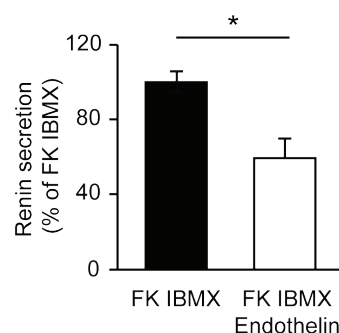
Because one of the proposed mechanisms for the mechanical gating of the TRPV4 ion channel is the indirect activation of the channel by endogenous lipids synthesis requiring PLA_2 activity, we applied a PLA_2 blocker during the mechanical stimulation (**additional FIGURE 2**). The response was significantly diminished (-49%, $P < 0.01$), arguing for an indirect, PLA_2 -dependent TRPV4 activation in our model.

Additional FIGURE 2. Quantification of the Ca^{2+} response to mechanical stimulation (flow-induced cell deformation) in presence of the PLA_2 blocker OBAA (10 μM). CTRL indicates the level of $[\text{Ca}^{2+}]_i$ transient observed in control conditions, before the addition of the blocker (n=6). ** $P < 0.01$



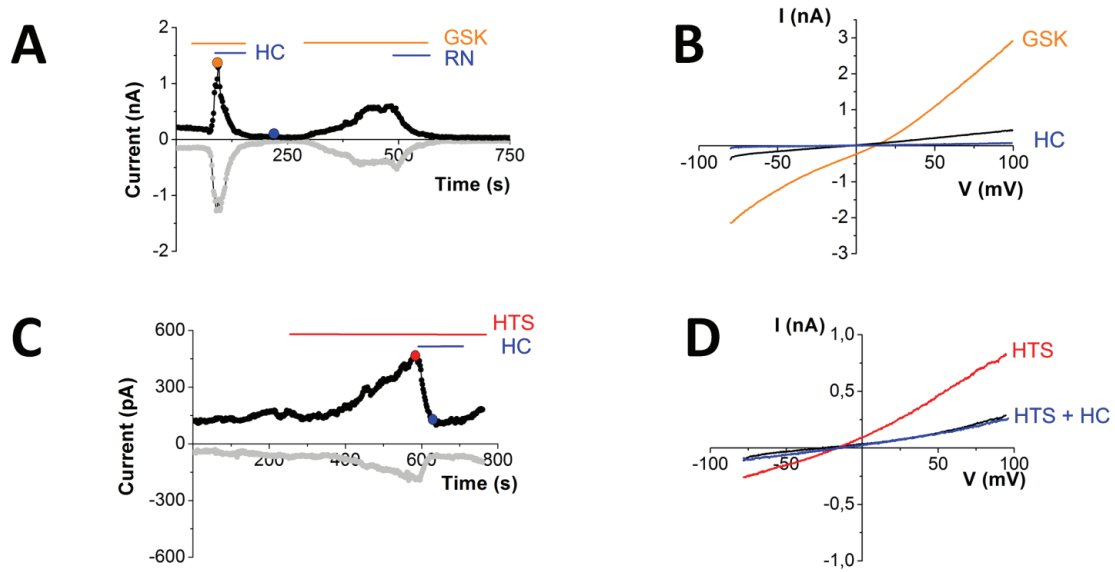
Before investigating the effect of TRPV4 activation on renin secretion in As4.1 cells, we verified that endothelin, known to inhibit renin secretion in a Ca^{2+} -dependent fashion [102, 187], could block the stimulation of renin secretion in our model (-41%, $P < 0.05$, **additional FIGURE 3**).

Additional FIGURE 3. Effect of endothelin (10nM) on renin secretion in As4.1 cells in the presence of FK (5 μM) and IBMX (100 μM) (n = 4 wells for each condition). * $P < 0.05$

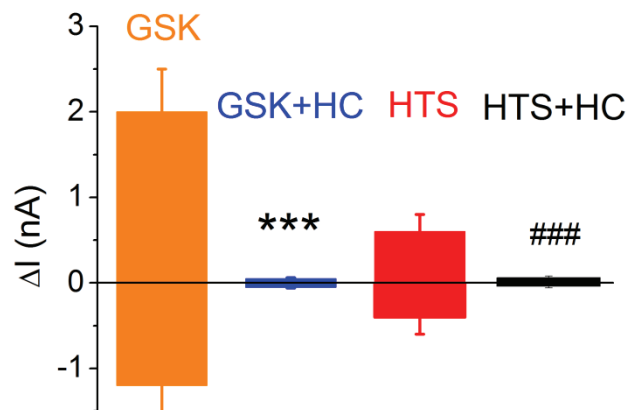


As shown in **additional FIGURE 4**, we also studied the electrophysiological response of As4.1 cells to TRPV4 activating stimuli. We measured robust current activation (both inward and outward) in response to the specific TRPV4 agonist GSK1016790A (100 nM). This current was completely blocked by the specific TRPV4 antagonists HC067047 (1 μM) and RN1734 (30 μM) (**additional FIGURE 4A-B**). Cells exposed to a hypotonic solution (~240 mOsm) also displayed a current, which was blocked by HC067047 (**additional FIGURE 4C-D**). Our results are quantified in **additional FIGURE 5**. Curiously, TRPV4 activation by hypotonic stimulus was not observed during $[\text{Ca}^{2+}]_i$ measurement experiments in As4.1 cells. However, the pattern of activation of TRPV4 during hypotonic shock in As4.1 cells is relatively slow, compared to the effect of the same stimulus in mPTCs (chapter II). This may explain why no significant Ca^{2+} transient was observed in As4.1 cells with this stimulus.

Results

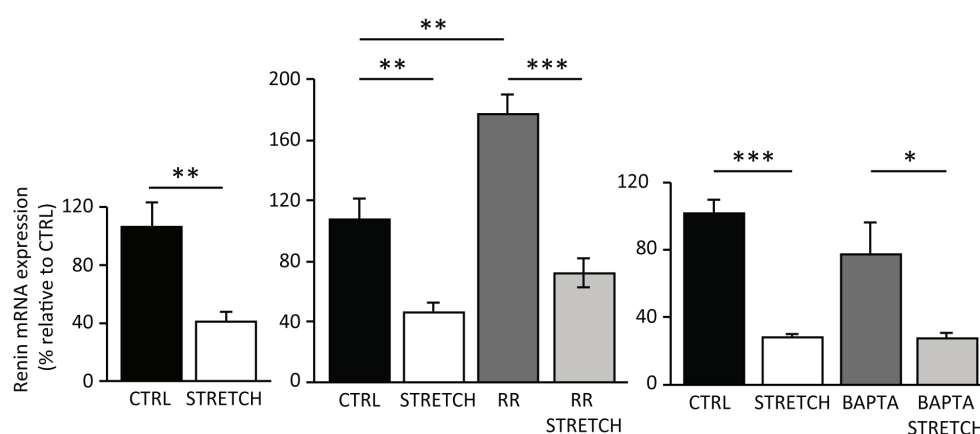


Additional FIGURE 4. Effect of TRPV4 agonist/antagonist and hypotonic solution stimulation on As4.1 cells in patch-clamp experiments. Representative time-courses at membrane potential of +50 mV (black curve, A) and -50 mV (grey curve, A) and I-V trace (B) of whole cell currents through As4.1 cells, during administration of GSK1016790A (100 nM), HC067047 (1 μ M) or RN1734 (30 μ M), at the indicated time intervals. Representative time-courses recorded at +50 mV (black curve, C) and -50 mV (grey curve, C) and I-V traces (D) of whole cell currents through As4.1 cells, during administration of hypotonic solution (HTS) and HC067047 (1 μ M), at the indicated time intervals. The black I-V traces showed in (B) and (D) are recorded in the absence of drugs (control)



Additional FIGURE 5. Pooled data of whole-cell currents (at +50 and -50 mV) through As4.1 cells. Currents evoked by 100 nM GSK1016790A (GSK), 100 nM GSK1016790A in the presence of 1 μ M HC067047 (GSK+HC), hypotonic solution (HTS), or hypotonic solution in the presence of 1 μ M HC067047 (HTS+HC). Each column represents mean $\pm$ SEM of $n > 5$ cells. *** $P < 0.001$ versus GSK; ### $P < 0.001$ versus HTS (unpaired Student's *t* test)

Beside renin secretion, regulation of renin mRNA expression has also been extensively studied in the As4.1 cell line, a model of JG cells. Renin expression is known to be inhibited by mechanical stimulation of the cells as well as renin secretion [162]. In order to test the possible involvement of mechanosensitive ion channels in renin expression regulation, we performed mRNA studies on mechanically stimulated cells. When the cells were cyclically stretched for 24h there was a decrease in renin mRNA content down to 40% of the expression level observed in non-stretched control cells. However, neither the RR blocker, nor the $[Ca^{2+}]_i$ buffer BAPTA-AM prevented the decrease in renin expression caused by cell stretching (**additional FIGURE 5**). These results indicate that, in contrast to renin secretion regulation, the transduction of mechanical forces into renin expression regulation does not involve a mechanosensitive Ca^{2+} channel, but rather a Ca^{2+} -independent mechanotransduction system.



Additional FIGURE 5. Mechanosensitive regulation of renin mRNA in As4.1 cells. Relative renin mRNA content in As4.1 JG cells exposed to 24h cyclic stretch. CTRL, unstretched control cells; STRETCH, stretched cells; RR, 30 μ M RR treatment; BAPTA, 20 μ M BAPTA-AM treatment. First panel, ** $P < 0.01$, Student's t-test. Second and last panels, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA and Bonferroni post-hoc test

CHAPTER IV

Implication of TRPV4 in osmoregulation by hypothalamus neurons

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Introduction

In chapter I, we reported a defective response to hyperosmolar states in the *Trpv4*^{-/-} mice. We assumed that this effect was attributed to the lack of neuronal TRPV4 in the hypothalamic osmosensory neurons. However, the osmosensitive channel TRPV4 is expressed in different organs and cell types involved in detection and regulation of osmotic challenges. In addition to being expressed in central osmosensory nuclei, it has been shown that TRPV4 is implicated in the activation of peripheral osmosensors whose impact on systemic osmoregulation is still incompletely understood [104, 109]. Moreover, astrocytes, which modulate neuronal activity, also respond to osmotic stimuli and they express TRPV4 [72, 104, 203].

Following the findings on the *Trpv4*^{-/-} mouse described in chapter I, we have created a model of conditional *Trpv4* knockout mouse, using the Cre-loxP recombination system. This will allow cell-specific study of the physiological roles of TRPV4, for instance in the hypothalamic neurons or in other cell types, and observation of the acute effects of TRPV4 deletion without possible developmental compensating mechanisms.

Methods

Trpv4lox mouse model design

Using AK7 mouse embryonic stem cells, we targeted the *Trpv4* gene by homologous recombination with a construct from the International KO Mice Consortium that bears *loxP* sites-flanked sixth exon (ID:79331) (**FIGURE 1**). After the selection of several correctly targeted clones, we injected them into C57BL/6J mice host blastocysts. The embryos were transferred into pseudopregnant CD1 mice. In the offspring, chimeric males were selected on basis of the coat colour. They were mated with C57BL/6J females in order to assess the transmission of the targeted allele. At this stage, we kept some mice harboring the targeted allele to allow non-immunological assessment of *Trpv4* histological expression, using the *lacZ* gene from the selection cassette as a reporter of expression. Other mice harboring the targeted allele were mated with *ROSA-Flp* females to have the selection cassette excised. The so obtained mice have the sixth exon of the *Trpv4* gene flanked with *loxP* sites (*Trpv4lox*). After getting mice homozygotes for the *Trpv4lox* allele (*Trpv4^{lox/lox}* mice), they can be crossed with a mouse line expressing the Cre recombinase under an inducible and/or tissue-specific promoter to selectively inactivate *Trpv4* expression. *Trpv4^{lox/lox}* mice were also crossed with a mouse line expressing the Cre recombinase under the PGK-1 promoter to obtain a constitutive *Trpv4* knockout mouse line (*Trpv4^{-/-}*). Expression of TRPV4 in the *Trpv4^{lox/lox}* and *Trpv4^{-/-}* mice was assessed by Western blotting on kidney extracts, confirming correct expression of TRPV4 in the *Trpv4^{lox/lox}* mice and functionality of the Cre-mediated recombination of the lox-flanked *Trpv4* exon (**FIGURE 2**).

Induction of the CamK-CreER^{T2} with tamoxifen

Trpv4^{lox/lox} mouse line was crossed with a mouse line harboring a *CamK-CreER^{T2}* transgene in order to achieve forebrain neuronal *Trpv4* knockout. To activate the recombinase, 1mg tamoxifen was injected intraperitoneally for 5 days twice a day. Tamoxifen was dissolved in ethanol and sunflower oil (5%-95% v/v) at 10mg/mL. Mice were used in experiments the second or the third week after tamoxifen injections.

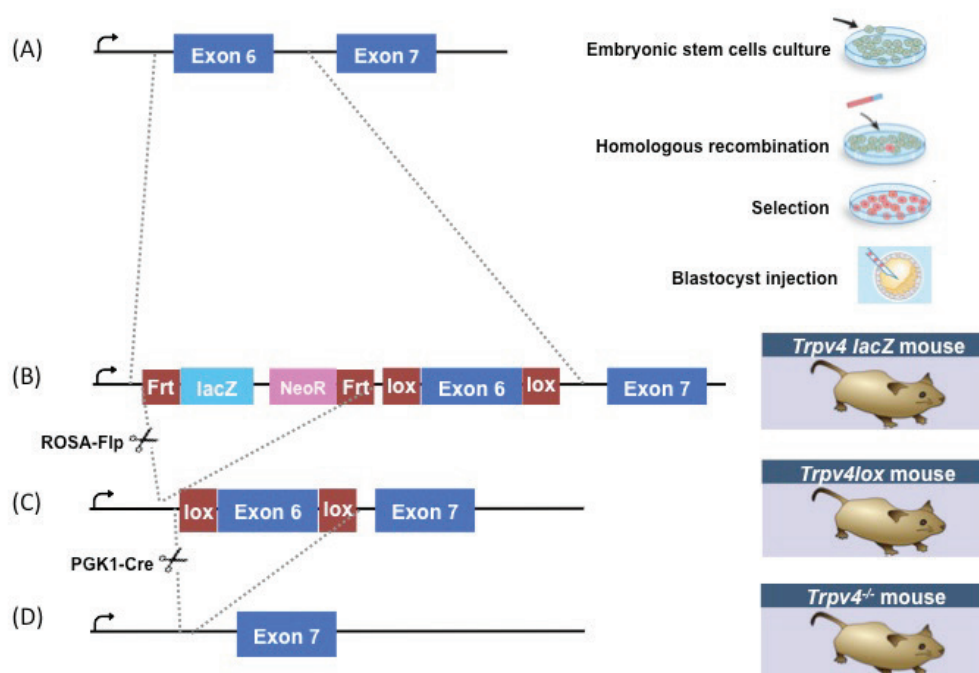
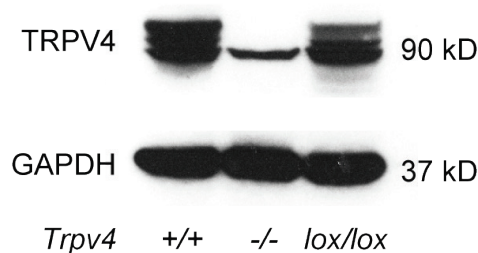


FIGURE 1. Creation of the *Trpv4*^{lox/lox} mouse line. (A) The *Trpv4* gene of embryonic stem cells is targeted by homologous recombination to create a loxP sites-flanked 6th exon. Correctly targeted cells are injected in host blastocysts. (B) Mice harboring the targeted allele allow non-immunological assessment of *Trpv4* expression, using the *lacZ* gene of the selection cassette as a reporter of expression. (C) Removal of the selection cassette restores TRPV4 expression in a new *Trpv4*^{lox/lox} mouse line. (D) A constitutive knockout line (*Trpv4*^{-/-}) is obtained by crossing the *Trpv4*^{lox/lox} mouse line with a line expressing the Cre recombinase under the PGK-1 promoter. Conditional knockout lines can be obtained using other Cre lines

FIGURE 2. Western blotting of total kidney extracts from control (+/+), knockout (-/-) and *Trpv4*^{lox/lox} (lox/lox) mice, showing the presence of the TRPV4 protein in the *Trpv4*^{lox/lox} mouse line and its absence in the *Trpv4*^{-/-} line



Physiociages and metabolic cages

Mice were housed in a light- and temperature-controlled room with *ad libitum* access to tap water and standard diet. Basal activity was evaluated in individual metabolic cages (Physiociage, Panlab-Bioseb, Vitrolles, France) during 48h after 24h of habituation. Overnight urine collections were obtained in individual metabolic cages, after appropriate training. To evaluate the central response to hyperosmotic stimuli, mice were challenged with an IP injection of a hyperosmotic solution (0.5 M NaCl, 0.04 mL/g body weight). Mice were single-housed, allowing the precise measurement of cumulative water intake (Physiociage testing).

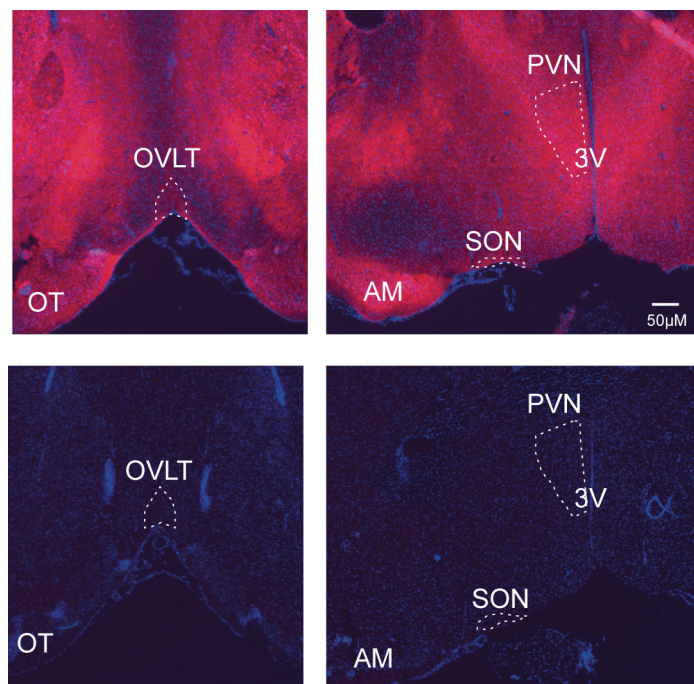
Results

Results

With the help of our conditional *Trpv4* knockout, we first focused on the role of TRPV4 in central osmoregulation. In order to specifically study the role of TRPV4 in hypothalamic neurons, we crossed our *Trpv4*^{lox/lox} mouse line with a mouse line harboring a *CamK-CreER*^{T2} transgene. This genomic construct expresses a modified Cre recombinase that is tamoxifen-activated, under the promoter of the *CaMKIIa* gene, allowing a tamoxifen-inducible gene inactivation in neurons of the adult mouse forebrain (cortex, hippocampus and hypothalamus) [204].

To confirm that recombination was occurring in the hypothalamus neurons, by breeding, we added in our *Trpv4*^{lox/lox} mouse line a *ROSA26*^{loxP-stop-loxP-Tomato} transgene. This transgene is a Cre recombinase activity reporter, providing expression of the red fluorescent protein tdTomato upon Cre recombinase activity in the cell (the transcription of the fluorescent protein is then unlocked). After final breeding with the *CamK-CreER*^{T2} mice, the progeny was administered tamoxifen in order to activate Cre recombination. Brain regions where recombination occurred were recognized by red fluorescence on brain sections. These comprised hypothalamic zones such as OVLT, PVN and SON. Notably, in absence of tamoxifen treatment, no recombination was detected (**FIGURE 3**). No recombination was observed in other organs (kidney and urinary bladder).

FIGURE 3. Brain sections of transgenic *Trpv4*^{lox/-} mice bearing *ROSA26*^{loxP-stop-loxP-Tomato} and *CamK-CreER*^{T2} transgenes. The brain in the upper panels originates from a tamoxifen-treated mouse whereas the lower panels illustrate the brain of a vehicle-treated littermate. Red fluorescence indicates tdTomato expression, signing *CamK-CreER*^{T2} activation. Blue color represents nuclear counterstaining with DAPI. OVLT, organum vasculosum laminae terminalis; OT, olfactory tubercle; PVN, paraventricular nucleus; SON, supraoptic nucleus; 3V, third ventricle; AM, amygdala

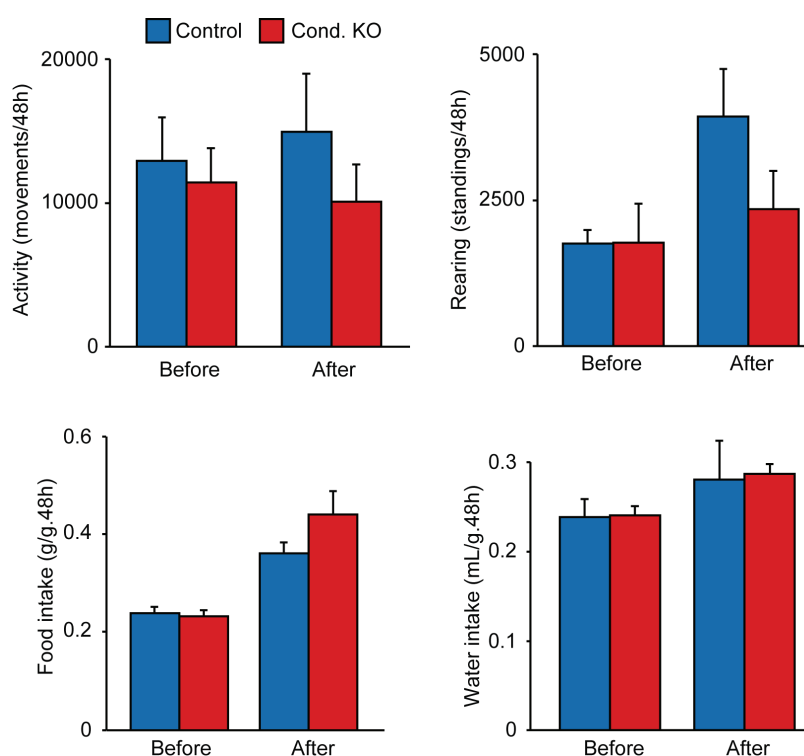


In order to maximize the absence TRPV4 in hypothalamic neurons, we chose to breed *Trpv4*^{lox/lox} mice with *CamK-CreER*^{T2} mice backcrossed on the *Trpv4*^{-/-} line in order to have one of the two copies of the *Trpv4* gene already constitutionally absent. We thus obtained heterozygous *Trpv4*^{lox/-} mice with or without the *CamK-CreER*^{T2} transgene. The experiments consisted in the comparison of tamoxifen-treated *Trpv4*^{lox/-} mice that were expressing the *CamK-CreER*^{T2} transgene versus littermates that did not.

We sought to measure the effect of *CamK-CreER*^{T2} activation on *Trpv4* mRNA levels in hypothalamus of *Trpv4*^{lox/-} mice. No significant difference could be observed, maybe because of the neuron-specific deletion of *Trpv4*, in case neurons would not be the major *Trpv4*-expressing cells in the hypothalamus. Immunostainings on brain sections gave unsatisfactory results, possibly because of the very low level of expression of *Trpv4* in the brain (*Trpv4* mRNA is expressed about 30 times less in the total brain than in the total kidney).

We tested the phenotype of conditional knockout mice (*Trpv4*^{lox/-} mice expressing the *CamK-CreER*^{T2} gene) and their controls (*Trpv4*^{lox/-} mice) at rest. As shown in **FIGURE 4**, no differences in general parameters such as activity, rearing, food and water intakes were observed between conditional knockout mice and controls, before or after Cre induction. No differences in weight were observed between the two groups.

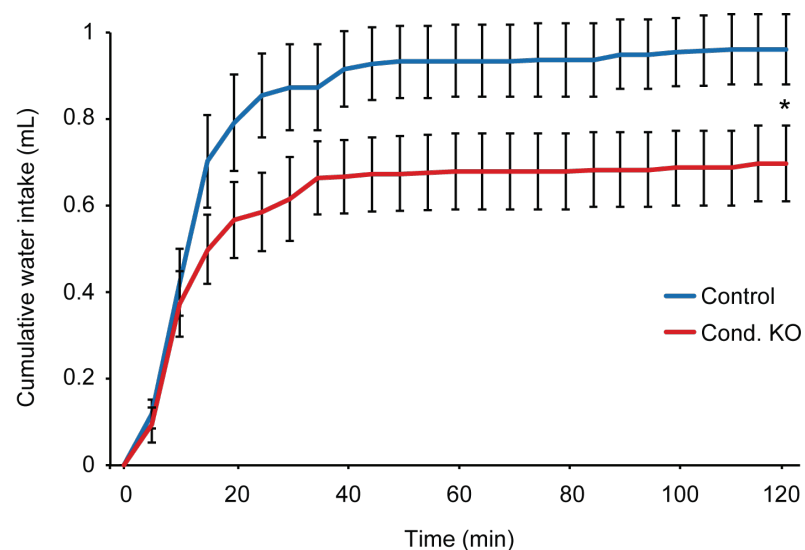
FIGURE 4. Comparison of general parameters of conditional knockout mice (*Trpv4*^{lox/-} mice expressing the *CamK-CreER*^{T2} gene) and their controls (*Trpv4*^{lox/-} mice), before and after Cre activation by tamoxifen administration (n=6 pairs)



Results

As we have shown in chapter I, *Trpv4*^{-/-} mice seem to have an impaired thirst mechanism because they drink less water upon hyperosmotic stimulation than controls. Using IP hyperosmotic saline injection, we sought to know whether this phenomenon was dependent on central neuronal TRPV4. Indeed, other TRPV4-expressing structures such as peripheral nerves, peritoneal cells or liver osmosensors could be involved in the response to this stimulus [109, 205], as our protocol involves penetration of the hypertonic bolus in the portal vein through mesenteric circulation. Another potential confounding variable is visceral pain that could subsequently alter thirst [206]. As shown in **FIGURE 5**, lack of central neuronal TRPV4 was associated with diminished water consumption after IP injection of hypertonic saline. This interesting finding indicates that central neuronal TRPV4 is required for thirst sensation. In this model, in contrast to **FIGURE 5** in chapter I, we did not observe an increased latency in the water intake of conditional knockout mice. This difference may reflect the participation of peripheral TRPV4 as a peripheral osmosensor or a nociceptor in the early response to IP hyperosmolar challenge.

FIGURE 5. Cumulative water intake of conditional knockout mice (*Trpv4*^{lox/-} mice expressing the *CamK-CreER*^{T2} gene) and their controls (*Trpv4*^{lox/-} mice) after IP hypertonic saline injection (n=10 pairs).
* *P*<0.05, Student's t-test



The *Trpv4*^{-/-} mice had an impaired VP secretion, as shown in chapter I, but urine concentration power was normal probably because of compensating mechanisms. In order to detect impairment of VP release related to the loss of expression of TRPV4 in hypothalamic neurons, urine concentration ability was assessed on single-housed mice in metabolic cages to collect their urine. During the resting time (which corresponds to our human daytime), when mice mainly sleep and do not drink water, the conditional knockout mice failed to drop their urine volume as much as did the control mice. This urine was also more diluted, indicating a defect in urine concentration (**FIGURE 6**). Whether this is accompanied by concomitant changes in plasma osmolality and VP levels remains to be determined.

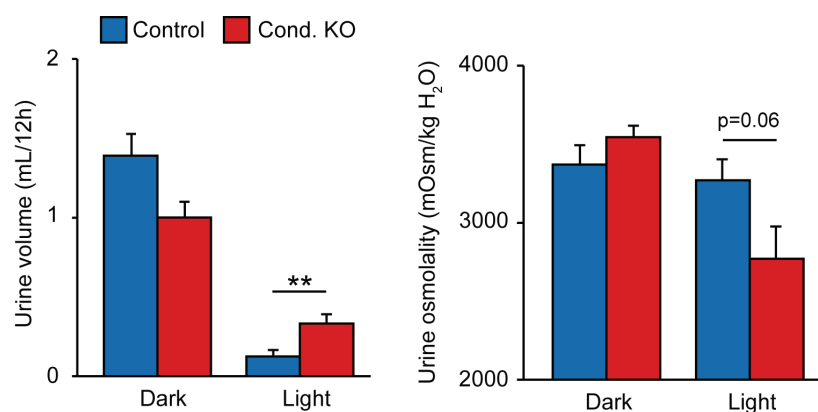


FIGURE 6. Urine volume and osmolality of tamoxifen-treated conditional knockout mice (*Trpv4^{lox/-}* mice expressing the *CamK-CreER^{T2}* gene) and their controls (*Trpv4^{lox/-}* mice). The collection was fractionated between the active (dark) and resting (light) time of the mice. ** $P < 0.01$, Student's t-test

Results

Perspective

The preliminary results obtained on our conditional *Trpv4* knockout model are suggestive of an involvement of TRPV4 in the response of central neurons to variations of systemic osmolality. We showed impaired thirst response in mice lacking TRPV4 specifically in the neurons of the forebrain, and a reduced urine concentration at rest.

Further advance is still needed in this study. Documentation of the recombination of the *Trpv4lox* locus is still lacking; it will be assessed at the genomic level in hypothalamic extracts. The characterization of the conditional knockout model parameters, in particular plasma osmolality and VP measurements will be added. To better understand the cellular role of TRPV4 in the central osmosensory neurons, electrophysiological studies of the hypothalamic nuclei will be performed in collaboration with Prof. Koji, Gunma University, Japan.

DISCUSSION

In our work, we have aimed to better understand the role of TRPV4 in the systemic osmoregulation and kidney physiology. In the first chapter, we detailed the expression profile of TRPV4 in mouse kidney: we demonstrated that TRPV4 is absent from the TAL but abundantly expressed in proximal and distal tubular segments. We analyzed the consequences of the genetic deletion of TRPV4 on the central and nephrogenic components of osmoregulation. Despite the widespread expression pattern of TRPV4 in the kidney, its deletion has no major effect on renal function parameters related to water and salt handling. Conversely, we showed that the absence of TRPV4 is associated with abnormal thirst regulation and VP release, demonstrating its participation in central osmoregulation. In this line, preliminary results obtained on our conditional *Trpv4* knockout model (in the fourth chapter), where TRPV4 is suppressed in the forebrain neurons only, are suggestive of an involvement of TRPV4 in the response of central neurons to variations of systemic osmolality. We indeed showed an impaired thirst response in these mice, and a reduced urine concentration at rest.

We also aimed to study the role of TRPV4 in the PT, where we described its expression for the first time. In the second chapter, experiments conducted on mPTCs and a mouse model of proteinuria suggested that TRPV4 modulates the endocytic capacity of the PT cells. On mPTCs, we showed that TRPV4 was activated by hypotonic cell swelling, and that it was a component of the response to mechanical cell stretch.

Finally, we focused on the mechanosensitivity of the JG cells (third chapter), identifying TRPV4 as a mechanically activated channel responsible for Ca^{2+} entry in these cells. Our data on cell cultures and isolated perfused kidneys demonstrated the involvement of TRPV4 in mechanotransduction in JG cells. We established a link between the increase in perfusion pressure and a TRPV4-dependent Ca^{2+} entry in JG cells, which inhibits renin secretion.

TRPV4 and osmoregulation

In chapter I, we observed that mice lacking TRPV4 present a defect in central functions related to the maintenance of systemic osmoregulation. We showed that *Trpv4*^{-/-} mice had reduced physiological responses to hyperosmolar states, which consist in diminished VP secretion and diminished water consumption under hypertonic stress. Echoing these research efforts, a human TRPV4 polymorphism has been associated with hyponatremia [113].

Another TRPV channel, TRPV1, has been involved in neuronal osmosensation. Indeed, it has been shown that isolated neurons from the OVLT and the SON respond to hypertonic and mechanical stimuli in a TRPV1-dependent manner. They increased their membrane cation conductance and enhanced action potential discharge. In OVLT neurons, involvement of TRPV4 was also assessed and it was not necessary for the modulation of electrical response in these conditions [41]. *Trpv1*^{-/-} mice behave very similarly to *Trpv4*^{-/-} mice, with a blunted VP secretion compared to controls at high plasma osmolality values, and a reduced drinking reflex upon hypertonic stress [135, 207]. These apparently parallel results may mask differences in the precise role of each channel. It is probable that a vital system such as osmoregulation relies on several sensors; a panel of sensors would allow transduction of diverse osmotic stimuli, varying in amplitude, direction or rate. The use of *Trpv4*^{-/-} mice combined with high magnitude and standardized tests is useful to determine the involvement of the sensor as a first step, but it will be then necessary to dissect further the mechanisms at play. For example, during central osmoregulatory processes, it is not known at this stage whether hypertonicity directly activates the channel or progressively inhibit it, neither in which cells it is at play (excitatory or inhibitory neurons, glial cells). Elucidation of the cellular mechanisms explaining TRPV4 participation in osmosensory neurons is still needed, and could be achieved using *in vivo* or *ex vivo* electrophysiological studies.

It is probable that several cell types are involved in osmosensing, beyond the well-studied MNCs and OVLT neurons that modify their action potential discharge when cell volume is modified on isolated neuron preparations. *In vivo*, in their close proximity, astrocytes also play a functional role in controlling the electrical activity of MNCs [106]. In particular, in the SON, their processes intercalated between the MNCs release taurine as an inverse function of osmolality. Taurine is an agonist of inhibitory glycine receptors, which decrease the action potential firing rate in MNCs [203]. Interestingly, TRPV4 is crucial for

cell volume control in astrocytes [72]. These findings reveal a new actor in the control on VP secreting neurons that needs further investigation. Another parameter in the control of central osmoregulatory structures is the peripheral osmosensing. The peripheral nervous system relays information about the osmotic environment in the alimentary tract via the vagus and the splanchnic nerves. Ingestion of anisotonic fluid causes a rapid change in VP secretion rate and modifies thirst sensation even before that plasma osmolality is altered. It further impacts the autonomous system by modulating blood pressure [104]. Some of these receptors are located in the nerve endings surrounding the portal vein branches. They are stimulated in a TRPV4-dependent manner when a hypotonic bolus is infused in the portal vein [109]. The relative contribution of peripheral osmoreceptors to the changes in VP secretion and thirst sensation apart from digestive stimuli has still to be determined.

While our results about the effect of hypertonicity on VP secretion in *Trpv4*^{-/-} mice are in line with previous observations [177], the results of other research groups are different, showing either upregulation of VP secretion in absence of TRPV4, or no effect [112, 206]. In Liedtke's model, the *Trpv4* gene was inactivated by a deletion of exon 12 that encodes the pore-loop domain and adjacent transmembrane domains 5 and 6 (Cre-mediated *loxP* site excision) [177]. The absence of the TRPV4 protein was verified with an antibody raised against the C-terminus. In Mizuno's model, the *Trpv4* gene was inactivated by disruption of exon 4 with a neo cassette [112]. Exons 3 to 6 encode the ARDs. The absence of the TRPV4 protein was verified with an antibody raised against the C-terminus. In chapter IV, we describe our own model of TRPV4 knockout, with a Cre-mediated *loxP* site excision of exon 6. The absence of the TRPV4 protein was also verified with an antibody raised against the C-terminus. Our first results obtained on this line are similar to the ones obtained from Liedtke's model. The differences observed between models are puzzling. The absence of the TRPV4 protein has been proved using antibodies raised against the C-terminus. The development of TRPV4 specific agonists has allowed proving that no TRPV4 current in any of these knockout models. One cannot formally exclude that some part of the TRPV4 protein would be expressed in one or other knockout model, and it is not known yet whether the TRPV4 protein could play physiological roles independently of its channel activity.

Advances are still needed in the study of TRPV4 as an actor in central osmoregulation to overcome the limits of the *Trpv4*^{-/-} mice. One possible issue with the constitutional *Trpv4*^{-/-} mice is the installation of compensating mechanisms [26, 208]. It is difficult to know in which

Discussion

way and to what extent the lack of this protein is compensated during the development. We have sought upregulation of other TRP isoforms at the mRNA level in the kidney and brain of 7 *Trpv4*^{-/-} mice, compared to 7 control littermates. No significant change in *Trpv2*, *Trpv3*, *Trpc1*, *Trpp2*, *Piezo1* or *Piezo2* mRNA level was observed. Surprisingly, *Trpv1* expression was strongly upregulated in one mouse (13 times more than the controls in the brain and 15 times more in the kidney), but not in the others. This strange finding suggests that compensatory mechanisms in knockout animals may be highly variable even between littermates. In order to bypass this difficulty, the use of the available specific pharmacological tools enables acute modulation of TRPV4 [132]. Furthermore, the characterization of the effects of inducible and cell-specific *in vivo* TRPV4 deletion will help identifying more precisely the cells involved in TRPV4-dependent modulation of osmoregulatory responses. For example, evaluation of neuronal (*CamK-CreER*^{T2}) and astrocytic (*GFAP-CreER*^{T2}) respective knockout is made possible by the use cell type-specific recombinase. In this line, we have developed a conditional *Trpv4* knockout mouse model. Our first results in an inducible forebrain- and neuron-specific *Trpv4* deletion (with the *CamK-CreER*^{T2} transgene) described in chapter IV, tend to indicate that neuronal TRPV4 from the central nervous system is involved in water craving upon hypertonic stress. We also observed in these mice a diminished urine concentration at rest. Additional experiments will be performed to better characterize the phenotype of this conditional mutant, in particular the participation of TRPV4 in osmosensing (electrophysiology on hypothalamus nuclei) and VP release.

TRPV4 and renal functions

The kidney is quantitatively the major site of expression of the TRPV4 ion channel [50, 123]. It is thus surprising that, in clinical medicine, the TRPV4 channelopathies causing inherited dominant skeletal and neurological defects do not seem to affect tissues and organs with a high expression of TRPV4 (kidney, bladder, blood vessels) [91]. However, the existence of a human TRPV4 variant associated with hyponatremia suggests that TRPV4 polymorphisms could also impact renal and cardiovascular parameters [113].

In the kidney, its pattern of expression extends from epithelial cells to the renal vessels branches. The most prominent TRPV4 expression in the kidney is found in the epithelial cells. In the renal vessels, it is expressed in the large branches of the renal arteries as well as in the vasa recta, although ten times lower than in the renal parenchyma [209]. Several TRP channels are expressed along the renal nephron, from the glomerulus to the inner medullary CD; their distinct activation mechanisms coupled to their segmental expression are suggestive of their specific functions in the kidney [47]. In chapter I, we have described in detail the TRPV4 expression pattern along the nephron, with no TRPV4 found in the glomerulus and the TAL, and an apical and basolateral localization in the PT and the distal nephron, comprising the DCT, the CN and the CD. Besides, we have shown in chapter III that TRPV4 is expressed and plays a role in the JG cells located in the wall of the afferent arterioles (**FIGURE 1**).

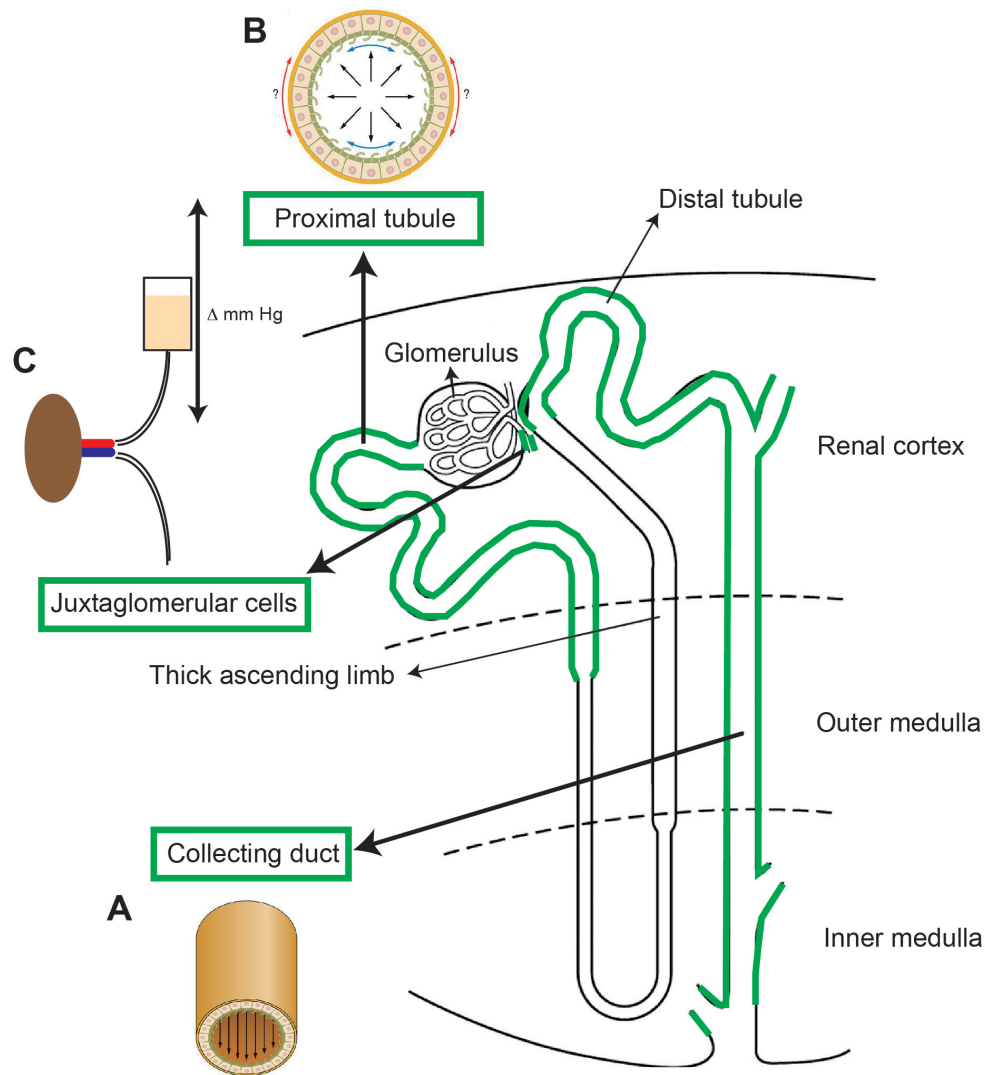


FIGURE 1. Sites of expression and functions of TRPV4 in the kidney. Renal tubules that express TRPV4 are highlighted in green. JG cells also express TRPV4. A. TRPV4 has been described as a flow sensor in the CD, and is involved in the flow-induced modulation of K^+ secretion in this segment [210]. B. We reported expression of TRPV4 in the PT, where we could activate it by cell swelling and stretching, and where it seemed to modulate the endocytic activity. C. We also detected TRPV4 in the JG cells, which respond to mechanical stimulation and inhibit their renin secretion in a TRPV4-dependent manner. Modified from [93, 211]

TRPV4 in CD cells physiology and renal regulation of water and salt handling

The role of apical TRPV4 in the distal nephron has been the subject of several *in vitro* studies. TRPV4 has been successfully activated by hypotonic stimulation and shear stress in renal CD cell lines and isolated CD [70, 122]. Functionally, it has been mainly described as an apical flow sensor. Its expression is regulated in an aldosterone-dependent manner, as is its apical localization [210].

The physiological role of an osmosensor in CD cells is unclear. It could act as a volume sensor and trigger regulatory volume changes to protect cell integrity as renal epithelial cells are subjected to osmotic stresses, facing changing urine osmolality and varying their water and solute permeability under the dependence of hormonal factors. TRPV4, in association with the cytoskeleton, has been shown to be necessary for the Ca^{2+} entry induced by hypotonicity and the subsequent RVD response [94]. On the other hand, as the osmotic forces are key players in the CD physiology, it was interesting to study whether the osmosensitive TRPV4 would play a role in VP-driven water reabsorption in the CD. As shown in chapter I, no defect in urine concentration was observed in the *Trpv4*^{-/-} mouse, in resting conditions, under water deprivation or loading and during stimulation of the VP receptor. Immunostainings and Western blots of AQP2 and its phosphorylated forms did not show any impairment of the AQP2 physiology in absence of TRPV4, pleading against its involvement in water reabsorption in the CD.

The CD transport functions are influenced by the luminal flow. Na^+ reabsorption and K^+ secretion are known to vary with CD flow changes [137]. It is known that the ENaC channel is flow sensitive [27]. This property led to hypothesize that the mechanosensitive TRPV4 would act as a urine flow sensor. Indeed, a TRPV4-dependent Ca^{2+} influx was observed in the isolated CD submitted to a luminal flow, with a small amplitude and a slow onset [122]. In chapter I, we compared the urinary Na^+ , K^+ and H^+ of normal and TRPV4-deficient mice, as markers of CD function. No differences were observed in resting conditions, under increased distal flow (furosemide administration), during Na^+ deprivation or acidic challenge. In their study, Mamenko *et al.* found that TRPV4 was involved in the upregulation of CD K^+ secretion during hyperkalemia [210]. They showed that TRPV4 is responsible for the flow-sensitive modulation of K^+ secretion via BK channels, which are apical Ca^{2+} -sensitive K^+ channels in the CD. Flow-sensitive K^+ secretion is a mechanism

Discussion

counteracting hyperkalemia, facilitated by the increase in diuresis observed during dietary K^+ increase, and achieved by CD BK channels [212]. *Trpv4*^{-/-} mice subjected to a K^+ rich diet were prone to elevated levels of plasma K^+ and decreased urinary K^+ , illustrating the role of TRPV4 in transducing the mechanical stimulus of increased flow to the BK channels [210].

While TRPV4 is a flow-activated channel in the CD, other ion channels may participate in the response to shear stress. Another member of the TRP family, TRPP2, was shown to be activated by increase in flow rate over the cell surface of mouse embryonic kidney cells. Mutations in the gene coding for polycystin-2 (TRPP2), as well as TRPP2 partners polycystin-1 (TRPP1) or fibrocystin are a common cause of polycystic kidney disease, the most frequent familial renal disease. The pathogenesis of PKD relies on the transformation of the renal epithelium to dedifferentiated secretory epithelium with aberrant polarization and high proliferation. Cysts then develop in the epithelium, mainly in the CD. It is thought that dedifferentiation and proliferation observed during PKD are related to the inability to sense mechanical stimuli. Interestingly, it has been demonstrated that TRPP2 associates with TRPV4 to form a mechanosensitive channel. At the subcellular level, TRPV4 has been described in the cilium of ciliated renal epithelial cell lines [88, 213]. The exact relation between these two channels in flow-induced phenomena remains to be elucidated [122].

TRPV4 in PT cells physiology

We reported TRPV4 expression in the PT. In the wake of this finding, we started looking at the possible functions and activating mechanisms of the channel in this nephron segment. Whereas TRPV4 played the role of a flow sensor in the CD, it has still to be determined whether PT cells respond to flow in a similar manner to CD cells. As opposed to CD cells, it was shown that it is rather the microvilli that transduce shear stress in PT cells, converting the augmentation of filtered volume into increased solute reabsorption [214]. However, beside mechanosensitive cytoskeletal structures, the presence of stretch-activated channels has been described in the PT [93, 156]. They could be functionally involved in other functions than solute reabsorption, such as PT metabolism or endocytic function. In this line, a recent study showed that the endocytic function of the PT was influenced by flow in a Ca^{2+} -dependent manner [138].

We have shown that *in vitro* activation of TRPV4 enhances the endocytic activity of PT cells. Accordingly, in a mouse model increasing urine volume and protein delivery to the PT, we highlighted a defect in albumin endocytosis in mice lacking TRPV4. Besides, at the cellular level, hypotonic cell swelling and cell stretching activated TRPV4 in PT cells. These features could indicate that TRPV4 is activated upon PT stretch. Overall, these results reveal a new role for TRPV4 in the PT that needs further understanding. In particular, the mechanism through which TRPV4 affects the endocytic process is not known and deserves further investigation.

Discussion

TRPV4 in JG cells physiology and blood pressure regulation

In chapter III, we have shown that mechanical activation of TRPV4 is involved in the control of renin secretion by the JG cells, a major site of blood pressure regulation.

TRPV4 has been previously involved in the physiological functions of several elements of the cardiovascular system. TRPV4 is known to be expressed in different components of the circulatory system: endothelial cells, vascular smooth muscle cells, cardiomyocytes, kidney, perivascular nerve endings [215]. This widespread pattern of expression renders the study of its *in vivo* activation or impairment difficult to assess.

Notably, the *Trpv4*^{-/-} mouse has a normal blood pressure [200]. The effect of TRPV4 activation or inhibition on systemic blood pressure has been investigated in mouse and rat using intravenous administration of 4 α PDD or GSK-1016790A, two TRPV4 agonists, or RR, a blocker of the TRPV channels [197, 199, 201, 216]. Knowing the multiple sites of TRPV4 expression, the mechanisms underlying the systemic response are not easy to interpret, but it would make sense to attribute the rapid changes (within minutes) to an effect on vascular or neuronal (perivascular) TRPV4. The systemic administration of RR has a hypertensive effect within minutes after the intravenous administration [197]. The overall effect of TRPV4 activation is a dose-dependent reduction in blood pressure that occurs rapidly and is attributed to the vascular TRPV4. At high doses of GSK1016790A, while modestly reducing further the systemic vascular resistances, the major effect of increasing TRPV4 activation is a diminution in the cardiac output secondary to a massive lung oedema [199]. There is actually a massive extravasation in the lungs, kidneys and intestine upon systemic TRPV4 agonist administration. TRPV4 activation triggers endothelial cell retraction and capillary leakage [199]. Similarly, in our experiments on isolated perfused kidneys, we noticed a dramatic fall in perfusion, and kidney swelling when GSK1016790A was added in the perfusate.

TRPV4 can be considered as an interesting pharmacological target in clinical medicine. Beside our field of investigation, bladder overactivity and lung oedema are two pathological conditions where TRPV4 activation has been shown to be deleterious. The effects of *in vivo* pharmacological targeting of TRPV4 on these pathologies has been studied in rodent models. By systemic administration of orally active TRPV4 blockers, the HC067047 and GSK2193874 compounds respectively, bladder overactivity and lung oedema

where improved, and no significant adverse effects were reported [87, 217]. Phase I and phase II clinical trials are currently conducted on another TRPV4 blocker, the GSK2798745 compound, to evaluate its tolerance and properties on healthy human volunteers and to test its effect on pulmonary function in patients with heart failure [218].

In our work, we have demonstrated that TRPV4 activation reduces renin secretion by JGC. We have also observed that its activation enhances the endocytic activity of the PT cells. From a clinical point of view, the use of a TRPV4 agonist would be helpful in the treatment of hypertension and proteinuria, two highly prevalent pathological states in human medicine. Unfortunately, the development of such a strategy would be tempered by the observation that systemic administration of the TRPV4 activator GSK1016790A causes endothelial dysfunction with profound circulatory collapse and death in rodents [199].

To better understand its physiological roles, TRPV4 has been studied in several isolated organs. So far, one of its best-known functions is a vasodilatory effect observed in several studies, upon activation in different isolated arterial branches (mesentery, carotid, coronary, aortic) [199, 215]. The effect on vascular resistances is attributed to various effects on endothelial, vascular smooth muscle and perivascular nerve endings TRPV4, and accounts for the fall in vascular resistance upon systemic TRPV4 activation and the opposite effects of its blockade.

Small arteries and arterioles act as the principal resistance vessels in the circulation, significantly determining systemic blood pressure. This resistance is regulated through contraction or relaxation of the vascular smooth muscle. The role of endothelial TRPV4 has been well studied. Hypotonic cell swelling and AA were shown to activate TRPV4 in endothelial cells [219]. It has also been observed in several *in vitro* experiments that flow (shear stress) induces a Ca^{2+} entry in the endothelial cells that is TRPV4-dependent and induces the arterial relaxation. Ca^{2+} entry through TRPV4 activates different intracellular effectors, such as NOS and Ca^{2+} -activated K^+ channels. Endothelial shear stress activation of TRPV4 seems to be a PLA_2 -dependent process [215, 220].

Beside its endothelial functions, TRPV4 is expressed within the vascular smooth muscle where its activation triggers the opening of Ca^{2+} -dependent K^+ channels, hyperpolarizing and relaxing the cells [200, 221].

TRPV4 is also expressed in sensory nerves, where it contributes to the transduction of mechanical hyperalgesia [222]. Its activation results in CGRP and substance P release, two nociceptive neuropeptides that are potent vasodilators [215, 223].

Discussion

In pulmonary artery smooth muscle cells, pulmonary hypertension-promoting factors such as hypoxia and serotonin produce their effects through TRPV4-dependent Ca^{2+} signaling pathways [224, 225].

TRPV4 is expressed in cardiomyocytes but there is limited evidence for a role in these cells [55]. Administration of GSK-1016790A on isolated perfused heart had no particular effect on rate or contractility [199].

Finally, TRPV4 is also expressed in the kidney, which hosts several major actors involved in blood pressure control. Interestingly, a mouse model that accumulates high levels of the endogenous TRPV4 activating lipid 5',6'-EET in the kidney displays low blood pressure with low aldosterone levels, indicating a primary down regulation of the RAS [197]. As shown in chapter I, TRPV4 is expressed in the renal epithelial cells, but our data indicate that it is probably not involved in modulation of sodium and water reabsorption, two factors also impacting blood pressure. We addressed the mechanism by which blood pressure exerts a negative feedback on renin secretion in the JG apparatus. Looking for the identity of the mechanosensor in JG cells, we now establish a novel role of TRPV4 in the control of renin secretion. Renin secretion is controlled by cytoplasmic cAMP levels, and the suppression of renin release by $[\text{Ca}^{2+}]_i$ is known to be mediated by an inhibition of AC5 and AC6, two AC isoforms that are inhibited by physiological concentrations of $[\text{Ca}^{2+}]_i$ [102]. $[\text{Ca}^{2+}]_i$ transients can be triggered by G protein-coupled receptors (*e. g.* AT2 and VP receptors as part of a hormonal feedback) [187], but we now show that a Ca^{2+} entry occurs in mechanically stressed JG cells and that pressure inhibition of renin secretion is mediated via a mechanosensitive TRPV4-dependent Ca^{2+} entry in the JG cells (**FIGURE 2**).

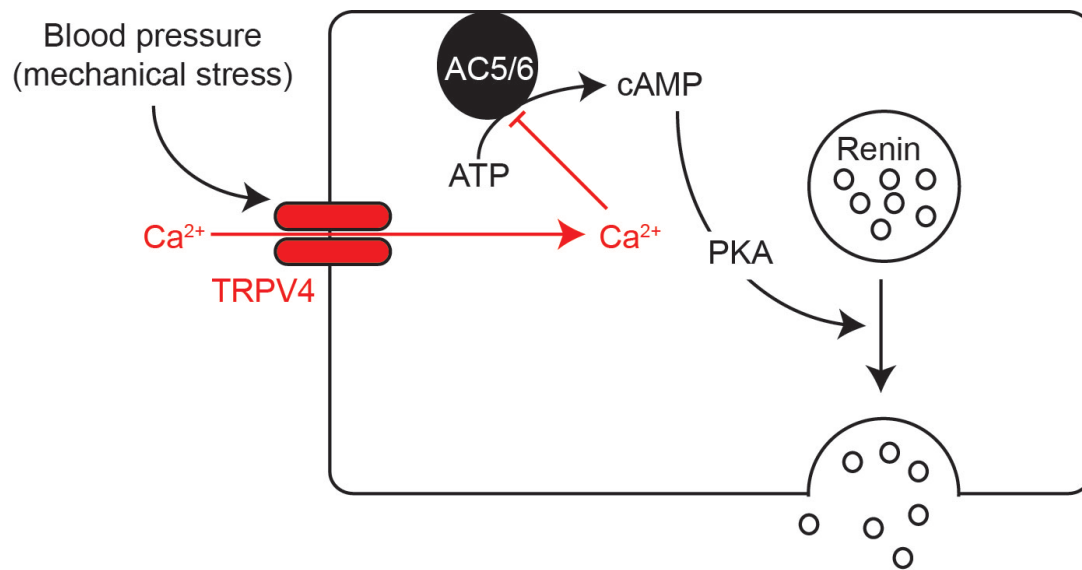


FIGURE 2. Schematic representation of the inhibitory effect of TRPV4 on renin secretion by the JG cells. The renin secretion stimulatory pathway (AC5/6, adenylate cyclase isotypes 5/6; PKA, protein kinase A) is inhibited by the entry of Ca^{2+} via the mechanically activated TRPV4 ion channel

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