SIGNALING PATHWAYS OF BRADYKININ-INDUCED CONTRACTIONS IN MURINE AND HUMAN DETRUSOR MUSCLE

PhD Thesis

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Table of Contents

List of Abbreviations:

α,β-meATP: α,β-methyleneadenosine 5’-triphosphate

AA: arachidonic acid

ACE: angiotensin-converting enzyme

ACh: acetylcholine

ATP: adenosine triphosphate

BK: bradykinin

CCh: carbachol

COX: cyclooxygenase

Cre-ERT2: Cre-recombinase with a modified estrogen receptor-binding domain

CRP: C-reactive protein

CTRL: control

DAG: diacylglycerol

DMSO: dimethyl sulfoxide

DO: detrusor overactivity

GPCR: G protein-coupled receptor

HBSS: Hank’s Balanced Salt Solution

HK: high-molecular-weight kininogen

IBS: irritable bowel syndrome

IP_3: inositol triphosphate

KO: knockout

LK: low-molecular-weight kininogen

LUT**:** lower urinary tract

MLC_{20}: regulatory myosin light chain

MLCK: myosin light-chain kinase

MLCP: myosin light-chain phosphatase

NA: noradrenaline

NGF: nerve growth factor

NO: nitric oxide

OAB: overactive bladder

PAG: periaqueductal gray

PG: prostaglandin

PGD_2: prostaglandin D_2

PGE_2: prostaglandin E_2

PGH_2: prostaglandin H_2

PLA_2: phospholipase A_2

PLC-ß: Phospholipase C-β

PMC: pontine micturition center

PPADS: pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate

ROCK: Rho-associated protein kinase, Rho-kinase

SEM: standard error of the mean

SMMHC: smooth muscle myosin heavy chain

TP: thromboxane-prostanoid receptor

TRP: transient receptor potential

TX: thromboxane

UBSM: urinary bladder smooth muscle

WT: wild-type

1. Introduction

The urinary bladder has two main physiological functions: accommodation to the urine produced continuously by the kidneys with minimal intravesical pressure increase, and to release it in a voluntarily controlled manner. Normally, one is not particularly aware of the filling of the bladder until the sensation of urinary urge appears, thanks to the large compliance of the bladder wall. The alternation of the storing and voiding phases is the result of a well-organized collaboration of autonomic and somatic neurons and the higher centers of the nervous system. The bladder is exposed to several stressors including mechanical (strain) and chemical stimuli (metabolites, inflammatory mediators, cytokines) as well as pathogens. Thus, the fine regulatory system of the bladder functions is vulnerable. Disruption of this network at any level of the hierarchy will harm bladder functions resulting in voiding disorders such as incomplete voiding, incontinence, or bladder overactivity causing frequent, often painful urge to urinate.

It is still unclear, whether the disorder originates from dysfunction of the nervous control, the bladder smooth muscle, or from the mucosa layer. Nevertheless, plenty of hypotheses have been established on the pathogenesis of bladder dysfunctions, most of them focusing on a specific aspect of the regulatory orchestra. Intriguingly, it has been implied lately that overactive bladder syndrome (OAB) and detrusor overactivity (DO) are linked to systemic inflammatory disorders. Accordingly, it is also hypothesized that inflammatory mediators, such as prostaglandins (PGs), isoprostanes, tachykinins and bradykinin (BK) have an important role in over-excitation of the bladder smooth muscle. As our knowledge is scarce in this topic and thus, pharmacological therapies are suboptimal, there is a pressing need for conducting research in this field. Therefore, our experiments aimed to investigate the potential role of inflammatory mediators in bladder smooth muscle contractions and to identify novel, potentially more specific therapeutic targets for treating bladder dysfunctions.

1.1. Brief anatomy of the urinary tract

The urinary system consists of two major parts: the upper and lower urinary tracts (LUT). The upper urinary tract includes the kidneys and ureters. The urine continuously produced by the kidneys is passed in boluses towards the bladder by the ureters' peristaltic movements. The ureters enter obliquely to the posterior part of the bladder and form the vesicoureteral orifices (ureterovesical junctions). The orifices are normally closed due to the oblique intersection and open only when ureter peristalsis increases pressure in the ureters (1).

The LUT consists of the urinary bladder, the urethra, and in males, the prostate gland as well *(Figure 1)*. The bladder, which is of the size and shape of the lemon, is a hollow organ located in the pelvis. Its position is stabilized by ligaments around the bladder neck. There are two major parts of the bladder that differ in their primary functions. The bladder body is above the vesicoureteral orifices and relaxes or contracts if needed. In contrast, the bladder base, which consists of the trigone (a triangle-shaped area marked by the two ureterovesicular junctions and the urethral opening) and the bladder neck, has a more passive, funneling role (2, 3). At the junction of the bladder neck and the urethra, there is a smooth muscle ring: the internal urethral sphincter, which is more developed in men and contracts gradually as the bladder fills to prevent urine dripping. The external urethral sphincter is composed of skeletal muscle, it is located at the end of the urethra in women, whereas in men, it is below the prostate. An important difference between the two sphincters is that the external sphincter can be controlled voluntarily, in contrast to the internal sphincter which is regulated by the autonomic nervous system (4).

1.2. Structure of the bladder wall

The urothelium is a unique epithelial lining, forming the innermost layer of the renal pelvis, ureters, urinary bladder, and the proximal urethra. It adapts to the distension of the bladder by reorganizing its layers, thus it is also known as transitional epithelium. It has a dual role, as it acts as a barrier but also takes part in signaling, conveying information on bladder volume, stretching, irritants to afferent nerves and smooth muscle cells (5, 6).

A basal membrane layer separates the urothelium from the lamina propria, which is also referred to as the suburothelium. This layer includes an extracellular matrix with elastic fibers, various cell types (including fibroblasts, myofibroblasts, smooth muscle cells, adipocytes, interstitial cells), nerve endings, and it is also rich in blood and lymph vessels (7). The muscular layer or detrusor muscle lays under the lamina propria and consists of interlacing, randomly oriented smooth muscle fibers in three distinguishable smooth muscle layers (outer longitudinal, middle circular, inner longitudinal). Around the bladder neck, the smooth muscle forms the internal sphincter (2, 8). The outermost layer of the bladder is covered by a serous membrane (tunica serosa), a thin connective tissue layer.

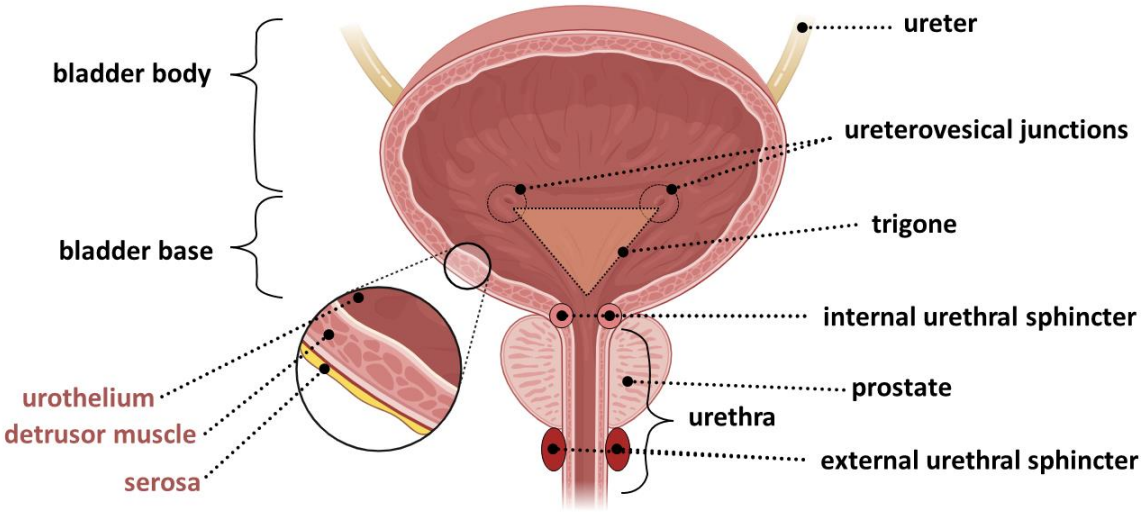

Figure 1. The structure of the lower urinary tract *(created with BioRender.com)*

1.3. Physiology and regulation of the lower urinary tract

1.3.1. Overview of the innervation of the lower urinary tract

A complex neural system involving neurons in the brain, spinal cord, and peripheral ganglia regulates the functions of urinary bladder, urethra, and the inner and outer sphincters. Information on bladder volume is conveyed to the central nervous system by Aδ- and C-type afferent nerve fibers. The LUT is innervated by both somatic and autonomic (sympathetic and parasympathetic) motoneurons. Parasympathetic efferents induce bladder contraction via acetylcholine (ACh)-mediated M_2 and M_3 receptor activation, whereas sympathetic efferent activation relaxes bladder smooth muscle through noradrenaline (NA) release, which binds to $ß_2$ and $ß_3$ adrenergic receptors. The external urethral sphincter is regulated by somatic motoneurons (9).

1.3.2. Neural regulation of micturition

During the filling phase, the bladder acts as a reservoir for the continuously produced urine. The average bladder capacity is about 500-600 ml at which the urine is stored at relatively low intravesical pressures. As the bladder volume increases, it expands relatively easily due to its viscoelastic properties, and in addition to this the increasing sympathetic and the simultaneously decreasing parasympathetic efferent nerve activity leads to a graded relaxation of the smooth muscle. Thus, the bladder has a large compliance and, under physiological conditions, there is no significant increase in the intravesical pressure up to 300-400 ml bladder volumes (10). As the bladder fills, the sympathetic nerves are activated leading to relaxation of the detrusor muscle, and the internal urethra sphincter contracts simultaneously to prevent urine leakage. Sympathetic efferent activation also leads to the inhibition of parasympathetic neurotransmission via presynaptic α_2 receptors, which also contributes to the relaxation and the high compliance of the bladder. During the storage phase, activation of the somatic (pudendal) motoneurons leads to contraction of the external urethral sphincter via nicotinic ACh receptor activation (9).

Normally, voiding is initiated centrally by the pontine micturition center (PMC), which is under the direct control of the periaqueductal gray matter (PAG). PMC is considered a hub that integrates information from various sources, as it receives projections from higher brain centers (prefrontal cortex, limbic system) and afferents from the periphery also convey information to the PAG about bladder stretching (11). Altogether, PAG determines whether voiding should be initiated or not. Once the PMC receives the "allowing signal", it activates descending pathways for initiating micturition (9).

1.3.3. The sensory role of the urothelium in bladder function

For a long time, the urothelium was considered merely a passive barrier. However, in the last two decades, it has become clear that it has sensory properties as well. This is proven partly by that the bladder urothelium expresses a diverse set of receptors that are usually associated with sensory neurons, such as BK receptors, purinergic receptors (P2X, P2Y), adrenergic (α, β) and cholinergic (muscarinic and nicotinic) receptors, PG receptors (EP, FP) and transient receptor potential (TRP) channels (TRPV1, TRPV2, TRPV4, TRPM8, TRPA1) (3). TRP channels are non-specific cationic ion channels and a number of them have been associated with bladder dysfunctions (cystitis, OAB) facilitating further studies on their signaling pathways (12). Another important family of cell membrane proteins expressed in the urothelium are Piezo channels. Both Piezo1 and Piezo2 channels have gained attention recently in the regulation of bladder function, as they have an important role in mechanosensation and also contribute to the release of inflammatory cytokines (13, 14).

Besides expressing numerous receptors, another proof of the active sensory role of urothelium is that it can secrete several signaling molecules including ATP, ACh, PGs, BK, nitric oxide (NO), cytokines conveying information to the underlying nerve terminals, smooth muscle, interstitial and various other cells about bladder stretching, irritation, inflammation (15). Among these mediators, ATP is considered one of the major messengers exerting its effects via binding to P2X or P2Y receptors, indicating bladder fullness and pain and it also excites interstitial cells and nerve terminals leading to detrusor muscle contraction (16).

1.3.4. Regulation of bladder function on the cellular level

Morphologically, the detrusor muscle cells are typical, spindle-shaped smooth muscle cells similar to those of other hollow organs (intestine, stomach). Smooth muscles are often classified as either single- or multi-unit, based on the level of interconnections between the cells. Single-unit smooth muscles are usually arranged into sheets or bundles and are coupled via gap-junctions, whereas multi-unit smooth muscles tend to form discrete fibers operating independently of each other and are densely innervated. The detrusor muscle has characteristics of both smooth muscle types. Effective cell-to-cell communication is essential for expelling urine properly, thus gap-junctions are present in the tissue, though their number is lower compared to classical single-unit smooth muscle tissues (17). The detrusor muscle is also densely innervated and depends on nervous control for initiating contraction, therefore, it shares some attributes of multi-unit smooth muscles as well (18). Intriguingly, this is a unique characteristic of the bladder, as efficient voiding contractions require densely interconnected smooth muscle cells to prevent residual urine. However, bladder contractions occur not only during voiding but in the filling phase as well. The purpose of these micro-contractions and how they are initiated are still under research but it is already known that they are localized to small parts (modules) of the bladder wall (19). These contractions assumingly take part in adjusting the muscle fibers and sensing bladder volume more accurately. Thus, perhaps, this modular aspect of bladder function can explain partly why the detrusor smooth muscle tissue shows features resembling to multi-unit tissues as well.

Regarding the intracellular mechanism, the general principles of smooth muscle contraction apply to the bladder smooth muscle. The contraction is usually initiated by an increase in the intracellular Ca^{2+} level either by Ca^{2+} entry via cell membrane ion channels or from the sarcoplasmic reticulum. Then, Ca^{2+} ions bind to calmodulin forming a complex, which activates the myosin light-chain kinase (MLCK). MLCK, in turn, phosphorylates the regulatory myosin light chain (MLC_{20}) leading to cross-bridge coupling between myosin and actin, and finally to smooth muscle contraction. MLC_{20} phosphorylation is also regulated by myosin light-chain phosphatase (MLCP), which dephosphorylates and inactivates MLC_{20} promoting relaxation of the smooth muscle (18).

There are two major G protein-coupled receptor (GPCR)-coupled pathways that lead to smooth muscle contraction and they can be activated in the bladder as well (18, 20):

1. **$G\alpha_{q/11}$-coupled** receptors stimulate phospholipase C-ß (PLC-ß) activity resulting in the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binding to its receptors on the SR is the stimulus for intracellular Ca^{2+} release, which in turn may induce the opening of store-operated Ca^{2+} channels of the plasma membrane resulting in Ca^{2+}-influx and finally, activation of the MLCK, thus MLC_{20} phosphorylation and contraction.

2. **$G\alpha_{12/13}$-coupled** receptor signaling involves activation of the small G protein RhoA and consequently, Rho-associated protein kinase (ROCK), which inactivates MLCP, leading to a sustained contraction. This pathway is often referred to as the Ca^{2+}-sensitizing pathway, as an increase in intracellular Ca^{2+} concentration is not needed for the contraction (21). It has to be noted that the ROCK enzyme has gained attention recently in disorders associated with LUT smooth muscle contractility (22, 23). In addition, ROCK activity may be enhanced under pathological conditions, for instance, in cystitis (24) and during aging (25), highlighting its possible role in bladder dysfunctions.

1.4. Overactivity of the detrusor muscle

As previously mentioned, storing and expelling urine requires a well-regulated cooperation between afferent and efferent nerves, cells of the mucosa layer, and the detrusor smooth muscle. Alteration at any level may lead to disruption of bladder function resulting in improper voiding or storing, such as incontinence, underactive bladder, DO and OAB syndrome. According to several studies conducted in Europe and in the US, the prevalence of OAB in the population aged 18 and over is about 16% without any significant sex differences. However, in the population aged 40-59, approximately 50% is affected by this disorder, indicating a close association between OAB and aging (26). OAB presents itself with storage abnormalities including urgency, frequency, and nocturia with or without incontinence (27). Urgency and frequency may be the result of involuntary detrusor muscle contractions (DO). DO is usually diagnosed during the standard urodynamic testing of patients with LUT symptoms (28) and it is closely

associated with OAB, as more than half of the OAB patients have DO as well (29). However, it is important to note, that despite that the two diseases often co-exist and the majority of OAB patients (about 70%) have DO (30) the two disorders may also exist independently from each other (31).

1.4.1. Pathophysiology of co-existent OAB and DO

The exact pathomechanism is unknown, as most cases of OAB and DO are idiopathic and it has become clear by now that we cannot identify a single cause that is solely responsible for the development of these disorders. However, several theories have emerged attempting to explain the pathomechanism approaching it from different aspects.

I. According to the **neurogenic hypothesis**, an altered neurological control either in the central nervous system (e.g. a frontal lobe lesion leading to diminished inhibition on the pontine micturition center) or at the level of the peripheral neurons (e.g. hyperreactivity of mechanosensitive or C-type neurons) underlie the bladder overactivity (32).

II. The **urotheliogenic hypothesis** states that OAB and DO symptoms are initiated from an altered bladder sensation resulting in increased release of mediators from the urothelium which in turn enhances afferent nerve firing (33).

III. The **myogenic hypothesis** is also a widely accepted hypothesis claiming that the cause of bladder overactivity originates from the detrusor muscle. This hypothesis is supported by the observation that bladder smooth muscle cells from DO patients have increased spontaneous activity and augmented contractile response to excitatory mediators [carbachol (CCh), ATP]. In addition, according to more recent theories, alteration in the initiation of contraction in different parts of the bladder (modules) and the propagation of these contractions are also of key importance in the development of bladder overactivity (34, 35).

1.4.2. Systemic inflammation and bladder function

Given that the prevalence of OAB increases with age, it is not uncommon for patients to develop aging-related conditions simultaneously with bladder dysfunctions. Moreover, these disorders often share some common pathogenic factors in their development. Therefore, a potential approach to understand OAB better is to investigate the underlying causes of the disorders that tend to co-occur with OAB, as they may provide valuable information for the pathomechanism of OAB as well.

a) Metabolic syndrome

Although it is still a highly debated topic, there are four commonly accepted elements of metabolic syndrome: central obesity, hypertension, dyslipidemia, and glucose intolerance. Studies investigating the direct link between metabolic syndrome and OAB are scarce, nevertheless, an association between these disorders has been suggested (36, 37). Moreover, there are numerous studies indicating links between OAB and one or more components of metabolic syndrome, showing that they, indeed, quite frequently occur simultaneously (38). Metabolic syndrome is associated with chronic, low-grade inflammation, leading to enhanced production of inflammatory mediators [C-reactive protein (CRP), PGs, cytokines] that may also be connected to bladder dysfunctions (36).

b) Obesity

A close association has been found between obesity and urological disorders, especially stress urinary incontinence, and bladder overactivity. Moreover, the correlation was strongest, if type II diabetes was present simultaneously with obesity in the patients. It was also shown that weight loss had a marked beneficial effect on obese patients' bladder function and symptoms of urgency, and incontinence eased, thus their quality of life improved (39). As obesity increases the concentration of various inflammatory mediators (e.g. PGE_2, PGD_2, BK) in the urine and in blood plasma, and these mediators may alter bladder function, they may provide a common link between obesity and bladder overactivity (40, 41).

c) Atherosclerosis

Systemic atherosclerosis has also emerged lately as a potential contributing factor to bladder dysfunctions. The rationale behind this assumption is that atherosclerosis damages bladder perfusion, thus, leading to patchy ischemia and oxidative stress in the bladder wall. Therefore, an inflammatory environment develops enhancing the production of isoprostanes and other inflammatory mediators that may impair bladder function eventually (42, 43).

d) Gastrointestinal disorders

The reported higher chance of bladder and intestine dysfunctions occurring together prompted research on bowel-bladder communication and a strong connection between the two organ systems has been recognized in the past decades. For example, the prevalence of irritable bowel syndrome (IBS) is 33% in patients with symptoms of urinary urgency and frequency (44), and the prevalence of chronic constipation in continent OAB and incontinent OAB is 18% and 27%, respectively (45). Recent research shows that the potential shared pathomechanism of OAB and IBS is multifactorial including systemic, low-grade inflammation, epithelial dysfunction and damaged urinary sensation (46). Furthermore, there is another intriguing finding, namely that OAB patients have an altered gut and bladder microbiome that may contribute to the development of the disorders. Although further research is needed on this topic, a complex communication network among the visceral organs, involving immune cells, inflammatory mediators, the microbiome, and the nervous system is suspected in the shared pathomechanism (47).

1.5. Contribution of inflammatory mediators to bladder dysfunctions

As mentioned previously, there is a strong relationship between disorders associated with systemic inflammatory processes (e.g. metabolic syndrome, atherosclerosis, IBS) and the development of OAB. Moreover, immune cell infiltration (e.g. mast cells, neutrophils) in the bladder tissue has also been proposed in the pathomechanism, thus, inflammatory mediators got into the limelight as possible contributors to bladder dysfunctions, especially OAB and DO. Previous studies on this topic showed that the concentrations of nerve growth factor (NGF), CRP, PGs, and BK are elevated in the urine and bladder tissue of patients with symptoms of OAB, verifying the presence of an

inflammatory environment (48-50). Thus, indeed, it is affirmed that inflammatory mediators appear to play important roles in the pathogenesis of bladder overactivity (51, 52).

1.5.1. Prostaglandins

Prostanoids have gained attention in bladder physiology and pathophysiology, as their concentration may elevate as a result of bladder distension, mechanical stress, inflammation, and damage to the mucosa (52, 53). These observations underline the importance of investigating the role of PGs in bladder smooth muscle dysfunctions.

The prostanoid family consists of thromboxanes (Txs) and PGs. The naturally occurring PGs are subdivided into families marked by different letters, namely: D (PGD), E (PGE), F (PGF), and I (PGI), and these groups are also further classified based on the double bonds in their structure indicated by an index number. The rate-limiting step in their synthesis is the release of arachidonic acid (AA), which is cleaved from the cell membrane lipids either by phospholipase A_2 (PLA_2) or DAG-lipase. AA is then converted and peroxidized by cyclooxygenases (COX) and peroxidases into PGH_2. It is then rapidly modified by specific PG synthases to the above-mentioned PGs, which are finally transported out of the cell via PG transporters (54, 55). The various (patho)physiological effects of prostanoids are exerted through GPCRs, specifically, TP or PG receptors (e.g. DP, IP, FP, EP) named according to their primary ligands (Tx, PGD, PGI, PGF, and PGE, respectively).

In human and rodent bladder, PGI_2 is produced in the largest amount followed by PGE_2, and $PGF_{2\alpha}$ (52, 56). Despite being the most abundant PG, PGI_2 has only a mild contractile effect in the bladder but otherwise its role is still vague. Research on what PGE_2 exerts in the bladder is more widespread. It has been demonstrated, for example, that its intravesical administration results in reduced bladder capacity and, in the human bladder, urgency as well (57, 58). An increase in PGE_2 expression in the bladder of rat models with OAB-like symptoms has also been reported and the bladder overactivity was alleviated by a COX-2 inhibitor (59). It has also been shown that PGE_2 concentration in the urine of OAB patients is increased and it showed a correlation with the detrusor

muscle's contractility and accordingly, its concentration was decreased in patients with underactive bladder (60, 61).

$PGF_{2\alpha}$, can also induce contractions in the bladder, thus, altogether PGE_2 and $PGF_{2\alpha}$ are the most likely candidates that contribute to bladder overactivity. PGE_2 and $PGF_{2\alpha}$ induce detrusor muscle contractions by activating TP, EP_1 and FP receptors mainly (62, 63). TP, EP_1 and FP receptors communicate through a number of G proteins, however, the G_q and the G_{12} family proteins are assumed to be the most common ones. Nevertheless, research on the intracellular signaling of PGs in the detrusor muscle is scarce and investigating it in details is of pivotal importance, given their potential role in the development of bladder dysfunctions.

1.5.2. Bradykinin

Bradykinin is a multifaceted inflammatory mediator that has attracted attention regarding LUT dysfunctions, as not only its concentration increases upon bladder distension, but its receptors are also upregulated in bladder disorders indicating its possible role in the pathomechanism (50, 51). Structurally, BK is a low-molecular-weight nonapeptide with the amino acid sequence of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (64). There are two major pathways of BK formation *(Figure 2)*:

1) The tissue kallikrein enzyme is present in various tissues (e.g. kidneys, blood vessels, neurons, bladder etc.) and it produces kallidin from its substrate, low-molecular-weight kininogen (LK). Then a plasma aminopeptidase cleaves a lysine from kallidin to form BK.

2) In the plasma, bradykinin is produced by the interaction of factor XII (aka the Hageman factor), prekallikrein and high-molecular-weight kininogen (HK) and anionic surfaces (e.g. endotoxins, silicates and heparin). Factor XII is autoactivated to factor XIIa, which has two substrates: prekallikrein and factor XI. These substrates form a complex with and attach to negatively charged surfaces leading to a conformation change allowing cleavage of prekallikrein to plasma kallikrein, which then cleaves HK to BK.

Two enzymes are responsible for degrading BK: kininase I and kininase II. Kininase I is also known as plasma carboxypeptidase N and its interaction with BK yields des-Arg^9-bradykinin, whereas kininase II (or angiotensin-converting enzyme, ACE) that produces a hepta- and eventually a pentapeptide from BK (65). Physiologically, the production of BK and its degradation is well-regulated, however, under pathological conditions, the balance of peptide release and degradation may be disturbed, and either overproduction or decreased degradation of the peptide may lead to an elevated BK concentration that could induce various symptoms depending on the affected site.

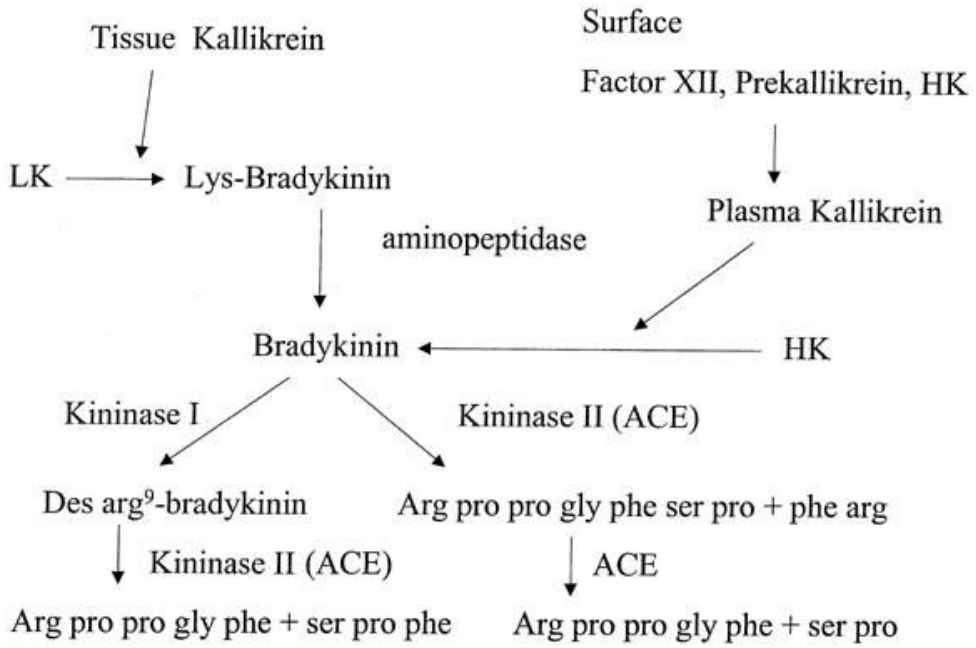

Figure 2 The formation and degradation of bradykinin[1] Abbreviations: ACE: angiotensin-converting enzyme, HK: high-molecular-weight kininogen, LK: low-molecular-weight kininogen (65)

As mentioned, BK is a versatile mediator having various effects in the body, such as vasodilation or -constriction, depending on the vessel type; regulating glucose homeostasis and natriuresis; sensitizing sensory neurons; regulating inflammatory processes (66). Besides blood vessels, BK is able to induce contractions in several

[1] Reprinted from the *Journal of Allergy and Clinical Immunology, Vol. 109, 2nd issue, Authors: Kaplan, Allen P.,Joseph, Kusumam Silverberg, Michael, Article title: Pathways for bradykinin formation and inflammatory disease, Pages: 195-209, Year: 2002* with permission from Elsevier.

other smooth muscle tissues as well, such as the prostate (67), the uterus (68) and the airways (69, 70).

These effects are mediated via two major receptor subtypes, namely B_1 and B_2. Despite sharing some structural similarities, their (patho)physiological roles differ markedly. B_1 receptors are usually barely detectable in healthy tissues but they are inducible, thus, as a consequence of tissue injury, inflammation, or endogenous factors, B_1 receptor expression may increase rapidly in the affected tissue (71). In contrast, B_2 receptors are constitutively expressed in various tissues (e.g. epithelium, endothelium, sensory nerves, smooth muscles) (72). The two receptor's ligand preference is also different: des-Arg^9-bradykinin has a high affinity to B_1 receptors, whereas BK preferentially binds to B_2 receptors. Once activated, both receptors couple to heterotrimeric G proteins. Generally to $G\alpha_{q/11}$, which induces PLC activation, IP_3 and DAG production, and elevation of the intracellular Ca^{2+} concentration (73). However, there are several other G proteins that may also be coupled to BK receptors, though less frequently, including $G\alpha_i$ (74), $G\alpha_{12/13}$ (75), and $G\alpha_s$ (76). After ligand binding, the two receptors follow separate ways, as B_2 receptors go through receptor internalization (77), whereas B_1 receptors are resistant to desensitization, thus they may signal for a longer period of time (78).

A role for BK receptors in regulating bladder function has been implicated, as they are expressed in practically all cell types of the bladder, including the mucosa, urinary bladder smooth muscle (UBSM), afferent nerve fibers as well as cells of innate and acquired immunity (79-82). Furthermore, as mentioned, BK induces contraction in various smooth muscle tissues, for example in the bronchi, a hollow organ similarly to the bladder. As these organs share some similarities in their contractile mechanisms (e.g. the contraction is largely dependent on M_3 receptors and the relaxation is induced by ß receptor-activation)(83) and in pathology as well (RhoA-ROCK signaling is enhanced)(84-86), the effect and signaling of BK may also share some similarities in the two organs. Indeed, a few studies have already shown that BK can evoke contraction in rat and rabbit bladders (87, 88). However, the exact role of BK's actions in the bladder and the signal transduction pathways regulating them are not quite clear yet. It is hypothesized that its role may gain significance in pathological, rather than physiological processes, as its receptors are upregulated in LUT disorders suggesting an important role for BK in these conditions (89).

2. Objectives

Bladder dysfunctions often occur simultaneously with systemic inflammatory disorders (atherosclerosis, obesity). Thus, inflammatory mediators, especially PGs, isoprostanes and BK, have emerged as potential contributors to OAB and DO. These molecules are not only promising biomarkers but may also play a substantial role in regulating bladder smooth muscle function and micturition.

Pharmacological therapy of OAB and DO has not yet been resolved, as the currently available treatments are symptomatic inducing severe adverse effects. Thus, research exploring the pathomechanism behind the disorders and identification of novel and more specific drug targets is needed for developing more effective therapies. Therefore, we aimed to outline the effect and signaling pathways of BK with an outlook on the intracellular signaling of PGs (specifically PGE_2 and $PGF_{2\alpha}$) in mouse and human UBSM tissues. Our main questions were the following:

1. Can we detect any effects of BK in murine and human UBSM and if yes, is it direct or indirect?
2. Which receptor(s) mediate the effect of BK and which intracellular signaling pathways are activated in the process?
3. Is there any common point within the signaling pathways activated by PGs and BK?
4. Is there any element in the signaling that may provide a potential target for the future therapy of bladder dysfunctions?

3. Materials and methods

3.1. Animals

All procedures were carried out according to the guidelines of the Hungarian Law of Animal Protection (28/1998) and were approved by the Government Office of Pest County (Permission number: PEI/001/2709-13/2014). Urinary bladders were obtained from adult (90-120-day-old, 30-35 g body weight) male wild-type (WT) mice with C57BL/6 genetic background as well as from knockout (KO) animals deficient in the cyclooxygenase (COX)-1 enzyme (COX-1-KO) or TP receptor (TP-KO) or from mice with induced smooth muscle-specific deficiency of $G\alpha_{q/11}$ or $G\alpha_{12/13}$ proteins, as described previously ($G\alpha_{q/11}$-KO and $G\alpha_{12/13}$-KO) (90, 91).

COX-1-KO mice were kindly provided by Ingvar Bjarnason (Department of Medicine, Guy's, King's College, St. Thomas' School of Medicine, London, United Kingdom), and prostanoid receptor-deficient mice were from Shuh Narumiya (Kyoto University, Kyoto, Japan). In the case of the COX-1-KO studies, littermate COX-$1^{+/+}$ animals, whereas in TP-KO experiments, WT C57BL/6N mice (from Charles River Laboratories, Isaszeg, Hungary) served as controls, as the TP-KO strain has been previously backcrossed with C57BL/6N mice for more than ten generations.

The mouse lines with smooth muscle-specific inducible deletion of the $G\alpha_{q/11}$ or $G\alpha_{12/13}$ proteins were generated on $G\alpha_{11}$-deficient ($G\alpha_{11}^{-/-}$) or $G\alpha_{12}$-deficient ($G\alpha_{12}^{-/-}$) background (92, 93) with floxed alleles of the genes coding $G\alpha_q$ (*Gnaq*$^{flox/flox}$) or Gα13 (*Gnα13*$^{flox/flox}$), and expressing a fusion protein of the Cre-recombinase with a modified estrogen receptor-binding domain (Cre-ERT2) (94) under the control of the smooth muscle myosin heavy chain (SMMHC) promoter. Deletion of *Gnaq* or *Gna13* was induced by intraperitoneal tamoxifen treatment (1 mg/day for five consecutive days) in SMMHC-CreERT2$^{+/-}$; *Gnaq*$^{flox/flox}$; *Gna11*$^{-/-}$ and SMMHC-CreERT2$^{+/-}$; *Gna12*$^{-/-}$; *Gna13*$^{flox/flox}$ mice, respectively as it was described earlier (90, 91). Mice with floxed alleles but without Cre expression served as controls. More precisely, tamoxifen-treated SMMHC-CreERT2$^{-/-}$; *Gnaq*$^{flox/flox}$; *Gna11*$^{+/+}$ and SMMHC-CreERT2$^{-/-}$; *Gna12*$^{+/+}$; *Gna13*$^{flox/flox}$ mice served as controls (CTRL) and are referred to as $G\alpha_{q/11}$-CTRL and $G\alpha_{12/13}$-CTRL.

3.2. Human tissues

All procedures involving human urinary bladder tissues have been approved by the Scientific and Research Committee of the Medical Research Council of Hungary (License No.: 21545-2/2019/EKU). Human urinary bladder tissues were obtained from 19 patients (15 males, 4 females; mean age of 65.5±9.3 years, range between 44-78 years) undergoing open radical cystectomy due to muscle-invasive bladder malignancy after having obtained written patient consent. None of the patients had any urodynamic disorders, symptoms of OAB syndrome, or was taking drugs for OAB.

Following the surgical removal of the bladders, they were immediately placed in a physiological saline solution and transported to the Department of Pathology, Forensic and Insurance Medicine of Semmelweis University, Budapest. Here, the healthy, tumor-free whole bladder wall tissue was excised by uro-pathologists within approximately 15-20 min following the removal of the bladders from patients as described previously (95). The bladder tissue was immediately placed into room temperature Hank's Balanced Salt Solution (HBSS) and transported to our myograph laboratory, where preparation of the smooth muscle strips was performed without any delay. Overall, myographic experiments started within 45-60 min following bladder removal from the patients.

3.3. Preparation of smooth muscle strips

Mice were euthanized by cervical dislocation under general anesthesia [i.p., ketamine (300mg/kg)+xylazine (30mg/kg)], and urinary bladders were removed from a lower midline incision 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 37 °C temperature. Under a dissection microscope (M3Z; Wild Heerbrugg AG, Gais, Switzerland), adipose and connective tissues were removed from the serosal surface. Bladders were cut into four strips of equal lengths and, the mucosa layer was also gently removed to prevent the potential release of paracrine factors from the mucosal epithelium or submucosa and avoid tension changes induced by myofibroblasts eventually. Human urinary bladder specimens were also placed into Krebs solution (same as described before, T = 37 °C) during the preparation. Under a dissection

microscope, the serosal tissue and the mucosal layer were removed. The isolated detrusor specimens were cut into equal, approximately 3x2x1 mm strips for myography.

3.4. Measurement of bladder contractility with myography

Both murine and human detrusor muscle strips were mounted on two parallel, horizontal stainless-steel tissue-holding needles of a myograph (needle diameter 200 µm, 610 M Multi-Wire Myograph System, Danish Myo Technology A/S, Aarhus, Denmark). Chambers were filled with 6 ml of 37 °C Krebs solution aerated with carbogen (mixture of 5% CO_2 and 95% O_2). Detrusor muscle contractions were recorded under isometric conditions. Every experiment started with a 60-minute resting period while the strips were stretched to and stabilized at a passive tension of 5 mN (murine) or 3 mN (human). After the resting period, UBSMs were challenged twice with 124 mM K^+-containing Krebs solution to examine the viability of the tissues. The contractile effect of 124 mM K^+ was comparable in the detrusor strips obtained from the WT and the genetically modified mouse lines (*Figure 3*).

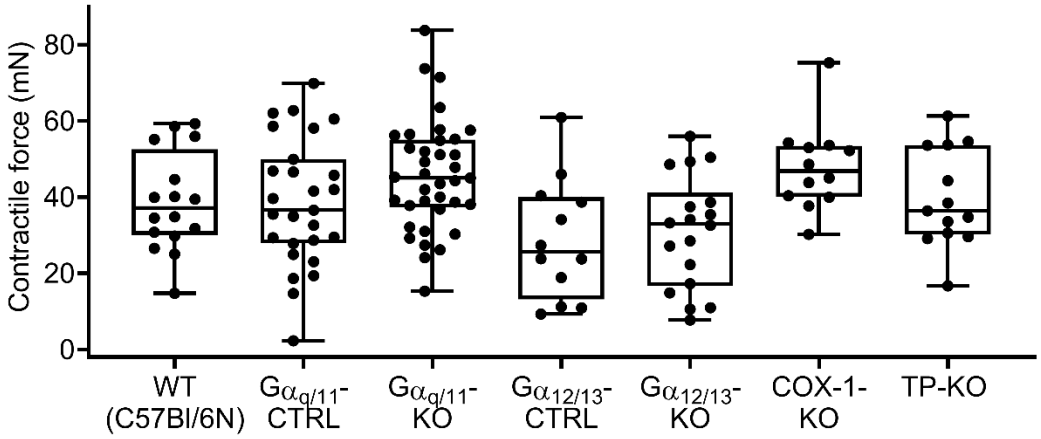

Figure 3. Comparison of contractions induced by 124 mM KCl solution in different mouse strains. Contractions induced by 124 mM KCl solution did not differ significantly in UBSM strips prepared from various mouse strains. (Kruskal-Wallis test, $p<0.05$ was considered as significant difference) Case numbers: C57BL/6N: n=16/4, $G\alpha_{q/11}$-CTRL: n=27/7, $G\alpha_{q/11}$-KO: n=37/8, $G\alpha_{12/13}$-CTRL: n=12/3, $G\alpha_{q/11}$-KO: n=18/4, COX-1-KO: n=12/4, TP-KO: n=13/4.

After several washes with normal Krebs solution, the contractile effects of BK (10^{-10} -10^{-4} M), Lys-[Des-Arg^9]-bradykinin (B_1 receptor agonist, 10^{-5} M), [Phe$^8\Psi$(CH-NH)-Arg^9]-bradykinin (B_2 receptor agonist, 10^{-5} M), CCh (10^{-6} M), α,β-methyleneadenosine 5'-triphosphate-[α,β-meATP, ATP-analogue (10^{-5} M)], PGE_2 (10^{-5} M), $PGF_{2\alpha}$ (10^{-5} M) was measured. Some of the strips were preincubated with one of the following inhibitors without washing out: R-715 (Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-DβNal-Ile, B_1 receptor antagonist, 10^{-6} M, 20 min), HOE-140 (icatibant, D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg, B_2 receptor antagonist, 10^{-6} M, 20 min), atropine (muscarinic ACh receptor antagonist, 10^{-6} M, 20 min), Y-27632 (ROCK inhibitor, 10^{-5} M, 20 min) pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS; P2 purinergic receptor antagonist, 10^{-5} M, 20 min), indomethacin (general COX inhibitor, 10^{-5} M, 20 min), *N*-[2-cyclohexyloxy-4-nitrophenyl]methanesulfonamide (NS-398, COX-2 inhibitor 10^{-5} M, 20 min). When acetic acid, dimethyl sulfoxide (DMSO), or saline was the solvent of the inhibitor, they were applied in matched concentrations as vehicle control. The final concentration of acetic acid in the tissue bath was 0.1 mM, while that of DMSO was 0.1%. Since repeated use of BK or its analogs in the same specimen is problematic due to receptor desensitization and the viability of the tissues may decline in a prolonged experiment, we performed unpaired, time-control experiments in this study. Finally, bladder strips were exposed to 124 mM K^+-containing Krebs solution to retest the viability of the detrusor strips. Agonist-induced tension changes were normalized to the reference contraction induced by 124 mM K^+-containing Krebs solution (second administration).

3.5. Drugs and solutions

BK was purchased from Bachem (Bubendorf, Switzerland) and dissolved in acetic acid (0.1 M) to stock solutions of 10^{-2} M. Lys-[Des-Arg^9]-bradykinin, [Phe$^8\Psi$(CH-NH)-Arg^9]-bradykinin, HOE-140, and R-715 were purchased from Tocris (Bristol, UK) and were dissolved in saline. Stock solutions of Lys-[Des-Arg^9]-bradykinin, [Phe$^8\Psi$(CH-NH)-Arg^9]-bradykinin, and R-715 were 10^{-3} M, whereas due to its poor solubility in water, stock solutions of HOE-140 were $5x10^{-4}$ M. CCh was from Sigma-Aldrich (St. Louis, MO) and dissolved in saline to a stock solution of $2x10^{-1}$ M. Atropine was purchased from Egis Pharmaceutical PLC (Budapest, Hungary) and was diluted in water to a stock

solution of $1.44x10^{-4}$ M. α,β-meATP, PPADS and Y-27632 were purchased from Cayman Chemical (Ann Arbor, MI) and all substances were dissolved in saline (α,β-meATP: 10^{-2} M, PPADS: 10^{-2} M and Y-27632: 10^{-3} M). Indomethacin was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO (10^{-2} M stock concentration), as its aqueous solutions are quite unstable (96). NS-398 was also purchased from Sigma-Aldrich (St. Louis, MO), and DMSO was applied as a solvent for preparing a 10^{-2} M stock solution. Both PGE_2 and $PGF_{2\alpha}$ were purchased from Cayman Chemical and dissolved in DMSO to produce a 10^{-2} M stock solution.

3.6. Data analysis

MP100 system and AcqKnowledge 3.9.2 software from Biopac System (Goleta, CA) were used for the acquisition and analysis of data in myographic measurements. The moving average smoothening function of the software was applied on recordings solely in order to eliminate the noises arising from the bubbling of the medium and to reduce the high frequency - low amplitude spontaneous tension oscillations. The parameters of the smoothening filter were carefully chosen in order to eliminate only the noises but not to alter the measured amplitude of the BK-induced responses - the baseline and peak values were always compared before and after the smoothing. The sample rate of the recordings was 10 samples/sec (10 Hz), the smoothing factor was between 10 and 40 samples. Spontaneous micro-contractions of the bladder strips were observed occasionally; however, they were not reproducible, thus these data were not considered for demonstration or evaluation in the present study.

The maximum contraction was defined as the peak value of tension developed after the addition of agonists. The individual values of the peak contractile force from each experiment are represented by boxplots, where the horizontal line shows the median value, the interquartile range is demonstrated by the lower and the upper part of the box (from 25^{th} to the 75^{th} percentiles, respectively). The whiskers on the boxplots represent the minimal and the maximal values. Average curves of individual contraction responses were also determined and presented on the left side of the figures, where they were plotted as mean values. In case of concentration-response curves, mean ± SEM values are demonstrated. For mouse concentration-response curve analysis, curves were fitted for data from each experiment, thus E_{max} and EC_{50} values were determined for each curve,

and the average values were calculated thereafter. In the case of human concentration-response correlation, curves were fitted on data gained from numerous experiments, as human tissues exhibit more variable responses which made curve-fitting from each individual experiment difficult.

For statistical analysis, data sets were subjected to nonparametric testing, as in the case of small sample sizes and skewed data, parametric testing might not be appropriate. In the case of comparing two data sets the Mann-Whitney test, whereas in the case of comparing several data sets, the Kruskal-Wallis test was performed to determine the corresponding p values. The following formula was used for demonstrating case numbers: n=x/y, where *x* represents the number of bladder strips and *y* indicates the number of bladders the strips were obtained from. Statistical analysis and graph plotting were performed with GraphPad Prism software (v.6.07; GraphPad Software Inc., La Jolla, CA, USA), and $p < 0.05$ was considered a statistically significant difference.

4. Results

4.1. Bradykinin induces concentration-dependent contractions in mouse and human bladders

First, we aimed to evaluate the effect of BK in murine and human urinary bladders. We found that BK induced a marked, transient contraction in the mouse bladder, although it did not reach the level of the response evoked by CCh, a stable analog of ACh *(Figure 4/A)*. The effect of BK was dose-dependent, with the EC_{50} of 1.24 μM and E_{max} of 52.4%, expressed as the percentage of the reference contraction induced by 124 mM KCl *(Figure 4/B)*.

In human bladder strips, BK induced comparable contractions with a similar ratio to the CCh's effect to that observed in mice *(Figure 4/C)*. BK-induced contractions were also dose-dependent in human bladders with an EC_{50} of 5.1 μM and E_{max} at 42.4% *(Figure 4/D)*. As repeated administration of BK appeared to desensitize BK receptors both in murine and human bladders, the dose-response curves were obtained by applying only one single concentration of BK to each muscle strip and we also avoided repeated administration in further experiments. Based on the dose-response relationship presented in the B and D panels of Figure 4, we decided to apply BK in the subsequent experiments in 10 μM, which induces a submaximal contractile effect enabling the analysis of the signaling pathways involved.

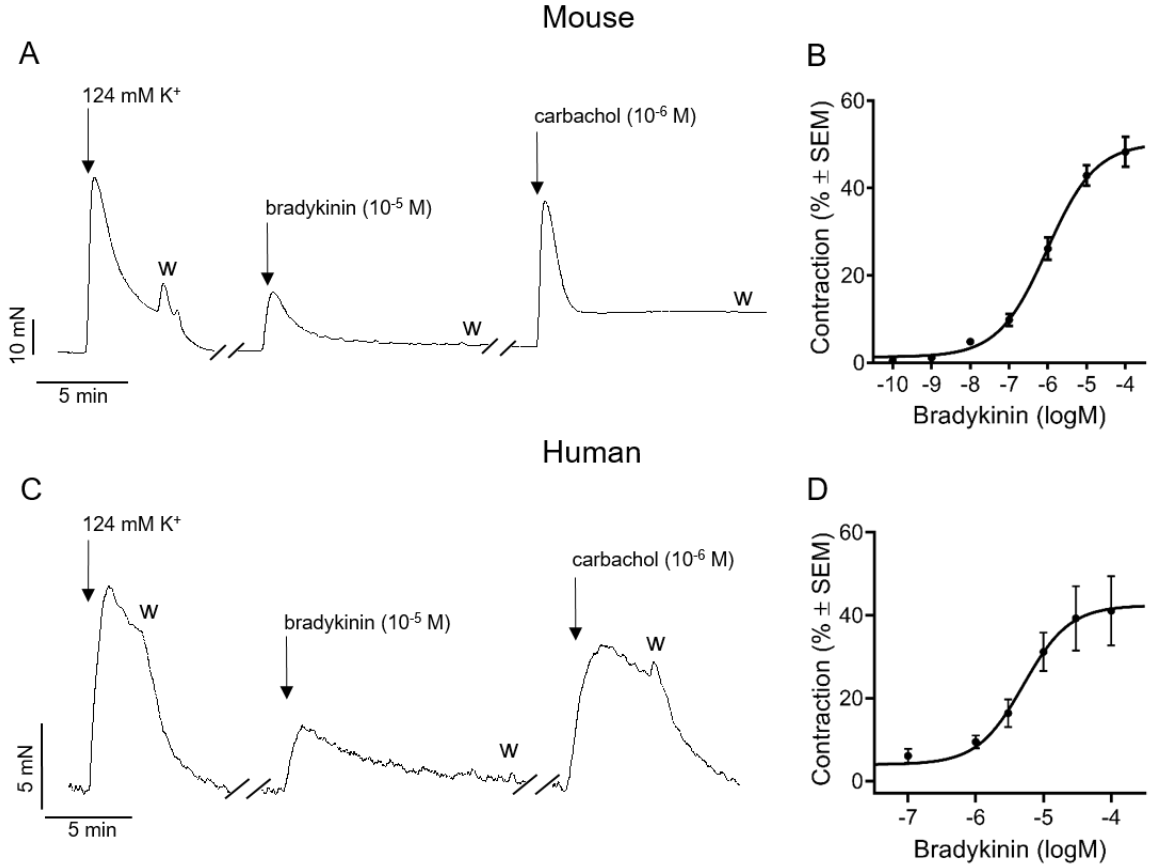

Figure 4. Bradykinin induces concentration-dependent contractions in mouse and human bladders with similar characteristics. A: Original trace: BK (10^{-5} M) evoked contractions in isolated murine detrusor smooth muscle strips which were comparable to the responses evoked by the muscarinic ACh receptor agonist, CCh (10^{-6} M). **B:** Concentration-response curve of BK in murine urinary bladder strips [E_{max}: 52.4% EC_{50}: 1.2 μM, case numbers: n(10^{-10}) = 2/2, n(10^{-9}) = 3/3, n(10^{-8}) = 3/3, n(10^{-7}) = 6/6, n(10^{-6}) = 9/7, n(10^{-5}) = 4/4. n(10^{-4}) = 10/10]. **C:** Original trace: In accordance with our results gained from murine bladder strips, BK (10^{-5} M) evoked contraction in human detrusor smooth muscle as well. Moreover, the amplitude of the contractile effect was comparable to that induced by the muscarinic-acetylcholine-receptor agonist CCh (10^{-6} M). **D:** Concentration-response curve representing BK's contractile effect in human detrusor smooth muscle. [E_{max}: 42.4%; EC_{50}: 5.1 μM, case numbers: n(10^{-7}) = 7/3, n(10^{-6}) = 7/3, n(3x10^{-6}) = 11/4, n(10^{-5}) = 13/6, n(3x10^{-5}) = 15/5, n(10^{-4}) = 11/4]

4.2. The signaling pathways of bradykinin-induced contractions of mouse bladder smooth muscle

4.2.1. Bradykinin-induced contractions are independent of purinergic and cholinergic neurotransmission and COX-derived prostanoids

To test whether secondary ACh or ATP release from parasympathetic nerve fibers may mediate the effects of BK, we applied the muscarinic ACh receptor antagonist atropine (10^{-6} M, 20 min incubation) (*Figure 5/A*) or the P2 purinergic receptor antagonist PPADS (10^{-5} M, 20 min) (*Figure 5/B*). Neither atropine nor PPADS altered the effect of BK in mouse detrusor muscle, suggesting that the BK-induced contractile responses are independent of secondary ACh or ATP release (*Figure 5 /A,B*). To verify the effectiveness of atropine and PPADS, we applied the specific purinergic agonist α,ß-meATP, and the muscarinic agonist CCh following a 20 min incubation period with either PPADS or atropine, respectively (*Figure 6/A,B)*. PPADS abolished the contractile effect of α,ß-meATP, likewise, atropine inhibited CCh-induced contractions, which confirmed the effectiveness of the two inhibitors.

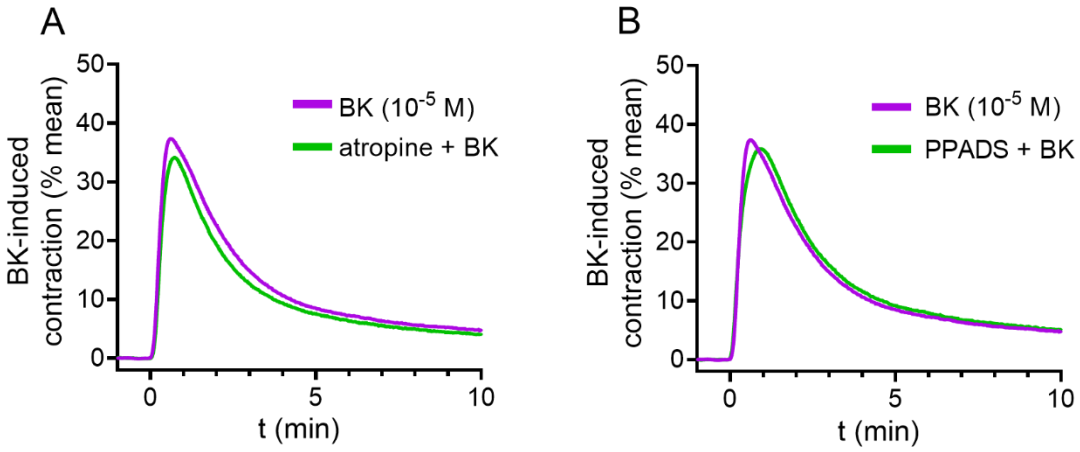

Figure 5. Bradykinin-induced detrusor muscle contraction is independent of purinergic or cholinergic neurotransmission. A, B: Neither inhibition of purinergic receptors with PPADS (10^{-5} M, 20 min incubation) nor the muscarinic receptor antagonist atropine (10^{-6} M, 20 min incubation) altered detrusor contraction induced by BK (10^{-5} M). Case numbers: A: BK: n=6/6, atropine + BK: n=7/7, B: BK: n=6/6, PPADS + BK: n=8/8.

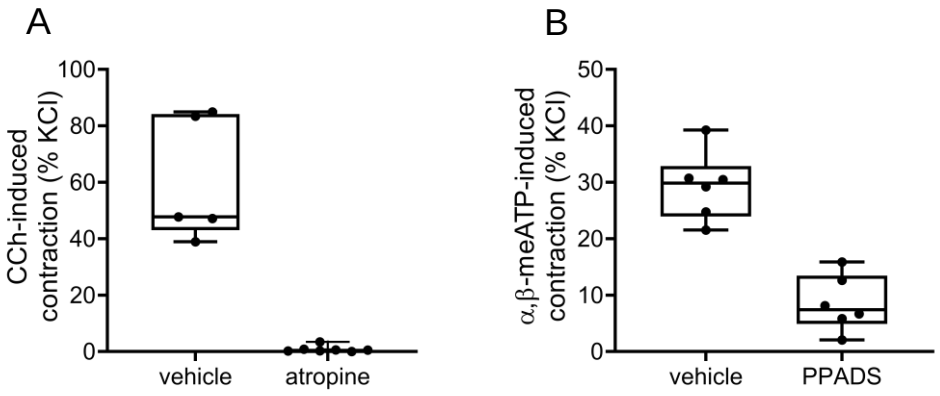

Figure 6. The muscarinic agonist carbachol- and the purinergic agonist α,β-meATP-induced contractions were abolished by the muscarinic (atropine) or purinergic (PPADS) antagonists, respectively. **A:** To verify the effectiveness of atropine (10^{-6} M, 20 min incubation), bladder strips were treated with the muscarinic agonist CCh (10^{-6} M). The presence of atropine inhibited CCh-induced contractions. **B:** The effectiveness of PPADS (10^{-5} M, 20 min incubation) was verified by administration of the purinergic agonist α,β-meATP (10^{-5} M) to UBSM strips. The contractile effect of α,β-meATP was diminished in the presence of PPADS. (A-B: Mann-Whitney test; ** $p < 0.01$) Case numbers: A: CCh: n=5/5, atropine+CCh: n=7/7, B: α,ß-meATP: n=6/6, PPADS+α,ß-meATP: 6/6.

In previous studies, data gained from human airway smooth muscle tissues suggested that BK may constrict bronchial smooth muscle through TxA_2 release resulting in TP receptor activation (97, 98). Thus, we also performed experiments with TP-KO mouse bladders and found that their contractile responses to BK were similar to those of the control bladder strips (*Figure 7/A*).

After proving that TP receptors do not mediate the effect of BK, we aimed to analyze the potential involvement of prostanoids more broadly, as other COX enzyme-derived molecules have also been suggested as potential mediators of BK's effects in smooth muscle (99-101). To test the function of COX enzymes, COX-1-KO mouse detrusor muscle tissues were subjected to BK, but its contractile effect remained unaltered (*Figure 7/B*). For further investigation, WT mouse bladders were incubated with either the general COX enzyme inhibitor indomethacin (10^{-5} M, 20 min) or the selective COX-2 inhibitor NS-398 (10^{-5} M, 20 min) but in both cases, the inhibitors failed to exert any effect on BK-evoked contractions. As the COX-1 enzyme deletion may be compensated by COX-2 enzyme upregulation (102-104), we also treated COX-1-KO bladders with NS-398 to address the possibility of such a compensatory mechanism. Affirming our previous findings, we found that the contractile effect of BK was unaltered, indicating

that neither COX-1 nor COX-2 appears to be involved in mediating the response. These results let us conclude that PGs do not play any significant role in BK-induced UBSM contractions in our experimental setup (*Figure 7/B*).

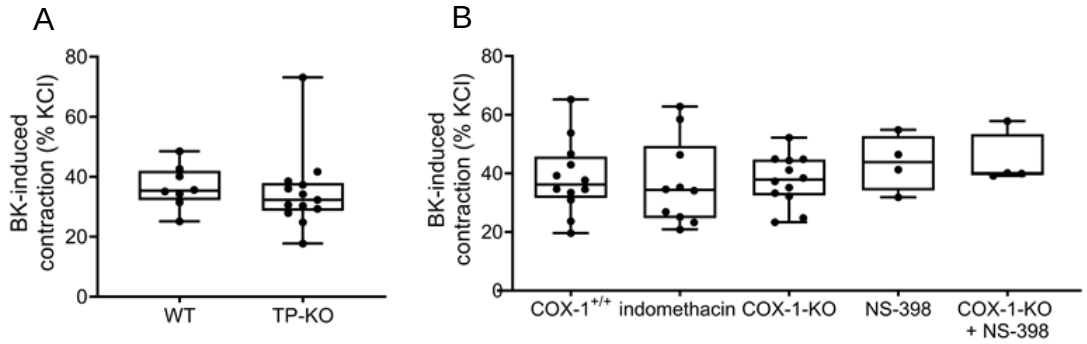

Figure 7. Bradykinin-induced detrusor muscle contractions are independent of COX-derived prostanoids. A: Contractile responses evoked by BK (10^{-5} M) were not altered in the bladder strips from mice deficient for TP receptors compared to those from WT mice. **B:** The presence of the nonspecific COX inhibitor indomethacin (10^{-5} M, 20 min) did not change BK-induced contractions. Furthermore, deficiency in COX-1 enzymes or treatment with the specific COX-2 inhibitor (NS-398, 10^{-5} M, 20 min), as well as their combination (NS-398+COX-1-KO), failed to influence the contractile effects elicited by BK. (A: Mann-Whitey test, B: Kruskal-Wallis test) Case numbers: A: WT: n=8/4, TP-KO: n=13/4, B: COX-$1^{+/+}$: n=12/4 indomethacin: n=10/5, COX-1-KO: n=12/4, NS-398: n=4/2, COX-1-KO + NS-398: n=4/2.

4.2.2. The role of B_2 receptors in bradykinin-induced mouse bladder contractions

Following verification of the direct contractile effect of BK in detrusor muscle, we investigated the role of B_1 and B_2 receptors in the contraction by applying the specific B_1 receptor antagonist R-715 (10^{-6} M, 20 min incubation) and B_2 receptor antagonist HOE-140 (10^{-6} M, 20 min incubation). BK-induced contractions were strongly inhibited by HOE-140, whereas R-715 failed to reduce them, implying that B_2 receptors play the main role in mediating the effect of BK in UBSM *(Figure 8/A*). Furthermore, the simultaneous application of the two inhibitors completely abolished the contractile responses. Smooth muscle strips were also treated with specific agonists of B_1 and B_2 receptors. B_1 agonist Lys-[Des-Arg^9]-bradykinin (10^{-5} M) elicited only minor smooth muscle tone elevation, whereas the B_2 agonist [$Phe^8\Psi$(CH-NH)-Arg^9]-bradykinin (10^{-5} M) had a potent constrictor effect in murine UBSM comparable to that of BK *(Figure 8/B*) verifying the predominant role of B_2.

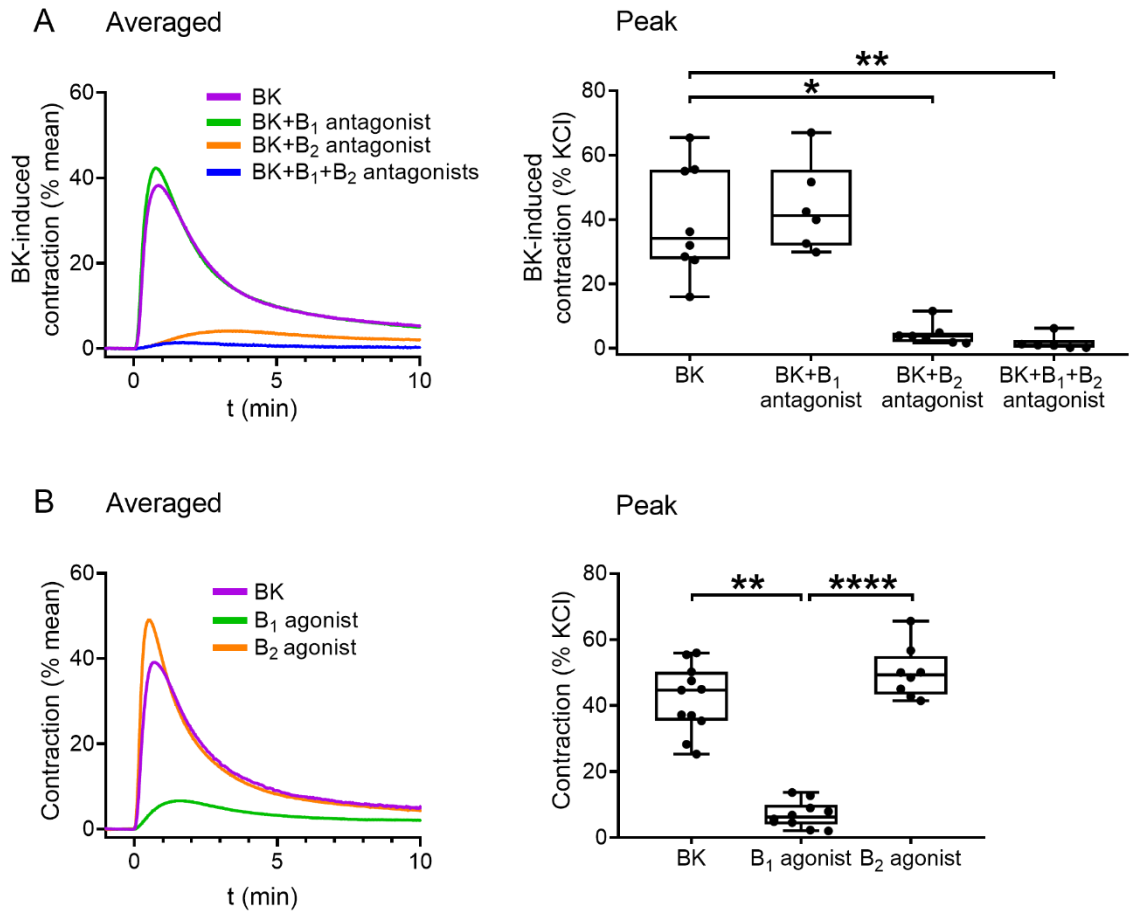

Figure 8. Role of B_2 receptors in mediating bradykinin-induced detrusor muscle contraction in murine urinary bladder strips. A: The BK (10^{-5} M)-induced contraction was abolished by the B_2 receptor-specific antagonist HOE-140 (10^{-6} M, 20 min), whereas the B_1 receptor antagonist R-715 (10^{-6} M, 20 min) failed to reduce it. **B:** The B_2 receptor agonist (10^{-5} M) induced contractions of the same magnitude as BK, whereas the B_1 receptor agonist (10^{-5} M) evoked only minor bladder contractions. (A-B: Kruskal-Wallis test, *$p < 0.05$, **$p < 0.01$, ****$p<0.0001$) Case numbers: A: BK: n=8/8, B_1 antagonist: n=6/6, B_2 antagonist: n=7/7, B_1 + B_2 antagonist: n=6/6, B: BK: n=11/11, B_1 agonist: n=10/8, B_2 agonist: n=8/7.

4.2.3. $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ proteins mediate bradykinin-induced contractions simultaneously in mouse bladder

Focusing on the intracellular signaling of B_2 receptors, we intended to examine the contribution of $G\alpha_{q/11}$- and $G\alpha_{12/13}$-mediated pathways to the constriction evoked by B_2 receptor activation. Contractile responses induced by BK (10^{-5} M) were reduced in $G\alpha_{q/11}$-deficient UBSM compared to those of $G\alpha_{q/11}$-CTRL murine bladders (*Figure 9/A*). Interestingly, despite that B_2 receptors are primarily coupled to either $G\alpha_{q/11}$ (most commonly) or $G\alpha_{12/13}$ proteins (less commonly) (73), BK-induced contractions also decreased in $G\alpha_{12/13}$-KO mouse bladder, suggesting that both pathways may contribute simultaneously to the effect (*Figure 9/B*).

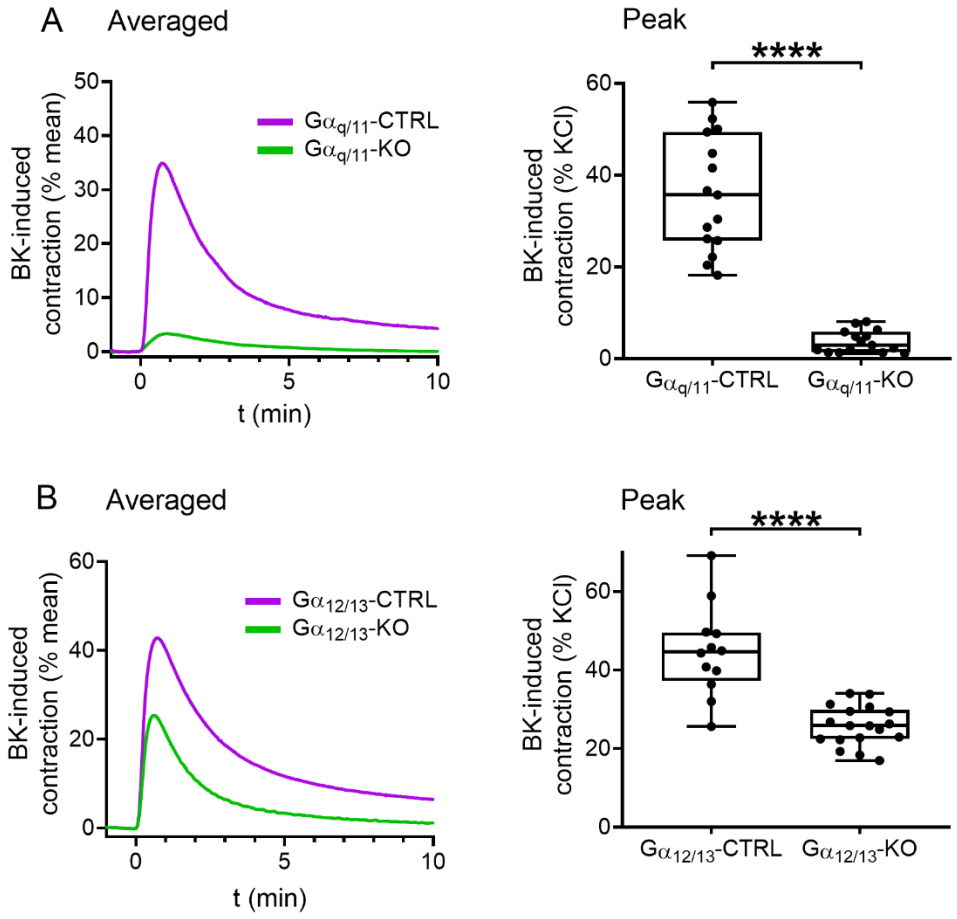

Figure 9. $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ proteins mediate the effect of bradykinin in murine urinary bladder detrusor muscle. A: Contractile responses evoked by BK (10^{-5} M) diminished in the UBSM from $G\alpha_{q/11}$-KO mice compared to bladder strips from $G\alpha_{q/11}$-CTRL. **B:** The detrusor contractions elicited by BK were also reduced in the UBSMs from $G\alpha_{12/13}$-KO compared to the strips from $G\alpha_{12/13}$-CTRL animals. (A-B: Mann-Whitney test, ****$p < 0.0001$) Case numbers: A: $G\alpha_{q/11}$-CTRL: n=15/4, $G\alpha_{q/11}$-KO: n=15/4, B: $G\alpha_{12/13}$-CTRL n=12/3, $G\alpha_{12/13}$-KO: n=18/4.

4.2.4. Role of the Gα12/13-RhoA-ROCK pathway in mediating bradykinin-induced contractions in murine detrusor muscle

As BK-evoked contractions decreased in Gα12/13-KO UBSM, we aimed to investigate the downstream signaling of Gα12/13-activation further via the application of the ROCK inhibitor Y-27632 (10^{-5} M, 20 min), which also reduced BK-evoked contractions in murine bladders (*Figure 10/A*). In addition, the presence of Y-27632 (10^{-5} M, 20 min) completely abolished BK-induced contractions in Gαq/11-KO UBSM (*Figure 10/B*).

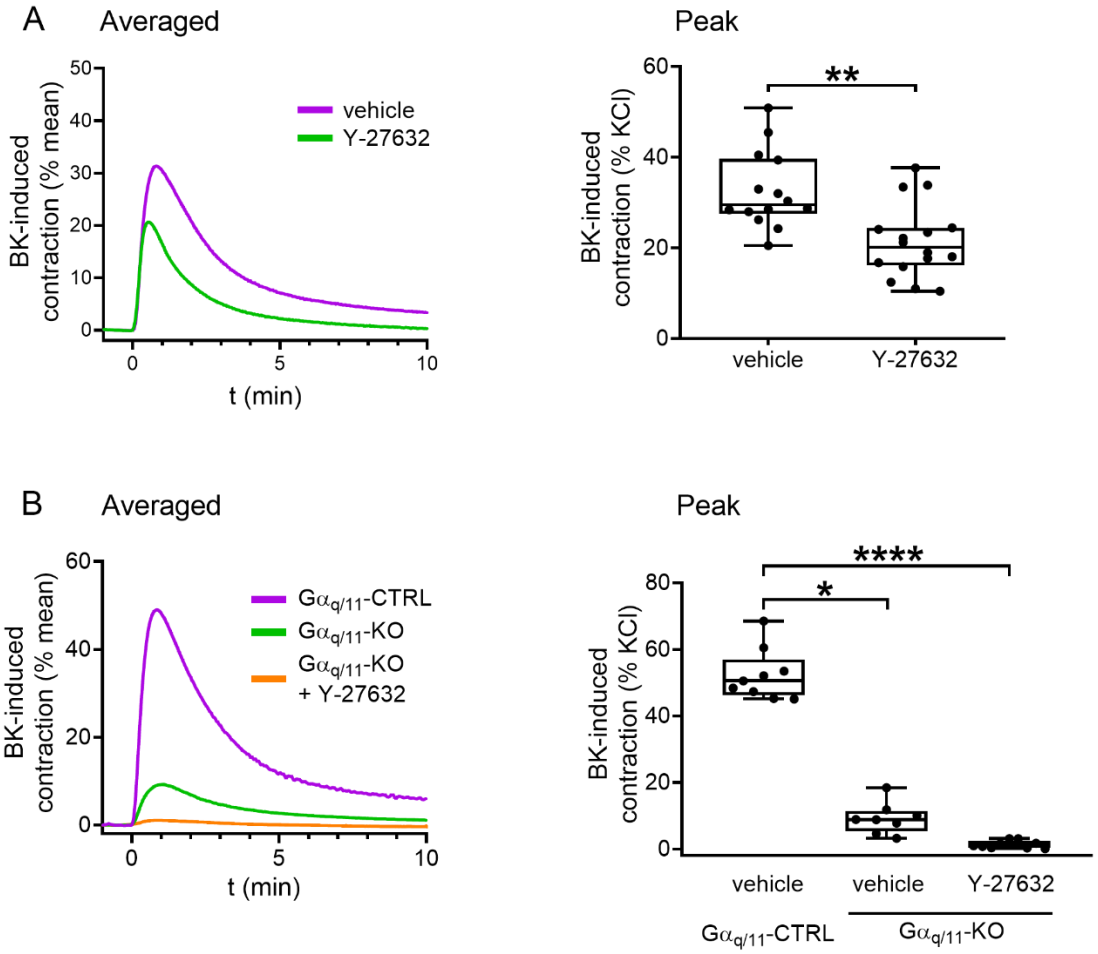

Figure 10. Role of Gα12/13-RhoA-ROCK pathway in mediating bradykinin-induced contractions in murine UBSM. A: The contractile responses induced by BK (10^{-5} M) were decreased in the presence of the ROCK inhibitor (Y-27632, 10^{-5} M, 20 min). **B:** The addition of Y-27632 (10^{-5} M, 20 min) completely suppressed the remaining BK-induced contractile responses in UBSM strips from Gαq/11-KO mice. (A: Mann-Whitney test, B: Kruskal-Wallis test, *p < 0.05, **p<0.01) Case numbers: A: vehicle: n=14/8, Y-27632: n=16/8, B: vehicle: n=9/4, Gαq/11-KO + vehicle: n=8/4, Gαq/11-KO + Y-27632: n=9/4.

4.2.5. $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins mediate contractions evoked by PGE_2 and $PGF_{2\alpha}$ in murine UBSM

The observation that the effect of BK is mediated by simultaneous activation of the $G\alpha_{q/11}$- and $G\alpha_{12/13}$-ROCK pathways prompted us to test whether a similar, dual signaling is involved in case of PGs, as they are also inflammatory mediators that induce murine UBSM contractions via activation of GPCRs (105). We found that $G\alpha_{q/11}$ deficiency markedly reduced and application of Y-27632 (10^{-5} M, min) to $G\alpha_{q/11}$-KO mouse UBSM completely abolished contractions evoked by PGE_2 and $PGF_{2\alpha}$, quite similarly to what we have seen in the case of BK *(Figure 11).*

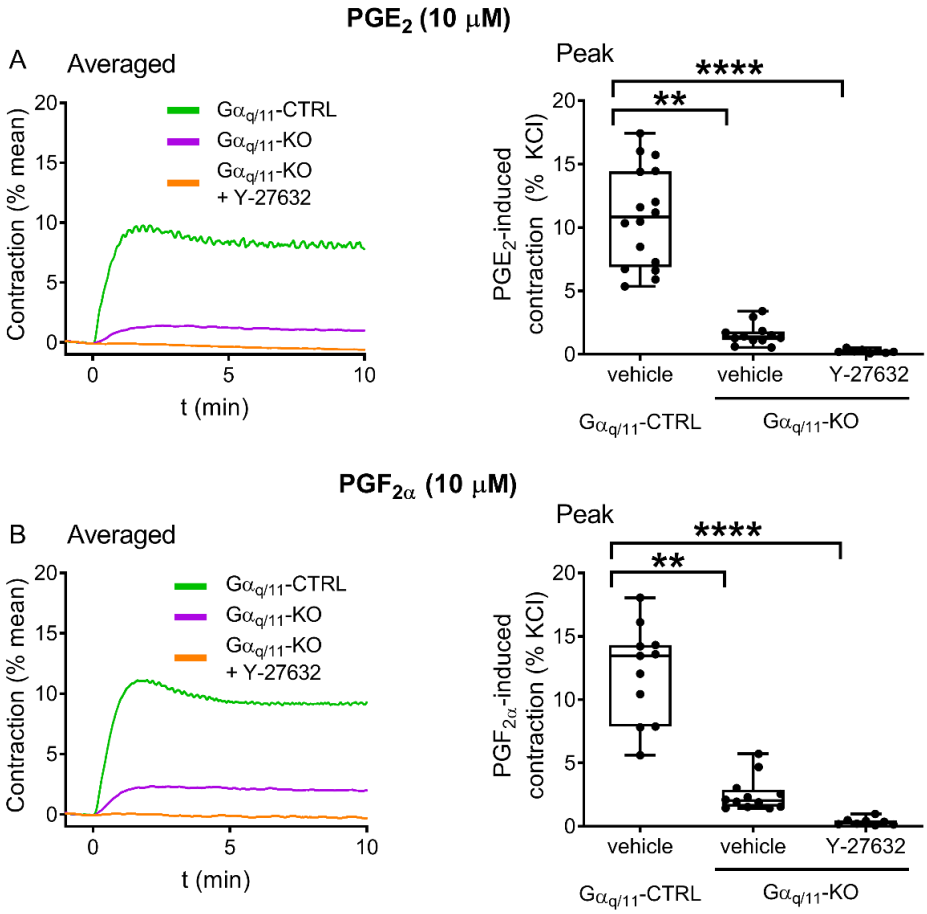

Figure 11. The role of $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ G protein-coupled pathways in PGE_2- and $PGF_{2\alpha}$-induced mouse bladder contractions (105).[2] $G\alpha_{q/11}$ deficiency markedly reduced, whereas administration of the ROCK-inhibitor Y-27632 (10^{-5} M, 20 min) to $G\alpha_{q/11}$-KO mouse UBSM strips completely abolished the contractile effect of PGE_2 **(A)** and $PGF_{2\alpha}$ **(B)**. (Kruskal-Wallis test, **p<0.01, ****p<0.001) Case numbers: A: vehicle: n=16/9, $G\alpha_{q/11}$-KO + vehicle: n=12/6, $G\alpha_{q/11}$-KO + Y-27632: n=8/5, B: vehicle: n=11/9, $G\alpha_{q/11}$-KO + vehicle: n=12/9, $G\alpha_{q/11}$-KO + Y-27632: n=8/5.

[2] Reprinted and modified from Molnar, P. J. et al. (2021). Supplementary Figure 3. Gαq/11 and the G12/13-Rho-ROCK signaling pathways mediate the effects of prostaglandin (PG) E2 and F2α in murine urinary bladder smooth muscle at https://doi.org/10.6084/m9.figshare.13377125.v1.

4.3. Bradykinin-induced smooth muscle contractions are mediated by B_2 receptors in human urinary bladder

Based on the promising murine results, we turned to investigate the signaling pathways of BK in the human bladder as well. The role of BK receptor subtypes was examined by applying the B_1 and B_2 receptor antagonists. The presence of the B_2 antagonist HOE-140 (10^{-6} M, 20 min) almost completely abolished BK-induced contractions, whereas the B_1 antagonist R-715 (10^{-6} M, 20 min) failed to alter contractions. This indicates that B_2 receptors play a prominent role in mediating BK-induced contractile responses of human UBSM as well, similar to our data gained from mouse experiments (*Figure 12/A*). Furthermore, the same selective B_1 and B_2 receptor agonists were applied to the human bladder strips as previously in murine UBSM. The B_2 agonist (10^{-5} M) evoked contractions approximately of the same magnitude as BK, however, the B_1 agonist (10^{-5} M) had only a minor constricting activity compared to them (*Figure 12/B*).

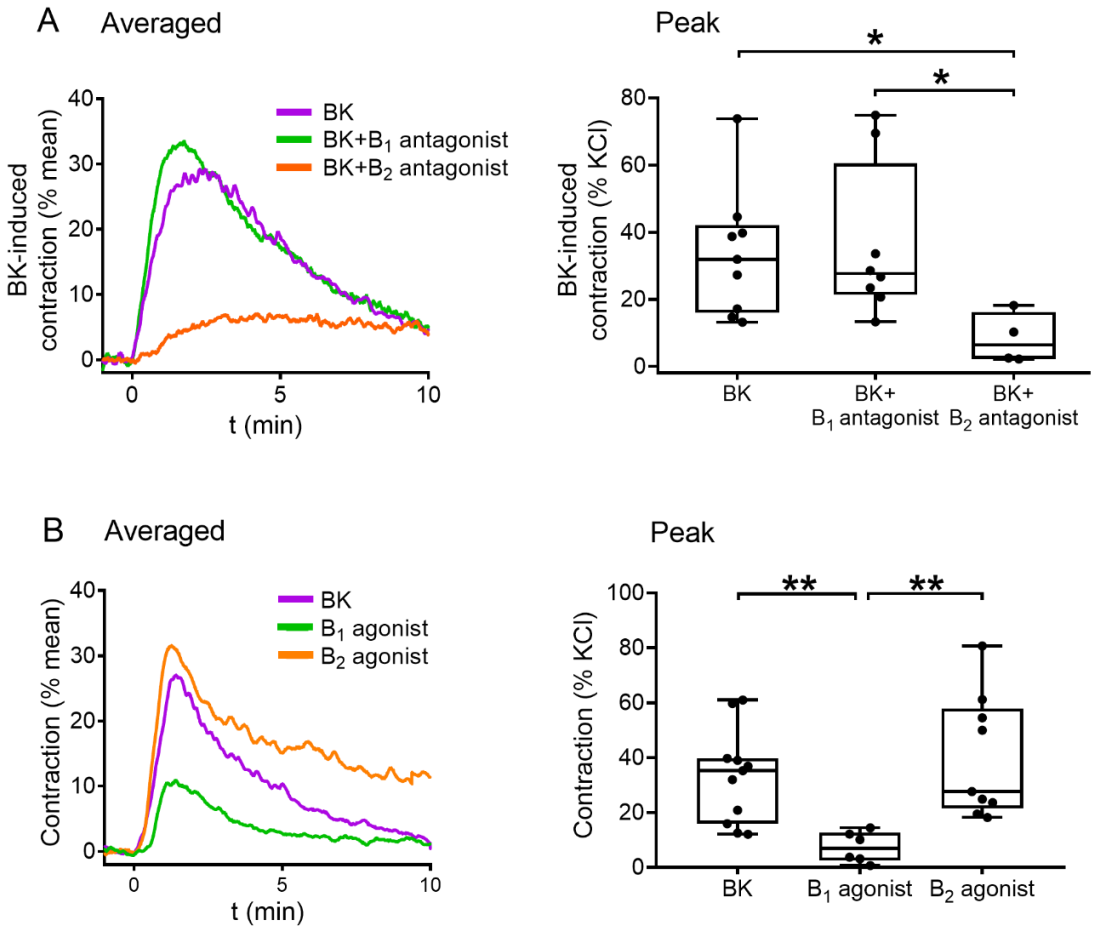

Figure 12. Bradykinin evokes concentration-dependent smooth muscle contraction in human urinary bladder mediated mostly by B_2 receptors. A: Contractile responses evoked by BK (10^{-5} M) in human detrusor muscle were almost completely abolished in the presence of the B_2 receptor antagonist HOE-140 (10^{-6} M, 20 min), whereas the B_1 receptor antagonist R-715 (10^{-6} M, 20 min) failed to reduce BK-induced contractions. **B:** The B_2 receptor agonist (10^{-5} M) induced contractions similarly to BK, whereas the B_1 receptor agonist (10^{-5} M) had only minor contractile activity in human detrusor muscle strips. (A-B: Kruskal-Wallis test, *$p < 0.05$) Case numbers: A: BK: n=9/3, B_1 antagonist: n=8/3, B_2 antagonist: n=4/3, B: BK: n=11/4, B_1 agonist: n=6/3, B_2 agonist: n=9/3.

4.4. Role of the RhoA-ROCK pathway in mediating human bladder contractions induced by bradykinin

Bladder strips were also treated with Y-27632 (10^{-5} M, 20 min) to examine the involvement of ROCK in mediating the effect of BK, and similarly to our observations in mice, the ROCK inhibitor decreased BK-induced contractile responses of the human detrusor muscle as well (*Fig. 13*).

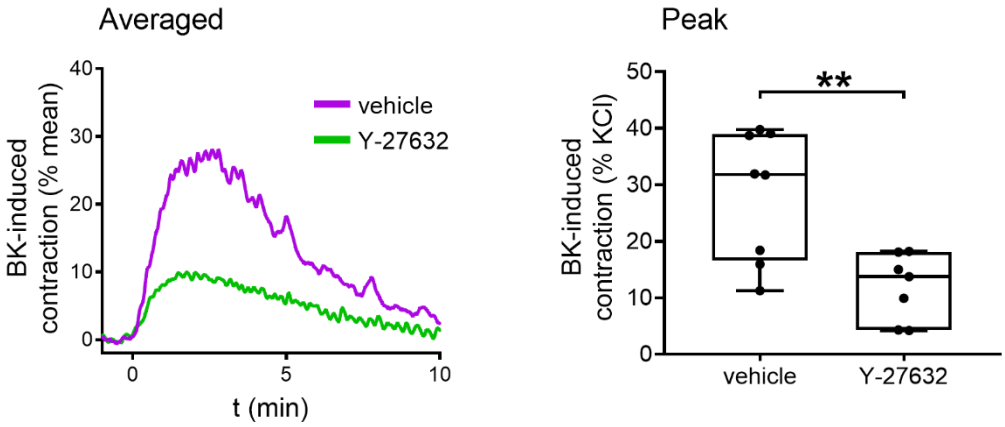

Figure 13. The role of the RhoA-ROCK pathway in mediating human detrusor contraction evoked by bradykinin. The ROCK inhibitor Y-27632 (10^{-5} M, 20 min) markedly reduced the contractions elicited by BK (10^{-5} M) in human UBSM. (Mann-Whitney test, **p < 0.01) Case numbers: vehicle: n=8/2, Y-27632: n=7/2.

5. Discussion

Various concepts have emerged over the years to explain the pathomechanism of DO and OAB, such as the myogenic (34, 35), the neurogenic (32) and the urotheliogenic hypotheses (33). Lately, these disorders have rather been considered as multifactorial, and it is accepted that the precisely regulated bladder functions (storage and voiding) may be disrupted at different levels of the control system (106). Although the underlying cause of OAB and DO is still unclear, uncontrolled detrusor smooth muscle contractions as a result of over-excitation via various stimulants are undoubtedly part of the etiology of these disorders (107). Therefore, our experiments focused primarily on the role of mediator molecules that may play a significant role in regulating micturition contractions and their signaling pathways in mouse and human UBSM.

Systematic inflammatory diseases (e.g. atherosclerosis, metabolic syndrome, IBS) are often associated with LUT dysfunctions (e.g. BPE, OAB), suggesting a possible overlap in the etiologies (e.g. intestine – bladder connection in IBS patients with OAB comorbidity or atherosclerosis associated with symptoms of DO) (39, 42, 47). It is still disputed, how close the connection between systemic inflammation and LUT dysfunctions is. Nevertheless, inflammatory mediators (e.g. PGs, BK, NGF) have an undisputable role in both. Therefore, there is a pressing need to outline the effects of inflammatory mediators in the urinary bladder, as it has become clear that they may play an important role in the development of bladder dysfunction. Bradykinin is an inflammatory mediator that has gained attention in the field of LUT dysfunctions, as it is present in the urine (108), its concentration increases in bladder dysfunctions (50) and induces smooth muscle contractions in several organs (intestine, prostate, trachea, gallbladder) (67, 109-111). Thus, we aimed to understand better and describe the effects and signaling pathways of BK in mouse and human UBSM.

Our research demonstrated that BK evokes concentration-dependent contractions in murine as well as in human UBSM. However, slight differences are noticeable in BK's effects in the bladders of the two species. BK contracted the mouse bladder samples with higher potency compared to the human UBSM (EC_{50}: $1.2x10^{-6}$ M and $5.1x10^{-6}$ M, respectively) and the maximal force of the contractions was also stronger in mice (E_{max}: 52% and 42%, respectively), although these differences are not significant

statistically. Interestingly, we also found interspecies-differences in the characteristics of contractile responses of UBSM tissues to KCl, CCh, BK, and α,ß-meATP, as the induced contractions were more prolonged in human as compared to the mouse bladders.

As BK may stimulate nerve endings to release various mediators, such as ACh and ATP (112), we aimed to investigate whether BK induces contractions directly in the UBSM, or perhaps, its effect is mediated by the release of ACh or ATP. Neither pharmacological inhibition of muscarinic ACh receptors by atropine, nor application of the purinergic receptor antagonist PPADS affected the contraction evoked by BK in our experimental setup. It has also been demonstrated by others that inhibiting nerve fiber activation with tetrodotoxin, thus inhibiting mediator release from nerve endings, fails to alter BK-induced detrusor muscle contractions (113). Taken together, BK appears to stimulate UBSM directly without the involvement or neurotransmitter release.

BK may also facilitate the release of other inflammatory mediators, like prostanoids, which may also induce detrusor muscle contractions (114, 115). Thus, our next goal was to explore the role of prostanoids in mediating BK-induced contractions in mouse bladders. Our data clearly shows that in our experimental setup, COX enzyme-derived mediators did not contribute to the contractile effect of BK, as the contractions remained unaltered in TP-KO or COX-1-KO mice, as well as following incubation with the non-isoenzyme-specific COX-inhibitor, indomethacin, or the specific COX-2 inhibitor NS-398. Given that COX-2 enzyme expression may increase in COX-1-KO mice to compensate for the lack of the enzyme (116), the effect of BK was also tested in COX-1-KO mouse bladders treated with NS-398. We concluded that in our experiments BK's contractile effect was independent of COX enzyme-derived mediators as well. Interestingly, secondary PG-release contributes reportedly to the effects of BK in some tissues including the bladder (117-119). However, besides the smooth muscle tissue, the mucosa layer is the major source of PGs (120), which was removed in our study, as we focused specifically on the effects and signaling of BK in the UBSM.

There is an intriguing question one may ask in the context of BK-induced UBSM contraction: What is the source of BK in the urinary bladder? As mentioned previously, it has been shown that BK is present in the urine, moreover, its concentration increases under pathological conditions, such as LUT dysfunctions (50). However, the primary source of BK is still a subject of debate. The urothelium is a tight barrier that is highly

impermeable to most of the compounds in the urine. The mucosa layer, however, has diverse functions in regulating bladder tone including the release of various mediators, which can easily reach their receptors in the UBSM (121). It has been reported that BK is released from the bladder mucosa under physiological conditions, moreover, the mucosa layer also plays a major role in degrading the peptide (122). Thus, it seems unlikely that BK diffuses through the urothelium and the mucosa layers reaching the UBSM to induce contraction. It is more plausible that BK released from the mucosa acts on smooth muscle, nerve endings, and interstitial cells. Nevertheless, the dispute is not settled completely, as the increased permeability of the urothelium in bladder pathologies should also be taken into consideration. In this case, it is possible for large molecules to reach the deeper layers of the bladder wall and thus, the UBSM. Therefore, in bladder dysfunctions, BK acting on the UBSM may come from the mucosa as well as the urine (123).

Once proven that BK acts directly, activating its receptors in the mouse bladder smooth muscle, we intended to assess the contribution of the BK receptors to the contractile effect and their intracellular signal transduction pathways. The B_2 receptor antagonist HOE-140 abolished contractions almost completely, whereas the B_1 receptor antagonist R-715 failed to alter them, indicating that B_2 receptors play the main role in mediating the response. These results were affirmed by the observations that application of the B_1 agonist induced only a mild increase in bladder tone. This is in contrast to the potent contractions evoked by the B_2 receptor agonist that resembled the effect of BK. As mentioned previously, B_2 receptors are more abundant in the bladder tissue under physiological conditions, compared to B_1 receptors, thus, our result is in accordance with the expression levels (124). However, it should be noted, that under pathological conditions, such as inflammatory environment or tissue injury, B_1 receptors are upregulated and therefore may gain more significance in bladder dysfunctions.

Subsequently, the downstream signaling pathways of B_2 receptors were in the focus of our research. As these receptors belong to the GPCR family and may readily couple to $G_{q/11}$ or, less frequently, to $G_{12/13}$ proteins, the effect of BK was investigated in $G\alpha_{q/11}$-KO or $G\alpha_{12/13}$-KO mouse UBSM. The results of these experiments indicated that both $G\alpha_{q/11}$- and $G\alpha_{12/13}$-coupled pathways contribute to the contractions, as they were markedly diminished in the bladder tissues of either KO mouse strains. The contribution of both

G proteins to the BK-induced contractions appears to be a unique characteristic of the UBSM, as it was reported that B_2 receptors exist mainly in complexes with $G\alpha_{q/11}$ proteins in the cell membrane, thus BK commonly acts exclusively via $G\alpha_{q/11}$ protein activation (125).

The unexpected finding that BK's effect is mediated partly by $G\alpha_{12/13}$ proteins prompted us to conduct further research on this pathway. The ROCK enzyme has received much attention recently in pathological processes of the LUT. Thus, we were curious whether it contributes to bladder contractions induced by BK. Our results indicated unambiguously that the contractions were inhibited by the ROCK-inhibitor Y-27632, suggesting an important role for this enzyme.

To further evaluate whether pathways other than $G_{q/11}$ and $G_{12/13}$-ROCK may also have a role in mediating the contractions, we treated $G\alpha_{q/11}$-KO mouse bladder strips with the ROCK enzyme inhibitor, Y-27632. As BK failed to elicit any contractions under these conditions, we concluded that the Ca^{2+}-dependent $G_{q/11}$ and the Ca^{2+}-sensitizing $G_{12/13}$-ROCK pathways simultaneously convey the effect of B_2 receptor activation, and they are the exclusive mediators of BK-evoked contractions in the mouse UBSM.

Additionally, we also planned experiments to investigate the contribution of $G_{q/11}$- and the $G_{12/13}$-coupled pathways to contractions evoked by PGs. PGs have also been proposed as important inflammatory mediators in LUT dysfunctions and overlapping in the signaling of different inflammatory-mediator-evoked UBSM contractions may provide target for designing new drugs for treatment. We found that the $G\alpha_{q/11}$- and the $G\alpha_{12/13}$-mediated pathways take part simultaneously in both PGE_2- and $PGF_{2\alpha}$-induced UBSM contractions. This implies that these two G protein-coupled pathways are of great significance, they are shared among different inflammatory mediator families in the UBSM, thus research aiming to elucidate their roles under physiological and pathological conditions is a promising approach for finding novel drug targets for treating bladder dysfunctions.

Evaluation of BK's intracellular signaling in mouse bladder built a solid foundation for continuing our research in human bladders. Experiments assessing the effect of BK in human bladder tissue are scarce. Thus, providing details on the signaling mechanism may aid understanding of this process better. Application of the same B_1 and B_2 receptor

antagonists as were used in our mouse bladder experiments proved that B_2 receptors are the main mediators of BK-evoked contractions in human UBSM as well. Our experiments with the B_1 and B_2 agonists also confirmed the major role of B_2 receptors, as, similarly to our mouse experiments, the contraction elicited by the B_2 agonist resembled that evoked by BK, whereas the B_1 agonist had only a minor effect in human UBSM. The expression profile of B_1 and B_2 receptors in the human bladder is very similar to that of the mouse bladder, as it was demonstrated that predominantly B_2 receptors are expressed in human cultured detrusor muscle cells (80) and our experiments gained with human samples are in accordance with this result. It should be noted again that the human bladder tissues used in the present study showed no sign of any obvious pathology. However, the receptor expression and thus the contribution of the different BK receptors to the contraction may alter in bladder disorders which means that further research, such as receptor expression analysis is needed, particularly in bladder tissues obtained from patients experiencing symptoms of OAB.

As tissue-specific, conditional gene deletion is not an option in the case of human bladder samples, the role of G protein-coupled pathways cannot be directly investigated. Nevertheless, the ROCK enzyme may be a promising drug target, given its increased expression under pathological conditions. As its inhibitor, Y-27632 reduced BK-evoked contractions markedly in mouse bladder tissues, we anticipated that ROCK may play a role in mediating contractions in human UBSM as well. Indeed, our predictions were supported by the experimental results, as Y-27632 inhibited BK-evoked contractions, proving that ROCK has a prominent contribution to BK's effect not only in mouse but also in human bladder.

Altogether, our results demonstrated that BK is a potent constrictor in mouse and human UBSM exerting its effect primarily via B_2 receptors in both species *(Figure 14)*. It was also proven by investigating genetically modified mice that both the $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins are activated following BK binding to B_2 receptors. Furthermore, ROCK enzyme, a downstream regulator within the $G\alpha_{12/13}$-coupled pathway, markedly decreased BK's contractile effect in murine as well as human UBSM. Administering the ROCK-inhibitor to $G\alpha_{q/11}$-KO mouse bladders abolished BK-evoked contractions, suggesting that the $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins are exclusive transducers between the B_2 receptor and its effectors. Moreover, we also proved that these two G protein-coupled

pathways play an important role in the signaling of other inflammatory mediators, namely PGE_2 and $PGF_{2\alpha}$.

As it has been implied that the ROCK enzyme may have an important role in pathological processes, and the inhibition of ROCK enzyme induces relaxation of the detrusor muscle, thus, increases bladder compliance. Consequently, ROCK enzyme together with B_2 receptors are appealing drug targets for treating bladder smooth muscle dysfunctions, especially OAB and DO.

However, besides the promising results, there are some limitations of the study that must be noted and assessed in further research. As previously mentioned, the proportion of B_1 and B_2 receptor-contribution to BK's effect may shift towards the B_1 receptors in tissue injury and inflammation, thus it would be interesting to repeat our experiments in mouse models of bladder dysfunctions (e.g. OAB, DO, urethral obstruction). In addition, our research focused specifically on UBSM, but one should note that BK is reported to induce the release of various mediators from the mucosa layer and it also activates somatosensory C-fibers (126). Thus, investigating its effect on the bladder under *in vivo* experimental conditions (with cystometry), where the neural circuits remain intact, would be also extremely intriguing and could provide valuable data. We are planning to address these problems and questions in our further experiments striving for a thorough understanding of the bladder regulation and its disorders.

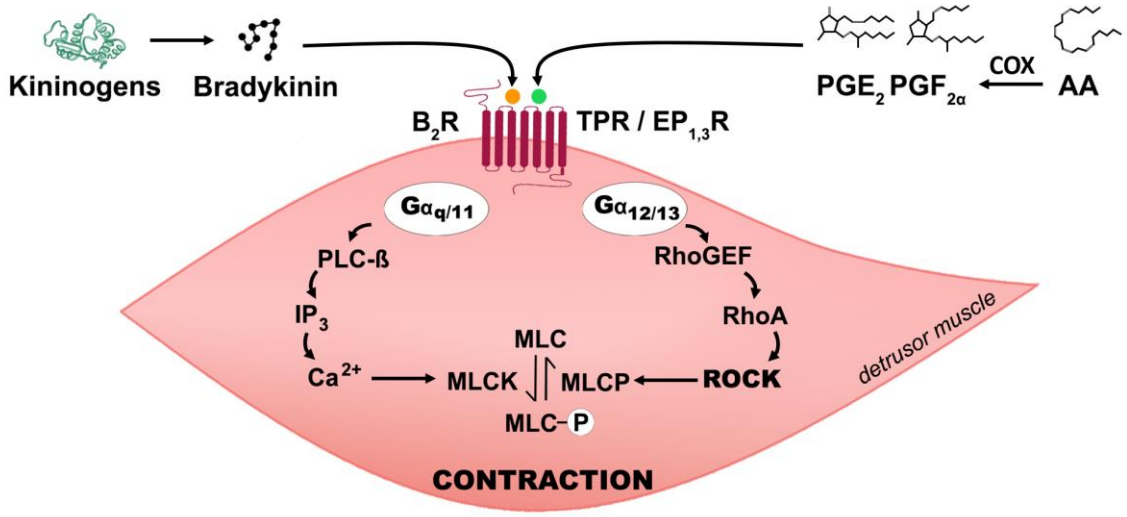

Figure 14. Summary. Bradykinin contracts both murine and human urinary bladder smooth muscle acting primarily on B_2 receptors. Along with the $G\alpha_{q/11}$-coupled pathway, the $G\alpha_{12/13}$-RhoA-ROCK cascade plays a significant role in mediating BK-induced contractions. In addition, both $G\alpha_{q/11}$ and $G\alpha_{12/13}$ protein-mediated pathways play an important role in PGE_2- and $PGF_{2\alpha}$-induced contractions, highlighting their possible role in regulating bladder smooth muscle tone under systemic inflammatory conditions. Altogether, B_2 receptors, prostanoid receptors and the ROCK enzyme may provide promising therapeutic targets for treating bladder dysfunctions.

6. Conclusion

The urinary bladder has two major functions: to store the increasing volume of urine while maintaining a relatively low intravesical pressure and to expel urine efficiently without any residual volume. This requires a finely regulated control system and if the complex network is damaged at any level of the hierarchy, it will result in LUT dysfunctions, such as OAB and DO. Pharmacological treatment of these disorders is still unsolved, as the currently applied gold standard therapy is symptomatic. Thus, seeking novel and more specific therapeutic targets is needed. Lately, systemic inflammation and inflammatory mediators have been implicated in the pathogenesis of bladder dysfunctions, especially with OAB. Our study focused on BK with an outlook on the intracellular signaling of PGs for comparison.

The novel findings of our research are listed below:

- It was proved that BK induces potent and dose-dependent constriction in both mouse and human detrusor muscle.
- The contraction is independent of the secondary release of other mediators (ACh, ATP, or COX enzyme-derived substances), thus BK appears to act directly in the UBSM.
- The BK-induced contractions are mediated primarily via activating B_2 receptors in both species.
- The intracellular signal transduction of B_2 receptors involves both the $G\alpha_{q/11}$ and $G\alpha_{12/13}$ protein-coupled pathways simultaneously and exclusively.
- It was also shown that $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins play an important role in PGE_2- and $PGF_{2\alpha}$-induced contractions, suggesting that these pathways may be involved in bladder pathologies associated with inflammatory mediators.
- The prominent role of the ROCK enzyme was demonstrated in mediating the BK-induced contractions in both mouse and human UBSM.

7. Summary

Our results indicate that BK elicits marked, dose-dependent contractions in both mouse and human UBSM, which effects are independent of the secondary release of ACh, ATP, or COX-derived mediators. The receptor preference of BK was also investigated and it was demonstrated that BK acts predominantly via activating B_2 receptors in both species in our experiments. Evaluation of mouse bladder experiments aiming to assess the downstream signaling of the B_2 receptors showed that the Ca^{2+}-dependent $G_{q/11}$- and the Ca^{2+}-sensitizing $G_{12/13}$-pathways both convey the effect of B_2 receptor activation. This sheds light on a unique feature of the UBSM, as BK's effects are usually mediated via only the $G_{q/11}$ and less frequently through the G_i, $G_{12/13}$, and G_s proteins. The role of the ROCK enzyme, which is part of the $G_{12/13}$ protein-coupled signaling pathway, was also affirmed in mediating BK-elicited mouse bladder contractions. As BK failed to induce any contractions in $G\alpha_{q/11}$-KO mouse bladders treated with the ROCK inhibitor, Y-27632, it was also proved that the $G_{q/11}$- and the $G_{12/13}$-coupled pathways exclusively mediate BK's effects in mouse UBSM. These pathways were also investigated in contractions evoked by other inflammatory mediators, namely PGE_2 and $PGF_{2\alpha}$. We proved that the two signaling cascades have an important role in PG-induced contractions, implying that their further research may be interesting regarding bladder dysfunctions associated with inflammatory mediators.

The contribution of ROCK enzyme to BK-evoked contractions was demonstrated in human bladder strips as well, suggesting that ROCK enzyme as well as B_2 receptors may provide promising, novel therapeutic targets for bladder smooth muscle dysfunctions, such as OAB and DO. Nevertheless, investigating the role of BK in disease models is essential in future studies, as our experiments were conducted on healthy tissues and BK receptor expression may alter in bladder disorders, especially in OAB and DO.

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Further publication:

H. Balla, **K. Borsodi**, P. Őrsy, B. Horváth, P. J. Molnár, Á. Lénárt, M. Kosztelnik, É. Ruisanchez, J. Wess, S. Offermanns, P. Nyirády, Z. Benyó "Intracellular signaling pathways of muscarinic acetylcholine receptor-mediated detrusor muscle contractions" AMERICAN JOURNAL OF PHYSIOLOGY: RENAL PHYSIOLOGY 325 : 5 pp. F618-F628. (2023) **IF: 4.20**

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