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K+ Channels and Beta3 Adrenergic Receptors as New Pharmacological Targets for Overactive Bladder Treatment

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K^+ CHANNELS AND BETA3 ADRENERGIC RECEPTORS AS NEW PHARMACOLOGICAL TARGETS FOR OVERACTIVE BLADDER TREATMENT

by

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DEDICATION

This journey started almost twenty years ago, when as a ten year old kid, I used to dream about owning a BMW like the local pharmacist of my neighborhood. Back then, all I could think of was getting out of poverty, becoming someone highly educated, respected, and wealthy who could one day come back to his hometown and give back to the community. Someone, who could tell the youngsters that everything is achievable with hard work, dedication, and passion, no matter where you come from. As I was trying to figure out how to achieve my dreams, sports and education became for me the only options. I became obsessed with basketball despite growing up in a country where soccer was king. I do still remember practicing for hours, working on my jump shots, my drives, and my post game every single day in order to be the best I could ever be. And during these long nights when my legs were cramping up so bad that I couldn't sleep anymore, my mother always came to my room with a glass of water for me to drink while my father was massaging my calves and thighs in order to make sure that I was ready for the next day of school and practice. All this hard work finally paid off when I received a full scholarship in 2005 to play at Kansas State University. Even though basketball will always remain my #1 passion, my aspiration of being involved in pharmaceutical research and make significant contribution to the health care system, especially in developing countries, is what drives me right now. And now my dreams of driving a BMW and owning a pharmacy store have transformed to owning a multi-national pharmaceutical company because as a gamecock, my drive has "NO LIMIT".

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ABSTRACT

Overactive bladder (OAB) is a pathophysiological condition which is characterized by the urinary bladder's failure to achieve proper storage of urine. OAB has a tremendous impact on the patient's quality of life by disturbing his sleep, work, and sexual activity. About 17% of Americans are affected with this disease and most patients experience symptoms of urgency, frequent urination, and abnormally elevated detrusor smooth muscle (DSM) contractility. Despite, numerous attempts to treat the disorder in the past with antimuscarinics and now with beta3 adrenergic receptors (beta3-AR agonists), OAB still remains a serious public health issue because the long term efficacy of these drugs remains questionable. Therefore, it is critical to explore new avenues and design new strategies in order to help improve the quality of life of more than 33 million people. K^+ channels and beta3-AR have been shown to be key regulators of smooth muscle excitability and contractility in various species; however, information about their expression and functional role in the DSM of humans was limited. In our study, we first investigated the molecular expression of several K^+ channels in human DSM cells using RT-PCR, qPCR, western blot, and immunostaining techniques, then tested their ability to affect DSM contractility by using isometric DSM tension recordings and selective pharmacological modulators. We found that two Ca^{2+}-activated K^+ channels including BK and SK3 channels as well as several voltage-gated K^+ channels including Kv2.1, Kv2.2, Kv7.4, and Kv7.5 channels were expressed in human DSM cells. We also found that pharmacological inhibition of BK or SK3 channels with iberiotoxin, a BK channel

blocker or apamin, a SK channel blocker, respectively, significantly increased human DSM spontaneous and nerve-evoked contractions *in vitro*. Similarly, pharmacological inhibition of Kv2 channels with stromatoxin-1, a Kv2 channel antagonist or Kv7 channels with XE991, a Kv7 channel antagonist, also increased human DSM spontaneous phasic and nerve-evoked contractions *in vitro*. On the other hand, application of retigabine, a Kv7 channel activator induced relaxation of human DSM strips spontaneous phasic and nerve-evoked contractions. As for the role of beta3-AR in human DSM, we found that pharmacological activation of beta3-AR with BRL37344, a beta3-AR agonist causes relaxation of human DSM nerve-evoked contractions *in vitro*. We further demonstrated that the beta3-AR mediated relaxation observed during nerve-evoked contractions was facilitated by BK channel activity. Finally, we obtained direct evidence suggesting that the decrease in BK channels expression and function is associated with symptoms of neurogenic detrusor overactivity.

In conclusion our study suggest that pharmacological modulation of K^+ channels (BK, SK3, Kv2, and Kv7 channels) or beta3-AR affect DSM contractility, therefore making K^+ channels and beta3-AR likely drug targets for the treatment of OAB.

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List of Abbreviations

ACh ..... Acetylcholine

Beta3-AR ..... beta3 adrenergic receptor

BK ..... Large-conductance voltage and Ca^{2+}-activated K^+ channel

BRL37344..... 2-(3-Chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid

DS ..... dissection solution

DSM ..... detrusor smooth muscle

EFS ..... electrical field stimulation

IK ..... intermediate-conductance Ca^{2+}-activated K^+ channel

Kv ..... voltage-gated K^+ channel

L-364373 ..... 5-(2-Fluorophenyl)-1,3-dihydro-3-(1*H*-indol-3-ylmethyl)-1-methyl-2*H*-1,4-benzodiazepin-2-one

L-755507 ..... 4-[[(Hexylamino)carbonyl]amino]-*N*-[4-[2-[[(2*S*)-2-hydroxy-3-(4-hydroxyphenoxy)propyl]amino]ethyl]phenyl]-benzenesulfonamide

LUT ..... lower urinary tract

NDO ..... neurogenic detrusor overactivity

OAB ..... overactive bladder

PSS ..... physiological saline solution

ScTx1 ..... stromatoxin-1

SK ..... small conductance Ca^{2+}-activated K^+ channel

TTX ..... tetrodotoxin

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

XE991 ..... 10,10-*bis*(4-Pyridinylmethyl)-9(10*H*)-anthracenone

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CHAPTER 1

INTRODUCTION

1.1 ANATOMY OF THE LOWER URINARY TRACT

The basic function of the lower urinary tract (LUT) is to secure urine storage in the bladder and achieve voluntary evacuation of the stored urine. The LUT is composed of two main parts: the urinary bladder and the urethra.

The urinary bladder is a distensible reservoir which initiates urine voiding by contracting its muscular wall composed essentially of detrusor smooth muscles (DSM) (102). The urinary bladder has a triangular pyramidal shape and is located in the anterior part of the pelvis (102). It is composed of the fundus, superior surface; the base, posterior surface; and two triangular side walls. The lowest part of the bladder where the lateral walls meet the base is called the bladder neck and the lowest part of the neck is the trigone (**Fig. 1.1.1**). The bladder has three distinct tissue layers which are the serosa, the DSM, and the urothelium. The serosa is the outer layer which is composed of adventitial connective tissue. The DSM is the middle layer- it comprises interlacing fibers which can either be randomly oriented or oriented in longitudinal, circumferential, and longitudinal patterns. The urothelium is the innermost layer- it is composed of transitional cell epithelium (**Fig. 1.1.1**).

The urethra is the portion of the lower urinary tract by which the urine is expelled. In females, the urethra is relative short (only few centimeters) while in males, the total

length approximates 20 cm. The urethra is composed of four sections in males which include prostatic, membranous, bulbar, and penile sections (**Fig. 1.1.1**). The membranous urethra is approximatively 2 cm long and is surrounded by the striated external urethral sphincter (EUS) which is innervated by pudental nerves. The external urethral sphincter is under the subject voluntary control and relaxes during urine voiding to allow urine flow.

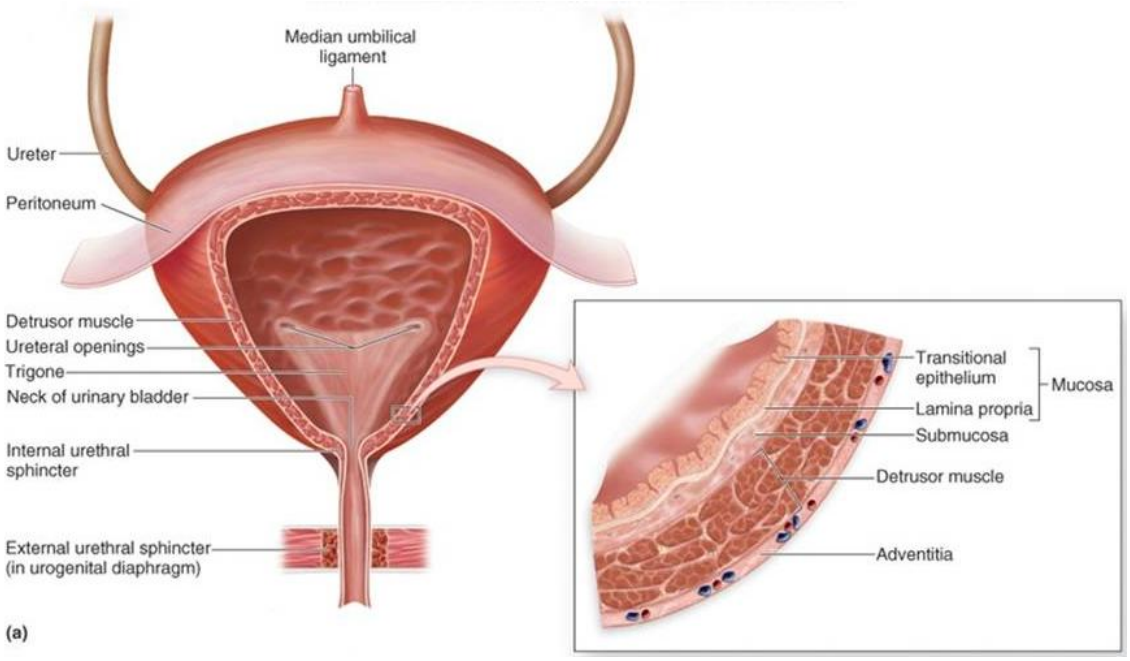

Figure 1.1.1 Anatomy of the urinary bladder. (a) Anterior view of the urinary bladder. Urine enters the urinary bladder via the ureters and exit via the urethra. Inset: cross-section of the bladder wall. Reprinted with permission from McGraw-Hill companies. http://academic.kellogg.edu/herbrandsonc/bio201_McKinley/f27-9a_urinary_bladder_c.jpg

1.2 THE MICTURITION PROCESS

The micturition process is a complex mechanism which requires a synergy of action between myogenic and neurogenic components. It includes a filling or storage phase and an emptying or voiding phase. The three major structures of the LUT which are the bladder, the urethra, and the EUS are innervated by afferent nerves which play different roles in urine storage and voiding (**Fig. 1.2.1**). During the bladder filling phase, information concerning the degree of distention of the bladder as well as the amount of stored urine is transmitted to the brain via several mechanosensitive afferent nerves present in the bladder (38). These nerves have been identified as Aδ afferent nerves. It has been reported that when the bladder is filled at 60-90% of its capacity, the desire to void exists but can be delayed by the subject if necessary. However, behind 90% capacity, a strong desire to void is reported and accidental urine leakage becomes common because the subject can no longer control his external urethral sphincter (38, 44).

Sympathetic Storage Reflexes

The filling phase of the micturition is an involuntary process shown to be under the control of sympathetic nerves input (38). During the filling phase, Aδ vesical afferents nerves are activated to induce the sacrolumbar intersegmental spinal reflex which in response causes an increase in urethral outflow resistance. This reflex also increases the tonic discharge of motoneurons innervating the striated muscles of the EUS (25, 152). Normally, this reflex pathway becomes inhibited when the intravesical pressure raises to the level required to producing voiding (**Fig. 1.2.1**). Inhibition of the sympathetic innervation can reduce urethral outflow resistance, reduce bladder capacity, and increase

the frequency and amplitude of bladder contractions (38). Therefore, this vesicosympathetic reflex represents a negative-feedback mechanism that allows the bladder to accommodate considerable amount of urine (38).

Voiding Reflexes

The voiding phase of the micturition has both voluntary and involuntary components which are regulated at the pontine micturition center (PMC) and the spinal cord levels, respectively (**Fig. 1.2.1**) (38). The involuntary/reflex pathway becomes activated when the mechanosensitive Aδ afferents present in the bladder are sensitized by the increase in intravesical pressure. The influx travels to the sacral spinal cord and initiate the voiding reflex (**Fig. 1.2.1**). During urine voiding, it has been shown that the urethral smooth muscles are relaxed and nitric oxide, an inhibitory neurotransmitter is released in the urethra. Additionally, PMC becomes activated which further leads to the inhibition of sympathetic storage reflexes. Finally, during micturition, the excitatory bladder reflex pathway is also activated by the flow of urine through the urethra and the sensitization of urethral afferents nerves (38).

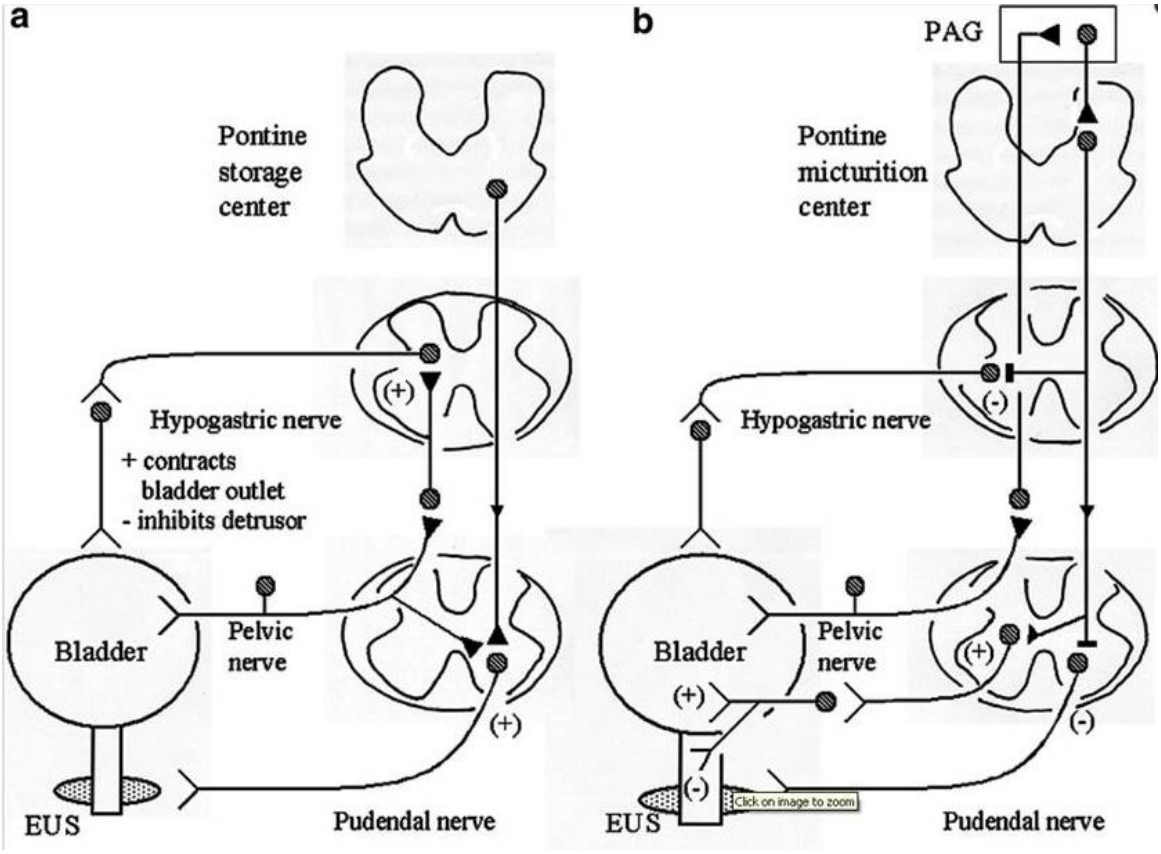

Figure 1.2.1 Neural circuits controlling continence and micturition. (**a**) Urine storage reflexes. During the storage of urine, distension of the bladder produces low-level afferent firing in the pelvic nerve, which in turn stimulates (1) the sympathetic outflow to the bladder outlet (base and urethra) and (2) pudendal outflow to the external urethral sphincter. These responses occur by spinal reflex pathways and represent guarding reflexes, which promote continence. Sympathetic firing also inhibits detrusor muscle and modulates transmission in bladder ganglia. A region in the rostral pons (the pontine storage center) increases external urethral sphincter activity. (**b**) Voiding reflexes. During elimination of urine, intense bladder afferent firing activates spinobulbospinal reflex pathways passing through the pontine micturition center, which stimulate the parasympathetic outflow to the bladder and internal sphincter smooth muscle and inhibit the sympathetic and pudendal outflow to the urethral outlet. Ascending afferent input from the spinal cord may pass through relay neurons in the periaqueductal gray (*PAG*) before reaching the pontine micturition center. Reprinted with authorization from (De Groat et al. 2009).

Neural and hormonal control of the micturition process

The contraction and relaxation of the urinary bladder are regulated by cholinergic, adrenergic, and non-adrenergic non-cholinergic (NANC) mechanisms.

The cholinergic mechanism is mainly involved in the urinary bladder emptying phase. This pathway is mediated via activation of muscarinic receptors (7). In most species, including humans, the M2/M3 muscarinic receptors are the most physiologically relevant (7). During the bladder emptying phase, acetylcholine (ACh) an excitatory neurotransmitter is released from nerve terminals and activates M2/M3 muscarinic receptors. Acetylcholine activation of M2 muscarinic receptors causes inhibition of adenylyl cyclase activity while activation of M3 receptors triggers the inositol 1,4,5-trisphosphate pathway (55, 104) (**Fig. 1.2.2**). In normal humans, nearly 100% of bladder contraction is due to muscarinic activation (11, 142).

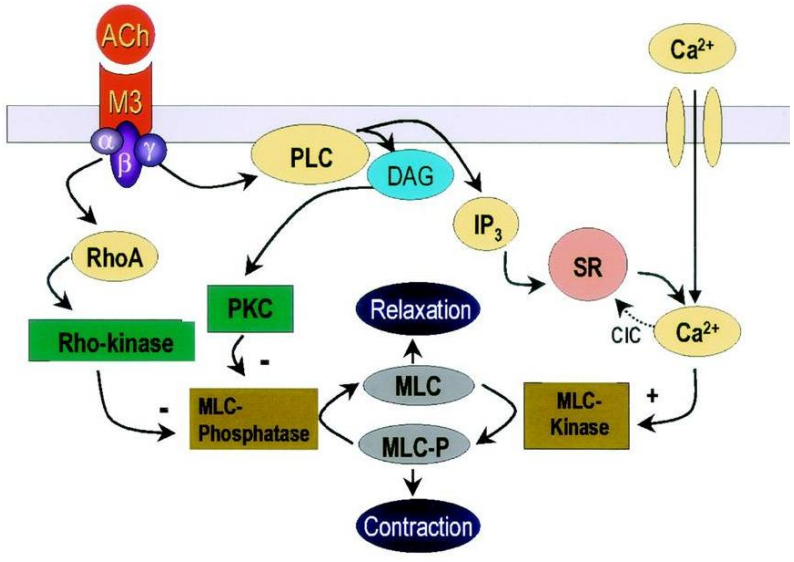

Figure 1.2.2 Transmitter signal pathway (1 – 4) involved in activation of detrusor contraction via muscarinic M3 receptors. Ach, acetylcholine; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MLC, myosin light chain; SR, sarcoplasmic reticulum; CIC, calcium-induced calcium release. There seem to be differences between species in the contribution of the different pathways in contractile activation. In human detrusor, Ca^{2+} influx (3) is of major importance. Reprinted from Andersson et *al*. 2004 with permission.

The adrenergic mechanism is mainly involved in the bladder filling phase. Norepinephrine is released from bladder nerve terminals and activates beta1-, beta2-, and beta3- adrenergic receptors to cause DSM relaxation (7, 79, 130). In the human DSM, beta3- ARs are suggested to be the most important as they represent more than 90% of all beta-AR (168). Activation of beta adrenergic receptors stimulates adenylyl cyclase to increase cAMP production. In turn, cAMP activates the protein kinase A pathway to further induce DSM relaxation (7, 79, 130). This beta3-AR induced relaxation of the DSM has recently been shown to be mediated by the large conductance-calcium activated K^+ channel (**Fig. 1.2.3**) (79, 130).

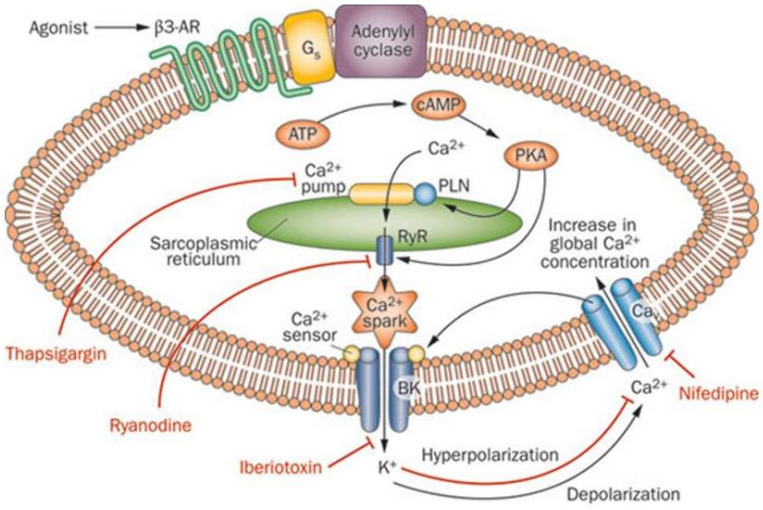

Figure 1.2.4. Schematic illustration of the functional coupling between the beta3-AR and the BK channel in the detrusor smooth muscle cell. The BK channel control the opening and the closing of the L-type Cav channels by depolarizing or hyperpolarizing the cell membrane, this regulating the global Ca^{2+} influx and spontaneous phasic contractions. BK channels are activated by localized Ca2+ signals ("Ca^{2+} sparks") caused by Ca^{2+} release from the RyRs of the SR, adjacent to the cell membrane. Stimulation of the beta3-AR with selective agonists leads to PKA activation, PLN phosphorylation, and activation of the SR Ca^{2+} pump and RyRs, which causes an increase in Ca^{2+} spark and TBKC activity. The increased TBKC frequency results in a sustained membrane hyperpolarization, closure of the L-type Cav channels, reduction in the global Ca^{2+} concentration, and detrusor smooth muscle relaxation. When Ca^{2+} spark are blocked with ryanodine and/or thapsigargin (a SR Ca^{2+}-pump inhibitor), the functional coupling between beta3-AR and BK channels is disrupted. Abbreviations: beta3-AR, beta3 adrenergic receptor; BK channel, large conductance voltage-activate and Ca^{2+}-activated K^+ channel; PKA, protein kinase A; PLN, phospholamban; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TBKC, transient BK current. Reprinted from Petkov (2012) with permission.

The NANC mechanism is mainly involved in the bladder emptying phase of the micturition process and is characterized by a fast contractile response upon neurotransmitter release (7). During the voiding phase of the micturition process, two major neurotransmitters ATP and acetylcholine are released from bladder nerves (59, 64, 119, 142, 161). In *in vitro* systems, neurotransmitters release can be achieved by applying electrical field stimulation (EFS) to DSM isolated strips (**Fig. 1.2.5**). While ACh is responsible for the cholinergic response, ATP will activate a NANC pathway, namely the purinergic pathway. Both pathways contributing to DSM contraction (24, 59, 64, 105, 119, 142, 160, 161). ATP activates purinergic (P2X1) receptors and therefore causes an increase in DSM cell excitability via an increase in Na^{+} and Ca^{2+} intracellular concentrations (59, 172). Evidence suggests that the contribution of the purinergic pathway to DSM contraction may become preponderant under pathophysiological condition of overactive bladder (OAB) in humans (6, 124, 171).

In addition to the release of ATP and ACh, calcitonin gene-related peptides and other substances such as nitric oxide are also released from nerves and have a relaxing effect on DSM (7, 48).

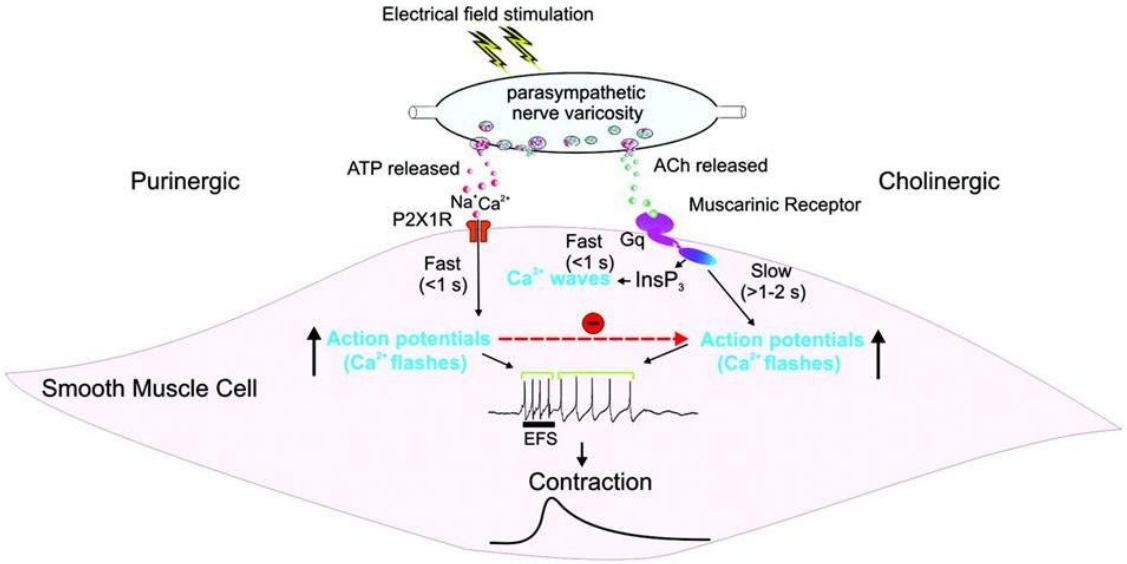

Figure 1.2.5. Schematic showing activation of purinergic and muscarinic pathways following release of neurotransmitters ATP and ACh from parasympathetic varicosities. Purinergic signalling rapidly evokes Ca^{2+} flashes during EFS whereas muscarinic signalling evokes Ca^{2+} waves during stimulation and Ca^{2+} flashes following stimulation. Purinergic activation appears to inhibit the muscarinic response. Ca^{2+} flashes from both purinergic and muscarinic pathways contribute to contraction. Reprinted from Heppner et *al*. (2009) with permission.

1.3 BLADDER DYSFUNCTIONS

The role of the LUT is to realize the storage and voiding of urine. This function is accomplished by a synergy of action between the autonomous and the central nervous systems, which control DSM relaxation and contraction during the filling and voiding phases, respectively (38). Any alterations in DSM contractility due to neuronal or muscular factors can affect the micturition process and render the LUT unable to accomplish its function. Bladder dysfunctions can be categorized into two groups based on the pathophysiological changes observed in the DSM. The first category includes bladder dysfunctions in which the DSM contractility is abnormally increased. Within this category, the most important pathophysiological condition is known as overactive bladder (OAB). The second category of bladder dysfunctions includes conditions in which the DSM contractility is decreased causing the LUT to retain urine for an abnormally long period of time. This condition is termed underactive bladder.

Overactive bladder is a pathophysiological condition which affects ~17% of the American population (147). Although, the disease affects people of all ages, studies report that the majority of OAB patients are 65 years old or more. OAB has a tremendous impact on patients' quality of life by affecting their sleep, work, and sexual activity. Patients affected with the disease, experience greater spontaneous phasic DSM contractions during the filling phase of the micturition process (16, 120) as well as nocturia, frequent urination, urgency, incontinence, and involuntary contractions of the DSM (147).

Underactive bladder is a less common pathophysiological condition which is defined as a contraction of reduced strength and/or duration resulting in prolonged or incomplete

emptying of the bladder (159). The disease has multiple origins including neuronal and muscular. Aging which causes decay in DSM activity is also seen as a contributing factor to this condition (159).

1.4 ION CHANNELS

Ions channels are protein structures anchored in the cell membrane to facilitate the flow of inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- down their electrochemical gradients (129). Ion channels are not only pores regulating the flux of ions through the cell membrane; they also play critical roles in numerous physiological processes including nerve and muscle excitation, action potential generation, muscle contraction, blood pressure regulation, cell proliferation, hormone secretion, learning and memory (129). In the human genome, more than 340 genes coding for ion channels have been identified and more than 60 ion channel targeting drugs are currently on the market to attest the importance of these proteins (129). Ion channels have two major functional properties-selectivity and gating.

Selectivity is the functional property which allows the channel to discriminate between ions by acting like a filter. With the exception of nonselective cation channels, most ion channels allow migration of a specific ion across the cell membrane (129).

Gating is the functional property that allows the channel to remain either in an open or closed state (129). When ion channel's gating is regulated by changes in the transmembrane voltage, they are termed voltage-gated ion channels (129). On the other hand, ion channels are named ligand-gated ion channels when the channel's gating is

regulated by a small molecule (ligand) which interact directly with the channel or with a receptor linked to the channel (129).

Classification and nomenclature of ion channels are based on the nature of their most important permeant ion. There are four major classes of ion channels- Na^+, K^+, Cl^-, and Ca^{2+} channels (129).

1.5 K^+ CHANNELS

K^+ channels are by far the largest and the most diverse group of ion channels. They are expressed in almost every cell type in which they play various physiological roles (129). Between 70 and 80 genes are known to code for K^+ channel proteins (129). In the DSM, the physiological role of K^+ channels is to regulate muscle contraction and relaxation (130). This function is achieved by a precise regulation of intracellular Ca^{2+} concentration which is controlled by Ca^{2+} entry via voltage-gated Ca^{2+} (Ca_v) channels (130). In general, K^+ channels' opening causes cell membrane hyperpolarization, interruption of Ca^{2+} entry via Ca_v channels, and DSM relaxation (129, 130). By contrast, K^+ channels' inhibition causes membrane depolarization, increase of Ca^{2+} entry via Ca_v, and DSM contraction (129, 130). Structurally, all K^+ channels form a pore-loop and can have 2 to 8 transmembrane (TM) domains (**Fig. 1.5.1**). K^+ channels are classified into four major families including voltage-gated (K_v), Ca^{2+}-activated (K_{Ca}), inward rectifying ATP-sensitive (K_{ir}), and two pore domain (K_{2P}) K^+ channels (129, 130).

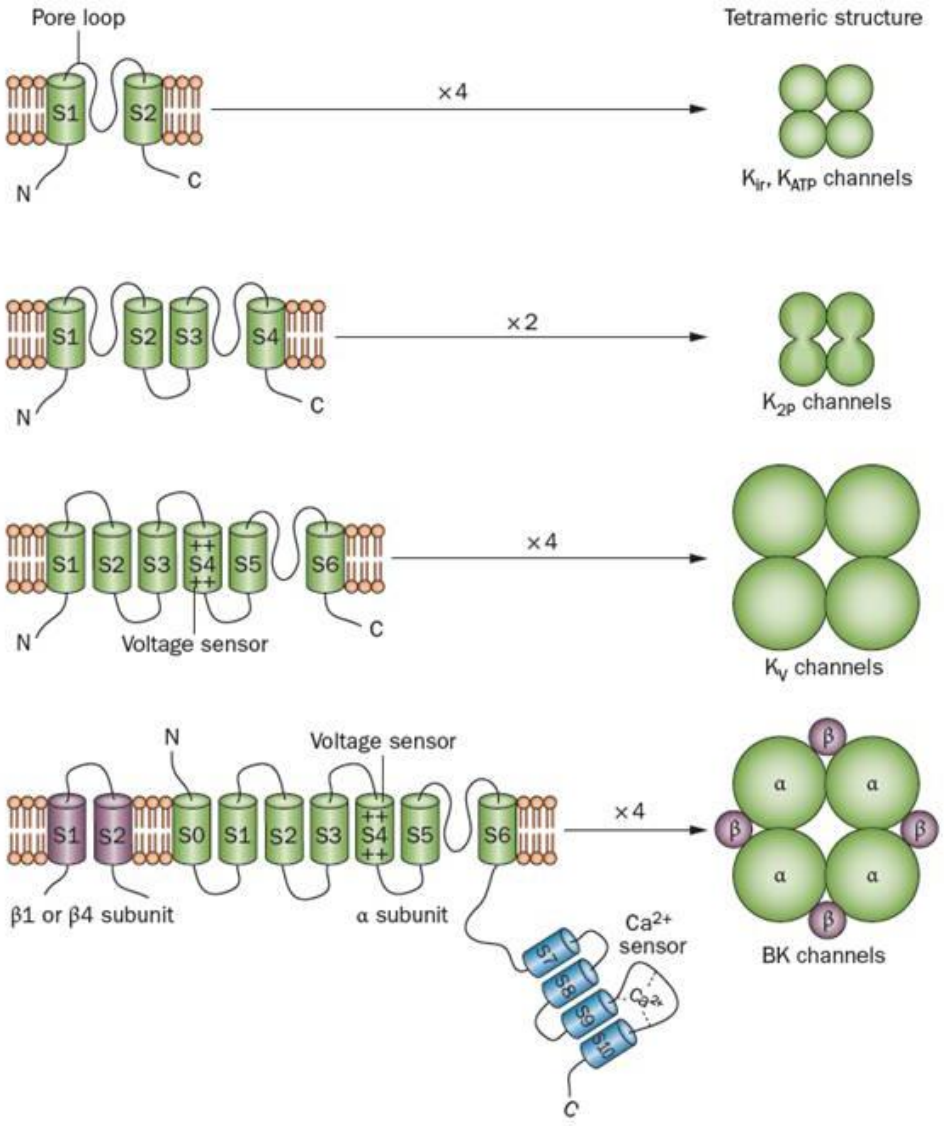

Figure 1.5.1. Illustration of the transmembrane architecture and subunit stoichiometry of the K^+ channel types expressed in detrusor smooth muscle cells. K_{ir} channels (represented by K_{ATP} channel) have the simplest K^+ channel structure, with two transmembrane segments (S) connected by a pore loop. Four such subunits form a functional tetrameric channel pore. K_{2P} channels form a tetrameric pore structure from two subunits each containing two pore loops. Kv channel subunits have six transmembrane segments with a voltage sensor in the S4 transmembrane domain. BK channels consist of four pore-forming α-subunits and the four regulatory β1 or β4 subunits. Abbreviations: BK channels, large-conductance voltage-activated and Ca^{2+}-activated K^+ channels; K_{2P}, two-pore-domain K^+ channels; K_{ATP}, channels, inward-rectifying ATP-sensitive K^+ channels; K_{ir} channels, inward-rectifying K^+ channels; Kv channels, voltage-gated K^+ channels. Reprinted from Petkov (2012) with permission.

Voltage-gated K^+ (Kv) channels form the most diverse family of K^+ channels and are encoded by more than 40 genes (130). Kv channels are known to participate in the repolarization phase of the action potential and in maintaining the cell's resting membrane potential (**Fig. 1.5.2**) (56, 60, 74, 77, 155). Kv channels are classified into 12 subfamilies (Kv1 – Kv12) based on the amino-acid sequence homology. Each Kv gene encodes a pore-forming α-subunit protein which assembles into tetramer to form a functional channel (130). While Kv1, Kv2, Kv3, Kv4, Kv7, Kv10, Kv11, and Kv12 channels can form their own homotetramers, Kv5, Kv6, Kv8, and Kv9 channels cannot but rather form heterotetramers by assembling with the Kv2 channel α-subunit (130). Despite, the significant number of Kv channel genes identified in the human genome, only a small fraction of Kv channels has been studied in the DSM suggesting that more work is needed to determine if these channels can potentially regulate DSM contraction and relaxation.

Ca^{2+}-activated K^+ (K_{Ca}) channel family has five members ($K_{Ca}1.1$, $K_{Ca}2.1$ $K_{Ca}2.2$, $K_{Ca}2.3$, and $K_{Ca}3.1$) which can be classified based on the single channel conductance (130). The large conductance voltage-activated and Ca^{2+}-activated K^+ ($K_{Ca}1.1$ or BK) channel is also known as big-K or maxi-K because of its high single channel conductance (100 – 250 pS) (130). The BK channel can be activated by both Ca^{2+} and voltage and is highly expressed in the DSM where it plays a role in maintaining the resting membrane potential and securing the repolarization phase of the action potential (60, 61, 67, 75, 80, 130). The small conductance Ca^{2+}-activated K^+ (SK) channels have a single channel conductance ranging from 5 to 20 pS (129, 130). There are 3 types of SK channels which

include SK1 ($K_{Ca}2.1$), SK2 ($K_{Ca}2.2$), and SK3 ($K_{Ca}2.3$) channels (129, 130). SK channels are voltage-insensitive and are regulated by Ca^{2+} and calmodulin (106). In the DSM, they are suggested to contribute to the afterhyperpolarization phase of the action potential (**Fig. 1.5.2**) (45, 56). The intermediate-conductance Ca^{2+}-activated K^+ ($K_{Ca}3.1$ or IK or SK4) channel has a single channel conductance ranging from 20 to 80 pS (129, 130). IK channels are activated by Ca^{2+} and insensitive to voltage changes (129, 130). Their role in the DSM remains unclear since most studies suggest that pharmacological modulation of these channels has no effects on DSM excitability and contractility (2, 127).

Inward-rectifying ATP-sensitive K^+ (K_{ir}) channels induce high amplitude of inward K^+ currents during the hyperpolarization phase of the action potential (129, 130). In the DSM, the K_{ir} channels are represented by the $K_{ir}6$ (K_{ATP}) channel which has a single channel conductance ranging from 11 to 12 pS (86, 87). These channels are activated by both low intracellular ATP and high ADP levels (129, 130). Whether K_{ATP} channels are physiologically relevant in the DSM remains controversial as evidence of their physiological role or not are conflicting (**Fig. 1.5.2**). Some studies argues in favor of K_{ATP} channels' ability to regulate DSM contractility and excitability (45, 58, 86, 87, 133, 141) while others suggest that activation of K_{ATP} channel with glibenclamide, a specific K_{ATP} channel inhibitor has not effects on DSM contractility and excitability (37, 133, 141).

Two-pore-domain K^+ (K_{2P}) channels has 15 members including ($K_{2P}1$ – $K_{2P}15$) which are mostly known for their role in stretch, oxygen, pH and temperature sensing (12, 129, 130). They play a role in maintaining the cell's resting membrane potential (**Fig. 1.5.2**). In the DSM, knowledge about K_{2P} channels is still at its infancy. The few studies which

investigated K_{2P} channels subtypes (TASK-1 and TASK-2) in the DSM suggest that these channels play an important role in stabilizing the DSM cell's resting membrane potential (12).

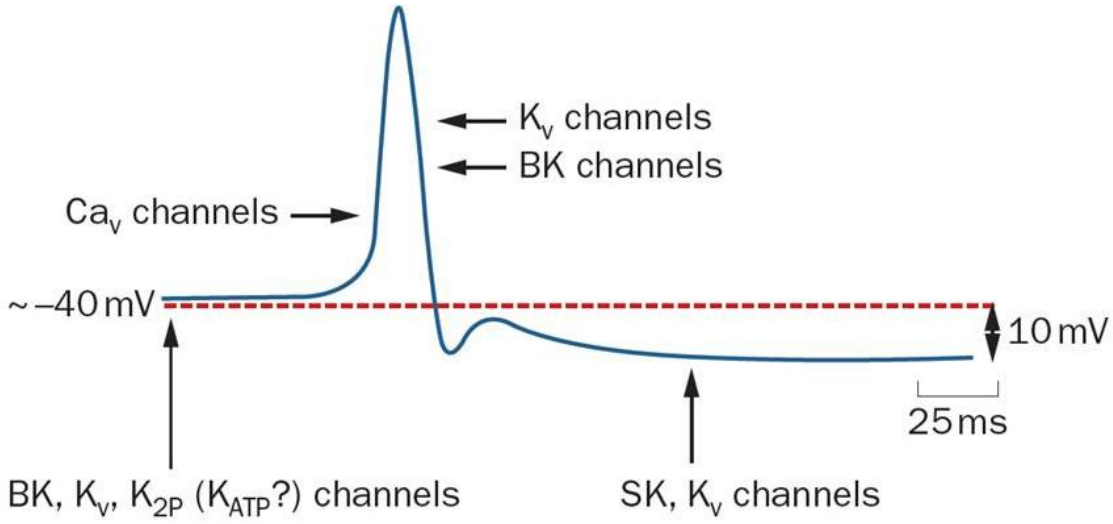

Figure 1.5.2. Schematic illustration of the detrusor smooth muscle action potential and the roles of various K^+ channels in determining resting membrane potential and action potential. BK, Kv, K_{2P}, and probably K_{ATP} channels determine the resting membrane potential. BK and Kv channels contribute to the initial repolarization phase of the action potential. SK and Kv channels contribute to the prolonged after-hyperpolarization phase of the action potential. Abbreviations: BK channels, large-conductance voltage-activated and Ca^{2+}-activated K^+ channels; Cav, voltage-gated Ca^{2+} channels; K_{2P}, two-pore-domain K^+ channels; K_{ATP}, channels, inward-rectifying ATP-sensitive K^+ channels; Kv channels, voltage-gated K^+ channels; SK channel, small conductance Ca^{2+}-activated K^+ channels;. Reprinted from Petkov (2012) with permission.

1.6 BETA3 ADRENERGIC RECEPTORS

Beta adrenergic receptors are receptor proteins which are expressed in a wide variety of cell types. They include three subtypes which are beta1-, beta2-, and beta3- adrenergic receptors. The molecular expression and functional role of beta-AR in the DSM varies from species to species. While in the guinea pig, beta1-ARs seem to be the most predominant and physiologically relevant (100); in rats, studies report that beta2- and beta3-AR are the most important (7). In the human DSM, although all three beta-AR have been identified (82, 83, 151, 168), qPCR data reveal that beta3-AR represent more than 90% all of beta-AR (168). Furthermore, the use of selective beta1- and/or beta2-AR on human DSM seems to have controversial effects (7). By contrast, pharmacological activation of beta3-AR has been shown to significant cause relaxation of the DSM contractions in humans (14, 27, 79, 81, 88, 90, 156). These studies suggest that beta3-ARs are key regulators of human DSM contractility. The mechanism by which β-AR activation causes relaxation of the DSM is thought to involve the cyclic cAMP (cAMP) signaling pathway (22, 115). cAMP activates protein kinase-A (PKA), which in turn phosphorylates various proteins involved in DSM excitation-contraction coupling and stimulates the BK channels to promote DSM relaxation (22, 58, 115). Beta3-AR agonists have been shown to increase the intracellular cAMP levels in native DSM tissues as well as in heterologously expressed systems (46, 69, 88, 94, 139, 150). Because of the preponderant role of beta3-AR in human DSM physiology, the FDA has approved mirabegron, a beta3-AR agonist for the treatment of overactive bladder (21, 27, 50, 51, 157).

CHAPTER 2
DISSERTATION OUTLINE

In the present chapter, I will describe the dissertation outline. I will first present the central hypothesis of my thesis and present the rationale that motivated my research during my PhD training. Next, I will described in details, the experimental approaches, methods, and techniques that I used to test my hypothesis. I employed two principal approaches in my research. The first approach consisted of identified the genetic markers of K^+ channels expression in the DSM. In this approach, I employed various techniques including RT-PCR, qPCR, western blot and immunostaining. These techniques were employed to identify the mRNA and the proteins expressions of specific K^+ channels. The second approach consisted of determining the functional role of the K^+ channels identified previously. The functional studies were performed using isometric DSM tension recording techniques and various pharmacological modulators. The majority of the data presented in this dissertation are based on these two principal approaches. In the context of my study, I will also present data collected by other members of the Petkov's lab that have been published with me as co-author. These additional data will provide further evidence for the role K^+ channels in the DSM.

2.1 CENTRAL HYPOTHESIS

Overactive bladder (OAB), a pathophysiological condition resulting from the urinary bladder's failure to achieve proper storage of urine which further lead to urgency, affects about 17% of Americans. For many years, the pharmacological treatment of OAB relied primarily on oral administration of antimuscarinic drugs which unfortunately have limited efficacy and dose-related side effects. Recently, the FDA has approved mirabegron, a beta3-ARs agonist, for the treatment of OAB. However, the ability of this drug to treat all forms of OAB remains uncertain. Intravesical injection of botulinium toxins has also been proposed but this approach is invasive, expensive, and uncomfortable for patients. Therefore, alternative treatment options are urgently needed. Numerous studies have demonstrated that various K^+ channels including the large-conductance Ca^{2+}-activated K^+ (BK) channels, the small (SK) and intermediate (IK) conductance Ca^{2+}-activated K^+ channels, and the voltage-dependent K^+ (K_V) channels are important regulators of the DSM excitability and contractility (2, 26, 29, 60, 65, 75, 77, 80, 120, 126, 130, 134, 145). Also, it has been revealed that during the urinary bladder filling phase, beta3-ARs are activated to induce DSM relaxation via the protein kinase A pathway. Therefore, we suggest that K^+ channels including K_V channels, K_{Ca} channels as well as beta3-AR could represent better pharmacological targets than antimuscarinics in the treatment of OAB. The objective of our study was to elucidate the physiological role and regulatory mechanisms of K^+ channels and β3-ARs in the DSM. **We hypothesized that K^+ channels and beta3-ARs play major roles in DSM excitability and contractility and could represent promising pharmacological targets for the treatment of various forms of OAB including neurogenic detrusor overactivity.**

2.2 EXPERIMENTAL APPROACHES, METHODS, AND TECHNIQUES

EXPERIMENTAL APPROACHES

To test our central hypothesis, we used various experimental approaches which allowed us to investigate the molecular expression of K^+ channels and beta3-ARs at the mRNA and protein level, and further assess the functional role of these proteins. Channels mRNA expression and relative quantity in DSM tissue was assessed using RT-PCR and qPCR techniques. We also developed a novel experimental approach of DSM single-cell enzymatic isolation to further determine mRNA messages in DSM cells. The single-cell RT-PCR and qPCR techniques allowed us to establish whether mRNA messages for various K^+ channels or beta3-ARs were expressed in DSM cells and not in other cells such as neurons, fibroblasts, interstitial cells which are also present in the DSM tissue. K^+ channels protein expressions were investigated by western blot and immunostaining techniques. Finally, the channels functional roles were addressed by using isometric DSM tension recordings and K^+ channels modulators.

METHODS AND TECHNIQUES

Human DSM tissue collection

All human studies presented in this dissertation were conducted according to the reviewed and approved institutional review board protocol HR#16918 of the Medical University of South Carolina (MUSC). Control human samples were collected from patients without a preoperative history of OAB symptoms. These control patients had an American Urological Association (AUA) symptom score<8 and did not have a clinically relevant neurological disease. NDO patients had overactive bladder (OAB) symptoms and were characterized by an American AUA symptom score >7. Human tissue samples were collected with patients' consent during surgeries such as radical cystectomy for bladder cancer and other open bladder surgeries for malignant or non-malignant conditions of the lower urinary tract. Two types of DSM samples were collected from each patient. The first sample was stored in ice-cold Ca^{2+}-free N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-buffered dissection solution (§Solutions and Drugs) and was used to conduct functional studies on DSM contractility, western blot, and immunostaining experiments. The second sample was kept in RNA-later (QIAGEN Sciences, Hilden, Germany) and was used for RT-PCR and qPCR experiments. Both samples were transported to the laboratory immediately after surgery.

Animal studies and DSM tissue harvesting

All animal studies including rat and guinea pig studies were performed according to the Animal Use Protocol #1747, reviewed and approved by the University of South Carolina

Institutional Animal Care and Use Committee. Sprague-Dawley rats and Hartley-Albino guinea pigs were euthanized by carbon dioxide inhalation followed by thoracotomy. The animal whole bladder was then removed by transversal incision above the bladder neck.

DSM cells isolation and collection

DSM cells were isolated from humans and guinea pigs DSM tissue as follows (1, 2, 22, 29, 30, 74, 75, 77, 79, 80, 127, 131, 133, 134, 143, 145, 164, 166): The bladder tissue was pinned on a sylgar-coated dish then the urothelium and the mucosa were removed under a dissection microscope using micro scissors. The DSM was then cut into strips (5–8 mm long, 2–4 mm wide), and 1–2 strips were placed in dissection solution (2 ml) supplemented with 1 mg/ml bovine serum albumin (BSA), 1 mg/ml papain, 1 mg/ml DL-dithiothreitol, and incubated for 25–30 min at 37 ºC. Next, the DSM strips were transferred into a dissection solution (2 ml) containing 1 mg/ml BSA, 1 mg/ml collagenase type II, and 100 µM $CaCl_2$ and incubated for another 5-6 min at 37 ºC. DSM strips were then washed with fresh BSA containing dissection solution. The individual DSM cells were dispersed when the enzyme-treated tissue was passed through the tip of a Pasteur pipette. Dispersed DSM cells were put in a glass chamber and allowed to settle at the bottom for about 20 min. Next, DSM cells were washed out 4-5 times using a perfusion system which removes the cells that do not adhere to the glass-bottom of the chamber. DSM cells are characterized by their spindle-shape, bright and shiny appearance under the Axiovert 40CFL microscope (Carl Zeiss®, Germany) as well as their responsiveness to mechanical stimulation which indicates the presence of functional contractile proteins and that they are physiologically active. DSM cells were collected

individually by suction into a glass micropipette using an MP-285/ROE micromanipulator (Sutter Instruments, San Rafael, California). A poll of approximately 100 DSM cells was collected for RNA extraction and further single-cell RT-PCR and qPCR experiments. The remaining DSM strips were kept in the dissection solution and used for functional studies and "whole tissue" RT-PCR and qPCR, western blot, and immunostaining.

RT-PCR and qPCR experiments

Using the RNeasy Mini Kit (Qiagen, Hilden, Germany), we extracted total RNA from whole DSM tissue, brain tissue, and a poll of enzymatically isolated DSM cells of humans and guinea pigs (1, 2, 74, 75). The extracted total RNA was reverse-transcribed into cDNA using M-MLV Reverse transcriptase (Promega, Madison, WI) and oligo d(T) primers. The amplification of the cDNA product was performed in the presence of GoTaq Green Master Mix (Promega, Madison, WI) and PCR primers for various K^+ channels and beta3-ARs, respectively using a mastercycler gradient from Eppendorf (Hamburg, Germany). Primers were designed based on the complete cDNA sequences of the K^+ channels and beta3-AR investigated and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). PCR products were loaded onto a 2% agarose ethidium bromide-stained gel and allowed to migrate via electrophoresis. The expected length of the fragment for each K^+ channels and beta3-ARs investigated was confirmed by using a 20 or 100 bp extended range DNA ladder from Lonza (Rockland, ME, USA). Guinea pig and human brain tissue were always used as positive control. For the qPCR experiments, we applied a two-step amplification followed by melting curve protocol using the IQ^{TM}5

Thermo Cycler system (Bio-Rad, California, USA). 10 – 100 ng/µL of whole DSM tissue or DSM isolated cells' cDNA was mixed with IQ SYBR Green Supermix (Bio-Rad, California, USA) and each individual K^+ channels primers separately then loaded onto a 96-wells plate. GAPDH was chosen as an internal control gene to analyze K^+ channel isoforms mRNA relative expression and each sample was run in duplicate or triplicate. All RT-PCR products from the whole DSM tissue, brain tissue, and DSM isolated cells of humans and guinea pigs were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Co., St. Louis, MO) and sequenced at the University of South Carolina Environmental Genomics Core Facility to confirm their identity.

Western blot

Approximatively 30 µg of total protein extracted from guinea pig whole DSM tissue was loaded into a 10 % Tris-HEPES-SDS precast polyacrylamide mini gels (Thermo scientific, Rockford, IL) and run at 150 V for 40 min using a PowerPac Universal power supply from Bio-Rad (Hercules, CA). Proteins were then transferred from the gel onto an immobilon-P PVDF membrane by semi-dry transfer technique using a Hoefer semi-dry blotter TE70XP (Fisher scientific). Next, the membrane was blocked by 5% non-fat dry milk in 0.5% TBST (0.5% Tween-20 in TBS) buffer for 1 h. Following the block, the membrane was incubated with the affinity-purified antibodies specific for each K^+ channel tested separately. Next, the membrane was washed with 0.5% TBST 4 times and re-incubated with secondary antibody (diluted to 1:1,000) in the blocking buffer for 1 h at room temperature. Bound antibodies were detected by the enhanced chemiluminescence substrate kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

To confirm the specificity of the primary antibody, additional Western blot experiments were conducted by incubating each primary antibody with its competing peptide.

Immunohistochemistry

Experiments were performed to determine whether K^+ channels proteins were expressed in DSM cells of humans and guinea pigs. DSM tissue was sliced into 2 X 2 mm and 6 µm thick sections using a vibratome Model G tissue slicer (Oxford Laboratories, Foster City, CA) and transferred in individual dishes for immunostaining. Tissue sections were incubated with primary antibody for each K^+ channel tested in 1% BSA/PBS at 37^{o}C for 1 h. Tissue sections were then rinsed multiple times with 1% BSA/PBS, incubated for one hour in 5% normal donkey serum in 1% BSA/PBS, then re-incubated with secondary antibody (1:100) in the dark for another hour. Tissue sections were subsequently washed with 1% BSA/PBS then PBS and then incubated with α smooth muscle actin (1:50) or phalloidin (1:50) in PBS for 2 h in the dark. Then DSM tissue sections were rinsed three more times with PBS, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:5000) in PBS for 15 min and then mounted with 1,4-diazabicyclo[2.2.2]octane **(**DABCO) (Sigma Aldrich, St. Louis, MO, USA). The specificity of the primary antibody was confirmed by incubating each K^+ channel antibody tested with its competing peptide. Tissue sections were visualized under a confocal microscope LSM 510 or 700 META (Carl Zeiss, Germany) 63X objective.

Isometric DSM tension recordings

Isometric DSM tension recordings experiments were performed as follows (1, 2, 22, 29, 74, 75, 77, 79, 80, 143, 145, 164, 166): DSM strips were clipped between a force displacement transducer and a hook then submerged into a thermostatically controlled (37^{o}C) 10 ml bath filled with Ca^{2+}-containing physiological saline solution (PSS) and aired with 95% oxygen- 5% carbon dioxide (**Fig. 2.2.1**).

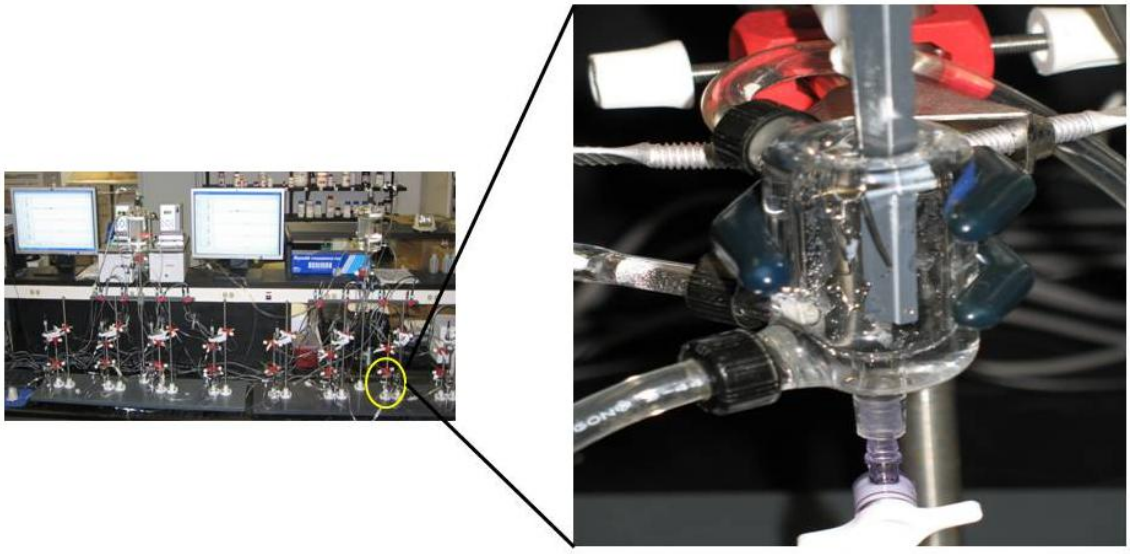

Figure 2.2.1. Isometric DSM tension recording setup used to study drug effect on DSM contractility *in vitro*.

DSM strips were then stretched to 1 g of tension to elicit spontaneous phasic contractions. Strips were washed out with fresh PSS every 15 min and allowed to equilibrate for a period 45 min to 1 h. On DSM isolated strips that exhibited spontaneous phasic contraction amplitude >0.1 g, tetrodotoxin (1 μM TTX), a neuronal Na^+ channels blocker was first added to the bath before a K^+ channels or beta3-AR modulator was applied. In DSM strips which exhibited spontaneous phasic contraction amplitude less than 0.1 g, contractions were induced by electrical field stimulation (EFS) and TTX (1 μM) was not added since TTX effectively suppresses EFS-induced contractions. Using a PHM-152I stimulator (Med Associates, Inc., Georgia, VT), we either applied stimulation of 10 Hz EFS frequency at 1 min intervals or we applied increasing EFS frequencies (0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, 50 Hz) at 3 min intervals. On the 10 Hz EFS-induced contractions, increasing concentrations of K^+ channel or beta3-AR activators and inhibitors were applied as described above. On the 0.5 – 50 Hz EFS-induced contractions, a single concentration of K^+ channel or beta3-AR modulator was applied. The EFS pulses had the following parameters: pulse amplitude (20 V), pulse width (0.75 ms), stimulus duration (3s), polarity was reversed for alternating pulses. The DSM response to EFS was recorded using MyoMed software (Med Associates).

Solutions and drugs

The Ca^{2+}-free dissection solution had the following composition (in mM): 80 monosodium glutamate; 55 NaCl; 6 KCl; 10 glucose; 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES); 2 $MgCl_2$; and pH 7.3 adjusted with NaOH. The Ca^{2+}-containing PSS was prepared daily and contained (mM): 119 NaCl; 4.7 KCl; 24

$NaHCO_3$; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 11 glucose; and aerated with 95% oxygen/5% carbon dioxide to obtain pH 7.4.

Data analysis and statistics

mRNA relative expressions was analyzed using the comparative threshold (Ct) method (ΔΔCt, delta-delta Ct) (101) after determining the Ct values for reference (GAPDH) and target genes for each sample. Fold changes in target mRNA expression level were calculated after normalization to GAPDH. We used MyoMed software (Med Associates, Inc., Georgia, VT) to record isometric DSM spontaneous phasic and nerve-evoked contractions. MiniAnalysis software (Synaptosoft, Inc., Decatur, GA) was used for data analysis of DSM spontaneous phasic and nerve-evoked contractions amplitude, muscle force (determined by integrating the area under the curve of the contractions), contraction frequency, contraction duration (determined at half width), and muscle tone (determined by contraction baseline curve).

For the spontaneous phasic contractions and the 10 Hz EFS-induced contractions, a 5 min period prior to the first compound application was taken as a control (100%) and the responses to subsequent compound application were normalized to that control. During cumulative addition of compounds, the effect of each compound concentration on contraction amplitude, muscle force, frequency, duration, and muscle tone was evaluated by analyzing a 5 or 10 min period prior to the following respective concentration application. For the 0.5–50 Hz EFS-induced DSM contractions, the contraction amplitude at EFS frequency of 50 Hz prior to compound application (control conditions) was taken to be 100% and the data were normalized. GraphPad Prism 4.03 software (GraphPad

Software, Inc., La Jolla, CA) was used for statistical analysis and CorelDraw Graphic Suite software (Corel Co, Ottawa, Ontario, Canada) was used for data illustration. In the summary data, **n** represents the number of individual DSM isolated strips or cells, and **N** is the number of guinea pigs, rats, or human subjects. Paired Student's t-test was used to analyze qPCR data, concentration-response, and frequency-response curves. All results are expressed as mean ± SEM; $p<0.05$ was considered to be statistically significant.

CHAPTER 3

Ca^{2+} -ACTIVATED K^+ CHANNELS IN THE DETRUSOR SMOOTH MUSCLE

Chapter 3 focuses on the physiological roles of Ca^{2+}-activated K^+ channels in the DSM. These K^+ channels comprise the large-conductance Ca^{2+}-and voltage-activated K^+ (BK) channel, the intermediate-conductance Ca^{2+}-activated K^+ (IK) channel, and the small-conductance Ca^{2+}-activated K^+ (SK1-3) channels. I have investigated all these ion channels individually and will present molecular and functional data collected in humans. A first section (chapter 3.1) addresses the role of BK channels in the pathology of neurogenic detrusor overactivity, a form of OAB. Data presented in this section have been submitted for publication and are currently under review. The second section (chapter 3.2) presents data pertaining to SK and IK channels expression and physiological roles in human DSM. These data have already been published (2) and have been slightly modified with authorization of the publisher to fit the format of this dissertation.

3.1. NEUROGENIC DETRUSOR OVERACTIVITY IS ASSOCIATED WITH DECREASED EXPRESSION AND FUNCTION OF THE LARGE CONDUCTANCE CA^{2+}-ACTIVATED K^+ CHANNELS IN DETRUSOR SMOOTH MUSCLE

Data presented in this section have been submitted for publication and are currently under revision. The original manuscript has been modified to fit the format of this dissertation. Human tissues were surgically obtained by Dr. Rovner's team from the Medical University of South Carolina. Dr. Rovner is a collaborator with the Petkov's lab

ABSTRACT

Neurogenic detrusor overactivity (NDO) is a form of bladder dysfunction which can affect patients suffering from various neurological diseases such as spinal cord injury, Parkinson's disease, and multiple sclerosis. Large-conductance Ca^{2+}-activated K^+ (BK) channels are key regulators of the detrusor smooth muscle (DSM) excitability and contractility. In the present study, we investigate the potential changes in BK channels expression and function in patients suffering from NDO in comparison to control non-NDO patients. We first used quantitative polymerase chain reactions (qPCR) to investigate BK channels mRNA relative expression in both control and NDO patients. Then, using isometric DSM tension recordings, we tested the DSM isolated strips physiological responses to pharmacological blockade of BK channels with iberiotoxin, a selective BK channel antagonist. DSM tissues were obtained from patients undergoing open bladder surgery for malignant or non-malignant conditions of the lower urinary tract. The qPCR experiments revealed that DSM tissues from NDO patients have lower BK channels mRNA expression in comparison to controls. The difference in BK channel

mRNA expression was statistically significant for the pore forming BKα-subunits but not for the accessory BKβ1 and BKβ4 subunits. Isometric DSM tension recordings experiments demonstrated that iberiotoxin significantly increase myogenic contractions in control DSM while DSM isolated strips from NDO patients were almost unresponsive to iberiotoxin treatment.

Our study suggests that a decrease in DSM BK channel expression and function might be the major reason for increased DSM contractility in NDO patients. BK channel dysfunction therefore plays a key role in NDO.

INTRODUCTION

Neurogenic detrusor overactivity (NDO) is a form of overactive bladder (OAB) which often develops in patients suffering from various neurological diseases such as spinal cord injury, Parkinson's diseases, spina bifida, and multiple sclerosis (8). In comparison to healthy patients, NDO patients experience greater spontaneous phasic detrusor smooth muscle (DSM) contractions during the filling phase of the micturition process (16, 120). Additionally, serious alteration in the DSM electrochemical properties of NDO patients is reported leading to significant increase in DSM contractility and excitability (8). NDO patients also experience the classic clinical symptoms which are associated with OAB including nocturia, frequent urination, urgency, incontinence, and involuntary contractions of the DSM (147). For many years, the mainstay of all forms of OAB treatment has been antimuscarinic drugs which have limited effectiveness and dose-related side effects (8). Recently, two new therapeutic approaches have been authorized by the FDA. These therapies include oral administration of mirabegron, a selective β3-adrenoceptor agonist (50, 157) and intravesical injection of botulinium toxin (33, 95, 96). Whether, these new therapies will be effective to treat OAB in a long term remains uncertain.

K^+ channels are known to be key regulators of the DSM contractility and excitability in various species (130). Amongst these K^+ channels, the large conductance voltage- and Ca^{2+} activated K^+ (BK) channel could be the most important because of its role in the regulation of the DSM action potential, resting membrane potential, and contractility (22, 60, 61, 67, 75, 80, 109, 130, 131, 134, 145, 146, 164). Structurally, BK channels are composed of four pore forming α-subunits and four regulatory β1, β2, β3, or β4 subunits

(130). The structural integrity of the BK channels are extremely important for their function since studies have demonstrated that loss of BKα subunits or BKβ1 subunits functions significantly increases DSM contractility and urination frequency causing detrusor overactivity (DO) in mouse (22, 109, 131, 154). It has also been shown that in a rat model of partial urethral obstruction, BKα subunit mRNA and protein expression were significantly reduced in comparison to sham-operated rats plus BK channel whole cell current was attenuated (9). A recent study by Petkov's laboratory has provided the first direct molecular evidence for BK channels expression and function in native human DSM (75). The aforementioned study demonstrated mRNA and protein expression for the pore forming BKα-subunit as well as the accessory BKβ1 and BKβ4 subunits in human DSM (75). Iberiotoxin, a selective BK channel blocker, inhibited the majority of the whole cell outward K^+ current, depolarized the DSM cell resting membrane potential, and increased the contractility of human DSM isolated strips (75). In the contrary, pharmacological activation of BK channels with NS1619, a BK channel agonist suppressed human DSM excitability and contractility (80). Other studies also demonstrated a direct involvement of BK channels in the etiology of OAB in patients with benign prostatic hyperplasia (BPH), DO, and NDO (26, 120). All of the above cited studies demonstrate the critical physiological role of BK channels in human DSM excitability and contractility, and further suggest that BK channels could be promising drug targets for OAB treatment (26, 32, 75, 80, 98, 107, 108, 120, 134, 145). In the present study, we investigated the changes in BK channels subunits expression and function in NDO patients using qPCR and isometric DSM tension recordings on native

human DSM isolated strips. The overall goal of the study was to determine whether NDO is linked to changes in the DSM BK channel expression and function.

MATERIALS AND METHODS

Human DSM tissue collection

Human DSM tissue was collected as described in chapter 2.2. For these studies, DSM specimens were isolated from 10 patients (7 controls and 3 NDO patients, 49-76 years old).

Quantification of BK channel subunits mRNA message by qPCR

Specific primers for BKα, BKβ1, BKβ4 subunits, and GAPDH were designed based on the cDNA complete sequences of human genes in Genbank and synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA) (**Table 3.1.1**). qPCR experiments were carried out on human DSM tissue cDNA (0.5 µg/µL) using IQ SYBR Green Supermix (Bio-Rad, California, USA). GAPDH was chosen as an internal control gene to analyze BKα, BKβ1, and BKβ4 subunits mRNA relative expression and each sample was run in triplicate. The parameters of the qPCR experiments were as follows: Cycle 1, 95^{o}C for 3 min; cycle 2, 95^{o}C for 10 s then 61^{o}C for 30 s (repeated 40 times); cycle 3, 95^{o}C for 1 min; cycle 4, 61^{o}C for 1 min; cycle 5, 61^{o}C for 10 s (repeated 81 times to generate a melting curve).

	Sense	Anti-sense	Product (bp)	Accession Number
BKα	TGCCTAAAGCATGATTTG	GCCGACATGCTAAATAAATTAG	400	NG012270
BKβ1	TGCCACCTGATTGAGACC	TGCGGAGAAGCAGTAGAAG	258	NM004137
BKβ4	CATTTGTGGTGGGCTTTCT	ACATGTTCCGCAGGTGG	168	NM170782
GAPDH	GGATTTGGTCGTATTGGG	GGAAGATGGTGATGGGATT	205	NM002046

Table 3.1.1. qPCR primers for BKα, BKβ1, BKβ4 subunits and GAPDH. bp-base pair

Isometric DSM tension recordings

Experiments were conducted as described in chapter 2.2.

Solutions and Drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS compositions are described in chapter 2.2. Iberiotoxin and TTX were purchased from Sigma-Aldrich Co.

Data analysis and statistics

Analysis was conducted as described in section 2.2.

RESULTS

The relative expression levels of BKα subunit mRNA is significantly decreased in NDO DSM

Recently, we established that human DSM cells express BKα, BKβ1, and BKβ4 subunits at both mRNA and protein levels (75). Here, we revealed that in patients with NDO, the BKα, BKβ1, and BKβ4 subunit mRNA expression was lower compared to control patients (**Fig. 3.1.1**). There was a 15.8-fold decrease in BKα subunit mRNA relative expression in NDO DSM (N=3) compared to controls (N=7) ($P<0.01$; **Fig. 3.1.1**). The changes in BKβ1 and BKβ4 subunits in NDO DSM compared to controls were minor and were evaluated at 1.1-fold and 1.6-fold decrease, respectively, ($P>0.05$; **Fig. 3.1.1**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and did not vary between DSM samples from control and NDO patients. These data suggest that in patients suffering from NDO, there is a statistically significant decrease in BKα subunit expression but not in BKβ1, and BKβ4 subunits expression.

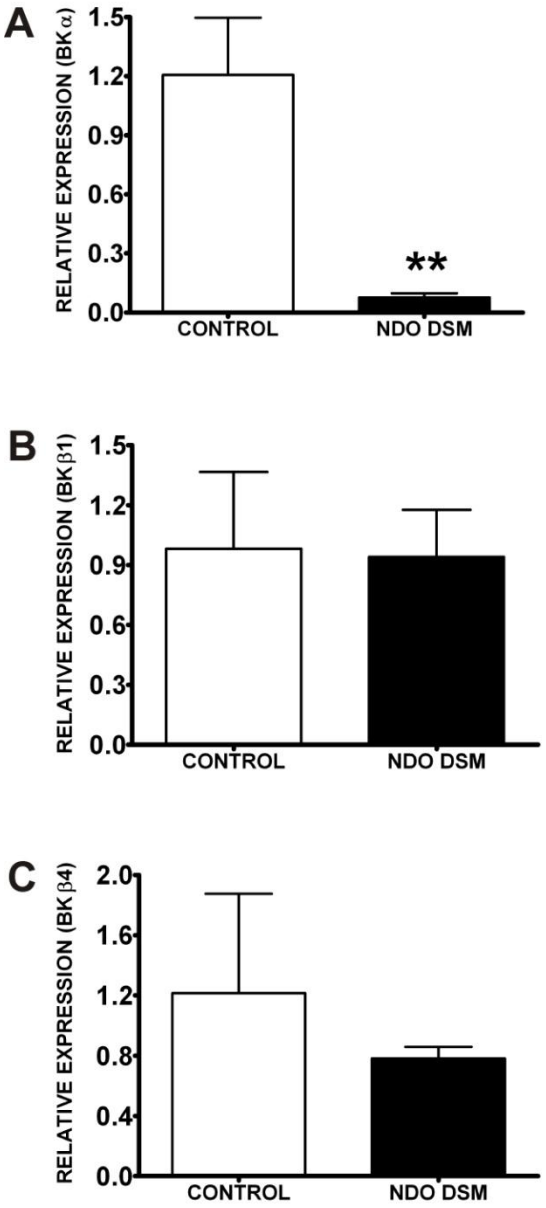

Figure 3.1.1. Patients with NDO have decreased BKα, BKβ1, and BKβ4 subunit mRNA expression in DSM. qPCR analyses showing a 15.8-fold, 1.1-fold, and 1.6-fold decrease in BKα (**A**), BKβ1 (**B**) and BKβ4 (**C**) mRNA expression in NDO DSM tissue (N=3) compared to controls (N=7). Data are shown as relative mRNA expression normalized to GAPDH**.**

Pharmacological inhibition of the BK channels with iberiotoxin had a reduced effect on the contractility of DSM strips isolated from patients with NDO

To restrain neuronal activity, tetrodotoxin (TTX, 1 µM), a selective voltage-gated Na^+ channel blocker, was added to the bath in the beginning of all experiments. In DSM strips isolated from control patients, iberiotoxin (200 nM) considerably increased the phasic contraction amplitude, muscle force integral, and muscle tone by 81.9±38.7%, 96.9±37.7%, and 14.4±8.3%, respectively (n=12, N=7; $P<0.05$; **Fig. 3.1.2A, C**). However, in DSM strips isolated from patients with NDO, iberiotoxin (200 nM) had no significant effects on any of the parameters of DSM contractility including phasic contraction amplitude, muscle force integral, and muscle tone (n=13, N=3; $P>0.05$; **Fig. 3.1.2B, C**). Collectively, these results suggest a decrease in BK channel activity in DSM from patients with NDO, which contributes to the increase in the spontaneous phasic contractions.

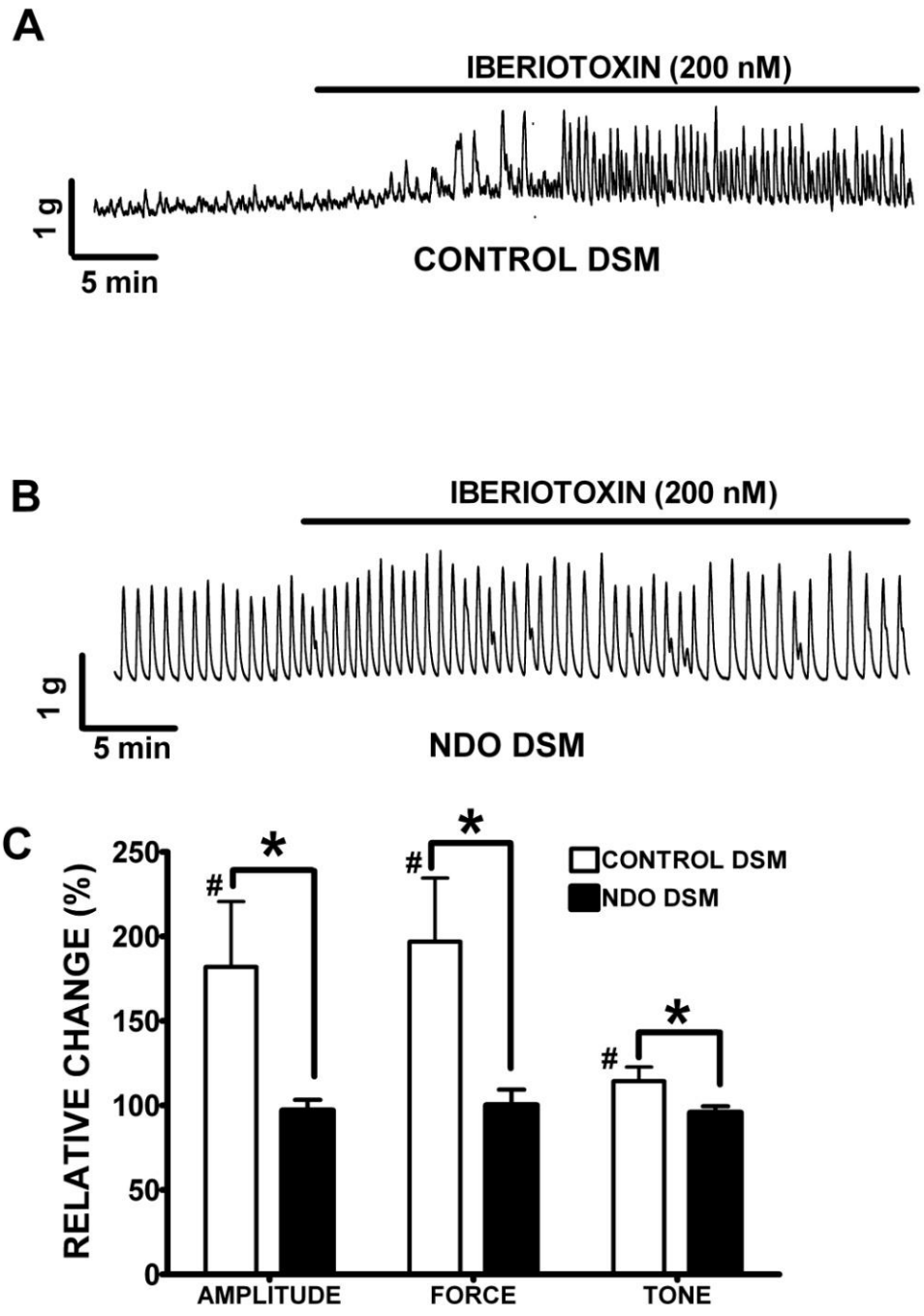

Figure 3.1.2. Inhibition of BK channels with iberiotoxin caused a significant increase in spontaneous phasic and tonic contractions in control DSM but not in NDO DSM isolated strips. Original DSM tension recording illustrating iberiotoxin (200 nM) effects on spontaneous phasic contraction in control (A) and NDO (B) DSM isolated strips, respectively. C. Summary data showing a statistically significant increase in spontaneous phasic contractions amplitude, muscle force integral, and muscle tone of control DSM strips (n=12, N=7, $P<0.05$) and lack of significant iberiotoxin effects in NDO DSM strips (n=13, N=3, $p>0.05$). #$P<0.05$ for control vs. iberiotoxin and *$P<0.05$ for control vs. NDO.

DISCUSSION

The present study establishes a clear correlation between BK channels expression and function and the abnormally elevated DSM contractility observed in NDO patients. We found that mRNA expression of the pore forming BKα subunit was significantly decreased in NDO DSM compared to controls. We also found that spontaneous phasic contractions were abnormally increased in NDO DSM compared to control DSM, and that pharmacological blockade of BK channels with iberiotoxin did not increased DSM contractility in NDO patients contrarily to controls.

The expression and physiological relevance of BK channels in human DSM has recently been demonstrated (75). RT-PCR and western blot data confirmed that the pore forming α-subunit as well as the regulatory β1 and β4 subunits are expressed at mRNA and protein level, respectively, in human DSM (75). The pore-forming α subunits ensure the movement of K^+ ions across the cell membrane, while the β1 subunits regulate the channel's activity by modulating BK channel Ca^{2+} sensitivity(22, 109, 130, 131, 146). The structural integrity of the BK channel confers to the channel its ability to determine the resting membrane potential and to contribute to the initial repolarization of the action potential (130). Any changes in BK channel subunits expression could significantly hamper the channel's ability to regulate DSM excitability and contractility. For instance, it has been demonstrated that in mouse model in which the BKα subunit was genetically deleted, DSM cells were depolarized and DSM contractility was abnormally increased, mimicking symptoms of DO (22, 109, 146, 154). Another study also showed that genetic deletion of the BKβ1 subunit causes an increase in DSM contractility in mice (131). One human study performed in patients with BPH and associated DO reported that mRNA

and protein expression for BKα and BKβ subunits were significantly reduced (26). Similarly to what is observed in BPH/DO patients, our study reported that in comparison to controls, NDO patients DSM also have significantly reduced BKα subunit mRNA expression and a moderate decrease in BKβ1 and BKβ4 subunits mRNA expression (**Fig. 3.1.1**). These studies support the concept that the NDO phenotype is associated with decreased BK channel expression in human DSM.

As we correlate the molecular findings with the functional data, we found that NDO patients DSM have abnormally elevated contractility in comparison to controls (**Fig. 3.1.2**). Additionally, unlike control DSM, inhibition of the BK channel with iberiotoxin did not significantly affect DSM contractility in NDO DSM isolated strips suggesting that in NDO patients DSM, the BK channel function might be altered (**Fig. 3.1.2**). Our data are in line with a previous report which demonstrates that in NDO patients, pharmacological activation of BK channels with NS1619, a BK channel agonist, has no effect on DSM phasic contractions (120).

Our data are further supported by recent findings which show that NS1619 significantly decreased DSM excitability and contractility in controls non-NDO patients (80).

CONCLUSION

We demonstrated that in NDO patient, the DSM contractility is abnormally elevated in comparison to controls and that this particular phenotype is most likely due to the significant reduction in BKα subunit expression. Therefore, we suggest that targeting BK channels by either pharmacological or genetic manipulation could be a promising therapeutic strategy to treat OAB, and especially NDO.

3.2 SK BUT NOT IK CHANNELS REGULATE HUMAN DETRUSOR SMOOTH MUSCLE SPONTANEOUS AND NERVE-EVOKED CONTRACTIONS

Data presented in this section have already been published and the original paper has been modified to fit the format of this dissertation with authorization of the publisher. Human tissues were surgically obtained by Dr. Rovner's team from the Medical University of South Carolina. Dr. Rovner is a collaborator with the Petkov's lab.

ABSTRACT

Animal studies suggest that the small (SK) and intermediate (IK) conductance Ca^{2+}-activated K^+ channels may contribute to detrusor smooth muscle (DSM) excitability and contractility. However, the ability of SK and IK channels to control DSM spontaneous phasic and nerve-evoked contractions in human DSM remains unclear. We first investigated SK and IK channels molecular expression in native human DSM and further assessed their functional role using isometric DSM tension recordings and SK/IK channels selective inhibitors. Quantitative PCR experiments revealed that SK3 channel mRNA expression in isolated DSM single cells was ~12 to 44-fold higher than SK1, SK2, and IK channels. RT-PCR studies at the single-cell level detected mRNA messages for SK3 channels but not SK1, SK2, and IK channels. Western blot and immunohistochemistry analysis further confirmed protein expression for the SK3 channel and lack of detectable protein expression for IK channel in whole DSM tissue. Apamin (1 μM), a selective SK channel inhibitor, significantly increased the spontaneous phasic contraction amplitude, muscle force integral, phasic contraction duration, and muscle

tone of human DSM isolated strips. Apamin (1 μM) also increased the amplitude of human DSM electrical field induced (EFS)-contractions. However, TRAM-34 (1 μM), a selective IK channel inhibitor, had no effect on the spontaneous phasic and EFS-induced DSM contractions suggesting a lack of IK channel functional role in human DSM. In summary, our molecular and functional studies revealed that the SK, particularly the SK3 subtype, but not IK channels are expressed and regulate the spontaneous and nerve-evoked contractions in human DSM.

INTRODUCTION

Overactive bladder (OAB), occurring in approximately 17% of the American population, is a pathophysiological condition that impairs quality of life (147). The current pharmacological treatment of OAB is primarily based on antimuscarinic drugs, which are not universally effective and have many unwanted side effects (5, 130). In order to develop novel effective pharmacological treatments for OAB, it is crucial to improve our understanding of the basic physiology, pathophysiology, and pharmacology of the bladder function. Previous studies have already demonstrated that various K^+ channels including the large-conductance Ca^{2+}-activated K^+ (BK) channel (22, 26, 35, 60, 75, 79, 80, 131, 145), the voltage-gated K^+ (K_V) channels (29, 57, 60, 74, 77), and the ATP-sensitive K^+ (K_{ATP}) channels (35, 132) are essential contributors to urinary bladder physiology. However, other important channels such as the small (SK) and intermediate (IK) conductance Ca^{2+}-activated K^+ channels remain largely unexplored in human detrusor smooth muscle (DSM) although some studies outline a potential role for these channels in DSM phasic contractions (31, 35, 58, 67, 68, 117, 120, 163). The SK channel family consists of three isoforms: SK1, SK2, and SK3 (129). IK channel, initially classified as SK4, is the only member of its family (129, 130). SK1-3 channels are selectively blocked by apamin, a peptide isolated from *Apis mellifera* (129, 153), whereas the IK channels are selectively blocked by TRAM-34 (163). In guinea pig DSM isolated strips, apamin significantly increased the amplitude and duration but decreased the frequency of the spontaneous phasic contraction (66). In pig DSM, which has similar characteristics to that of human DSM (34), apamin increased both amplitude and frequency of the DSM spontaneous contractions (23). In mice, genetic ablation of the

SK3 channels caused an increase in the spontaneous phasic contraction *in vitro* and non-voiding contractions *in vivo* (68).

Notably, it has been demonstrated that activation of SK and IK channels has the opposite effect in the DSM of various species. NS4591, a non-selective SK and IK channel opener, inhibited electrical field stimulation (EFS)-induced and carbachol-induced phasic contractions in rat, pig, and human DSM (117). In rats, an increase in bladder capacity and micturition volume as well as a reduction in bladder overactivity was observed after application of NS309, an activator of SK/IK channels (125). Similarly, in rats and cats, NS4591 (30 mg/kg) inhibited bladder overactivity induced by capsaicin and acetic acid *in vivo* (73). Furthermore, SKA-31, the most potent SK/IK channel activator known to date (137) has been shown to decrease excitability and contractility in guinea pig DSM via selective SK channel activation (127). Collectively, these data suggest an important role played by SK and IK channels in DSM contractility in non-human tissues. Indeed, the majority of the current knowledge about the functional role of SK and IK channels comes principally from animals and is likely not directly translatable to humans due to species differences in DSM ion channel expression, action potential shape, and pattern of contractility. Furthermore, the limited number of functional studies performed on human DSM specimens have not addressed the role of SK and IK channels under normal physiological conditions of spontaneous activity but rather after addition of depolarizing agents such as KCl or carbachol (35, 117, 120). An investigation of human DSM strips exhibiting spontaneous phasic contractions would provide a more physiologically relevant condition for the determination of the role of SK and IK channels.

The aim of this study was two-fold: 1) to investigate the molecular expression of SK and IK channels in human DSM using RT-PCR, quantitative PCR (qPCR), Western blot, and immunohistochemistry and, 2) to evaluate the functional role of SK and IK channels in spontaneous and nerve-evoked contractions using isometric DSM tension recordings and the selective SK and IK channels inhibitors, apamin and TRAM-34, respectively.

MATERIALS AND METHODS

Human DSM tissue collection

Human tissue collection experiment is described in chapter 2.2. In total, we used DSM tissue samples from 36 (25 males and 11 females) patients (28-85 years old; average age 61.9±2.2) including 29 Caucasians and 7 African-Americans without a history of OAB.

Detection and quantification of mRNA message by RT-PCR and qPCR

Experiments were conducted as described in chapter 2.2. Specific primers for SK1-4 channels and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**Table 3.2.1**) were designed based on the cDNA complete sequences of human genes in Genbank. The parameters of the qPCR experiments were as follows: Cycle 1, 95 ^{o}C for 3 min; cycle 2, 95 ^{o}C for 10 s then 55 ^{o}C for 30 s (repeated 40 times); cycle 3, 95 ^{o}C for 1 min; cycle 4, 55 ^{o}C for 1 min; cycle 5, 55 ^{o}C for 10 s (repeated 81 times to generate a melting curve). The parameter for the RT-PCR experiments were as follows: cDNA was heated for 3 min at 94^{o}C then amplified by 35 cycles (94 ^{o}C for 30 s, 60.1 ^{o}C for 30 s, 72 ^{o}C for 15 s) followed by 5 min of extension at 72 ^{o}C. Total RNA extracted from human brain was purchased from Clontech (Mountain view, CA, USA) and used as a positive control.

Channel	Sense	Anti-sense	Product (bp)	Accession Number
SK1	TCAAATGCCTCATCAGCCTCTCCA	GCGCGTGTTGAAGGTGATCTTGTT	389	NM002248
SK2	TGGTGGACAATGGAGCAGATGACT	AACCAAGAGTACAGTTCCTGGGCA	359	NM170775
SK3	CTGCCTGTGGGAAATTGAATGGCA	AGGAGCACCATTCTTGGGACATGA	470	NM170782
IK	CTCATCGTGGCCTTTCATGCCAAA	CATGTAAAGCTTGGCCACGAACCA	378	NM002250
GAPDH	GGATTTGGTCGTATTGGG	GGAAGATGGTGATGGGATT	205	NM002046

Table 3.2.1. RT-PCR and qPCR primers for SK and IK channels identification. bp- base pair

Detection and localization of SK and IK channel proteins by Western blot and immunohistochemistry

Western blot and immunohistochemistry experiments are described in chapter 2.2. We used the following primary antibodies: affinity-purified rabbit polyclonal antibodies anti-K_{Ca}2.3 (SK3) (1:200) and anti-K_{Ca}3.1 (IK) (1:300) (Alomone Labs, Jerusalem, Israel). Goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted to 1:1,500) was used as a secondary antibody. Human heart protein medley was purchased from Clontech Laboratories (Mountain View, CA) and used as a positive control.

Isometric DSM tension recordings

Experiments were performed as described in chapter 2.2.

Solutions and drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS composition are described in chapter 2.2. Apamin, TRAM-34, and TTX were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Data analysis and statistics

Analysis was performed as described in chapter 2.2. Fold changes in target mRNA expression level were calculated after normalization to GAPDH and the SK3 channel expression level was arbitrarily chosen as the calibrator.

RESULTS

Detection and relative expression level of mRNA messages for SK1, SK2, SK3, and IK channels in human DSM

First, qPCR experiments were performed in order to evaluate the mRNA relative expression for all SK1-3 and IK channels. In human whole DSM tissue, we found that SK1 and SK3 channel mRNA expressions were higher compared to SK2 and IK channels (**Fig. 3.2.1A**). SK3 channel mRNA expression was 3-fold and 7-fold higher than SK2 and IK channel mRNA expression, respectively (n=14, N=7, $p<0.001$; **Fig. 3.2.1A**). However, SK1 and SK3 channel mRNA expression was comparatively similar in whole DSM tissue (n=14, N=7, $p<0.05$; **Fig. 3.2.1A**). Next, we conducted qPCR experiments on human DSM freshly isolated single cells to confirm that the mRNA messages detected in the whole DSM tissue originated directly from DSM cells but not from other non-DSM cell types such as neurons, fibroblasts, vascular, and endothelial cells present within the DSM layers (29, 30, 74, 75). The single-cell qPCR experiments confirmed a significantly higher SK3 channel mRNA relative expression compared to other SK and IK channels. SK3 channel mRNA expression was 12-fold, 41-fold, and 44-fold higher than SK1, SK2, and IK channels, respectively (n=16, N=8, $p<0.001$; **Fig. 3.2.1B**). These results demonstrate that in human DSM cells, the SK3 channel expression is predominant among SK and IK channels at the mRNA level**.**

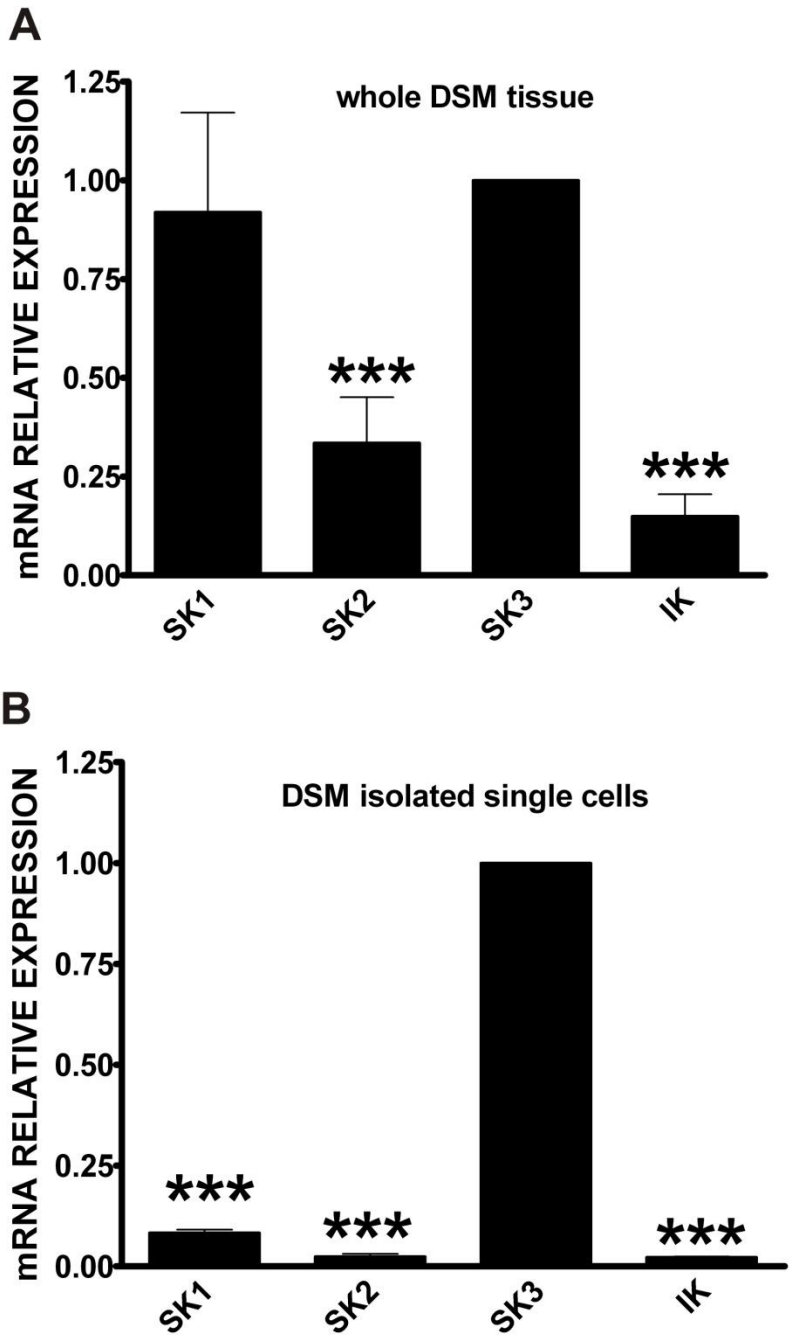

Figure 3.2.1. qPCR analyses for SK1, SK2, SK3, and IK channel mRNA expression in (A) human whole DSM tissue (n=14, N=7); and (B) human DSM isolated single cells (n=16, N=8). Data were normalized to GAPDH and SK3 mRNA message was arbitrarily chosen as the calibrator. Ct values are expressed as mean ± SE. SK and IK channel mRNA levels which are statistically different from SK3 channel mRNA level are indicated by (***) for $p<0.005$.

Next, we investigated SK1, SK2, SK3, and IK channel mRNA message expression using RT-PCR experiments on both human whole DSM tissue and freshly isolated DSM single cells. In human whole DSM tissue, our RT-PCR experiments demonstrated detectable expression of all SK and IK channel subtype mRNA messages (n=6-14, N=3-7; **Fig. 3.2.2**). At the DSM single-cell level, only the SK3 channel mRNA message was detected. There was no detection of SK1, SK2, or IK channels which is consistent with our qPCR data in human isolated DSM single cells (n=8-12, N=4; **Fig. 3.2.2**). RT-PCR experiments were conducted on human DSM cDNAs equivalent to 100 ng of starting mRNA. Negative control experiments performed in the absence of the reverse transcriptase (-RT) demonstrated an absence of genomic DNA contamination. These data suggest that the SK3 channel is highly expressed at the mRNA level, whereas SK1, SK2, and IK channel mRNA is expressed at very low, perhaps non-physiological, levels in human DSM cells.

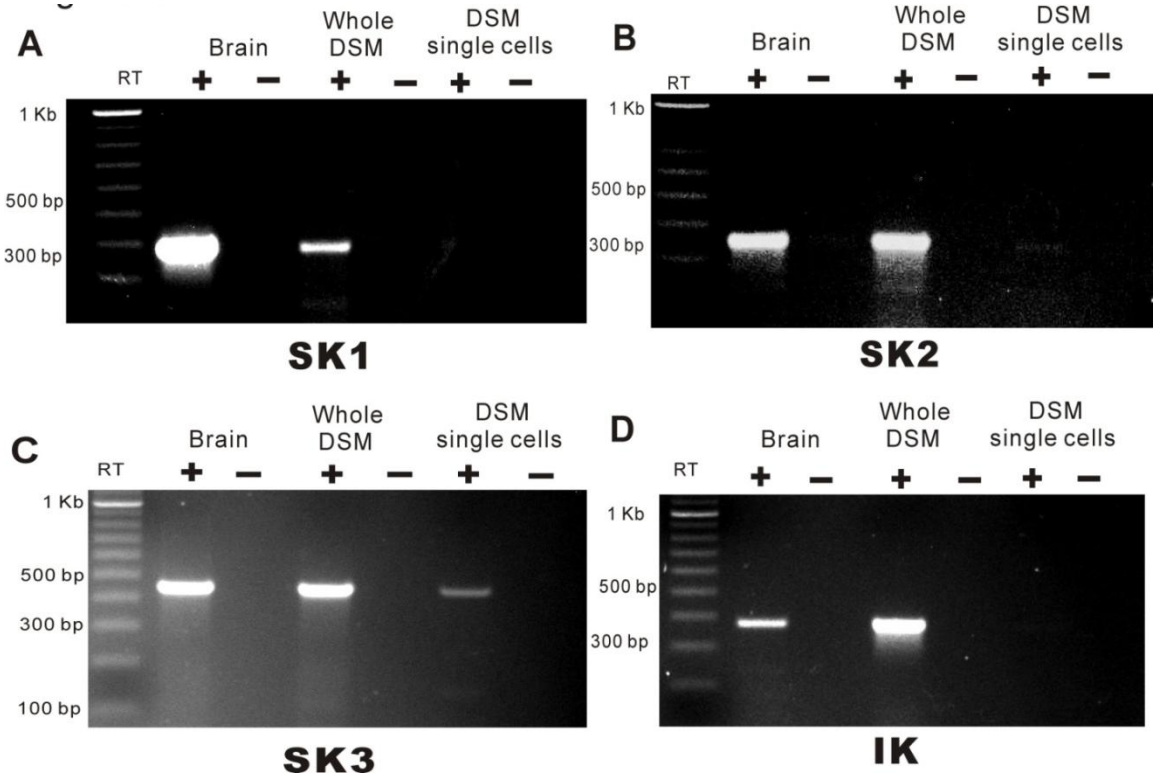

Figure 3.2.2. RT-PCR detection of SK1 (A), SK2 (B), SK3 (C), and IK (D) channel mRNA messages in human whole DSM tissue (n=6-14, N=3-7) and isolated DSM single cells (n=8-12, N=4). No product was observed in the negative controls (-RT) in which reverse transcriptase was not added to the reaction.

Native human whole DSM tissue expresses SK3 but not IK channel protein

To further investigate the expression of SK/IK channel protein in native human DSM tissue, we performed Western blot experiments using specific antibodies against the SK3 or IK channels. The data showed that human whole DSM tissue expresses SK3 channel protein (**Fig. 3.2.3A**) but not IK channel protein (**Fig. 3.2.4A**). These data verified our RT-PCR results which showed an SK3 but not an IK channel mRNA message in human DSM single cells. Further immunohistochemical analysis also confirmed the expression of SK3 channel protein (**Fig. 3.2.3B**) and ruled out the expression of IK channel protein (**Fig. 3.2.4B**) in human whole DSM tissue. Western blot and immunohistochemistry control experiments were further conducted by pre-absorption of the primary antibody with its antigenic competing peptide to verify the specificity of the antibodies for their intended epitope.

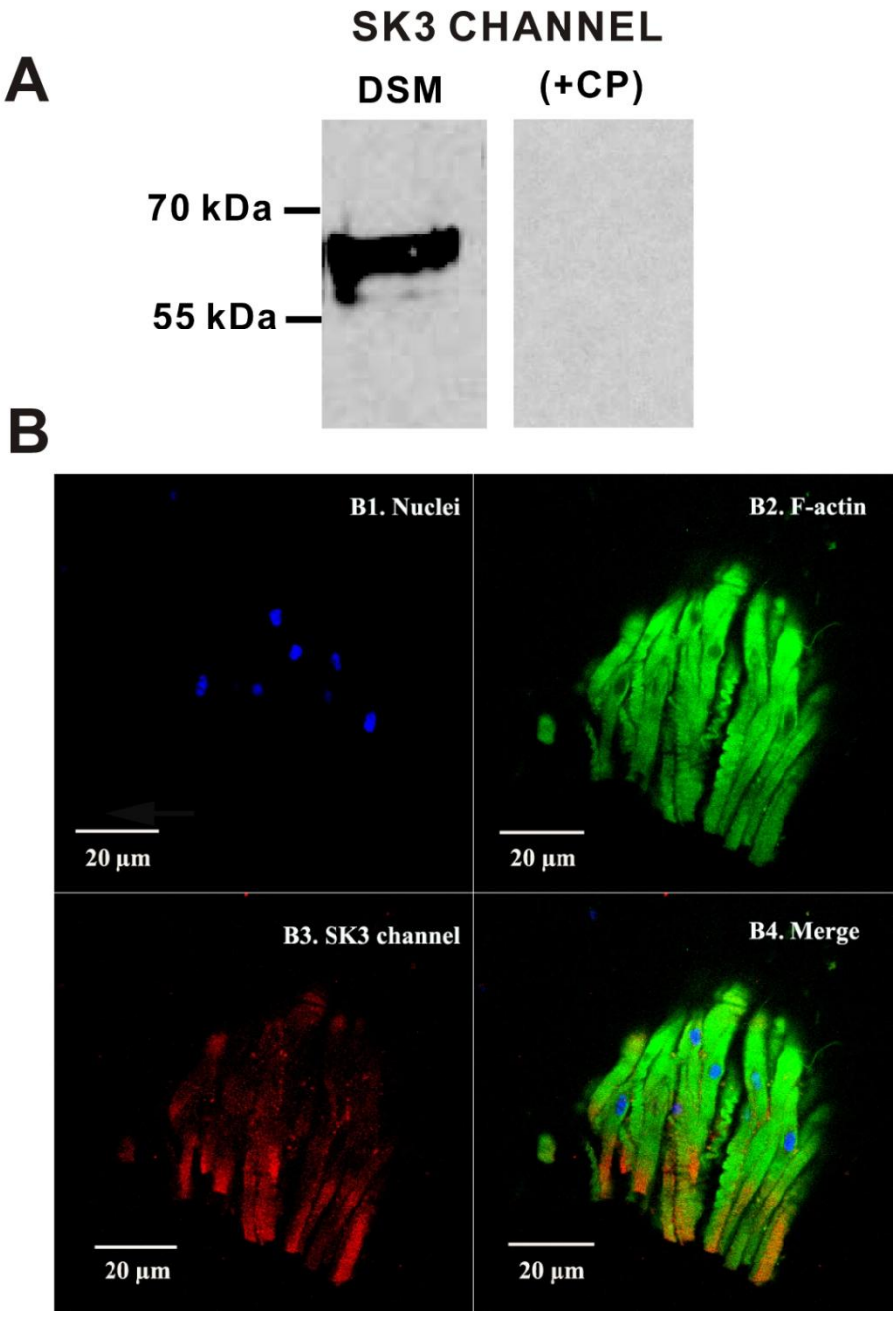

Figure 3.2.3. **Western blot and immunohistochemical detection of SK3 channel protein expression in native human whole DSM tissues**. A) Protein expression for SK3 channels was detected by Western blot in native human whole DSM tissue. The immunoreactive band was eliminated by a competing peptide (+CP). Experiments were conducted in 3 separate Western blot reactions using protein isolated from 3 patients. B) Immunohistochemical detection of SK3 channels in mucosa-free human whole DSM tissue using SK3 channel specific antibody. Cells' nuclei are shown in blue (B1); F-actin is shown in green (B2). Red staining indicates detection of the SK3 channels (B3). The merged images of the nuclei, F-actin, and the expected SK3 channels are illustrated in B4. Images were captured with a Carl Zeiss LSM 510 META confocal microscope. Experiments were conducted on tissue samples isolated from 4 different patients.

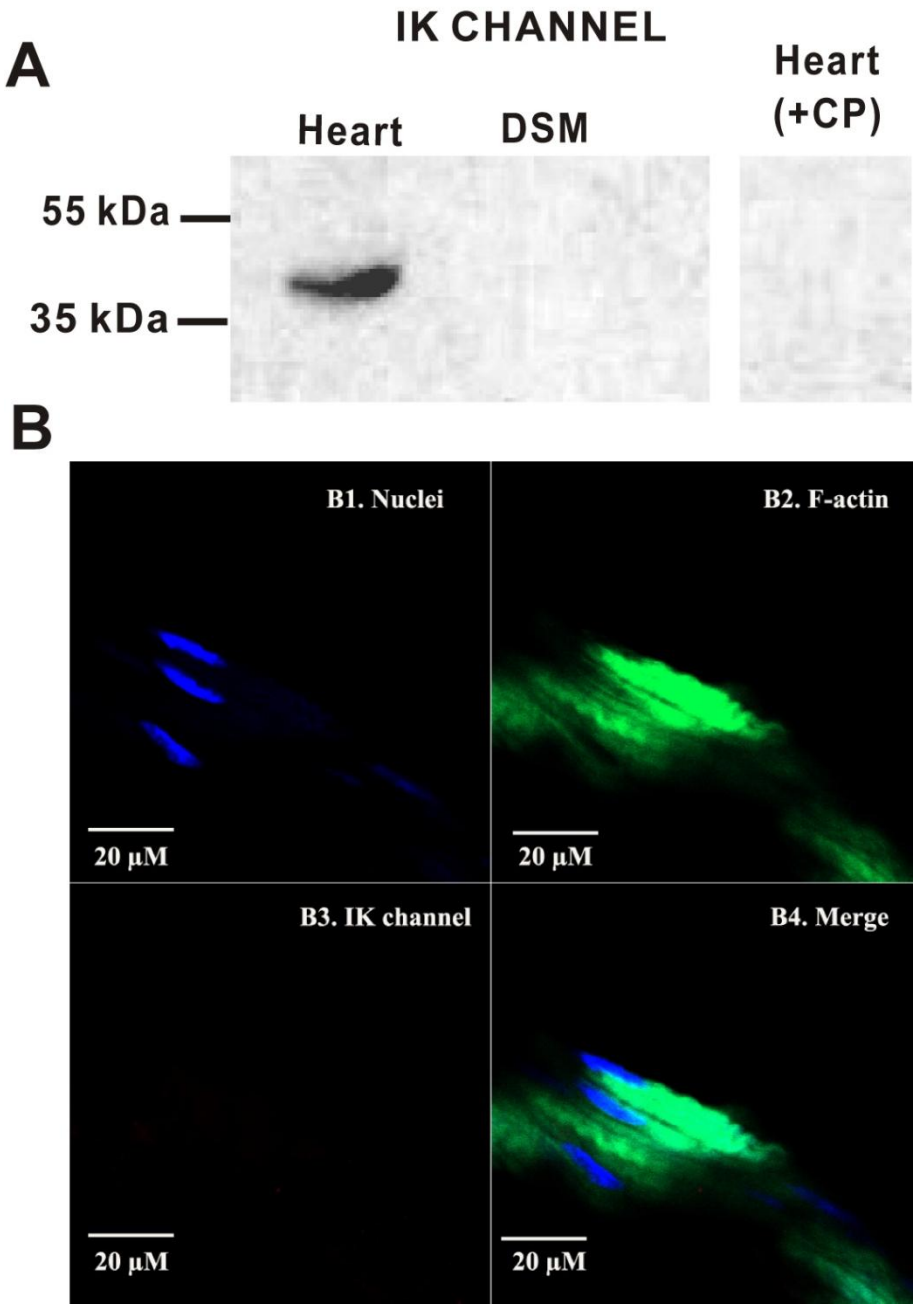

Figure 3.2.4. Western blot and immunohistochemical detection of IK channel showing the lack of IK channel protein expression in native human whole DSM tissues. A) Protein expression for IK channels was detected by Western blot in human heart protein medley (positive control) but not in native human whole DSM tissue. The immunoreactive band in human heart protein medley was eliminated by a competing peptide (+CP). Experiments were conducted in 3 separate Western blot reactions using protein isolated from 4 patients. B) IK channel protein was not detected in mucosa-free human whole DSM tissue following immunohistochemical reaction using IK channel specific antibody. Cells' nuclei are shown in blue (B1); F-actin is shown in green (B2). Lack of red staining in panel B3 indicates no expression of IK channel. The merged images of the nuclei and F-actin are illustrated in B4. Images were captured with a Carl Zeiss LSM 510 META confocal microscope. Experiments were conducted on tissue samples isolated from 4 different patients.

Role of SK and IK channels in spontaneous (myogenic) phasic and tonic DSM contractions Here, we sought to evaluate the roles of SK and IK channels in human DSM spontaneous contractions by using the SK and IK channel specific inhibitors, apamin and TRAM-34, respectively. Apamin (1 μM) increased the DSM spontaneous phasic and tonic contractions and had a biphasic effect on DSM spontaneous contraction (**Fig. 3.2.5A**). Within the first 10 min, apamin (1 μM) increased the DSM spontaneous phasic contraction amplitude by 162.1 ± 41.7%, muscle force integral by 171.8 ± 45.5%, and muscle tone by 11.7 ± 1.8% (n=28, N=13, $p<0.005$), without any significant effect on the spontaneous phasic contraction frequency and duration (**Fig. 3.2.5B**). We also evaluated the changes in the spontaneous phasic contractions, 30-40 min after apamin addition. During this second time period, the spontaneous phasic contraction amplitude was increased by 116.4 ± 39.2%, muscle force integral by 136.5 ± 50.7%, and phasic contraction duration by 13.8 ± 3.9% (n=28, N=13, $p<0.005$); no significant effect was observed on the phasic contraction frequency and muscle tone (**Fig. 3.2.5C**). On the other hand, TRAM-34 (1 μM) did not have any significant effect on the spontaneous phasic and tonic contractions in the first 10 min or 30-40 min after addition (n=4, N=4, $p>0.05$; **Fig. 3.2.6**). Collectively, these results suggest that SK channels but not IK channels regulate human DSM myogenic activity.

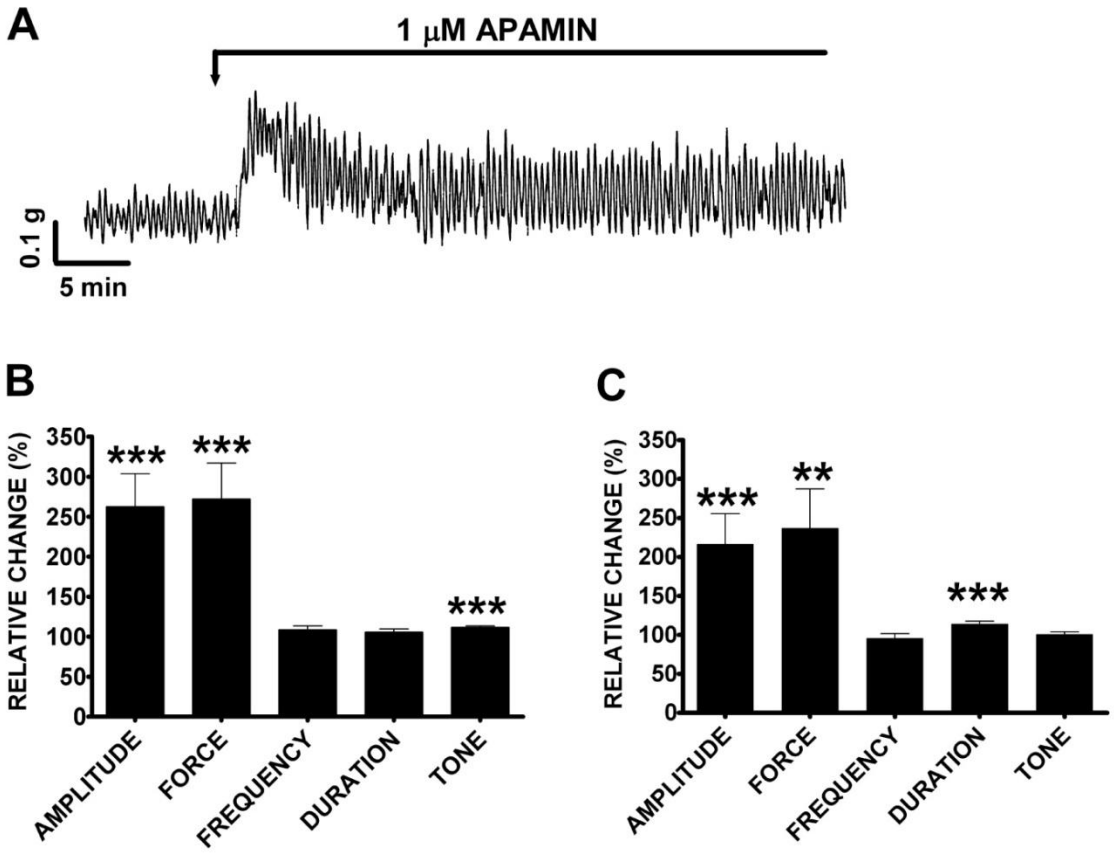

Figure 3.2.5. **Apamin increases the spontaneous phasic contraction amplitude, muscle force integral, phasic contraction duration, and muscle tone in human DSM isolated strips**. A) Original DSM tension recordings illustrating apamin (1 μM) effect on human DSM isolated strips. B) Summary data showing significant increase in human DSM spontaneous phasic contractions amplitude, muscle force integral, and muscle tone within 10 min following apamin (1 μM) addition. C) Summary data showing a significant increase in human DSM spontaneous phasic contractions amplitude, muscle force integral, and phasic contraction duration 30-40 min following apamin (1 μM) addition. The 10-min control period prior to apamin addition was taken to be 100 % and data were normalized. Values are means ± SE (n=28, N=13; **p<0.01, ***p<0.005). TTX (1 μM) was present throughout the experiments.

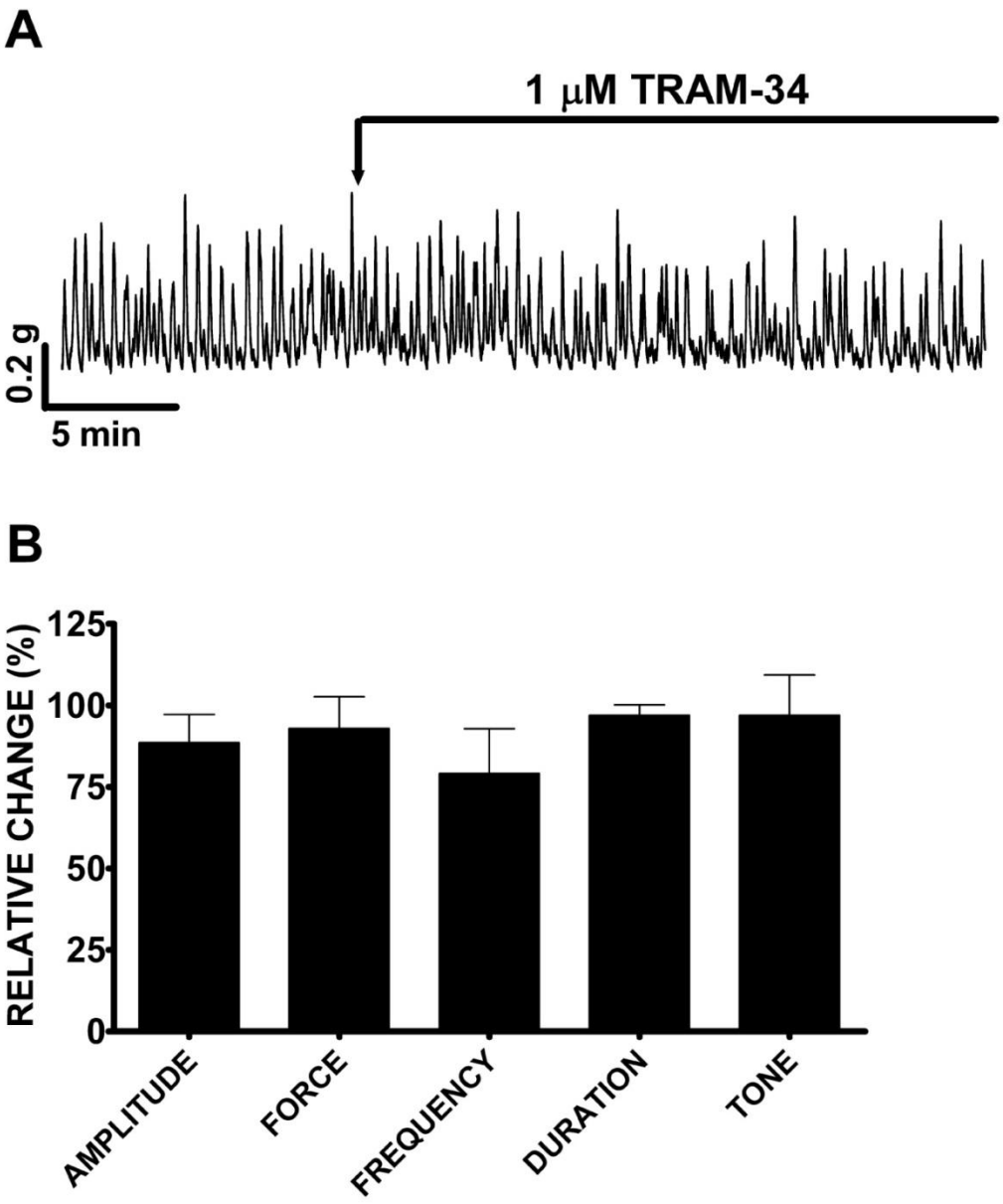

Figure 3.2.6. **TRAM-34 does not affect the spontaneous phasic and tonic contractions in human DSM isolated strips**. A) Original DSM tension recordings illustrating the lack of TRAM-34 (1 μM) effect on human DSM isolated strips. B) Summary data showing that TRAM-34 (1 μM) has no effect on any of the spontaneous phasic and tonic contractions parameters. TRAM-34 effect was evaluated within 10 min following TRAM-34 addition. The 10-min control period prior to TRAM-34 addition was taken to be 100 % and data were normalized. Values are means ± SE (n=4, N=4; $p>0.05$). TTX (1 μM) was present throughout the experiments.

Role of SK and IK channels in nerve-evoked contractions

In this experimental series, we investigated the effects of apamin (1 μM) and TRAM-34 (1 μM or 30 μM) on nerve-evoked contractions of human DSM isolated strips (**Fig. 3.2.7-8**). Nerve-evoked contractions were generated by EFS and characterized in response to increasing EFS frequencies (0.5–50 Hz) in the presence or absence of apamin or TRAM-34, respectively. Increasing EFS frequencies (0.5, 2.0, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, 50 Hz) were first applied to each human DSM strip as a control. Then, apamin (1 μM) or TRAM-34 (1 μM or 30 μM) was added to the bath for 30 min. Next, the same EFS protocol (0.5-50 Hz) was applied to evaluate how these SK and IK channel inhibitors modulate EFS-induced contractions. The frequency-response curves showed that apamin (1 μM) significantly increased the amplitude of the EFS-induced contractions at stimulation frequencies ranging from 3.5 to 50 Hz ($n=23$, $N=9$, $p<0.01$; **Fig. 3.2.7B**). At the maximal stimulation frequency of 50 Hz, apamin increased the EFS-induced contraction amplitude by 23.5 ± 6.1% ($n=23$, $N=9$, $p<0.01$). However, TRAM-34 (1 μM) did not have any significant effect on EFS-induced contraction at all stimulation frequencies (0.5-50 Hz) ($n=7$, $N=2$, $p>0.05$). This lack of effect was also observed at TRAM-34 concentration as high as 30 μM ($n=21$, $N=7$, $p>0.05$; **Fig. 3.2.8B**) suggesting that IK channels do not play a role in the nerve-evoked contractions.

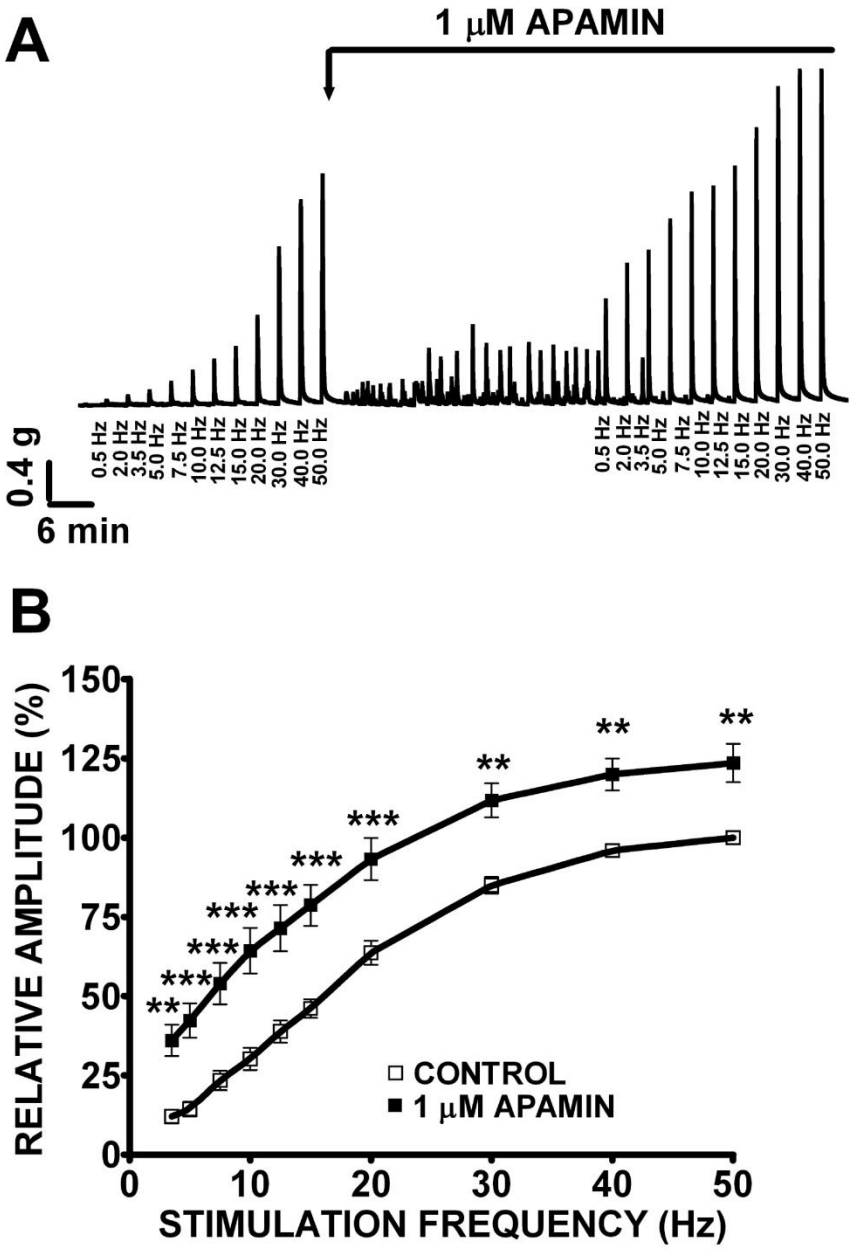

Figure 3.2.7. **Apamin increases the amplitude of the electrical field stimulation (EFS)-induced contractions in human DSM isolated strips.** A) Original recordings illustrating apamin (1 μM) effect on EFS-induced (0.5-50 Hz) contractions. As shown, apamin (1 μM) also induced myogenic contractions, consistent with data illustrated in Fig. 4. B) Frequency-response curves showing a significant increase in the amplitude of the EFS-induced contractions following application of apamin (1 μM). The maximal EFS-contraction amplitude at a stimulation frequency of 50 Hz under control conditions was taken to be 100 % and the contractions were normalized. Values are means ± SE (n=23, N=9; **p<0.01, ***p<0.005).

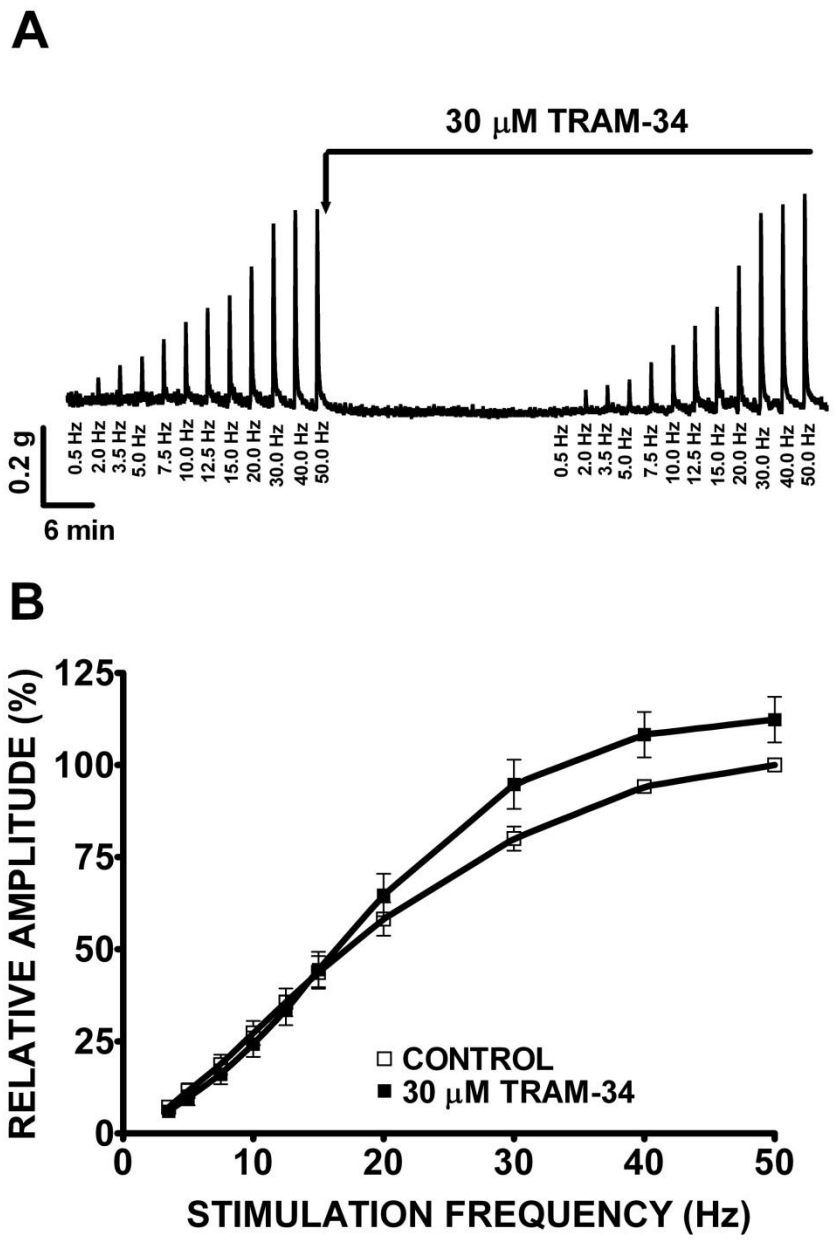

Figure 3.2.8. **TRAM-34 has no effect on the amplitude of the electrical field stimulation (EFS)-induced contractions in human DSM isolated strips.** A) Original recordings illustrating the lack of TRAM-34 (30 μM) effect on EFS-induced (0.5-50 Hz) contractions. B) Frequency-response curves showing no significant change in the amplitude of the EFS-induced contractions following application of TRAM-34 (30 μM). The maximal EFS-contraction amplitude at a stimulation frequency of 50 Hz under control conditions was taken to be 100 % and the contractions were normalized. Values are means ± SE (n=21, N=7; $p>0.05$).

DISCUSSION

In the present study, we used combined molecular (RT-PCR, qPCR, Western blot, and immunohistochemistry), functional (isometric DSM tension recordings), and pharmacological (SK and IK channel inhibitors) approaches to investigate the molecular expression of all SK and IK channels in freshly isolated human DSM cells and further evaluated their functional role in human DSM contractility. Single-cell RT-PCR experiments confirmed the expression of only the SK3 channel subtype at the mRNA level. qPCR experiments indicated that the SK3 channel is the most predominant subtype in native human DSM single cells at mRNA level. Western blot and immunostaining further confirmed SK3 channel protein expression. Functional studies using apamin and TRAM-34 suggest that the SK channels but not the IK channels have an important role in human DSM contractility.

SK and IK channels mRNA expression in human DSM cells

In human whole DSM tissue, RT-PCR showed that all SK and IK channels were expressed. Furthermore, the qPCR experiments not only confirmed mRNA expression for all SK and IK channel subtypes in whole DSM but also indicated that SK1 and SK3 channels are the most predominant. However, at the DSM single-cell level, only the SK3 channels were detected by RT-PCR, in all patients tested. Moreover, qPCR data from isolated DSM single cells indicate a much higher expression level for the SK3 channel. The difference in mRNA relative expression between whole DSM tissue and isolated DSM single cells could be explained by the fact that the detection of mRNA messages in isolated DSM single cells is a more accurate approach. The unique advantage of using

freshly isolated DSM single cells in RT-PCR and qPCR is that this approach eliminates any possible contamination by other non-DSM cell types including neurons, fibroblasts, endothelial cells, and vascular cells present within the DSM layers (29, 30, 74, 75). It is possible that the detected SK1 channel mRNA in whole DSM originated from neuronal cells present in the DSM layers consistent with the findings from Chen et *al*. (31). The aforementioned study using qPCR experiments, revealed that the SK3 channel is the most predominant SK channel subtype in human whole bladder tissue and that the SK1 channel is highly expressed in human neuronal tissues (brain) (31). This observation was confirmed by our RT-PCR, Western blot, and immunohistochemistry data showing mRNA message and protein expression for SK3 channel in native human DSM single cells and whole DSM tissue, respectively (**Figs. 3.2.1-3**). The presence of the SK3 channel protein in human whole DSM tissue confirms that SK3 channels play a critical physiological role in human bladder physiology, similar to rodent models (68). Studies have shown that selective suppression of SK3 channel expression increased the phasic contractions of mouse isolated DSM strips and increased non-voiding contractions *in vivo* (68). Suppression of SK3 channel expression by genetic manipulations further elevated micturition pressure compared to control mice. Transgenic mice overexpressing the SK3 channels had greatly elevated bladder capacities and urine output compared to wild-type mice (68). In the present study, we detected high levels of SK3 channel mRNA message in human DSM single cells and further confirmed the SK3 channel protein expression in whole DSM tissue. Therefore, it is likely that SK3 channels play an important functional role in human DSM contractility.

SK channels regulate human DSM spontaneous phasic contractions

Using channel specific inhibitors, apamin and TRAM-34, we determined the SK and IK channel functional roles in human DSM spontaneous phasic contractions. Our data show that apamin but not TRAM-34 significantly increased the phasic contraction amplitude, muscle force integral, phasic contraction duration and muscle tone of the DSM spontaneous contraction suggesting that SK channels but not IK channels are effective modulators of human DSM contractility. Consistent with our human data, studies in guinea pig DSM have shown that apamin but not TRAM-34 increases DSM spontaneous contractions (127). Furthermore, apamin but not TRAM-34 blocked guinea pig DSM relaxation induced by SKA-31, a novel and potent SK channel opener, suggesting that IK channels have no functional role in guinea pig DSM physiology as compared to SK channels (127). A previous study has established a correlation between spontaneous contractile activity and electrical activity in guinea pig DSM (58). The study showed that the inhibition of SK channels with apamin increased spontaneous phasic contraction amplitude and duration, reduced phasic contraction frequency while converting the electrical activity from individual action potentials into bursts without changing either the action potential shape or afterhyperpolarization (58). Apamin also increased guinea pig and mouse DSM action potential frequency suggesting that SK channels are important regulators of DSM excitability (45, 60). These animal data are consistent with our human data since apamin increased spontaneous phasic contraction amplitude and duration without affecting the phasic contraction frequency of human DSM isolated strips. We propose that the initial burst in action potentials could explain the initial jump observed in spontaneous contraction observed immediately after apamin was added into the baths

(**Fig. 3.2.5A**). Earlier studies further showed that apamin increased human DSM phasic contractions induced by 10 mM KCl, suggesting the involvement of SK channels in KCl-induced contractions (35). Studies by others also reported that apamin can partially reverse the relaxation caused by the selective SK channel opener, NS4591, in rat, pig and human DSM strips stimulated by 1 μM carbachol (117). The major advantage of our work compared to those conducted previously on human DSM is that herein we evaluated the SK and IK channels functional role on human DSM strips exhibiting spontaneous phasic contractions. The unique advantage of using these spontaneously contracting DSM strips is their direct physiological relevance compared to those in which contractions are artificially induced by depolarizing agents such as KCl or carbachol (35, 117).

SK channel role in human DSM nerve-evoked contractions

During micturition, parasympathetic nerves release ATP and acetylcholine which activate purinergic P2X receptors and muscarinic receptors to cause DSM contraction. Using EFS, we activated the cholinergic and purinergic nerves present in the human DSM strips to induce contractions and then evaluated if apamin or TRAM-34 could modulate EFS-induced contractions. Our data show that apamin but not TRAM-34 significantly increased the amplitude of the EFS-induced contraction at all stimulation frequencies between 3.5 and 50 Hz suggesting that SK channels but not IK channels modulate human DSM nerve-evoked contraction. Our data on human DSM are consistent with previous studies on mouse and guinea pig DSM isolated strips (65, 127). In mouse and guinea pig, blocking SK channels with apamin resulted in an increase in the amplitude of nerve-evoked contractions (65, 127). However, in guinea pig DSM, inhibition of IK channels

with TRAM-34 had no effect on nerve-evoked contractions suggesting the lack of a functional role for IK channels (127). Our findings suggest that SK channels participate in a negative feedback loop which limits the DSM excitability and contractility in response to nerve stimulation consistent with previous mouse and guinea pig DSM studies (65, 66, 68, 127).

In summary, the SK3 channels are the only SK channel subtype expressed in human DSM single cells at the mRNA level. SK3 channel protein expression was also confirmed in whole DSM tissue. DSM contractility studies using apamin and TRAM-34 showed that the SK channels but not the IK channels have a critical role in human DSM function. Taken together, we suggest that the SK channels, the SK3 subtype in particular, are important modulators of human DSM contractility. SK3 channels could therefore represent novel therapeutic targets for the pharmacological treatment of OAB.

CHAPTER 4

FUNCTIONAL LINK BETWEEN BK CHANNELS AND BETA3 ADRENERGIC RECEPTORS DURING NERVE-EVOKED CONTRACTIONS

Chapter 4 addresses the functional link between BK channels and beta3-AR during nerve-evoked contractions. Because human material is limited, we first designed strategies to test our hypothesis by using laboratory animals. We investigated the role of beta3-AR in guinea pigs (chapter 4.1) and rats (chapter 4.2), two commonly used laboratory animals. We found that contrarily to guinea pigs, beta3-AR played a more physiologically-relevant role in rat DSM nerve-evoked contractions and that rats are better animal models than guinea pig to study beta3-AR functions in the DSM. We further found that there is a functional link between BK channels and beta3-AR during nerve-evoked contractions in rat DSM (chapter 4.2). The data in rat DSM served as a basis to further investigate the mechanism in humans (chapter 4.3).

4.1 DO BETA3-ADRENERGIC RECEPTORS PLAY A ROLE IN GUINEA PIG DETRUSOR SMOOTH MUSCLE EXCITABILITY AND CONTRACTILITY?

Data presented in this section have already been published and the original paper has been modified to fit the format of this dissertation with authorization of the publisher. Electrophysiological data were collected by Dr. Hristov, a post-doctoral fellow in Dr. Petkov's lab.

ABSTRACT

In many species, beta3-ARs have been reported to play a primary role in pharmacologically-induced detrusor smooth muscle (DSM) relaxation. However, their role in Guinea pig DSM remains controversial. The aim of this study was to investigate if beta3-ARs are expressed in Guinea pig DSM and to evaluate how BRL37344 and L-755,507, two selective beta3-AR agonists, modulate Guinea pig DSM excitability and contractility. We used a combined experimental approach including RT-PCR, patch-clamp electrophysiology, and isometric DSM tension recordings. Beta3-AR mRNA message was detected in freshly isolated Guinea pig DSM single cells. BRL37344 but not L-755,507 caused a slight decrease in DSM spontaneous phasic contraction amplitude and frequency in a concentration-dependent manner. In the presence of atropine (1 μM), only the spontaneous phasic contractions frequency was inhibited by BRL37344 at higher concentrations. Both BRL37344 and L-755,507 significantly decreased DSM carbachol-induced phasic and tonic contractions in a concentration-dependent manner. However,

only BRL37344 inhibitory effect was partially antagonized by SR59230A (10 μM), a beta3-AR antagonist. In the presence of atropine, BRL37344 and L-755,507 had no inhibitory effect on electrical field stimulation-induced contractions. Patch-clamp experiments showed that BRL37344 (100 μM) did not affect the DSM cell resting membrane potential and K^+ conductance. Although beta3-ARs are expressed at the mRNA level, they play a minor to no role in Guinea pig DSM spontaneous contractility without affecting cell excitability. However, BRL37344 and L-755,507 have pronounced inhibitory effects on Guinea pig DSM carbachol-induced contractions. The study outlines important DSM beta3-ARs species differences.

INTRODUCTION

For many years, pharmacological treatment of OAB and associated urinary incontinence (UI) has relied exclusively on antimuscarinic drugs (5, 114, 170). However, beta-ARs) could represent alternative targets for OAB because pharmacological stimulation of β-ARs causes DSM relaxation (22, 79). Among all beta-ARs subtypes (beta1, beta2, and beta3), the beta3-ARs may be the most promising new pharmacological targets for OAB treatment because they represent 97% of all beta-ARs expressed in human DSM (168). Such predominant expression in DSM suggests that drugs targeting beta3-ARs will potentially have fewer side effects compared to antimuscarinic drugs, which can cause dry mouth, constipation, headaches, blurred vision, etc. (5, 14, 170). The activation of beta-ARs stimulates adenylyl cyclase which converts ATP into cAMP. Increased intracellular cAMP activates protein kinase A (PKA), which then can phosphorylate various proteins and relax DSM (111, 134). In mouse and Guinea pig, stimulation of beta-ARs by isoproterenol, a non-selective beta-AR agonist, relaxes DSM by activation of the large-conductance calcium-activated potassium (BK) channels (22, 134). Similarly in rat, stimulation of beta3-AR by the selective beta3-AR agonist, BRL37344, relaxes DSM by activation of the ryanodine receptors (RyRs) and BK channels (79). The increase in RyRs activity in rat DSM by BRL37344 and in Guinea pig DSM by isoproterenol leads to an increase in transient BK currents (TBKCs) frequency, which causes membrane hyperpolarization and thus relaxation (79, 134). Furthermore, in DSM of BK channel knockout mice, beta-AR-mediated relaxation is significantly compromised (22). However, to our knowledge, beta3-AR mRNA or protein expression has not yet been confirmed in Guinea pig DSM, and the functional role of beta3-ARs in Guinea pig

DSM remains controversial (100, 169). Clarification of this role is important because the Guinea pig is a widely used animal model to study DSM function.

Our goal was to provide molecular evidence for beta3-AR mRNA message expression in Guinea pig DSM using RT-PCR and establish whether beta3-ARs have a functional role in Guinea pig DSM excitability and contractility. Our molecular studies indicate the presence of the beta3-AR mRNA message in Guinea pig DSM cells, and that the selective beta3-AR agonists, BRL37344 and L-755,507 substantially inhibit carbachol-induced contractions. The compounds have a negligible inhibitory effect on spontaneous contractions, and no inhibitory effect on the nerve-evoked contractions of Guinea pig DSM in the presence of atropine. Finally, with the patch-clamp technique, we established that BRL37344 had no effect on Guinea pig resting membrane potential, voltage-step induced steady-state K^+ currents, and TBKCs.

METHODS

Animal studies and DSM tissue harvesting

DSM tissue harvesting was conducted as described in chapter 2.2. We used 76 adult (63 males, and 13 females) Hartley-Albino guinea pigs, weighing on average 366.6 ± 21.1 g.

DSM single-cell isolation and individual collection

DSM single cells collection for electrophysiology and RT-PCR experiments were collected as described in chapter 2.2.

RNA Extraction/RT-PCR/Sequencing

Specific primers sequences for beta3-AR were obtained from a previous publication (70) and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The sense and antisense primers were 5′-GTGGGAGGCAACCTGCTGGT-3′ and 5′-CGCCACCACTGGCTCAT-3′, respectively. RT-PCR experiments were conducted as described in chapter 2.2. cDNA was heated for 15 min at 95 ^{o}C then amplified by 35 cycles (95 ^{o}C for 30 s, 60 ^{o}C for 30 s, 72 ^{o}C for 30 s) followed by a 5-min extension at 72 ^{o}C. The expected length of the fragment was 387 bp.

Isometric DSM tension recordings

Isometric tension recordings experiments are described in section 2.2. Increasing BRL37344 or L-755,507 concentrations (1 nM–100 μM) were applied at 10-min intervals directly into the bath in the absence or presence of SR59230A (10 μM), a

selective β3-AR antagonist (22, 39, 71, 138). On a separate set of strips, BRL37344 (100 μM) was added into the tissue baths containing DSM strips exhibiting spontaneous phasic contractions in the presence or absence of atropine (1 μM), a muscarinic receptor antagonist. For the EFS experimental series, we separated the cholinergic component of the EFS-induced contractions from the purinergic component by blocking purinergic receptors with α,β-methylene-ATP and suramin. DSM isolated strips were pre-incubated with suramin (10 μM) and α,β-methylene-ATP (10 μM) for 15 min prior to the EFS-control protocol. The purinergic component of the EFS-induced contractions was assessed by addition of 1 μM atropine to block the cholinergic component. This experimental series was also performed in the presence or absence of SR59230A (10 μM).

Electrophysiological (patch-clamp) experiments

These experiments were performed by Dr. Hristov, a post-doctoral fellow in Dr. Petkov's laboratory. To preserve the physiological environment of the DSM cells, amphotericin-perforated whole-cell mode of the patch-clamp technique was applied in all electrophysiological experiments (54, 72). Whole-cell currents and membrane potential were recorded as previously described (22, 75, 79, 91). Briefly, freshly isolated single DSM cells were placed onto an experimental chamber for 20 min to allow them to adhere to the glass bottom, and were then washed with extracellular solution. The ionic currents and resting membrane potential were recorded using Axopatch 200 B amplifier; pCLAMP version 10.2 software; Digidata 1322A and Digidata 1440A (Molecular Devices, Union City, CA). The signals were filtered using an eight-pole Bessel filter

(model 900CT/9L8L; Frequency Devices, Ottawa, IL). Patch-clamp pipettes were pulled from borosilicate glass (Sutter Instruments, Novato, CA) using a Narishige PP-830 vertical puller, coated with sticky dental wax to reduce capacitance, and then polished with a Micro Forge MF-830 fire polisher (Narishige Group, East Meadow, NY) to give a final tip resistance of 4–6 MΩ. Leak currents were not subtracted. TBKCs were recorded at a holding potential of -40 mV or -20 mV (corrected for junction potential) for at least a 10-min period in the absence (control), and then in the presence of BRL37344 (100 μM). Voltage-step protocols were used to elicit steady-state K^+ outward current. Resting membrane potentials of DSM cells were recorded using the current clamp mode of the perforated patch-clamp technique. To determine the mean amplitude and frequency of TBKCs, analysis was performed off-line using Clampfit of the pCLAMP version 10.2 and MiniAnalysis software (Synaptosoft, Inc., Decatur, GA). All patch-clamp experiments were conducted at room temperature (22–23 °C).

Solutions and drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS compositions are described in chapter 2.2. The extracellular (bath) solution used for electrophysiological recordings, and the patch pipette solution were prepared daily and had the same composition as previously described (22, 79, 91). BSA and amphotericin-B were purchased from Fisher Scientific (Pittsburgh, PA). Papain was obtained from Worthington Biochemical Co. (Lakewood, NJ). L-755,507 was purchased from Tocris Bioscience (Ellisville, MO). The rest of the drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO). BRL37344 stock solution was prepared daily in double distilled

H_2O and was heated to 60 ^oC to fully dissolve the drug according to the manufacturer's instructions. L-755,507 was also prepared daily and dissolved in dimethyl sulfoxide (DMSO) at a final DMSO concentration not greater than 0.1%.

Data analysis and statistics

Analyses for isometric DSM tension recordings were analyzed as previously described in chapter 2.2. Paired Student's t-test was used to analyze electrophysiology data.

RESULTS

Detection of mRNA message for β3-AR in Guinea pig DSM

RT-PCR experiments detected a β3-AR mRNA message in whole DSM and isolated DSM single cells (**Fig. 4.1.1**). Using isolated DSM single cells is advantageous, eliminating possible contamination from other cell types such as neurons, fibroblasts, vascular myocytes, and other interstitial cells present within the DSM layers (29, 30, 91). These results demonstrate that Guinea pig isolated DSM cells express β3-AR mRNA message. Negative controls performed in the absence of reverse transcriptase (-RT) demonstrated an absence of genomic DNA contamination. Next, we sought to evaluate the β3-AR functional role in Guinea pig DSM contractility.

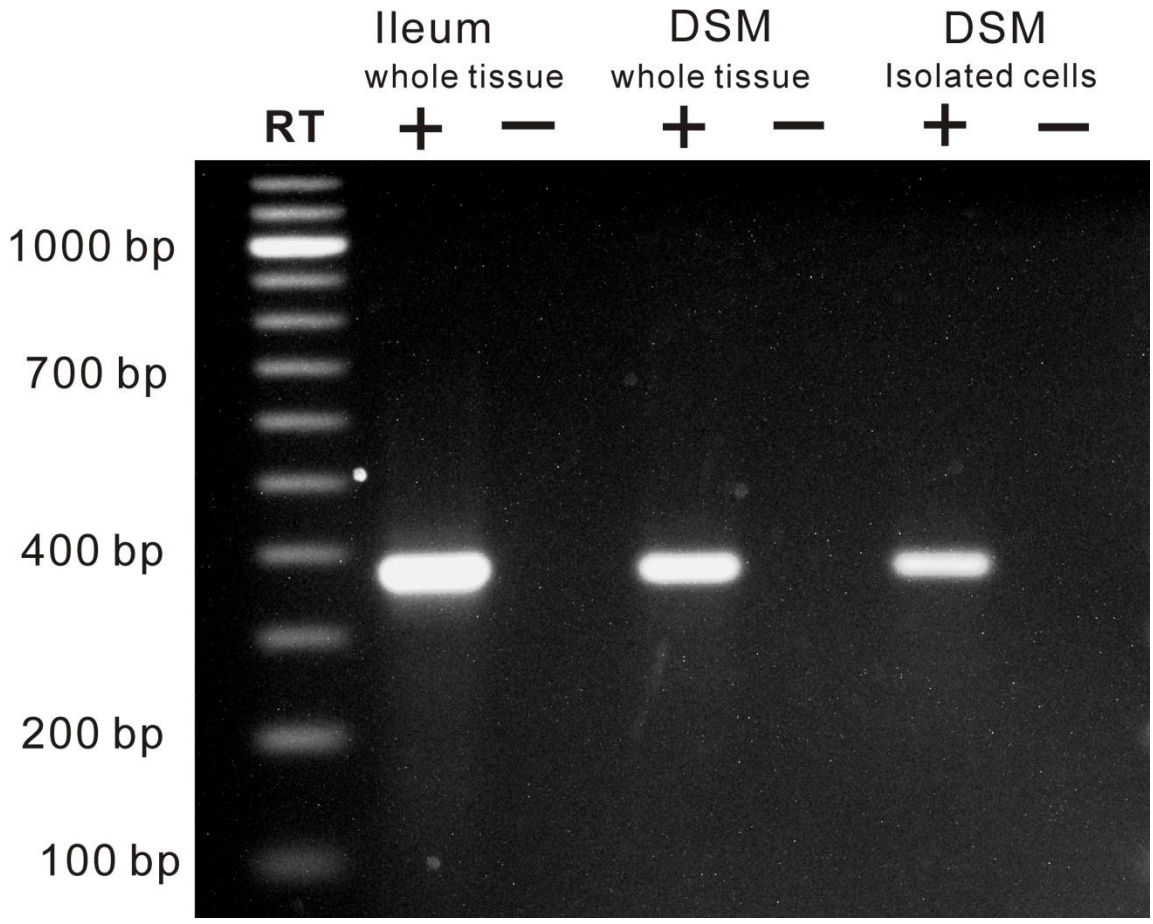

Figure 4.1.1. RT-PCR detection of β3-AR mRNA in DSM whole tissue and isolated DSM single cells. The expected size of the product was 387 bp. RT (+): reverse transcription, RT (-): no reverse transcription. Guinea pig ileal tissue was used as a positive control. Illustrated gel image is a selected representation of at least 5 independent RT-PCR experiments based on RNA extracted from 4 animals

BRL37344 but not L-755,507 has a minor inhibitory effect on the spontaneous phasic and tonic contractions in Guinea pig DSM isolated strips

Under control conditions before drug (BRL37344, L-755,507, or SR59230A) application (taken to be 100%), Guinea pig DSM spontaneous phasic contractions average amplitude was 0.4 ± 0.1 g, force was 1.4 ± 0.5 g/s, frequency was 3.0 ± 0.01 contractions/min, duration was 8.0 ± 0.9 s/contraction, and tone was 0.2 ± 0.03 g (n=41 , N=23). In the absence of SR59230A, BRL37344 (1 nM-100 μM) slightly inhibited the spontaneous phasic contraction amplitude and frequency without statistically significant effects on muscle force integral, phasic contraction duration, and muscle tone (**Fig. 4.1.2A**). At the maximal concentration of 100 μM, BRL37344 significantly reduced the phasic contractions amplitude by 17.6 ± 9.7%, and frequency by 20.7 ± 8.5% (n=7, N=7, $p<0.05$; **Fig. 4.1.2C and E**). In the presence of SR59230A (10 μM), the BRL37344 (100 μM) inhibitory effect on the spontaneous phasic contraction frequency was antagonized (n=8, N=4, $p<0.05$; **Fig. 4.1.2E**) but no antagonistic effect on the spontaneous phasic contraction amplitude was observed (n=8, N=4, $p>0.05$; **Fig. 4.1.2C**). SR59230A alone increased the amplitude and muscle force integral of the spontaneous phasic contractions. SR59230A increased the spontaneous phasic contraction amplitude by 196.0 ± 20.7% (n=8, N=4; $p<0.005$) and muscle force integral by 166.0 ± 57.6% (n=8, N=4; $p<0.01$) with no significant effect on the phasic contraction frequency, duration, or muscle tone (n=8, N=4; **Fig. 4.1.2B**). Unlike BRL37344, the other selective β3-AR agonist, L-755,507 (1 nM-100 μM) had no significant effects on any of the five parameters of the spontaneous contractions (n=12, N=6, $p>0.05$; **Fig. 4.1.2C-G**).

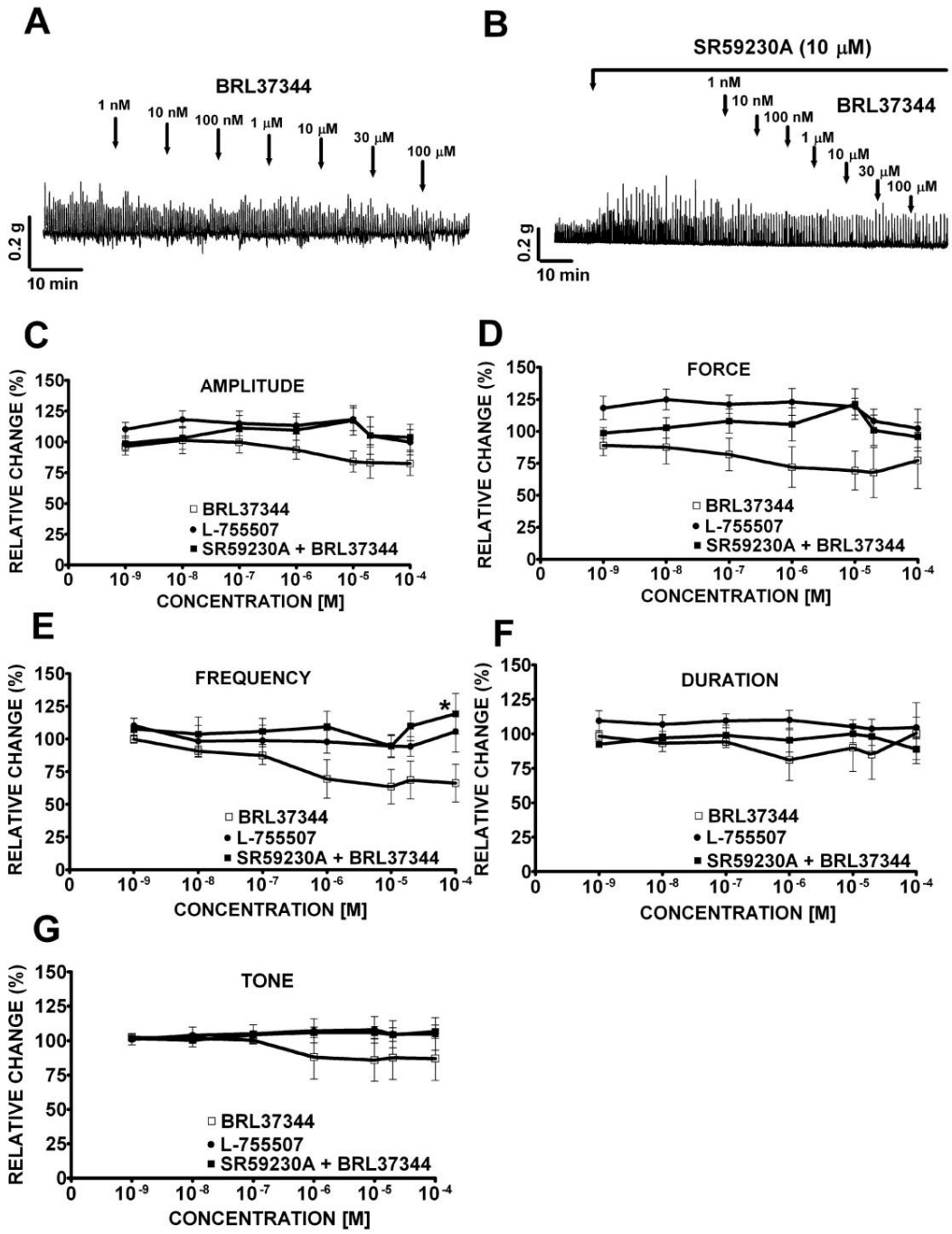

Figure 4.1.2. Effects of BRL37344 and L-755,507 on the spontaneous phasic and tonic contractions of Guinea pig DSM isolated strips. A-B. Original recordings of DSM spontaneous phasic contractions illustrating BRL37344 inhibitory effect (1 nM–100 μM) in the absence (A) or presence (B) of SR59230A (10 μM). C-G. Concentration-response curves for BRL37344 (1 nM-100 μM) inhibitory effects on the spontaneous phasic contraction amplitude (C), muscle force integral (D), phasic contraction frequency (E), phasic contraction duration (F), and muscle tone (G), in the presence or absence of SR59230A (10 μM). Concentration-response curves for L-755,507 (1 nM–100 μM) showed no inhibitory effect on the spontaneous phasic and tonic contractions of DSM isolated strips (C-G). Spontaneous contractions were taken to be 100% (n=7, N=7 for BRL37344; n=12, N=6 for L-755,507; and n=8, N=4 for SR59230A + BRL37344; *P<0.05 for the SR59230A effect). TTX (1 μM) was present throughout the experiments.

To prove that the BRL37344 inhibitory effect on spontaneous contractions in the concentration-response experiments was not due to a run-down of the preparation or a desensitization of the β3-AR, we further examined the effect of a single supramaximal BRL37344 concentration (100 μM) on spontaneous contractions. BRL37344 (100 μM) significantly decreased spontaneous phasic contraction amplitude, muscle force integral, phasic contraction frequency, but not phasic contraction duration or muscle tone. The amplitude of the spontaneous phasic contractions decreased by 31.0 ± 9.1%, muscle force integral by 33.2 ± 10.0%, and phasic contraction frequency by 16.6 ± 5.5% (n=14, N=5, $p<0.05$; **Fig. 4.1.3A and B**). BRL37344 has been reported to have some antimuscarinic effects in addition to its β3-AR agonist properties (79, 93). To separate these two effects, we used the cholinergic antagonist atropine. In the presence of 1 μM atropine, BRL37344 (100 μM) significantly inhibited only the DSM spontaneous contraction frequency by 21.2 ± 3.8% without any significant effect on phasic contraction amplitude, muscle force integral, phasic contraction duration, and muscle tone (n=9, N=5, $p<0.05$; **Fig. 4.1.3C and D**). These results suggest that BRL37344 but not L-755,507 has a minor inhibitory effect on the spontaneous contractions of guinea pig DSM isolated strips. This BRL37344 inhibitory effect is partially blocked by atropine (**Fig. 4.1.3**).

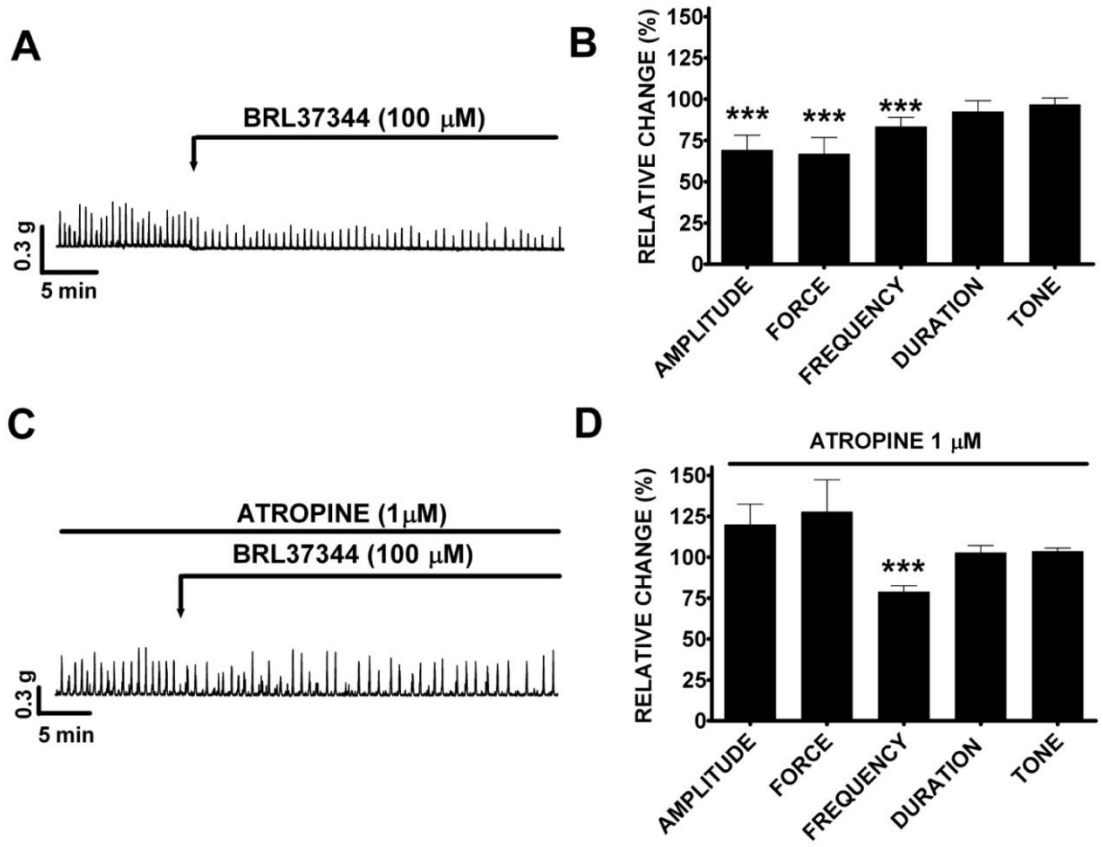

Figure 4.1.3. Application of a single concentration of BRL37344 (100 μM) decreased the Guinea pig DSM spontaneous phasic contraction amplitude, muscle force integral, and phasic contraction frequency in the absence of atropine. A, C. Original recording of DSM contractions illustrating BRL37344 inhibitory effect in the absence (A) or presence of 1 μM atropine (C). B, D. Summary data showing BRL37344 (100 μM) inhibitory effect in the absence (B) (n=14, N=5, ***P<0.005) or presence of 1 μM atropine (D) (n=9, N=5, ***P<0.005). Spontaneous contractions were taken to be 100%. TTX (1 μM) was present throughout the experiments.

Both BRL37344 and L-755,507 inhibit carbachol-induced contractions in Guinea pig DSM isolated strips

Under control conditions (taken to be 100%), Guinea pig DSM carbachol-induced phasic contraction average amplitude was 1.4 ± 0.3 g, force was 6.8 ± 1.3 g/s, frequency was 3.0 ± 0.01 contractions/min, duration was 11.0 ± 0.6 s, and tone was 0.4 ± 0.06 g (n=59, N=21). BRL37344 (1 nM–100 μM) completely inhibited all five parameters of the carbachol-induced contractions (**Fig. 4.1.4A and 4.1.5**). L-755,507 (1 nM-100 μM) caused a less dramatic—but still statistically significant—decrease in all five parameters of the carbachol-induced contractions (**Fig. 4.1.4B**). However, only at concentrations higher than 10 μM L-755,507 did significantly inhibit DSM carbachol-induced contractions. At a concentration of 100 μM, L-755,507 decreased the phasic contraction amplitude by 26.4 ± 4.8%, muscle force integral by 53.6 ± 4.0%, phasic contraction frequency by 16.1 ± 6.6%, phasic contraction duration by 27.4 ± 3.8%, and muscle tone by 20.3 ± 7.0% (n=12, N=3, $p<0.05$; **Fig. 4.1.5**).

In the presence of SR59230A (10 μM), BRL37344 still inhibited DSM carbachol-induced contractions; however, its inhibitory effect was partially antagonized (**Fig. 4.1.4C and Fig. 4.1.5**). BRL37344 (100 μM) inhibitory effect was partially reduced as follows: phasic contraction amplitude was reduced by only 47.1 ± 3.1% (**Fig. 4.1.5A**); muscle force integral was reduced by 30.4 ± 2.2% (**Fig. 4.1.5B**); phasic contraction frequency was reduced by 51.6 ± 6.7% (**Fig. 4.1.5C**); phasic contraction duration was reduced by 57.1 ± 5.7% (**Fig. 4.1.5D**); muscle tone was reduced by 64.2 ± 6.2% (**Fig. 4.1.5E**); (n=14, N=6 for control and n=25, N=6, in presence of 10 μM SR59230A;

p<0.01). There was no significant change in the inhibitory effect of L-755,507 (1 nM–100 μM) on the carbachol-induced contractions in the presence of 10 μM SR59230A (n=7, N=3, p>0.05; **Fig. 4.1.5**).

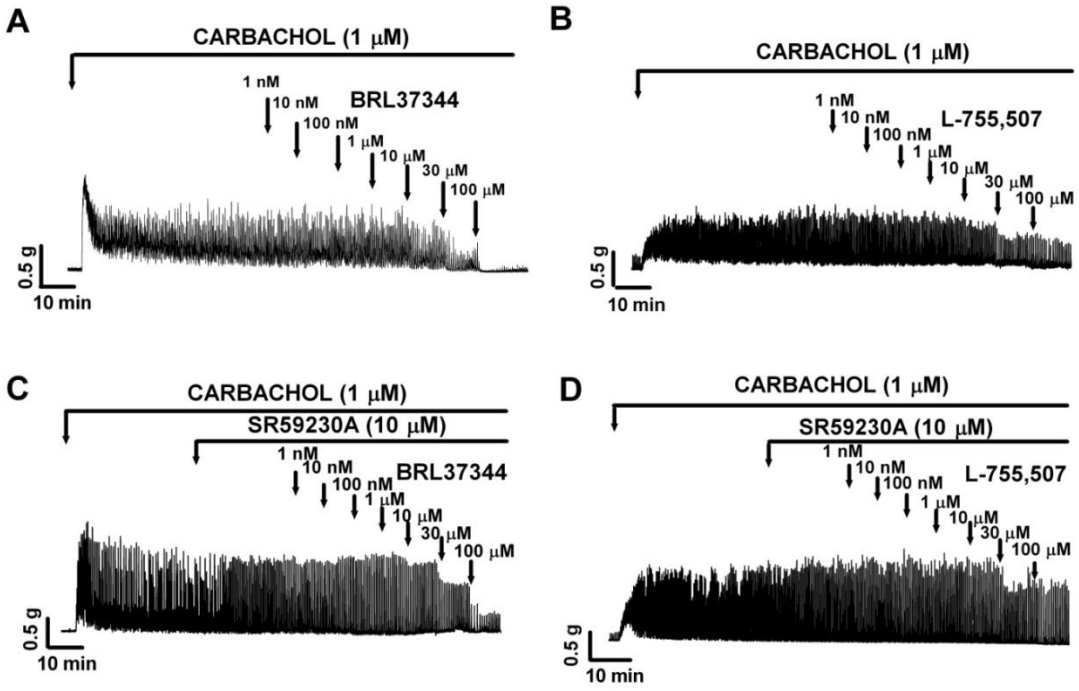

Figure 4.1.4. Original DSM contraction recordings illustrating BRL37344 (1 nM–100 μM) and L-755,507 (1 nM-100 μM) concentration-dependent inhibitory effects on carbachol-induced phasic and tonic contractions of DSM isolated strips in the absence (A, B) or presence (C, D) of SR59230A (10 μM). TTX (1 μM) was present throughout the experiments.

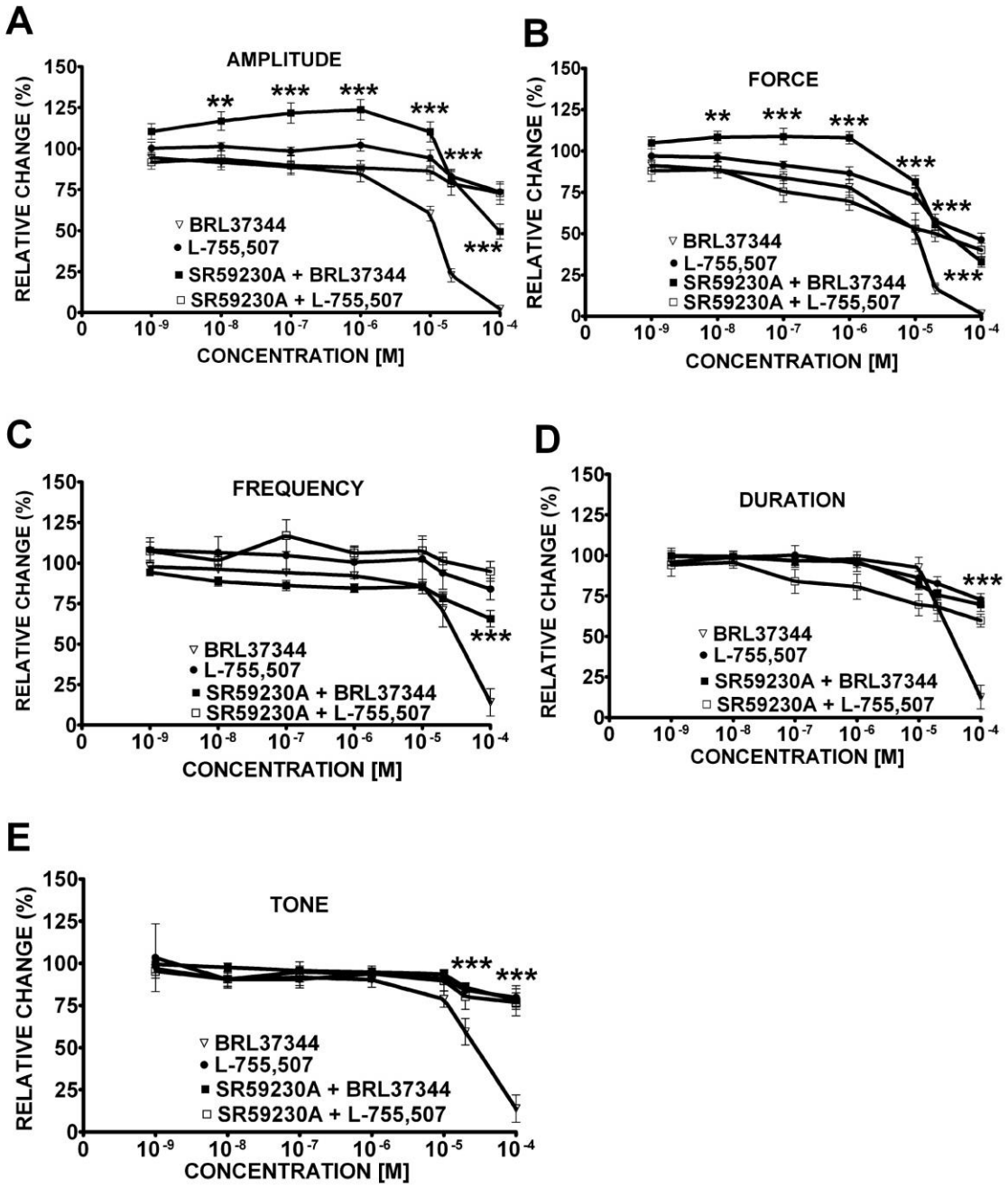

Figure 4.1.5. Concentration-response curves for BRL37344 (1 nM–100 µM) and L-755,507 (1 nM–100 µM) inhibitory effects on carbachol-induced phasic contraction amplitude (**A**), muscle force integral (**B**), phasic contraction frequency (**C**), phasic contraction duration (**D**), and muscle tone (**E**) in the presence or absence of SR59230A (10 µM). Carbachol-induced contractions were taken to be 100% (n=14, N=6 for BRL37344; n=25, N=6 for BRL37344 + SR59230A; **p<0.01, ***p<0.005 for the SR59230A effect) and (n=12, N=3 for L-755,507; n=7, N=3 for L-755,507 + SR59230A; p>0.05 for the SR59230A effect). TTX (1 µM) was present throughout the experiments

We further examined the effect of a single supramaximal concentration of L-755,507 (100 μM) on carbachol-induced contractions. L-755,507 (100 μM) significantly decreased the amplitude, muscle force integral, duration, and tone but not the frequency of carbachol-induced contractions (**Fig. 4.1.6A**). The amplitude of carbachol-induced phasic contractions decreased by 18.1 ± 6.2%, muscle force integral by 34.4 ± 5.2%, phasic contraction duration by 18.0 ± 5.1%, and muscle tone by 12.2 ± 5.0% (n=12, N=3, $p<0.05$; **Fig. 4.1.6B**). These data suggest that BRL37344 and L-755,507 can substantially reduce carbachol-induced contractions. Next, we evaluated whether pharmacological activation of β3-ARs can modulate nerve-evoked contractions of Guinea pig DSM isolated strips.

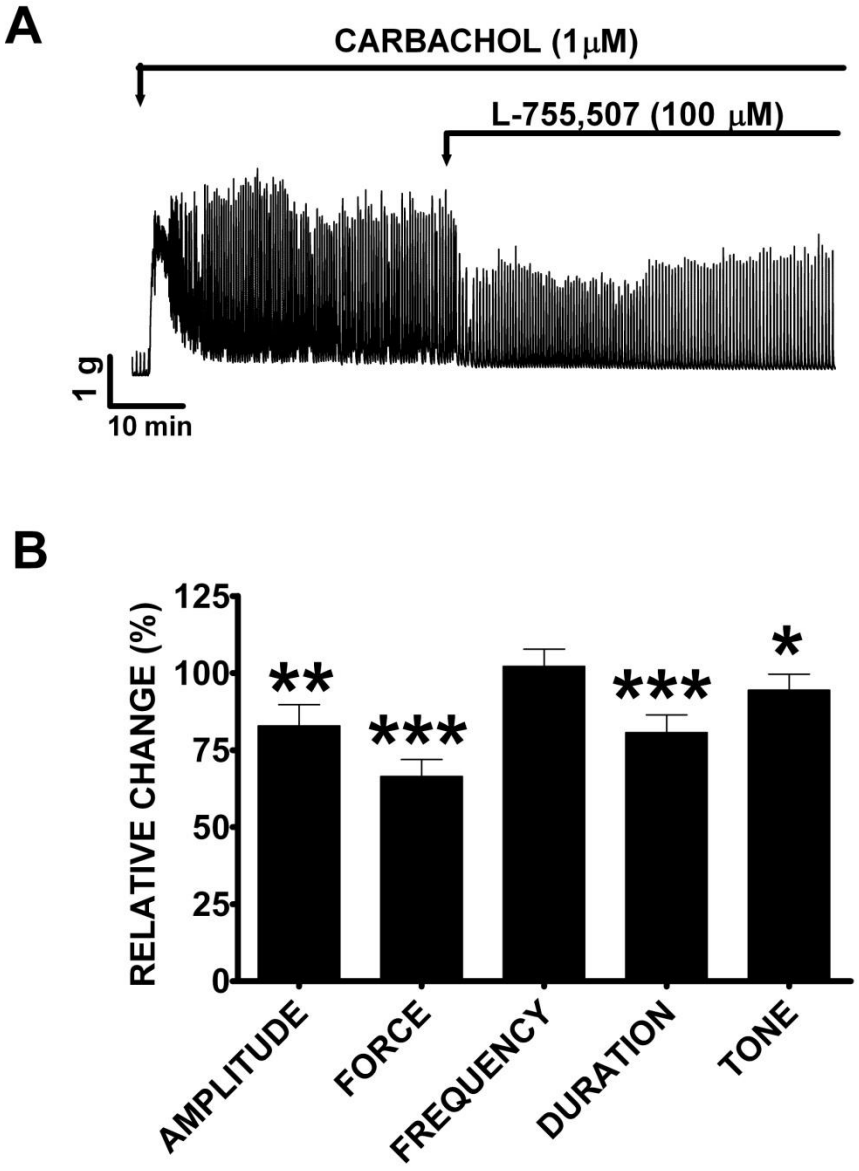

Figure 4.1.6. L-755,507 (100 μM) significantly reduced carbachol-induced phasic and tonic contractions of DSM isolated strips. A. Original DSM contraction recordings illustrating L-755,507 (100 μM) inhibitory effect in the absence of SR59230A (10 μM). B. Summary data showing that L-755,507 (100 μM) significantly reduced the amplitude, muscle force integral, phasic contraction duration, and muscle tone (n=12, N=3, *$p<0.05$, **$p<0.01$, ***$p<0.005$). TTX (1 μM) was present throughout the experiments.

BRL37344 decreases the amplitude of the EFS-induced contractions in Guinea pig DSM isolated strips

Under control conditions at 50 Hz (taken to be 100%), Guinea pig DSM EFS-induced contractions average amplitude was 4.0 ± 0.3 g (n=36, N=12). In the absence of atropine, BRL37344 (100 μM) significantly reduced the amplitude of the EFS-induced contractions at all stimulation frequencies between 7.5 and 50 Hz (**Fig. 4.1.7A**). At 50 Hz, BRL37344 (100 μM) significantly reduced the amplitude of the EFS-induced contraction by 22.5 ± 6.2% (n=10, N=4, $p<0.005$; **Fig. 4.1.7B**). In the presence of SR59230A (10 μM), BRL37344 (100 μM) still decreased the amplitude of the EFS-induced contractions (**Fig. 4.1.7C**). At 50 Hz, in the presence of SR59230A (10 μM), BRL37344 inhibited EFS-induced contractions by 29.3 ± 5.4% (n=11, N=5, $p<0.005$; **Fig. 4.1.7D**). SR59230A (10 μM) alone had no effect on the EFS-induced contractions as illustrated in **Fig. 4.1.7E and F** (n=9, N=6, $p>0.05$). These results suggest that BRL37344 inhibitory effect on EFS-induced contractions is not mediated by β3-ARs but it is most likely due to BRL37344 antimuscarinic effects.

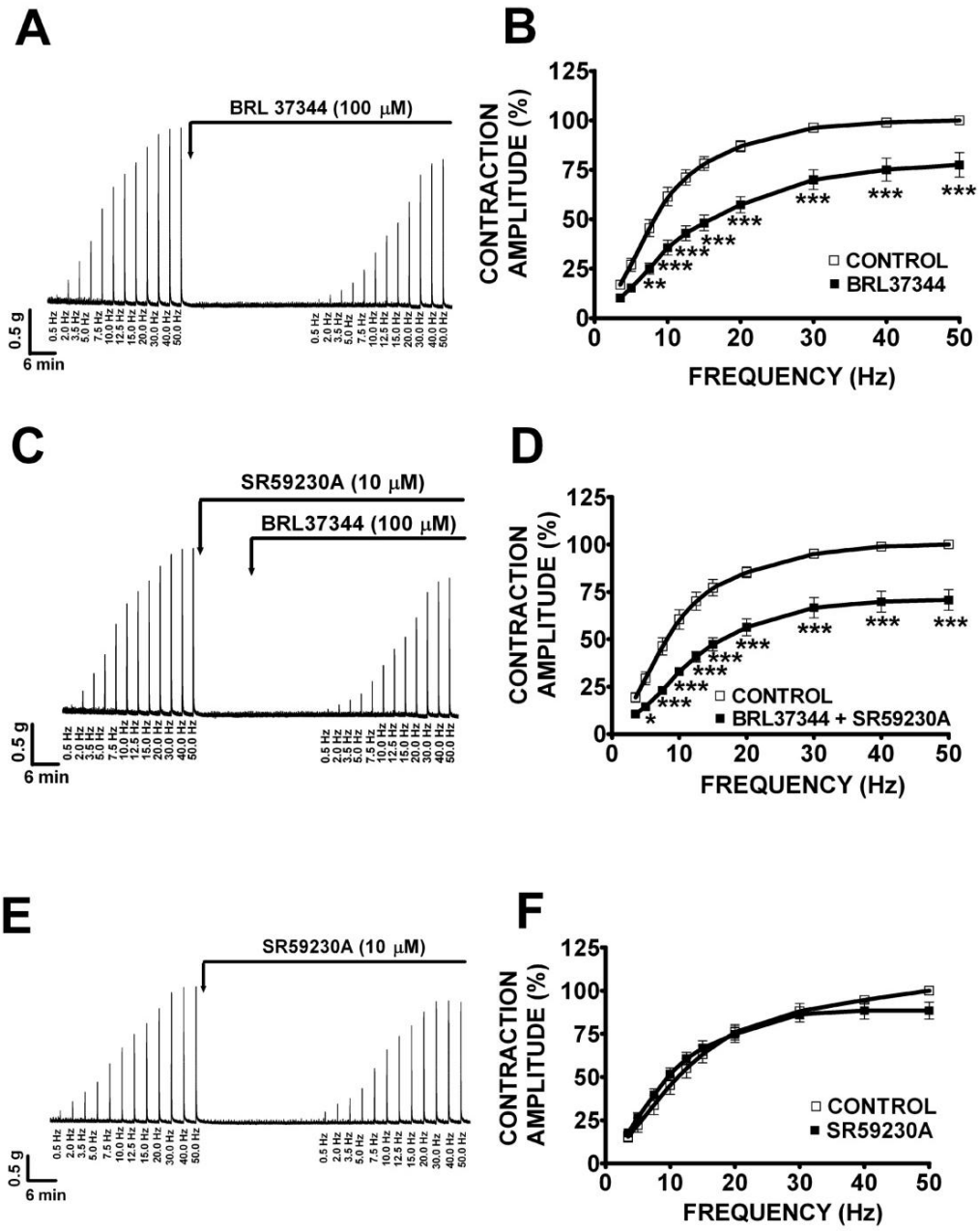

Figure 4.1.7. BRL37344 (100 μM) significantly decreases the amplitude of the EFS-induced contractions of Guinea pig DSM isolated strips in the absence or presence of SR59230A (10 μM). Original recordings illustrating EFS-induced (0.5–50 Hz) contractions of DSM isolated strips in response to 100 μM BRL37344 (A), 10 μM SR59230A and 100 μM BRL37344 combined (C), 10 μM SR59230A (E). Frequency-response curves for the EFS-induced (0.5-50 Hz) contractions in response to 100 μM BRL37344 (B), 10 μM SR59230A and 100 μM BRL37344 combined (D), 10 μM SR59230A (F) (n=10, N=4 for B; n=11, N=5 for D; n=9, N=6, p>0.05 for F; *p<0.05, **P<0.01, ***P<0.005). EFS-induced contraction amplitude at 50 Hz under control conditions was taken to be 100%.

In the presence of suramin (10 μM) and α,β-methylene-ATP (10 μM) which were used to block the purinergic component of EFS-induced contraction, BRL37344 (100 μM) still decreased the amplitude of the EFS-induced contractions as shown in **Figure 4.1.8A**. In the presence of suramin and α,β-methylene-ATP, Guinea pig DSM EFS-induced contraction average amplitude at 50 Hz was 2.4 ± 0.4 g and was taken to be 100% (n=20, N=10). At the maximal stimulation frequency of 50 Hz, BRL37344 (100 μM) significantly reduced the amplitude of the EFS-induced contractions by 36.9 ± 8.5% (n=10, N=5, $p<0.005$; **Fig. 4.1.8B**). SR59230A (10 μM) again did not block the BRL37344 inhibitory effect on contraction amplitude at EFS stimulation frequencies between 10 and 50 Hz (**Fig. 4.1.8C**). At the maximal stimulation frequency of 50 Hz, in the presence of SR59230A (10 μM), BRL37344 (100 μM) decreased the EFS-induced contraction amplitude by 52.7 ± 5.0% (n=10, N=5, $p<0.005$; **Fig. 4.1.8D**).

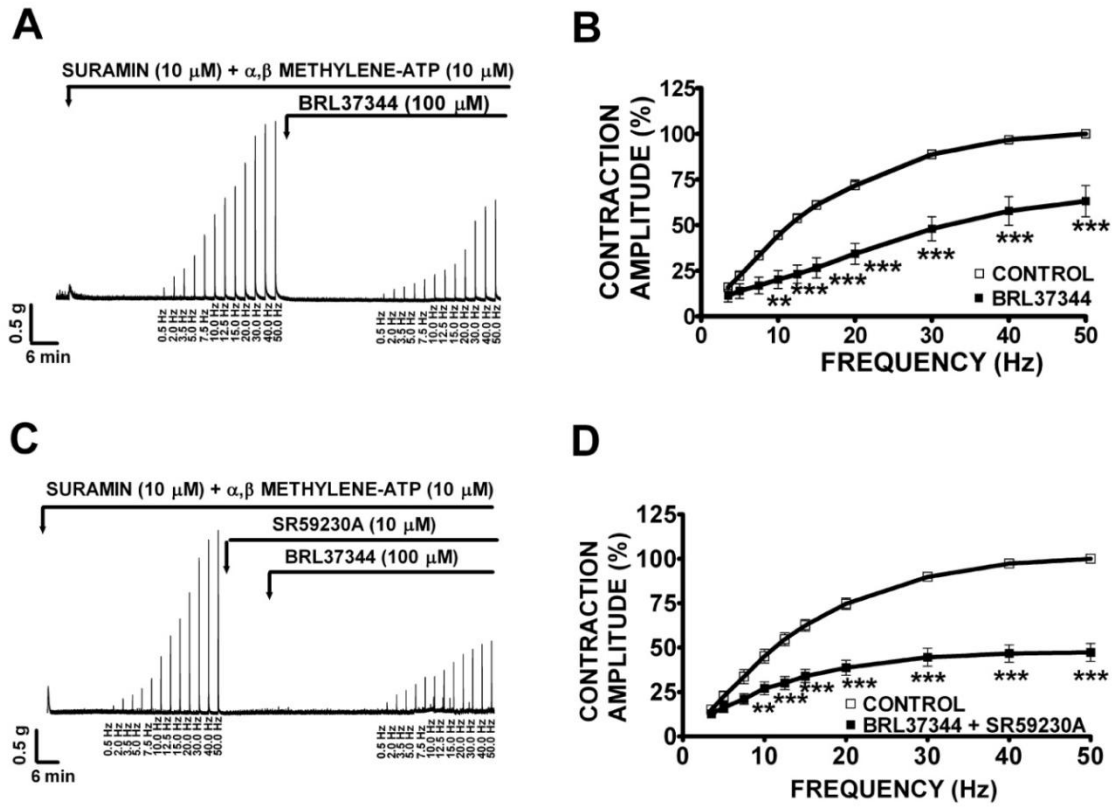

Figure 4.1.8. In the presence of suramin (10 μM) and α,β-methylene-ATP (10 μM), BRL37344 (100 μM) decreased the amplitude of the EFS-induced (0.5-50 Hz) contractions in DSM isolated strips. A, C. Original EFS-induced contraction recordings showing BRL37344 (100 μM) inhibitory effect in the absence (A) or presence (C) of SR59230A (10 μM). B, D. Frequency-response curves showing BRL37344 inhibitory effect on EFS-induced contraction amplitude in the absence (n=10, N=5, **P<0.01, ***P<0.005) (B) or presence (D) of 10 μM SR59230A (n=10, N=5, **P<0.01, ***P<0.005). EFS-induced contraction amplitude at 50 Hz under control conditions was taken to be 100%.

In the presence of atropine (1 μM), which was used to block the cholinergic component of the EFS-induced contraction, BRL37344 (100 μM) had no effect on the EFS-induced contractions in the absence (n=13, N=6, $p>0.05$) or presence of 10 μM SR59230A (n=12, N=5, $p>0.05$; **Fig. 4.1.9**). In the presence of atropine, Guinea pig DSM EFS-induced contraction average amplitude at 50 Hz was 2.3 ± 0.2 g and was taken to be 100% (n=25, N=11). These data also show that BRL37344's inhibitory effect on EFS-induced contractions is not mediated by β3-AR but is rather due to BRL37344 antimuscarinic properties. Next, we sought to evaluate L-755,507 effect on EFS-induced contractions.

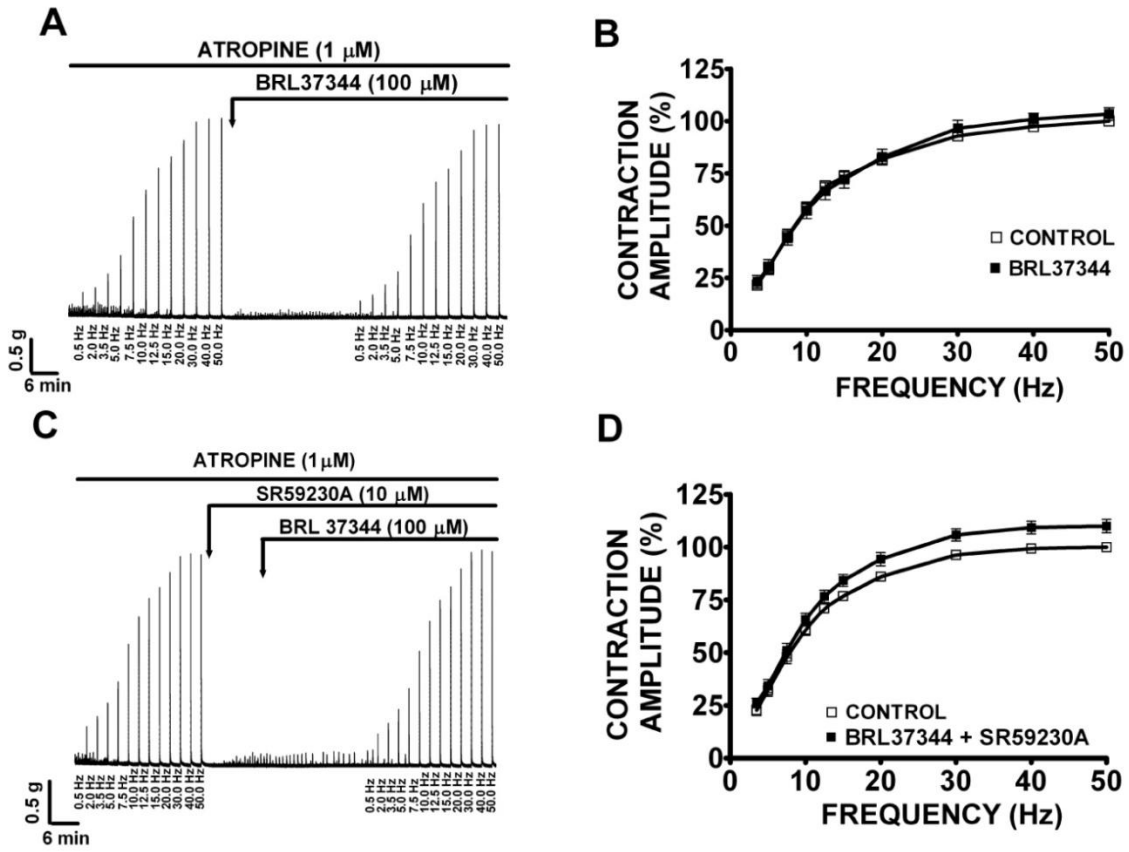

Figure 4.1.9. In the presence of atropine (1 μM), BRL37344 (100 μM) does not affect the amplitude of the EFS-induced (0.5-50 Hz) contractions of DSM isolated strips. A, C. Original recordings illustrating the lack of BRL37344 (100 μM) effect on EFS-induced contractions in the absence (A) or presence (C) of SR59230A (10 μM). B, D. Frequency-response curves showing the lack of BRL37344 effect on the EFS-induced contraction amplitude in the absence (B) (n=13, N=6, $p>0.05$) or presence (D) of 10 μM SR59230A (n=12, N=5, $p>0.05$).

L-755,507 has no effect on the EFS-induced contractions of Guinea pig DSM isolated strips

In this experimental series we investigated the effect of L-755,507 on EFS-induced contractions. We observed that L-755,507 (100 μM) did not significantly inhibit EFS-induced contractions at all stimulation frequencies (0.5-50 Hz) in the absence (n=9, N=4, p>0.05) or presence of 1 μM atropine (n=6, N=4, p>0.05; **Fig. 4.1.10**).

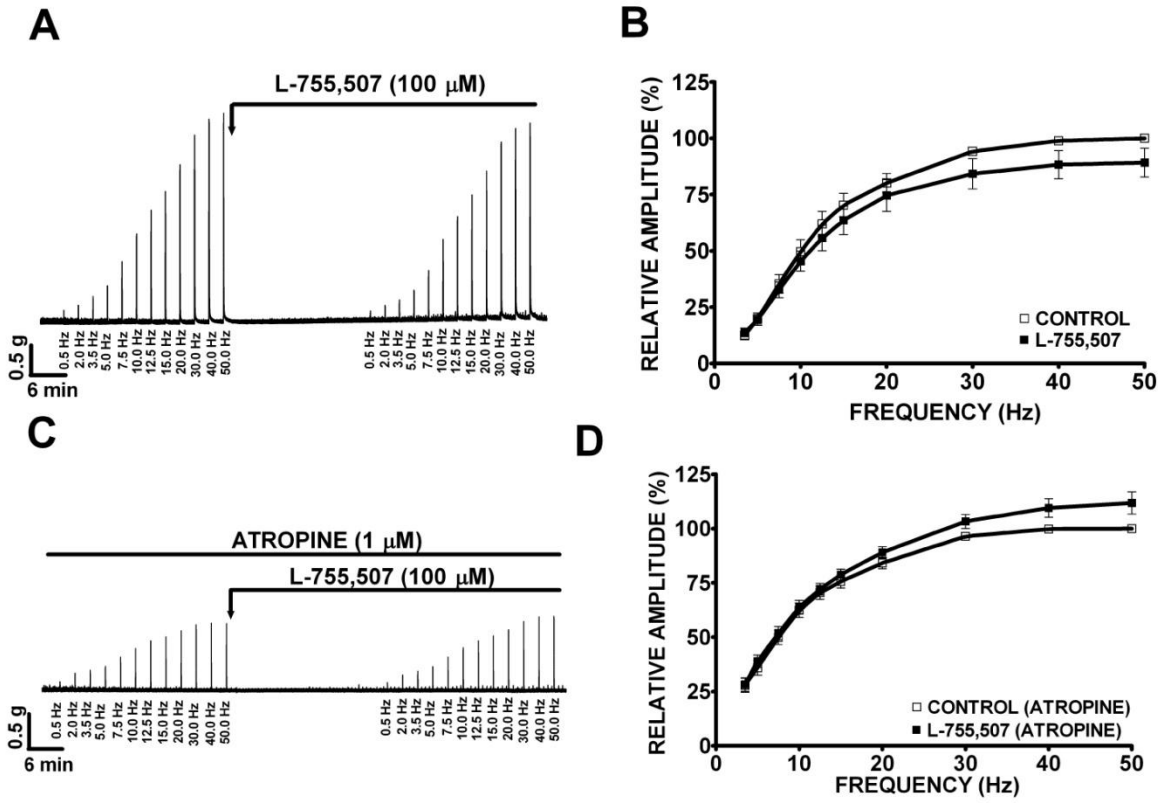

Figure 4.1.10. L-755,507 (100 μM) does not affect the amplitude of the EFS-induced (0.5–50 Hz) contractions of DSM isolated strips. A, C. Original recordings illustrating a lack of L-755,507 (100 μM) effect on EFS-induced contractions in the absence (A) or presence (C) of atropine (1 μM). B, D. Frequency-response curves showing the lack of inhibitory effect on EFS-induced contractions by L-755,507 in the absence (B) (n=9, N=4, p>0.05) or presence (D) of 1 μM atropine, (n=6, N=4, p>0.05).

Pharmacological activation of beta3-ARs with BRL37344 does not modulate cell excitability in Guinea pig DSM freshly isolated cells

BRL37344 was preferred over L-755,507 due to its more pronounced effects on Guinea pig DSM contractility. We previously reported that in rat DSM, BRL37344 (100 μM) induces relaxation by activation of TBKCs and membrane hyperpolarization (79).

The average capacitance of Guinea pig DSM cells was 34.7±0.8 pF (n=123 cells, N=30). In the first patch-clamp experimental series, we investigated BRL37344 (100 μM) effect on Guinea pig DSM TBKCs amplitude and frequency. Our results showed no statistically significant change in the mean amplitude and frequency of TBKCs after application of 100 μM BRL37344 (n=6-9, N=5-7, $p>0.05$; **Table 4.1.1**). Next, we examined whether the application of BRL37344 (100 μM) activates the voltage step induced steady-state K^+ current in single DSM cells. From a holding potential -70 mV, we applied a voltage-step protocol from 0 mV to +80 mV in 20 mV increments and 200 ms duration. BRL37344 (100 μM) did not activate the depolarization-induced steady-state outward K^+ currents (n=4, N=2, $p>0.05$; **Table 4.1.1**). In current clamp mode, we tested the effect of BRL37344 (100 μM) on the resting membrane potential of freshly isolated Guinea pig DSM cells, and BRL37344 did not affect the resting membrane potential (n=8, N=4, $p>0.05$; **Table 4.1.1**). These data suggest that activation of β3-ARs with BRL37344 in Guinea pig DSM does not modulate basal cell excitability.

		Control	BRL37344 (100 μM)	Statistics
Transient BK currents (TBKCs)	**Amplitude -40 mV**	24.0±4.6 pA	26.3±6.5 pA	n=9; N=7 p>0.05
	Amplitude -20 mV	19.8±2.4 pA	18.16±1.1 pA	n=6; N=5 p>0.05
	Frequency -40 mV	50.2±22.8 TBKCs/min	65.76±30.4 TBKCs/min	n=9; N=7 p>0.05
	Frequency -20 mV	45.3±18.2 TBKCs/min	74.23±31.2 TBKCs/min	n=6; N=5 p>0.05
Voltage step induced whole-cell current	**0 mV**	2.3±1.0 pA/pF	1.1±0.3 pA/pF	n=4; N=2 p>0.05
	20 mV	3.5±1.1 pA/pF	1.8±0.2 pA/pF	n=4; N=2 p>0.05
	40 mV	5.9±1.7 pA/pF	3.6±0.6 pA/pF	n=4; N=2 p>0.05
	60 mV	11.4±3.3 pA/pF	10.8±1.9 pA/pF	n=4; N=2 p>0.05
	80 mV	22.8±7.5 pA/pF	22.7±5.1 pA/pF	n=4; N=2 p>0.05
Resting membrane potential recorded in current clamp mode		-22.8±3.5 mV	-21.6±4.0 mV	n=8; N=4 p>0.05

Table 4.1.1. Summarized data illustrating a lack of effect of BRL37344 (100 μM) on cell membrane excitability in Guinea pig DSM freshly isolated cells. Perforated whole-cell patch-clamp experiments revealed no effect of 100 μM BRL37344 on TBKCs amplitude and frequency recorded at -40 and -20 mV. Whole-cell outward K^+ current amplitude was also unchanged after application of 100 μM BRL37344. Current-clamp experiments revealed that 100 μM BRL37344 did not significantly change the level of the resting membrane potential in Guinea pig DSM freshly isolated cells. *These data were collected by Dr. Hristov.*

DISCUSSION

In this study, we applied a multi-disciplinary approach including molecular, cellular, and tissue studies to evaluate the expression and potential functional role of β3-ARs in Guinea pig DSM. Because beta3-ARs are considered viable therapeutic targets for OAB, it is crucial to understand beta3-ARs expression and function in the bladder of Guinea pig, a commonly used animal research model. Our data suggest that beta3-ARs are expressed at the mRNA level but are minor contributors (if at all) in Guinea pig DSM function. Unlike earlier studies (169), the novelty of our study is that we systematically spanned for all five parameters of the phasic contractions including amplitude, muscle force integral, frequency, duration, and muscle tone in Guinea pig DSM isolated strips.

The physiological significance of our study derives from the fact that beta3-AR have been shown to be important facilitators of bladder relaxation by controlling spontaneous phasic and nerve-evoked contractions as well as TBKC that contribute to DSM excitability (22, 79, 134). Previous studies have established a functional link between the beta3-AR and the BK channel, which plays a key role in DSM function (22, 75, 79, 91). These findings have lead researchers to suggest that beta3-ARs could be suitable targets for the pharmacological treatment of OAB. As a consequence, there are now a number of beta3-AR agonists, which are currently under investigation for the treatment of OAB. In anesthetized mice, CL316,243, a selective beta3-AR agonist, given intravenously at doses of 0.03 and 0.1 mg/kg, significantly increases bladder capacity and threshold pressure without a significant change in bladder compliance (39). CL316,243 also induces a significant decrease in the amplitude of both micturition and non-voiding contractions

(39). In anesthetized rats, intravenous injection of selective beta3-AR agonists such as BRL37344, TAK-677, and FK175 (0.1-500 µg/kg) have been proven to significantly decrease voiding frequency, bladder pressure, and the amplitude of bladder contractions (94).

Whether beta3-ARs are expressed in Guinea pig DSM has been debated: no reports identify their presence at the single-DSM-cell level. Pharmacological studies on Guinea pig DSM using various beta3-AR agonists and antagonists yielded controversial results (49, 100, 169). Clarification of beta3-AR functional role has a strong physiological significance because the Guinea pig is an established animal model to study DSM function added to the fact that species differences could also be a determining factor. Some authors suggest that the beta1-AR is the most predominant beta-AR in Guinea pig DSM, and thus is mainly responsible for Guinea pig DSM relaxation (100, 111, 169). Other studies show that the selective beta3-AR agonist BRL37344 (300 µM) reduced the amplitude of phasic rises in intravesical pressure of Guinea pig isolated whole bladder, suggesting a role of beta3-ARs in Guinea pig DSM relaxation (49). Our molecular data support the latter publication that the beta3-AR mRNA message is expressed in isolated single cells of Guinea pig DSM (**Fig. 4.1.1**). The advantage of using isolated single cells for RT-PCR is that this approach eliminates any possible contamination by other cell types present within the DSM (29, 30, 75, 79). Due to the lack of specific antibodies for Guinea pig beta3-ARs (111), we performed no Western blot or immunohistochemistry studies at this time to further prove beta3-AR protein expression. To our knowledge, this is the first report in which molecular evidence for the presence of beta3-ARs in Guinea pig DSM has been provided.

The functional role of beta-ARs activation in DSM is to induce relaxation (22, 79, 91, 134, 169), which is suggested to be mediated via activation of the beta-AR/PKA pathway (79, 111, 134). Our results indicate that at higher concentrations BRL37344 induced a slight concentration-dependent reduction of the amplitude and frequency of the spontaneous phasic contractions of Guinea pig DSM isolated strips (**Fig. 4.1.2**). Furthermore, a single supramaximal concentration of BRL37344 (100 μM) also decreased the amplitude, muscle force integral, and frequency of the spontaneous phasic contractions of Guinea pig DSM isolated strips (**Fig. 4.1.3**). The use of this supramaximal concentration of 100 μM is justified by the fact that BRL37344 concentrations less than or equal to 30 μM seems insufficient to induce inhibition of spontaneous DSM contractions (22, 49, 169). However, at these high concentrations BRL37344 may also non-selectively activate beta1-ARs and/or beta2-ARs (150).

Studies on rat DSM have shown that BRL37744 also exhibit some antimuscarinic properties (22, 93, 110). In our study, we addressed this issue by using the muscarinic receptor antagonist atropine. In the presence of atropine, BRL37344 (100 μM) lost its inhibitory effect on the DSM phasic contraction amplitude and muscle force integral (**Fig. 4.1.3**), further supporting its antimuscarinic properties. Our data (consistent with early findings) (169), indicate that in the Guinea pig, beta3-ARs have a limited effects on DSM relaxation because their pharmacological activation with BRL37344 significantly modulates only one (frequency) spontaneous phasic contraction parameter and only at high concentrations (10-100 μM). At these high concentrations BRL37344 may also non-selectively activate beta1-ARs and/or beta2-ARs (150).

In DSM, the beta adrenergic relaxation is primarily mediated by activation of the BK channel (22, 79, 134). Our electrophysiological data supported the idea that beta3-ARs, although expressed in Guinea pig DSM, do not regulate the DSM excitability. Perforated patch-clamp experiments revealed no effect of BRL37344 on whole-cell steady-state K^+ current, TBKCs amplitude and frequency, and the resting membrane potential (**Table 4.1.1**). These findings clearly suggest species differences. Our previous studies on rat DSM showed that the activation of beta3-AR by BRL37344 increased TBKCs frequency, and hyperpolarized the cell membrane (79). In rat DSM, a functional link between beta3-ARs and BK channel is mediated by the RyRs of the sarcoplasmic reticulum (79). Our study shows that in Guinea pig DSM, unlike rat and human DSM, beta3-ARs are not the primary regulator of DSM function.

Since the relationship between length and force can be converted to volume and pressure using the law of Laplace (7), the evaluation of muscle force integral provides information on the increase in bladder volume and intravesical pressure during bladder filling and voiding phases. It is known that the BK channels shape Guinea pig DSM spontaneous action potentials that determine the spontaneous phasic contractions including contraction amplitude, duration, frequency, muscle force integral, and tone (22, 58, 66, 76, 79, 132). Pharmacological inhibition of DSM BK channels increases the action potential duration and frequency, causes membrane potential depolarization (62, 76), and increases the amplitude of phasic contractions (22, 66, 76, 132). The increase in the amplitude of a phasic contraction is provoked by the increase in intracellular Ca^{2+} during the action potential depolarization phase and activation of L-type voltage-gated Ca^{2+} channels, whereas the duration of a phasic contraction depended on the duration of the single action

potential or a whole burst of action potentials (58, 62). Thus, there is a clear association between the action potential parameters, BK channel activity, and the related phasic contraction parameters. However, our data on Guinea pig DSM show that BRL37344 does not affect BK channel activity (**Table 4.1.1**).

We also evaluated the effects of beta3-AR agonists during cholinergic stimulation using carbachol, a stable pharmacological analogue of acetylcholine, as previously done by others (49, 91). Under such conditions, BRL37344 and L-755,507 significantly decreased all phasic and tonic contraction parameters in a concentration-dependent manner (**Figs. 4.1.4-5**). BRL37344 inhibitory effect could be explained by its pronounced antimuscarinic properties since this inhibitory effect on spontaneous contractions was partially blocked by atropine (**Fig. 4.1.3**). Consistent with our results, previous studies by Klausner *et al.* (2009) showed that isoproterenol, a non-selective beta3-AR agonist, decreased the contractions induced by low concentrations of carbachol in rabbit DSM strips suggesting the involvement of beta-ARs (91). Using arecaidine, a muscarinic receptor agonist, to induce contractions in Guinea pig isolated whole bladder, Gillespie (2004) also showed that BRL37344 (300 µM) could significantly reduce the amplitude of phasic rises in intravesical pressure of Guinea pig isolated whole bladder (49). Furthermore, SR59230A did not antagonize L-755,507 inhibitory effect on the carbachol-induced contractions (**Figs. 4.1.4-5**) suggesting that L-755,507-induced relaxation of the carbachol-induced contractions is not mediated through beta3-AR. Indeed, at higher concentrations (10-100 µM), L-755,507 may also act on beta1-/ beta2-AR directly. L-755, 507 EC_{50} values for beta3-, beta1-, and beta2-AR are 0.43 nM, 530 nM, and >10 µM, respectively (43, 128). Therefore, the inhibitory effect of L-755,507 observed at

such high concentrations (10–100 μM) on carbachol-induced contractions could be due to its direct effects on beta1-and/or beta2-ARs (128), which tend to corroborate previous finding by others (100, 169). One explanation for BRL37344 inhibitory effects on carbachol-induced contractions is that during cholinergic stimulation with carbachol, muscarinic M2 receptors, which are linked to the cAMP/PKA inhibitory pathway, are activated. M2 receptors and beta3-ARs have opposing effects at the cAMP/PKA level which may account for the inhibitory effects of BRL37344 and L-755,507 on carbachol-induced contractions. We find that the relaxation of Guinea pig DSM isolated strips by BR37344 and L-755,507 is predominant during cholinergic stimulation with carbachol and minor during spontaneous contractions, consistent with previous studies (169).

Nerve stimulation also plays an important role in the micturition process during which acetylcholine and ATP are released from parasympathetic nerve terminals and act on DSM muscarinic (M2/M3) and purinergic (P2X) receptors to generate DSM contractions (59, 65). The observed decrease of the EFS-induced contraction amplitude by BRL37344 can be misleading (**Fig. 4.1.7**). This BRL37344 inhibitory effect was suppressed by atropine, suggesting that BRL37344 acted directly on muscarinic receptors rather than β3-ARs during nerve stimulation (**Fig. 4.1.9**). In addition to DSM cells, muscarinic receptors are also present in bladder nerves (89). It has been reported that presynaptic muscarinic receptors on parasympathetic nerve terminals in DSM strips are involved in an autofacilitatory mechanism that enhances acetylcholine release during continuous EFS (15). BRL37344 may therefore block muscarinic receptors in both DSM cells and bladder nerves. Furthermore, the beta3-AR agonist, L-755,507, at concentration as high as 100 μM has no effect on the EFS-induced contractions in the absence or presence of atropine

suggesting that beta3-ARs do not play any role in the nerve-evoked contraction of Guinea pig DSM (**Fig. 4.1.10**). This latter observation confirms the concept that the decrease in EFS-induced contraction caused by BRL37344 was a direct antimuscarinic effect. An alternative explanation is that the beta3-ARs in Guinea pig DSM have a distinct pharmacological profile compared to beta3-ARs in other species or that beta3-ARs might not be functionally expressed in Guinea pig DSM.

In summary, four key points can be drawn from our study: 1) beta3-ARs are expressed at the mRNA level but play a negligible functional role, if any, in Guinea pig DSM relaxation; 2) BRL37344 inhibitory effect on Guinea pig DSM spontaneous and nerve-evoked contractions is primarily mediated through its antimuscarinic properties; 3) BRL37344 and L-755,507 have significant inhibitory effects on Guinea pig carbachol-induced contractions; 4) BRL37344 does not modulate Guinea pig basal cell membrane excitability. Collectively, our data suggest that unlike rat and human DSM (79, 140, 151, 168), in Guinea pig DSM beta3-ARs have a minor to no functional role in DSM excitability and spontaneous contractility.

We recommend particular prudence when interpreting data generated with BRL37344 because of its pronounced antimuscarinic effects. We also suggest that, although the Guinea pig DSM is widely used for experimental studies, future investigations on beta3-ARs should not use this animal model. Our study does not rule out β3-ARs functional role in Guinea pig urothelium and other non-DSM bladder cells (49, 168).

4.2 FUNCTIONAL BK CHANNELS FACILITATE THE BETA3 ADRENERGIC RECEPTOR AGONIST-MEDIATED RELAXATION OF NERVE-EVOKED CONTRACTIONS IN RAT DETRUSOR SMOOTH MUSCLE ISOLATED STRIPS

Data presented in this section have been submitted for publication and are currently under revision. The original manuscript has been modified to fit the format of this dissertation.

ABSTRACT

The large-conductance voltage- and Ca^{2+}-activated K^+ (BK) channel is a major regulator of detrusor smooth muscle (DSM) contractility. Recent findings suggest that activation of beta3 adrenergic receptors causes relaxation of the DSM. However, it is unknown whether the beta3 adrenergic receptor-mediated relaxation of DSM is BK channel-dependent during nerve-evoked contractions. To test this hypothesis, we induced nerve-evoked contractions in rat DSM isolated strips by using a tissue bath system equipped with platinum electrodes for electrical field stimulation (EFS). BRL37344, a beta3 adrenergic receptor agonist, significantly decreased the amplitude and muscle force of the 20 Hz EFS-induced DSM contractions in a concentration-dependent manner. The BRL37344 inhibitory effect was significantly antagonized by SR59230A, a selective beta3 adrenergic receptor antagonist. We further isolated the cholinergic from the purinergic component of the 0.5–50 Hz EFS-induced DSM contractions by using selective inhibitors, atropine as well as suramin and α,β-methylene-ATP, respectively. We found that BRL37344 inhibited both the purinergic and cholinergic components of

the nerve-evoked contractions in rat DSM isolated strips. The pharmacological blockade of the BK channels with iberiotoxin, a selective BK channel inhibitor, increased the amplitude and muscle force of the 20 Hz EFS-induced contractions in rat DSM isolated strips. In the presence of iberiotoxin, there was a significant reduction of the BRL37344-induced maximal inhibition of the 20 Hz EFS-induced contractions in rat DSM isolated strips. These latter findings suggest that BK channels play a critical role in the beta3 adrenergic receptor-mediated relaxation of rat DSM nerve-evoked contractions.

INTRODUCTION

The ability of beta3 adrenergic receptor agonists such as BRL37344, mirabegron (YM178), solabegron (GW427353), FK-175, TAK677, TRK-380, CL316243, and CGP12177A to inhibit detrusor smooth muscle (DSM) myogenic contractions has been well documented (14, 46, 69, 79, 88, 94, 99, 139, 150, 151, 156). These beta3 adrenergic receptor agonists have been shown to increase the intracellular cAMP levels in native DSM tissues as well as in heterologously expressed systems (46, 69, 88, 94, 139, 150). Specifically, in rat DSM isolated strips, FK175 was shown to increase the intracellular cAMP level by ~30% (46). The recent approval of mirabegron as the first beta3 adrenergic receptor agonist for the treatment of overactive bladder (OAB) further outlines the importance of beta3 adrenergic receptors as pharmacological targets for OAB. Since OAB has myogenic and neurogenic origins with diverse mechanisms and etiology, it is still unknown whether this new therapeutic approach will be effective to treat all forms of OAB. There has been significant effort aiming to investigate the mechanism by which beta3 adrenergic receptor agonists induce relaxation of DSM, especially during neurogenic contractions. However, the exact mechanism remains uncertain. Experiments conducted on DSM myogenic contractions suggest that the mechanism underlining the beta3 adrenergic receptor-mediated relaxation of the DSM involves the cAMP signaling pathway and the large-conductance Ca^{2+}-activated K^+ (BK) channels (79, 111, 130, 134, 164, 166). Our group revealed a functional link between BK channels and beta3 adrenergic receptors during myogenic contractions, a mechanism which utilizes the ryanodine receptors of the sarcoplasmic reticulum to induce DSM relaxation (79). The pharmacological blockade of BK channels with iberiotoxin, a selective BK channel

inhibitor, caused a rightward shift of the concentration-response curve of the BRL37344 relaxant effects on myogenic contractions of rat DSM isolated strips. The BRL37344-induced hyperpolarization of freshly isolated DSM cells was eliminated by iberiotoxin or ryanodine suggesting that activation of beta3 adrenergic receptors increases the BK channel activity and ultimately leads to DSM relaxation (79). It has been demonstrated that in mouse DSM isolated strips, selective inhibition of the BK channels and protein kinase-A with iberiotoxin and H-89, respectively, reduced the relaxant effect of isoproterenol, a non-selective beta adrenergic receptor agonist (22). The fundamental role of the BK channels in the beta-adrenergic/protein kinase-A-mediated relaxation of DSM is demonstrated by the observations that genetic ablation of BK channel in a transgenic mouse model leads to development of a compensatory adaptive upregulation of the beta-adrenergic/cAMP/protein kinase-A signal transduction pathway (22). However, these novel mechanisms have yet to be investigated in nerve-evoked DSM contractions. The present study utilizes tissue baths equipped with electrodes for electrical field stimulation (EFS), DSM strips isolated from rat urinary bladder, and various pharmacological modulators to assess beta3 adrenergic receptor and BK channel functional role in the nerve-evoked contractions.

METHODS

Isometric DSM tension recordings

Isometric DSM tension recording experiments were performed as described in chapter 2.2. In total, 41 male Sprague-Dawley rats (10-16 weeks old) weighing 361.7 ± 5.2 g on average were used.

Solutions and Drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS compositions are given in chapter 2.2. Iberiotoxin, atropine, suramin, and α,β-methylene-ATP were purchased from Sigma-Aldrich (St. Louis, MO). BRL37344 and SR59230A were purchased from Tocris Bioscience (Bristol, UK). BRL37344 was prepared daily in double-distillated water and heated at 60^{o}C to be completely dissolved as suggested by the manufacturer**.**

Data analysis and statistics

The data analysis was performed as described in chapter 2.2.

RESULTS

BRL37344 inhibitory effect on EFS-induced DSM contractions is antagonized by SR59230A, a selective beta3 adrenergic receptor antagonist

We investigated how beta3 adrenergic receptor stimulation with BRL37344 affects nerve-evoked contractions of rat DSM isolated strips. We found that BRL37344 decreased the amplitude and muscle force of the 20 Hz EFS-induced contractions of rat DSM isolated strips in a concentration-dependent manner. BRL37344 (10 nM – 1 μM) had no significant inhibitory effect, however, BRL37344 at concentrations of 10 μM and 100 μM statistically significantly decreased the amplitude of the 20 Hz EFS-induced DSM contractions by 22.3±9.3% and 48.4±7.0%, respectively and the muscle force by 26.0±8.6% and 52.6±6.2%, (n=8, N=6, $p<0.05$; **Fig. 4.2.1A, C, and D**). Pre-incubation of rat DSM strips with SR59230A (10 μM), a selective beta3 adrenergic antagonist, significantly reduced the BRL37344 inhibitory effect on the 20 Hz EFS-induced contractions **(Fig. 4.2.1B-D)**. SR59230A attenuated the relaxant effect of BRL37344 at 10 μM and 100 μM on the contraction amplitude from 22.3±9.3% to 5.5±0.7%, and from 48.4±7.0% to 31.0±3.5%, respectively; and also on the contraction force from 26.0±8.6% to 10.0±2.6%, and from 52.6.0±6.2% to 33.5±6.0%, respectively (n=11, N=6, $p<0.05$; **Fig. 4.2.1C, D**). Time control experiments performed in the absence of BRL37344 and SR59230A showed no statistically significant difference in the 20 Hz EFS-induced contraction amplitude and force (n=6, N=6, $p>0.05$; data not illustrated). These findings suggest that the inhibitory effects on EFS-induced contractions induced by the beta3 adrenergic receptor agonist, BRL37344, can be antagonized by SR59230A.

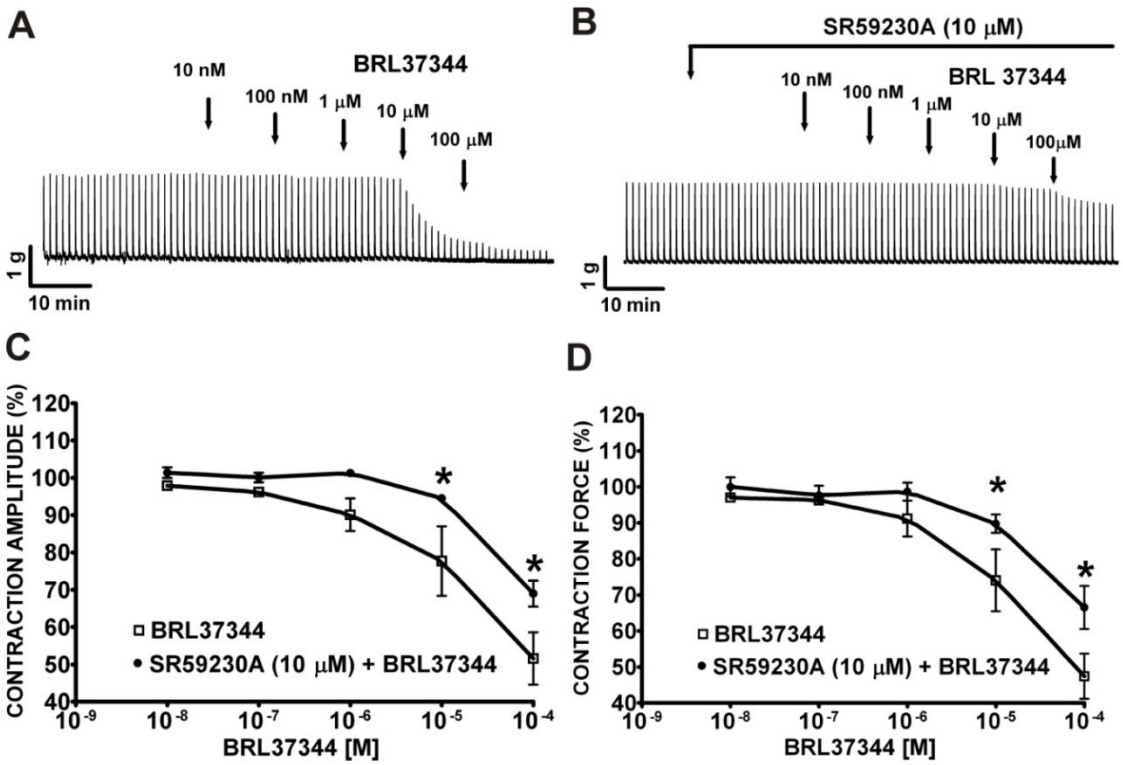

Figure 4.2.1. Activation of beta3 adrenergic receptors with BRL37344 decreases the amplitude and muscle force of the 20 Hz EFS-induced contractions in rat DSM isolated strips. A) Original DSM tension recordings illustrating BRL37344 (10 nM – 100 μM) inhibitory effects on 20 Hz EFS-induced contractions. This particular original recording shows a bit greater degree of inhibition than the average observed. B) Original DSM tension recordings illustrating the attenuation of BRL37344 (10 nM – 100 μM) inhibitory effects on rat DSM 20 Hz EFS-induced contractions in the presence of SR59230A (10 μM). C-D) Cumulative concentration-response curves illustrating SR59230A antagonistic effects against BRL37344 inhibitory effects on EFS-induced contraction amplitude and force, respectively (n=11, N=6; *$p<0.05$)

We also investigated how BRL37344 modulates the two main components of the nerve-evoked contractions, namely the cholinergic and the purinergic components, under a wide range of stimulation frequencies (0.5 – 50 Hz). First, we evaluated the changes in the amplitude of the rat DSM EFS-induced contractions following application of BRL37344 (100 µM). We found that BRL37344 significantly inhibited EFS-induced contractions at frequencies ranging from 3.5 to 50 Hz (**Fig. 4.2.2A, C**). At the maximal stimulation frequency of 50 Hz, BRL37344 caused a 30.3±6.0% decrease in the amplitude of the EFS-induced DSM contractions (n=12, N=6, $p<0.005$; **Fig. 4.2.2C**). This BRL37344 inhibitory effect was significantly antagonized by SR59230A (10 µM) at low stimulation frequencies (3.5 – 12.5 Hz) but not at high frequencies (15 – 50 Hz) (n=8, N=3, $p<0.005$; **Fig. 4.2.2B, D**). SR59230A (10 µM) *per se* did not have any effect on the nerve-evoked DSM contractions (n=8, N=3, $p>0.05$). These data suggest that stimulation of beta3 adrenergic receptors with BRL37344 decreases rat DSM nerve-evoked contractions.

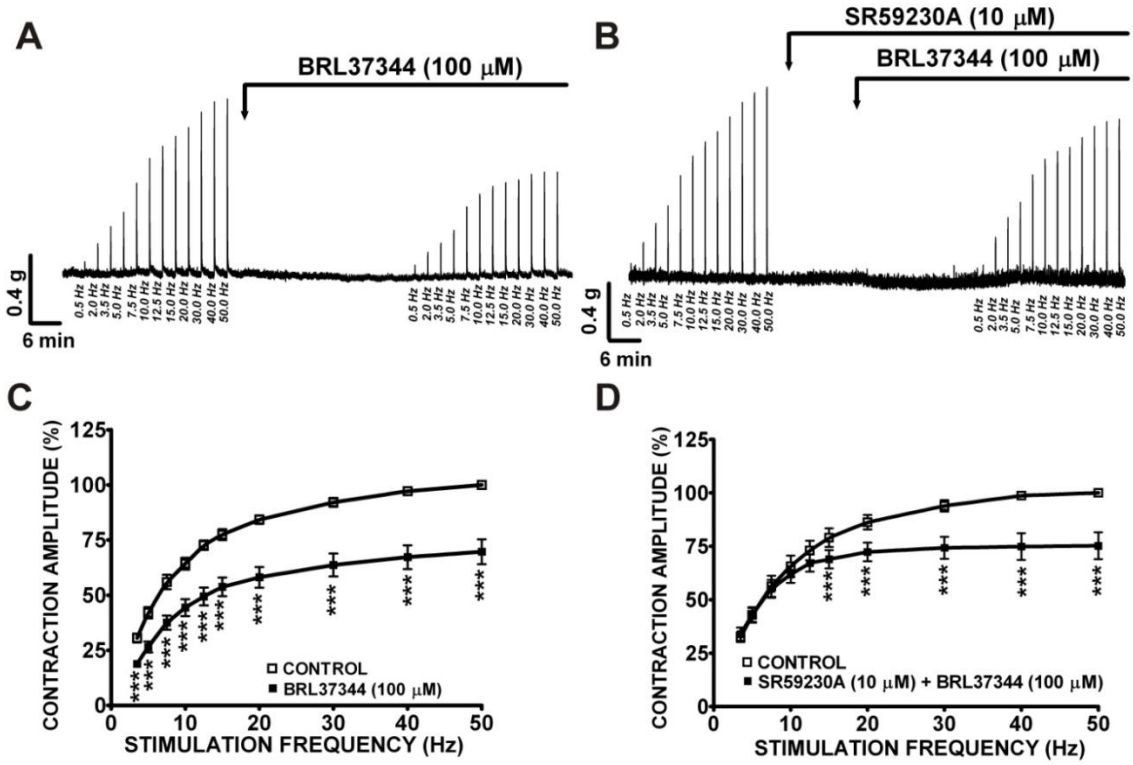

Figure 4.2.2. Stimulation of beta3 adrenergic receptors with BRL37344 reduces rat DSM EFS-induced contractions generated by a wide range of stimulation frequencies (0.5 – 50 Hz). Original DSM tension recordings illustrating BRL37344 (100 μM) inhibitory effects on rat DSM EFS-induced contractions in the absence (A) or presence (B) of SR59230A (10 μM). C) Frequency-response curves for the 0.5 – 50 Hz EFS-induced DSM contractions in response to 100 μM BRL37344 (n=12, N=6). D) EFS frequency-response curves for BRL37344 (100 μM) inhibitory effects on 0.5 – 50 Hz EFS-induced DSM contractions in the presence of SR59230A (10 μM) (n=8, N=3; ***p<0.005).

BRL37344 inhibitory effect on nerve-evoked contractions of rat DSM isolated strips: Role of cholinergic and purinergic components

We further separated the cholinergic component from the purinergic component of the nerve-evoked contractions by using inhibitors of these two components. In the presence of atropine (1 μM), which was used to block the cholinergic component of the nerve-evoked contraction, BRL37344 (100 μM) significantly decreased the amplitude of the EFS-induced DSM contractions at EFS stimulation frequencies ranging from 3.5 Hz to 50 Hz (**Fig. 4.2.3A**). In the presence of atropine, at the maximal stimulation frequency of 50 Hz, BRL37344 (100 μM) caused a 25.4±6.6% decrease in the amplitude of the EFS-induced contractions (n=15, N=8, $p<0.005$; **Fig. 4.2.3C**). This BRL37344 inhibitory effect was antagonized by SR59230A (10 μM) at all EFS stimulation frequencies (3.5 – 50 Hz) (n=13, N=5; $p>0.05$; **Fig. 4.2.3B, D**). These data suggest that stimulation of beta3 adrenergic receptors with BRL37344 relaxes the EFS-induced DSM contractions of rat DSM isolated strips via inhibition of the purinergic component of the EFS-induced DSM contractions.

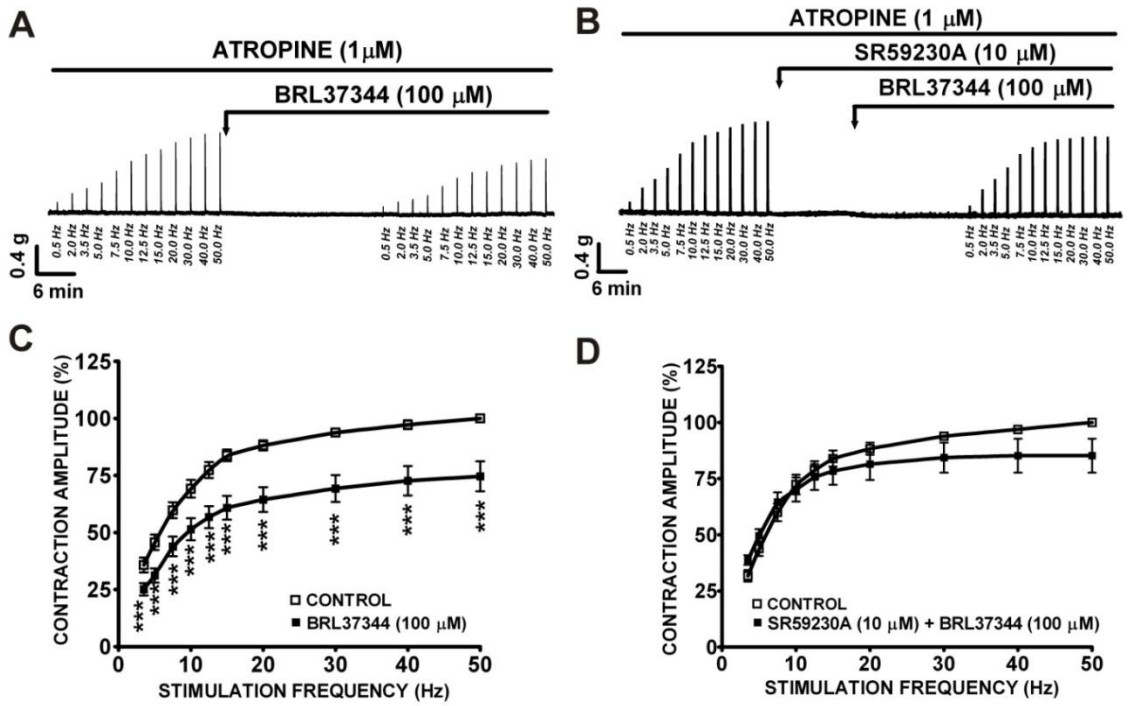

Figure 4.2.3. In the presence of atropine, BRL37344 significantly inhibited the amplitude of the 0.5 – 50 Hz EFS-induced contractions of rat DSM isolated strips. Original DSM tension recordings illustrating BRL37344 (100 μM) inhibitory effects on rat DSM EFS-induced purinergic contractions in the absence (**A**) or presence (**B**) of SR59230A (10 μM). **C**) EFS frequency-response curves showing the BRL37344 inhibitory effects on the nerve-evoked contractions in the presence of 1 μM atropine (n=15, N=8; ***p<0.005). **D)** EFS frequency-response curves showing that SR59230A blocks BRL37344 inhibitory effects on the EFS-induced contractions (n=13, N=5, p>0.05).

In order to investigate the cholinergic component of the EFS-induced contractions, we blocked the purinergic component of the EFS-induced contractions with suramin (10 μM) and α,β-methylene-ATP (10 μM) (63, 64, 145, 154, 161). These two inhibitors have different mechanism of action. While suramin inhibits the purinergic receptor directly, α, β-methylene-ATP first activates the receptors (it also acts as a receptor agonist) but then quickly desensitizes the receptor. Thus, the combined use of both drugs secures higher degree of purinergic receptor inhibition. It has been shown that the combination of these two purinergic inhibitors decreases the number of spontaneous global Ca^{2+} flashes and also nearly abolished the local Ca^{2+} transients evoked by EFS suggesting that these two compounds combined completely block the purinergic component of the nerve-evoked contractions in DSM (63). In the presence of suramin (10 μM) and α,β-methylene-ATP (10 μM), BRL37344 significantly decreased the amplitude of the EFS-induced contractions in rat DSM isolated strips at a wide range of EFS stimulation frequencies (3.5 – 50 Hz) suggesting that BRL37344 inhibited the cholinergic component of the EFS-induced contractions (**Fig. 4.2.4A, C**). BRL37344 (100 μM) inhibited EFS-induced contraction amplitude by 42.3±4.5% at the maximal stimulation frequency of 50 Hz (n=12, N=5, $p<0.005$; **Fig. 4.2.4C**). This BRL37344 inhibitory effect on the cholinergic component was slightly reduced at low stimulation frequency (3.5 – 20 Hz) by SR59230A (10 μM) (n=9, N=5; $p<0.005$; **Fig. 4.2.4B, D**).

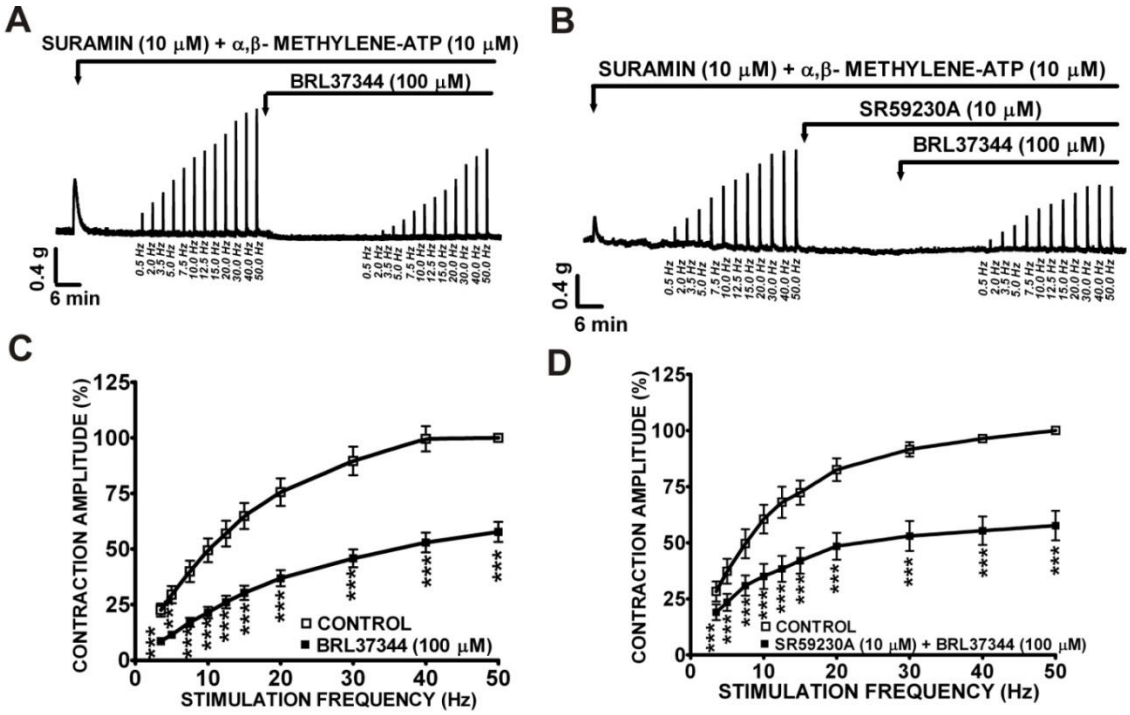

Figure 4.2.4. BRL37344 effects on the amplitude of the 0.5 – 50 Hz EFS-induced contractions of rat DSM isolated strips in the presence of suramin and α,β-methylene-ATP. Original DSM tension recordings of EFS-induced DSM cholinergic contractions illustrating BRL37344 (100 μM) inhibitory effects in the absence (A) or presence (B) of SR59230A (10 μM). EFS frequency-response curves showing BRL37344 inhibitory effect on EFS-induced contraction amplitude in the absence (n=12, N=5) (C) or presence (D) of 10 μM SR59230A (n=9, N=5; ***$p<0.005$). Application of α,β-methylene-ATP, a purinergic receptor agonist caused a transient contraction (A and B) of the DSM followed by desensitization of the receptor.

BRL37344 inhibitory effect on EFS-induced contractions is antagonized by iberiotoxin, a selective BK channel blocker

In this experimental series, we investigated whether the BK channel plays a role during the beta3 adrenergic receptor-mediated relaxation of nerve-evoked contractions in rat DSM. We pretreated rat DSM isolated strips with iberiotoxin (200 nM) prior to BRL37344 (10 nM – 100 μM) application (**Fig. 4.2.5A**). We found that blocking BK channels with iberiotoxin significantly antagonized BRL37344 inhibitory effects on the amplitude and muscle force of the 20 Hz EFS-induced contractions **(Fig. 4.2.5B-C)**. The BRL37344 inhibitory effects on 20 Hz EFS-induced DSM contractions were significantly reduced as illustrated by reduction in the maximal BRL37344 inhibitory effect on the EFS-induced contraction amplitude and force ($n=7$, $N=5$, $p<0.05$; **Fig. 4.2.5B-C**). These findings suggest that BK channels and beta3 adrenergic receptors work in synergy to oppose EFS-induced contractions in rat DSM isolated strips.

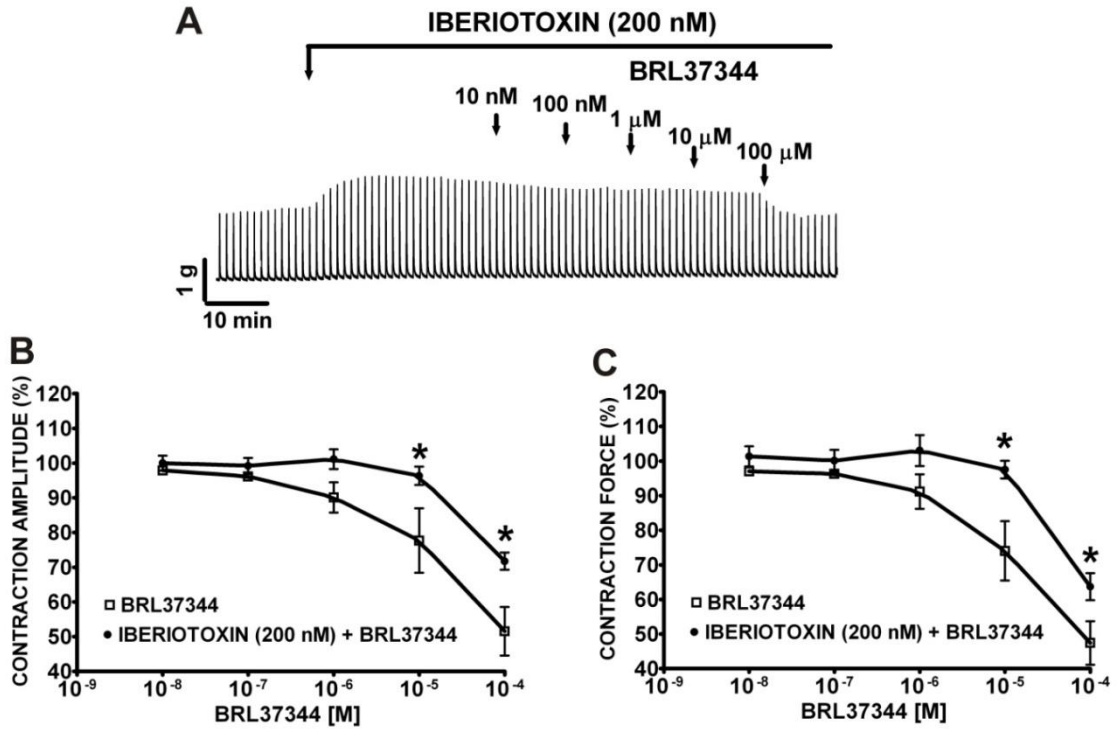

Figure 4.2.5. Blockade of BK channels with iberiotoxin reduces the BRL37344 inhibitory effects on 20 Hz EFS-induced contractions of rat DSM isolated strips. A) Original DSM tension recordings illustrating the reduction of BRL37344 (10 nM – 100 μM) inhibitory effects on rat DSM 20 Hz EFS-induced contractions by BK channels blockade with 200 nM iberiotoxin. Cumulative concentration-response curves showing that pretreatment of rat DSM isolated strips with 200 nM iberiotoxin opposes BRL37344 inhibitory effects on DSM EFS-induced contraction amplitude (B) and force (C) (n=7, N=5; *p<0.05)

DISCUSSION

The present study provides evidence that the beta3 adrenergic receptor agonist BRL37344-mediated relaxation of rat DSM nerve-evoked contraction is regulated by the BK channels. This BRL37344-induced DSM relaxation involved inhibition of both cholinergic and purinergic components of the nerve-evoked contractions.

Stimulation of beta3 adrenergic receptors with BRL37344 reduces nerve-evoked contractions in rat DSM isolated strips: role of purinergic and cholinergic components. During the voiding phase of the micturition process, the parasympathetic nerves become activated and acetylcholine and ATP, the two main excitatory neurotransmitters in the bladder are released (7). ATP activates purinergic P2X receptors while acetylcholine activates M2/M3 muscarinic cholinergic receptors to induce DSM contractions and facilitate voiding (7). In our study, bladder nerves were stimulated with a wide range of EFS frequencies in order to activate these two neurogenic pathways. It has been demonstrated that the activation of both purinergic and cholinergic pathways is EFS frequency-dependent (64). At low stimulation frequencies ($\leq$ 20 Hz), the purinergic pathway is predominant, contrary to the cholinergic component which predominates at high frequencies ($\geq$ 20 Hz) (18, 64, 161).

In a previous report, we showed in guinea pig DSM isolated strips BRL37344's inhibitory effect on EFS-induced contractions was not mediated by beta3 adrenergic receptors suggesting important species differences (1). We further showed that L-755,507, a selective beta3 adrenergic receptor agonist failed to inhibit the nerve-evoked contractions in guinea pig DSM isolated strips suggesting that in this particular species beta3 adrenergic receptors play a minor to no role at all during nerve-evoked contractions in

guinea pig DSM (1). Here, we found that contrarily to guinea pig DSM, stimulation of beta3 adrenergic receptors with BRL37344 caused a significant decrease of the nerve-evoked contraction amplitude and force in rat DSM isolated strips in the presence or absence of atropine (**Figs. 4.2.2-3**). BRL37344 (10 and 100 μM) caused a ~26% and ~52% decrease in rat DSM nerve-evoked contractions force, respectively. The BRL37344 inhibitory effect reported here is similar to that in previous reports on human DSM (151). The aforementioned study showed that BRL37344 (pEC_{50}=6.25) caused a 22% and a 47% inhibition of human DSM carbachol-induced tone when used at concentrations of 10 μM and 100 μM, respectively (151). Similar to Takeda et al. study, the BRL37344 inhibitory effects that we report here at 10 and 100 μM could also be mediated via activation of beta2 adrenergic receptors as recently suggested by Baker et al. (10). We further studied the purinergic and the cholinergic components separately by using inhibitors of these two components. In the presence of atropine, which was used to block the cholinergic component of the nerve-evoked contractions, we found that BRL37344 decreased the amplitude of the nerve-evoked contractions at all stimulation frequencies tested suggesting that BRL37344 inhibits the purinergic component of the nerve-evoked contractions (**Fig. 4.2.3A, C**). This BRL37344 inhibitory effect was completely blocked at all EFS stimulation frequencies by SR59230A, which suggests that BRL37344 inhibits the purinergic component of the nerve-evoked contractions. Our data also showed that in the presence of α,β-methylene-ATP and suramin which were used to block the purinergic component of the nerve-evoked contractions, BRL37344 decreased the amplitude of the EFS-induced contractions at all stimulation frequencies tested suggesting that BRL37344 inhibits the cholinergic component of the nerve-evoked

contractions. However, the BRL37344 inhibitory effect on the cholinergic component was not completely antagonized by SR59230A suggesting that BRL37344 might exhibit some non-selective effects by also activating the beta2 adrenergic receptors (10). Taken together, these data suggest that BRL37344 inhibitory effects on rat DSM nerve-evoked contractions were mediated via inhibition of both purinergic and cholinergic pathways.

Functional BK channels regulate beta3 adrenergic receptor-mediated relaxation of rat DSM neurogenic contractions

Previous studies, both in human and rodents, have shown that the BK channel plays an important regulatory role in DSM physiology (22, 67, 75, 80, 130, 131, 134, 145, 164, 166). Activation of BK channels hyperpolarizes the cell membrane and prevents Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels, which ultimately causes DSM relaxation (75, 79, 130, 134, 145). This BK channel-mediated relaxation of the DSM has made BK channel-targeting compounds potential drug candidates for the treatment of bladder dysfunction (26, 80, 98, 130, 145). Along with BK channels, beta3 adrenergic receptors have also been demonstrated to be promising targets for the pharmacological treatment of OAB (13, 22, 50, 99, 157). Our group has recently provided evidence that there is a functional link between beta3 adrenergic receptors and BK channels (79). We have previously demonstrated that the pharmacological blockage of BK channels with iberiotoxin opposes beta3 adrenergic receptor-mediated relaxation of rat DSM myogenic contractions (79). In the present study, the beta3 adrenergic receptor agonist, BRL37344, attenuates the contraction amplitude and muscle force of the 20 Hz EFS-induced contractions in a concentration-dependent manner (**Fig. 4.2.5**). We further observed that pharmacological blockade of BK channels with iberiotoxin antagonized the beta3

adrenergic receptor-mediated relaxation of rat DSM nerve-evoked contraction amplitude and force (**Fig. 4.2.5**). Using immunohistochemical analysis, Dr. Mark Nelson's group has demonstrated that BK channels are expressed in DSM but not in the bladder nerves that innervate DSM (161). These findings clearly suggest that all of the iberiotoxin effects reported in our study were not mediated via activation of efferent nerve terminals to increase neurotransmitter release but rather solely through direct inhibition of BK channels which are highly expressed in DSM (75, 131). Collectively, our data suggest that BK channel activity is critical for the beta3 adrenergic receptor-induced attenuation of the nerve-evoked contractions.

CONCLUSION

The present study reveals that the beta3 adrenergic receptor agonist, BRL37344, inhibited both the purinergic and cholinergic components of the nerve-evoked contractions in rat DSM. We also found that functional BK channels play a critical role in the BRL37344-induced inhibition of the nerve-evoked contractions in rat DSM.

4.3 BRL37344, A BETA3-ADRENERGIC RECEPTOR AGONIST, DECREASES NERVE-EVOKED CONTRACTIONS IN HUMAN DETRUSOR SMOOTH MUSCLE ISOLATED STRIPS: ROLE OF BK CHANNELS

Data presented in this section have been submitted for publication and is currently under revision. The original manuscript has been modified to fit the format of this dissertation. Human tissues were surgically obtained by Dr. Rovner's team from the Medical University of South Carolina. Dr. Rovner is a collaborator with the Petkov's lab

ABSTRACT

The goal of the study was to investigate the mechanism by which BRL37344, a beta3-AR agonist, facilitate the inhibition of nerve-evoked contractions in human DSM isolated strips and to identify a role for the large-conductance Ca^{2+}-activated K^{+} (BK) channels in this process.

Human DSM specimens were obtained from open bladder surgeries on patients without preoperative history of OAB symptoms. Isometric DSM tension recordings were conducted using force-displacement transducers and thermostatically controlled tissue baths. Nerve-evoked contractions were generated by electrical field stimulation (EFS).

BRL37344, a beta3-AR agonist, significantly decreased the amplitude and muscle force of the DSM contractions induced by 20 Hz EFS, in a concentration-dependent manner. This BRL37344-mediated inhibition of the amplitude and muscle force of the nerve-evoked DSM contraction was significantly reduced by iberiotoxin, a selective inhibitor of the BK channel, revealing a role for BK channels in the beta3-AR-induced inhibition of human DSM nerve-evoked contractions. We further used atropine, α,β-methylene-ATP,

and suramin to separate the cholinergic and purinergic components of human DSM nerve-evoked contractions. We found that the beta3-AR agonist, BRL37344, inhibited both components of the EFS-induced (0.5–50 Hz) DSM contractions.

In conclusion, this study supports the concept that beta3-AR agonists inhibit nerve-evoked contractions in human DSM. We have further revealed that BK channels play a critical role in BRL37344-mediated relaxation of nerve-evoked contractions in human DSM. The study suggests that in addition to beta3-ARs, BK channels could also be promising pharmacological targets in the treatment of urinary bladder dysfunction.

INTRODUCTION

Overactive bladder (OAB) is a symptom based condition characterized by frequent urination, urinary urgency, with or without urinary incontinence. The current therapeutic approaches for OAB treatment which rely primarily on behavioral therapies in combination with antimuscarinic drugs and a number of surgical techniques, have variable outcomes and numerous potential adverse effects. These drawbacks in treating OAB have led researchers to investigate alternative therapeutic approaches with novel mechanisms of action, better efficacy, and fewer unfavorable effects.

Beta3-ARs, and the beta3-AR in particular, have emerged as targets for the treatment of OAB as pharmacological activation of these receptors results in detrusor smooth muscle (DSM) relaxation (22, 79). Beta3-AR agonists including BRL37344 **(**[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium hydrate**),** mirabegron (YM178), solabergon (GW427353), TRK-380, and ritobegron (KUC-7483) have been demonstrated to induce relaxation of human and rodent DSM spontaneous, nerve-evoked, and pharmacologically-induced phasic contractions (14, 27, 79, 81, 88, 90, 156). In human DSM isolated strips, BRL37344 has been shown to inhibit carbachol-induced tone in a concentration-dependent manner (pEC_{50}=6.25)(151). In fact, BRL37344 caused a 22% and 47% inhibition of human DSM carbachol-induced tone when used at a concentration of 10 µM and 100 µM, respectively (151). Furthermore, in phase 3 clinical studies on patients with OAB, mirabegron has demonstrated both efficacy and safety (27, 90). Based on these aforementioned studies which demonstrate the prominent role of beta3-ARs in human DSM relaxation both *in vitro* and *in vivo*, mirabegron has been approved as the first beta3-AR agonist for OAB in the US (27, 90).

The mechanism by which beta-AR activation causes relaxation of the DSM is thought to involve the cyclic cAMP (cAMP) signaling pathway (22, 115). cAMP activates protein kinase-A (PKA), which in turn phosphorylates various proteins involved in DSM excitation-contraction coupling and stimulates the BK channels to promote DSM relaxation (22, 58, 115). In animal species, the BK channel is one of the most important physiologically relevant K^+ channels that controls DSM function (22, 58, 66, 79, 130, 134). Recent studies have further revealed a critical role for the BK channel in regulating human detrusor excitability and contractility (75, 80). Increases in intracellular cAMP levels via phosphodiesterase inhibition also stimulate BK channel activity and leads to DSM relaxation (164, 166). Collectively, studies have established that there is a clear functional link between the beta-AR/cAMP/PKA signal transduction pathway and BK channels during DSM relaxation (22, 79, 134).

However, the potential functional link between beta3-ARs and BK channels during nerve-evoked contractions is not well defined. To investigate this mechanism, we used thermostatically-controlled tissue baths equipped with platinum electrodes for electrical field stimulation (EFS) to generate nerve-evoked contractions in human DSM isolated strips. The relationship between the effects of BRL37344, a beta3-AR agonist, and iberiotoxin, a selective BK channel inhibitor, were examined in order to elucidate the underlying mechanisms involved in the beta3-AR agonist-induced relaxation of human DSM.

METHODS

Human DSM tissue collection

Human studies were conducted as described in chapter 2.2.

Isometric DSM tension recordings

Isometric DSM tension recordings were conducted as described in chapter 2.2.

Solutions and Drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS compositions are described in chapter 2.2. All drugs were purchased from Sigma-Aldrich Co. (St. Louis, MO), unless specified otherwise. BRL37344 was prepared daily in double-distillated water and heated at 60^{o}C to be completely dissolved at the concentration of 10 mM as a stock solution as recommended by the manufacturer.

Data analysis and statistics

Data analysis was performed as described in chapter 2.2.

RESULTS

BRL37344 inhibits EFS-induced contractions of human DSM strips in a concentration-dependent manner

We investigated the effect of BRL37344 on the amplitude and muscle force of EFS-induced DSM contractions generated by 20 Hz/min EFS in human DSM isolated strips. BRL37344 (10 nM–100 μM) was very effective at inhibiting the amplitude and force of the 20 Hz EFS-induced contractions of human DSM isolated strips. BRL37344 (10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) reduced the amplitude of the EFS-induced contractions by 11.6±3.9%, 22.9±8.2%, 33.3±10.3%, 45.5±10.4%, and 71.9±6.1%, respectively (n=4, N=3; **Fig. 4.3.1A, C**). BRL37344 (10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) also decreased the force of the EFS-induced contractions by 7.0±5.2%, 33.2±4.9%, 46.2±9.1%, 57.7±7.9%, and 78.4±4.4%, respectively (n=4, N=3; **Fig. 4.3.1A, D**). These data suggest that BRL37344 effectively decreases nerve-evoked contractions in human DSM isolated strips.

BRL37344 inhibitory effect on EFS-induced contractions is reduced by iberiotoxin, a selective BK channel blocker

In this experimental series, we investigated the relationship between β3-ARs and BK channels in nerve-evoked DSM contractions. We found that in the presence of iberiotoxin (200 nM), BRL37344 (10 nM–100 μM) inhibitory effects on the 20 Hz EFS-induced contractions amplitude and force of human DSM isolated strips were significantly reduced (**Fig. 4.3.1B, C, and D)**. The maximal antagonistic activity of iberiotoxin was observed at 1 μM BRL37344 where the BRL37344 inhibitory effects on the EFS-induced contraction amplitude and force were reduced from 32.4±10.3% to only 2.4±0.3% and

from 46.2±9.1% to 7.4±4.2%, respectively (**Fig. 4.3.1C and D**). These findings suggest that BK channels and β3-ARs work in synergy to oppose nerve-evoked contractions in human DSM.

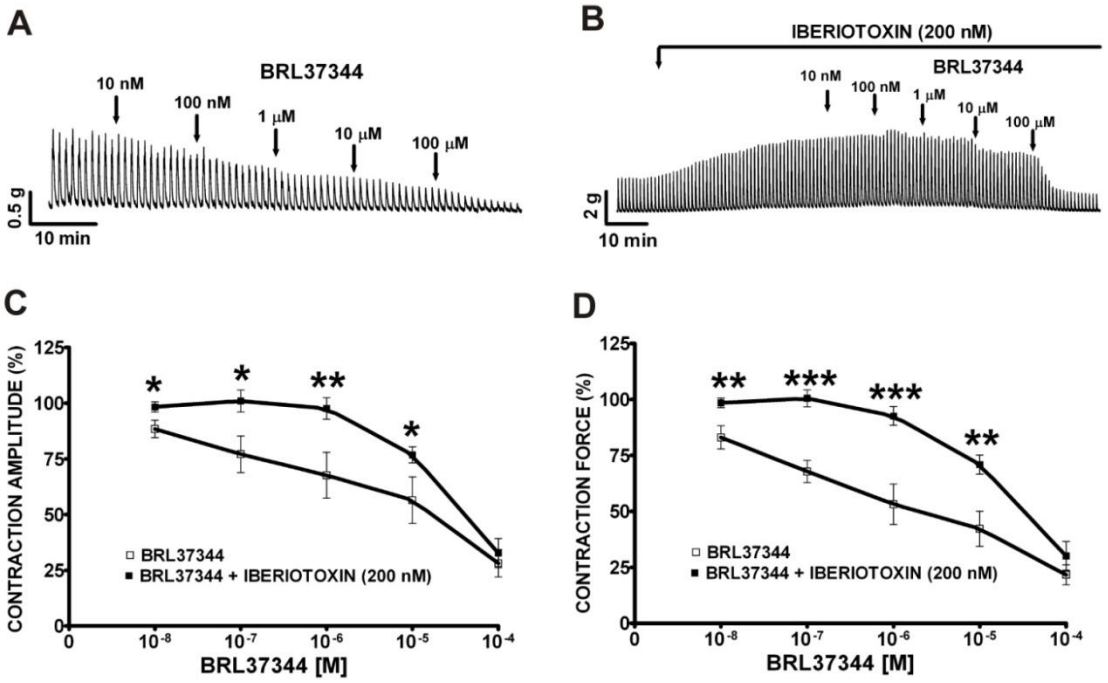

Figure 4.3.1. Iberiotoxin, a BK channel blocker, significantly reduced the BRL37344 inhibitory effects on 20 Hz EFS-induced contractions in human DSM isolated strips. A) An original DSM tension recording illustrating BRL37344 (10 nM-100 μM) inhibitory effects on 20 Hz EFS-induced contractions in a human DSM isolated strip. B) An original DSM tension recording illustrating that iberiotoxin (200 nM) increased the 20 Hz EFS-induced contractions in human DSM and that the BRL37344 (10 nM-100 μM) inhibitory effects on the EFS-induced contraction amplitude and force were significantly attenuated in the presence of iberiotoxin (200 nM). C-D) Cumulative concentration-response curves illustrating iberiotoxin (200 nM) antagonistic action on BRL37344 inhibitory effects on EFS-induced contraction amplitude (C) and force (D), respectively (n=4, N=3; *P<0.05, **P<0.01, ***P<0.005).

BRL37344 decreases the amplitude of the EFS-induced DSM contractions in a wide range of stimulation frequencies

Here, we investigated how the β3-AR agonist, BRL37344, modulates DSM nerve-evoked contractions in response to a wide range of EFS frequencies in human DSM isolated strips. We first applied increasing EFS frequencies (0.5–50 Hz) as a control protocol, followed by the addition of a single concentration of BRL37344 (10 μM). Then, a second EFS protocol was applied to evaluate BRL37344 effects on the nerve-evoked contractions. In human DSM isolated strips, BRL37344 (10 μM) significantly decreased the amplitude of the EFS-induced contractions (**Fig. 4.3.2A**). At the maximum EFS frequency of 50 Hz, BRL37344 (10 μM) decreased human DSM EFS-induced contraction amplitude by 38.1±6.6% (n=8, N=4; **Fig. 4.3.2B**). We also applied a higher concentration of BRL37344 (100 μM) which caused almost a complete inhibition of the EFS-induced DSM contractions. At the maximum EFS frequency of 50 Hz, BRL37344 (100 μM) decreased human DSM EFS-induced contraction amplitude by 85.4±2.1% (n=11, N=3; data not illustrated). These data suggest that BRL37344 reduces human DSM nerve-evoked contractions induced by EFS at a wide range of stimulation frequencies.

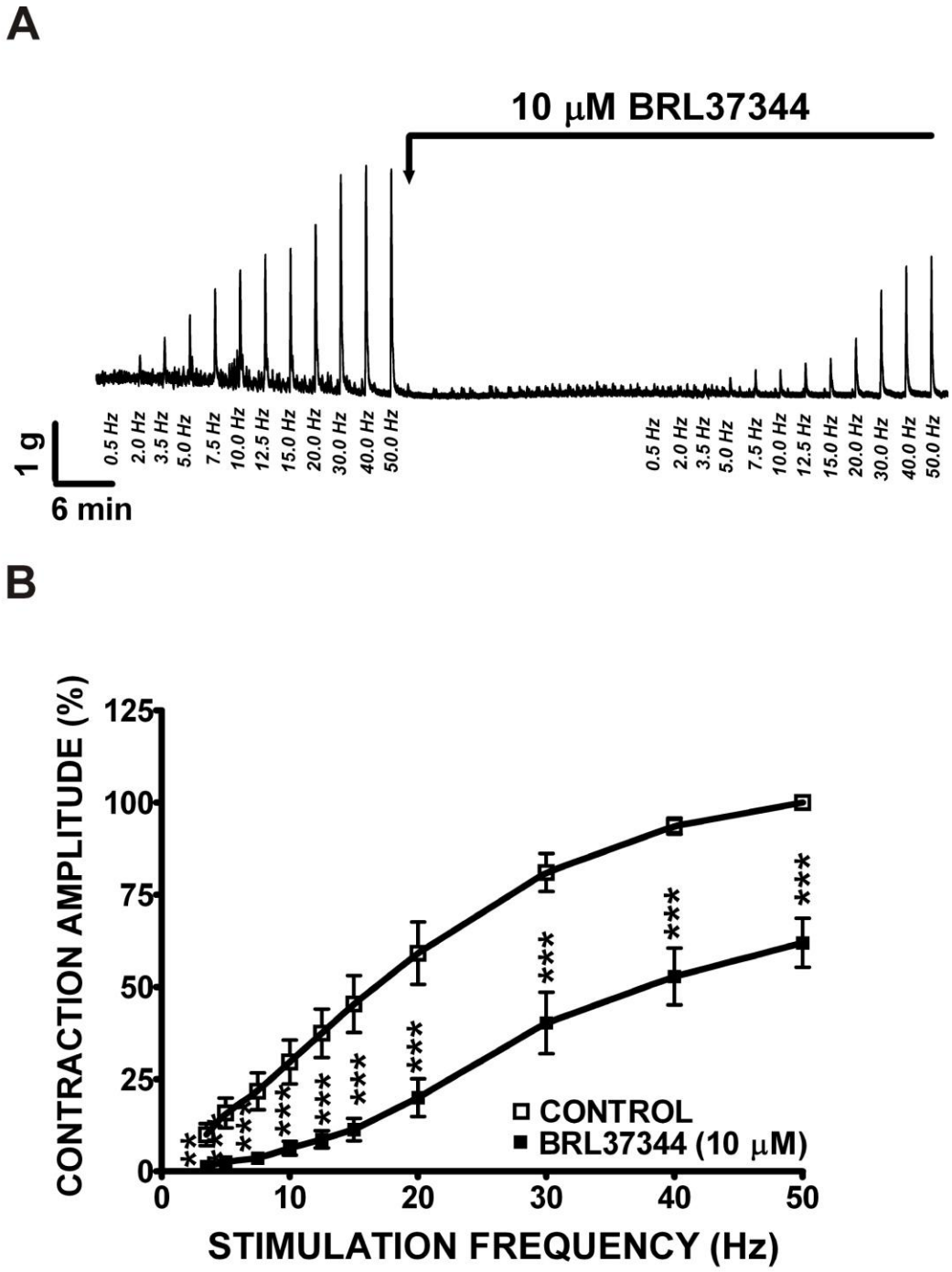

Figure 4.3.2. BRL37344 decreases the amplitude of the EFS-induced contractions in human DSM isolated strips in a wide range of stimulation frequencies. A) An original DSM tension recording illustrating the inhibitory effects of 10 μM BRL37344 on 0.5–50 Hz EFS-induced contractions of human DSM. B) Frequency-response curves showing 0.5–50 Hz EFS-induced contractions in response to 10 μM BRL37344 (n=8, N=4; **P<0.01, ***P<0.005).

BRL37344 attenuates both purinergic and cholinergic components of EFS-induced contractions in human DSM isolated strips

In this experimental series, we applied an experimental approach that allowed us to separate the cholinergic component from the purinergic component during EFS-induced DSM contractions. The cholinergic component of the EFS-induced DSM contractions was assessed by blocking purinergic receptors with α,β-methylene-ATP, a desensitizing agonist; and suramin, a purinergic receptor antagonist. Human DSM isolated strips were pre-incubated with suramin (10 μM) and α,β-methylene-ATP (10 μM) for 15 min prior to applying a 0.5–50 Hz EFS control protocol. Next, BRL37344 was added in the bath for 30 min followed by a second EFS protocol. The purinergic component of the EFS-induced contractions, on the other hand, was assessed by pre-treating the DSM strips with 1 μM atropine for 15 min prior to applying a 0.5–50 Hz EFS control protocol in the presence or absence of BRL37344. In human DSM isolated strips, BRL37344 (10 μM) caused a significant decrease in the amplitude of the EFS-induced contraction in the presence of atropine at frequencies ranging from 3.5 to 50 Hz (**Fig. 4.3.3A, B**). At the maximal stimulation frequency of 50 Hz, BRL37344 (10 μM) caused a 35.6±9.6% decrease in the amplitude of DSM EFS-induced purinergic contractions in human DSM (n=4, N=3; **Fig. 4.3.3B**). BRL37344 (100 μM) had a more pronounced effect compared to BRL37344 (10 μM), and reduced the EFS-induced purinergic contraction amplitude of human DSM strips by 68.6±12.1% at 50 Hz-frequency (n=4, N=3; data not illustrated).

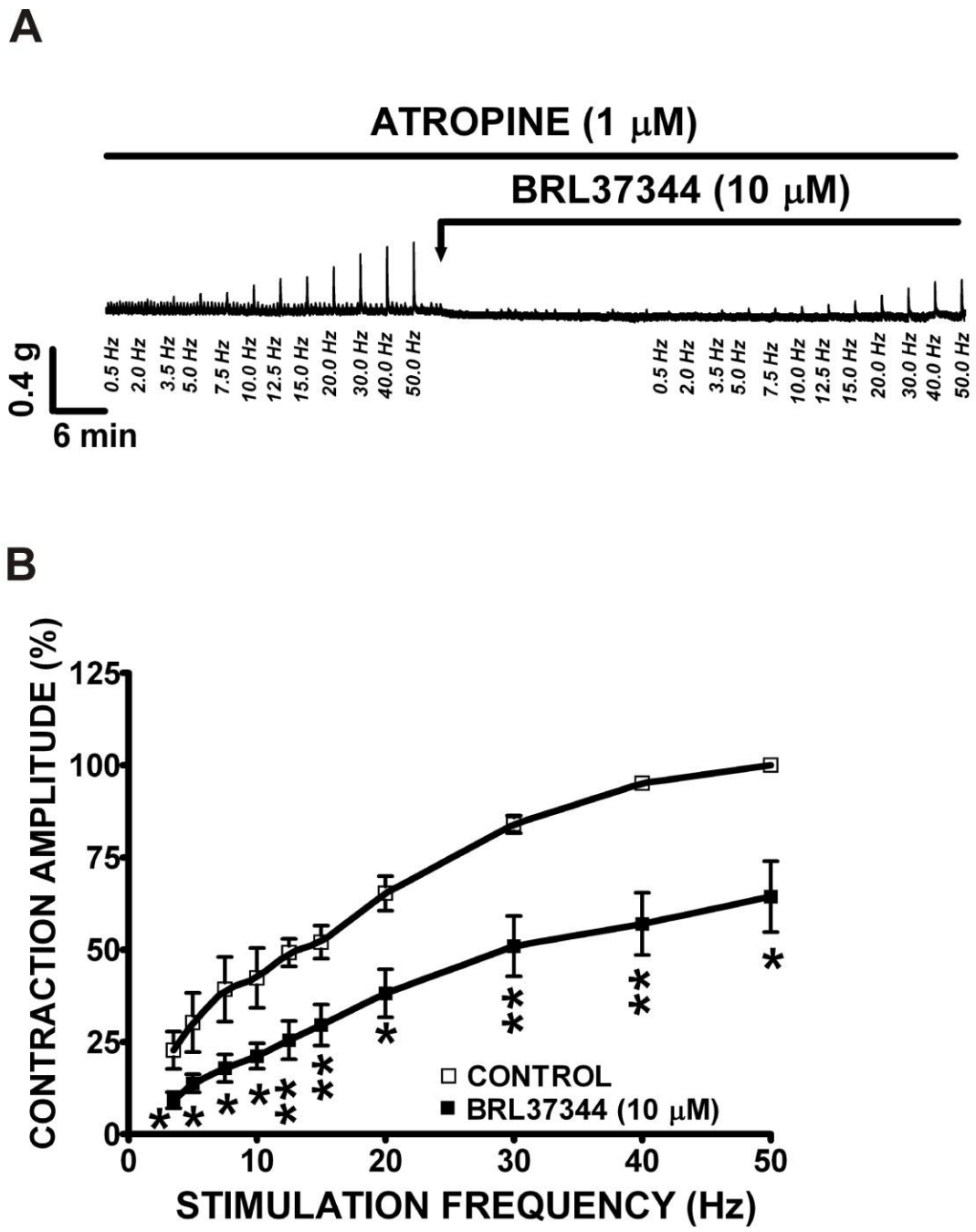

Figure 4.3.3. BRL37344 significantly inhibited the purinergic component of the 0.5–50 Hz EFS-induced contractions in human DSM isolated strips. A) An original DSM tension recording illustrating BRL37344 (10 μM) inhibitory effects on EFS-induced contractions of human DSM isolated strips in the presence of atropine (1 μM). B) EFS frequency-response curves showing 10 μM BRL37344 inhibitory effects on nerve-evoked contractions of human DSM isolated strips (n=4, N=3; *P<0.05, **P<0.01).

In the presence of suramin (10 μM) and α,β-methylene-ATP (10 μM), BRL37344 also significantly decreased the amplitude of the EFS-induced cholinergic contractions in human DSM isolated strips at a wide range of EFS frequencies (3.5–50 Hz) (**Fig. 4.3.4A and B**). At the maximal frequency of 50 Hz, BRL37344 (10 μM and 100 μM) decreased the EFS-induced cholinergic contraction amplitude of human DSM strips by 26.5±9.7% (n=8, N=5, $P<0.05$; **Fig. 4.3.4B**) and 91.0±5.3% (n=4, N=3; data not illustrated), respectively. These data suggest that BRL37344 inhibits both the cholinergic and the purinergic components of the EFS-induced contractions in human DSM.

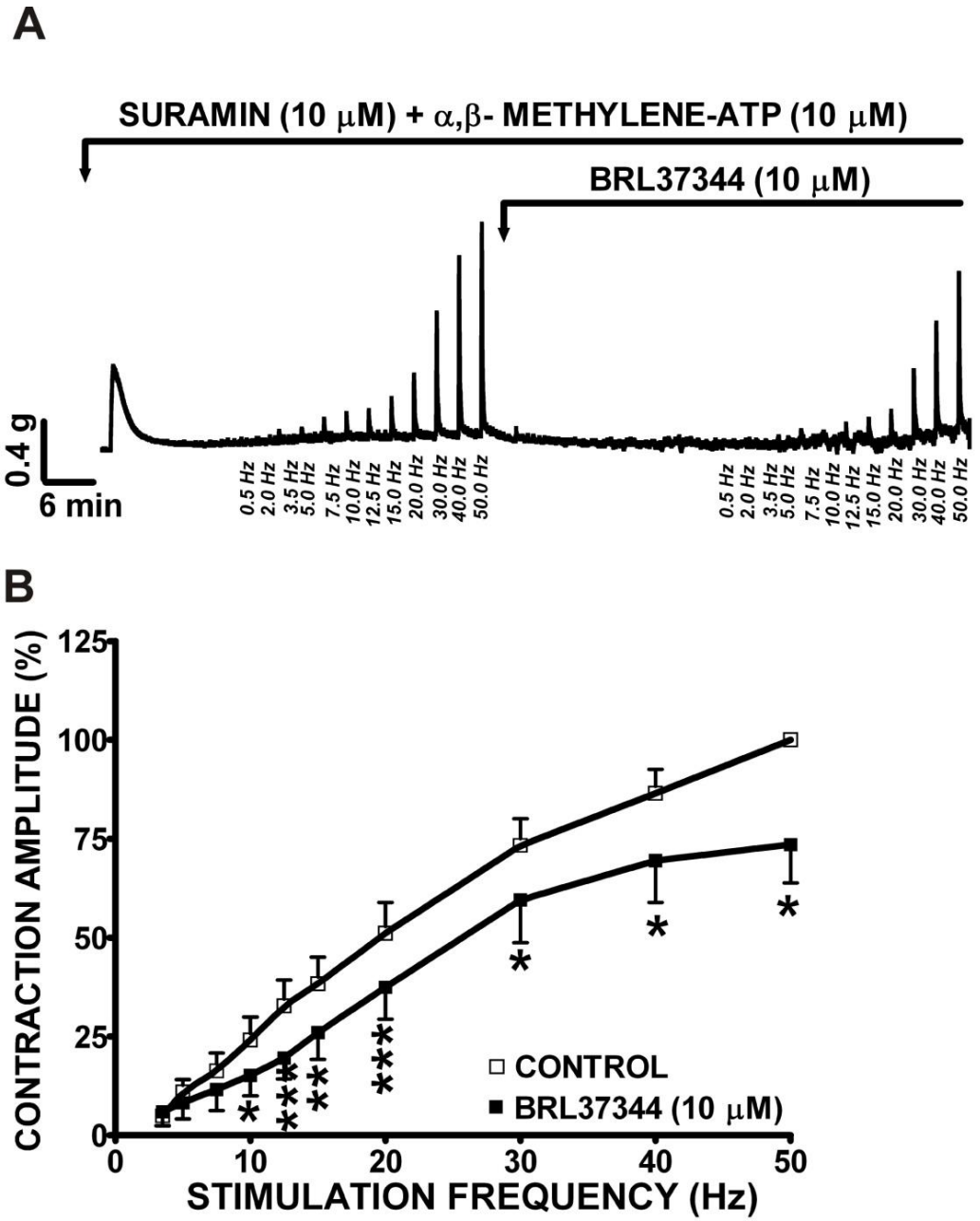

Figure 4.3.4. BRL37344 reduced the cholinergic component of the 0.5–50 Hz EFS-induced contractions of the human DSM. A) An original DSM tension recording illustrating BRL37344 (10 μM) inhibitory effects on human DSM EFS-induced contractions in the presence of suramin (10 μM) and α,β-methylene-ATP (10 μM). B) Frequency-response curves illustrating BRL37344 (10 μM) inhibitory effects on EFS-induced contraction amplitude of human DSM isolated strips in the presence of suramin and α,β-methylene-ATP (n=8, N=5; *P<0.05, **P<0.01, ***P<0.005).

DISCUSSION

The present study demonstrates that beta3-AR agonist, BRL37344, effectively inhibits both the purinergic and cholinergic components of human DSM nerve-evoked contractions. This BRL37344 inhibitory effect was significantly reduced by iberiotoxin, a BK channel selective inhibitor suggesting that functional BK channels play a critical role in the beta3-AR mediated relaxation of human DSM nerve-evoked contractions.

In mouse, rat, guinea pig, and human DSM, it has been shown that physiological DSM nerve-evoked contractions are due to the combined action of two main excitatory neurotransmitters, acetylcholine and ATP, released from the parasympathetic nerves(59, 64, 119, 142, 161). ATP activates P2X receptors while acetylcholine stimulates M2/M3 muscarinic receptors to induce DSM contractions(24, 59, 64, 105, 119, 142, 160, 161). Acetylcholine activation of M2 muscarinic receptors causes inhibition of adenylyl cyclase activity while activation of M3 receptors triggers the inositol 1,4,5-trisphosphate pathway (55, 104). This dual muscarinic receptor action ultimately leads to DSM contraction. We found that low concentrations of BRL37344 (10 nM–1 μM) decreased the amplitude of nerve-evoked contractions in human DSM consistent with previous studies demonstrating that BRL37344 could inhibit human DSM carbachol-induced tone (151). Our study suggests that beta3-ARs play an important role in opposing human DSM nerve-evoked contractions. Our data are also consistent with previous findings showing that KUC-7322 and GW427353, two other selective beta3-AR agonists, also decreased carbachol-induced and nerve-evoked contractions in human DSM (14, 81, 156). Unlike the current study, these latter studies did not address the potential involvement of BK channels in this process. Previously, we showed that BRL37344 effectively inhibits

spontaneous phasic contractions of rat DSM isolated strips (79). This BRL37344 inhibitory effect was further demonstrated to be antagonized by SR59230A, a selective beta3-AR antagonist, and H89, a PKA inhibitor, suggesting that the beta3-AR-mediated relaxation of the DSM tone utilized the cAMP/PKA pathway(79).

Recently, our laboratory and others have demonstrated the critical role played by BK channels in regulating DSM spontaneous phasic contractions (22, 66, 75, 79, 80, 130, 164, 166). BK channels, which are activated by Ca^{2+} sparks released from the sarcoplasmic reticulum through the ryanodine receptors, regulate DSM function by opposing the phasic contractions induced by Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels (79, 130, 134). Our group was the first to provide evidence for a functional link between β3-ARs and BK channels (79). In guinea pig, isoproterenol, a non-selective beta-AR agonist, activates cAMP/PKA pathway, increases Ca^{2+} spark activity which activates BK channels and induces relaxation of the DSM (134). Iberiotoxin shifts the concentration-response curves for the BRL37344 inhibitory effects on the spontaneous phasic contraction amplitude, muscle force integral, and muscle tone, to the right suggesting that the pharmacological blockage of BK channels opposes beta3-AR-mediated relaxation of rat DSM myogenic contractions (79). However, until now this mechanism has never been investigated in DSM nerve-evoked contractions. Here, we reveal for the first time the role of BK channels in the beta3-AR-induced relaxation of human DSM nerve-evoked contractions. BRL37344 decreased human DSM nerve-evoked contractions in a concentration-dependent manner (**Fig. 4.3.1**). At concentrations ranging between 10 nM and 10 μM, the BRL37344 inhibitory effect was significantly antagonized by iberiotoxin suggesting that functional BK channels play an important role

in the beta3-AR mediated relaxation of human DSM nerve-evoked contractions (**Fig. 4.3.1B, C and D**). It has been suggested that at concentrations equal or higher than 10 μM, BRL37344 may also activate beta2-ARs(10). Regardless of the potential BRL37344 effect on beta2-ARs, one would anticipate that the majority of the BRL37344 effect was mediated via activation of the beta3-ARs since beta3-ARs represent ~97% of all beta-AR mRNA expressed in the human bladder(167).

Using selective inhibitors, we further separated the purinergic and cholinergic pathways which both contribute to DSM nerve-evoked contractions. Previous studies have established that at low stimulation frequencies (≤ 20 Hz), the purinergic pathway plays a greater role whilst at high stimulation frequencies (≥ 20 Hz), the cholinergic component predominates (64, 161). In the presence of atropine, which was used to block the cholinergic component of the nerve-evoked contractions, we found that beta3-AR activation decreased the amplitude of the nerve-evoked contractions in human DSM in a wide range of stimulation frequencies (**Fig. 4.3.3**). In the presence of suramin and α,β methylene-ATP which were used to block the purinergic component, BRL37344 also significantly decreased the amplitude of the nerve-evoked contractions in human DSM (**Fig. 4.3.4**). Taken together, these data suggest that BRL37344 inhibits both cholinergic and purinergic contractions of human DSM.

CONCLUSION

The present study reveals that beta3-AR agonist, BRL37344, is very effective at low concentrations in reducing human DSM nerve-evoked contractions. We reveal for the first time that the beta3-AR-agonist-mediated relaxation of human DSM nerve-evoked

contractions is BK channel-dependent, emphasizing the critical role of BK channels in human DSM physiology. Future studies using DSM tissue from patients with OAB and detrusor overactivity are anticipated to demonstrate a similar functional relationship between beta3-ARs and the BK channel, which would provide further impetus for studying potential pharmacologic targets in this area for the treatment of OAB.

CHAPTER 5

VOLTAGE-GATED K^+ (KV) CHANNELS IN THE DETRUSOR SMOOTH MUSCLE

Chapter 5 discusses the expression pattern and functional roles of several Kv channels in the DSM. We focused on two Kv channel families including Kv2 and Kv7 channels. The study of Kv2 channels were performed in human DSM (chapter 5.1) as a follow up study of the role of these channels in guinea pig. The data presented here have already been published with me as a co-author and the original article has been modified to fit the format of this dissertation after obtaining authorization from the publisher. In chapter 5.2, we investigated Kv7 channels in guinea pig, a commonly used laboratory animal, to assess how pharmacological modulation of these channels affects DSM contractility (chapter 5.2). Based on the guinea pig data, we confidently initiated the investigation of Kv7 channels roles in human DSM (chapter 5.3). The preliminary data presented in chapter 5.3 need further investigation to clearly demonstrate the regulatory role of Kv7 channels in human DSM.

5.1 EXPRESSION AND FUNCTION OF KV2-CONTAINING CHANNELS IN HUMAN DETRUSOR SMOOTH MUSCLE

Data presented in this section have already been published with me as a co-author and the original paper has been modified to fit the format of this dissertation after obtaining authorization from the publisher. Molecular biology data were collected by Dr. Muyan Chen, a former post-doctoral fellow in Dr. Petkov's lab. Electrophysiological data were collected by Dr. Kiril Hristov, a post-doctoral fellow in Dr. Petkov's lab. All isometric DSM tension recordings experiments were performed by me. Ca^{2+}-imaging data were obtained by Qiuping Chen, a former researcher associate in Dr. Petkov's lab. Human tissue harvesting was achieved by Dr. Eric Rovner's urology team at the Medical University of South Carolina. Dr. Eric Rovner is a collaborator with the Petkov's lab.

ABSTRACT

The functional role of the voltage-gated K^+ (K_V) channels in human detrusor smooth muscle (DSM) is largely unexplored. Here, we provide molecular, electrophysiological, and functional evidence for the expression of $K_V2.1$, $K_V2.2$, and the electrically silent $K_V9.3$ subunits in human DSM. Stromatoxin-1 (ScTx1), a selective inhibitor of $K_V2.1$, $K_V2.2$, and $K_V4.2$ homotetrameric channels and of $K_V2.1/9.3$ heterotetrameric channels, was used to examine the role of these channels in human DSM function. Human DSM tissues were obtained during open bladder surgeries from patients without a history of overactive bladder. Freshly isolated human DSM cells were studied using RT-PCR, immunocytochemistry, live-cell Ca^{2+} imaging, and the perforated whole cell patch-clamp technique. Isometric DSM tension recordings of human DSM isolated strips were

conducted using tissue baths. RT-PCR experiments showed mRNA expression of $K_V2.1$, $K_V2.2$, and $K_V9.3$ (but not $K_V4.2$) channel subunits in human isolated DSM cells. $K_V2.1$ and $K_V2.2$ protein expression was confirmed by Western blot analysis and immunocytochemistry. Perforated whole cell patch-clamp experiments revealed that ScTx1 (100 nM) inhibited the amplitude of the voltage step-induced K_V current in freshly isolated human DSM cells. ScTx1 (100 nM) significantly increased the intracellular Ca^{2+} level in DSM cells. In human DSM isolated strips, ScTx1 (100 nM) increased the spontaneous phasic contraction amplitude and muscle force, and enhanced the amplitude of the electrical field stimulation-induced contractions within the range of 3.5–30 Hz stimulation frequencies. These findings reveal that ScTx1-sensitive K_V2-containing channels are key regulators of human DSM excitability and contractility and may represent new targets for pharmacological or genetic intervention for bladder dysfunction.

INTRODUCTION

Increased detrusor smooth muscle (DSM) contractions are often associated with overactive bladder (OAB), a pathophysiological condition that affects millions of individuals by disturbing their sleep, work, quality of life, and social interactions (7, 8). The exact mechanism that underlies human DSM contractility still remains unclear. Potassium (K^+) channels play a critical role in controlling DSM electrical activity, and therefore contractility (7, 22, 75, 77, 126, 130, 131, 134, 145, 155). Inhibition of certain types of K^+ channels leads to membrane depolarization, activation of the L-type voltage-gated Ca^{2+} (Ca_V) channels, and DSM contraction. Activation of K^+ channels leads to hyperpolarization of the cell membrane, inhibition of Ca_V-channels, a decrease in intracellular Ca^{2+} concentration, and DSM relaxation (130).

Among K^+ channels that control cell excitability and contractility, the K_V channel superfamily is the most diverse, but the least investigated in human DSM (130). In the human genome the K_V channel superfamily is represented by more than 40 genes encoding the pore-forming α-subunits (52, 53, 130). K_V channels are classified into 12 subfamilies based on their amino acid sequence homology. Each functional K_V channel is composed of four α-subunits. The members of K_V1–4, K_V7, and K_V10–12 channel subfamilies can form functional homotetrameric channels, whereas the members of K_V5, K_V6, K_V8, and K_V9 channel subfamilies do not form their own homotetramers, but rather form heterotetrameric channels with K_V2 channel subunits (130). K_V channels that contain K_V2 (K_V2.1 or K_V2.2) subunits are referred to as "K_V2-containing channels." K_V2-containing channels could form their own homotetramers or heterotetramers with electrically silent K_V (K_V5, K_V6, K_V8, and K_V9) subunits.

At least several K_V channels are expressed in DSM of mammals (29, 36, 47, 77, 122, 155). Expression of K_V2.1, K_V5.1, K_V6.1, K_V6.2, and K_V6.3 channel subunits has been shown in mouse DSM (155). The expression of K_V2.1 and K_V2.2 channels has been shown in rat DSM (29), and the expression of K_V2.1 and electrically silent K_V channel subunits has been detected in guinea pig DSM (77). However, it is questionable whether these findings in animal models could be directly translated to humans, since species differences in human and rodent DSM function are well documented (1, 7, 17, 29, 57, 77, 130).

Knowledge about K_V channels in human DSM is limited and only the molecular expression of some K_V1 channels has been proven (36). There is a lack of information about expression, function, and regulation of each individual K_V channel in human DSM. Microelectrode studies have shown that the nonselective K_V channel inhibitor 4-aminopyridine (4-AP) affects the after-hyperpolarization phase of the action potential and increases the frequency of the action potentials in human DSM (57). However, because of the nonselectivity of 4-AP, these experiments do not provide any information about the physiological role of individual members of the K_V channel family in human DSM. The lack of K_V channel subtype selective inhibitors with high affinity and selectivity has hampered the study of these channels not only in human DSM but also in other tissues. Recently, highly specific K_V channel inhibitors have emerged enabling researchers to examine individual K_V channel subtypes and determine their functional roles in DSM (130).

Stromatoxin-1 (ScTx1), a peptide isolated from African tarantulas, has been shown to selectively inhibit K_V2.1, K_V2.2, and K_V4.2 homotetramers, as well as K_V2.1/6.3 and

K_V2.1/9.3 heterotetrameric channels (40, 41, 113). Recently, we showed that ScTx1 is a useful pharmacological tool to study the function of K_V channels in DSM of animal species (29, 77). We demonstrated that K_V2-containing channels sensitive to ScTx1 regulate rat and guinea pig DSM excitability and contractility (29, 77). However, the expression and function of the K_V2 channels have never been studied in human DSM. Dr. Alison Brading, a world-renowned expert in ion channel DSM research, has emphasized that "It would seem particularly important for those studying the ion channels in a particular species to extend their studies to correlate channel properties with function, and there are still far too few basic studies on human material" (17). A recent review article further outlines the urgent need of studies on native human DSM (130).

The aim of this study was to examine the expression of ScTx1-sensitive K_V2-containing channels in human DSM, and to reveal their functional role in human DSM excitability and contractility. To achieve this aim, we applied a multidisciplinary approach using molecular biology techniques, live-cell Ca^{2+} imaging, patch-clamp electrophysiology, and isometric DSM tension recordings of human DSM isolated strips. Because human is the target species of interest for therapeutic intervention, the present study on native human DSM is critical to validate animal models reflecting human bladder function in the development of novel therapeutic strategies for the treatment of bladder pathologies.

MATERIALS AND METHODS

Human DSM tissue collection

All human studies were performed as previously described in chapter 2.2. In this study we used tissues from 27 patients (21 Caucasians and 6 African-Americans), of which there were 16 males and 11 females aged 24 to 85 (average age 59.6 ± 3.2 yr). The clinical diagnoses of the patients were as follows: 22 patients had urothelial carcinoma, 1 had urethral adenocarcinoma, 1 had enterovesical fistula, and 3 patients had reconstructive lower urinary tract surgeries.

Fresh human DSM single cell isolation for RT-PCR, immunocytochemistry, live-cell Ca^{2+} imaging, and patch-clamp experiments

Fresh human DSM single cells were isolated as previously described in chapter 2.2.

RNA extraction/RT-PCR/sequencing

RT-PCR experiments were conducted as described in chapter 2.2 and performed by Dr. Muyan Chen, a former post-doctoral fellow in Dr. Petkov's lab. All primers specific for $K_V2.1$, $K_V2.2$, $K_V4.2$, and $K_V9.3$ were designed on the basis of the cDNA complete sequences of human in GenBank. Specific primer pair sequences are listed in **Table 5.1.1**.

Channel	Sense	Anti-sense	Product (bp)
K_V2.1	ATGCGAAGTATGTCAAGCA	CATGGTACATCCCTAGAACG	334
K_V2.2	ATGACCACTGTTGGCTATG	GTTTGGGTGAGAATGAGG	537
K_V4.2	ATAGATACGGTAAGAGCCA	TGTGATATTAGTCCCAGTCA	417
K_V9.3	GCACTCGGTAGGACTTCG	AGGATCGCTCACCACAAT	481

Table 5.1.1. RT-PCR primers for the subunits of the K_V channels sensitive to ScTx1 and the silent K_V9.3 channel subunit. bp-base pair. *RT-PCR primers were designed by Dr. Muyan Chen.*

Western blot analysis

These experiments were performed by Dr. Muyan Chen. Western blotting analysis was conducted as described in chapter 2.2. Blots were incubated with the affinity-purified rabbit polyclonal primary antibodies: anti-K_V2.1 (DRK1 1:400), anti-K_V2.2 (CDRK 1:2,000), anti-K_V4.2 (Kcnd2 1:200) (Alomone Labs, Jerusalem, Israel) and anti-K_V9.3 [K_V9.3(K-12) 1:200] (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-K_V9.3 (KCNS3 1:200) (Abcam) overnight at 4°C. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as secondary antibody.

Immunocytochemistry on human DSM isolated cells

These experiments were performed by Dr. Muyan Chen. Human DSM cells were enzymatically isolated from fresh human DSM tissue, and dropped on a glass coverslip to settle at room temperature, then processed for the following steps. Cells were fixed with prewarmed 4% paraformaldehyde for exactly 10 min. Cells were then washed twice in PBS, blocked, and permeabilized in PBS containing 10% normal donkey serum and 0.1% Triton X-100 for 30 min. Once again, cells were washed in PBS and incubated with rabbit polyclonal primary antibodies: anti-K_V2.1, anti-K_V2.2, and anti-K_V4.2 (DRK1, CDRK and Kcnd2, 1:100, Alomone Labs) at 37°C for 1 h. After that, cells were washed two times with PBS and labeled with secondary antibodies Cy3-conjugated anti-rabbit IgG, at 1:200, PBS-3% normal donkey serum-0.01% Triton X-100 (Jackson ImmunoResearch, West Grove, PA) for 1 h in the dark. After labeling, cells were washed with PBS and incubated with phalloidin for 2 h in the dark. Cells were then washed two more times and incubated with 4′,6-diamidino-2-phenylindole for 15 min and washed again, then mounted onto slides with DABCO. Control treatments were carried out as

follows: *1*) omission of the primary antibody for confirming the specificity of the secondary antibody, and *2*) absorption of the primary antibody by a competing peptide for confirming the specificity of the primary antibody. Confocal images were acquired with an LSM 510 META confocal microscope (Carl Zeiss).

Electrophysiological (patch-clamp) recording

Patch-clamp experiments were performed and by Dr. Kiril Hristov, a post-doctoral fellow in Dr. Petkov's lab. The amphotericin-B perforated patch-clamp technique was applied in all electrophysiological experiments to preserve the native physiological environment of the human DSM cells. Several drops (~0.5 ml) of the DS containing the isolated single cells were placed in an experimental chamber. Cells were allowed to adhere to the glass bottom of the chamber for ~20 min and after that were washed with extracellular solution. Most cells were elongated and had a bright, shiny appearance when examined with a phase-contrast microscope. Cells were used for patch-clamp recordings within 24 h after isolation. Axopatch 200B amplifier, Digidata 1440 A, and pCLAMP 10.2 software (Molecular Devices) were used for electrophysiological recordings of the ionic currents. The signal was filtered with eight-pole Bessel filter 900CT/9L8L (Frequency Devises, Ottawa, IL). The glass pipettes used for patch-clamp experiments were made from Borosilicate glass (Sutter Instruments, Novato, CA) and pulled using a Narishige PP-830 vertical puller (Narishige Group, Tokyo, Japan) to give a final tip resistance of 4–6 MΩ. The pipettes were polished with a Micro Forge MF-830 fire polisher (Narishige Group). All patch-clamp experiments were performed in voltage-clamp mode, in the presence of the large-conductance Ca^{2+}-activated K^{+} (BK) channel selective inhibitor iberiotoxin (200

nM). The leak current was not subtracted during or after the experiments. The average value of the current amplitude during the last 50 ms of the 250-ms depolarization step was taken to evaluate the steady-state K_V current. For the control current, at least five pulses within 10 min were recorded and averaged. Only cells with stable control currents for at least 10 min were used to examine the effect of ScTx1. All patch-clamp experiments were carried out at room temperature.

Ca^{2+} imaging experiments

These experiments were performed by Qiuping Cheng, a research associate in Dr. Petkov's lab. For intracellular Ca^{2+} imaging experiments, 250 μl poly-l-lysine was added to each 35 mm diameter glass-bottom dish, incubated at 37°C for 1 h, then dishes were aspirated and washed with 2 ml sterile water three times. DSM cell suspension was added into the dishes and kept for 30 min to allow cell adherence to the bottom of the dishes. Fura-2 AM solution was diluted with bath solution to a final concentration of 2 μM. Supernatant was then removed from the dishes, 250 μl fura-2 AM working solution was added into the dishes, and samples were stored in a dark room for 30 min. Fura-2 AM bath solution was then removed and cells were washed with bath solution three times. Ca^{2+} imaging recordings were acquired with an OLYMPUS IX81 motorized inverted research microscope, with 40 × oil objective, equipped with the MetaFluor 7.7.2.0 software. All Ca^{2+} imaging experiments were carried out at room temperature.

Isometric human DSM tension recordings

Isometric tension recordings were performed as described in chapter 2.2.

Solutions and drugs

The Ca^{2+}-free DS and the Ca^{2+}-containing PSS compositions are described in chapter 2.2. The extracellular (bath) solution used in the electrophysiological and Ca^{2+}-imaging experiments contained (in mM) 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA, pH adjusted to 7.2 with NaOH and supplemented with freshly dissolved (every 1–2 h) 200 μg/ml amphotericin-B. TTX was purchased from Sigma-Aldrich (St. Louis, MO). ScTx1 was purchased from Alomone Labs. TTX was dissolved in citrate buffer and ScTx1 was dissolved in distilled water.

Data analysis and statistics

Data analysis was performed as described in chapter 2.2.

RESULTS

RT-PCR.

The expression of mRNA messages for K_V2.1, K_V2.2, K_V4.2 and K_V9.3 channel subunits was detected in human DSM whole tissue (**Fig. 5.1.1**). Other cell types such as neurons, fibroblasts, and vascular myocytes are also present within the DSM layer. Thus, it is plausible that K_V2.1, K_V2.2, K_V4.2, and K_V9.3 channel detection may come from cell types other than DSM cells. To eliminate the possible interference from non-DSM cell types, we applied single-cell RT-PCR experiments on freshly isolated human DSM cells. This experimental approach allows us to use selectively only the isolated DSM cells while avoiding any contamination from non-DSM cells (1, 28, 30, 75, 77, 126). DSM cells were characterized by their elongated, spindle shape, with a bright and shiny contour. Single-cell RT-PCR experiments confirmed the mRNA expression of K_V2.1, K_V2.2, and K_V9.3 channel subunits in human DSM single cells (**Fig. 5.1.1**). mRNA expression of the K_V4.2 channel subunit was not confirmed in single DSM cells, suggesting that this channel subunit is not expressed in human DSM cells. A lack of genomic DNA contamination was also confirmed by performing negative control experiments in the absence of the reverse transcriptase (−RT). All RT-PCR purified products from the intact human DSM tissue and isolated human DSM cells were sequenced to confirm their identity. PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich) and sequenced at the University of South Carolina Environmental Genomics Core Facility to confirm their identity. Results were verified in at least 30 different preparations obtained from 10 patients.

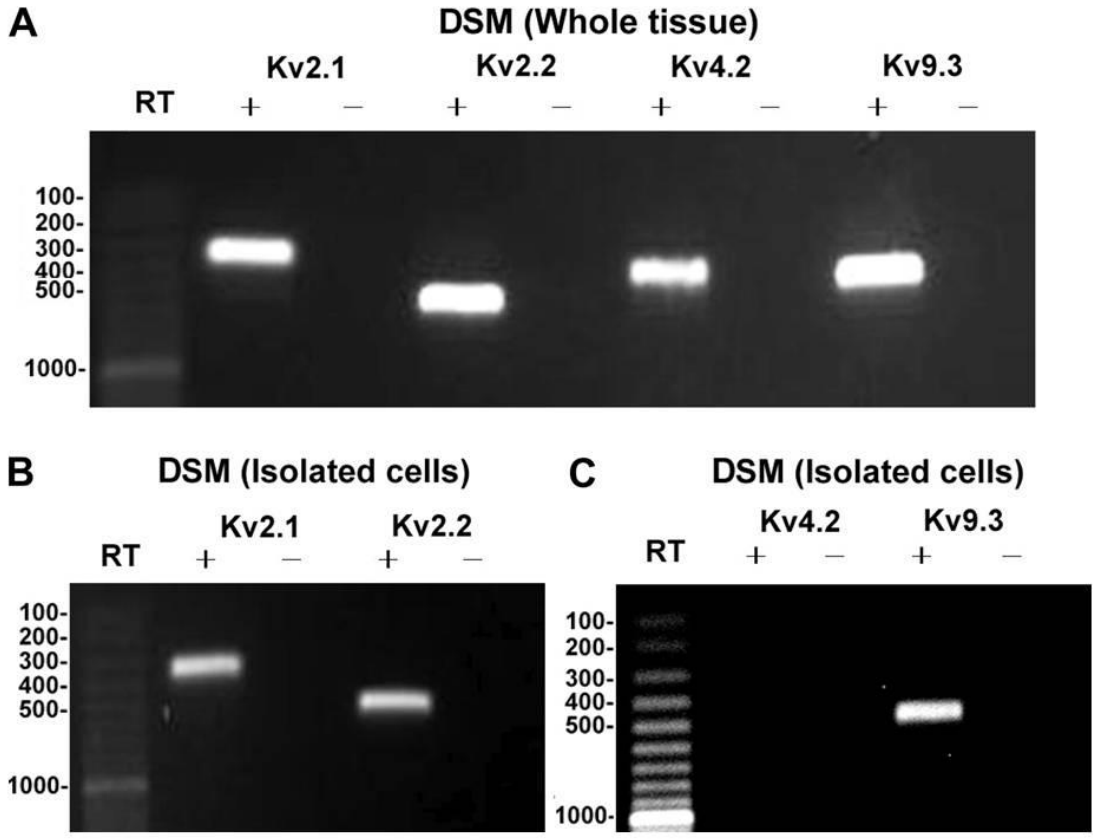

Figure 5.1.1. RT-PCR detection of mRNA messages for voltage-gated K^+ channel subunits $K_V2.1$, $K_V2.2$, $K_V4.2$, and $K_V9.3$ in human detrusor smooth muscle (DSM) whole tissue (A) and freshly isolated single cells (B and C). $K_V2.1$, 334 bp; $K_V2.2$, 537 bp; $K_V4.2$, 417 bp; and $K_V9.3$, 481 bp. No products were observed in the negative controls (−RT) in which reverse transcriptase was left out of the reaction**.** *Data collected by Dr. Muyan Chen***.**

Western blot analysis

To confirm the expression of these K_V2-containing channels at the protein level in human DSM tissue, we applied Western blot analysis. The presence of the $K_V2.1$ and $K_V2.2$ proteins in human DSM tissue was confirmed by using channel subunit-specific antibodies (**Fig. 5.1.2**). Consistent with our single cell RT-PCR data, no $K_V4.2$ protein was detected in whole human DSM tissue. The specific antibodies for $K_V9.3$ channel did not show any signal in whole human DSM (data not illustrated). Preabsorption of the primary antibody with its antigenic competing peptide indicated the specificity of the antibodies for their intended epitope (**Fig. 5.1.2**). The results were verified in three separate Western blot reactions using proteins isolated from three patients.

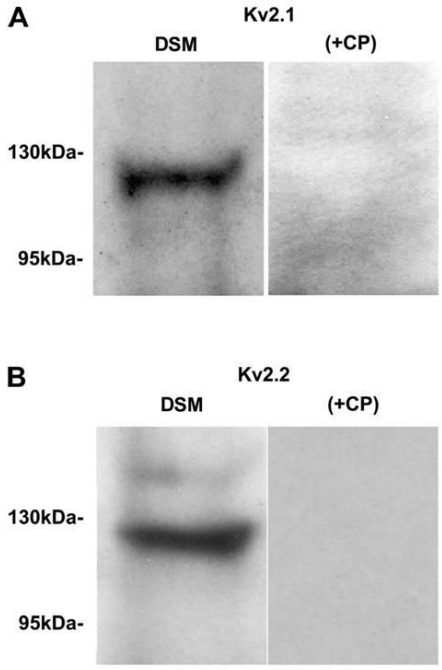

Figure 5.1.2. Western blot detection of $K_V2.1$ and $K_V2.2$ channel protein expression in human DSM whole tissue. A: $K_V2.1$ channel. B: $K_V2.2$ channel. The immunoreactive band was eliminated by a competing peptide (+CP). *Data collected by Dr. Muyan Chen*.

Immunocytochemistry

Immunocytochemical labeling was further applied to confirm the specific expression of $K_V2.1$, and $K_V2.2$ proteins at the level of single DSM cells and to demonstrate that the K_V2 proteins were localized to the cell membrane (**Fig. 5.1.3**). The results were carefully controlled for specificity using the omission of the primary antibody or absorption of the primary antibody by a competing peptide (**Fig. 5.1.3**). The data were verified in 12 single DSM cells freshly isolated from three patients.

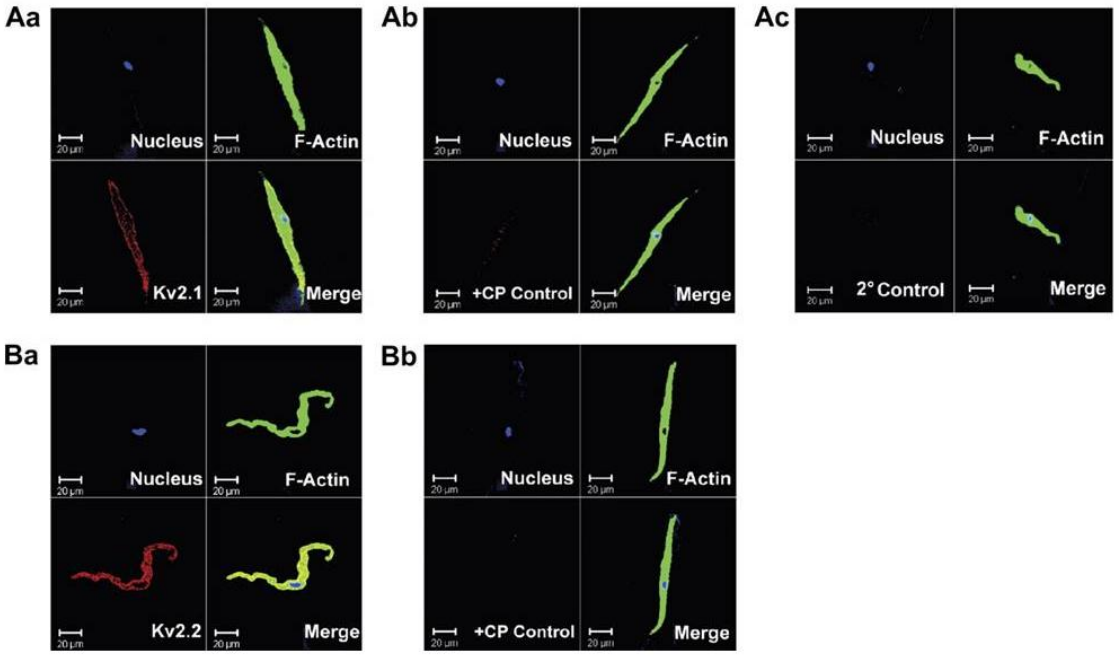

Figure 5.1.3. Immunocytochemical detection of $K_V2.1$ (A) and $K_V2.2$ (B) channel in freshly isolated single human DSM cells using $K_V2.1$ and $K_V2.2$ channel-specific antibody. Red staining (*bottom left panels*) indicates detection of $K_V2.1$ channel (Aa) and $K_V2.2$ channel (Ba); after absorption of the primary antibody with a competing peptide (Ab and Bb); and after the primary antibody was omitted and cells were incubated with the secondary antibody only (2°Control) (Ac). Cell nuclei are shown in blue (*top left* panels); F-actin is shown in green (*top right* panels). The merged images (*bottom right*) illustrate the overlap of all 3 images. Images were obtained via confocal microscope at ×63 and clearly demonstrate that the K_V2 channel proteins were localized to the cell membrane. *Data collected by Dr. Muyan Chen*.

ScTx1-sensitivity of the whole cell K_V current in human DSM cells

Our recent study revealed that K_V2.1 and electrically silent K_V channels sensitive to ScTx1 are likely important regulators of guinea pig DSM excitability (77). Here, we went one step further to evaluate the contribution of the K_V2 channels sensitive to ScTx1 in human DSM excitability. Whole cell voltage-clamp experiments were performed in the presence of 200 nM iberiotoxin to eliminate the contribution of the BK current to the total whole cell current. The average capacitance of the human DSM cells was 26.5 ± 6.1 pF (n = 4, N = 4). The cells were held at −70 mV, and then a 250-ms-long series of potentials between −70 mV and +30 mV were applied in 10-mV increments, and then the cells were repolarized back to −40 mV for another 250 ms. With increasing depolarization of the membrane potential, the human DSM cells responded with gradual increases of the whole cell current amplitude. Tail currents were recorded upon repolarization back to −40 mV (**Fig. 5.1.4**). Under these experimental conditions, ScTx1 (100 nM) was applied to study the contribution of the ScTx1-sensitive current to the total K_V current in human DSM. The results showed that ScTx1 (100 nM) caused significant inhibition of the whole cell current amplitude at positive voltages from +10 mV to +30 mV (n = 4, N = 4; P < 0.05; **Fig. 5.1.4**). The remaining whole cell current, after application of the ScTx1, was probably determined by other types of K_V channels. Current-voltage relationships of the control current and ScTx1-insensitive current are illustrated in **Fig. 5.1.4B**. **Figure 5.1.4C** represents the current-voltage relationship of the ScTx1-sensitive current (n = 4, N = 4).

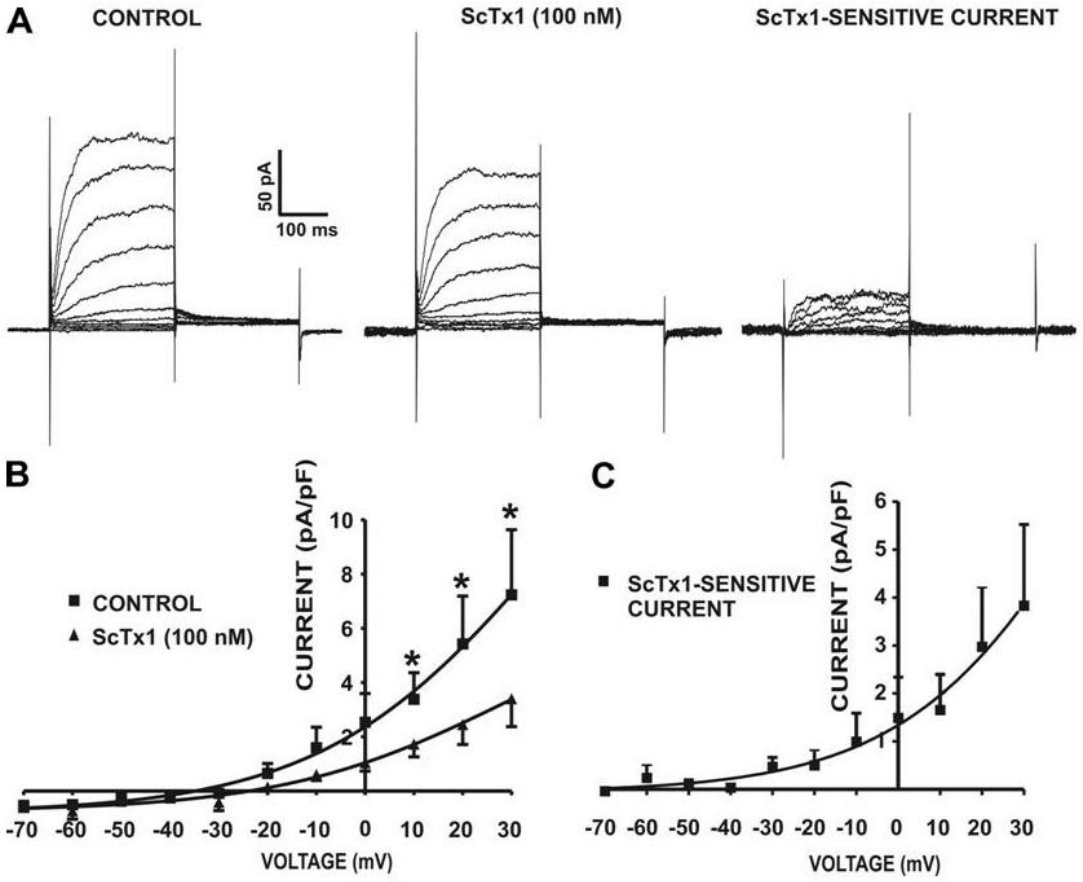

Figure 5.1.4. Stromatoxin-1 (ScTx1) sensitivity of voltage-dependent whole cell K_V current in freshly isolated human DSM cells. A: representative recordings of whole cell outward K_V currents elicited by a depolarizing voltage step protocol (−70 mV to +30 mV in 10-mV increments) depicting the effect of ScTx1. ScTx1 (100 nM) significantly decreased the voltage step-induced outward current in human DSM cells. ScTx1-sensitive current was obtained after subtraction of the remaining ScTx1-insensitive current from the control current. B: current-voltage relationship in the presence or absence of 100 nM ScTx1. ScTx1 (100 nM) significantly decreased the whole cell outward K_V current in human DSM cells (n = 4, N = 4; *P < 0.05). C: current-voltage relationship of the subtracted ScTx1-sensitive current in human DSM cells (n = 4, N = 4). Values are means ± SE. All experiments were performed in the presence of the large-conductance Ca^{2+} activated K^+ (BK) channel selective inhibitor iberiotoxin (200 nM). *Data collected by Dr. Kiril Hristov*.

Inhibition of ScTx1-sensitive K_V2-containing channels increases intracellular Ca^{2+} levels

To determine the role of ScTx1-sensitive K_V2-containing channels on the intracellular Ca^{2+} levels, live-cell real-time Ca^{2+} imaging was carried out with fura-2. As shown in **Fig. 5.1.5**, the intracellular Ca^{2+} level was significantly increased following application of 100 nM ScTx1 (**Fig. 5.1.5A**). Under control conditions, the fluorescence ratio (340/380 nM) was 0.84 ± 0.01 (n = 31, N = 7), and in the presence of 100 nM ScTx1, the fluorescence ratio increased to 0.92 ± 0.01 (n = 31, N = 7; **Fig. 5.1.5B**). Intracellular Ca^{2+} level reached a plateau within 20 min following 100 nM ScTx1 application (**Fig. 5.1.5A**). Thus, inhibition of ScTx1-sensitive K_V2-containing channels increases intracellular Ca^{2+} levels in freshly isolated human DSM cells.

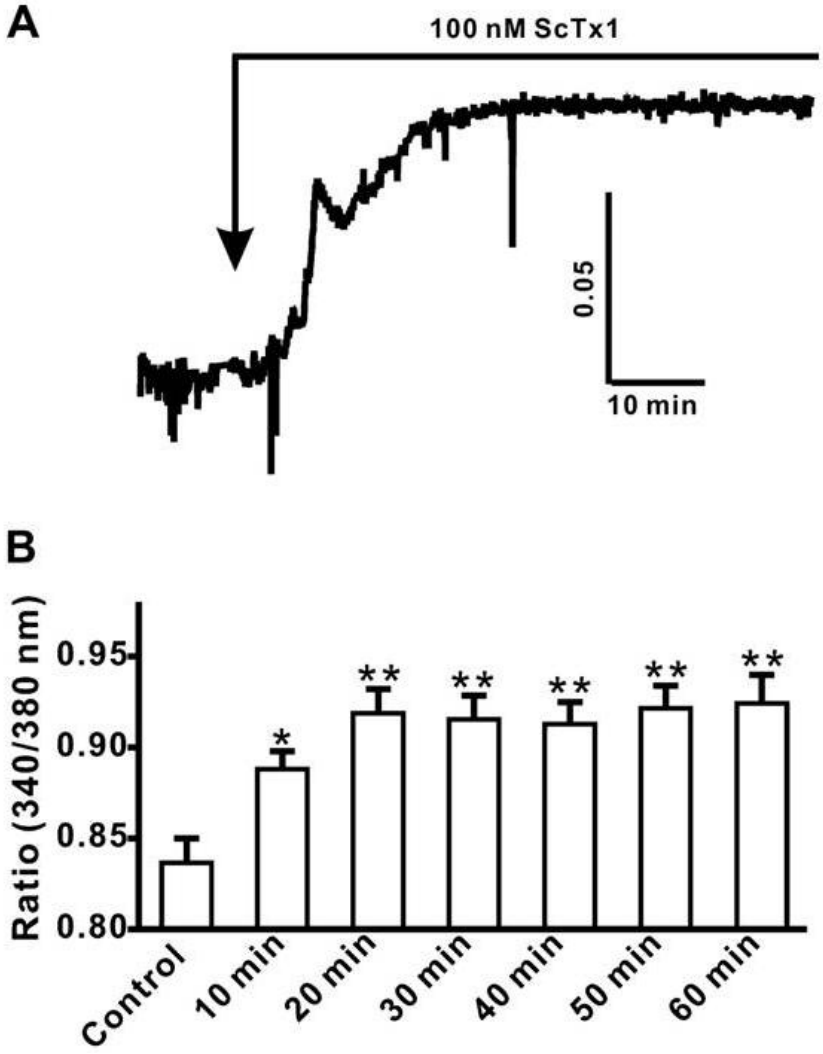

Figure 5.1.5. ScTx1 increases the intracellular free Ca^{2+} level in freshly isolated human DSM cells. *A*: original trace illustrating the increase in the intracellular free Ca^{2+} level following the application of 100 nM ScTx1. *B*: summary data showing a significant increase in the intracellular free Ca^{2+} level after application of 100 nM ScTx1. This increase in Ca^{2+} levels reached a plateau within the first 20 min following ScTx1 application. Values are means ± SE (n = 31, N = 7; *P < 0.05; **P < 0.01). *Data collected by Mr. Qiuping Cheng*.

Role of ScTx1-sensitive K_V2-containing channels in myogenic human DSM contractions

ScTx1 was employed to investigate the potential functional role of K_V2.1, K_V2.2, and K_V2.1/9.3 channels in human DSM spontaneous phasic contractions. Blocking these K_V channels with ScTx1 (100 nM) significantly increased the DSM spontaneous phasic contraction amplitude and force (**Fig. 5.1.6**). Within the first 10-min period after ScTx1 (100 nM) addition, the DSM spontaneous phasic contraction amplitude increased by 9.4 ± 4.3% and muscle force integral increased by 19.8 ± 10.9% (n = 16, N = 11; $P < 0.05$; **Fig. 5.1.6**). ScTx1 did not have any significant effects on the spontaneous phasic contraction frequency, phasic contraction duration, and muscle tone (n = 16; N = 11; $P > 0.05$; **Fig. 5.1.6**).

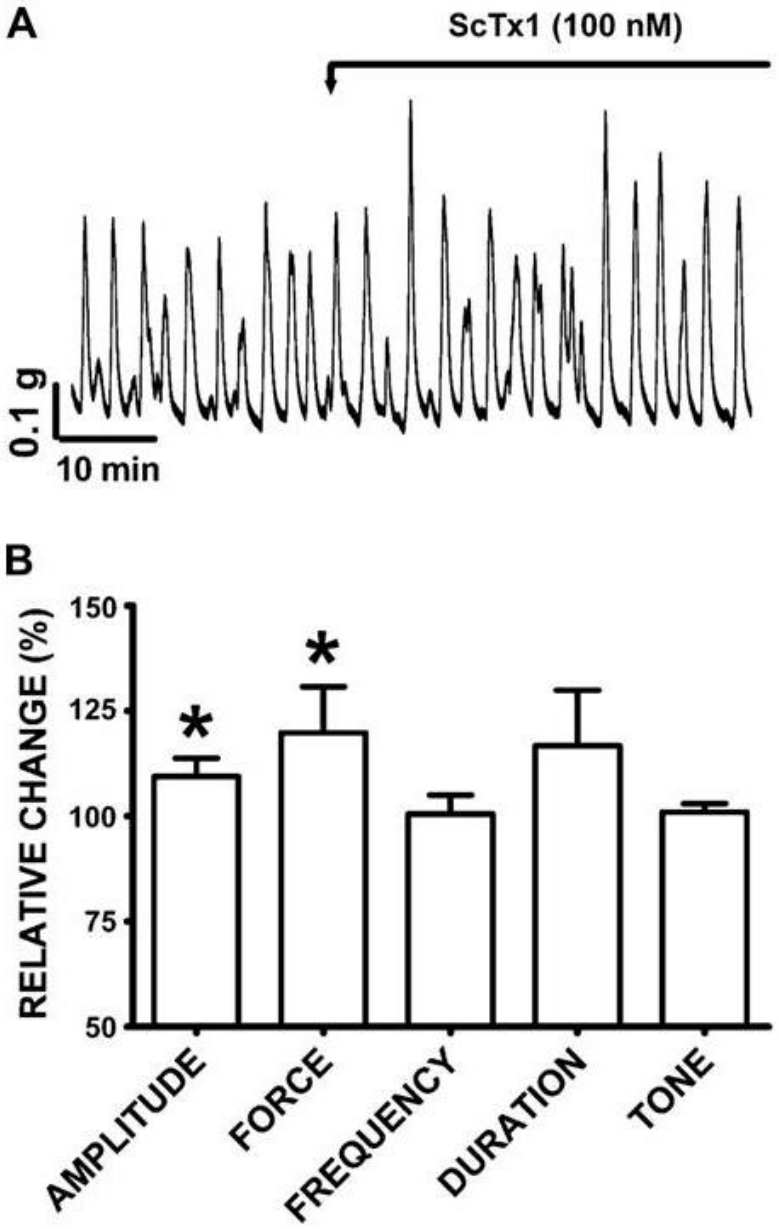

Figure 5.1.6. ScTx1 increases the amplitude and force of the spontaneous phasic contractions in human DSM isolated strips. A: original DSM tension recordings illustrating the effect of ScTx1 (100 nM) on the spontaneous phasic contractions of human DSM isolated strips. B: summary data showing significant increases in human DSM spontaneous phasic contraction amplitude and force upon ScTx1 (100 nM) application. A 10-min period before ScTx1 application was taken as a control. The spontaneous contractions under control conditions were taken to be 100% and data were normalized. The effect of ScTx1 was evaluated during the first 10 min following ScTx1 application. Values are means ± SE (n = 16; N = 11; *P < 0.05). TTX (1 μM) was present throughout the experiments.

Role of ScTx1-sensitive K_V2 containing channels in nerve-evoked human DSM contractions

In this experimental series, ScTx1 (100 nM) was employed to investigate the functional implication of $K_V2.1$, $K_V2.2$, and $K_V2.1/9.3$ channels inhibition on EFS-induced contractions of human DSM strips (**Fig. 5.1.7**). ScTx1 (100 nM) significantly increased the amplitude of the EFS-induced contractions at all stimulation frequencies ranging from 3.5 to 30 Hz (n = 24; N = 11; $P < 0.05$; **Fig. 5.1.7**). At 20 Hz EFS, ScTx1 (100 nM) increased the amplitude of the EFS-induced contraction by 18.6 ± 4.7% (n = 24, N = 11; $P < 0.05$; **Fig. 5.1.7**).

Collectively, these results suggest that $K_V2.1$, $K_V2.2$, and $K_V2.1/9.3$ channels work to oppose human DSM myogenic and nerve-evoked contractions.

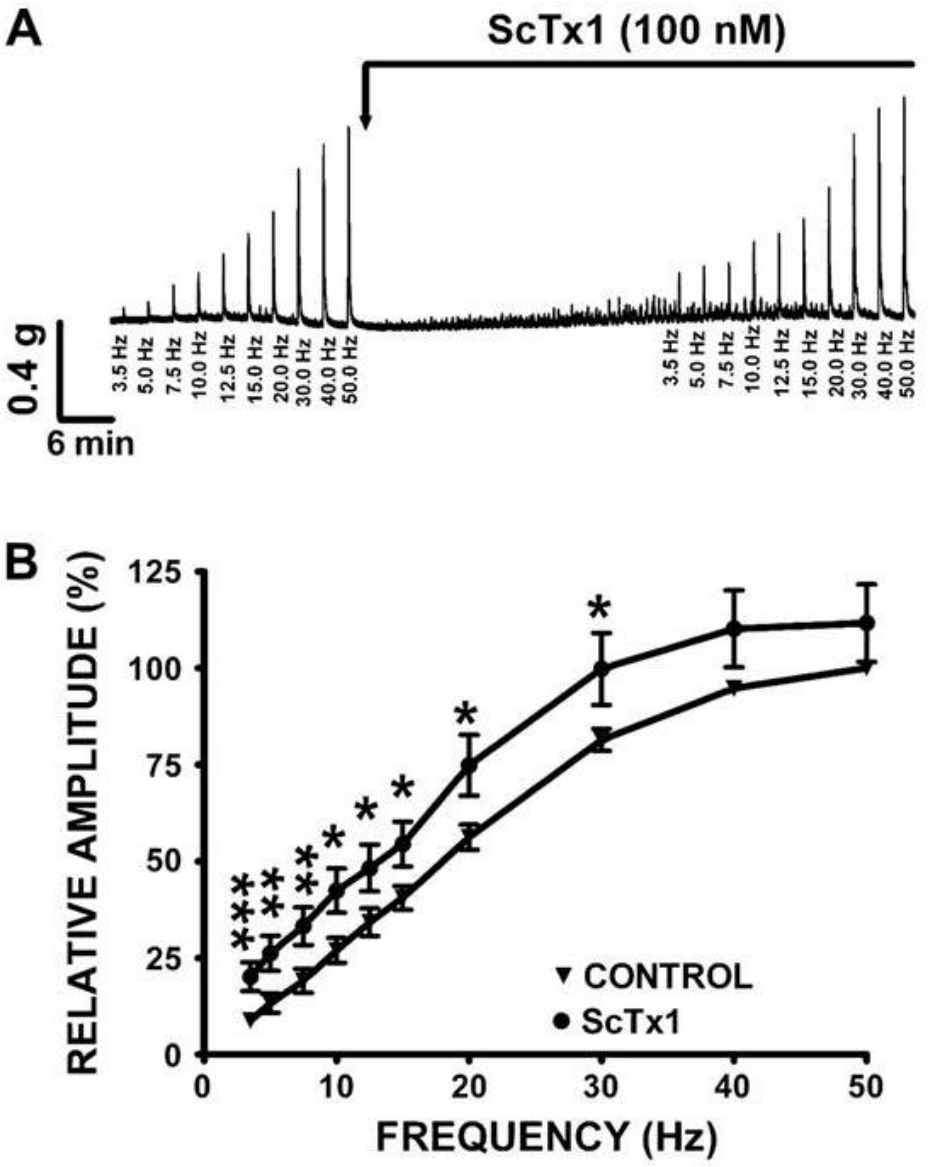

Figure 5.1.7. ScTx1 increases the amplitude of the electrical field stimulation (EFS)-induced contractions in human DSM isolated strips. A: original traces of EFS-induced contractions (3.5–50 Hz) in the absence or presence of 100 nM ScTx1 illustrating an increase of the amplitude of the contractions following 100 nM ScTx1 application. As illustrated, ScTx1 also induces spontaneous phasic contractions, consistent with the results illustrated in Fig. 6. B: frequency-response curves showing a significant increase in the amplitude of the EFS-induced contractions following application of ScTx1 (100 nM). The EFS-induced contraction amplitude at a stimulation frequency of 50 Hz under control conditions was taken to be 100%. Values are means ± SE (n = 24, N = 11; *P < 0.05, **P < 0.01, ***P < 0.005).

DISCUSSION

This study showed for the first time the expression and function of K_V2-containing channels sensitive to ScTx1 in native human DSM. Specifically, we used a multidisciplinary experimental approach to demonstrate the expression of $K_V2.1$ and $K_V2.2$ channel subunits and the electrically silent $K_V9.3$ channel subunit as well as their functional role in human DSM excitability and contractility.

Our RT-PCR experiments revealed mRNA expression of $K_V2.1$, $K_V2.2$, $K_V4.2$, and $K_V9.3$ channel subunits in whole human DSM tissue (**Fig. 5.1.1**). To avoid interference of mRNA signals from other cell types such as neurons, fibroblasts, and interstitial cells, we conducted single-cell RT-PCR experiments on freshly isolated human DSM cells. Unlike RT-PCR experiments on whole human DSM tissue, single-cell RT-PCR experiments revealed only the mRNA expression of $K_V2.1$, $K_V2.2$ and the electrically silent $K_V9.3$ channel subunits (**Fig. 5.1.1**). These results indicated clearly that human DSM cells express the $K_V2.1$, $K_V2.2$ and the silent $K_V9.3$ channel subunits, but not $K_V4.2$ channel subunit. Furthermore, our Western blot experiments confirmed the expression of $K_V2.1$ and $K_V2.2$ channel subunits at the protein level (**Fig. 5.1.2**). Immunocytochemical experiments also demonstrated the protein expression of $K_V2.1$ and $K_V2.2$ channel in single human DSM cells and its cell membrane localization (**Fig. 5.1.3**). Although expressed at mRNA level, detection of the electrically silent $K_V9.3$ subunit protein was not possible at this time because no selective $K_V9.3$ antibody is commercially available to further confirm $K_V9.3$ protein expression. Thus, we conclude that human DSM expresses $K_V2.1$ and $K_V2.2$ channel subunits, and at least mRNA of the electrically silent $K_V9.3$ channel subunit.

The next step was to investigate the role of K_V2-containing channels in human DSM excitability. Earlier studies conducted by conventional patch-clamp technique indicated the presence of Ca^{2+} independent K^+ current in human DSM cells, which was inhibited by the nonselective K_V channel blocker 3,4-diaminopyridine (36). Here, for the first time, we provide electrophysiological data on K_V2-containing channels in freshly isolated human DSM cells, using the amphotericin-perforated patch-clamp technique in physiological conditions with an intact intracellular environment. The main focus of our patch-clamp experiments was to evaluate the contribution of K_V2-containing channels sensitive to ScTx1 in human DSM excitability. These experiments were performed following inhibition of the BK channels with 200 nM iberiotoxin. Under such experimental conditions the inhibitory effect of ScTx1 was significant at positive voltages from +10 mV to +30 mV (**Fig. 5.1.4B**), corresponding to the peak of the action potential in human DSM (57, 112). The results showed that ScTx1 significantly decreased the amplitude of the whole cell K_V current in human DSM cells (**Fig. 5.1.4**). At more negative voltages, which correspond to the resting membrane potential in human DSM, ScTx1 did not significantly inhibit the whole cell K_V current. This observation supports the concept that in human DSM cells K_V2-containing channels most likely contribute to the repolarization phase of the action potential, rather than the resting membrane potential. The assumption that K_V2 channels contribute to the repolarization phase of the action potential is consistent with recent reports, which showed no effect of ScTx1 on the resting membrane potential in guinea pig and mouse DSM (60, 77). Collectively, the findings suggest that K_V2-containing channels sensitive to ScTx1 contribute to human DSM excitability and are most likely involved in control of the

repolarization phase of the action potential in human DSM.

In human DSM, Ca_V-channels are responsible for the initiation of the action potential (57, 112). Here, we demonstrated that inhibition of the K_V2-channels with ScTx1 significantly increases the intracellular Ca^{2+} levels in freshly isolated human DSM cells (**Fig. 5.1.5**). This increase of the intracellular Ca^{2+} is caused by inhibition of the ScTx1-sensitive K_V2 channels and most likely subsequent activation of Ca_V channels in human DSM cells. Our findings are supported by earlier studies showing that nonselective blockade of the K_V channel with 4-AP causes an inhibition of the slow after-hyperpolarization, and increases the action potential frequency by activation of the Ca_V channels in human DSM (57). These results indicate a functional role of K_V2-containing channels in providing a link between human DSM excitability, intracellular Ca^{2+} levels, and therefore contractility in human DSM.

An increase in intracellular Ca^{2+} concentration is a key process required for activation of DSM spontaneous contractions. Several studies have demonstrated the importance of Ca^{2+} influx via Ca_V channels in the DSM contraction induced by muscarinic and purinergic stimulation (7, 158, 162). DSM spontaneous phasic contractions are myogenic in origin, and TTX (1 μM) does not affect these spontaneous phasic contractions (7, 60, 145). Our data were consistent with these previous reports. For the purpose of the current study, we used TTX only as a precaution to block the transmission of the nerve impulses within the autonomic nerves. Our studies on human DSM contractility reveal that ScTx1 significantly increased the spontaneous phasic contraction amplitude and force in human DSM isolated strips, consistent with our previous findings in rat and guinea pig DSM (29, 77). However, ScTx1 did not cause any significant effect on spontaneous phasic

contraction frequency, phasic contraction duration, and muscle tone. In experimental animals a burst of action potentials triggers a single phasic contraction (58). If a similar mechanism operates in human DSM, one should expect that blocking of the K_V2-containing channel with ScTx1 would increase the frequency of the action potentials, and therefore the amplitude of the phasic contraction without any effect on the phasic contraction frequency.

Davies et al. (36) showed an increase in the amplitude of the spontaneous phasic contractions of human DSM strips, without any effect on the phasic contraction frequency or muscle tone following K_V1 channel inhibition with correolide or agiotoxin-2. Collectively, our data indicate that by decreasing membrane excitability, K_V2-containing channels also oppose human DSM spontaneous phasic contractions under physiological conditions. Interestingly, ScTx1 had more profound effects on the spontaneous phasic contractions in guinea pig as compared with human DSM (77). This could be explained by the differential expression of the K_V2-containing channels. Human DSM expresses both $K_V2.1$ and $K_V2.2$ channels whereas guinea pig DSM expresses only $K_V2.1$ but not $K_V2.2$ channels (77).

We studied the nerve-evoked contractions by stimulating the DSM nerves with EFS (**Fig. 5.1.7**). In both animal and human DSM, TTX (1 μM) completely eliminates the EFS-induced contractions (8, 29, 56, 60). The TTX-sensitivity confirms the neuronal origin underlying the EFS-induced contractions. Our results also show that ScTx1 significantly increases the amplitude of EFS-induced contractions over a range of stimulation frequency from 3.5 to 30 Hz, indicating the functional role of these channels in human DSM nerve-evoked contractions. This is consistent with our earlier animal studies (29,

77), and revealed that K_V2-containing channels also work to oppose human DSM contractility in response to excitatory neurotransmitters.

Phosphorylation of the K_V2-containing channels is now recognized as a major means of regulating their functional activity (84, 92). Our previous studies indicate a major role for protein kinase A in DSM function (22, 79, 134). It is likely that phosphorylation of the K_V2-containing channels by protein kinase A also plays a role in regulating human DSM function, but this topic requires further investigation.

The pathophysiology of OAB associated with detrusor overactivity is poorly understood, and the current treatment is limited to antimuscarinics that have many adverse effects (8). Therefore, a better understanding of the basic physiology of DSM is critical for developing novel therapeutic approaches against OAB. Compelling lines of evidence by our group and others have led us to now consider the K_V2-containing channels as promising pharmacological targets for some types of bladder dysfunctions. In rat and guinea pig DSM, our laboratory has shown that pharmacological blockade of K_V2-containing channels with ScTx1 increased DSM contractility (29, 77). Other studies have also shown that rat detrusor hyperreflexia was associated with a significant decrease in $K_V2.1$ channel mRNA levels (47). Therefore, mutations in K_V2-containing channels would be expected to cause bladder dysfunction.

The clinical significance of modulating the K_V2-containing channels is twofold. Targeting the K_V2-containing channels with channel opening agents will reduce detrusor overactivity and alleviate OAB. Alternatively, targeting K_V2-containing channels with selective inhibitors may have the potential for increasing DSM contractility, and thus have clinical application for the treatment of some types of urinary retention due to

detrusor underactivity. Moreover, increasing K_V2 channels expression by genetic manipulation can potentially reduce detrusor overactivity. To facilitate these novel therapeutic approaches, we first need to better understand K_V2-containing channel expression, function, and regulation in human DSM. The present study has validated the family of K_V2-containing channels as important physiological regulators of human DSM function. Importantly, such channels are potential pharmacological targets for the treatment of some highly prevalent urological disorders such as OAB and/or some types of urinary retention. Current pharmacological therapy for OAB consists of primarily antimuscarinic therapy which has limited efficacy and is associated with considerable side effects. Cholinergic agonists, the primary therapy for detrusor underactivity, have almost no efficacy. The development of K_V2-channel modulators would have potential favorable effects on both of these conditions. However, development of such agents would require some degree of selectivity for the lower urinary tract as K_V2-containing channel expression has been reported in some other smooth muscle tissues (3, 97, 135) as well as some other human tissues such as pulmonary artery (42, 135), neurons (84), and pancreatic delta cells (19). Thus, application of such agents for lower urinary tract conditions may result in collateral effects elsewhere. Clinical trials of K_V2-containing channel modulators would need to be performed to validate such assumption and assess the efficacy and therapeutic safety window.

CONCLUSION

In conclusion, ScTx1-sensitive K_V2-containing channels are expressed in human DSM. They control human DSM excitability, intracellular Ca^{2+} levels, and myogenic and nerve-

evoked contractions. The K_V2-containing channels may represent new targets for pharmacological or genetic manipulation of human DSM. This study is a fundamental basis for future investigation on human DSM tissues from patients with bladder pathologies which may help reveal the role of K_V2-containing channels in the etiology of these bladder pathologies.

5.2 FUNCTIONAL IMPLICATIONS OF KV7 CHANNELS EXPRESSION IN GUINEA PIG DETRUSOR SMOOTH MUSCLE CONTRACTILITY

ABSTRACT

There has been increasing interest in Kv7 channels targeting drugs for the treatment of bladder dysfunctions since retigabine, a Kv7.2-7.5 channel activator normally prescribed in the treatment of epilepsy was shown to also cause urinary retention. In the present study, we examined the expression and functional roles of Kv7 channels in the detrusor smooth muscle (DSM) of guinea pigs. Kv7 channels molecular expressions were investigated using RT-PCR, qPCR, Western blot, and immunohistochemistry while their functional roles were investigated using isometric DSM tension recordings and Kv7 channels modulators. In DSM isolated cells, we found that Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels mRNA expression was relatively higher compared to the Kv7.4 channel which was detected at non-physiological level. Immunohistochemical analysis revealed protein expression for Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels but not the Kv7.4 channel in DSM cells. L-364373, a selective Kv7.1 channel activator and retigabine, induced relaxation of spontaneous phasic contraction amplitude, muscle force, duration, frequency, and muscle tone as well as the 10 Hz electrical field stimulation (EFS)-induced contraction amplitude and muscle force of DSM isolated strips in a concentration-dependent manner. Linopiridine and XE991, two Kv7 channel inhibitors, increased the spontaneous phasic as well as the 10 Hz EFS-induced contractions amplitude and muscle force of DSM isolated strips in a concentration-dependent manner. On EFS-induced contractions generated by a wide range of stimulation frequency (0.5 – 50 Hz), we found that L-364373 (10 μM) and retigabine (10 μM) application decreased

the contraction amplitude while XE991 (10 μM) application increased the contraction amplitude. In summary, we revealed that among Kv7 channels, Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels but not the Kv7.4 channel are expressed in guinea pig DSM cells and that these channels regulate DSM spontaneous and nerve-evoked contractions.

INTRODUCTION

The physiological roles of voltage-activated K^+ (Kv) channels, one of the most diverse K^+ channel family, in the detrusor smooth muscle (DSM) of mammals is emerging. More than 40 genes coding for Kv channels have been identified in the human genome (52, 53), however, information about the expression and the functional roles of Kv channels in the DSM concerns only a fraction of these ion channels. In mouse and rat DSM, molecular, electrophysiological, and functional studies have demonstrated the expression and physiological roles of Kv2.1, Kv5.1, Kv6.1, Kv6.2, Kv6.3, Kv7.1, Kv7.4, and Kv7.5 channels (29, 36, 47, 122, 136, 148, 149, 155). In guinea pig DSM isolated cells, studies suggest that Kv2.1, Kv6.2, Kv6.3, Kv8.2, Kv9.1, Kv9.2, and Kv9.3 channels are expressed at the mRNA level and that only Kv2-containing channels are physiologically active (74). Like in guinea pig DSM, the expression and physiological role of Kv channels in humans DSM is only limited to Kv2-containing channels (74). In humans, molecular evidence has confirmed the mRNA and protein expression of Kv2.1 and Kv.2.2 channels only (74). Regardless of the type of Kv channel investigated in the DSM, the general consensus suggest that activation of Kv channels hyperpolarizes the cell membrane and diminishes DSM excitability and contractility while their inhibition causes membrane depolarization and increase in DSM contractility via an elevation of intracellular Ca^{2+} concentration (4, 29, 36, 47, 74, 118, 122, 136, 148, 149). In recent years, retigabine, a third generation antiepileptic drug has become the focus of new investigation for bladder dysfunction. Retigabine is a Kv7.2-7.5 channel activator which is routinely prescribed to epileptic patients in order to restore physiological levels of neuronal excitability which is abnormally elevated in those patients (51, 85, 123). In

addition to retigabine effects on the central nervous system, clinical studies have reported that retigabine affects peripheral organs such as the urinary bladder by causing relaxation of the DSM which in certain patients lead to urinary retention (21, 51, 85, 123). This latter retigabine effect on the DSM has lead researchers to believe that Kv7 channels targeting compounds such as retigabine could potentially be useful in the treatment of overactive bladder (OAB), a pathophysiological condition which affects about 17% of the American population (85, 147). The Kv7 channel is a subfamily of the Kv channel family, and has five members or subtypes which are Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels. Structurally, Kv7 channels are composed of six transmembrane domains and a pore loop forming one α-subunit that can assemble to form homo- or heterotetramer channels (51-53). Unlike the Kv7.1 channel, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels possess a hydrophobic pocket outside the channel's gate which allows allosteric binding of their modulators (51). In the present study, we investigated the molecular (mRNA messages and proteins) expression of all Kv7 channels subtypes (Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels) in the DSM of guinea pigs. We further used Kv7 channel activators and blockers to determine whether the pharmacological modulation of Kv7 channels affects DSM spontaneous and nerve-evoked contractions. The overall objective of this study was to investigate Kv7 channels expression and function in guinea pig DSM and therefore assess whether Kv7 channels could be promising drug target for the treatment of bladder dysfunctions.

MATERIAL AND METHODS

Animal studies and DSM tissue harvesting

Animal experiments were conducted as described in chapter 2.2. For this study, we used 40 adult males Hartley-Albino guinea pigs, weighing on average 422.3±6.7 g.

DSM cells isolation and collection

Guinea pig DSM cells were isolated and collected as described in chapter 2.2.

RT-PCR and qPCR experiments

RT-PCR and qPCR experiments were conducted as previously described in chapter 2.2. We used previously published guinea pig primers sequences for Kv7.1 – 7.5 channels (116). The primers sequences and the size of the amplicon were as follows: Kv7.1- F: 5′-ATTGTCCTGGTGGTGTTCTTTG-3′ and R: 5′-CCCCTGATGGCTGATGTGG-3′ (206 bp); Kv7.2- F: 5′-TCTACGCCACCAACCTGTC-3′ and R: 5′-TACATGGGCACCGTGACC-3′ (79 bp); Kv7.3- F: 5'-CTTGAAAACCGTCATCTGC-3' and R: 5'-CAAGTTCACAGGGTCGTG-3' (124 bp); Kv7.4- F: 5'-CGATGTCATGCCTGCTGTG-3' and R: 5'-GGTGTCCTGCTGAATACTGC-3' (137 bp); Kv7.5- F: 5'-CGTCCGCACTCAGAAGTC-3' and R: 5'-TCCAATGTACCAGGCTGTG-3' (137 bp); GAPDH- F: 5′-TACGACAAGTCCCTCAAGATTG-3′ and R: 5′-TCTGGGTGGCAGTGATGG-3′ (139 bp) (116). The parameters of the RT-PCR experiments were as follows: 5 min at 95 ^{o}C then amplified by 35 cycles (95 ^{o}C for 30 s, 58 ^{o}C for 30 s, 72 ^{o}C for 30 s) followed by a

5-min extension at 72 ^{o}C. The parameter of the qPCR experiments were as follows: Cycle 1, 95 ^{o}C for 3 min; cycle 2, 95 ^{o}C for 10 s then 58 ^{o}C for 30 s (repeated 40 times); cycle 3, 95 ^{o}C for 1 min; cycle 4, 58 ^{o}C for 1 min; cycle 5, 58 ^{o}C for 10 s with 0.5 ^{o}C increment (repeated 81 times to generate a melting curve).

Western blot and immunohistochemistry

Our western blot and immunohistochemistry experiments were conducted as described in chapter 2.2. We used the following primary antibodies: affinity-purified goat polyclonal antibodies anti-KCNQ1 (Kv7.1) (1:100), anti-KCNQ2 (Kv7.2) (1:100), anti-KCNQ3 (Kv7.3) (1:100), anti-KCNQ4 (Kv7.4) (1:100), or anti-KCNQ5 (Kv7.5) (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For the western blot experiment, donkey anti-goat IgG conjugated with horseradish peroxidase (diluted to 1:1,000) was used as a secondary antibody while Cy3-conjugated anti-goat IgG (1:100) (Jackson ImmunoResearch, West Grove, PA) was the secondary antibody used in the immunostaining experiments. Tissue sections were visualized under a confocal microscope LSM 700 META (Carl Zeiss, Germany) 63X objective.

Isometric DSM tension recordings

Isometric DSM tension recordings experiments are described in chapter 2.2. We activated Kv7 channels by applying increasing concentrations of L-364373 (100 nM – 100 μM), a selective Kv7.1 channel agonist or retigabine (100 nM – 100 μM), a Kv7.2 – 7.5 channel agonist at 10-min intervals. We also evaluated DSM spontaneous phasic contractions responses to Kv7 channels blockade with XE991 (100 nM – 30 μM), a Kv7.1 – 7.5

channel blocker; or linopiridine (100 nM – 30 μM), another Kv7.1 – 7.5 channel antagonist. In DSM strips which exhibited spontaneous phasic contraction amplitude less than 0.1 g, contractions were induced by electrical field stimulation (EFS) and TTX (1 μM) was not added since TTX effectively suppresses EFS-induced contractions. On the 10 Hz EFS-induced contractions, increasing concentrations (100 nM – 100 μM) of Kv7 channel activators and inhibitors were applied as described above. On the 0.5 – 50 Hz EFS-induced contractions, a single concentration of Kv7 channel modulator was applied.

Solutions and drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS composition are provided in chapter 2.2. L-364373, retigabine, XE991, and linopiridine were purchased from Tocris Bioscience (Bristol, UK). Stock solutions of L-364373, retigabine, and XE991 were prepared daily in dimethyl sulfoxide (DMSO). The final DMSO concentration in the bath was not greater than 0.1%. Linopiridine was also prepared daily and dissolved in double-distillated water.

Data analysis and statistics

Data analysis and statistics were performed as described in chapter 2.2.

RESULTS

Detection and relative expression level of mRNA messages for Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels in guinea pig DSM

We conducted RT-PCR and qPCR experiments on guinea pig DSM tissue and a poll of isolated DSM cells to investigate Kv7 channels mRNA expressions. We used guinea pig brain as a positive control since previous studies have established that all Kv7 channel isoforms are expressed in guinea pig brain at the mRNA level (116). The RT-PCR experiments revealed mRNA expression for all Kv7 channel isoforms in guinea pig whole DSM tissue (N=4; **Fig. 5.2.1A**). Next, we conducted RT-PCR experiments on guinea pig DSM freshly isolated single cells to confirm that the mRNA messages detected in the whole DSM tissue originated from DSM cells but not from other non-DSM cell types such as neurons, fibroblasts, vascular, and endothelial cells present within the DSM layers (1, 78, 127, 143, 165). Unlike whole DSM tissue, our RT-PCR analysis on guinea pig DSM isolated single cells showed mRNA expression for all Kv7 channel isoforms except the Kv7.4 channel (N=4; **Fig. 5.2.1A**). RT-PCR experiments were conducted on DSM cDNAs equivalent to 100 ng of starting mRNA. Negative control experiments performed in the absence of the reverse transcriptase (-RT) demonstrated an absence of genomic DNA contamination. Following the RT-PCR experiments, we conducted qPCR analysis to determine the relative mRNA expression level for each Kv7 channel isoform in guinea pig DSM whole tissue and isolated single cells. In whole DSM tissue, we found that Kv7 channel isoforms relative mRNA level was as follows: Kv7.1 ≈ Kv7.2 > Kv7.3 ≈ Kv7.5 > Kv7.4 (N=4; $p<0.05$; **Fig. 5.2.1B**). When qPCR analysis was performed on a poll of isolated DSM cells, the relative mRNA

level for Kv7 channel isoforms was as follows: Kv7.1 ≈ Kv7.2 > Kv7.5 > Kv7.3 ≈ Kv7.4 (N=4; $p<0.05$; **Fig. 5.2.1B**). These latter single-cell qPCR experiments confirmed the previous RT-PCR data and further suggest that the Kv7.1, Kv7.2, Kv7.3, and Kv7.5 are highly expressed at the mRNA level, whereas the Kv7.4 channel may not be physiologically expressed in guinea pig DSM cells.

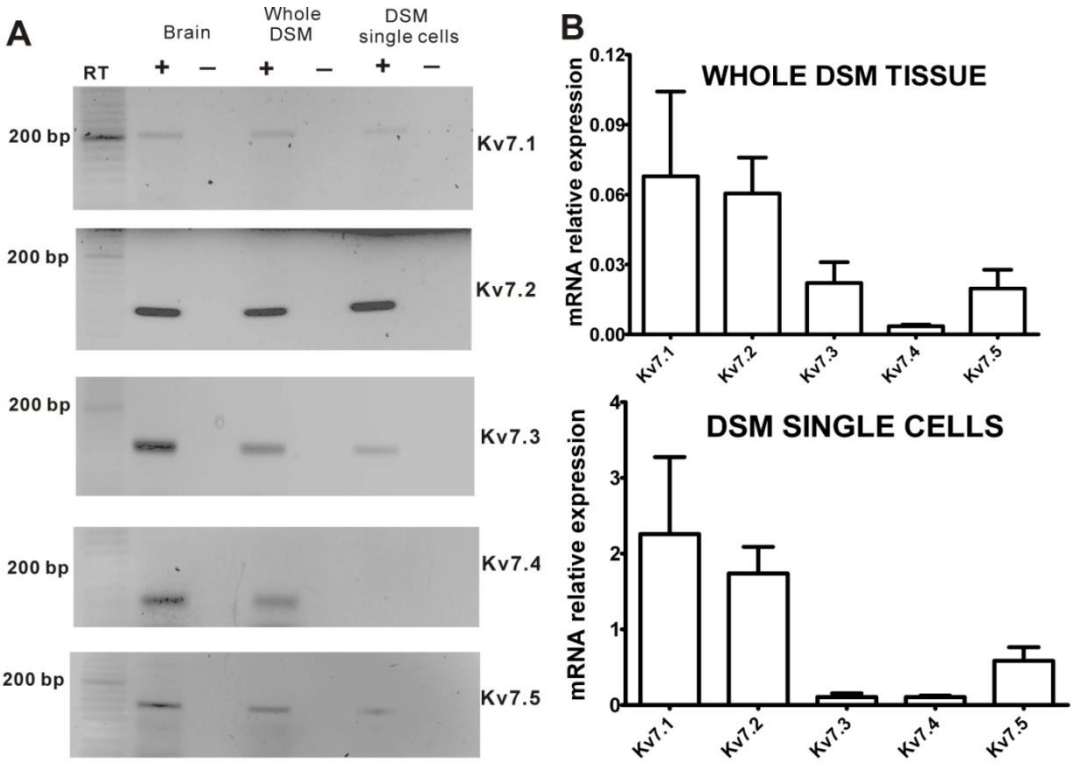

Figure 5.2.1. **Kv7 channels mRNA expression in guinea pig DSM. A) Gel electrophoresis images illustrate RT-PCR detection of all Kv7 channel subtypes including Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 mRNA messages in whole DSM tissue (N=4)**. At the DSM single cell level, unlike other Kv7 channel isoforms (Kv7.1, Kv7.2, Kv7.3, and Kv7.5), the Kv7.4 channel mRNA message was not detected. Guinea pig brain was used as a positive control. No product was observed in the negative controls (-RT) in which reverse transcriptase was not added to the reaction. B) qPCR analysis show relatively low Kv7.4 channel mRNA expression in both DSM whole tissue (N=4) and DSM single cells (N=4). Data were normalized to GAPDH using the ΔCt method. Ct values are expressed as mean ± SE.

Guinea pig DSM cells express Kv7.1, Kv7.2, Kv7.3, and Kv7.5 but not Kv7.4 channel proteins

To assess whether the Kv7 channels previously found at the mRNA level using RT-PCR experiments were also expressed at the protein level, we performed both Western blot and immunostaining analysis on guinea pig whole DSM tissue. Western blot and immunostaining analysis were performed using specific antibodies against each Kv7 channel isoform. Our Western blot data revealed that all Kv7 channel isoforms including Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels proteins are expressed in guinea pig whole DSM tissue (N=3; **Fig. 5.2.2**) consistently with the RT-PCR data in guinea pig whole DSM tissue (**Fig. 5.2.1A**).

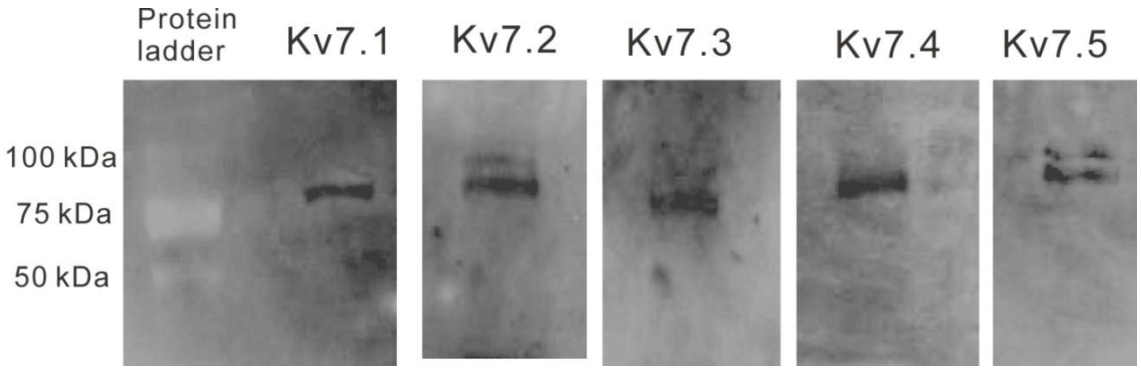

Figure 5.2.2. Detection of Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5 channels protein expression in guinea pig whole DSM tissues by the Western blot technique. The expected molecular weight (MW) for all Kv7 channels isoforms including Kv7.1 (75 kDa), Kv7.2 (90 kDa), Kv7.3 (77 kDa), Kv7.4 (77 kDa), and Kv7.5 (99 kDa) channels was confirmed by the use of a protein ladder. Experiments were conducted in 3 separate Western blot reactions using protein isolated from 3 guinea pig whole DSM

Immunohistochemical experiments were performed to determine whether these Kv7 channel proteins were localized in DSM cells. We used α smooth muscle actin antibody (ab21027, Abcam) to specifically stain DSM cells as previously published (143). We found protein expression for Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels in guinea DSM (**Fig. 5.2.3**). However, we did not detect protein expression for Kv7.4 channel in guinea pig DSM cells (**Fig. 5.2.4**) consistent with our single-cells RT-PCR data (N= 3; **Fig. 5.2.1A**). The specificity of every Kv7 channel isoform antibody was tested by incubating the primary antibody with its competing peptide (**Fig. 5.2.5**).

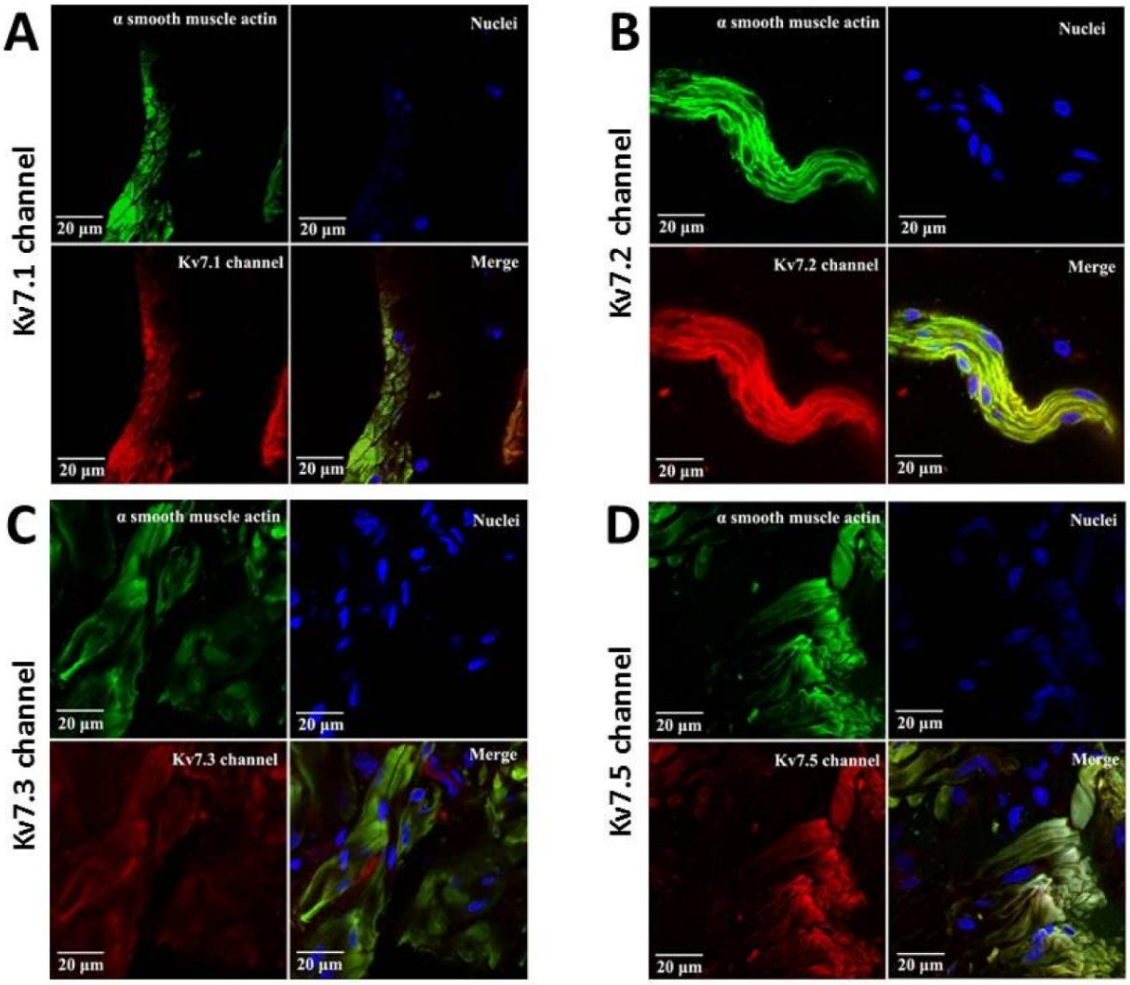

Figure 5.2.3. **Confocal images illustrate Kv7.1 (A), Kv7.2 (B), Kv7.3 (C), and Kv7.5 (D) channels protein expression in mucosa-free guinea pig whole DSM tissue**. Kv7 channels proteins were detected by immunohistochemical reaction using channel's specific antibodies. In all panels, α smooth muscle actin is shown in green; cells' nuclei are shown in blue; the specific Kv7 channel subtype protein expression is represented by the red staining. The merged images of α smooth muscle actin, nuclei, and the Kv7 channel's protein expression are illustrated in quadrant labeled "Merge". Images were captured with a Carl Zeiss LSM 700 META confocal microscope (63x objective). Experiments were conducted on tissue samples isolated from 3 different guinea pigs.

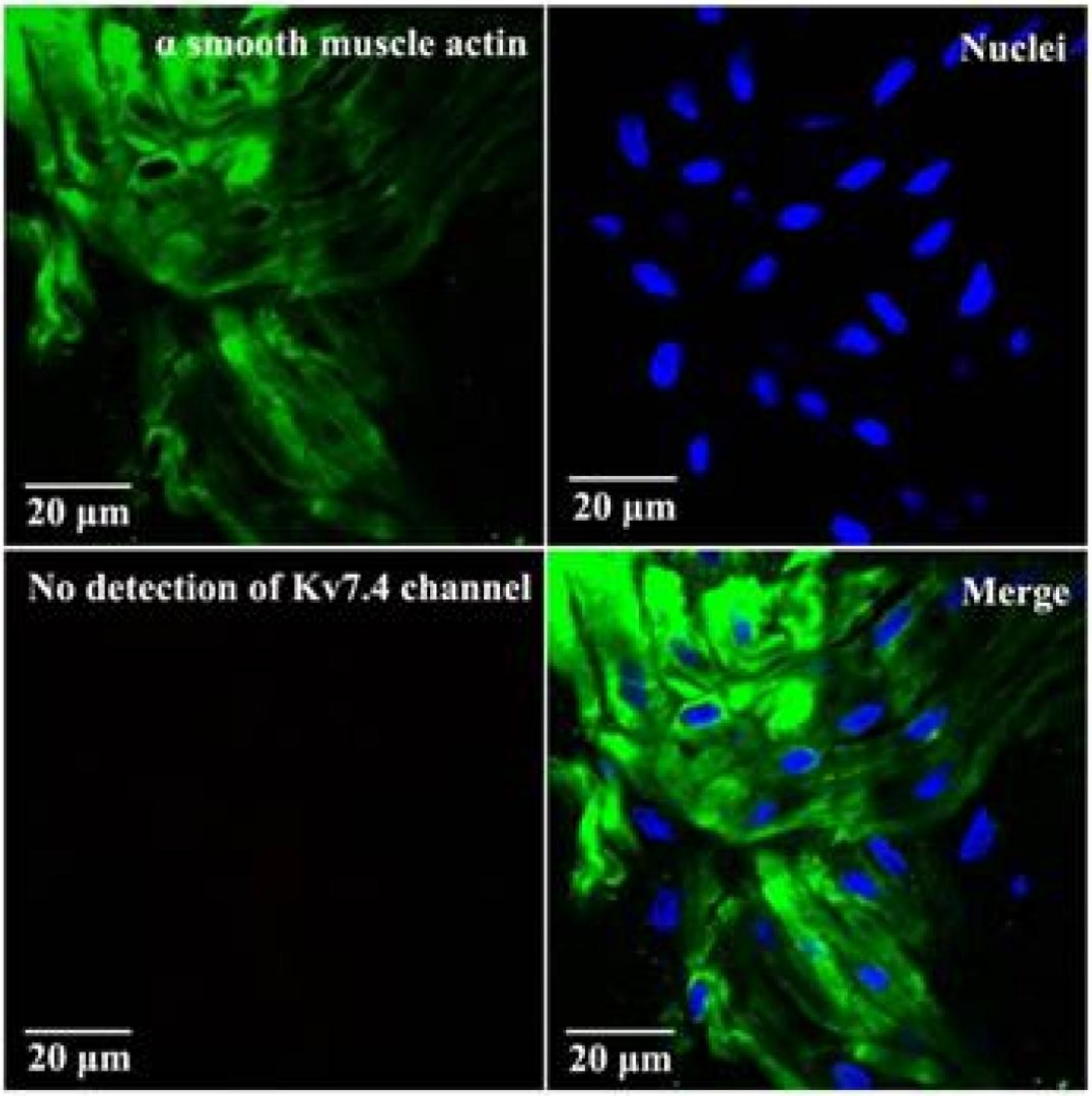

Figure 5.2.4. Confocal images illustrate the lack of Kv7.4 channel protein detection in guinea pig DSM tissue. α smooth muscle actin is shown in green; Cells' nuclei are shown in blue; the absence of red staining confirm that Kv7.4 was not detected. The merged images of α smooth muscle actin and nuclei are illustrated in quadrant labeled "Merge". Images were captured with a Carl Zeiss LSM 700 META confocal microscope (63x objective). Experiments were conducted on tissue samples isolated from 3 different guinea pigs.

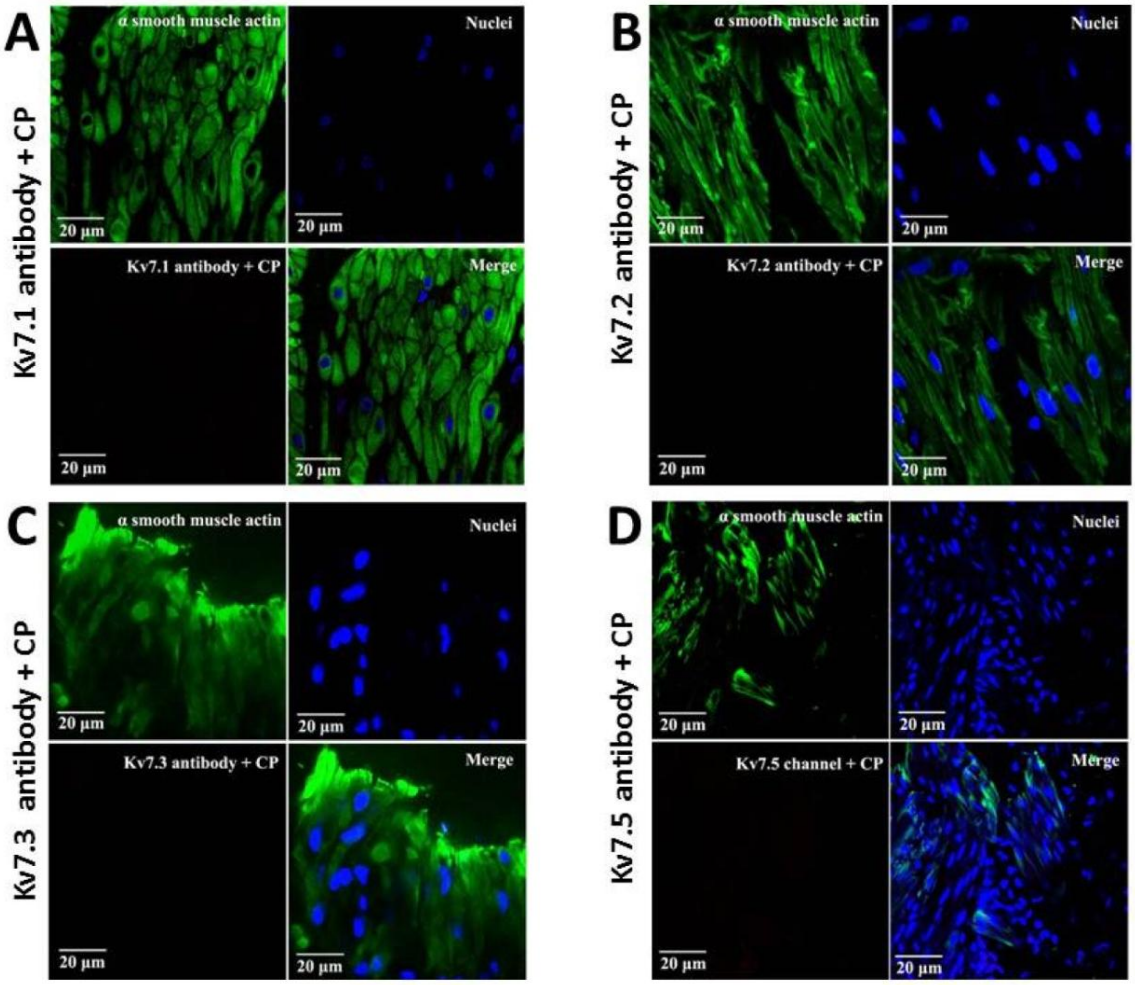

Figure 5.2.5. **Confocal images illustrate the lack of protein detection in the presence of competing peptides confirming the specificity of the primary antibody for Kv7.1 (A), Kv7.2 (B), Kv7.3 (C), and Kv7.5 (D) channels protein, respectively**. In all panels, α smooth muscle actin is shown in green; Cells' nuclei are shown in blue; the absence of red staining confirm the successful block of the primary antibody by its competing peptide. The merged images of α smooth muscle actin and nuclei are illustrated in quadrant labeled "Merge". Images were captured with a Carl Zeiss LSM 700 META confocal microscope (63x objective). Experiments were conducted on tissue samples isolated from 3 different guinea pigs.

Pharmacological activation of Kv7 channels causes relaxation of spontaneous phasic and tonic contractions of guinea pig DSM isolated strips

We investigated the spontaneous phasic and tonic contractions responses to Kv7 channels activation with L-364373, a selective Kv7.1 channel agonist, and retigabine, a Kv7.2-7.5 channel agonist in guinea pig DSM isolated strips. We found that L-364373 (100 nM – 100 μM) effectively decreased the spontaneous phasic and tonic contractions of guinea pig DSM isolated strips (**Fig. 5.2.6**). Our data showed that L-364373 (100 nM – 100 μM) attenuated the spontaneous phasic contractions amplitude, muscle force, frequency, duration, and muscle tone of guinea pig DSM isolated strips in a concentration-dependent manner (n=7, N=4; **Fig. 5.2.6B** and **Table 5.2.1**).

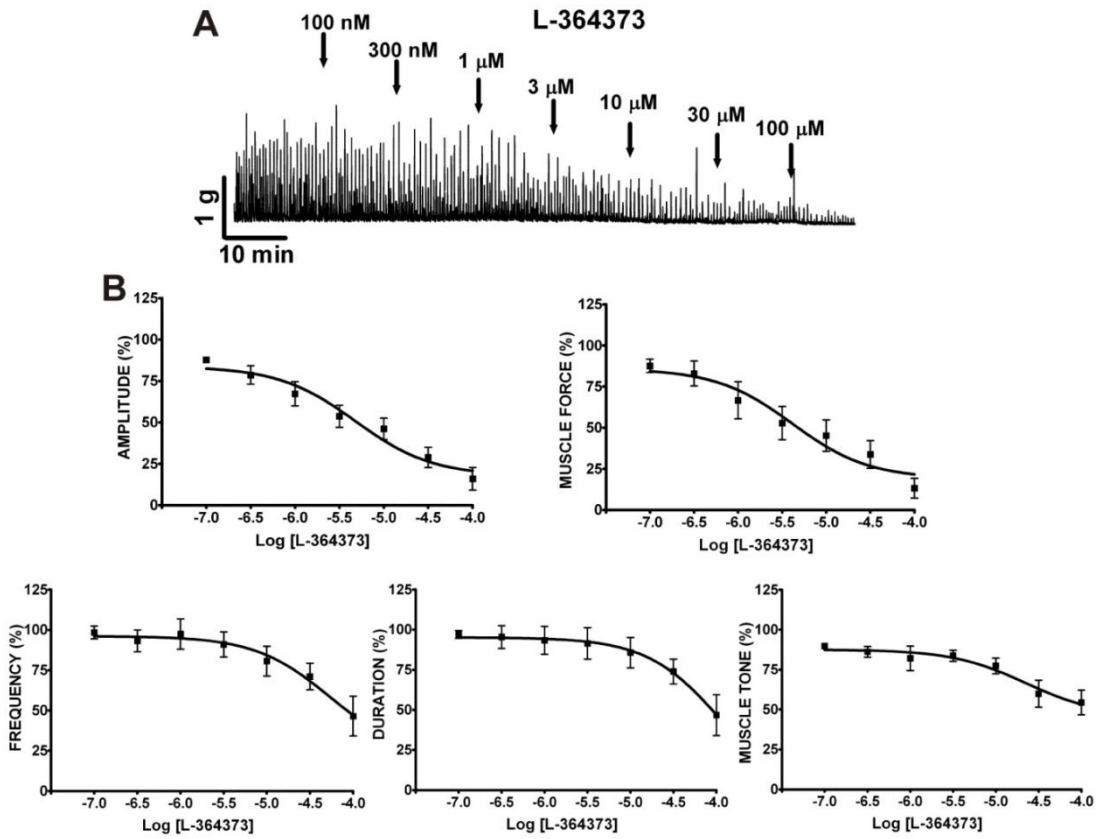

Figure 5.2.6. L-364373, a selective Kv7.1 channel activator causes relaxation of spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone in guinea pig DSM isolated strips. A) This original DSM tension recording illustrates L-364373 (100 nM – 100 µM) relaxant effect on spontaneous phasic contractions of DSM isolated strips in a concentration-dependent manner. B) Cumulative concentration-response curves showing significant reduction in DSM spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone following L-364373 application. The 5-min control period prior to L-364373 addition was taken to be 100 % and data were normalized (n=7, N=4). TTX (1 µM) was present throughout the experiments.

Compound	Amplitude	Muscle Force	Frequency	Duration	Muscle tone
L-364373	4.8 (2.0 - 11.7) µM 16.0 ± 6.8%	4.1 (1.2 - 13.9) µM 13.2 ± 6.0%	N/A 46.4 ± 12.2%	N/A 46.8 ± 12.7%	N/A 54.4 ± 7.7%
Retigabine	2.3 (1.4 – 3.5) µM 0.7 ± 0.7%	2.1 (1.3 – 3.4) µM 0.2 ± 0.2%	14.0 (4.3 – 46.1) µM 11.2 ± 11.2%	8.7 (5.6 – 13.4) µM 0.4 ± 0.4%	3.5 (0.1–17.3) µM 76.4 ± 14.9%
XE991	4.5 (0.4 – 54.6) µM 212.9 ± 42.2%	2.3 (0.1 – 49.3) µM 233.3 ± 66.2%	No effect	No effect	No effect
Linopiridine	11.5 (1.0 – 127.0) µM 415.1 ± 75.9%	10.7 (0.9 – 132.5) µM 415.1 ± 110.4%	No effect	No effect	No effect

Table 5.2.1. IC_{50} values and maximum effects of Kv7 channels modulators on spontaneous phasic and tonic contractions of guinea pig isolated strips. IC_{50} values (in µM) are reported as means (95% confidence interval); Maximum inhibition values for L-364373 and retigabine were determined at concentrations equal to 100 µM. Maximum increase values for XE991 and linopiridine were determined at concentrations equal to 30 µM. Values are reported as percentages of means ± SEM in comparison to control condition (taken to be 100%). N/A = not applicable.

In comparison to L-364373, retigabine had a more pronounced effect on DSM spontaneous phasic and tonic contractions. Retigabine (100 nM – 100 μM) inhibited the spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone of guinea pig DSM isolated strips in a concentration-dependent manner (**Fig. 5.2.7**). At the maximal concentration of 100 μM, retigabine almost completely abolished the phasic contractions amplitude, muscle force, frequency, and duration while the effect on muscle tone was less pronounced (n=7, N=6, **Fig. 5.2.7B** and **Table 5.2.1**). Table 5.2.1 shows the different IC_{50} values for L-364373 and retigabine effects on guinea pig DSM isolated strips spontaneous phasic contractions amplitude, muscle force, frequency, duration, and muscle tone. These results suggest that Kv7 channels activation leads to inhibition of the spontaneous phasic and tonic contractions in guinea pig DSM isolated strips.

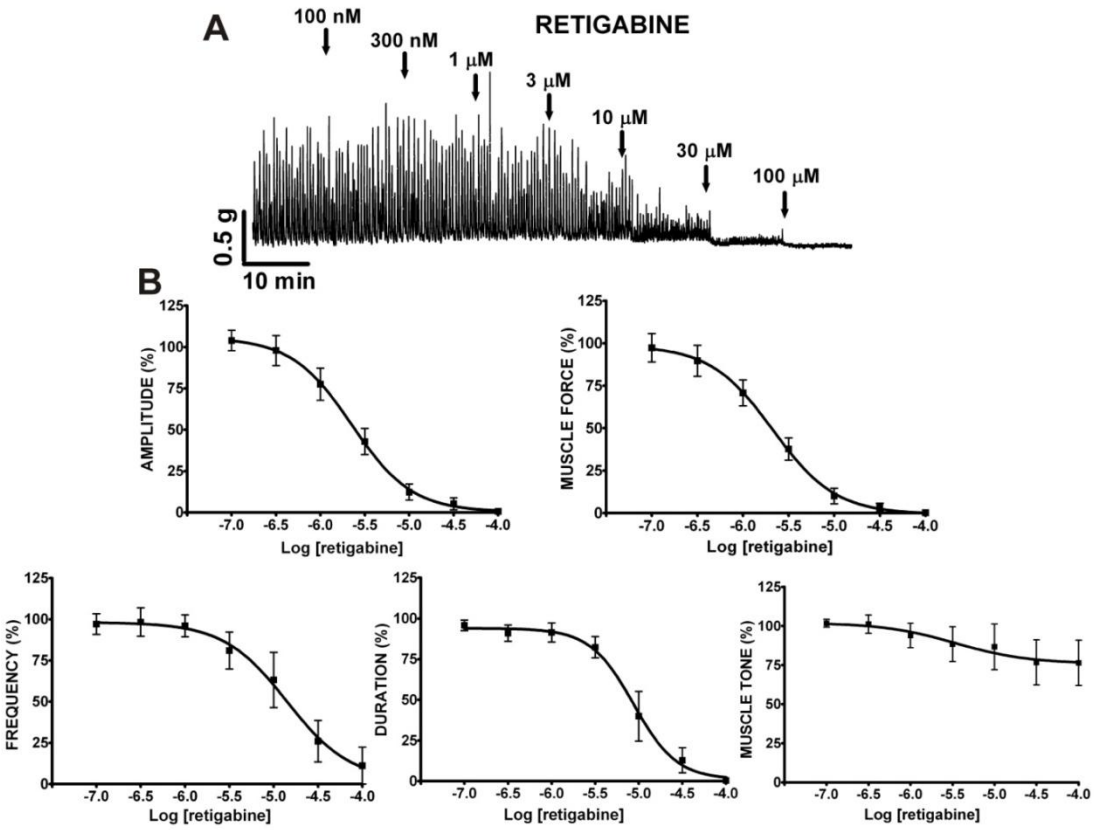

Figure 5.2.7. Retigabine, a Kv7.2 – 7.5 channel activator causes relaxation of spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone in guinea pig DSM isolated strips. A) This original DSM tension recording illustrates retigabine (100 nM – 100 μM) inhibitory effect on spontaneous phasic contractions of DSM isolated strips in a concentration-dependent manner. B) Cumulative concentration-response curves showing significant reduction in DSM spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone following retigabine application. The 5-min control period prior to retigabine addition was taken to be 100 % and data were normalized (n=7, N=6). TTX (1 μM) was present throughout the experiments.

Pharmacological blockade of Kv7 channels enhances spontaneous phasic and tonic contractions of guinea pig DSM isolated strips

We investigated the effects of pharmacological inhibition of Kv7 channels on spontaneous phasic and tonic contractions of DSM isolated strips. We used XE991 and linopiridine, two Kv7.1-7.5 channel antagonists. We found that XE991 (100 nM – 30 μM) enhanced the spontaneous phasic contraction amplitude and muscle force of DSM isolated strips in a concentration-dependent manner without statistically significant effects on contraction frequency, duration, and muscle tone (**Fig. 5.2.8A-B**). At the maximal concentration of 30 μM, XE991 effects on the spontaneous phasic contractions amplitude and force compared to control were 212.9±42.2 % and 233.3±66.2%, respectively (n=8, N=5, **Fig. 5.2.8B** and **Table 5.2.1**). We found that pharmacological blockade of Kv7 channels with linopiridine (100 nM – 100 μM) also increased phasic contraction amplitude and muscle force in a concentration-dependent manner (**Fig. 5.2.8C-D**). At the maximal concentration of 30 μM, linopiridine effects on the spontaneous phasic contraction amplitude and muscle force compared to control were 415.1±75.9% and 415.0±110.4%, respectively (n=7, N=5, **Fig. 5.2.8D** and **Table 5.2.1**). Table 5.2.1 shows the different IC_{50} values for XE991 and linopiridine effects on spontaneous phasic contractions amplitude and muscle force of DSM isolated strips. These data suggest that Kv7 channels blockade enhances the spontaneous phasic contractions in guinea pig DSM isolated strips.

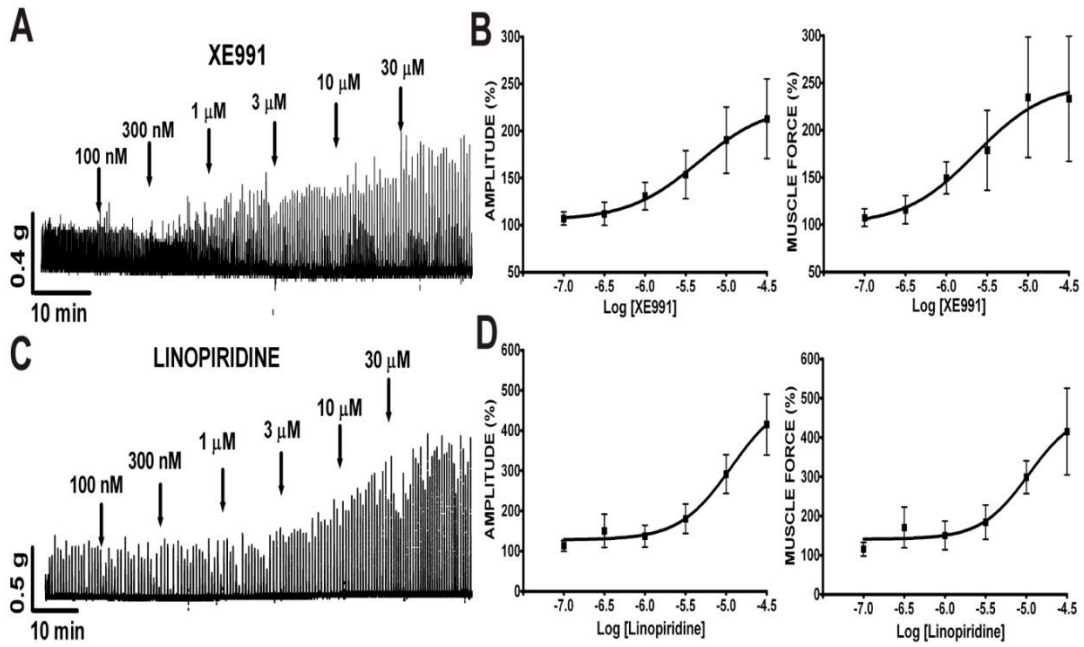

Figure 5.2.8. XE991 and linopiridine, two Kv7.1 – 7.5 channel blockers increase spontaneous phasic contraction amplitude and muscle force in guinea pig DSM isolated strips. Original DSM tension recordings illustrate that XE991 (100 nM – 30 μM) (A) and linopiridine (100 nM – 30 μM) (C) application enhance spontaneous phasic contractions in guinea DSM isolated strips in a concentration-dependent manner. Cumulative concentration-response curves show significant increase in DSM spontaneous phasic contractions amplitude and muscle force following XE991 (B; n=8, N=5) and linopiridine (D; n=7, N=5) application. The 5-min control period prior to XE991 or linopiridine addition was taken to be 100 % and data were normalized. TTX (1 μM) was present throughout the experiments**.**

Pharmacological activation of Kv7 channels inhibits EFS-induced contractions of guinea pig DSM strips in a concentration-dependent manner

We investigated the effects of L-364373 and retigabine on the amplitude, muscle force, duration, and muscle tone of EFS-induced DSM contractions generated by 10 Hz/min EFS in DSM isolated strips. We found that L-364373 attenuated 10 Hz EFS-induced contractions of DSM isolated strips in a concentration-dependent manner (**Fig. 5.2.9A-B**). L-364373 (100 nM-100 µM) decreased the amplitude and muscle force of the 10 Hz EFS-induced contractions without any significant effect on contraction duration and muscle tone (n=6, N=4; **Fig. 5.2.9B** and **Table 5.2.2**). Similarly to L-364373 effect, retigabine (100 nM–100 µM) also decreased the 10 Hz EFS-induced contraction amplitude and muscle force in a concentration-dependent manner (**Fig. 5.2.9C-D**). At higher concentrations, retigabine was more effective than L-364373 at reducing the amplitude and muscle force of the 10 Hz EFS-induced contractions of DSM isolated strips since retigabine (100 µM) completely inhibited the amplitude and muscle force of the 10 Hz EFS-induced contractions (n=9, N=8; **Fig. 5.2.9D** and **Table 5.2.2**). These data suggest that pharmacological activation of Kv7 channels attenuates nerve-evoked contractions in guinea pig DSM isolated strips.

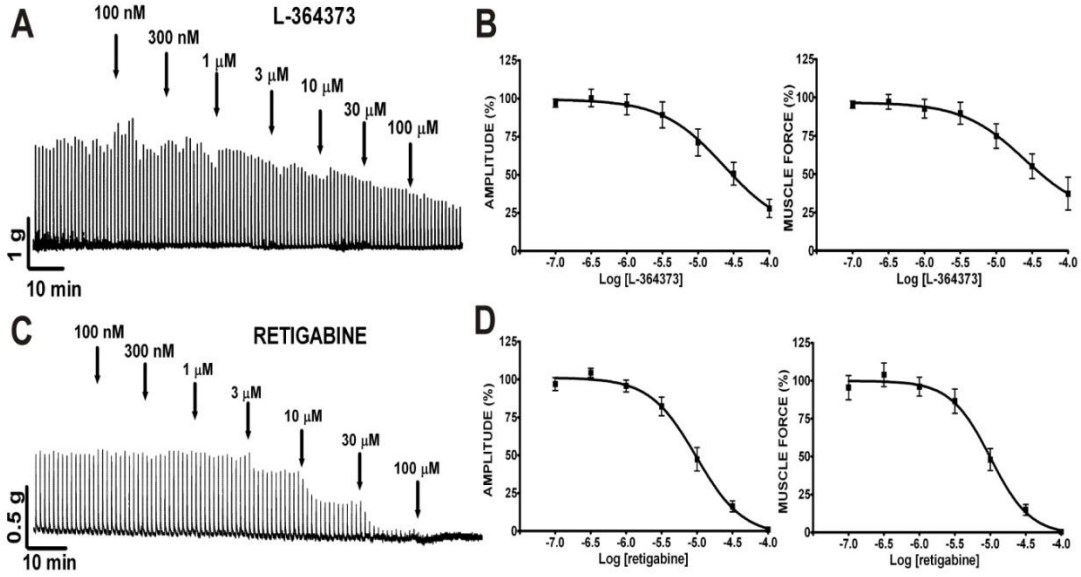

Figure 5.2.9. **Kv7 channels activation with L-364373 or retigabine reduces the 10 Hz EFS-induced contractions in guinea pig DSM isolated strips**. Original DSM tension recordings illustrate L-364373 (100 nM – 100 μM) (A) and retigabine (100 nM – 100 μM) (C) inhibitory effects on DSM 10 Hz EFS-induced contractions, respectively. Cumulative concentration-response curves show that L-364373 (B; n=6, N=4) and retigabine (D; n=9, N=8) attenuate guinea pig DSM 10 Hz EFS-induced contraction amplitude and muscle force in a concentration dependent-manner.

Compound	Amplitude	Muscle force	Duration	Muscle tone
L-364373	23.1 (8.7 – 61.2) µM 28.0 ± 6.0%	24.9 (7.3 – 84.3) µM 37.2 ± 10.7%	No effect	No effect
Retigabine	9.7 (6.9 – 13.9) µM 1.0 ± 0.7%	10.1 (6.7 – 15.1) µM 0.3 ± 0.2%	No effect	No effect
XE991	0.6 (0.1 – 5.6) µM 155.3 ± 16.4%	1.4 (0.2 – 9.8) µM 180.5 ± 24.2%	No effect	No effect
Linopiridine	N/A 144.0 ± 20.1%	N/A 130.1 ± 13.1%	No effect	No effect

Table 5.2.2. IC_{50} values and maximum effects of Kv7 channels modulators on 10 Hz EFS induced contractions of guinea pig isolated strips. IC_{50} values (in µM) are reported as means (95% confidence interval); Maximum inhibition values for L-364373 and retigabine were determined at concentrations equal to 100 µM. Maximum increase values for XE991 and linopiridine were determined at concentrations equal to 30 µM. Values are reported as percentages of means ± SEM in comparison to control condition (taken to be 100%). N/A = not applicable.

Pharmacological blockade of Kv7 channels increases EFS-induced contractions of guinea pig DSM strips in a concentration-dependent manner: We used XE991 and linopiridine, two Kv7 channel blockers to assess the change in 10 Hz EFS-induced contractions in guinea pig DSM isolated strips upon Kv7 channels blockade. The data showed that XE991 (100 nM–30 μM) effectively enhanced the amplitude and muscle force of the 10 Hz EFS-induced contractions of guinea pig DSM isolated strips in a concentration-dependent manner (**Fig. 5.2.10A-B**). At the maximal concentration of 30 μM, XE991 effects on the 10 Hz EFS-induced contractions amplitude and muscle force compared to control were 155.3±16.4% and 180.5±24.2%, respectively (n=9, N=7, **Fig. 5.2.10B** and **Table 5.2.2**). We also found that similarly to XE991, linopiridine (100 nM–30 μM) also increased guinea pig DSM isolated strips 10 Hz EFS-induced contraction amplitude and muscle force in a concentration-dependent manner (n=6, N=5; **Fig. 5.2.10C-D** and **Table 5.2.2**). At the maximal concentration of 30 μM, linopiridine effects on the 10 Hz EFS-induced contraction amplitude, force, and muscle tone compared to control were 144.0±20.1%, 130.1±13.1%, and 159.3±28.6%, respectively (n=6, N=5, **Fig. 5.2.10D** and **Table 5.2.2**). These data suggest that pharmacological blockade of Kv7 channels enhances nerve-evoked contractions in guinea pig DSM isolated strips.

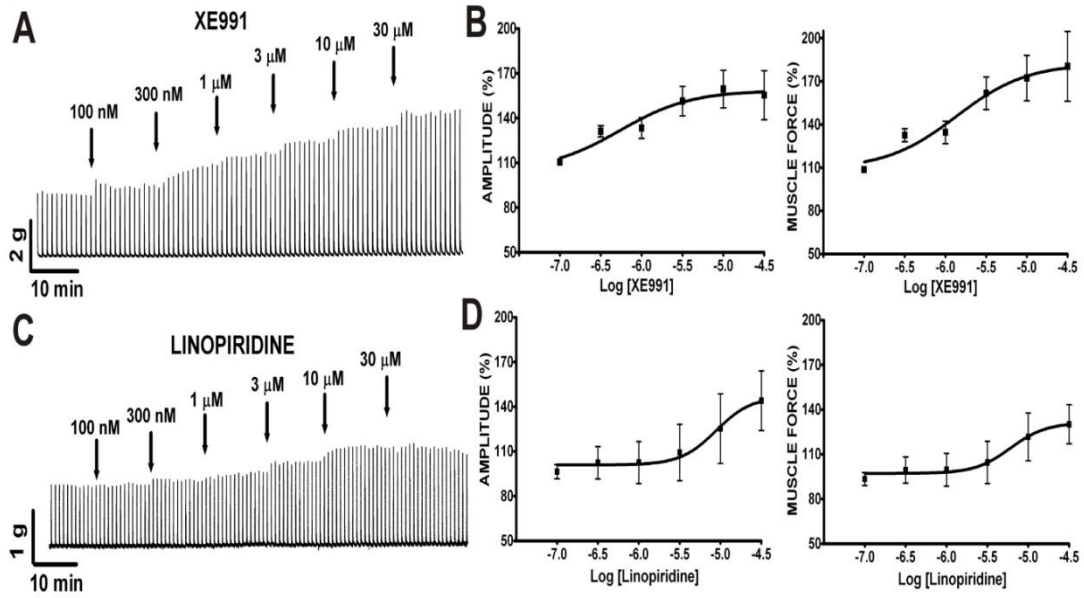

Figure 5.2.10. Kv7.1 – 7.5 channels blockade with XE991 or linopiridine increases the 10 Hz EFS-induced contractions in guinea pig DSM isolated strips. The original DSM tension recordings illustrate that XE991 (100 nM – 30 μM) (A) and linopiridine (100 nM – 30 μM) (C) enhance guinea pig DSM 10 Hz EFS-induced contractions. Cumulative concentration-response curves show that XE991 (B; n=9, N=7) and linopiridine (D; n=6, N=5) increases DSM 10 Hz EFS-induced contraction amplitude and muscle force in a concentration dependent-manner.

Activation of Kv7 channels with retigabine and L-364373 decreases the amplitude of the EFS-induced DSM contractions in a wide range of stimulation frequencies

We addressed how activation of Kv7 channels modulates DSM nerve-evoked contractions in response to a wide range of EFS frequencies in guinea pig DSM isolated strips. We first applied increasing EFS frequencies (0.5–50 Hz) as a control protocol, followed by the addition of a single concentration of L-364373 (10 μM) or retigabine (10 μM) (**Fig. 5.2.11 A-B**). Then, a second EFS protocol was applied to evaluate the compound effects on the EFS-induced contractions. We found that in guinea pig DSM isolated strips, L-364373 (10 μM) inhibited the 0.5 – 50 Hz EFS-induced contractions. L-364373 (10 μM) significantly attenuated the amplitude of the EFS-induced contractions at stimulation frequencies ranging between 7.5 and 50 Hz (n=7, N=4, $p<0.05$; **Fig. 5.2.11A**). At the maximum EFS frequency of 50 Hz, L-364373 (10 μM) reduced the contraction amplitude to 67.3±4.8% in comparison to control (n=7, N=4; $p<0.05$; **Fig. 5.2.11A**). Retigabine (10 μM) significantly decreased the amplitude of the EFS-induced contractions at stimulation frequencies ranging between 12.5 and 50 Hz (n=6, N=4, $p<0.05$; **Fig. 5.2.11B**). At the maximum EFS frequency of 50 Hz, retigabine (10 μM) reduced the contraction amplitude to 76.4±10.8% in comparison to control (taken to be 100%) (n=6, N=4; $p<0.05$; **Fig. 5.2.11B**). These data suggest that activation of Kv7 channels reduces guinea pig DSM nerve-evoked contractions induced by EFS at a wide range of stimulation frequencies.

Kv7 channels inhibition with XE991 increases the amplitude of the 0.5 – 50 Hz EFS-induced DSM contractions: XE991, a Kv7.1-7.5 channel blocker was used to investigate nerve-evoked contractions responses to Kv7 channel blockade. We employed

the same experimental approach as described above for the Kv7 channel activators (**Fig. 5.2.11A-B)**. The data showed that in DSM isolated strips, XE991 (10 μM) significantly increased the amplitude of the EFS-induced contractions at stimulation frequencies ranging between 5.0 and 20 Hz (n=8, N=5, p<0.05; **Fig. 5.2.11C**). The maximum increase in the amplitude of the EFS-induced contraction was observed at 12.5 Hz frequency where XE991 (10 μM) increased the contraction amplitude by ~20% (n=8, N=5; p<0.05; **Fig. 5.2.11C**). These data suggest that inhibition of Kv7 channels enhances guinea pig DSM nerve-evoked contractions induced by EFS at a wide range of stimulation frequencies.

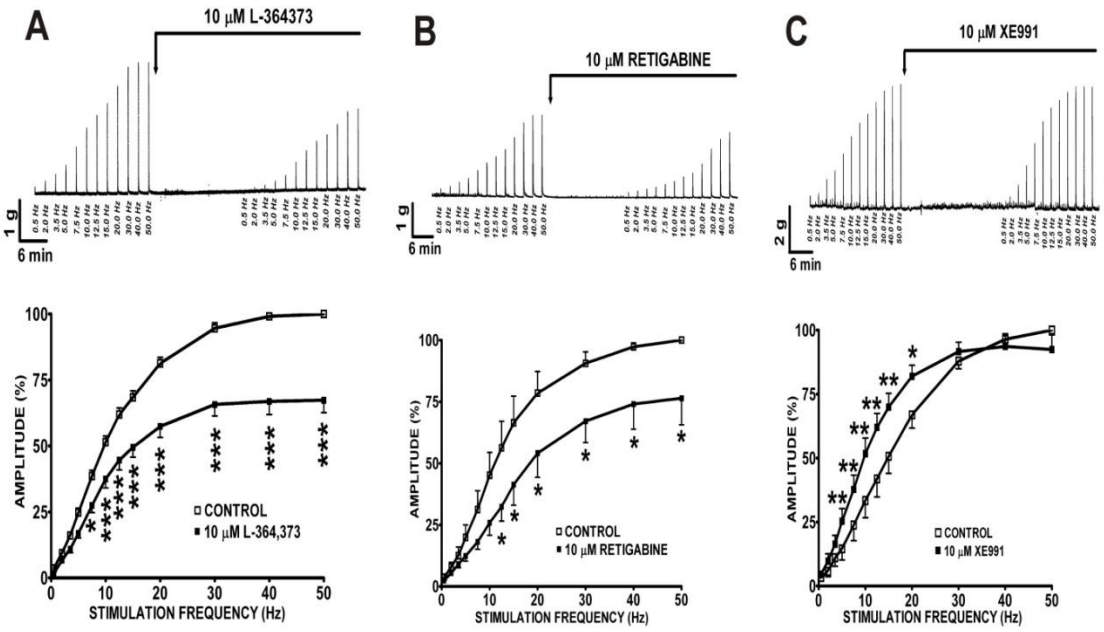

Figure 5.2.11. Effects of Kv7 channel modulators on the 0.5 – 50 Hz EFS-induced contractions in guinea pig DSM isolated strips. Original DSM tension recordings and frequency-response curves illustrate L-364373 (**A**; n=7, N=4) and retigabine (**B**; n=6, N=4) inhibitory effect on the 0.5 – 50 Hz EFS-induced contractions amplitude. **C**) Original DSM tension recordings and frequency-response curves reveal that XE991 enhances the 0.5 – 50 Hz EFS-induced contraction amplitude (n=8, N=5). The maximal EFS-induced contraction amplitude at a stimulation frequency of 50 Hz under control conditions was taken to be 100 % and the contractions were normalized (*p<0.05, **P<0.01, ***P<0.005).

DISCUSSION

The present study reveals that Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels are expressed at both mRNA and protein levels in guinea pig DSM cells. Additionally, we demonstrate that these channels are functional as evidenced by the decrease in DSM contractility upon channels activation with L-364373 and retigabine; and the increase in DSM contractility when Kv7 channels were blocked by XE991 or linopiridine. The lack of Kv7.4 channel expression in guinea pig DSM cells at both mRNA and protein level further suggest that the observed changes in DSM contractility subsequent to various Kv7 channels modulators application were mediated via the other Kv7 channel isoforms (Kv7.1, Kv7.2, Kv7.3, and Kv7.5) but not the Kv7.4 channel.

Our RT-PCR experiments performed on a poll of enzymatically-isolated guinea pig DSM cells demonstrate the mRNA expression of Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels but not the Kv7.4 channel. Further qPCR experiments validated the RT-PCR findings since the relative mRNA message expression of the Kv7.4 channel was extremely low compared to the others Kv7 channel subtypes. These two experimental series combined suggest that in the guinea pig DSM cells, only Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels are expressed at the mRNA level. The Kv7 channels mRNA expression pattern that we revealed in the present study is slightly different from previous studies in rat DSM where only Kv7.4, Kv7.5, and arguably Kv7.1 channels are suggested to be expressed at the mRNA level (121, 149). The protein expression of Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels in guinea pig DSM cells was further demonstrated via immunostaining. Using a specific antibody against each Kv7 channel isoform, α smooth muscle marker, and a confocal microscope, we demonstrated Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels protein

expression in guinea pig DSM cells. Consistently with the single-cell RT-PCR and qPCR data, we find no protein expression for the Kv7.4 channel in guinea pig DSM cell using immunostaining approach suggesting that only the Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels are expressed in guinea pig DSM cells at the protein level. In absolute contrast to the Kv7 channel protein expression pattern in guinea pig DSM cell, Svalo et al. have found that in rat DSM, only the Kv7.4 channel protein was expressed, outlining important species differences (149). Our findings represent the first molecular evidence for Kv7 channels expression in guinea pig DSM.

Next, we investigated the physiological roles of these Kv7 channels in the DSM of guinea pigs. In most mammals including guinea pigs, the micturition process comprises a filling and a voiding phase. During the filling phase, the DSM appears relaxed allowing the bladder to store large amount of urine while during the voiding phase, the DSM contracts forcefully to expel the stored urine (7, 15, 130). K^+ channels have been demonstrated to be involved in both phases of the micturition process. Studies suggest that during the filling phase, K^+ channels remain opened causing cell membrane hyperpolarization and therefore limiting Ca^{2+} influx via L-type Ca_v channels (7, 15, 130). The low intracellular Ca^{2+} level is responsible for keeping the DSM in a relaxed state. However, during the voiding phase, K^+ channels are closed causing cell membrane depolarization; L-type Ca_v channels become activated and opened to allow influx of Ca^{2+}. The increase of intracellular Ca^{2+} concentration will further lead to DSM contraction (7, 15, 130). Our study reports that pharmacological activation of Kv7 channels with L-364373 and retigabine relaxes guinea pig DSM spontaneous phasic and tonic contractions. We also report that Kv7 channels blockade with XE991 or linopiridine

increases DSM spontaneous phasic contractions. Therefore, our findings suggest that Kv7 channels are major regulators of guinea pig DSM contractility and that Kv7 channels are involved in both phases of the micturition process. Our findings are in line with previous reports which demonstrated that intravesical administration of retigabine increased micturition volume and voiding intervals in freely moving conscious rats (148). In conscious rats with capsaicin-induced irritated bladders, retigabine was shown to strongly reduce bladder rings contractility, reduce bladder pressure, and delay voiding, plus the retigabine effect on bladder rings was blocked by XE991 (149).

Kv7 channels regulatory roles on neurogenic contractions were also addressed in our study. It is well-established that DSM contractions also have neurogenic origins. In mouse, rat, guinea pig, and human, DSM contractions can be initiated by the combined action of acetylcholine and ATP, two major excitatory neurotransmitters released from the parasympathetic nerves (59, 64, 161). ATP activates P2X receptors while acetylcholine stimulates M2/M3 muscarinic receptors to induce DSM contractions (24, 59, 64, 105, 119, 161). We found that Kv7 activation with L-364373 and retigabine decreased the amplitude and muscle force of the guinea pig DSM EFS-induced contractions in a concentration-dependent manner. Neurogenic contractions were generated by EFS frequency of 10 Hz/min. We also found that pharmacological blockade of Kv7 channels with XE991 or linopiridine enhanced DSM 10 Hz EFS-induced contractions amplitude and muscle force in a concentration-dependent manner. Therefore our data suggest that Kv7 channels play an important regulatory role in guinea pig DSM neurogenic contractions. The retigabine effect on EFS-induced contractions reported in our study is somehow similar to previous studies in rats DSM (136). The aforementioned

study reported that retigabine (1 – 30 μM) inhibited EFS-induced contraction amplitude generated by 20 Hz in a concentration-dependent manner (136). The authors also reported that the retigabine relaxant effects on the EFS-induced contractions were antagonized by 10 μM XE991 suggesting that Kv7 channels, more precisely Kv7.4 and Kv7.5 channels, regulate rat DSM neurogenic contractions since only Kv7.4 and Kv7.5 but not Kv7.1, Kv7.2, and Kv7.3 channels were present at the mRNA level in rat bladder (121, 136).

We also found that Kv7 channel regulate both the cholinergic and the purinergic components of the guinea pig DSM EFS-induced contractions. Studies have demonstrated that at EFS frequencies ≤ 20 Hz, the purinergic (ATP) pathway plays a greater role whilst at EFS frequencies ≥ 20 Hz, the cholinergic (acetylcholine) component predominates (64, 161). Our data showed that L-364373 (10 μM) and retigabine (10 μM) inhibited the 0.5 – 50 Hz EFS-induced contraction amplitude while XE991 (10 μM) application enhanced the 0.5 – 50 Hz EFS-induced contraction amplitude. The ability of these Kv7 channels modulators to affect guinea pig DSM EFS-induced contractions (0.5 – 50 Hz) commands that Kv7 channels control the purinergic and the cholinergic components of the neurogenic contractions in guinea pig DSM.

CONCLUSION

In summary, our study reveals that Kv7 channels are major regulators of guinea pig DSM physiology as evidenced by their ability to control spontaneous phasic and EFS-induced contractions. The changes in guinea pig DSM contractility observed after application of various Kv7 channels modulators were mediated via Kv7.1, Kv7.2, Kv7.3, and Kv7.5

channels but not Kv7.4 channel since our molecular data showed mRNA and protein expression for Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels but not Kv7.4 channel in guinea pig DSM cells. Kv7 channels could therefore represent promising drug targets for the treatment of bladder dysfunctions.

5.3. MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF KV7 CHANNELS IN HUMAN DETRUSOR SMOOTH MUSCLE

OBJECTIVES

In the previous section (chapter 5.2), we examined the expression and functional roles of Kv7 channels in the detrusor smooth muscle (DSM) of guinea pigs using RT-PCR, qPCR, Western blot, immunohistochemistry and isometric DSM tension recordings approaches. We found that among Kv7 channels, Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels but not the Kv7.4 channel were expressed at both mRNA and protein level in guinea pig DSM cells. We also found that their pharmacological activation with L-364373 and retigabine or their inhibition with XE991 and linopiridine affected DSM spontaneous and nerve-evoked contractions suggesting that these channels play a key role in guinea pig DSM contractility. We therefore proposed that Kv7 channels could represent promising drug targets for the treatment of bladder dysfunctions. In the present section, we went a step further by testing our hypothesis in humans. Given that human is the ultimate subject of study and that animal findings cannot always been translated into humans because of species differences, here, we investigated the expression and the functional roles of all Kv7 channels in human DSM tissue. In the following paragraphs, preliminary data on the expression and roles of Kv7 channels will be succinctly presented.

MATERIALS AND METHODS

Human DSM tissue collection

DSM tissue collection is described in chapter 2.2. In total, we used DSM tissue samples from 11 (8 males and 3 females) patients (55-79 years old; average age 65.8±2.2) without a history of OAB.

Detection and quantification of Kv7 channels mRNA message by RT-PCR and qPCR

RT-PCR and qPCR experiments were conducted as previously described in chapter 2.2. The primers sequences and the size of the amplicon were as follows: Kv7.1- F: 5′-CTTGATGCGCACCATGAGGTTG-3′ and R: 5′- ACACCCATCACCCACATCTCAC-3′ (180 bp); Kv7.2- F: 5′- CGAAGTACTCCACGCCAAACAC-3′ and R: 5′-TTCCTCTACAACGTGCTGGAGC-3′ (190 bp); Kv7.3- F: 5'-GAGACGTAGCATCACGCGAGAC-3' and R: 5'- TCCGTGTCTGTGTCCGTCTCAC-3' (195 bp); Kv7.4- F: 5'- TGCTGGCTTCGCCTTACTGG-3' and R: 5'-TACCAGGTGGCTGTCAGGTAGG-3' (201 bp); Kv7.5- F: 5'-ACGCGCTTAGCCCTACTATGC-3' and R: 5'- CAGATGCTTGATGGCTGGGAG-3' (173 bp); GAPDH- F: 5′- GGATTTGGTCGTATTGGG-3′ and R: 5′-GGAAGATGGTGATGGGATT-3′ (205 bp) . The parameters of the qPCR experiments were as follows: Cycle 1, 95 ^{o}C for 3 min; cycle 2, 95 ^{o}C for 10 s then 58 ^{o}C for 30 s (repeated 40 times); cycle 3, 95 ^{o}C for 1 min; cycle 4, 58 ^{o}C for 1 min; cycle 5, 58 ^{o}C for 10 s with 0.5 ^{o}C increment (cycle 5 was repeated 81 times to generate a melting curve).

Isometric DSM tension recordings

These series of experiments were performed as previously described in chapter 2.2.

Solutions and drugs

The Ca^{2+}-free dissection solution and the Ca^{2+}-containing PSS had the same compositions as previously described in chapter 2.2. Retigabine and XE991 were purchased from Tocris Bioscience (Bristol, UK) and stock solutions were prepared daily in dimethyl sulfoxide (DMSO). The final DMSO concentration in the bath was not greater than 0.1%.

Data analysis and statistics

Data analysis and statistical analysis were performed as previously described in chapter 2.2.

RESULTS

Kv7.4 and Kv7.5 channels but not Kv7.1, Kv7.2, and Kv7.3 channels are expressed in human DSM cells at the mRNA level

The RT-PCR experiments revealed that amongst Kv7 channels, only Kv7.1, Kv7.4, and Kv7.5 channels was found in human whole DSM tissue while at the DSM single cells level, Kv7.4 and Kv7.5 channels but not Kv7.1 channel was identified at the mRNA level (N=4; **Fig. 5.3.1A**). The unique advantage of conducting RT-PCR experiments on freshly isolated single cells is that this approach eliminates possible contamination from other non-DSM cells such as neurons, fibroblasts, vascular, and endothelial cells present within the DSM layers (2, 74, 75, 127, 143, 165). RT-PCR experiments were conducted on DSM cDNAs equivalent to at least 50 ng of starting mRNA. Negative control experiments performed in the absence of the reverse transcriptase (-RT) demonstrated an absence of genomic DNA contamination. In whole DSM tissue, our qPCR data showed that Kv7 channels relative mRNA level was as follows: Kv7.1 > Kv7.5 ≈ Kv7.4 (N=4; **Fig. 5.3.1B**) while at the single cell level, Kv7.5 > Kv7.4 >> Kv7.1 (N=4; **Fig. 5.3.1B**). Taken together these RT-PCR and qPCR data suggest that in human DSM cells, only Kv7.4 and Kv7.5 are expressed at the mRNA level, and that the Kv7.5 channel could be the most highly expressed.

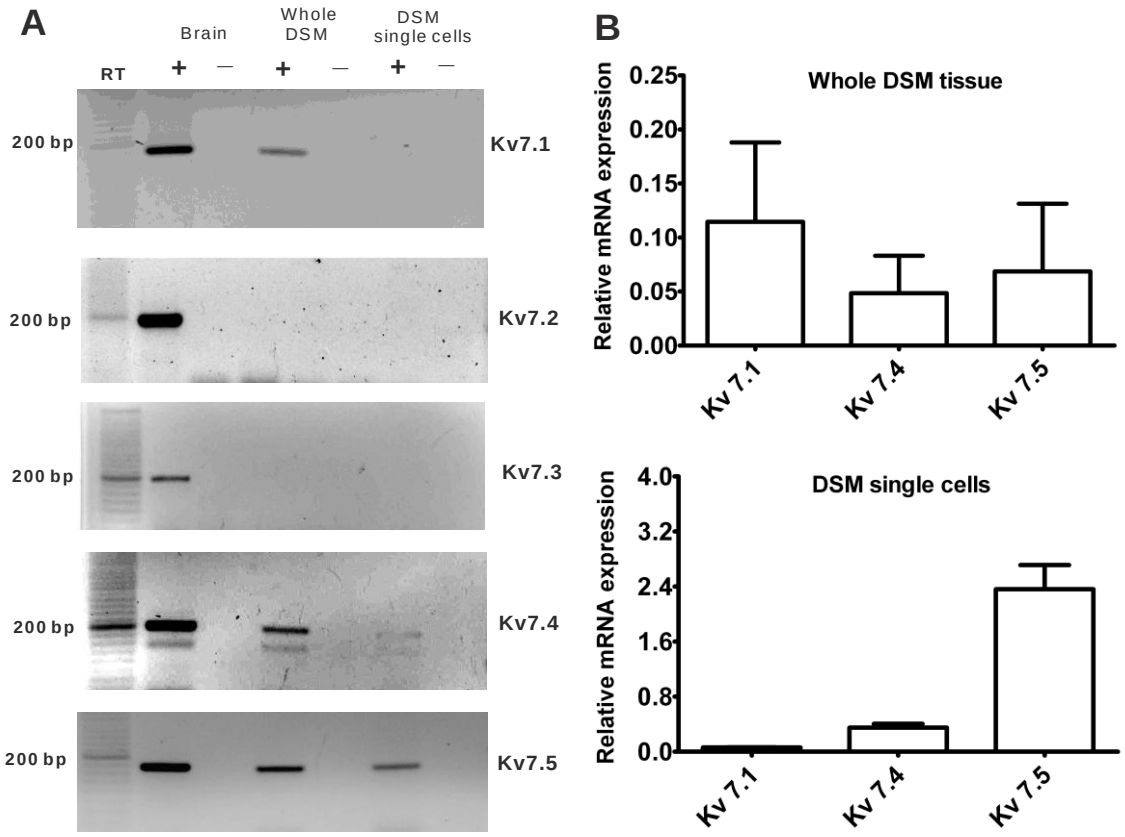

Figure 5.3.1. **Kv7 channels mRNA expression in human DSM.** A) Gel electrophoresis images illustrate RT-PCR detection of Kv7.1, Kv7.4, and Kv7.5 mRNA messages in whole DSM tissue (N=4). At the DSM single cell level, only Kv7.4 and Kv7.5 channels mRNA messages were detected. Human brain was used as a positive control. No product was observed in the negative controls (-RT) in which reverse transcriptase was not added to the reaction. B) qPCR analysis show relative expression of Kv7.1, Kv7.4, and Kv7.5 channel mRNA expression in both DSM whole tissue (N=4) and DSM single cells (N=4). Data were normalized to GAPDH using the ΔCt method. Ct values are expressed as mean ± SE.

Role of Kv7 channels in spontaneous phasic and tonic DSM contractions in humans

We used retigabine, a Kv7.2-7.5 channel activator and XE991, a Kv7.1-7.5 channel activator to investigate the roles of Kv7 channels in human DSM spontaneous contractions. Previous studies in guinea pig DSM demonstrated that retigabine was very effective at reducing the spontaneous phasic contraction at lower micromolar concentrations. In the present study, we found that retigabine (10 μM) significantly decreased the human DSM spontaneous phasic and tonic contractions (**Fig. 5.3.2A**). Retigabine (10 μM) effects on the spontaneous phasic contraction amplitude, muscle force, frequency, duration, and muscle tone compared to control were 19.6±9.9%, 17.5±8.7%, 37.8±22.4%, 48.1±24.7%, and 43.1±21.3%, respectively (n=6, N=4, $p<0.05$; **Fig. 5.3.2A**).

On the other hand, pharmacological blockade of Kv7 channels with XE991 enhanced DSM spontaneous contractions amplitude, muscle force, and muscle tone without any significant effects on contraction duration and frequency (**Fig. 5.3.2B**). XE991 (10 μM) effects DSM on spontaneous phasic contraction amplitude, force, and tone compared to control were 197.6±44.9%, 237.0±72.8%, and 159.3±21.0%, respectively (n=5, N=3, $p<0.05$; **Fig. 5.3.2B**). Collectively, these results suggest that Kv7 channels regulate human DSM myogenic activity.

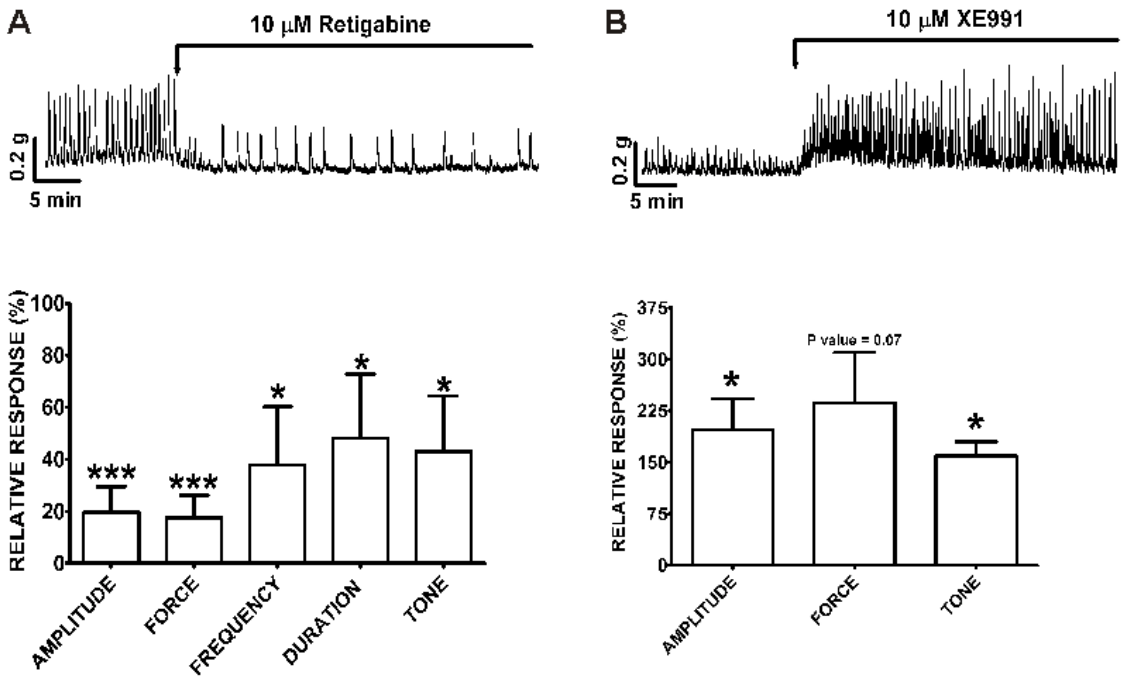

Figure 5.3.2. **Pharmacological modulation of Kv7 channels with retigabine (10 μM) or XE991 (10 μM) affects human DSM spontaneous phasic and tonic contractions**. A) Original DSM tension recordings and summary data showing retigabine (10 μM) relaxant effects on human DSM isolated strips. Retigabine significantly decreased all 5 parameters of the DSM spontaneous phasic and tonic contractions including amplitude, muscle force, frequency, duration, and muscle tone (n=6, N=4). B) Original DSM tension recordings and summary data showing that XE991 (10 μM) enhances human DSM spontaneous phasic and tonic contractions. XE991 effects were more pronounced on DSM contraction amplitude and muscle tone and at a lesser extent on muscle force (n=5, N=3). The 10-min control period prior to apamin addition was taken to be 100 % and data were normalized. TTX (1 μM) was present throughout the experiments. *p<0.05, ***p<0.005

Role of Kv channels in nerve-evoked contractions in humans

We further investigated the effects of retigabine (10 µM) and XE991 (10 µM) on nerve-evoked contractions of human DSM isolated strips (**Fig. 5.3.3-4**). We first applied a wide range of EFS frequencies (0.5 – 50 Hz) as a control followed by retigabine (10 µM) or XE991 (10 µM) application for approximatively 30 min. Next, the same EFS protocol (0.5 – 50 Hz) was applied to evaluate how these Kv channel modulators affect EFS-induced contractions. The frequency-response curves showed that retigabine (10 µM) significantly attenuated the amplitude of the EFS-induced contractions at stimulation frequencies ranging from 10 to 50 Hz (n=7, N=4, $p<0.05$; **Fig. 5.3.3**). At the maximal stimulation frequency of 50 Hz, retigabine effect on DSM EFS-induced contraction amplitude compared to control was 57.4±7.6% (n=7, N=4, $p<0.05$; **Fig. 5.3.3B**). Pharmacological inhibition of Kv7 channels with XE991 (10 µM) increased the amplitude of the EFS-induced contractions at stimulation frequencies ranging from (3.5 – 15 Hz) (n=6, N=5, $p<0.05$; **Fig. 5.3.4**). XE991 effect on DSM EFS-induced contraction amplitude compared to control was greater at stimulation frequency of 12.5 Hz where XE991 (10 µM, induced ~20% increase (n=6, N=5, $p<0.05$; **Fig. 5.3.4B**). These latter EFS data suggest that Kv7 channels play a role in the nerve-evoked contractions.

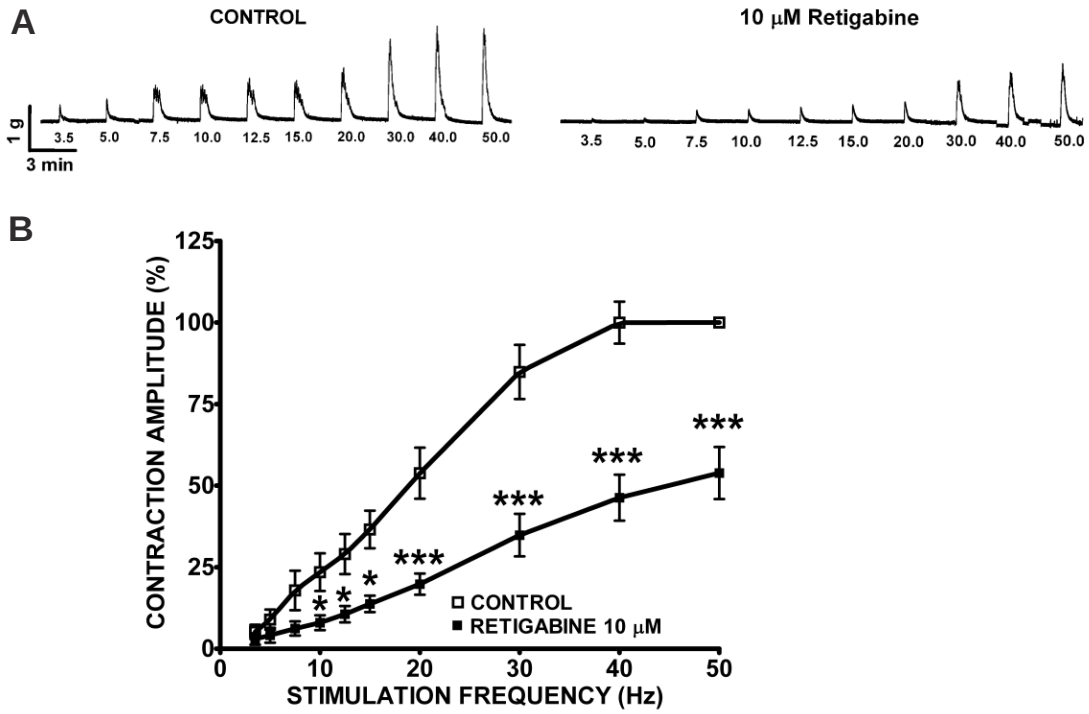

Figure 5.3.3. Retigabine decreases the amplitude of the EFS-induced contractions in human DSM isolated strips in a wide range of stimulation frequencies. A) An original DSM tension recording illustrating the inhibitory effects of 10 μM retigabine on 3.5–50 Hz EFS-induced contractions of human DSM. B) Frequency-response curves showing that retigabine (10 μM) significantly decreased EFS-induced contractions at frequencies 10–50 Hz (n=7, N=4; *P<0.05, ***P<0.005).

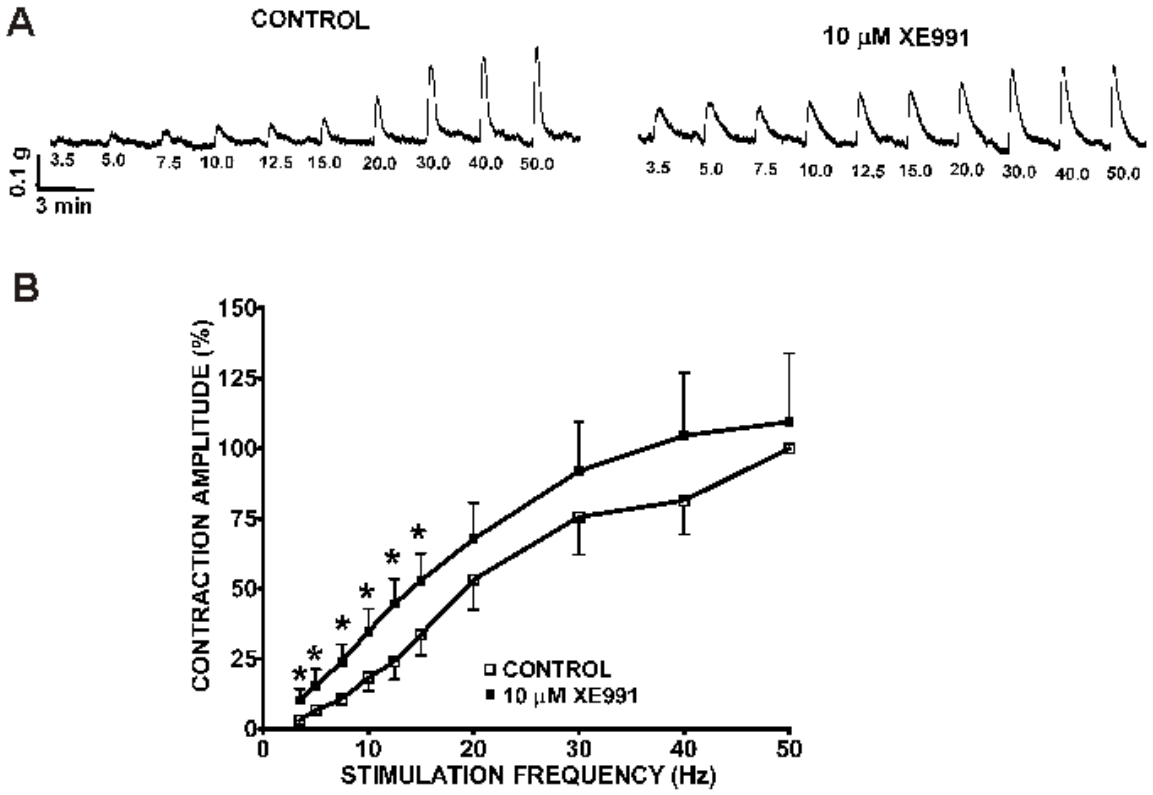

Figure 5.3.4. XE991 enhances the amplitude of the EFS-induced contractions in human DSM isolated strips in a wide range of stimulation frequencies. A) An original DSM tension recording showing that XE991 (10 μM) effects on 3.5–50 Hz EFS-induced contractions of human DSM. B) Frequency-response curves showing XE991 significantly increases EFS-induced contractions at stimulation frequencies ranging from 3.5–15 Hz (n=6, N=5; *P<0.05).

REMARKS

The above preliminary data are encouraging and suggest that several Kv7 channels namely Kv7.4 and Kv7.5 channels are expressed in human DSM and play important physiological roles in DSM spontaneous and nerve-evoked contractions. However, additional evidence for these Kv7 channel proteins expression in human DSM should be provided to clearly establish their functionality. The implications of these findings with respect to the treatment of functional disorders of lower urinary tract such as overactive bladder are that the Kv7 channels could be promising pharmacological targets to control urinary bladder function.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The pathophysiology of OAB is a serious condition characterized by urgency, frequent urination, and abnormal increase in DSM contractility. Despite, numerous attempts to treat the disorder with antimuscarinics and now with beta3-AR agonists, OAB still remains a serious public health issue because of the tremendous impact that it has on patients' quality of life. In an effort to alleviate the discomfort of millions of patients, we initiated these studies which aimed to propose an alternative therapeutic approach for the treatment of OAB. K^+ channels have been shown to be key regulators of smooth muscle excitability and contractility in various species, however, information about their expression and functional role in the DSM was limited. Plus, there was a tremendous gap in knowledge about the role of these ion channels in the DSM of humans. Our hypothesis suggests that K^+ channels and beta3-AR are major regulators of DSM contractility and excitability and could represent promising pharmacological targets for the treatment of OAB. Throughout my PhD training, I collected data to test our hypothesis and prove our concept. Four major conclusions can be drawn from our studies. **1**) Several Ca^{2+}-activated K^+ channels including BK and SK3 channels are key regulators of human DSM spontaneous and nerve-evoked contractions. **2**) We found a strong correlation between the decrease in BKα subunit expression and the lack of BK channel activity in the pathophysiology of NDO. **3**) We demonstrated that there is a functional link between

beta3-AR and BK channels during nerve-evoked contractions in rat and human DSM and that beta3-AR play a minor role in guinea pig DSM. **4**) Finally, several Kv channels including Kv2 and Kv7 channels regulate DSM excitability and contractility. These data suggest that pharmacological modulation of K^+ channels (BK, SK3, Kv2, and Kv7 channels) or beta3-AR affect DSM contractility and excitability, therefore making K^+ channels and beta3-AR likely drug targets for the treatment of OAB.

Ca^{2+}-activated K^+ channels expression and regulation of human DSM spontaneous phasic and nerve-evoked contractions.

In order to assess whether Ca^{2+}-activated K^+ channels play a role in human DSM spontaneous and nerves evoked contractions, we first had to demonstrate their molecular expression in the DSM. For this purpose, we employed, RT-PCR, qPCR, western blot and immunostaining techniques to show mRNA and protein expression of these ion channels. A previous work published by our laboratory has already demonstrated the mRNA and protein expression of the pore-forming BKα-subunit as well as the regulatory β1 and β4 subunits in human DSM (75). However, evidence for SK and IK channels in human DSM was still lacking. Here, we successfully identified SK and IK channels molecular expression in human DSM. More specifically, we demonstrated via qPCR analysis that among SK and IK channels, the SK3 channel was the most highly expressed at the mRNA level in human DSM cells (**Fig. 3.2.1)**. The qPCR data were further confirmed by protein expression of SK3 channel and lack of IK channel protein expression in human DSM (**Fig. 3.2.3-4**). The protein expression of these K^+ ion channels in the DSM of humans suggests at first glance that the channels might be

physiologically active. We then used several modulators to test these Ca^{2+}-activated K^+ channels individually. In human DSM, isolated strips, we found that pharmacological inhibition of BK channels with iberiotoxin, a BK channel blocker, increased DSM spontaneous phasic contraction amplitude, muscle force, and tone (**Fig. 3.1.2**). Similarly, pharmacological blockade of SK channels with apamin, a SK channel blocker increased human DSM spontaneous phasic contraction amplitude, muscle force, duration, and muscle tone (**Fig. 3.2.5**). We also found that TRAM-34, a selective IK channel inhibitor had no effect on human DSM contractility (**Fig. 3.2.6**). The key role of BK channels in human DSM has further been demonstrated by a recent study which suggests that pharmacological activation of BK channels with NS1619, a BK channel activator decreases human DSM excitability and contractility (80). Similar findings on SK channels also revealed that pharmacological activation of SK channels with SKA-31, a SK channel activator hyperpolarizes the cell membrane and decreases DSM contractility in humans (144). Taken together, the aforementioned data suggest that Ca^{2+}-activated K^+ channels including BK and SK3 channels are major regulators of human DSM contractility and excitability. The ability of BK and SK3 channels to increase human DSM contractility upon inhibition and decrease DSM contractility upon activation represents a unique opportunity to consider these ion channels in the treatment of bladder dysfunctions including overactive and underactive bladder.

After determining the role of both BK and SK3 channels in human DSM under normal physiological conditions, we expanded our studies by investigating BK channels roles under pathophysiological conditions of NDO. The molecular analysis of BK channel mRNA expressions by qPCR revealed a discrepancy in BKα subunit mRNA expression

between NDO and control patients. We found that in NDO patients the BKα mRNA relative expression level was significantly lower (~15.8 – fold) compared to control patients (**Fig. 3.1.1**). Given the fact that the pore-forming α subunits ensure the movement of K^+ ions across the cell membrane and therefore DSM excitability, it is expected that any changes in BKα channel subunits expression could significantly hamper the channel's ability to regulate DSM excitability and contractility (22, 109, 130, 131, 146). Consistent with this assumption, we found that NDO DSM had abnormally elevated contractility in comparison to controls (**Fig. 3.1.2**). Additionally, unlike in control DSM, inhibition of the BK channel with iberiotoxin failed to affect DSM contractility in NDO DSM suggesting that in NDO patients DSM, the BK channel function might be altered (**Fig. 3.1.2**). Therefore, we suggest that the abnormal increase in DSM contractility observed in NDO patients is due to the significant reduction in BKα subunit expression. As a consequence, we propose that targeting BK channels by either pharmacological or genetic manipulation could be a promising therapeutic strategy to treat OAB, and especially NDO.

The beta3-AR induced relaxation of the DSM nerve-evoked contraction is mediated via activation of BK channels.

Beta3-ARs are known to be important facilitators of bladder relaxation because of their ability to control DSM spontaneous phasic and nerve-evoked contractions as well as TBKC that contribute to DSM excitability (22, 79, 134). Recently, the FDA has approved the use of mirabegron, a selective beta3-AR agonist for the treatment of OAB (27, 90, 157). The mechanism by which beta3-AR induces relaxation of the DSM myogenic contraction is suggested to be mediated via activation of BK channels (79, 130).

However, the functional link between beta3-ARs and BK channels during nerve-evoked contractions has never been investigated. In our study, we address this mechanism by first testing our approach in rodents followed by humans.

The first animal model that we chose was the guinea pig. Guinea pigs are commonly used laboratory animals which based on our own experience have DSM tissue that usually generate stable spontaneous phasic contractions. Additionally, guinea pig DSM cells are relatively easy to isolate for single-cell RT-PCR/qPCR or patch-clamp electrophysiology. The molecular expression of beta3-ARs in guinea pig DSM cell has never gain consensus. To clarify whether beta3-ARs are expressed in guinea DSM cells, we first developed a technique called "single-cell" RT-PCR. This approach allow us to conduct RT-PCR experiments on a poll of isolated DSM cells only while eliminating contamination from other cell types such as neurons, fibroblasts, interstitial cells, which are present within the DSM tissue (1, 2, 30, 74, 75, 77). Our results showed that beta3-ARs are expressed in guinea pig DSM cells at the mRNA level (**Fig. 4.1.1**); however, we could not confirm their protein expression because no specific beta3-AR antibody was commercially available. The pharmacological activation of beta3-ARs with BRL37344, a beta3-AR agonist had limited effects on guinea DSM spontaneous contractions (**Fig. 4.1.3**). The BRL37344 effect on spontaneous contractions was partially suppressed in the presence of atropine, a muscarinic receptor antagonist, suggesting that the BRL37344 effect could have been mediated via inhibition of muscarinic receptors directly. Such effects have previously been reported by other groups (22, 93, 111). Furthermore, it has been suggested that at high concentrations BRL37344 may also non-selectively activate beta1-ARs and/or beta2-ARs (150). Additional electrophysiological experiments

performed by Dr. Hristov supported the idea that beta3-ARs, although expressed in guinea pig DSM, do not regulate the DSM excitability. Perforated patch-clamp experiments revealed no effect of BRL37344 on whole-cell steady-state K^+ current, TBKCs amplitude and frequency, and the resting membrane potential (**Table 4.1.1**). We further assessed BRL37344 effects on guinea pig DSM nerve-evoked contractions. Although, we observed a significant decrease in contraction amplitude by BRL37344 (**Fig. 4.1.7**), this BRL37344 effect was once again suppressed by atropine, suggesting that BRL37344 acted directly on muscarinic receptors rather than beta3-ARs during nerve stimulation (**Fig. 4.1.9**). Moreover, we employed another beta3-AR agonist, L-755,507, and found that this latter compound had no effect on the nerve-evoked contractions in the absence or presence of atropine suggesting that beta3-ARs do not play any role in the nerve-evoked contraction of guinea pig DSM (**Fig. 4.1.10**). Based on the aforementioned observations, we concluded that although expressed in guinea pig DSM cells at the mRNA level, beta3-ARs play a minor to no functional role or that in guinea pig DSM beta3-ARs have distinct pharmacological profiles compared to beta3-ARs in other species. As a consequence, we concluded that the potential functional link between beta3-AR and BK channels could not be investigated in guinea pig DSM.

Next, we addressed the functional link between beta3-AR and BK channels in rat DSM. Rat was chosen because previous studies have shown that beta3-ARs are expressed in rat DSM tissue (46). Additionally, it has been proven that in rat DSM, activation of beta3-AR with BRL37344 increased TBKCs frequency, and hyperpolarized the cell membrane (79). Our data showed that unlike in guinea pig DSM, stimulation of beta3-AR receptors with BRL37344 caused a significant decrease of the nerve-evoked contraction in rat

DSM isolated strips in the presence or absence of atropine (**Figs. 4.2.1-3**). We also found that the BRL37344 effect was mediated via inhibition of both the cholinergic and purinergic components of the nerve-evoked contraction. In the presence of atropine, which was used to block the cholinergic component of the nerve-evoked contractions, we found that BRL37344 decreased the amplitude of the nerve-evoked contractions at all stimulation frequencies tested suggesting that BRL37344 inhibits the purinergic component of the nerve-evoked contractions (**Fig. 4.2.3**). In the presence of α,β-methylene-ATP and suramin which were used to block the purinergic component of the nerve-evoked contractions, BRL37344 decreased the amplitude of the EFS-induced contractions at all stimulation frequencies tested suggesting that BRL37344 inhibits the cholinergic component of the nerve-evoked contractions (**Fig. 4.2.4**). After confirming that beta3-ARs regulate rat DSM nerve-evoked contractions, we next addressed the functional link between beta3-AR and BK channels during nerve-evoked contractions. During myogenic contractions, it was previously shown that pharmacological blockage of BK channels with iberiotoxin opposes beta3-AR-mediated relaxation in rats DSM (79). We found that similar to what was observed during myogenic contractions, the BRL37344 inhibitory effects on rat DSM nerve-evoked contractions was significantly decreased when BK channel was antagonized by iberiotoxin (**Fig. 4.2.5**). The iberiotoxin effects reported here originates from direct inhibition of BK channels in DSM since immunohistochemical analysis have previously shown that BK channels are expressed in DSM but not in the bladder nerves that innervate the DSM (161). Collectively, our data suggest that BK channel activity is critical for the beta3 adrenergic receptor-induced attenuation of the nerve-evoked contractions in rat DSM.

After developing our strategy in laboratory animals, we could then confidently proceed to the investigation of the functional link between beta3-AR and BK channels in human DSM. Previous studies have demonstrated that beta3-AR are expressed in human DSM (46) and that beta3-ARs could represent as much as 97% of all beta-AR expressed in human DSM (168). We found that low concentrations of BRL37344 (10 nM–1 μM) decreased the amplitude of nerve-evoked contractions in human DSM suggesting that beta3-ARs play an important role in opposing human DSM nerve-evoked contractions. Our data are also consistent with previous findings showing that KUC-7322 and GW427353, two other selective beta3-AR agonists, also decreased carbachol-induced and nerve-evoked contractions in human DSM (14, 81, 156). Like in rat DSM, we found that in the presence of atropine, BRL37344 decreased the amplitude of the nerve-evoked contractions in human DSM in a wide range of stimulation frequencies (**Fig. 4.3.3**). Also, in the presence of suramin and α,β methylene-ATP, BRL37344 also significantly decreased the amplitude of the nerve-evoked contractions in human DSM (**Fig. 4.3.4**). These latter findings suggest that BRL37344 inhibits both cholinergic and purinergic contractions of human DSM. Similarly to our findings in rat DSM nerve-evoked contractions, iberiotoxin shifted the concentration-response curves for the BRL37344 inhibitory effects on human DSM nerve-evoked contractions suggesting that functional BK channels play an important role in the beta3-AR mediated relaxation of human DSM nerve-evoked contractions (**Fig. 4.3.1**).

Taken together, we were finally able to demonstrate that the beta3-AR-agonist-mediated relaxation of DSM nerve-evoked contractions is BK channel-dependent, emphasizing the critical role of BK channels in DSM physiology. While the functional link between

beta3-AR and BK channels is prominent in rats and humans, this was not the case in guinea pig DSM since the role of beta3-AR during nerve-evoked contractions could not be demonstrated. These findings suggest critical species difference between rats, humans, and guinea pigs.

Kv2 and Kv7 channels expression and regulation of DSM contractility.

Kv channels represent the largest and the most diverse group of K^+ channels identified in the human genome with more than 40 members. Kv channels have the potential to become promising drug targets for the treatment of various pathologies because of their ability to regulate excitability and contractility in smooth muscles as well as in neurons. Unfortunately, information about their physiological role in numerous organs more specifically in the DSM is scarce. What makes the investigation of individual Kv channels difficult in any organ is the lack of selective pharmacological tools that will allow investigators to study each channel separately. During my PhD training, I investigated the expression and physiological roles of several Kv channels including Kv2 and Kv7 channels in the DSM. These studies were made possible because selective Kv2 and Kv7 channels have been recently discovered and made commercially available.

Before addressing the functional roles of both Kv2 and Kv7 channels in the DSM, molecular expression of these channels had to be demonstrated. The investigation of Kv2 channels molecular expression in human DSM cells was performed by Dr. Chen. She revealed the expression of Kv2.1 and Kv2.2 channels in human DSM cells at both mRNA and protein levels (**Fig. 5.1.1-3**). In human DSM cells, I provided the first molecular evidence for Kv7.4 and Kv7.5 channels at the mRNA level (**Fig. 5.3.1**). The expression

pattern of Kv2 channels in human were similar to that of rat where Kv2.1 and Kv2.2 were also found in DSM cells (29). Comparable observation was made about Kv7 channels expression between human and rats. Like our findings in human DSM, studies by others revealed Kv7.4, Kv7.5, and arguably Kv7.1 channels mRNA expression in rat DSM (121, 149). Unlike rats and humans, guinea pig DSM cells expressed Kv7.1, Kv7.2, Kv7.3, and Kv7.5 channels at both mRNA and protein levels (**Fig. 5.2.1-3**). These results indicate that the expression pattern of Kv2 and Kv7 channels in the DSM may vary depending on the species.

To assess the Kv2 channels functional roles in human DSM, we used stromatoxin-1 (ScTx1), a selective Kv2.1 and Kv2.2 channel blocker. Pharmacological blockade of Kv2 channels in human DSM isolated strips significantly increased the spontaneous phasic contraction amplitude and force in human DSM isolated strips (**Fig. 5.1.6**), consistent with our previous findings in rat and guinea pig DSM (29, 77). Our results also show that ScTx1 significantly increases the amplitude of nerve-evoked contractions in human DSM isolated strips **(Fig. 5.1.7)** indicating the functional role of these channels in human DSM nerve-evoked contractions. These data are consistent with our earlier animal studies (29, 77), and revealed that K_V2-containing channels also work to oppose human DSM contractility under physiological conditions.

In human DSM isolated strips, we also found that pharmacological activation of Kv7 channels with retigabine, a Kv7.2-7.5 channels activator, induced relaxation of the spontaneous phasic and nerve-evoked contractions (**Fig. 5.3.2-4)**. Similar retigabine effects were also observed in guinea pig DSM spontaneous phasic and nerve-evoked contractions (**Fig. 5.2.6 and 5.2.11)**. Additionally, L-364373, a selective Kv7.1 channel

activator was shown to attenuate the guinea pig DSM isolated strips spontaneous phasic and nerve-evoked contractions (**Fig. 5.1.7 and 5.2.11)**. On the other hand, pharmacological blockade of Kv7 channels increases DSM contractility. In human DSM isolated strips, XE991, a non-selective Kv7 channel blocker was shown to enhance spontaneous phasic and nerve-evoked contractions. And in guinea pig DSM isolated strips, application of XE991 or linopiridine, another Kv7 channel blocker was reported to increase spontaneous phasic and nerve-evoked contractions. The retigabine effects reported in our studies are in line with previous reports which demonstrated that intravesical administration of retigabine increased micturition volume and voiding intervals in freely moving conscious rats (148). Also in conscious rats with capsaicin-induced irritated bladders, retigabine was shown to strongly reduce bladder rings contractility, reduce bladder pressure, and delay voiding, plus the retigabine effect on bladder rings was blocked by XE991 (149). The retigabine effect on nerve-evoked contractions reported in our study is somehow similar to previous studies in rats DSM (136). The aforementioned study reported that retigabine (1 – 30 μM) inhibited EFS-induced contraction amplitude generated by 20 Hz in a concentration-dependent manner (136). The authors also reported that the retigabine relaxant effects on the EFS-induced contractions were antagonized by 10 μM XE991 suggesting that Kv7 channels, more precisely Kv7.4 and Kv7.5 channels, regulate rat DSM neurogenic contractions since only Kv7.4 and Kv7.5 but not Kv7.1, Kv7.2, and Kv7.3 channels were present at the mRNA level in rat bladder (121, 136).

IMPLICATIONS AND FUTURE DIRECTIONS

The pathophysiology of OAB is generally poorly understood and the current pharmacological treatments which include antimuscarinics and beta3-AR agonists have yet to be proven infallible. Throughout my PhD training, I explored several K^+ channels and have found that BK, SK3, Kv2, and Kv7 channels are major regulators of human DSM contractility. I also found that pharmacological activation of beta3-AR induced relaxation of DSM nerve-evoked contractions in humans. These data suggest that the aforementioned K^+ channels and beta3-AR could represent pharmacological targets for some types of bladder dysfunctions.

There could be two direct clinical benefits for modulating the aforementioned K^+ channels in the DSM. Targeting BK, SK3, Kv2, or Kv7 channels with channel opening agents will decrease DSM contractility and contribute to alleviate detrusor overactivity observed in OAB patients. Alternatively, targeting BK, SK3, Kv2, and Kv7 channels with selective inhibitors will increase DSM contractility, and thus have clinical application for the treatment of detrusor underactivity. The major contribution of our studies was that we provided for the first time, evidence for a direct correlation between NDO and BK channel expression and function. Therefore, it is possible that by increasing BK channels expression in the DSM through genetic manipulation, detrusor overactivity can be reduced in NDO patients.

It is critical that future studies be initiated to develop agents which could selectively target one or more of these K^+ channels in the lower urinary tract exclusively in order to reduce potential collateral effects as BK, SK3, Kv2, and Kv7 channels expression has been reported in other human tissues (19, 20, 31, 42, 84, 103, 135). Further studies should

investigate molecular differences between the aforementioned K^+ channels in the DSM and other organs in order to design organ specific compounds with limited side effects, therefore providing a unique advantage over antimuscarinic drugs. Compound such as retigabine which is currently used for epilepsy treatment could find use in the treatment of OAB if its molecular structure is modified to make it impermeable to the blood-brain barrier and therefore reduce potential side effects in the brains. Clinical trials for selective BK, SK3, Kv2, or Kv7 channels agents would need to be performed to validate such assumption and assess the efficacy and therapeutic safety window.

REFERENCES

1. **Afeli SA, Hristov KL, and Petkov GV**. Do beta3-adrenergic receptors play a role in guinea pig detrusor smooth muscle excitability and contractility? *Am J Physiol Renal Physiol* 302: F251-263, 2012.

2. **Afeli SA, Rovner ES, and Petkov GV**. SK but not IK channels regulate human detrusor smooth muscle spontaneous and nerve-evoked contractions. *Am J Physiol Renal Physiol* 303: F559-568, 2012.

3. **Amberg GC, and Santana LF**. Kv2 channels oppose myogenic constriction of rat cerebral arteries. *Am J Physiol Cell Physiol* 291: C348-356, 2006.

4. **Anderson UA, Carson C, and McCloskey KD**. KCNQ currents and their contribution to resting membrane potential and the excitability of interstitial cells of Cajal from the guinea pig bladder. *J Urol* 182: 330-336, 2009.

5. **Andersson KE**. Pharmacotherapy of the overactive bladder. *Discov Med* 8: 118-124, 2009.

6. **Andersson KE**. Storage and voiding symptoms: pathophysiologic aspects. *Urology* 62: 3-10, 2003.

7. **Andersson KE, and Arner A**. Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol Rev* 84: 935-986, 2004.

8. **Andersson KE, and Wein AJ**. Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence. *Pharmacol Rev* 56: 581-631, 2004.

9. **Aydin M, Wang HZ, Zhang X, Chua R, Downing K, Melman A, and DiSanto ME**. Large-conductance calcium-activated potassium channel activity, as determined by whole-cell patch clamp recording, is decreased in urinary bladder smooth muscle cells from male rats with partial urethral obstruction. *BJU Int* 110: E402-408, 2012.

10. **Baker JG**. The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. *Br J Pharmacol* 160: 1048-1061, 2010.

11. **Bayliss M, Wu C, Newgreen D, Mundy AR, and Fry CH**. A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol* 162: 1833-1839, 1999.

12. **Beckett EA, Han I, Baker SA, Han J, Britton FC, and Koh SD**. Functional and molecular identification of pH-sensitive K+ channels in murine urinary bladder smooth muscle. *BJU Int* 102: 113-124, 2008.

13. **Bhide AA, Digesu GA, Fernando R, and Khullar V**. Use of mirabegron in treating overactive bladder. *Int Urogynecol J* 23: 1345-1348, 2012.

14. **Biers SM, Reynard JM, and Brading AF**. The effects of a new selective beta3-adrenoceptor agonist (GW427353) on spontaneous activity and detrusor relaxation in human bladder. *BJU Int* 98: 1310-1314, 2006.

15. **Biers SM, Reynard JM, Doore T, and Brading AF**. The functional effects of a c-kit tyrosine inhibitor on guinea-pig and human detrusor. *BJU Int* 97: 612-616, 2006.

16. **Brading AF**. A myogenic basis for the overactive bladder. *Urology* 50: 57-67; discussion 68-73, 1997.

17. **Brading AF**. Spontaneous activity of lower urinary tract smooth muscles: correlation between ion channels and tissue function. *J Physiol* 570: 13-22, 2006.

18. **Brading AF, and Williams JH**. Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha,beta-methylene ATP. *Br J Pharmacol* 99: 493-498, 1990.

19. **Braun M, Ramracheya R, Amisten S, Bengtsson M, Moritoh Y, Zhang Q, Johnson PR, and Rorsman P**. Somatostatin release, electrical activity, membrane currents and exocytosis in human pancreatic delta cells. *Diabetologia* 52: 1566-1578, 2009.

20. **Brereton MF, Wareing M, Jones RL, and Greenwood SL**. Characterisation of k(+) channels in human fetoplacental vascular smooth muscle cells. *PLoS One* 8: e57451, 2013.

21. **Brickel N, Gandhi P, VanLandingham K, Hammond J, and DeRossett S**. The urinary safety profile and secondary renal effects of retigabine (ezogabine): a first-in-class antiepileptic drug that targets KCNQ (K(v)7) potassium channels. *Epilepsia* 53: 606-612, 2012.

22. **Brown SM, Bentcheva-Petkova LM, Liu L, Hristov KL, Chen M, Kellett WF, Meredith AL, Aldrich RW, Nelson MT, and Petkov GV**. Beta-adrenergic relaxation of mouse urinary bladder smooth muscle in the absence of large-conductance Ca^{2+}-activated K^+ channel. *Am J Physiol Renal Physiol* 295: F1149-1157, 2008.

23. **Buckner SA, Milicic I, Daza AV, Coghlan MJ, and Gopalakrishnan M**. Spontaneous phasic activity of the pig urinary bladder smooth muscle: characteristics and sensitivity to potassium channel modulators. *Br J Pharmacol* 135: 639-648, 2002.

24. **Burnstock G, and Williams M**. P2 purinergic receptors: modulation of cell function and therapeutic potential. *J Pharmacol Exp Ther* 295: 862-869, 2000.
25. **Chang HY, Cheng CL, Chen JJJ, and de Groat WC**. Serotonergic drugs and spinal cord transections indicate that different spinal circuits are involved in external urethral sphincter activity in rats. *Am J Physiol-Renal* 292: F1044-F1053, 2007.

26. **Chang S, Gomes CM, Hypolite JA, Marx J, Alanzi J, Zderic SA, Malkowicz B, Wein AJ, and Chacko S**. Detrusor overactivity is associated with downregulation of large-conductance calcium- and voltage-activated potassium channel protein. *Am J Physiol Renal Physiol* 298: F1416-1423, 2010.

27. **Chapple CR, Kaplan SA, Mitcheson D, Klecka J, Cummings J, Drogendijk T, Dorrepaal C, and Martin N**. Randomized Double-blind, Active-controlled Phase 3 Study to Assess 12-Month Safety and Efficacy of Mirabegron, a beta(3)-Adrenoceptor Agonist, in Overactive Bladder. *Eur Urol* 63: 296-305, 2013.

28. **Chen M, Kellett WF, and Petkov GV**. Voltage-gated K(+) channels sensitive to stromatoxin-1 regulate myogenic and neurogenic contractions of rat urinary bladder smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 299: R177-184, 2010.

29. **Chen M, Kellett WF, and Petkov GV**. Voltage-gated K^+ channels sensitive to stromatoxin-1 regulate myogenic and neurogenic contractions of rat urinary bladder smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 299: R177-184, 2010.

30. **Chen M, and Petkov GV**. Identification of large conductance calcium activated potassium channel accessory beta4 subunit in rat and mouse bladder smooth muscle. *J Urol* 182: 374-381, 2009.

31. **Chen MX, Gorman SA, Benson B, Singh K, Hieble JP, Michel MC, Tate SN, and Trezise DJ**. Small and intermediate conductance Ca^{2+}-activated K^+ channels confer distinctive patterns of distribution in human tissues and differential cellular localisation in the colon and corpus cavernosum. *Naunyn Schmiedebergs Arch Pharmacol* 369: 602-615, 2004.

32. **Christ GJ, Day NS, Day M, Santizo C, Zhao W, Sclafani T, Zinman J, Hsieh K, Venkateswarlu K, Valcic M, and Melman A**. Bladder injection of "naked" hSlo/pcDNA3 ameliorates detrusor hyperactivity in obstructed rats in vivo. *Am J Physiol Regul Integr Comp Physiol* 281: R1699-1709, 2001.

33. **Conte A, Giannantoni A, Proietti S, Giovannozzi S, Fabbrini G, Rossi A, Porena M, and Berardelli A**. Botulinum toxin A modulates afferent fibers in neurogenic detrusor overactivity. *Eur J Neurol* 19: 725-732, 2012.

34. **Crowe R, and Burnstock G**. A histochemical and immunohistochemical study of the autonomic innervation of the lower urinary tract of the female pig. Is the pig a good model for the human bladder and urethra? *J Urol* 141: 414-422, 1989.

35. **Darblade B, Behr-Roussel D, Oger S, Hieble JP, Lebret T, Gorny D, Benoit G, Alexandre L, and Giuliano F**. Effects of potassium channel modulators on human detrusor smooth muscle myogenic phasic contractile activity: potential therapeutic targets for overactive bladder. *Urology* 68: 442-448, 2006.

36. **Davies AM, Batchelor TJ, Eardley I, and Beech DJ**. Potassium channel KV alpha1 subunit expression and function in human detrusor muscle. *J Urol* 167: 1881-1886, 2002.

37. **Davis-Taber R, Molinari EJ, Altenbach RJ, Whiteaker KL, Shieh CC, Rotert G, Buckner SA, Malysz J, Milicic I, McDermott JS, Gintant GA, Coghlan MJ, Carroll WA, Scott VE, and Gopalakrishnan M**. [125I]A-312110, a novel high-affinity 1,4-dihydropyridine ATP-sensitive K+ channel opener: characterization and pharmacology of binding. *Mol Pharmacol* 64: 143-153, 2003.

38. **de Groat WC, and Yoshimura N**. Afferent nerve regulation of bladder function in health and disease. *Handb Exp Pharmacol* 91-138, 2009.

39. **Deba A, Palea S, Rouget C, Westfall TD, and Lluel P**. Involvement of beta(3)-adrenoceptors in mouse urinary bladder function: role in detrusor muscle relaxation and micturition reflex. *Eur J Pharmacol* 618: 76-83, 2009.

40. **Escoubas P, Diochot S, Celerier ML, Nakajima T, and Lazdunski M**. Novel tarantula toxins for subtypes of voltage-dependent potassium channels in the Kv2 and Kv4 subfamilies. *Mol Pharmacol* 62: 48-57, 2002.

41. **Escoubas P, and Rash L**. Tarantulas: eight-legged pharmacists and combinatorial chemists. *Toxicon* 43: 555-574, 2004.

42. **Firth AL, Remillard CV, Platoshyn O, Fantozzi I, Ko EA, and Yuan JX**. Functional ion channels in human pulmonary artery smooth muscle cells: Voltage-dependent cation channels. *Pulm Circ* 1: 48-71, 2011.

43. **Fisher MH, Amend AM, Bach TJ, Barker JM, Brady EJ, Candelore MR, Carroll D, Cascieri MA, Chiu SH, Deng L, Forrest MJ, Hegarty-Friscino B, Guan XM, Hom GJ, Hutchins JE, Kelly LJ, Mathvink RJ, Metzger JM, Miller RR, Ok HO, Parmee ER, Saperstein R, Strader CD, Stearns RA, MacIntyre DE, and et al.** A selective human beta3 adrenergic receptor agonist increases metabolic rate in rhesus monkeys. *J Clin Invest* 101: 2387-2393, 1998.

44. **Fowler CJ, Griffiths D, and de Groat WC**. The neural control of micturition. *Nat Rev Neurosci* 9: 453-466, 2008.

45. **Fujii K, Foster CD, Brading AF, and Parekh AB**. Potassium channel blockers and the effects of cromakalim on the smooth muscle of the guinea-pig bladder. *Br J Pharmacol* 99: 779-785, 1990.

46. **Fujimura T, Tamura K, Tsutsumi T, Yamamoto T, Nakamura K, Koibuchi Y, Kobayashi M, and Yamaguchi O**. Expression and possible functional role of the beta3-adrenoceptor in human and rat detrusor muscle. *J Urol* 161: 680-685, 1999.

47. **Gan XG, An RH, Bai YF, and Zong DB**. Expressions of voltage-gated K+ channel 2.1 and 2.2 in rat bladder with detrusor hyperreflexia. *Chin Med J (Engl)* 121: 1574-1577, 2008.

48. **Gillespie JI**. Inhibitory actions of calcitonin gene-related peptide and capsaicin: evidence for local axonal reflexes in the bladder wall. *BJU Int* 95: 149-156, 2005.

49. **Gillespie JI**. Noradrenaline inhibits autonomous activity in the isolated guinea pig bladder. *BJU Int* 93: 401-409, 2004.

50. **Gras J**. Mirabegron for the treatment of overactive bladder. *Drugs Today (Barc)* 48: 25-32, 2012.

51. **Gunthorpe MJ, Large CH, and Sankar R**. The mechanism of action of retigabine (ezogabine), a first-in-class K+ channel opener for the treatment of epilepsy. *Epilepsia* 53: 412-424, 2012.

52. **Gutman GA, Chandy KG, Adelman JP, Aiyar J, Bayliss DA, Clapham DE, Covarriubias M, Desir GV, Furuichi K, Ganetzky B, Garcia ML, Grissmer S, Jan LY, Karschin A, Kim D, Kuperschmidt S, Kurachi Y, Lazdunski M, Lesage F, Lester HA, McKinnon D, Nichols CG, O'Kelly I, Robbins J, Robertson GA, Rudy B, Sanguinetti M, Seino S, Stuehmer W, Tamkun MM, Vandenberg CA, Wei A, Wulff H, and Wymore RS**. International Union of Pharmacology. XLI. Compendium of voltage-gated ion channels: potassium channels. *Pharmacol Rev* 55: 583-586, 2003.

53. **Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA, Robertson GA, Rudy B, Sanguinetti MC, Stuhmer W, and Wang X**. International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. *Pharmacol Rev* 57: 473-508, 2005.

54. **Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ**. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 391: 85-100, 1981.

55. **Harriss DR, Marsh KA, Birmingham AT, and Hill SJ**. Expression of muscarinic M3-receptors coupled to inositol phospholipid hydrolysis in human detrusor cultured smooth muscle cells. *J Urol* 154: 1241-1245, 1995.

56. **Hashitani H, and Brading AF**. Electrical properties of detrusor smooth muscles from the pig and human urinary bladder. *Br J Pharmacol* 140: 146-158, 2003.

57. **Hashitani H, and Brading AF**. Ionic basis for the regulation of spontaneous excitation in detrusor smooth muscle cells of the guinea-pig urinary bladder. *Br J Pharmacol* 140: 159-169, 2003.

58. **Hashitani H, Brading AF, and Suzuki H**. Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder. *Br J Pharmacol* 141: 183-193, 2004.

59. **Hashitani H, Bramich NJ, and Hirst GD**. Mechanisms of excitatory neuromuscular transmission in the guinea-pig urinary bladder. *J Physiol* 524 Pt 2: 565-579, 2000.

60. **Hayase M, Hashitani H, Kohri K, and Suzuki H**. Role of K^+ channels in regulating spontaneous activity in detrusor smooth muscle in situ in the mouse bladder. *J Urol* 181: 2355-2365, 2009.

61. **Heppner TJ, Bonev AD, and Nelson MT**. Ca^{2+}-activated K^+ channels regulate action potential repolarization in urinary bladder smooth muscle. *Am J Physiol* 273: C110-117, 1997.

62. **Heppner TJ, Bonev AD, and Nelson MT**. Ca(2+)-activated K+ channels regulate action potential repolarization in urinary bladder smooth muscle. *Am J Physiol* 273: C110-117, 1997.

63. **Heppner TJ, Bonev AD, and Nelson MT**. Elementary purinergic Ca^{2+} transients evoked by nerve stimulation in rat urinary bladder smooth muscle. *J Physiol* 564: 201-212, 2005.

64. **Heppner TJ, Werner ME, Nausch B, Vial C, Evans RJ, and Nelson MT**. Nerve-evoked purinergic signalling suppresses action potentials, Ca^{2+} flashes and contractility evoked by muscarinic receptor activation in mouse urinary bladder smooth muscle. *J Physiol* 587: 5275-5288, 2009.

65. **Herrera GM, Etherton B, Nausch B, and Nelson MT**. Negative feedback regulation of nerve-mediated contractions by KCa channels in mouse urinary bladder smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 289: R402-R409, 2005.

66. **Herrera GM, Heppner TJ, and Nelson MT**. Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. *Am J Physiol Regul Integr Comp Physiol* 279: R60-68, 2000.

67. **Herrera GM, and Nelson MT**. Differential regulation of SK and BK channels by Ca^{2+} signals from Ca^{2+} channels and ryanodine receptors in guinea-pig urinary bladder myocytes. *J Physiol* 541: 483-492, 2002.

68. **Herrera GM, Pozo MJ, Zvara P, Petkov GV, Bond CT, Adelman JP, and Nelson MT**. Urinary bladder instability induced by selective suppression of the murine small conductance calcium-activated potassium (SK3) channel. *J Physiol* 551: 893-903, 2003.

69. **Hicks A, McCafferty GP, Riedel E, Aiyar N, Pullen M, Evans C, Luce TD, Coatney RW, Rivera GC, Westfall TD, and Hieble JP**. GW427353 (solabegron), a novel, selective beta3-adrenergic receptor agonist, evokes bladder relaxation and increases micturition reflex threshold in the dog. *J Pharmacol Exp Ther* 323: 202-209, 2007.

70. **Horinouchi T, Tanaka Y, and Koike K**. Function of beta1-adrenoceptors and mRNA expression of beta1- and beta2-adrenoceptors in guinea-pig esophagus. *Eur J Pharmacol* 473: 79-82, 2003.

71. **Horinouchi T, Yamamoto Y, and Koike K**. Effect of SR59230A on atypical beta-adrenoceptor mediating relaxation in the Guinea Pig gastric fundus. *Pharmacology* 62: 98-102, 2001.

72. **Horn R, and Marty A**. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* 92: 145-159, 1988.

73. **Hougaard C, Fraser MO, Chien C, Bookout A, Katofiasc M, Jensen BS, Rode F, Bitsch-Norhave J, Teuber L, Thor KB, Strobaek D, Burgard EC, and Ronn LC**. A positive modulator of K Ca 2 and K Ca 3 channels, 4,5-dichloro-1,3-diethyl-1,3-dihydro-benzoimidazol-2-one (NS4591), inhibits bladder afferent firing in vitro and bladder overactivity in vivo. *J Pharmacol Exp Ther* 328: 28-39, 2009.

74. **Hristov KL, Chen M, Afeli SA, Cheng Q, Rovner ES, and Petkov GV**. Expression and function of K(V)2-containing channels in human urinary bladder smooth muscle. *Am J Physiol Cell Physiol* 302: C1599-1608, 2012.

75. **Hristov KL, Chen M, Kellett WF, Rovner ES, and Petkov GV**. Large-conductance voltage- and Ca^{2+}-activated K^+ channels regulate human detrusor smooth muscle function. *Am J Physiol Cell Physiol* 301: C903-912, 2011.

76. **Hristov KL, Chen M, Kellett WF, Rovner ES, and Petkov GV**. Large conductance voltage- and Ca2+-activated K+ channels regulate human detrusor smooth muscle function. *Am J Physiol Cell Physiol* 2011.

77. **Hristov KL, Chen M, Soder RP, Parajuli SP, Cheng Q, Kellett WF, and Petkov GV**. KV2.1 and electrically silent KV channel subunits control excitability and contractility of Guinea pig detrusor smooth muscle. *Am J Physiol Cell Physiol* 2011.

78. **Hristov KL, Chen M, Soder RP, Parajuli SP, Cheng Q, Kellett WF, and Petkov GV**. KV2.1 and electrically silent KV channel subunits control excitability and contractility of guinea pig detrusor smooth muscle. *Am J Physiol Cell Physiol* 302: C360-372, 2012.

79. **Hristov KL, Cui X, Brown SM, Liu L, Kellett WF, and Petkov GV**. Stimulation of beta3-adrenoceptors relaxes rat urinary bladder smooth muscle via activation of the large-conductance Ca^{2+}-activated K^+ channels. *Am J Physiol Cell Physiol* 295: C1344-1353, 2008.

80. **Hristov KL, Parajuli SP, Soder RP, Cheng Q, Rovner ES, and Petkov GV**. Suppression of human detrusor smooth muscle excitability and contractility via pharmacological activation of large conductance Ca^{2+}-activated K^+ channels. *Am J Physiol Cell Physiol* 302: C1632-1641, 2012.

81. **Igawa Y, Schneider T, Yamazaki Y, Tatemichi S, Homma Y, Nishizawa O, and Michel MC**. Functional investigation of beta-adrenoceptors in human isolated detrusor focusing on the novel selective beta3-adrenoceptor agonist KUC-7322. *Naunyn Schmiedebergs Arch Pharmacol* 385: 759-767, 2012.

82. **Igawa Y, Yamazaki Y, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, and Andersson KE**. Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. *Br J Pharmacol* 126: 819-825, 1999.

83. **Igawa Y, Yamazaki Y, Takeda H, Kaidoh K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, and Andersson KE**. Relaxant effects of isoproterenol and selective beta3-adrenoceptor agonists on normal, low compliant and hyperreflexic human bladders. *J Urol* 165: 240-244, 2001.

84. **Ikematsu N, Dallas ML, Ross FA, Lewis RW, Rafferty JN, David JA, Suman R, Peers C, Hardie DG, and Evans AM**. Phosphorylation of the voltage-gated potassium channel Kv2.1 by AMP-activated protein kinase regulates membrane excitability. *Proc Natl Acad Sci U S A* 108: 18132-18137, 2011.

85. **Jepps TA, Olesen SP, and Greenwood IA**. One man's side effect is another man's therapeutic opportunity: targeting Kv7 channels in smooth muscle disorders. *Br J Pharmacol* 168: 19-27, 2013.

86. **Kajioka S, Nakayama S, Asano H, Seki N, Naito S, and Brading AF**. Levcromakalim and MgGDP activate small conductance ATP-sensitive K+ channels of

K+ channel pore 6.1/sulfonylurea receptor 2A in pig detrusor smooth muscle cells: uncoupling of cAMP signal pathways. *J Pharmacol Exp Ther* 327: 114-123, 2008.

87. **Kajioka S, Shahab N, Asano H, Morita H, Sugihara M, Takahashi-Yanaga F, Yoshihara T, Nakayama S, Seki N, and Naito S**. Diphosphate regulation of adenosine triphosphate sensitive potassium channel in human bladder smooth muscle cells. *J Urol* 186: 736-744, 2011.

88. **Kanie S, Otsuka A, Yoshikawa S, Morimoto T, Hareyama N, Okazaki S, Kobayashi R, Hasebe K, Nakao K, Hayashi R, Mochizuki H, Matsumoto R, and Ozono S**. Pharmacological effect of TRK-380, a novel selective human beta3-adrenoceptor agonist, on mammalian detrusor strips. *Urology* 79: 744 e741-747, 2012.

89. **Kennedy C, Tasker PN, Gallacher G, and Westfall TD**. Identification of atropine- and P2X1 receptor antagonist-resistant, neurogenic contractions of the urinary bladder. *J Neurosci* 27: 845-851, 2007.

90. **Khullar V, Amarenco G, Angulo JC, Cambronero J, Hoye K, Milsom I, Radziszewski P, Rechberger T, Boerrigter P, Drogendijk T, Wooning M, and Chapple C**. Efficacy and Tolerability of Mirabegron, a beta(3)-Adrenoceptor Agonist, in Patients with Overactive Bladder: Results from a Randomised European-Australian Phase 3 Trial. *Eur Urol* 63: 283-295, 2013.

91. **Klausner AP, Rourke KF, Miner AS, and Ratz PH**. Potentiation of carbachol-induced detrusor smooth muscle contractions by beta-adrenoceptor activation. *Eur J Pharmacol* 606: 191-198, 2009.

92. **Ko EA, Park WS, Firth AL, Kim N, Yuan JX, and Han J**. Pathophysiology of voltage-gated K+ channels in vascular smooth muscle cells: modulation by protein kinases. *Prog Biophys Mol Biol* 103: 95-101, 2010.

93. **Kubota Y, Nakahara T, Yunoki M, Mitani A, Maruko T, Sakamoto K, and Ishii K**. Inhibitory mechanism of BRL37344 on muscarinic receptor-mediated contractions of the rat urinary bladder smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* 366: 198-203, 2002.

94. **Kullmann FA, Limberg BJ, Artim DE, Shah M, Downs TR, Contract D, Wos J, Rosenbaum JS, and de Groat WC**. Effects of beta3-adrenergic receptor activation on rat urinary bladder hyperactivity induced by ovariectomy. *J Pharmacol Exp Ther* 330: 704-717, 2009.

95. **Kuo HC, Liao CH, and Chung SD**. Adverse events of intravesical botulinum toxin a injections for idiopathic detrusor overactivity: risk factors and influence on treatment outcome. *Eur Urol* 58: 919-926, 2010.

96. **Kuo YC, and Kuo HC**. Botulinum toxin injection for lower urinary tract dysfunction. *Int J Urol* 2012.

97. **Kyle B, Bradley E, Ohya S, Sergeant GP, McHale NG, Thornbury KD, and Hollywood MA**. Contribution of Kv2.1 channels to the delayed rectifier current in freshly dispersed smooth muscle cells from rabbit urethra. *Am J Physiol Cell Physiol* 301: C1186-1200, 2011.

98. **Layne JJ, Nausch B, Olesen SP, and Nelson MT**. BK channel activation by NS11021 decreases excitability and contractility of urinary bladder smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 298: R378-384, 2010.

99. **Leon LA, Hoffman BE, Gardner SD, Laping NJ, Evans C, Lashinger ES, and Su X**. Effects of the beta 3-adrenergic receptor agonist disodium 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole -2,2-dicarboxylate (CL-316243) on bladder micturition reflex in spontaneously hypertensive rats. *J Pharmacol Exp Ther* 326: 178-185, 2008.

100. **Li JH, Yasay GD, and Kau ST**. Beta-adrenoceptor subtypes in the detrusor of guinea-pig urinary bladder. *Pharmacology* 44: 13-18, 1992.

101. **Livak KJ, and Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.

102. **MacKinnon PCB, Morris JF, and Beste A**. Oxford Textbook Of Functional Anatomy: Volume 2: Thorax And Abdomen. *Oxford University Press, USA* 2: 169-178, 2005.

103. **Martelli A, Testai L, Breschi MC, Lawson K, McKay NG, Miceli F, Taglialatela M, and Calderone V**. Vasorelaxation by hydrogen sulphide involves activation of K7 potassium channels. *Pharmacol Res* 70: 27-34, 2013.

104. **Matsui M, Griffin MT, Shehnaz D, Taketo MM, and Ehlert FJ**. Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contractions in smooth muscle from M2 receptor knockout mice. *J Pharmacol Exp Ther* 305: 106-113, 2003.

105. **Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, Takahashi S, and Taketo MM**. Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. *Proc Natl Acad Sci U S A* 97: 9579-9584, 2000.

106. **Maylie J, Bond CT, Herson PS, Lee WS, and Adelman JP**. Small conductance Ca^{2+}-activated K^+ channels and calmodulin. *J Physiol* 554: 255-261, 2004.

107. **Melman A, Bar-Chama N, McCullough A, Davies K, and Christ G**. Plasmid-based gene transfer for treatment of erectile dysfunction and overactive bladder: results of a phase I trial. *Isr Med Assoc J* 9: 143-146, 2007.

108. **Melman A, Biggs G, Davies K, Zhao W, Tar MT, and Christ GJ**. Gene transfer with a vector expressing Maxi-K from a smooth muscle-specific promoter restores erectile function in the aging rat. *Gene Ther* 15: 364-370, 2008.

109. **Meredith AL, Thorneloe KS, Werner ME, Nelson MT, and Aldrich RW**. Overactive bladder and incontinence in the absence of the BK large conductance Ca^{2+}-activated K^+ channel. *J Biol Chem* 279: 36746-36752, 2004.

110. **Michel MC, Ochodnicky P, and Summers RJ**. Tissue functions mediated by beta(3)-adrenoceptors-findings and challenges. *Naunyn Schmiedebergs Arch Pharmacol* 382: 103-108, 2010.

111. **Michel MC, and Vrydag W**. Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate. *Br J Pharmacol* 147 Suppl 2: S88-119, 2006.

112. **Montgomery BS, Thomas PJ, and Fry CH**. The actions of extracellular magnesium on isolated human detrusor muscle function. *Br J Urol* 70: 262-268, 1992.

113. **Moreno-Dominguez A, Cidad P, Miguel-Velado E, Lopez-Lopez JR, and Perez-Garcia MT**. De novo expression of Kv6.3 contributes to changes in vascular smooth muscle cell excitability in a hypertensive mice strain. *J Physiol* 587: 625-640, 2009.

114. **Muhlstein J, and Deval B**. [Anticholinergic drugs in overactive bladder]. *Gynecol Obstet Fertil* 36: 90-96, 2008.

115. **Nakahira Y, Hashitani H, Fukuta H, Sasaki S, Kohri K, and Suzuki H**. Effects of isoproterenol on spontaneous excitations in detrusor smooth muscle cells of the guinea pig. *J Urol* 166: 335-340, 2001.

116. **Navarro-Lopez J, Jimenez-Diaz L, Geranton SM, and Ashmore JF**. Electrophysiological and molecular analysis of Kv7/KCNQ potassium channels in the inferior colliculus of adult guinea pig. *J Mol Neurosci* 37: 263-268, 2009.

117. **Nielsen JS, Rode F, Rahbek M, Andersson KE, Ronn LC, Bouchelouche K, Nordling J, and Bouchelouche P**. Effect of the SK/IK channel modulator 4,5-dichloro-1,3-diethyl-1,3-dihydro-benzoimidazol-2-one (NS4591) on contractile force in rat, pig and human detrusor smooth muscle. *BJU Int* 108: 771-777, 2011.

118. **Ning Lo W, and Liang W**. Blockade of voltage-sensitive K+ channels increases contractility more in transverse than in longitudinal rat detrusor strips. *Urology* 73: 400-404, 2009.

119. **O'Reilly BA, Kosaka AH, Knight GF, Chang TK, Ford AP, Rymer JM, Popert R, Burnstock G, and McMahon SB**. P2X receptors and their role in female idiopathic detrusor instability. *J Urol* 167: 157-164, 2002.

120. **Oger S, Behr-Roussel D, Gorny D, Bernabe J, Comperat E, Chartier-Kastler E, Denys P, and Giuliano F**. Effects of potassium channel modulators on myogenic spontaneous phasic contractile activity in human detrusor from neurogenic patients. *BJU Int* 108: 604-611, 2011.

121. **Ohya S, Asakura K, Muraki K, Watanabe M, and Imaizumi Y**. Molecular and functional characterization of ERG, KCNQ, and KCNE subtypes in rat stomach smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 282: G277-287, 2002.

122. **Ohya S, Tanaka M, Watanabe M, and Maizumi Y**. Diverse expression of delayed rectifier K+ channel subtype transcripts in several types of smooth muscles of the rat. *J Smooth Muscle Res* 36: 101-115, 2000.

123. **Orhan G, Wuttke TV, Nies AT, Schwab M, and Lerche H**. Retigabine/Ezogabine, a KCNQ/K(V)7 channel opener: pharmacological and clinical data. *Expert Opin Pharmacother* 13: 1807-1816, 2012.

124. **Palea S, Artibani W, Ostardo E, Trist DG, and Pietra C**. Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. *J Urol* 150: 2007-2012, 1993.

125. **Pandita RK, Ronn LC, Jensen BS, and Andersson KE**. Urodynamic effects of intravesical administration of the new small/intermediate conductance calcium activated potassium channel activator NS309 in freely moving, conscious rats. *J Urol* 176: 1220-1224, 2006.

126. **Parajuli SP, Soder RP, Hristov KL, and Petkov GV**. Pharmacological activation of SK channels with SKA-31 decreases Guinea pig detrusor smooth muscle excitability and contractility. *J Pharmacol Exp Ther* 2011.

127. **Parajuli SP, Soder RP, Hristov KL, and Petkov GV**. Pharmacological Activation of Small Conductance Calcium-Activated Potassium Channels with Naphtho[1,2-d]thiazol-2-ylamine Decreases Guinea Pig Detrusor Smooth Muscle Excitability and Contractility. *J Pharmacol Exp Ther* 340: 114-123, 2012.

128. **Parmee ER, Ok HO, Candelore MR, Tota L, Deng L, Strader CD, Wyvratt MJ, Fisher MH, and Weber AE**. Discovery of L-755,507: a subnanomolar human beta 3 adrenergic receptor agonist. *Bioorg Med Chem Lett* 8: 1107-1112, 1998.

129. **Petkov GV**. Ion channels. In: *Pharmacology: Principles and Practice,*edited by Hacker M, Messer W, Bachmann K. New York, NY: Elsevier 2009, chapt. 16, p. 385–425.

130. **Petkov GV**. Role of potassium ion channels in detrusor smooth muscle function and dysfunction. *Nat Rev Urol* 9: 30-40, 2012.

131. **Petkov GV, Bonev AD, Heppner TJ, Brenner R, Aldrich RW, and Nelson MT**. Beta1-subunit of the Ca^{2+}-activated K^+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J Physiol* 537: 443-452, 2001.

132. **Petkov GV, Heppner TJ, Bonev AD, Herrera GM, and Nelson MT**. Low levels of K(ATP) channel activation decrease excitability and contractility of urinary bladder. *Am J Physiol Regul Integr Comp Physiol* 280: R1427-1433, 2001.

133. **Petkov GV, Heppner TJ, Bonev AD, Herrera GM, and Nelson MT**. Low levels of K_{ATP} channel activation decrease excitability and contractility of urinary bladder. *Am J Physiol Regul Integr Comp Physiol* 280: R1427-1433, 2001.

134. **Petkov GV, and Nelson MT**. Differential regulation of Ca2+-activated K+ channels by beta-adrenoceptors in guinea pig urinary bladder smooth muscle. *Am J Physiol Cell Physiol* 288: C1255-1263, 2005.

135. **Platoshyn O, Remillard CV, Fantozzi I, Mandegar M, Sison TT, Zhang S, Burg E, and Yuan JX**. Diversity of voltage-dependent K+ channels in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 287: L226-238, 2004.

136. **Rode F, Svalo J, Sheykhzade M, and Ronn LC**. Functional effects of the KCNQ modulators retigabine and XE991 in the rat urinary bladder. *Eur J Pharmacol* 638: 121-127, 2010.

137. **Sankaranarayanan A, Raman G, Busch C, Schultz T, Zimin PI, Hoyer J, Kohler R, and Wulff H**. Naphtho[1,2-d]thiazol-2-ylamine (SKA-31), a new activator of KCa2 and KCa3.1 potassium channels, potentiates the endothelium-derived hyperpolarizing factor response and lowers blood pressure. *Mol pharmacol* 75: 281-295, 2009.

138. **Sarma DN, Banwait K, Basak A, DiMarino AJ, and Rattan S**. Inhibitory effect of beta3-adrenoceptor agonist in lower esophageal sphincter smooth muscle: in vitro studies. *J Pharmacol Exp Ther* 304: 48-55, 2003.

139. **Sato M, Horinouchi T, Hutchinson DS, Evans BA, and Summers RJ**. Ligand-directed signaling at the beta3-adrenoceptor produced by 3-(2-Ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propan ol oxalate (SR59230A) relative to receptor agonists. *Mol Pharmacol* 72: 1359-1368, 2007.

140. **Sato M, Hutchinson DS, Evans BA, and Summers RJ**. The beta3-adrenoceptor agonist 4-[[(Hexylamino)carbonyl]amino]-N-[4-[2-[[(2S)-2-hydroxy-3-(4-hydroxypheno xy)propyl]amino]ethyl]-phenyl]-benzenesulfonamide (L755507) and antagonist (S)-N-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]amino]-ethyl] phenyl]benzenesulfonamide (L748337) activate different signaling pathways in Chinese hamster ovary-K1 cells stably expressing the human beta3-adrenoceptor. *Mol Pharmacol* 74: 1417-1428, 2008.

141. **Shieh CC, Feng J, Buckner SA, Brioni JD, Coghlan MJ, Sullivan JP, and Gopalakrishnan M**. Functional implication of spare ATP-sensitive K(+) channels in bladder smooth muscle cells. *J Pharmacol Exp Ther* 296: 669-675, 2001.

142. **Sibley GN**. A comparison of spontaneous and nerve-mediated activity in bladder muscle from man, pig and rabbit. *J Physiol* 354: 431-443, 1984.

143. **Smith AC, Hristov KL, Cheng Q, Xin W, Parajuli SP, Earley S, Malysz J, and Petkov GV**. Novel role for the transient potential receptor melastatin 4 channel in guinea pig detrusor smooth muscle physiology. *Am J Physiol Cell Physiol* 2013.

144. **Soder RP, Parajuli SP, Hristov KL, Rovner ES, and Petkov GV**. SK channel-selective opening by SKA-31 induces hyperpolarization and decreases contractility in human urinary bladder smooth muscle. *Am J Physiol Regul Integr Comp Physiol* 304: R155-163, 2013.

145. **Soder RP, and Petkov GV**. Large conductance Ca^{2+} -activated K^{+} channel activation with NS1619 decreases myogenic and neurogenic contractions of rat detrusor smooth muscle. *Eur J Pharmacol* 670: 252-259, 2011.

146. **Sprossmann F, Pankert P, Sausbier U, Wirth A, Zhou XB, Madlung J, Zhao H, Bucurenciu I, Jakob A, Lamkemeyer T, Neuhuber W, Offermanns S, Shipston MJ, Korth M, Nordheim A, Ruth P, and Sausbier M**. Inducible knockout mutagenesis reveals compensatory mechanisms elicited by constitutive BK channel deficiency in overactive murine bladder. *Febs J* 276: 1680-1697, 2009.

147. **Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, Hunt TL, and Wein AJ**. Prevalence and burden of overactive bladder in the United States. *World J Urol* 20: 327-336, 2003.

148. **Streng T, Christoph T, and Andersson KE**. Urodynamic effects of the K+ channel (KCNQ) opener retigabine in freely moving, conscious rats. *J Urol* 172: 2054-2058, 2004.

149. **Svalo J, Hansen HH, Ronn LC, Sheykhzade M, Munro G, and Rode F**. K(v) 7 Positive Modulators Reduce Detrusor Overactivity and Increase Bladder Capacity in Rats. *Basic Clin Pharmacol Toxicol* 2011.

150. **Takasu T, Ukai M, Sato S, Matsui T, Nagase I, Maruyama T, Sasamata M, Miyata K, Uchida H, and Yamaguchi O**. Effect of (R)-2-(2-aminothiazol-4-yl)-4'-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide (YM178), a novel selective beta3-adrenoceptor agonist, on bladder function. *J Pharmacol Exp Ther* 321: 642-647, 2007.

151. **Takeda M, Obara K, Mizusawa T, Tomita Y, Arai K, Tsutsui T, Hatano A, Takahashi K, and Nomura S**. Evidence for beta3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods. *J Pharmacol Exp Ther* 288: 1367-1373, 1999.

152. **Thor KB, Hisamitsu T, Roppolo JR, Tuttle P, Nagel J, and Degroat WC**. Selective Inhibitory Effects of Ethylketocyclazocine on Reflex Pathways to the External Urethral Sphincter of the Cat. *J Pharmacol Exp Ther* 248: 1018-1025, 1989.

153. **Thorneloe KS, Knorn AM, Doetsch PE, Lashinger ESR, Liu AX, Bond CT, Adelman JP, and Nelson MT**. Small-conductance, Ca^{2+}-activated K^{+} channel 2 is the key functional component of SK channels in mouse urinary bladder. *Am J Physiol Regul Integr Comp Physiol* 294: R1737-R1743, 2008.

154. **Thorneloe KS, Meredith AL, Knorn AM, Aldrich RW, and Nelson MT**. Urodynamic properties and neurotransmitter dependence of urinary bladder contractility in the BK channel deletion model of overactive bladder. *Am J Physiol Renal Physiol* 289: F604-610, 2005.

155. **Thorneloe KS, and Nelson MT**. Properties and molecular basis of the mouse urinary bladder voltage-gated K+ current. *J Physiol* 549: 65-74, 2003.

156. **Tyagi P, Thomas CA, Yoshimura N, and Chancellor MB**. Investigations into the presence of functional Beta1, Beta2 and Beta3-adrenoceptors in urothelium and detrusor of human bladder. *Int Braz J Urol* 35: 76-83, 2009.

157. **Tyagi P, and Tyagi V**. Mirabegron, a beta(3)-adrenoceptor agonist for the potential treatment of urinary frequency, urinary incontinence or urgency associated with overactive bladder. *IDrugs* 13: 713-722, 2010.

158. **Uchida W, Masuda N, Shirai Y, Shibasaki K, Satoh N, and Takenada T**. The role of extracellular Ca2+ in carbachol-induced tonic contraction of the pig detrusor smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* 350: 398-402, 1994.

159. **van Koeveringe GA, Vahabi B, Andersson KE, Kirschner-Herrmans R, and Oelke M**. Detrusor underactivity: a plea for new approaches to a common bladder dysfunction. *Neurourol Urodyn* 30: 723-728, 2011.

160. **Vial C, and Evans RJ**. P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. *Br J Pharmacol* 131: 1489-1495, 2000.

161. **Werner ME, Knorn AM, Meredith AL, Aldrich RW, and Nelson MT**. Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels. *Am J Physiol Regul Integr Comp Physiol* 292: R616-624, 2007.

162. **Wu C, Sui G, and Fry CH**. The role of the L-type Ca(2+) channel in refilling functional intracellular Ca(2+) stores in guinea-pig detrusor smooth muscle. *J Physiol* 538: 357-369, 2002.

163. **Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, and Chandy KG**. Design of a potent and selective inhibitor of the intermediate-conductance Ca2+-activated K+ channel, IKCa1: a potential immunosuppressant. *Proc Natl Acad Sci U S A* 97: 8151-8156, 2000.

164. **Xin W, Cheng Q, Soder RP, and Petkov GV**. Inhibition of phosphodiesterases relaxes detrusor smooth muscle via activation of the large-conductance voltage- and Ca^{2+}-activated K^+ channel. *Am J Physiol Cell Physiol* 302: C1361-1370, 2012.

165. **Xin W, Cheng Q, Soder RP, and Petkov GV**. Inhibition of phosphodiesterases relaxes detrusor smooth muscle via activation of the large-conductance voltage- and Ca(2)(+)-activated K(+) channel. *Am J Physiol Cell Physiol* 302: C1361-1370, 2012.

166. **Xin W, Cheng Q, Soder RP, Rovner ES, and Petkov GV**. Constitutively active phosphodiesterase activity regulates urinary bladder smooth muscle function: critical role of KCa1.1 channel. *Am J Physiol Renal Physiol* 303: F1300-1306, 2012.

167. **Yamaguchi O**. Beta3-adrenoceptors in urinary bladder. *Urology* 59: 25-29, 2002.

168. **Yamaguchi O, and Chapple CR**. Beta3-adrenoceptors in urinary bladder. *Neurourol Urodyn* 26: 752-756, 2007.

169. **Yamamoto Y, Mori A, and Koike K**. Beta-adrenoceptors in the detrusor of guinea pig bladder. *J Smooth Muscle Res* 34: 233-242, 1998.

170. **Yarker YE, Goa KL, and Fitton A**. Oxybutynin. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic use in detrusor instability. *Drugs Aging* 6: 243-262, 1995.

171. **Yoshida M, Miyamae K, Iwashita H, Otani M, and Inadome A**. Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. *Urology* 63: 17-23, 2004.

172. **Young JS, Meng E, Cunnane TC, and Brain KL**. Spontaneous purinergic neurotransmission in the mouse urinary bladder. *J Physiol* 586: 5743-5755, 2008.