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

PhD Thesis

Kinga Mihályi-Borsodi, PharmD

Theoretical and Translational Medicine Doctoral School

Semmelweis University

Supervisor: Zoltán Benyó, MD, DSc

Official reviewers: Tamás Kaucsár, MD, PhD

Balázs István Tóth, MD, PhD

Head of the Complex Examination Committee:

György Losonczy, MD, DSc

Members of the Complex Examination Committee:

Andrea Székely, PhD

Ákos Jobbágy, PhD, DSc

Budapest

2024

1. Introduction

Physiologic bladder function 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 detrusor overactivity (DO) and overactive bladder syndrome (OAB) syndrome. These conditions affect about 16% of the adult population in Europe. The etiology of the disorders is unknown, however, they often occur simultaneously with systemic inflammatory disorders (atherosclerosis, obesity). Thus, inflammatory mediators, especially prostaglandins (PGs) and bradykinin (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.

Bradykinin is a peptide mediator and besides its various effects in the human body, it induces smooth muscle contraction in various organs (e.g. prostate, intestines, stomach, gall bladder) via activating its B_1 or B_2 G protein coupled receptors (GPCR). A role for BK receptors in regulating bladder function has been implicated, as they are expressed in practically all cell types of the urinary bladder smooth muscle (UBSM). In addition, BK receptor expression is upregulated in bladder dysfunctions, highlighting their possible role in the development of bladder overactivity.

Prostaglandins are produced enzymatically from arachidonic acid via cyclooxygenase (COX) enzymes. The concentration of PGs is elevated in the urine of OAB patients, moreover, they also induce contraction in the UBSM. In the bladder, their effects are exerted mainly via EP_1, EP_2, TP, GPCRs.

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. Then, the Ca^{2+}-calmodulin complex is formed, activating the myosin light-chain kinase (MLCK) that leads to the phosphorylation of regulatory myosin light chain (MLC_{20}) and

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. Detrusor muscle contraction may occur via G protein-coupled receptor (GPCR) activation as well. There are two major GPCR-coupled pathways for inducing smooth muscle contraction. The $G\alpha_{q/11}$-coupled pathway includes activation of phospholipase C-ß which cleaves inositol-triphosphate that induces the release of Ca^{2+} from intracellular stores, which leads to the contraction. $G\alpha_{12/13}$-coupled receptor activation involving activation of the small G protein RhoA and consequently, Rho-kinase (ROCK), which inactivates MLCP, leading to a sustained contraction.

2. Objectives

As inflammatory processes have been implied in the etiology of bladder functions, our research focused on the effects and signaling pathways of inflammatory mediators, especially 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. 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 ($G\alpha_{q/11}$-KO and $G\alpha_{12/13}$-KO).

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.

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. 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 (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. 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. After several washes with normal Krebs solution, the effects of different agonist and antagonists were tested (see section 3.5).

3.5. Drugs and solutions

BK was 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 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 dissolved in saline to a stock solution of $2x10^{-1}$ M. Atropine was diluted in water to a stock solution of $1.44x10^{-4}$ M. α,β-meATP, PPADS and Y-27632 were dissolved in saline (α,β-meATP: 10^{-2} M, PPADS: 10^{-2} M and Y-27632: 10^{-3} M). Indomethacin was dissolved in DMSO (10^{-2} M stock concentration), as its aqueous solutions are quite unstable. NS-398 was dissolved in DMSO to prepare a 10^{-2} M stock solution. Both PGE_2 and $PGF_{2\alpha}$ were 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 maximum contraction was defined as the peak value of tension developed after the addition of agonists. 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. All data are presented with the median values except concentration-response curves, in which cases mean ± SEM was used. 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

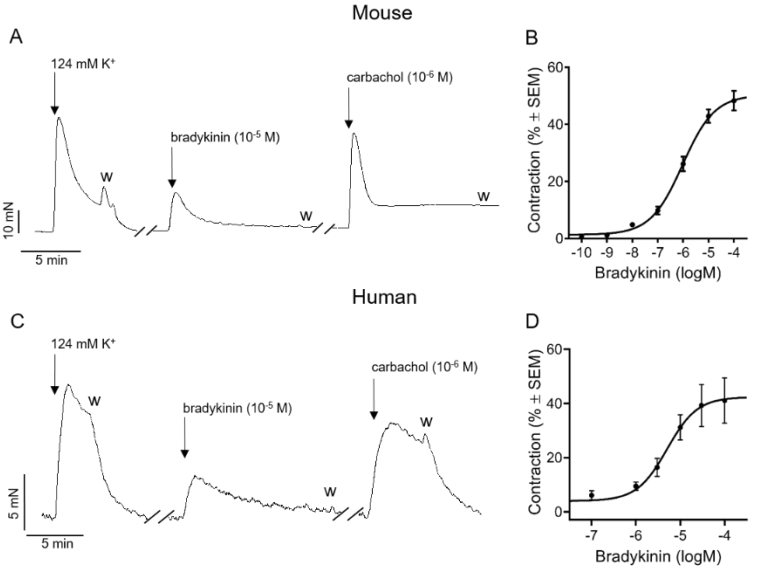

Figure 1. Bradykinin induces concentration-dependent contractions in mouse and human bladders with similar characteristics. A: Original trace: BK (10^{-5} M) evoked contractions in isolated murine detrusor smooth muscle strips which were comparable to the responses evoked by the muscarinic ACh receptor 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]

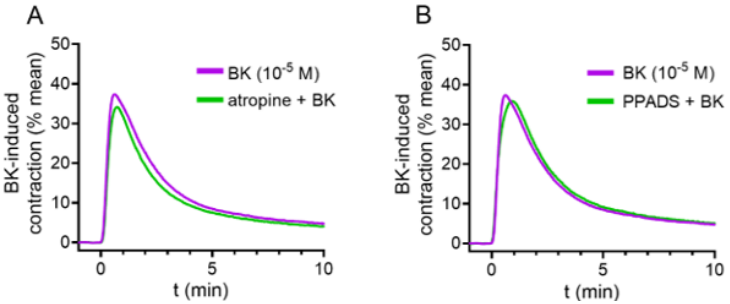

Figure 2. Bradykinin-induced detrusor muscle contraction is independent of purinergic or cholinergic neurotransmission. A, B: Neither inhibition of purinergic receptors with PPADS (10^{-5} M, 20 min incubation) nor the muscarinic receptor antagonist atropine (10^{-6} M, 20 min incubation) altered detrusor 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.

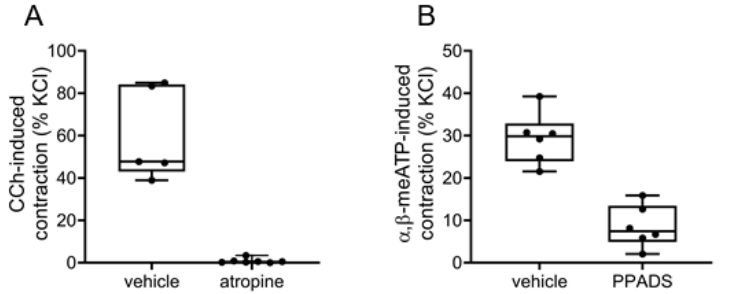

Figure 3. The muscarinic agonist carbachol- and the purinergic agonist α,β-meATP-induced contractions were abolished by the muscarinic (atropine) or purinergic (PPADS) antagonists, respectively. **A:** To verify the effectiveness of atropine (10^{-6} M, 20 min incubation), bladder strips were treated 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.

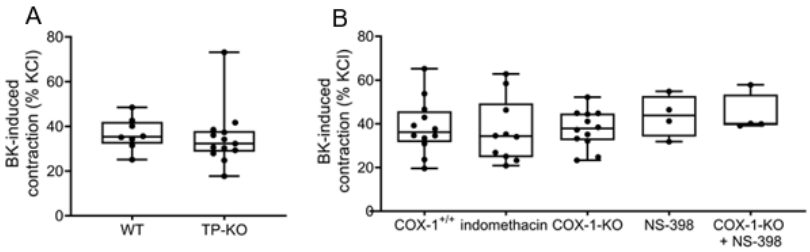

Figure 4. Bradykinin induces detrusor muscle contractions independently of COX-derived prostanoids. A: Contractile responses evoked by BK (10^{-5} M) were not altered in the bladder strips from mice deficient for TP receptors compared to those from WT mice. **B:** The presence of the nonspecific COX inhibitor indomethacin (10^{-5} M, 20 min) did not change BK-induced contractions. Furthermore, deficiency for 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.

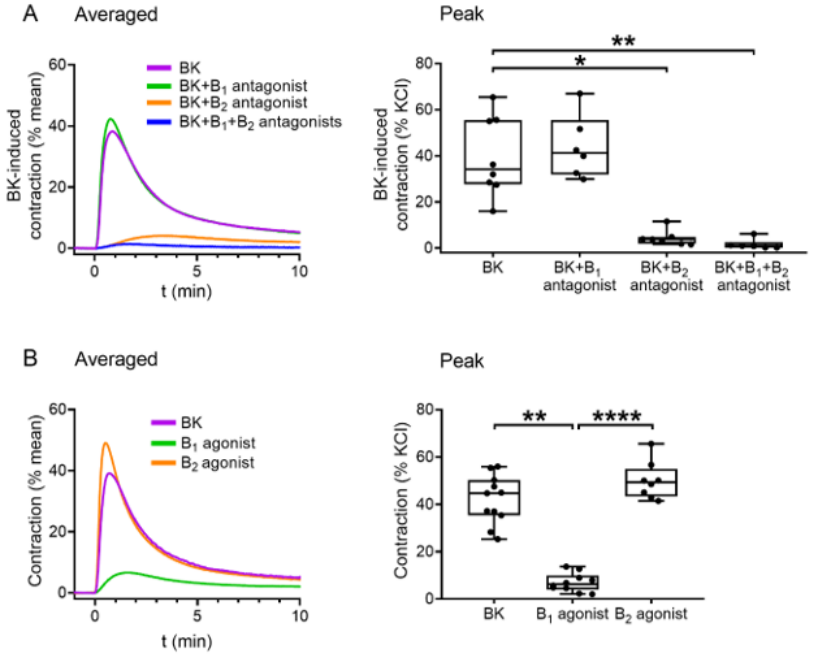

Figure 5. Role of B_2 receptors in mediating bradykinin-induced detrusor muscle contraction in murine urinary bladder strips. A: The BK (10^{-5} M)-induced contraction was abolished by the B_2 receptor-specific antagonist HOE-140 (10^{-6} M, 20 min), whereas the B_1 receptor antagonist R-715 failed to reduce it (10^{-6} M, 20 min). **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.

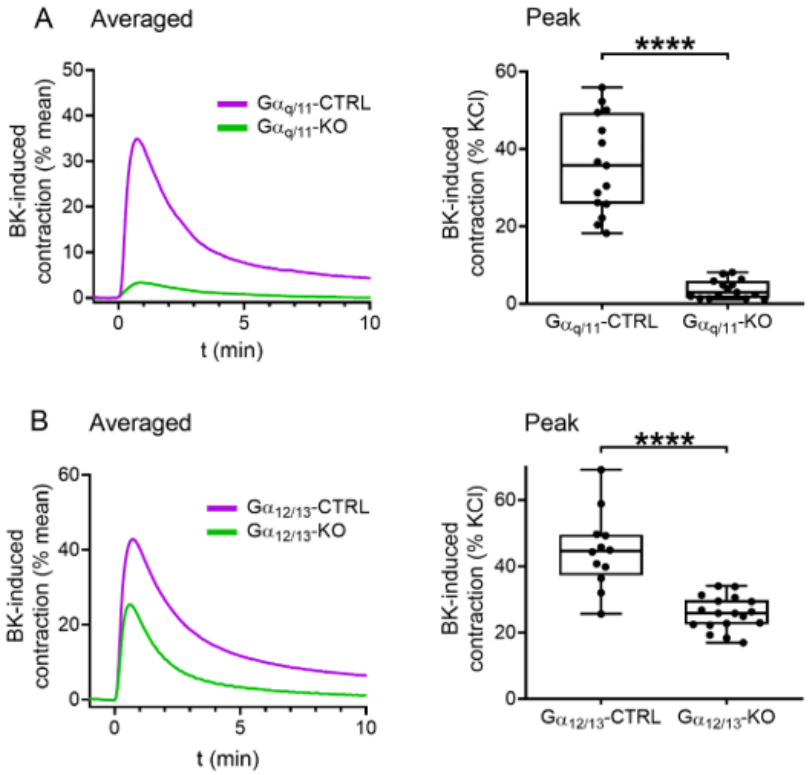

Figure 6. $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ G proteins mediate the effects of bradykinin in murine urinary bladder detrusor muscle. A: Contractile responses evoked by BK (10^{-5} M) were diminished in the UBSM from $G\alpha_{q/11}$-KO mice compared to bladder strips from $G\alpha_{q/11}$-CTRL. **B:** The detrusor contractions elicited by BK were also markedly 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.

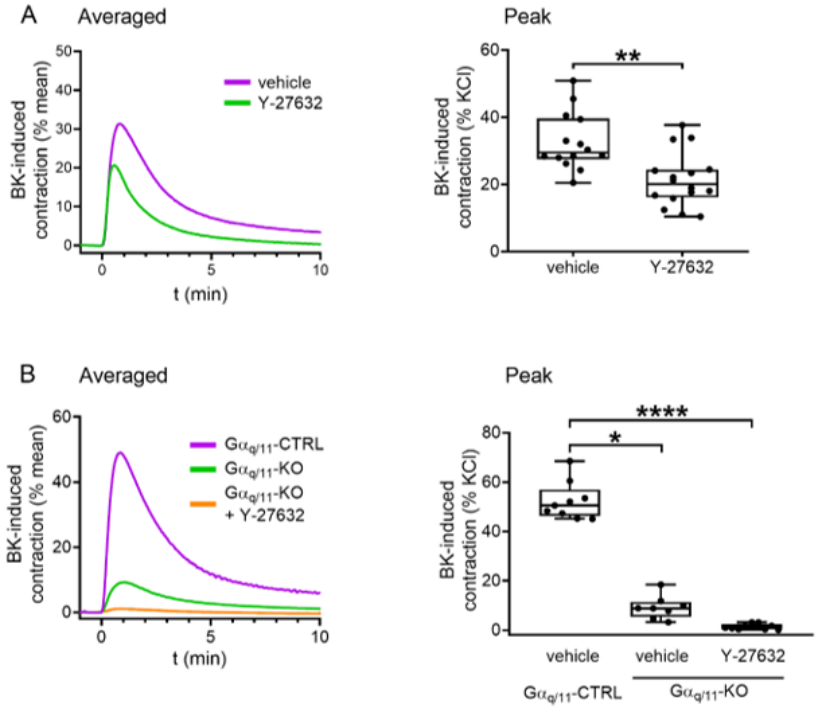

Figure 7. Role of Gα$_{12/13}$-RhoA-ROCK pathway in mediating bradykinin-induced contractions in murine UBSM. A: The contractile responses induced by BK (10^{-5} M) were decreased in the presence of the ROCK inhibitor (Y-27632, 10^{-5} M, 20 min). **B:** The addition of Y-27632 (10^{-5} M, 20 min) completely suppressed the remaining BK-induced contractile responses in UBSM strips from Gα$_{q/11}$-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, Gα$_{q/11}$-KO + vehicle: n=8/4, Gα$_{q/11}$-KO + Y-27632: n=9/4.

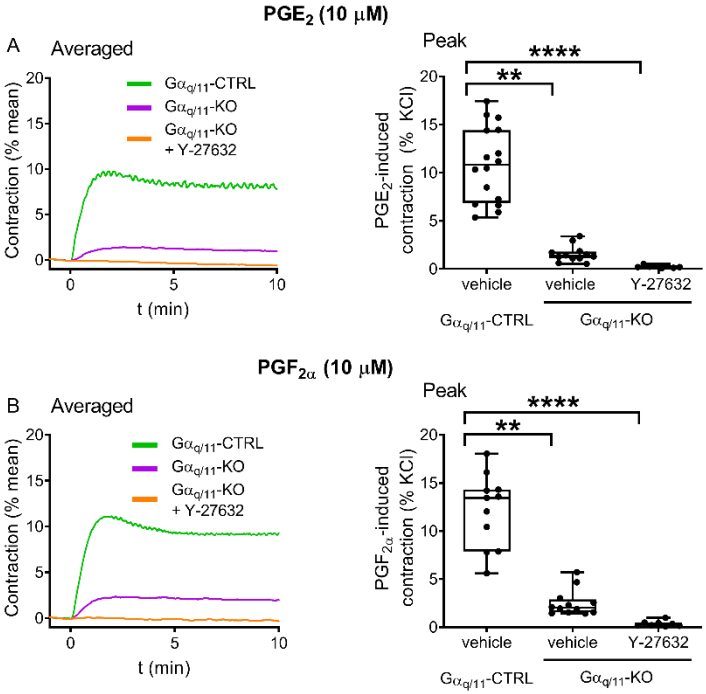

Figure 8. The role of $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ G protein-coupled pathways in PGE_2- and $PGF_{2\alpha}$-induced mouse bladder contractions. $G\alpha_{q/11}$ deficiency markedly reduced, whereas administration of the ROCK-inhibitor Y-27632 (10^{-5} M, 20 min) to $G\alpha_{q/11}$-KO mouse 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.

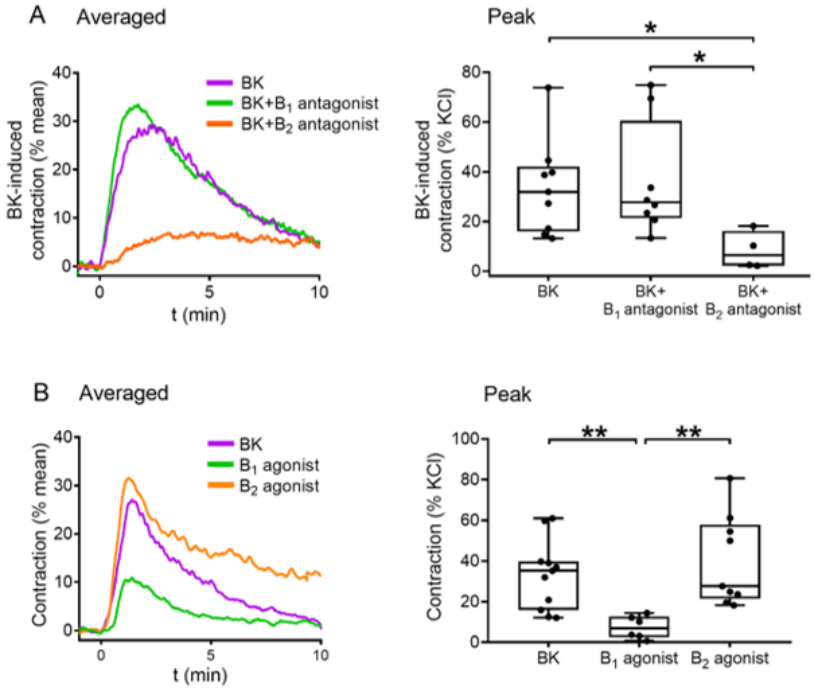

Figure 9. Bradykinin evokes concentration-dependent smooth muscle contraction in human urinary bladder mediated mostly by B_2 receptors. A: Contractile responses evoked by BK (10^{-5} M) in human detrusor muscle were almost completely abolished in the presence of the B_2 receptor antagonist HOE-140 (10^{-6} 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.

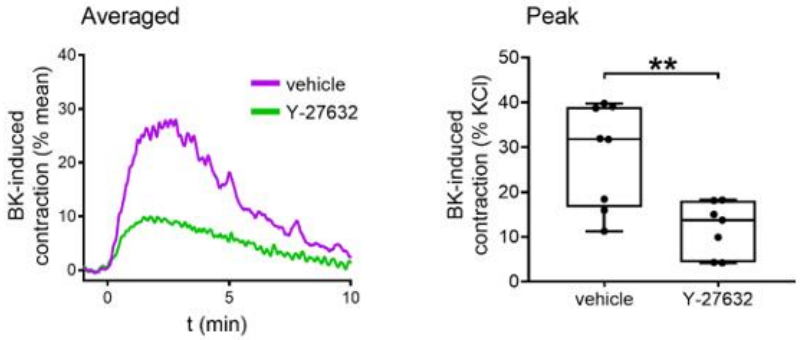

Figure 10. The role of the RhoA-ROCK pathway in mediating human detrusor contraction evoked by bradykinin. The ROCK inhibitor Y-27632 (10^{-5} M, 20 min) markedly reduced the contractions elicited by BK (10^{-5} M) in human UBSM. (Mann-Whitney test, **$p < 0.01$) Case numbers: vehicle: n=8/2, Y-27632: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

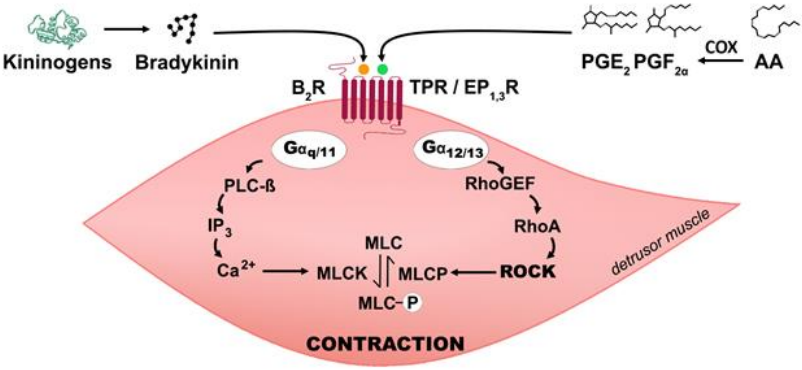

Figure 11. Summary. Bradykinin contracts both murine and human urinary bladder smooth muscle acting primarily on B_2 receptors. Along with the $G\alpha_{q/11}$-coupled pathway, the $G\alpha_{12/13}$-RhoA-ROCK cascade plays a significant role in mediating BK-induced contractions, therefore B_2 receptors and ROCK enzyme may provide a promising therapeutic target for bladder dysfunctions.

5. Conclusions

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 current first-line 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.

6. Bibliography of the candidate's publications

Publications related to the thesis:

K. Borsodi, H. Balla, P.J. Molnár, Á. Lénárt, I. Kenessey, A. Horváth, A. Keszthelyi, M. Romics, A. Majoros, P. Nyirády, S. Offermanns, Z. Benyó "Signaling Pathways Mediating Bradykinin-Induced Contraction in Murine and Human Detrusor Muscle" FRONTIERS IN MEDICINE 8 Paper: 745638 , 13 p. (2022) **IF: 3.90**

P. J. Molnár, B. Dér, K. Borsodi, H. Balla, Z. Borbás, K. Molnár, É. Ruisanchez, I. Kenessey, A. Horváth, A. Keszthelyi, A. Majoros, P. Nyirády, S. Offermanns, and Z. Benyó, "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, vol. 320, no. 4, pp. F537 F547, (2021) **IF:4.097**

Publications not related to the thesis:

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: 3.7**

∑IF: 11.697