SIGNALING PATHWAYS OF TACHYKININS IN THE REGULATION OF URINARY BLADDER SMOOTH MUSCLE CONTRACTION

PhD thesis

Bálint Dér MD

Doctoral School of Theoretical and Translational Medicine

Semmelweis University

Supervisor: Zoltán Benyó, MD, PhD, DSc

Official reviewers: Pál Tod, PhD
Gábor Turu, PhD

Head of the Complex Examination Committee:
György Reusz, MD, PhD, DSc

Members of the Complex Examination Committee:
Mihály Kovács, PhD, DSc
Zoltán Prohászka, MD, PhD, DSc

Budapest
2023

Table of Contents

1.0 List of Abbreviations

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

CNS: central nervous system

CTRL: control

COX: cyclooxygenase

DAG: diacylglycerol

IP_3: inositol trisphosphate

KO: knockout

LUT: lower urinary tract

NKA: neurokinin A

NKB: neurokinin B

NK1R: NK1 receptor

NK2R: NK2 receptor

NK3R: NK3 receptor

OAB: overactive bladder

PLC-β: phospholipase C β

PKC: protein kinase C

Prostaglandin E_2: PGE_2

Prostaglandin $F_{2\alpha}$: $PGF_{2\alpha}$

ROCK: Rho kinase

SMMHC: smooth muscle myosin heavy chain

RhoGEF: Rho guanine nucleotide exchange factor

SP: substance P

TRPV4: transient receptor potential vanilloid-type 4

TK: tachykinin

UBSM: urinary bladder smooth muscle

VDCC: voltage-dependent Ca^{2+} channel

2.0 Introduction

Medical students are exposed to a wide variety of disease conditions during their clinical rotations. We frequently heard that a disease is categorized as severe if it significantly shortens the lifespan of the patients (e.g. a malignant tumor) or they experience unbearable levels of pain (e.g. severe headache). After these unique experiences, the question arises: what does the severity of the disease mean? Is it the accompanying pain? Is it the level of invasiveness of the treatment? Is it the reduction of the anticipated lifespan or the duration of recovery? In my opinion, all the above can contribute to the severity of a disease, yet, we need to consider how much it impacts everyday activities, general well-being, and the quality of life of the patients.

Voiding disorders, despite not affecting the lifespan directly, impair the quality of life severely. The focus of my undergraduate and graduate research was on the understanding of the signaling pathways of the detrusor muscle with the hope that this knowledge will elucidate the pathomechanism of its disorders and contribute to the development of novel therapies.

2.1 Overview

The urinary bladder is a luminous contractile organ under the control of complex neuroregulatory circuits. As a part of the lower urinary tract (LUT), the bladder function ensures the ability to store higher volumes of urine and void varying amounts on demand as well. The balance of these alternating functions is essential for healthy bladder control. The malfunction of the urinary bladder can lead to both health-related and social issues. Besides the neuronal regulation, locally released mediators – the family of tachykinins and prostanoids/isoprostanes – also affect the function of the urinary bladder. In our study, we characterized the effects and the signal transduction pathways of these two mediator families in inducing the contraction of the urinary bladder.

2.2 The Tachykinin Peptides

The mammalian tachykinins (TKs) represent one of the largest families of neuropeptides and are widely expressed neurotransmitters within the nervous system (1,

2). Their common structural motif is the C-terminal Phe-X-Gly-Leu-Met-NH_2 amino acid sequence, where X indicates an amino acid with a hydrophobic side chain (3). There are three classical members of the mammalian TKs: neurokinin A (NKA), neurokinin B (NKB) and substance P (SP) (4-6).

The first discovered TK was SP: Von Euler and Gaddumn characterized the spasmogenic and hypotensive effect of SP in 1931, later the peptide was isolated and sequenced by Chang et al (4). In 1973, Erspamer and Melchiorri observed that a group of amphibian peptides sharing the Phe-X-Gly-Leu-Met-NH_2 motif can elicit rapid, twitching contractile movements in smooth muscle, therefore the term TK is coined based on their physiological effect (7). The discovery of NKA and NKB happened in the mid-1980s, and the coding genes of the TKs and their receptors were identified in the same decade (8).

Initially, NKA and SP are thought to be expressed in the primary afferent sensory nerve endings that innervate peripheral organs (9-11), however, TKs can be expressed in non-neuronal tissues too. SP and/or NKA are also present in enterochromaffin cells, intestines, airways and the female reproductive organ system (12-16). NKB is expressed in the brain and spinal cord (17-19). Hemokinin-1 and endokinin A, B, C or D (are more recently discovered TKs) in non-neuronal cells regulate lymphopoesis or have hemodynamic effects, respectively (20-22).

2.2.1 The Biosynthesis of Tachykinins

The biosynthesis of the TKs will be highlighted through the example of NKA based on Pennefather et al, 2004 (1). NKA is encoded by the sixth exon of the preprotachykinin-A gene (along with SP, neuropeptide K and neuropeptide γ), which is located on the 7q21–q22 chromosomal location (6, 23). The third exon of preprotachykinin-A gene encodes SP (6). The synthesis of NKA occurs simultaneously with the synthesis of SP – SP and NKA frequently released together from the nerve endings of neurons (24-26). After the transcription of PPT-A gene, the pre-mRNA is synthesized that has four splice variants. The coding sequence of NKA is presented in the β and γ isoforms (6, 27, 28).

After the translation of mature mRNA, the prepropeptide is generated that consists of a signal peptide, the neuropeptide(s) and spacers. The signal peptide sequence is 16-30 amino acids long and located on the N-terminal of the peptide. It facilitates the

translocation of the forming peptide during the synthesis from the cytoplasmatic ribosomes to the endoplasmic reticulum. At the end of synthesis, the signal peptide sequence is cleaved off resulting in the propeptide. The propeptide is transported to the Golgi apparatus then spacer regions are removed. The borders of the final active neuropeptide are indicated by the proximity of two adjacent basic amino acids or by a single arginine or lysin residue (20, 22, 24, 29). The biologically active neuropeptides are packaged into secretory granules at the Golgi apparatus and they are delivered to the nerve terminals via axonal transport (24, 29). The amino acid sequence of processed NKA is His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2, where the phenyl-alanine, glycine and leucine are the common motives of TKs.

2.2.2 Tachykinin Receptors

TKs can bind to three distinct receptor subtypes: NK1R, NK2R, and NK3R (1). At the „Substance P and Neurokinins" conference in Montreal in 1986, a nomenclature for the receptors was created based on the following criteria 1) ranking order of the potency of the different mammalian and non-mammalian natural TKs 2) the effect of different C-terminal TK fragments 3) the availability of selective agonists which are specifically activated one type of the TK receptor (3).

NK1R is constitutively expressed in the central and peripheral nervous system (CNS and PNS) in addition to vascular endothelial cells, smooth muscle and immune cells (30-34). NK1R expression is inducible in the bone marrow (35). In the peripheral nerve system, NK1R mediates several classic TK effects, e.g. the increase of vascular permeability and protein extravasation or mucus secretion in airways (31, 36). Intrathecally administered NK1R antagonist resulted in the overfilling of the bladder and dribbling incontinence suggesting that NK1R is involved in the control of micturition at the level of the spinal cord (37). SP exerts its contractile effects in the urinary bladder via NK1R (38, 39).

NK2Rs are abundant in peripheral tissues outside the CNS mediating contractile responses in the uterus, intestinal muscle strips, bronchi and notably urinary bladder (30, 38-42), however, within the CNS it is expressed in the hippocampus, thalamus and septum (43). Despite the expression of NK2R being weaker than NK1R and NK3R in the CNS (44), their function seems to be significant in regulating complex neuronal functions:

the intrastriatal administration of NK2R-specific agonist-induced turning behavior in mice, which was dose-dependently inhibited by NK2R specific antagonists (45).

In contrast, the NK3R is predominantly expressed in the CNS along with a few peripheral tissues – skeletal muscle, lung and liver (30, 46). It plays a role in modulating intestinal nociceptive signals and there is an emerging role of NK3R antagonists in the treatment of menopausal hot flashes (47, 48). All three TK receptors belong to the superfamily of G protein-coupled receptors (1). G protein-coupled receptors have common structural elements: seven hydrophobic transmembrane helices (TM I-VII) are connected via three extracellular (E_1, E_2 and E_3) and three intracellular loops (C_1, C_2 and C_3). The N-terminal is extracellular and the C-terminal is within the intracellular space (3, 49, 50).

2.2.3 The Role of Neuropeptides in the Lower Urinary Tract

The urinary bladder, urethra, internal and external urethral sphincters are functional and anatomical units of the LUT. Neuropeptides are co-transmitters and paracrine signaling molecules released together with the classic cholinergic/adrenergic neurotransmitters from the nerve terminals (51). Several neuropeptides – along with TKs – regulate the function of the LUT, like VIP (vasoactive intestinal peptide),– PACAP (pituitary adenylate cyclase activated polypeptide) and CGRP (calcitonin gene-related peptide). Besides nerve terminals, neuropeptides appear in non-neuronal tissues as well: e.g. the urothelium of the urinary bladder. Under physiological conditions, neuropeptides are localized in afferent and autonomic efferent nerves innervating the urinary bladder, urethra and urothelium (52, 53). Inflammation, injury, or disease of the LUT can alter the tissue-specific distribution of neuropeptides in these pathological conditions that can result in the dysfunction of the LUT with frequent and/or urgent urination, nycturia or pain. These symptoms might be associated with the amplification of neuropeptide signaling during the operation of the voiding reflex (51).

The neuronal circuits that control the voiding of the urinary bladder are located in the brain and spinal cord. The efferent nerve fibers innervate the smooth and striated muscle fibers of the effector organs in the LUT. The regulation of the voiding reflex is similar to the mechanism of a toggle switch: the urinary bladder is in either the filling or voiding phase. The contraction of the detrusor muscle and urethral sphincter has to follow a

reciprocal pattern to maintain the normal function of LUT (54). The urinary bladder's smooth muscle has to be in a relaxed state during the filling phase, while the urethral outlet has to be closed (55). During the voiding phase, the contraction of the urinary bladder and the relaxation of the sphincter is necessary (56). The imbalance of these mutually exclusive functions can develop the pathological condition of detrusor sphincter dyssynergia (57).

During intrauterine development and the first year of newborn life, urine is collected in the bladder until it reaches the intravesical pressure threshold when involuntary voiding happens. Newborns can retain urine during their first two years of life which is termed continence. Acquiring continence is a critical milestone in social behavior. The synchronized operation of the elements of LUT allows the storage of urine at the lowest possible pressure. During voiding, the urine travels from the direction of higher pressure towards lower hydrodynamic resistance. Intact regulatory neuronal circuits and a certain level of elasticity of the urinary bladder are required for normal LUT functioning (58).

The micturition reflex is controlled by three nerve outlets of the CNS: 1) sacral parasympathetic (pelvic) nerves that innervate the urinary bladder with excitatory nerve fibers 2) thoracolumbar (hypogastric) nerves send inhibitory signals towards the urinary bladder detrusor muscle, and excitatory signals towards the bladder neck and urethra 3) the sacral somatic (pudendal) nerve innervates the perineal muscles and the external urethral sphincter. All three regulatory systems contain afferent (sensory) axons as well. The center of LUT reflex arches is located in the lumbosacral spinal cord under the hierarchical control of supraspinal structures. The reflex pathway of filling is organized on the spinal cord level (59). The transneuronal virus tracing approach showed that the nuclei of lumbosacral reflexes are connected to supraspinal centers, e.g. the Barrington's nucleus (the pontine micturition center) or raphe nuclei (59, 60).

The afferent fibers of the urinary bladder are formed from myelinated Aδ fibers and unmyelinated C-fibers. The Aδ fibers sense the state of bladder fullness and active contraction. The C-fibers are inactive under physiological circumstances and hence named "silent fibers", normally they do not send excitatory signals toward the CNS. However, different noxious stimuli (chemical irritation, high mechanical pressure) can trigger the firing of these fibers. The Aδ and C-fibers innervate the urothelium, the suburothelial space and the muscle layers of the bladder (61, 62). The neurons of the

afferent nerves are located in the dorsal root ganglia of the S2–S4 and T11–L2 spinal segments. The afferent neuronal pathways project to the dorsal commissure and the superficial dorsal horn of the spinal cord, and to the sacral parasympathetic nucleus which consists of parasympathetic preganglionic neurons (63).

The afferent and sensory neurons in the urinary bladder are identified by immunohistochemistry labeling based on the expression of neuropeptides: calcitonin gene-related peptide, vasoactive intestinal peptide, and NKA along with SP as the members of the TK family (64). There are increasing number of evidence suggesting that during pathological conditions abnormal afferent nerve activity can cause detrusor overactivity and neuropeptides can disturb the regulatory balance with the cholinergic/adrenergic systems. Further pathohistological observations support this hypothesis: patients with detrusor overactivity exhibit increased density of suburothelial afferent nerves that are immunoreactive for CGRP and SP; in addition, botulinum toxin-treated OAB patients with restored bladder activity have normalized afferent nerve density (54, 65).

TKs are involved in both afferent and efferent reflex pathways in the urinary bladder (2, 51). Their sensory functions include the regulation of mechanosensation (66), micturition threshold on the supraspinal level (67), and visceral pain (68, 69). On the efferent side, TKs can regulate smooth muscle cell activity, nerve excitability, and vascular functions (blood flow and plasma protein extravasation) (53). The local application of capsaicin can stimulate afferent nerves and release TKs in the urinary bladder, which can induce contraction, implicating the dual function of sensory nerves in both afferent and efferent pathways (70).

NKA and SP (released as cotransmitters from capsaicin-sensitive sensory nerves) are reported to induce detrusor muscle contraction through NK1Rs and NK2Rs in rats (38). NK2Rs are expressed in the human urinary bladder as well and have been shown to mediate the contractile effects of NKA and its synthetic analog in the detrusor muscle (71, 72). Based on the effects of intravesical NKA/SP administration, NK2Rs are also present in the urothelium (66, 73).

The role of NK2R in the micturition reflex is controversial under physiological conditions (66, 74). NK2R was shown to be involved in the regulation of bladder voiding reflexes (74), and its inhibition reduced the amplitude of detrusor muscle contractions in

experimental bladder obstruction and cystitis models (37, 74, 75). Furthermore, in capsaicin-induced hyperactivity model in rats, the NK2R antagonist SR 48965 ameliorated the hyperactive reflexes without interfering physiological micturition (76). This indicates the importance of NK2R in bladder overactivity (77). Under pathological conditions (e.g., injury and inflammation), TKs have been reported to alter their expression pattern in neurons; hence, TK and NK2R signaling might be amplified in bladder dysfunctions (e.g., OAB) (51, 78).

2.3 Review of the Functions, Biosynthetic Enzymes and Receptors of Prostanoids

Prostanoids are a family of lipid mediators which includes prostaglandins (PGE_2, $PGF_{2\alpha}$, PGI_2, PGD_2) and thromboxane A_2. They have relevant roles in both physiological and pathological conditions acting as either autocrine or paracrine signaling molecules. Prostanoids are involved in complex and diverse (patho)physiological processes, like inflammation, pain sensation, regulation of renal function, cardiovascular homeostasis, platelet aggregation and asthma (79).

Prostanoids are derived from arachidonic acid and a key step of the synthesis is an enzymatic conversion by cyclooxygenases (COX), lipoxygenases, or epoxygenases. The COX enzyme has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells and plays a major role in the housekeeping functions of prostanoids (e.g. hemostasis). COX-2 is inducible and its expression and activity increase during inflammation and potentially in oncogenesis, however, the functions of the COX enzymes can overlap.

Prostaglandins bind and activate seven transmembrane G protein-coupled receptors similar to TKs. The prostanoid receptor subfamily is formed from eight members (DP, EP1–4, FP, IP, and TP), with the more recent addition of CRTH2. Their classification is based on the ligand binding affinity to different prostanoids. The coding genes of receptors share 20-30% homologous sequence among the different types. The (patho)physiological actions of prostanoids can be highly variable, depending on the binding affinity of ligands, the receptor expression of target cells, the intracellular signaling of receptors, and the microenvironment where the target cell is exposed to prostanoids (80).

2.3.1 The Role of Prostanoids in the Lower Urinary Tract

The forthcoming overview on the role of prostanoids in bladder physiology is based on the review by Rahnama'I et al, 2012 (81). Animal studies carried out in the early 1970s have shown that prostaglandins were released into circulation as a result of bladder distension (82). Mechanical irritation of the bladder epithelium also triggers the release of prostanoids (83). Intraarterial infusion of prostaglandin E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) decreases the threshold micturition volume suggesting a regulatory role of prostaglandins in bladder contraction under physiological conditions (84).

Human detrusor muscle produces PGE_2 and $PGF_{2\alpha}$-like substances (85). There are conflicting findings about PGE_2 as a biomarker for OAB syndrome: increased urinary levels of PGE_2 and $PGF_{2\alpha}$ show positive correlation with the incidence of OAB syndrome, however, other studies failed to show any significant association with detrusor overactivity, increased bladder sensation and interstitial cystitis (86, 87). In addition, E. coli-induced inflammation increased COX-2 expression and PGE_2 synthesis in the urinary bladder (88, 89). COX-2 inhibition prevents and reverses the urodynamic changes in the inflammatory cystitis model of rats induced by surgery, lipopolysaccharides or cyclophosphamide (90). Different prostanoid receptor antagonists showed efficiency in reducing and restoring bladder overactivity in animal models (EP1, EP3, EP4) (91-93) shedding light on potential future pharmacological intervention targets to treat malfunctional voiding. PGE_2 can act on capsaicin-sensitive sensory nerves as well highlighting a further link between prostanoid and TK signaling (94). Notably, these experiments and observations suggest that both prostaglandins and TKs play a pathogenic role in bladder hyperactivity during inflammation.

2.4 G protein-coupled Signaling Pathways Related to Smooth Muscle Contraction

The seven transmembrane domain G protein-coupled receptors (a receptor family that includes NK and prostanoid receptors too) can elicit contraction via two main signaling pathways in smooth muscle cells (*Figure 1.)* (95).

Smooth muscle contraction is mediated via Ca^{2+}-dependent or independent pathways, eventually by the simultaneous activation of both. The Ca^{2+}-dependent pathway is

initiated by the binding of $G_{q/11}$-protein to the cytoplasmic domain of seven transmembrane receptors. Consecutively, the $G_{q/11}$-protein acquires the active conformational state by binding and hydrolyzing GTP. The $G_{q/11}$-protein activates phospholipase C β (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol-trisphosphate (IP_3) and diacyl-glycerol (DAG). IP_3 binds the IP_3 receptor on the sarcoplasmic reticulum, which releases Ca^{2+} into the cytoplasm. After binding four Ca^{2+} ions, calmodulin activates the myosin light chain kinase and turns on the cross-bridge cycle. Simultaneously, DAG activates protein kinase C (PKC) that participates indirectly in eliciting contraction.

In Ca^{2+} independent pathway, the starting point is similarly the activated membrane receptor, but instead of the $G_{q/11}$-proteins, the $G_{12/13}$-proteins contribute to the contraction of smooth muscle cells. In further detail, the $G_{12/13}$-protein activates the guanine nucleotide exchange factor of the Rho small GTP binding protein (RhoGEF). RhoGEF accelerates the switch of GDP to GTP of Rho which facilitates its transition to the active conformation. Rho activates the Rho kinase (ROCK). ROCK phosphorylates and therefore inhibits the myosin light chain phosphatase. Finally, the myosin light chain phosphatase is not able to remove the phosphate group from the myosin light chain, thus maintaining the activity of the cross-bridge cycle. Crosstalk can take place between the Ca^{2+}-dependent and independent pathways via CPI-17 (95).

2.5 The Epidemiology and the Current Treatment Options of Overactive Bladder Syndrome

OAB syndrome is a clinical condition that is related to our research project (96). OAB is defined as the symptoms of frequency (> 8 micturition / 24 h) and urgency, with/without incontinence (97, 98). An additional criterium is the absence of local pathological (e.g. urinary tract infection, stones, interstitial cystitis) or metabolic conditions (e.g. diabetes mellitus) that can explain the symptoms of OAB (98-100). OAB impairs the quality of life of the affected patients (101). In human urinary bladders, NKA, NKB, and SP induce detrusor muscle contraction under physiological conditions; furthermore, their potency is amplified in the neurogenic bladders of patients with spinal cord lesions (102). Interestingly, NK2R agonists have been recently considered as a potential therapy to elicit on-demand voiding in individuals with spinal cord injuries (103).

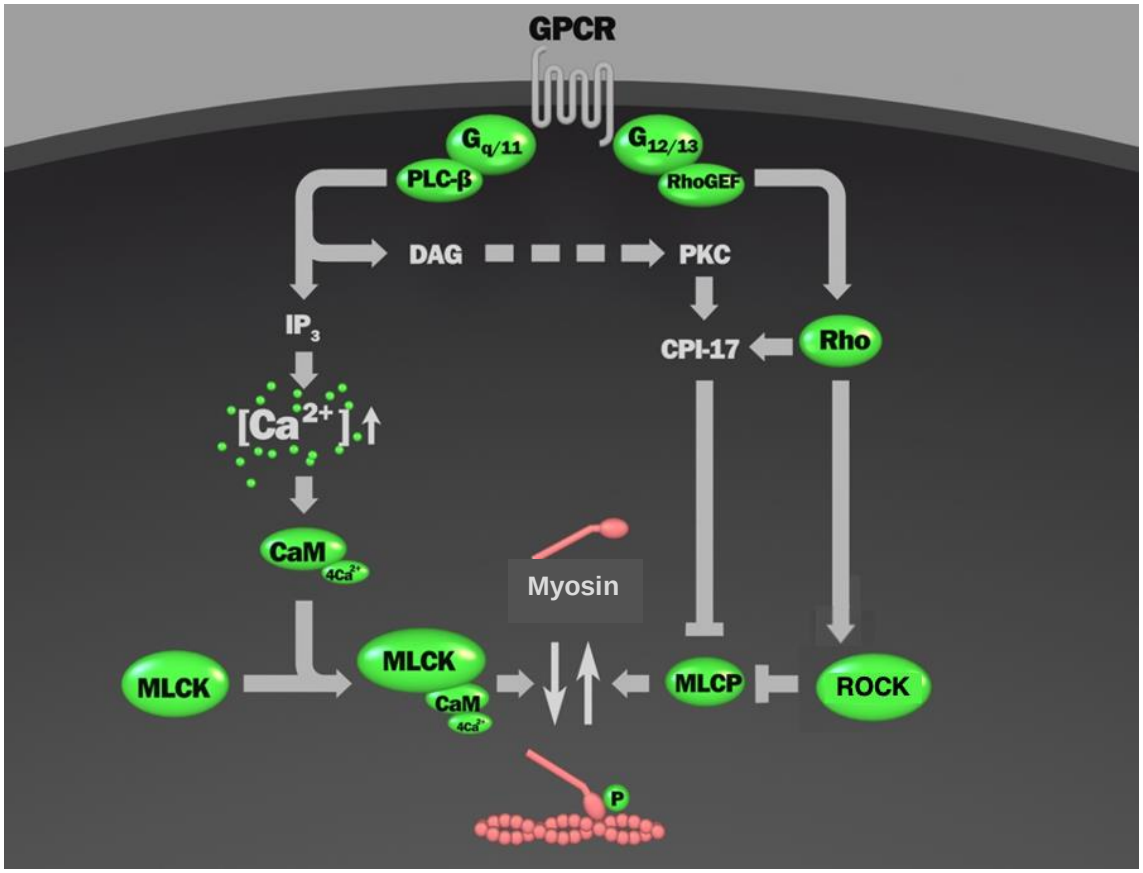

Figure 1. G protein-coupled signal transduction pathways lead to the activation of the cross-bridge cycle during smooth muscle contraction.
The $G_{q/11}$-protein induces contraction in a Ca^{2+} dependent manner whereas the $G_{12/13}$-protein activates the Ca^{2+}-independent pathway. The pathways are connected via CPI-17 protein. GPCR: G protein-coupled receptor, PLC-β: phospholipase C β, CaM: calmodulin, MLCK: myosin light chain kinase, MLCP: myosin light chain phosphatase, ROCK: Rho kinase.

The epidemiology of OAB syndrome has been investigated by multiple studies. In the European population, the prevalence of at least one OAB-related symptom was 16.6% based on telephone and in-person surveys. The prevalence is increased in both sexes with age. The overall prevalence of OAB was higher in women compared to men (women: 17.4% vs. men: 15.6%) (98).

The National Overactive Bladder Evaluation program surveyed the population of the United States by telephone. The predicted overall prevalence by the program was 16.9% in women and 16.0% in men. An additional difference between the sexes was the higher association rate of urging incontinence with OAB syndrome in women than men. OAB

syndrome was associated with lower quality of life, higher depression scores, and poorer quality of sleep compared to control groups (104). To sum up, we can state that both studies estimated the prevalence of OAB syndrome in a similar range (16–17%).

OAB syndrome makes everyday activities burdensome, including occupation, social activities, traveling, sexual life, and even sleep. Further consequences of the syndrome can be additional fear of odor, anxiety and frustration (105).

There is no available definitive and curative solution for OAB syndrome currently. Treatments are aimed to reduce the frequency and severity of symptoms. First-line treatment is commonly behavioral therapy that is technically side effect free. The second line of treatment is pharmacological management with potential adverse events. The widely used anti-muscarinergic compounds can have numerous adverse events, e.g. dry mouth, constipation or dyspepsia since muscarinergic receptors are widely expressed in different organ systems too. For moderate and severe OAB syndrome, invasive approaches represent the third line of interventions, e.g. injection of onabotulinumtoxin into detrusor muscle, sacral neuromodulation, or the stimulation of peripheral tibial nerve (106).

3.0 Objectives

In the urinary bladder, the exact mechanism of TK signal transduction is still unexplored. Data in the literature indicate that NK2Rs play an important role in the pathogenesis of OAB (37, 51, 77). Therefore, our main aim was to characterize the signaling of TKs, specifically NK2Rs, in the urinary bladder smooth muscle (UBSM), providing a theoretical basis for the development of future medications for bladder disorders, including OAB.

4.0 Methods

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Hungarian National Scientific Ethical Committee on Animal Experimentation (PEI/001/2709-13/2014).

Experiments were carried out on urinary bladders of adult male wild-type (C57BL/6 mice from Charles River Laboratories, Isaszeg, Hungary) and genetically modified mice. The mouse line with smooth muscle-specific inducible deletion of the $G_{q/11}$ signaling pathway [$G\alpha_{-q/11}$ knockout (KO)] was generated on the $G\alpha_{q/11}$-deficient ($G_{11}^{-/-}$) background (107) with floxed alleles of the genes coding $G\alpha_q$ ($Gnaq^{flox/flox}$) and expressed a fusion protein of the Cre recombinase with a modified estrogen receptor binding domain (Cre-ERT2) (108) under the control of the smooth muscle myosin heavy chain (SMMHC) promoter (109). Deletion of *Gnaq* was induced by intraperitoneal tamoxifen treatment (1 mg/day for 5 consecutive days) as previously described (109). Tamoxifen-treated SMMHC-$CreERT2^{-/-}$;$Gnaq^{flox/flox}$;$Gna11^{+/+}$ mice served as controls and are referred to as $G\alpha_{q/11}$-CTRL. Similarly, $G_{12/13}$-KO mice were generated with the floxed alleles of $G\alpha_{13}$ (*Gna13*) on the background of null alleles of Gα12 (*Gna12*). The smooth muscle-specific deletion of $G\alpha_{13}$ was achieved by the SMMHC promoter-driven Cre expression (110).

Mice were euthanized by cervical dislocation under anesthesia, and the urinary bladders were removed and placed into Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 0.03 mM EDTA, and 10 mM glucose, pH 7.4) at room temperature. Under a dissection microscope, the serosal surface was cleaned from adipose and connective tissue. The urothelial layer was also removed to prevent the potential release of paracrine factors from the mucosal epithelium as a result of tachykinin administration (36) and to avoid tension changes related to myofibroblasts (47). Bladders were pinned down at the trigonal area. We cut from the dome of the bladder towards the urethral opening ensuring that the majority of a strip consists of the detrusor muscle. Bladders were cut into four strips of equal length for myography, whereas for the RhoA activity assay, half-bladders were used. Intracellular Ca^{2+} measurements were performed on incised whole urinary bladders without urothelium as well.

Detrusor muscle contraction force was registered by myographs (type 610 M, Danish Myo Technology, Aarhus, Denmark) under isometric conditions. Smooth muscle strips

were suspended in chambers filled with 8 ml of 37°C Krebs solution equilibrated with a gas mixture of 5% CO_2-95% O_2. Before every experiment, urinary bladder strips were rested for 30 min during which passive tension was adjusted to 5 mN. After this equilibration period, bladder strips were exposed to 124 mmol/l K^+-containing Krebs solution (made by iso-osmolar replacement of Na^+ by K^+ to test the reactivity of the smooth muscle). After several washes with normal Krebs solution, the contractile effect of TKs [NKA/NKB/SP/[β-Ala8]-NKA(4–10) at 10^{-5} M], carbamylcholine chloride [carbachol (10^{-6} M)] was determined. Some of the strips were preincubated with one of the following inhibitors: MEN10376 [NK2R-specific antagonist, 3 X 10^{-5} M, 15 min, (111)], U-73122 [PLC-β inhibitor, 10^{-5} M, 35 min, (112)], edelfosine [PLC-β inhibitor, 10^{-5} M, 35 min, (113)], thapsigargin [SERCA inhibitor, 2 X 10^{-6} M, 20 min, (114)], Y-27632 [Rho kinase (ROCK) inhibitor, 10^{-5} M, 5 min, (115)], diltiazem [blocker of L-type voltage-dependent Ca^{2+} channels (VDCC), 5 X 10^{-5} M, 15 min, (116)], or Ca^{2+}-free Krebs solution (10 min). When DMSO or ethanol was the solvent of the inhibitor, the vehicle was applied in matched concentration as a control. Finally, bladder strips were exposed to 124 mmol/l K^+-containing Krebs solution to retest the reactivity of the smooth muscle. TK-induced tension changes were normalized to the reference contraction induced by 124 mM K^+-containing Krebs solution. MP100 system and AcqKnowledge3.72 software from Biopac System (Goleta, CA) were used for the acquisition and analysis of myographic measurements. To eliminate tension oscillations, the smoothening function of the software was applied to recordings.

To measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), freshly dissected tissue was prepared as described above, urinary bladders were slightly stretched and fixed on plastic rings with needles. For loading with fluorescent dye, samples were incubated for 60 min in HBSS with 5 μM fluo-4-AM (with added 0.02 m/v% pluronic F-127 and 1.8 mM probenecid), which was followed by a one hour washout period. During both processes, the plate shaker was set to 300 rpm at 35°C. Ca^{2+} signals evoked by [β-Ala^8]-NKA(4–10) were normalized to the amplitude of $[Ca^{2+}]_i$ induced by ionomycin (5 X 10^{-7} M). The viability of bladder samples was tested by the administration of ATP (10^{-5} M) in those cases when [β-Ala^8]-NKA(4–10) failed to induce any Ca^{2+} signal. The excitation wavelength of fluorescence was 494 nm, and the detection wavelength was 516 nm. To

acquire images and analyze data, a Nikon Eclipse Ti-S microscope linked to a TILL Oligochrome light source and TILL Photonics Live Acquisition software was used.

Activated RhoA protein was quantified with a pull-down ELISA kit. Briefly, urinary bladder strips were prepared as described above. After 30 min of equilibration, bladder strips were stretched to 5 mN in myograph chambers containing normal Krebs solution. After the addition of 10^{-5} M [β-Ala^{8}]-NKA(4–10), when bladders reached maximal contraction, they were snap-frozen in liquid N_2. To quantify the amount of activated and total RhoA protein, a RhoA G-LISA Activation Assay Kit and Total RhoA ELISA Assay Kit were used, respectively. In addition to the recommended assay protocols, lysis buffer was supplemented with protease and phosphatase inhibitors (1% Cytoskeleton protease inhibitor cocktail, 50 mM sodium fluoride, 1% phosphatase inhibitor cocktail, and 1 mM 4-nitrophenyl phosphate). Activated RhoA-GTP values were normalized to the total RhoA content. Baseline and its paired NK2R-activated sample RhoA activity measurements were performed from the two halves of the same urinary bladder.

During data analysis, "phasic contraction" was defined as the peak value of developed tension, whereas "tonic contraction" was characterized by the amplitude of contraction at the fifth minute after the addition of TKs. The same evaluation protocol was used for both myography and intracellular Ca^{2+} level measurements. Representative original traces as well as average curves of contractions and Ca^{2+} responses are presented on the left side of the figures. For the description of case numbers, the following formula was used: n x/y, where x represents the number of bladder strips/specimens obtained from y animals. All data are presented as means SD.

For statistical analysis, all data sets were first subjected to the D'Agostino-Pearson normality test. If the normal distribution of the data was verified, the *P* values were determined by a Student's unpaired *t*-test or one-way ANOVA, depending on the number of experimental groups, whereas the Mann-Whitney test or Kruskal-Wallis test was used if the normality test failed. Statistical analysis and graph plotting were performed with GraphPad Prism software (v.6.07, GraphPad Software, La Jolla, CA). $P < 0.05$ was considered a statistically significant difference.

Fluo-4-AM, probenecid, and ionomycin were purchased from ThermoFisher Scientific (Waltham, MA). Fluo-4-AM and probenecid were dissolved freshly before experiments. Ionomycin was dissolved in ethanol and stored at 10^{-3} M concentration.

NKA trifluoroacetate, NKB trifluoroacetate, SP acetate, MEN10376, and [β-Ala^8]-NKA(4–10) were purchased from Bachem (Bubendorf, Switzerland). NKA trifluoroacetate, NKB trifluoroacetate, and SP acetate are referred to as NKA, NKB, and SP, respectively, in this article. NKA, NKB, and [β-Ala^8]-NKA(4–10) were dissolved in DMSO to make a 1,000-fold concentrated stock solution. SP was dissolved in distilled water and MEN10376 was dissolved in saline (stock solution concentrations: 10^{-2} M for SP and 3 x 10^{-3} M for MEN10376). U-73122 and edelfosine were purchased from Tocris (Bristol, UK). U-73122 was dissolved in DMSO and edelfosine in ethanol both to 10^{-2} M stock solutions. Y-27632 and thapsigargin were purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in saline (Y-27632: 10^{-3} M, and thapsigargin: 10^{-2} M. ATP was dissolved in HBSS, and the concentration of its stock solution was 10^{-3} M. RhoA G-LISA Activation Assay Kit (Colorimetric format, catalog no. BK124), Total RhoA ELISA Assay (catalog no. BK150), and Cytoskeleton protease inhibitor cocktail (catalog no. PIC02) were purchased from Cytoskeleton (Denver, CO). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Diltiazem was dissolved in saline immediately before use. Carbachol was dissolved in saline to 10^{-3} M stock solution. PGE_2 and PGF_2 were purchased from Cayman Chemical and stock solutions were constituted at 10^{-2} M in DMSO. HBSS without phenol red was purchased from Lonza (Basel, Switzerland). When organic solvents were used, vehicle treatment served as a control.

5.0 Results

First, we evaluated the role of NK2R in mediating the effects of TKs. NKA, NKB, and SP induced strong contractions *(Figure 2./A–C)* in which a phasic and a tonic component could be discriminated. The phasic contractions, characterized by the maximal elevation of the tension, were markedly inhibited by the NK2R-specific antagonist MEN10376 in the case of all three TKs *(Figure 2./A–C)*. In contrast, the amplitude of the tonic contractions, determined at 5 mins, remained unaltered in the presence of MEN10376 in the case of NKA and NKB, whereas it was abolished in the case of SP. Since all TKs increased UBSM tension in a MEN10376-reversible manner, it was concluded that the NK2R is likely to play an important role in the control of urinary bladder functions.

Since the main goal of the present study was to investigate the intracellular signaling pathways mediating the detrusor muscle contraction in response to NK2R activation, we next tested the effects of NK2R-specific agonist [β-Ala^8]-NKA(4–10). In our experimental setup, we found that it induced dose-dependent contractions with an EC_{50} value of 3.18 μM and a maximum effect (E_{max}) value of 36.1%. (normalized to K^+-induced contraction) Importantly, both phasic and tonic components of the contraction induced by the submaximal concentration (10^{-5} M) of [β-Ala^8]-NKA(4–10) were diminished by MEN10376 *(Figure 2./D)*. Therefore, in further experiments, 10^{-5} M [β-Ala^8]-NKA(4–10) was used to activate specifically NK2R-mediated signaling in the UBSM.

Next, we aimed to identify the G-proteins mediating the effect of NK2Rs in UBSM. To examine the contribution of the G_q/G_{11} signaling pathway, the consequences of $G\alpha_{q/11}$ deletion on NK2R-mediated smooth muscle contraction and Ca^{2+} signaling were analyzed. In $G\alpha_{q/11}$-KO animals, both phases of the contraction response induced by [β-Ala^8]-NKA(4–10) were eliminated (*Figure 3/A*). [β-Ala^8]-NKA(4–10) not only elicited contraction but also triggered $[Ca^{2+}]_i$ increase, which also disappeared in $G\alpha_{q/11}$-KO bladders (*Figure 3/B*). Taken together, these results indicate that $G_{q/11}$ proteins have an exclusive role in mediating the intracellular signaling of NK2Rs in the UBSM.

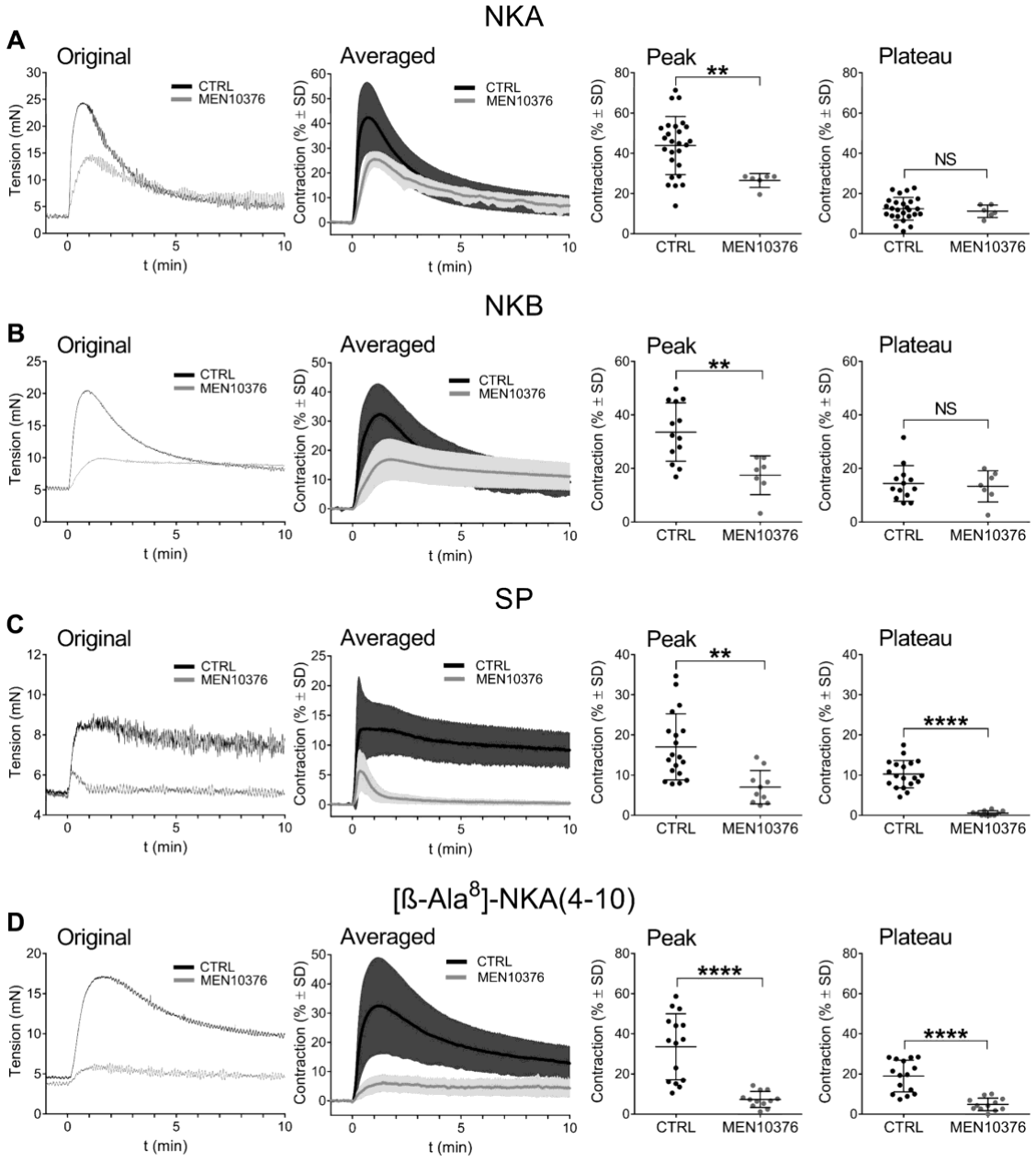

Figure 2. Role of NK2Rs in mediating tachykinin-induced detrusor muscle contraction.

NKA, NKB, and SP induced contraction in the mouse urinary bladder, which was attenuated markedly by the NK2R-specific antagonist MEN10376 (*A–C)*. The NK2R-specific agonist-induced similar contraction, an effect that was abolished by MEN10376 (*D*). Individual traces are presented on the *left* followed by the averaged curves. NS, not significant. $^{**}P < 0.01$; $^{****}P < 0.0001$ (*A* and *B*: Mann-Whitney test; *C* and *D*: Student's *t*-test). Case numbers were as follows: in *A*, *n* = 26/14 for CTRL and *n* = 6/6 for MEN10376; in *B*, n = 13/12 for CTRL and *n* = 6/6 for MEN10376; in *C*, *n* = 19/15 for CTRL and *n* = 10/7 for MEN10376; and in *D*, *n* = 15/12 for CTRL and *n* = 12/9 for MEN10376.

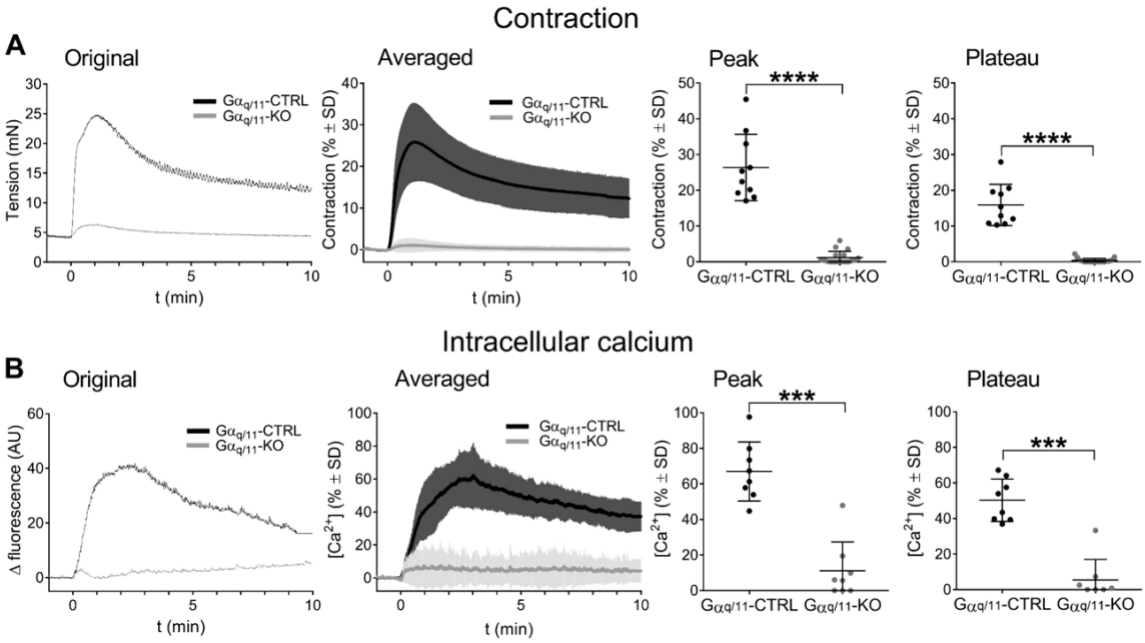

Figure 3. $G_{q/11}$ proteins mediate intracellular signaling of NK2R in the UBSM muscle.

A: in $G\alpha_{q/11}$-KO mice, the contraction completely disappeared in both phases. *B*: both the peak and plateau of the intracellular Ca^{2+} signal were eliminated in $G\alpha_{q/11}$-KO urinary bladders. ***P < 0.001; ****P < 0.0001 (Mann-Whitney test). Case numbers were as follows: in *A*, n = 10/9 for the $G\alpha_{q/11}$ CTRL group and n = 17/9 for the $G\alpha_{q/11}$-KO group; and in *B*, n = 8/4 for the $G\alpha_{q/11}$ CTRL group and n = 8/4 for the $G\alpha_{q/11}$-KO group.

To exclude the potential overlapping function of multiple G proteins in eliciting contraction, we investigated the effects of genetic $G_{12/13}$ deletion on [β-Ala^8]-NKA(4–10) induced contraction. To answer our question, we generated $G\alpha_{12/13}$-KO mice with a similar approach as we applied in $G\alpha_{q/11}$-KO. Bladders of $G_{12/13}$-KO mice showed no significant difference in the phasic or tonic phase of contraction compared to $G_{12/13}$-CTRL mice (*phasic*: $G\alpha_{12/13}$-CTRL: 32.0±2.7% vs. $G\alpha_{12/13}$-KO: 29.8±2.9%, P = 0.578; *tonic*: $G\alpha_{12/13}$-CTRL: 20.2±2.0% vs. $G\alpha_{12/13}$-KO: 15.7±1.5%, P = 0.094; n=12 bladder strips, three biological replicates each group; Student's *t*-test). It appears that $G_{q/11}$ solely mediates NK2R signaling in urinary bladder smooth muscle *ex vivo*.

Although NK2R is coupled exclusively to $G_{q/11}$ *ex vivo* in detrusor muscle, in other systems $G_{12/13}$ protein and the Ca^{2+} independent pathway can also elicit smooth muscle contraction. Prostaglandins can induce contraction via the FP receptor under physiological conditions (117). TP receptor has been shown to exert its effects via $G_{12/13}$ proteins in platelets (118). We hypothesized prostaglandins mediate contraction via the

$G\alpha_{12/13}$-Rho-ROCK pathway in the urinary bladder smooth muscle. The contractile responses evoked by prostaglandins (PGE_2 and $PGF_{2\alpha}$, 10^{-5} M) were reduced in $G\alpha_{12/13}$-KO animals and in the presence of ROCK inhibitor Y-27632 to a similar magnitude suggesting the conventional activation of Ca^{2+} independent pathway (*Figure 4.*). Therefore, the predominance of the $G_{q/11}$ pathway, as observed in the case of NK2R, is not a general phenomenon for all GPCR-mediated UBSM contractions.

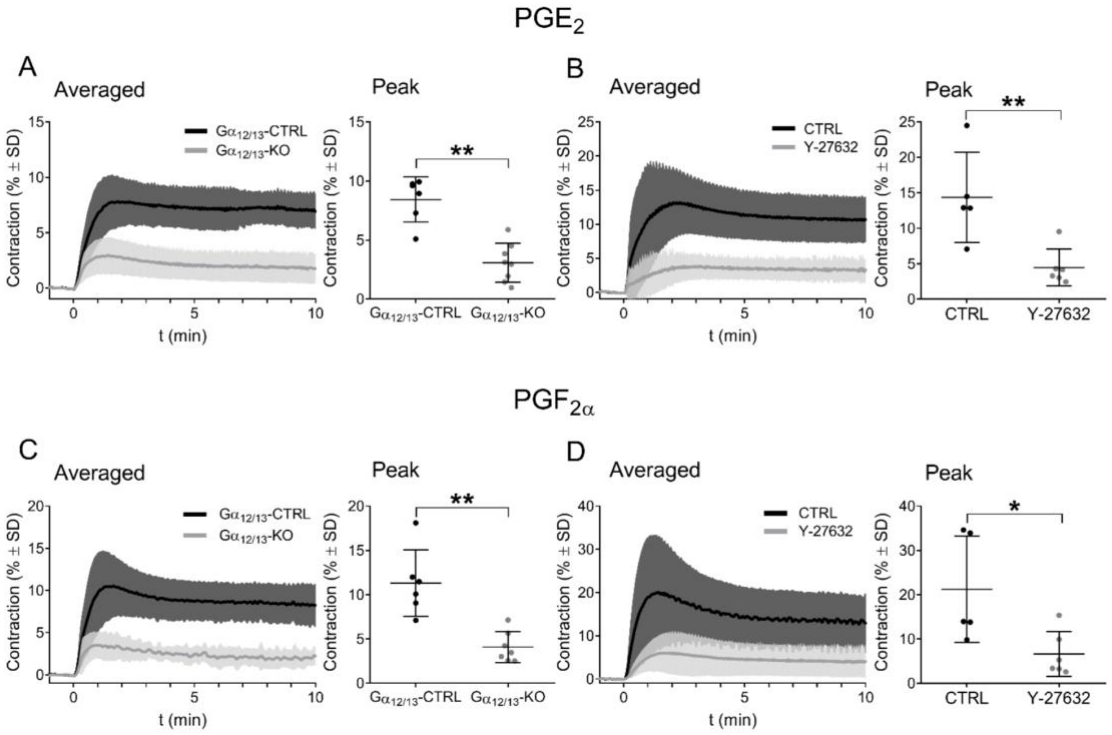

Figure 4. Prostaglandin E_2 and $F_{2\alpha}$ induce urinary bladder smooth muscle contraction primarily via $G\alpha_{12/13}$-Rho-ROCK pathway.
A, C: In $G\alpha_{12/13}$-KO mice the contractile effects of PGE_2 and $PGF_{2\alpha}$ were reduced compared to $G\alpha_{12/13}$-CTRL animals. *B, D*: Rho-kinase inhibition by Y-27632 (10^{-5} M, 20 min incubation) comparably decreased the amplitude of contraction responses to $G\alpha_{12/13}$-KO mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Mann-Whitney test). Case numbers were as follows: A: n = 6-8, B: n = 5-6, C: n = 6-7, D: n = 5-6. PGE_2 and $PGF_{2\alpha}$: prostaglandin E_2 and $F_{2\alpha}$.

Our next aim was to identify the downstream signaling partner(s) of $G_{q/11}$ proteins in mediating NK2R-induced UBSM contraction. $G_{q/11}$ proteins are linked primarily to PLC-β, which can evoke intracellular Ca^{2+} release by producing IP_3. However, two different types of PLC-β inhibitors (U-73122 and edelfosine) both failed to have any effect on contraction force, either in the acute (*Figure 5./A*) or during the tonic (*Figure 5./B*) phase

of contraction. Therefore, PLC-β does not appear to be involved in the $G_{q/11}$-mediated signal transduction pathway of the UBSM.

Next, we aimed to figure out whether the source of the Ca^{2+} signal is intracellular or extracellular. Intracellular Ca^{2+} store depletion by thapsigargin, which inhibits SERCA, did not alter the contractile effect of [β-Ala^8]-NKA(4–10) (*Figure 5./A and B*). In contrast, the removal of Ca^{2+} from Krebs solution resulted in a marked reduction of the contraction force in both the phasic and tonic phase of the response (*Figure 6.*), indicating the predominant role of Ca^{2+} influx from the extracellular space in mediating UBSM contraction upon activation of NK2Rs.

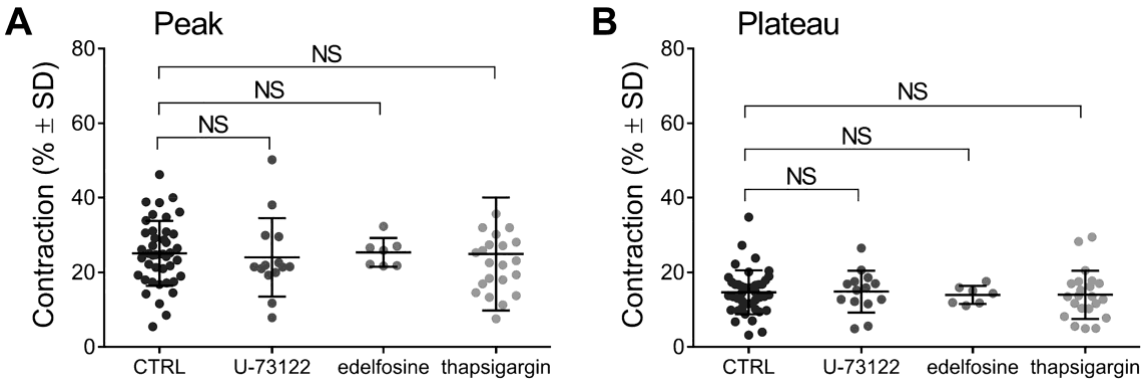

Figure 5. Ineffectiveness of PLC-β inhibition or intracellular Ca^{2+} store depletion on NK2R-mediated detrusor muscle contraction.
Neither PLC-β inhibitors (U-73122 and edelfosine) nor the depletion of intracellular Ca^{2+} stores (by thapsigargin) altered the contractile response to [β-Ala^8]-NKA(4–10) administration. NS, not significant (one-way ANOVA). Case numbers were as follows: *n* = 42/29 for CTRL, *n* = 14/11 for U-73122, *n* = 7/7 for edelfosine, and *n* = 21/11 for thapsigargin.

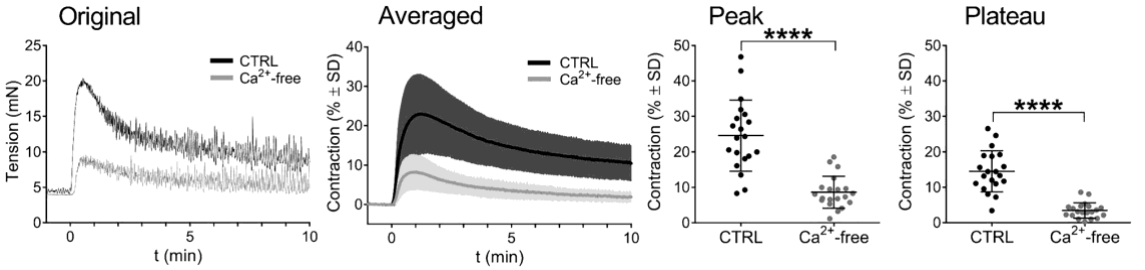

***Figure 6.* Role of extracellular Ca^{2+} in mediating [β-Ala^8]-NKA(4–10)-induced urinary bladder contraction.**
Lack of extracellular Ca^{2+} markedly reduced both the "peak" and "plateau phase" of contraction force. ****$P < 0.0001$ (Student's *t*-test). Case numbers were as follows: *n* = 20/20 for CTRL and *n* = 20/20 for Ca^{2+}-free medium.

Different Ca^{2+} channels have been implicated in regulating the tone of the UBSM, of which VDCCs appear to have the most widespread biological function (119). Therefore, we aimed to evaluate their role in NK2R-mediated UBSM contraction. The VDCC blocker diltiazem induced similar inhibition of the contractile effect of [β-Ala^8]-NKA(4–10) as was observed in the absence of extracellular Ca^{2+} (*Figure 7.*). Taken together, these results indicate that Ca^{2+} influx through VDCCs mediates the contractile effect of NK2R stimulation.

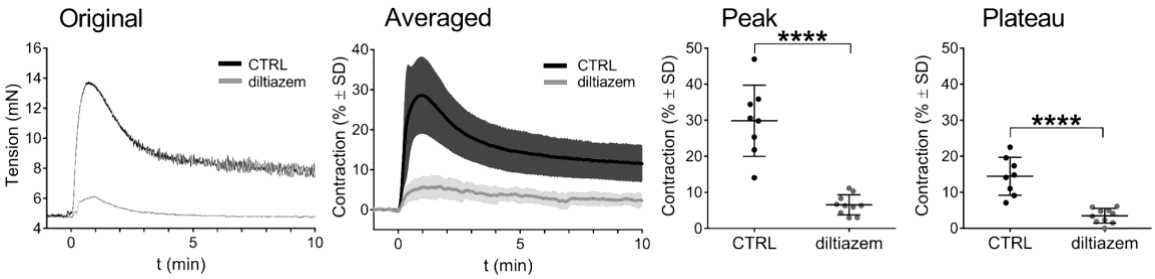

Figure 7. Involvement of VDCCs in NK2R-mediated detrusor muscle contraction. Urinary bladder strips treated with the VDCC blocker diltiazem showed diminished contraction in both phases. ****$P < 0.0001$ (Student's *t*-test). Case numbers were as follows: $n = 8/8$ for CTRL and $n = 10/10$ for diltiazem.

Interestingly, neither removal of extracellular Ca^{2+} nor administration of diltiazem was able to diminish the effect of [β-Ala^8]-NKA(4–10) as completely as the deletion of $G\alpha_{q/11}$ proteins did, indicating the presence of an alternative intracellular signaling mechanism. We hypothesized that this might be the RhoA-ROCK signaling pathway since a previous study indicated its importance in the regulation of UBSM tone (120). Indeed, administration of [β-Ala^8]-NKA(4–10) increased RhoA activity in the UBSM of CTRL mice, whereas this effect was absent in the urinary bladders of $G\alpha_{q/11}$-KO animals (*Figure 8./A*). In addition, the ROCK inhibitor Y-27632 reduced the amplitude of [β-Ala^8]-NKA(4–10)-induced contraction during both the phasic and tonic phases (*Figure 8./B*). The combination of Y-27632 with the removal of extracellular Ca^{2+} or administration of diltiazem abolished completely the contractile effect of [β-Ala^8]-NKA(4–10) (*Figure 8./B*). Therefore, our results indicate that NK2Rs simultaneously activate VDCC-mediated Ca^{2+} influx and the RhoA-ROCK signaling pathway, both of which contribute significantly to UBSM contraction.

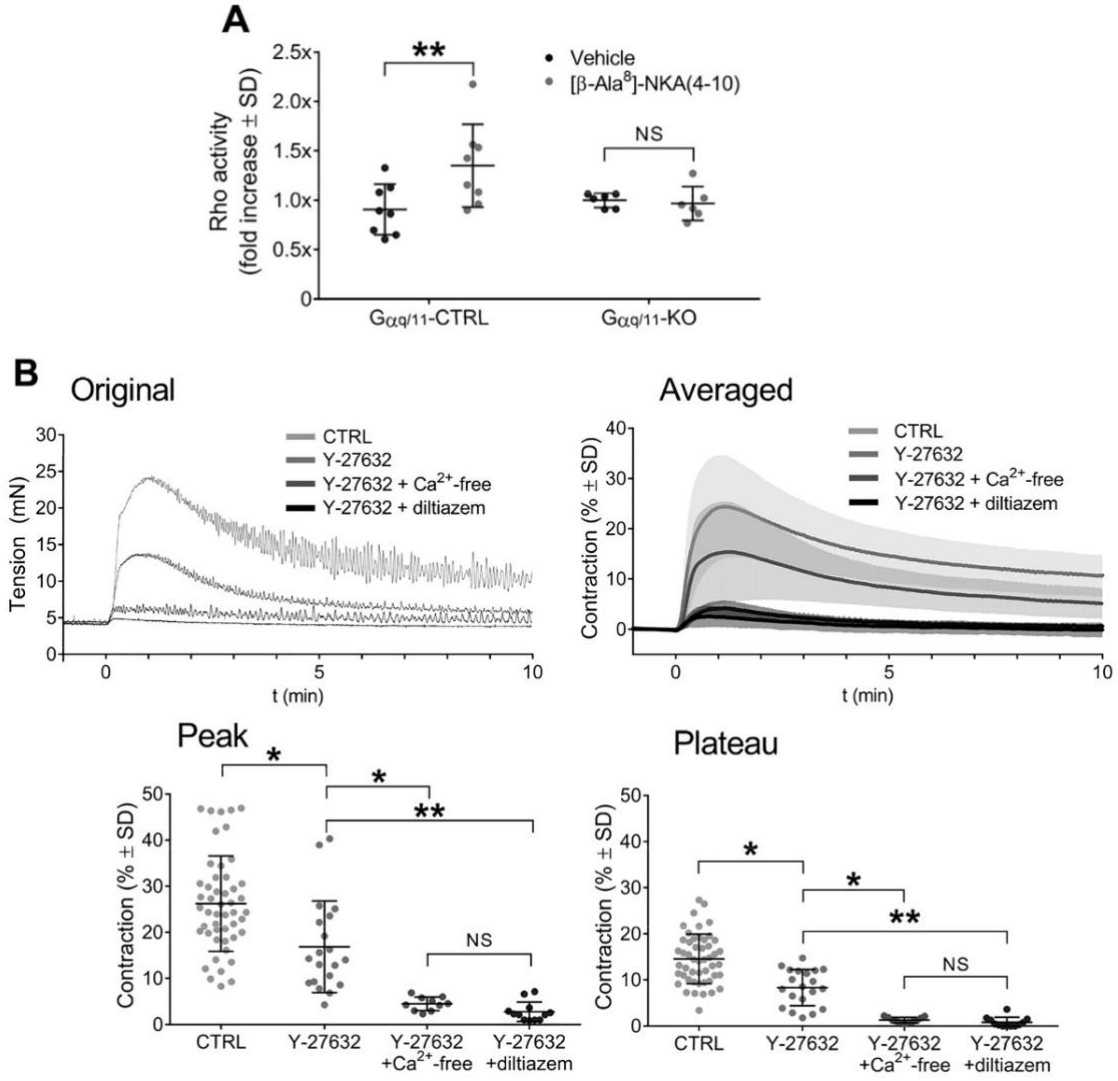

Figure 8. Role of the RhoA-Rho kinase pathway in mediating detrusor contraction by [β-Ala^{8}]-NKA(4–10).

A: effects of $G\alpha_{q/11}$ protein deficiency on RhoA activation. RhoA activity was expressed as normalized to baseline. *B*: inhibitory effects of the Rho kinase inhibitor Y-27632 alone or in combination with Ca^{2+}-free medium or diltiazem. *$P < 0.05$; **$P < 0.01$ (*A*: Kruskal-Wallis test; *B*: two-way ANOVA with a Bonferroni's post hoc test). Case numbers were as follows: in *A*, n = 49/49 for CTRL, n = 20/19 for Y-27632, n = 10/10 for Y-27632 + Ca^{2+}-free medium, and n = 12/12 for Y-27632 + diltiazem; and in *B*, n = 8/8 for the $G\alpha_{q/11}$ CTRL group and n = 6/6 for the $G\alpha_{q/11}$-KO group.

6.0 Discussion

The contractile effect of TKs in the UBSM has been previously described; however, there are only a few studies that have provided a mechanistic explanation of their intracellular signaling under physiological conditions. We demonstrated that NKA, NKB, and SP exert sustained contraction through NK2Rs on smooth muscle cells in mouse urinary bladder. Selective NK2R activation induced a Ca^{2+} signal with matching kinetics of the contraction curve that is mediated by $G_{q/11}$ proteins. In contrast, prostaglandins elicit contraction mostly via the $G_{12/13}$-Rho-ROCK pathway. Surprisingly, inhibitors of the PLC-β-mediated intracellular Ca^{2+}-releasing pathway (U-73122, edelfosine, and thapsigargin) failed to influence the amplitude of contraction force either in phasic or tonic phase. Contrariwise, our results indicate that extracellular Ca^{2+} enters the cytoplasm via VDCCs and activates the cross-bridge cycle upon activation of NK2Rs. In addition, $G_{q/11}$-mediated activation of the RhoA-ROCK pathway has a major contribution to detrusor muscle contraction.

TKs play a complex role in the regulation of detrusor muscle tone. Their effects are mediated by NK receptors of the urothelium and nerve fibers as well as the UBSM itself (2, 121, 122), of which the latter was the focus of the present study. Experiments with urothelium-denuded smooth muscle strips excluded urothelium-mediated indirect actions in our experimental setup. Treatment of urinary bladders with pharmacological inhibitors of muscarinergic and purinergic receptors did not alter the NK2R-induced contraction response suggesting that nerve fiber-derived acetylcholine or ATP does not contribute (data not presented in the dissertation).

NKA, NKB, and SP induced characteristic contraction response with two distinct phases: 1) a phasic, transient phase and 2) a tonic, sustained response consistently with a previous publication (38). The rank order of potency was NKA, NKB, and SP, analogous to humans (123). MEN10376 inhibited the first phase of the NKA and NKB response. Furthermore, in SP-induced contraction, NK2Rs contributed to both phasic and tonic contraction (38, 124). Interesting to observe the different inhibitory effects of MEN10376: this suggests that NK2R is involved in the tonic phase of the SP-induced contraction as well, or there is receptor desensitization involved in the SP induced response. Consequently, the NK2R is involved in mediating the effect of all three TKs

and therefore could potentially be involved in bladder hyperactivity in those cases when TK and/or NK2R expression is upregulated. As expected from the *in vivo* experiments (77), selective activation of NK2Rs with [β-Ala^8]-NKA(4–10) resulted in a contraction curve similar to TK-evoked responses and was abolished by MEN10376, enabling us to selectively examine the intracellular signaling pathways of the NK2R in our experiments with this compound. Importantly, our unpublished observations indicated a very similar contractile effect of [β-Ala^8]-NKA(4–10), both in terms of amplitude and temporal pattern, in human detrusor muscle to that shown in the present study, underlining the human relevance of our findings.

In *in vitro* systems, it has been previously described that, depending on their actual conformational state, NK2Rs can couple to both G_s and $G_{q/11}$ proteins (125, 126). We examined the relevance of G proteins in the urinary bladder in an *ex vivo* model with conditional KO mice and provided direct evidence, for the first time, that downstream signaling of NK2Rs in detrusor muscle solely depends on the $G_{q/11}$ pathway, which is a critical element of both NK2R-induced $[Ca^{2+}]_i$ increase, RhoA activation, and contraction force.

PLC-β is classically regulated by $G_{q/11}$ proteins (127) and hydrolyzes phosphatidylinositol 4,5-bisphosphate to IP_3 and DAG. According to the well-established concept, IP_3 binds to the IP_3 receptor of intracellular Ca^{2+} stores and triggers the release of Ca^{2+} from the sarcoplasmic reticulum, leading to the activation of the cross-bridge cycle. Catalioto et al. (128) expressed NK2Rs in Chinese hamster ovary cells and demonstrated its linkage to PLC-β. Consequently, receptor activation increased the intracellular level of IP_3. In contrast to our expectations based on data obtained through *in vitro* experiments, inhibition of PLC-β failed to alter NK2R-mediated detrusor muscle contraction. Interestingly, carbachol-induced detrusor muscle contraction, which is mediated by $G_{q/11}$-coupled M_3 muscarinic receptors, is also resistant to pharmacological inhibition of PLC-β (129, 130), indicating the presence of a similar alternative pathway in both cholinergic and tachykinin-induced intracellular signaling. In addition, thapsigargin treatment was also ineffective, indicating little or no involvement of sarcoplasmic Ca^{2+} release in mediating the effect of NK2R activation. Therefore, the common and classical motives of $G_{q/11}$ signaling, PLC-β and intracellular Ca^{2+} release from the sarcoplasmic reticulum, do not appear to be linked to NK2Rs in the UBSM.

Quinn et al. (131) found that Ca^{2+}-free medium fully abolished, whereas nifedipine decreased the NKA and SP-induced contraction in rat detrusor smooth muscle tissue *in vitro*. In hamster urinary bladder strips, nifedipine almost completely suppressed the contractile response to [β-Ala^{8}]-NKA(4–10) (122). Most recently, Grundy et al. (132) found in isolated mouse bladders that NKA simultaneously increased intravesical pressure and afferent nerve firing, effects that were both sensitive to the NK2R antagonist GR159897 and nifedipine. Therefore, whole tissue and organ systems appear to operate with completely different signaling pathways compared with Chinese hamster ovary cells, in which the application of verapamil did not influence NKA-induced responses (128). Based on previous data from the literature, we assumed that Ca^{2+} entry through VDCCs from the extracellular space is the major source of the internal Ca^{2+} increase when NK2Rs are activated. In our experiments, both Ca^{2+}-free Krebs solution and the VDCC inhibitor diltiazem reduced the contraction markedly, although they failed to induce complete blockade as observed in rat detrusor muscle (131). These discrepancies can be attributed to differences in the animal species or experimental protocols. Our results are in line with the previously established role of extracellular Ca^{2+} in inducing contraction force via VDCCs in response to cholinergic stimulation (133). The relationship between NK2Rs and VDCCs might explain why diltiazem improved the symptoms and restored impaired voiding parameters in patients with OAB (134).

An intriguing question, which unfortunately could not be answered by our experiments, is the link between NK2R-activated $G_{q/11}$ proteins and VDCCs. Transient receptor potential vanilloid-type 4 (TRPV4) channels have been recently implicated to play a major role in the physiological regulation and dysfunctions of micturition (135-137). Activation of TRPV4 channels induces detrusor muscle contraction in a VDCC-dependent manner (138), and it has been recently shown that stimulation of $G_{q/11}$ protein-coupled receptors may activate TRPV4 channels either via IP_3 production or depletion of plasma membrane phosphatidylinositol 4,5-bisphosphate (139, 140). However, both IP_3 production and phosphatidylinositol 4,5-bisphosphate depletion require PLC-β activation, which appears not to be involved in NK2R-mediated UBSM contraction. Interestingly, $G_{q/11}$-coupled M_3 muscarinic receptor-mediated detrusor muscle contraction has also been reported to develop in a PLC-β-independent and VDCC-dependent manner (129, 133, 141). In that case, a signaling complex consisting of VDCC,

PKC, and phospholipase D has been proposed to mediate VDCC activation (142). However, in our experimental setting, the potent and specific PKC inhibitor GF109203X was able to alter NK2R-mediated detrusor contractions only in micromolar concentrations, which simultaneously inhibited the contractile effect of 124 mM KCl, indicating a PKC-independent nonspecific action. Therefore, further studies will be required to identify the signaling between NK2R-activated $G_{q/11}$ proteins and VDCCs.

As neither extracellular Ca^{2+} depletion nor VDCC blockade was sufficient to abolish NK2R-mediated detrusor muscle contraction, the potential role of a Ca^{2+}-independent signaling was also addressed in the present study. Wibberley et al. (143) demonstrated a high expression level of ROCK isoforms in the rat urinary bladder. In addition, the RhoA-ROCK pathway has been recently implicated in both the physiology and pathophysiology of urinary bladder functions (144-146). Since NKA also activates ROCK in the urinary bladder (147), we hypothesized that NK2R might mediate RhoA activation. We have demonstrated the coupling of NK2R to RhoA-ROCK in two ways: with the application of the pharmacological inhibitor of ROCK (Y-27632) and by direct measurement of activated RhoA levels. Taken together, our results indicate that $G_{q/11}$ mediates RhoA activation in the urinary bladder in response to NK2R stimulation. A recently described and specifically $G_{q/11}$-activated RhoGEF in smooth muscle, p63RhoGEF, might be a potential link between $G_{q/11}$ proteins and the RhoA-ROCK pathway (148).

Activated RhoGEFs promote the exchange of GTP to GDP of RhoA protein. By binding and hydrolyzing GTP, RhoA can act as a downstream effector in indirectly inhibiting the dephosphorylation of myosin to induce smooth muscle contraction. Multiple RhoGEF mRNA isoforms are expressed in rat detrusor, trigonal and urethral tissue: e.g. p115RhoGEF, PDZ-RhoGEF and LARG. Western blot analysis confirms the presence of these proteins, albeit these proteins are traditionally linked to the $G_{12/13}$ pathway (149). The expression of p63RhoGEF – a different isotype – is also characterized in human J82 bladder carcinoma cells (149). However, there is no current study investigating its presence either on mRNA or protein levels in UBSM. Momotani *et al.*, 2022 showed that p63RhoGEF is expressed in multiple mouse and human vascular smooth muscle cell lines, derived from the aorta, pulmonary artery, brain vascular area, etc. The suppression of p63RhoGEF expression by the short hairpin RNA approach inhibited the $G_{q/11}$-linked ET-1 mediated contraction response suggesting that

p63RhoGEF is part of the $G_{q/11}$ downstream signaling cascade. In the same study, introducing the p63RhoGEF$^{331–580}$ mutant form (the binding domain to $G_{q/11}$ protein based on crystallographic studies) to the smooth muscle cells resulted in decreased levels of RhoA-GTP as well (148). A similar link can also connect $G_{q/11}$ and RhoA in the detrusor muscle, which requires further experiments to prove its existence even utilizing smooth muscle-specific KO mouse models.

Prostaglandins (PGE_2 and $PGF_{2\alpha}$) mediated detrusor contraction *ex vivo* correspondingly with previously published results from animal and human studies (84, 94, 150). We can conclude from the $G_{12/13}$-KO and ROCK inhibition experiments that prostanoids mediate contraction via the Ca^{2+} independent pathway in contrast to the NK2R-initiated response. Notably, since neither the genetic ($G_{12/13}$-KO) nor the pharmacological (Y-27632) interventions resulted in the complete abolishment of the contractile response evoked by prostaglandins, we can speculate that alternative routes must exist along with the Ca^{2+} independent pathway. Interestingly, ROCK activation appears to be context independent in UBSM: both $G_{q/11}$ and $G_{12/13}$ initiated signaling converge towards the same regulatory pathway.

In conclusion, TKs have a unique signal transduction pathway in the urinary bladder, which involves $G_{q/11}$ proteins, VDCCs, RhoA, and ROCK but not the common $G_{q/11}$-mediated downstream signaling mechanism via PLC-β and sarcoplasmic reticulum Ca^{2+} release (*Figure 9.*). The detailed characterization of UBSM NK2R signaling may help to better understand the pathomechanism of OAB syndrome. Our work provided a framework to be further validated in the animal models of OAB syndrome. In the future, local or systemic small molecule inhibitor or genetic targeting of NK2R, VDCC and ROCK in the UBSM might be favorable treatment strategies.

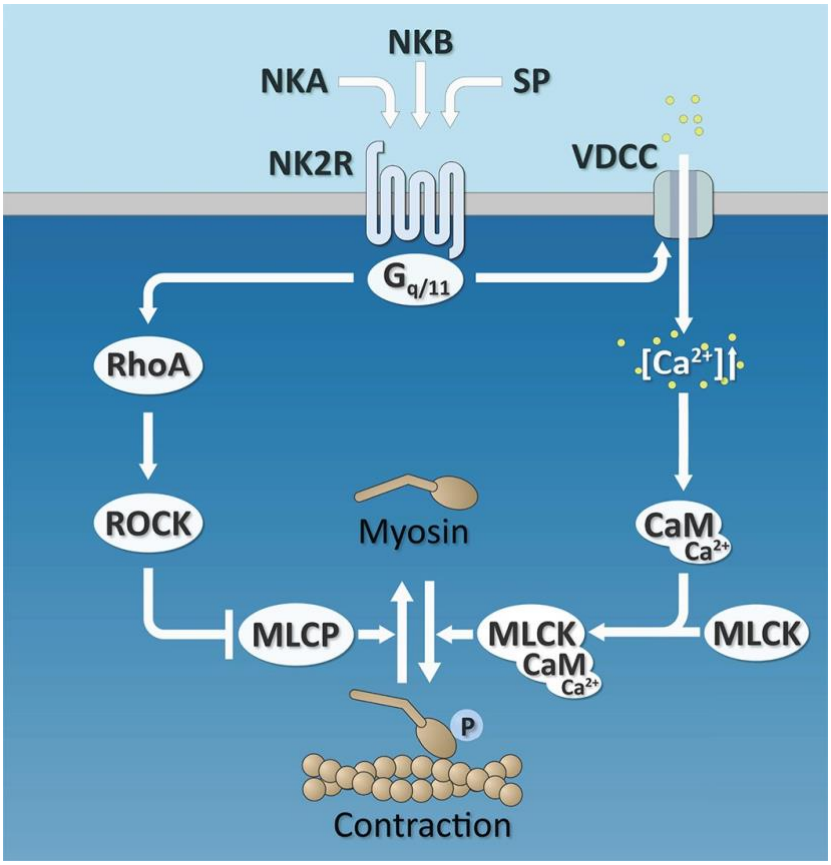

Figure 9. Summary of NK2R signal transduction in the UBSM muscle.
Tachykinins (NKA, NKB, SP) activate seven transmembrane NK2Rs on smooth muscle cells, which transmit signals exclusively through $G_{q/11}$ protein, evoking an $[Ca^{2+}]_i$ increase and contraction. Surprisingly, neither of the classical $G_{q/11}$ signaling elements (PLC-β or sarcoplasmic reticulum Ca^{2+} release) are involved in the contraction. Ca^{2+} enters the cytoplasm from the extracellular space through VDCCs and activates the cross-bridge cycle, which results in contraction. $G_{q/11}$ proteins activate RhoA, probably via p63RhoGEF, which amplifies the response. The signal transduction of the NK2R in the urinary bladder is different in many points from classical $G_{q/11}$ signaling. ROCK: Rho kinase; MLCP: myosin light-chain phosphatase; MLCK: myosin light-chain kinase; CaM: calmodulin.

7.0 Conclusions

In our experiments, we aimed to investigate the intracellular signaling of TK-induced urinary bladder smooth muscle contraction under physiological conditions.

Our results indicate that:

- All three TKs (NKA, NKB and SP) elicit detrusor contraction via NK2R which is coupled exclusively to $G_{q/11}$ protein
- PLC-β and intracellular Ca^{2+} release is not involved in the contractile response
- VDCC mediates contraction induced by NK2R activation
- Both the enzymatic assay readout and treatment with pharmacological inhibitor suggest that $G_{q/11}$ protein activates the Rho-ROCK pathway
- Prostaglandins mediate UBSM contraction via $G_{12/13}$-Rho-ROCK signaling.

8.0 Summary

TKs are involved in both the physiological regulation of urinary bladder functions and the development of OAB syndrome. The aim of our project was to investigate the signal transduction pathways of TKs in the detrusor muscle to provide potential pharmacological targets for the treatment of bladder dysfunctions associated with enhanced TK production. Contraction force, intracellular Ca^{2+} concentration, and RhoA activity were measured in the mouse UBSM. TKs and the NK2R-specific agonist [β-Ala^8]-NKA(4–10) evoked contraction, which was inhibited by the NKR2 antagonist MEN10376. In $G\alpha_{q/11}$-deficient mice, [β-Ala^8]-NKA(4–10)-induced contraction and the intracellular Ca^{2+} concentration increase were abolished. In $G\alpha_{12/13}$-KO mice, the contractile response was unaltered. Although $G_{q/11}$ proteins are linked principally to PLC-β and IP_3-mediated Ca^{2+} release from intracellular stores, we found that PLC-β inhibition and sarcoplasmic reticulum Ca^{2+} depletion failed to have any effect on contraction induced by [β-Ala^8]-NKA(4–10). In contrast, lack of extracellular Ca^{2+} or blockade of VDCC suppressed contraction. Furthermore, [β-Ala^8]-NKA(4–10) increased RhoA activity in the UBSM in a $G_{q/11}$-dependent manner and inhibition of Rho kinase with Y-27632 decreased contraction force, whereas the combination of Y-27632 with either VDCC blockade or depletion of extracellular Ca^{2+} resulted in complete inhibition of [β-Ala^8]-NKA(4–10)-induced contractions. In summary, our results indicate that NK2Rs are linked exclusively to $G_{q/11}$ proteins in the UBSM and that the intracellular signaling involves the simultaneous activation of VDCC and the RhoA-Rho kinase pathway. These findings may help to identify potential therapeutic targets of bladder dysfunctions associated with the upregulation of TKs.

9.0 References

1. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. Life Sci. 2004;74(12):1445-63.
2. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev. 2014;94(1):265-301.
3. Maggi CA. The mammalian tachykinin receptors. Gen Pharmacol. 1995;26(5):911-44.
4. Chang MM, Leeman SE, Niall HD. Amino-acid sequence of substance P. Nat New Biol. 1971;232(29):86-7.
5. Kangawa K, Minamino N, Fukuda A, Matsuo H. Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. Biochem Biophys Res Commun. 1983;114(2):533-40.
6. Nawa H, Kotani H, Nakanishi S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. Nature. 1984;312(5996):729-34.
7. Erspamer V, Melchiorri P. Active polypeptides of the amphibian skin and their synthetic analogues. Pure and Applied Chemistry. 1973;35(4):463-94.
8. Nakanishi S. Substance P precursor and kininogen: their structures, gene organizations, and regulation. Physiol Rev. 1987;67(4):1117-42.
9. Holzer P. Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience. 1988;24(3):739-68.
10. Maggi CA, Meli A. The sensory-efferent function of capsaicin-sensitive sensory neurons. Gen Pharmacol. 1988;19(1):1-43.
11. Lundberg JM. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev. 1996;48(1):113-78.
12. Simon C, Portalier P, Chamoin MC, Ternaux JP. Substance P like-immunoreactivity release from enterochromaffin cells of rat caecum mucosa. Inhibition by serotonin and calcium-free medium. Neurochem Int. 1992;20(4):529-36.

13. Khan I, Collins SM. Fourth isoform of preprotachykinin messenger RNA encoding for substance P in the rat intestine. Biochem Biophys Res Commun. 1994;202(2):796-802.

14. Maghni K, Michoud MC, Alles M, Rubin A, Govindaraju V, Meloche C, et al. Airway smooth muscle cells express functional neurokinin-1 receptors and the nerve-derived preprotachykinin-a gene: regulation by passive sensitization. Am J Respir Cell Mol Biol. 2003;28(1):103-10.

15. Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martín JD, et al. Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol. 2003;139(3):523-32.

16. Pintado CO, Pinto FM, Pennefather JN, Hidalgo A, Baamonde A, Sanchez T, et al. A role for tachykinins in female mouse and rat reproductive function. Biol Reprod. 2003;69(3):940-6.

17. Minamino N, Masuda H, Kangawa K, Matsuo H. Regional distribution of neuromedin K and neuromedin L in rat brain and spinal cord. Biochem Biophys Res Commun. 1984;124(3):731-8.

18. Moussaoui SM, Le Prado N, Bonici B, Faucher DC, Cuiné F, Laduron PM, et al. Distribution of neurokinin B in rat spinal cord and peripheral tissues: comparison with neurokinin A and substance P and effects of neonatal capsaicin treatment. Neuroscience. 1992;48(4):969-78.

19. Goubillon ML, Forsdike RA, Robinson JE, Ciofi P, Caraty A, Herbison AE. Identification of neurokinin B-expressing neurons as a highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. Endocrinology. 2000;141(11):4218-25.

20. Kurtz MM, Wang R, Clements MK, Cascieri MA, Austin CP, Cunningham BR, et al. Identification, localization and receptor characterization of novel mammalian substance P-like peptides. Gene. 2002;296(1-2):205-12.

21. Zhang Y, Lu L, Furlonger C, Wu GE, Paige CJ. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. Nat Immunol. 2000;1(5):392-7.

22. Page NM, Bell NJ, Gardiner SM, Manyonda IT, Brayley KJ, Strange PG, et al. Characterization of the endokinins: human tachykinins with cardiovascular activity. Proc Natl Acad Sci U S A. 2003;100(10):6245-50.

23. Carter MS, Krause JE. Structure, expression, and some regulatory mechanisms of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K, and neuropeptide gamma. J Neurosci. 1990;10(7):2203-14.

24. Krause JE, Chirgwin JM, Carter MS, Xu ZS, Hershey AD. Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(3):881-5.

25. Takeda Y, Takeda J, Smart BM, Krause JE. Regional distribution of neuropeptide gamma and other tachykinin peptides derived from the substance P gene in the rat. Regul Pept. 1990;28(3):323-33.

26. Maggi CA. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. Regul Pept. 2000;93(1-3):53-64.

27. Kawaguchi Y, Hoshimaru M, Nawa H, Nakanishi S. Sequence analysis of cloned cDNA for rat substance P precursor: existence of a third substance P precursor. Biochem Biophys Res Commun. 1986;139(3):1040-6.

28. Harmar AJ, Hyde V, Chapman K. Identification and cDNA sequence of delta-preprotachykinin, a fourth splicing variant of the rat substance P precursor. FEBS Lett. 1990;275(1-2):22-4.

29. Holmgren S, Jensen J. Evolution of vertebrate neuropeptides. Brain Res Bull. 2001;55(6):723-35.

30. Tsuchida K, Shigemoto R, Yokota Y, Nakanishi S. Tissue distribution and quantitation of the mRNAs for three rat tachykinin receptors. Eur J Biochem. 1990;193(3):751-7.

31. Patacchini R, Maggi CA. Peripheral tachykinin receptors as targets for new drugs. Eur J Pharmacol. 2001;429(1-3):13-21.

32. Stewart-Lee A, Burnstock G. Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp6,L-Pro9]SP6-11 are potent agonists for endothelial neurokinin-1 receptors. Br J Pharmacol. 1989;97(4):1218-24.

33. Ho WZ, Lai JP, Zhu XH, Uvaydova M, Douglas SD. Human monocytes and macrophages express substance P and neurokinin-1 receptor. J Immunol. 1997;159(11):5654-60.

34. Lai JP, Douglas SD, Ho WZ. Human lymphocytes express substance P and its receptor. J Neuroimmunol. 1998;86(1):80-6.

35. Bandari PS, Qian J, Yehia G, Seegopaul HP, Harrison JS, Gascon P, et al. Differences in the expression of neurokinin receptor in neural and bone marrow mesenchymal cells: implications for neuronal expansion from bone marrow cells. Neuropeptides. 2002;36(1):13-21.

36. Piedimonte G, Bertrand C, Geppetti P, Snider RM, Desai MC, Nadel JA. A new NK1 receptor antagonist (CP-99,994) prevents the increase in tracheal vascular permeability produced by hypertonic saline. J Pharmacol Exp Ther. 1993;266(1):270-3.

37. Ishizuka O, Igawa Y, Lecci A, Maggi CA, Mattiasson A, Andersson KE. Role of intrathecal tachykinins for micturition in unanaesthetized rats with and without bladder outlet obstruction. Br J Pharmacol. 1994;113(1):111-6.

38. Maggi CA, Patacchini R, Santicioli P, Giuliani S. Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission. Br J Pharmacol. 1991;103(2):1535-41.

39. Meini S, Maggi CA. Evidence for a capsaicin-sensitive, tachykinin-mediated, component in the NANC contraction of the rat urinary bladder to nerve stimulation. Br J Pharmacol. 1994;112(4):1123-31.

40. Pennefather JN, Zeng XP, Gould D, Hall S, Burcher E. Mammalian tachykinins stimulate rat uterus by activating NK-2 receptors. Peptides. 1993;14(2):169-74.

41. Croci T, Aureggi G, Manara L, Emonds-Alt X, Le Fur G, Maffrand JP, et al. *In vitro* characterization of tachykinin NK2-receptors modulating motor responses of human colonic muscle strips. Br J Pharmacol. 1998;124(6):1321-7.

42. Naline E, Devillier P, Drapeau G, Toty L, Bakdach H, Regoli D, et al. Characterization of neurokinin effects and receptor selectivity in human isolated bronchi. Am Rev Respir Dis. 1989;140(3):679-86.

43. Saffroy M, Torrens Y, Glowinski J, Beaujouan JC. Presence of NK2 binding sites in the rat brain. J Neurochem. 2001;79(5):985-96.

44. Saffroy M, Torrens Y, Glowinski J, Beaujouan JC. Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. Neuroscience. 2003;116(3):761-73.

45. Poncelet M, Gueudet C, Emonds-Alt X, Brelière JC, Le Fur G, Soubrié P. Turning behavior induced in mice by a neurokinin A receptor agonist: stereoselective blockade by SR 48968, a non-peptide receptor antagonist. Neurosci Lett. 1993;149(1):40-2.
46. Weston-Bell N, Gj G, Gibbins J, Pj L, Page N. The activation of peripheral neurokinin receptors in the pathology of pre-eclampsia. Endocrine Abstracts. 2001
47. Fioramonti J, Gaultier E, Toulouse M, Sanger GJ, Bueno L. Intestinal anti-nociceptive behaviour of NK3 receptor antagonism in conscious rats: evidence to support a peripheral mechanism of action. Neurogastroenterol Motil. 2003;15(4):363-9.
48. Prague JK. Neurokinin 3 receptor antagonists - prime time? Climacteric. 2021;24(1):25-31.
49. Regoli D, Boudon A, Fauchére JL. Receptors and antagonists for substance P and related peptides. Pharmacol Rev. 1994;46(4):551-99.
50. Krause J, Blount, P, Sachais, BS. Molecular Biology of receptors. Structures, Expression and Regulatory Mechanisms. 1st ed. Buck S, editor. Totowa, NJ: Humana Press Inc.
51. Arms L, Vizzard MA. Neuropeptides in lower urinary tract function. Handb Exp Pharmacol. 2011(202):395-423.
52. Andersson KE. Bladder activation: afferent mechanisms. Urology. 2002;59(5 Suppl 1):43-50.
53. Maggi CA. The dual function of capsaicin-sensitive sensory nerves in the bladder and urethra. Ciba Found Symp. 1990;151:77-83; discussion-90.
54. Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nat Rev Neurosci. 2008;9(6):453-66.
55. Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nature Reviews Neuroscience. 2008;9(6):453-66.
56. Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol Rev. 2004;84(3):935-86.
57. Stoffel JT. Detrusor sphincter dyssynergia: a review of physiology, diagnosis, and treatment strategies. Transl Androl Urol. 2016;5(1):127-35.
58. Fonyó A. Principles of Medical Physiology. Budapest: Medicina Publishing House Co.; 2011. p. 301 p.

59. de Groat W C SWD. Autonomic regulation of the urinary bladder and sex organs. In: Loewy AD SK, (Eds), editor. Central Regulation of Autonomic Functions. 1st edn. ed. Oxford: Oxford University Press; 1990. p. 310–33.

60. Nadelhaft I, Vera PL, Card JP, Miselis RR. Central nervous system neurons labeled following the injection of pseudorabies virus into the rat urinary bladder. Neurosci Lett. 1992;143(1-2):271-4.

61. Kullmann FA, Artim DE, Birder LA, de Groat WC. Activation of muscarinic receptors in rat bladder sensory pathways alters reflex bladder activity. The Journal of neuroscience: the official journal of the Society for Neuroscience. 2008;28(8):1977-87.

62. Andersson KE, Wein AJ. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. Pharmacol Rev. 2004;56(4):581-631.

63. de Groat WC, Yoshimura N. Afferent nerve regulation of bladder function in health and disease. Handbook of experimental pharmacology. 2009(194):91-138.

64. Smet PJ, Moore KH, Jonavicius J. Distribution and colocalization of calcitonin gene-related peptide, tachykinins, and vasoactive intestinal peptide in normal and idiopathic unstable human urinary bladder. Lab Invest. 1997;77(1):37-49.

65. Apostolidis A, Popat R, Yiangou Y, Cockayne D, Ford AP, Davis JB, et al. Decreased sensory receptors P_2X_3 and TRPV1 in suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. J Urol. 2005;174(3):977-82; discussion 82-3.

66. Grundy L, Chess-Williams R, Brierley SM, Mills K, Moore KH, Mansfield K, et al. NKA enhances bladder-afferent mechanosensitivity via urothelial and detrusor activation. Am J Physiol Renal Physiol. 2018;315(4):F1174-f85.

67. Gu BJ, Ishizuka O, Igawa Y, Nishizawa O, Andersson KE. Role of supraspinal tachykinins for micturition in conscious rats with and without bladder outlet obstruction. Naunyn Schmiedebergs Arch Pharmacol. 2000;361(5):543-8.

68. Abelli L, Conte B, Somma V, Maggi CA, Giuliani S, Geppetti P, et al. The contribution of capsaicin-sensitive sensory nerves to xylene-induced visceral pain in conscious, freely moving rats. Naunyn Schmiedebergs Arch Pharmacol. 1988;337(5):545-51.

69. Maggi CA. Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. Prog Neurobiol. 1995;45(1):1-98.

70. Maggi CA, Geppetti P, Santicioli P, Frilli S, Giuliani S, Furio M, et al. Tachykinin-like immunoreactivity in the mammalian urinary bladder: correlation with the functions of the capsaicin-sensitive sensory nerves. Neuroscience. 1988;26(1):233-42.

71. Burcher E, Zeng XP, Strigas J, Shang F, Millard RJ, Moore KH. Autoradiographic localization of tachykinin and calcitonin gene-related peptide receptors in adult urinary bladder. J Urol. 2000;163(1):331-7.

72. Zeng XP, Moore KH, Burcher E. Characterization of tachykinin NK2 receptors in human urinary bladder. J Urol. 1995;153(5):1688-92.

73. Ishizuka O, Mattiasson A, Andersson KE. Tachykinin effects on bladder activity in conscious normal rats. J Urol. 1995;154(1):257-61.

74. Lecci A, Giuliani S, Patacchini R, Maggi CA. Evidence against a peripheral role of tachykinins in the initiation of micturition reflex in rats. J Pharmacol Exp Ther. 1993;264(3):1327-32.

75. Lecci A, Giuliani S, Santicioli P, Maggi CA. Involvement of spinal tachykinin NK1 and NK2 receptors in detrusor hyperreflexia during chemical cystitis in anaesthetized rats. European Journal of Pharmacology. 1994;259(2):129-35.

76. Lecci A, Giuliani S, Santicioli P, Maggi CA. Involvement of spinal tachykinin NK1 and NK2 receptors in detrusor hyperreflexia during chemical cystitis in anaesthetized rats. Eur J Pharmacol. 1994;259(2):129-35.

77. Maggi CA, Giuliani S, Ballati L, Lecci A, Manzini S, Patacchini R, et al. In vivo evidence for tachykininergic transmission using a new NK-2 receptor-selective antagonist, MEN 10,376. J Pharmacol Exp Ther. 1991;257(3):1172-8.

78. Callsen-Cencic P, Mense S. Expression of neuropeptides and nitric oxide synthase in neurons innervating the inflamed rat urinary bladder. J Auton Nerv Syst. 1997;65(1):33-44.

79. Overactive Bladder and Its Treatments Consensus Conference. London, United Kingdom, July 4, 1999. Urology. 2000;55(5A Suppl):1-84.

80. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther. 2004;103(2):147-66.
81. Rahnama'i MS, van Kerrebroeck PEV, de Wachter SG, van Koeveringe GA. The role of prostanoids in urinary bladder physiology. Nature Reviews Urology. 2012;9(5):283-90.
82. Gilmore NJ, Vane JR. Hormones released into the circulation when the urinary bladder of the anaesthetized dog is distended. Clin Sci. 1971;41(1):69-83.
83. Downie JW, Karmazyn M. Mechanical trauma to bladder epithelium liberates prostanoids which modulate neurotransmission in rabbit detrusor muscle. J Pharmacol Exp Ther. 1984;230(2):445-9.
84. Khalaf IM, Ghoneim MA, Elhilali MM. The effect of exogenous prostaglandins F2 alpha and E2 and indomethacin on micturition. Br J Urol. 1981;53(1):21-8.
85. Abrams PH, Sykes JA, Rose AJ, Rogers AF. The synthesis and release of prostaglandins by human urinary bladder muscle in vitro. Invest Urol. 1979;16(5):346-8.
86. Kim JC, Park EY, Seo SI, Park YH, Hwang TK. Nerve growth factor and prostaglandins in the urine of female patients with overactive bladder. J Urol. 2006;175(5):1773-6; discussion 6.
87. Liu HT, Tyagi P, Chancellor MB, Kuo HC. Urinary nerve growth factor but not prostaglandin E2 increases in patients with interstitial cystitis/bladder pain syndrome and detrusor overactivity. BJU Int. 2010;106(11):1681-5.
88. Poljakovic M, Svensson ML, Svanborg C, Johansson K, Larsson B, Persson K. Escherichia coli-induced inducible nitric oxide synthase and cyclooxygenase expression in the mouse bladder and kidney. Kidney Int. 2001;59(3):893-904.
89. Wheeler MA, Yoon JH, Olsson LE, Weiss RM. Cyclooxygenase-2 protein and prostaglandin $E_{(2)}$ production are up-regulated in a rat bladder inflammation model. Eur J Pharmacol. 2001;417(3):239-48.
90. Lecci A, Birder LA, Meini S, Catalioto RM, Tramontana M, Giuliani S, et al. Pharmacological evaluation of the role of cyclooxygenase isoenzymes on the micturition reflex following experimental cystitis in rats. Br J Pharmacol. 2000;130(2):331-8.

91. Lee T, Hedlund P, Newgreen D, Andersson K-E. Urodynamic Effects of a Novel EP(1) Receptor Antagonist in Normal Rats and Rats With Bladder Outlet Obstruction. Journal of Urology. 2007;177(4):1562-7.
92. Jugus MJ, Jaworski JP, Patra PB, Jin J, Morrow DM, Laping NJ, et al. Dual modulation of urinary bladder activity and urine flow by prostanoid EP3 receptors in the conscious rat. Br J Pharmacol. 2009;158(1):372-81.
93. Chuang YC, Yoshimura N, Huang CC, Wu M, Tyagi P, Chancellor MB. Expression of E-series prostaglandin (EP) receptors and urodynamic effects of an EP4 receptor antagonist on cyclophosphamide-induced overactive bladder in rats. BJU Int. 2010;106(11):1782-7.
94. Khanna OP, Barbieri EJ, McMichael R. Effects of prostaglandins on vesicourethral smooth muscle of rabbit. Therapeutic implications. Urology. 1978;12(6):674-81.
95. Webb RC. Smooth muscle contraction and relaxation. Advances in Physiology Education. 2003;27(4):201-6.
96. Nitti VW. Clinical impact of overactive bladder. Rev Urol. 2002;4 Suppl 4(Suppl 4):S2-6.
97. Abrams P WA. Introduction: overactive bladder and its treatments. BJU Int. 2000;85:1-2.
98. Milsom I, Abrams P, Cardozo L, Roberts RG, Thüroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. BJU Int. 2001;87(9):760-6.
99. Abrams P WA. Overactive bladder. Urology. 1998;51(8).
100. Abrams PWAJ. The overactive bladder: a widespread but treatable condition. Stockholm, Sweden: Erik Sparre Medical AB; 1998.
101. Jackson S. The patient with an overactive bladder-symptoms and quality-of-life issues. Urology. 1997;50(6A Suppl):18-22; discussion 3-4.
102. Ohmura M, Kondo A, Saito M. Response of isolated human neurogenic bladders to tachykinins. Urol Int. 1997;59(4):221-5.
103. Marson L, Thor KB, Katofiasc M, Burgard EC, Rupniak NMJ. Prokinetic effects of neurokinin-2 receptor agonists on the bladder and rectum of rats with acute spinal cord transection. Eur J Pharmacol. 2018;819:261-9.

104. Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, et al. Prevalence and burden of overactive bladder in the United States. World J Urol. 2003;20(6):327-36.

105. Abrams P, Kelleher CJ, Kerr LA, Rogers RG. Overactive bladder significantly affects quality of life. Am J Manag Care. 2000;6(11 Suppl):S580-90.

106. Lightner DJ, Gomelsky A, Souter L, Vasavada SP. Diagnosis and Treatment of Overactive Bladder (Non-Neurogenic) in Adults: AUA/SUFU Guideline Amendment 2019. J Urol. 2019;202(3):558-63.

107. Offermanns S, Zhao LP, Gohla A, Sarosi I, Simon MI, Wilkie TM. Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. Embo j. 1998;17(15):4304-12.

108. Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, et al. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. Nucleic Acids Res. 1999;27(22):4324-7.

109. Wirth A, Benyó Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. Nat Med. 2008;14(1):64-8.

110. Moers A, Nieswandt B, Massberg S, Wettschureck N, Grüner S, Konrad I, et al. G13 is an essential mediator of platelet activation in hemostasis and thrombosis. Nat Med. 2003;9(11):1418-22.

111. Zagorodnyuk V, Santicioli P, Maggi CA. Tachykinin NK1 but not NK2 receptors mediate non-cholinergic excitatory junction potentials in the circular muscle of guinea-pig colon. Br J Pharmacol. 1993;110(2):795-803.

112. Frazier EP, Braverman AS, Peters SLM, Michel MC, Ruggieri MR. Does Phospholipase C Mediate Muscarinic Receptor-Induced Rat Urinary Bladder Contraction? Journal of Pharmacology and Experimental Therapeutics. 2007;322(3):998.

113. MacMillan D, Kennedy C, McCarron JG. ATP inhibits $Ins_{(1,4,5)}P_3$-evoked Ca^{2+} release in smooth muscle via P_2Y_1 receptors. J Cell Sci. 2012;125(Pt 21):5151-8.

114. Takahashi N, Aizawa H, Fukuyama S, Inoue H, Nishima S, Hara N. Thapsigargin, a Ca^{2+}-ATPase inhibitor, relaxes guinea pig tracheal smooth muscle by producing

epithelium-dependent relaxing factors. European Journal of Pharmacology. 2000;410(1):61-8.

115. Schneider T, Fetscher C, Krege S, Michel MC. Signal Transduction Underlying Carbachol-Induced Contraction of Human Urinary Bladder. Journal of Pharmacology and Experimental Therapeutics. 2004;309(3):1148.

116. Nausch B, Heppner TJ, Nelson MT. Nerve-released acetylcholine contracts urinary bladder smooth muscle by inducing action potentials independently of IP_3-mediated calcium release. Am J Physiol Regul Integr Comp Physiol. 2010;299(3):R878-88.

117. Stromberga Z, Chess-Williams R, Moro C. Prostaglandin E_2 and F_{2alpha} Modulate Urinary Bladder Urothelium, Lamina Propria and Detrusor Contractility via the FP Receptor. Frontiers in Physiology. 2020;11.

118. Offermanns S, Laugwitz KL, Spicher K, Schultz G. G proteins of the G12 family are activated via thromboxane A_2 and thrombin receptors in human platelets. Proceedings of the National Academy of Sciences. 1994;91(2):504-8.

119. Brading AF, Brain KL. Ion Channel Modulators and Urinary Tract Function. In: Andersson K-E, Michel MC, editors. Urinary Tract. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 375-93.

120. Anjum I. Calcium sensitization mechanisms in detrusor smooth muscles. J Basic Clin Physiol Pharmacol. 2018;29(3):227-35.

121. Grundy L, Chess-Williams R, Brierley S, Mills K, Moore K, Mansfield K, et al. NKA enhances bladder afferent mechanosensitivity via urothelial and detrusor activation. American Journal of Physiology-Renal Physiology. 2018;315.

122. Tramontana M, Catalioto RM, Lecci A, Maggi CA. Role of prostanoids in the contraction induced by a tachykinin NK2 receptor agonist in the hamster urinary bladder. Naunyn Schmiedebergs Arch Pharmacol. 2000;361(4):452-9.

123. Maggi CA, Santicioli P, Patacchini R, Cellerini M, Turini D, Barbanti G, et al. Contractile response of the human isolated urinary bladder to neurokinins: involvement of NK-2 receptors. Eur J Pharmacol. 1988;145(3):335-40.

124. Lecci A, Giuliani S, Meini S, Maggi CA. In vivo effects of neurokinin B on rat urinary bladder motility: involvement of tachykinin NK1 and NK2 receptors. Neuropeptides. 1994;27(1):53-62.

125. Alexander SP, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA, et al. The Concise Guide to Pharmacology 2017/18: G protein-coupled receptors. Br J Pharmacol. 2017;174 Suppl 1(Suppl Suppl 1):S17-s129.

126. Palanche T, Ilien B, Zoffmann S, Reck MP, Bucher B, Edelstein SJ, et al. The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. J Biol Chem. 2001;276(37):34853-61.

127. Exton JH. Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. Annu Rev Pharmacol Toxicol. 1996;36:481-509.

128. Catalioto RM, Cucchi P, Renzetti AR, Criscuoli M, Maggi CA. Independent coupling of the human tachykinin NK2 receptor to phospholipases C and A_2 in transfected Chinese hamster ovary cells. Naunyn Schmiedebergs Arch Pharmacol. 1998;358(4):395-403.

129. Frazier EP, Braverman AS, Peters SL, Michel MC, Ruggieri MR, Sr. Does phospholipase C mediate muscarinic receptor-induced rat urinary bladder contraction? J Pharmacol Exp Ther. 2007;322(3):998-1002.

130. Frei E, Hofmann F, Wegener JW. Phospholipase C mediated Ca^{2+} signals in murine urinary bladder smooth muscle. Eur J Pharmacol. 2009;610(1-3):106-9.

131. Quinn T, Collins C, Baird AW. Mechanisms of neurokinin A- and substance P-induced contractions in rat detrusor smooth muscle in vitro. BJU International. 2004;94(4):651-7.

132. Grundy L, Chess-Williams R, Brierley SM, Mills K, Moore KH, Mansfield K, et al. NKA enhances bladder-afferent mechanosensitivity via urothelial and detrusor activation. American Journal of Physiology-Renal Physiology. 2018;315(4):F1174-F85.

133. Wegener JW, Schulla V, Lee T-S, Koller A, Feil S, Feil R, et al. An essential role of Cav1.2 L-type calcium channel for urinary bladder function. The FASEB Journal. 2004;18(10):1159-61.

134. Faustini S, Salvini A, Pizzi P, Conti M, Magistretti MJ, Vescovini R. Experimental study on the action of diltiazem on detrusor muscle and clinical evaluation in patients with detrusor hyperactivity. Arzneimittelforschung. 1989;39(8):899-903.

135. Donkó A, Ruisanchez E, Orient A, Enyedi B, Kapui R, Péterfi Z, et al. Urothelial cells produce hydrogen peroxide through the activation of Duox1. Free Radic Biol Med. 2010;49(12):2040-8.

136. Andersson KE. TRP Channels as Lower Urinary Tract Sensory Targets. Med Sci (Basel). 2019;7(5).

137. Franken J, Uvin P, De Ridder D, Voets T. TRP channels in lower urinary tract dysfunction. Br J Pharmacol. 2014;171(10):2537-51.

138. Thorneloe KS, Sulpizio AC, Lin Z, Figueroa DJ, Clouse AK, McCafferty GP, et al. N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I. J Pharmacol Exp Ther. 2008;326(2):432-42.

139. Harraz OF, Longden TA, Hill-Eubanks D, Nelson MT. $PIP_{(2)}$ depletion promotes TRPV4 channel activity in mouse brain capillary endothelial cells. Elife. 2018;7.

140. Hong K, Cope EL, DeLalio LJ, Marziano C, Isakson BE, Sonkusare SK. TRPV4 (Transient Receptor Potential Vanilloid 4) Channel-Dependent Negative Feedback Mechanism Regulates $G_{(q)}$ Protein-Coupled Receptor-Induced Vasoconstriction. Arterioscler Thromb Vasc Biol. 2018;38(3):542-54.

141. Frei E, Hofmann F, Wegener JW. Phospholipase C mediated Ca^{2+} signals in murine urinary bladder smooth muscle. European Journal of Pharmacology. 2009;610(1):106-9.

142. Huster M, Frei E, Hofmann F, Wegener JW. A complex of CaV1.2/PKC is involved in muscarinic signaling in smooth muscle. The FASEB Journal. 2010;24(8):2651-9.

143. Wibberley A, Chen Z, Hu E, Hieble JP, Westfall TD. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. Br J Pharmacol. 2003;138(5):757-66.

144. Bing W, Chang S, Hypolite JA, DiSanto ME, Zderic SA, Rolf L, et al. Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho kinase. Am J Physiol Renal Physiol. 2003;285(5):F990-7.

145. Christ GJ, Andersson KE. Rho-kinase and effects of Rho-kinase inhibition on the lower urinary tract. Neurourol Urodyn. 2007;26(6 Suppl):948-54.

146. Peters SL, Schmidt M, Michel MC. Rho kinase: a target for treating urinary bladder dysfunction? Trends Pharmacol Sci. 2006;27(9):492-7.

147. Wibberley A, Chen Z, Hu E, Hieble JP, Westfall TD. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. British Journal of Pharmacology. 2003;138(5):757-66.

148. Momotani K, Somlyo AV. p63RhoGEF: a new switch for $G_{(q)}$-mediated activation of smooth muscle. Trends Cardiovasc Med. 2012;22(5):122-7.

149. Teixeira CE, Jin L, Priviero FB, Ying Z, Webb RC. Comparative pharmacological analysis of Rho-kinase inhibitors and identification of molecular components of Ca^{2+} sensitization in the rat lower urinary tract. Biochem Pharmacol. 2007;74(4):647-58.

150. Andersson KE, Ek A, Persson CG. Effects of prostaglandins on the isolated human bladder and urethra. Acta Physiol Scand. 1977;100(2):165-71.

10.0 Bibliography of the candidate's publications

Publications related to the dissertation

Dér B, Molnár PJ, Ruisanchez É, Őrsy P, Kerék M, Faragó B, Nyírády P, Offermanns S, Benyó Z. (2019)
NK2 Receptor-Mediated Detrusor Muscle Contraction Involves $G_{q/11}$-Dependent Activation of Voltage-Dependent Ca^{2+} Channels and the RhoA-Rho Kinase Pathway. American Journal of Physiology - Renal Physiology, 317(5), 1154-1163. **IF = 3.191**

Molnár PJ, **Dér B**, Borsodi K, Balla H, Borbás Z, Molnár K, Ruisanchez É, Kenessey I, Horváth A, Keszthelyi A, Majoros A, Nyírády P, Offermanns S, Benyó Z. (2021) Isoprostanes Evoke Contraction of the Murine and Human Detrusor Muscle via Activation of the Thromboxane Prostanoid TP Receptor and Rho Kinase. American Journal of Physiology - Renal Physiology, 320(4), 537-547. **IF = 4.097**

Publications not related to the dissertation

Gyarmati G, Kadoya H, Moon J, Burford JL, Ahmadi N., Gill IS, Hong Y, **Dér B**, Peti-Peterdi J. (2018)
Advances in Renal Cell Imaging. Seminars in Nephrology, 38(1), 52-62. **IF = 3.629**

Tran T, Lindstrom NO, Ransick A, Brandine GDS, Guo Q, Kim AD, **Dér B**, Peti-Peterdi J, Smith AD, Thornton M, Grubbs B, McMahon JA, McMahon, AP. (2019)
In Vivo Developmental Trajectories of Human Podocyte Inform *In Vitro* Differentiation of Pluripotent Stem Cell-Derived Podocytes. Developmental Cell, 50(1), 102-116. **IF = 10.092**

Dér B, Sanford D, Hakim R, Vanstrum E, Nguyen JH, Hung AJ. (2021)
Efficiency and Accuracy of Robotic Surgical Performance Decayed Among Urologists During COVID-19 Shutdown. Journal of Endourology, 35(6), 888-890. **IF = 2.619**

Desposito D, Schiessl IM, Gyarmati G, Riquier-Brison A, Izuhara AK, Kadoya H, **Dér B**, Shroff UN, Hong Y, Peti-Peterdi J. (2021)
Serial Intravital Imaging Captures Dynamic and Functional Endothelial Remodeling with Single-Cell Resolution. JCI Insight, 6(10), e123392. **IF = 9.496**

Sanford DI, **Dér B**, Haquee, TF, Ma R, Hakim R, Nguyen JH, Cen S, Hung AJ. (2021) Technical Skill Impacts the Success of Sequential Robotic Suturing Sub-Steps. Journal of Endourology, 36(2), 273-278. **IF = 2.619**

Tran T, Song CJ, Nguyen T, Cheng S, McMahon JA, Yang R, Guo Q, **Dér B**, Lindstrom NO, Lin DC, McMahon, AP. (2022)
A scalable organoid model of human autosomal dominant polycystic kidney disease for disease mechanism and drug discovery. Cell Stem Cell, 29(7), 1083-1101. **IF = 25.269** (from the year 2021)

11.0 Acknowledgments

I would like to acknowledge my mentor, professor and friend, Dr. Zoltán Benyó for supporting me all along from the very beginning of my medical and scientific training. He wisely pointed out the directions to proceed, let me make my own experimental mistakes, and created an environment where reproducible and rigorous data collection and interpretation are highly expected.

I am grateful to Dr. Bernadett Faragó and Zoltánné Nagy for teaching me new methodology and their data collection. I would like to acknowledge László Hricisák, Ágnes Fülöp, and Ildikó Murányi for their contribution to the experimental work. Dr. András Kucsa made the graphic design of the schematic figures. Last, but not least, I am expressing my gratitude to Dr. Péter Molnár, my friend and research fellow, who has been working with me in the field of urology research in the laboratory.

The scientific research would not have been possible without the unconditional support of my family, including my parents, sister, and the broader family as well. I would like to say a special thank you to my girlfriend, Momo for creating an inspiring environment for thesis writing.

This work was funded by the Hungarian National Research, Development and Innovation Office (Grants OTKA H07-B-74286, K-112964, K-125174, and NVKP_16-1-2016-0042) as well as by the Higher Education Institutional Excellence Program of the Ministry of Human Capacities in Hungary, within the framework of the Molecular Biology thematic program of the Semmelweis University and supported by Grant EFOP-3.6.3-VEKOP-16-2017-00009.

No conflicts of interest, financial or otherwise, are declared hereby.