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INVESTIGATION OF INTRACELLULAR MECHANISMS OF SPHINGOSINE-1-PHOSPHATE (S1P)-INDUCED CONTRACTIONS IN DETRUSOR SMOOTH MUSCLE OF RATS HAVING CYCLOPHOSPHAMIDE-INDUCED CYSTITIS

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PhD THESIS

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"Investigation of Intracellular Mechanisms of Sphingosine-1-Phosphate (S1P)-Induced Contractions in Detrusor Smooth Muscle of Rats having Cyclophosphamide-Induced Cystitis"

Irfan Anjum

This study has been approved and accepted as a PhD dissertation in the program of "Pharmacology" by the examining committee, whose members are listed below, on 30.05.2017.

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0 7 Haziran 2017

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In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor, Assoc. Professor N. Tugba KANDILCI and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences .

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ABSTRACT

Anjum I., Investigation of Intracellular Mechanisms of Sphingosine-1-Phosphate (S1P)-Induced Contractions in Detrusor Smooth Muscle of Rats having Cyclophosphamide-Induced Cystitis, Hacettepe University, Institute of Health Sciences, Doctor of Philosphy in Pharmacology, Ankara 2017. Interstitial cystitis is a chronic disease characterized by lower abdominal pain and an increase in urinary frequency and urgency. Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid that controls smooth muscle tone via G-protein coupled receptors ($S1P_{1-3}$ receptors). The intracellular mechanism of S1P-induced contractile response was investigated in β-escin permeabilized detrusor smooth muscle of rats having cyclophosphamide-induced cystitis. S1P-induced contraction and calcium sensitization response were significantly increased in cystitis. According to our data, both $S1P_2$ and $S1P_3$ receptors are involved in S1P-induced augmented contractile response. Both Rho kinase (ROCK) and protein kinase C (PKC) pathways of calcium sensitization play a role in that increase. Furthermore, sarcoplasmic reticulum calcium stores and calcium storing lysosome-related organelles participate in S1P-induced contraction. The investigation of intracellular mechanisms of mediators which has a role in detrusor innervation under pathologic conditions may be of importance in developing new drugs for the treatment of bladder dysfunction.

Key words: Sphingosine 1-phosphate, cystitis, permebilized, detrusor smooth muscle, rat

ÖZET

Anjum İ., Siklofosfamid ile sistit oluşturulan sıçanların detrusor düz kasında sfingosin 1-fosfat (S1P) ile indüklenen kasılma yanıtlarının mekanizmasının incelenmesi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmakoloji Programı Doktora Tezi, Ankara 2017.

İnterstisyel sistit abdominal ağrı ve işeme sıklığı ve aciliyetinde artış ile karakterize kronik bir hastalıktır. Sfingosin 1-fosfat (S1P), G-protein kenetli reseptörler ($S1P_{1-3}$ reseptörler) aracılı düz kas tonusunu kontrol eden bir biyoaktif sfingolipiddir. Bu tez çalışmasında, Siklofosfamid ile sistit oluşturulan sıçanların β-eskin ile permeabilize edilen detrusor düz kasında S1P ile indüklenen kasılma yanıtlarının hücre içi mekanizması incelendi. S1P ile indüklenen ve kalsiyum sensitizasyonu kasılma yanıtları sistitte artmaktadır. Bulgularımız göre S1P ile indüklenen artan kasılma yanıtları $S1P_2$ ve $S1P_3$ reseptörleri ile ilişkilidir. Kalsiyum sensitizasyonu yolakları Rho kinaz (ROCK) ve protein kinaz C (PKC) bu artışta rol oynamaktadır. Ayrıca, sarkoplazmik retikulum ve lizozom gibi kalsiyum depoları da S1P ile indüklenen kasılmada rol oynamaktadır. Patolojik durumlarda detrusor inervasyonunda rolü olan mediyatörlerin hücre içi mekanizmalarının incelenmesi mesane disfonksiyonu tedavisinde yeni ilaç moleküllerinin geliştirilebilmesi açısından önemlidir.

Anahtar kelimeler: Sfingosin 1-fosfat, sistit, permeabilizasyon, detrusor düz kası, sıçan

CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
cADPR	Cyclic adenosine diphosphate ribose
CPA	Cyclopiazonic acid
CPI-17	Phosphate inhibitor protein-17kDa
CYP	Cyclophosphamide
DMSO	Dimethyl sulfoxide
FCCP	Carbonyl cyanide p-trifluromethoxyphenylhydrazone
GTP	Guanosine 5 ′-triphosphate
GTP-β-S	Guanosine-5′-O-(2-thio-triphosphate) sodium salt
IP$_3$	Inositol triphosphate
NAADP	Nicotinic acid adenine dinucleotide phosphate
PKC	Protein kinase C
S1P	Sphingosine 1-phosphate

FIGURES

1. INTRODUCTION

Urinary bladder is one of the largest hollow smooth muscle organ of the body. Storage and emptying of urine are two main functions performed by urinary bladder. Storage of urine takes place during filling phase, which is characterized by the relaxation of the bladder at low pressure while emptying of urine takes place by the coordinated contraction of bladder creating high pressure and by relaxation of the urethra. Thus, these functions depend upon the movement of the smooth and striated muscles in the urinary bladder, urethra and external urethral sphincter. Three groups of nerves (parasympathetic, sympathetic and somatic) exiting from the sacral and thoracolumbar regions of the spinal cord, coordinate these movements (1, 2). Detrusor smooth muscle is the major component of the urinary bladder wall. These muscle fibers must have the ability to elongate and reorganize themselves to permit an increase in the volume of the bladder without allowing the rise in intravesical pressure during the storage of urine. Detrusor smooth muscle fibers must then contract synchronously to generate the pressure for expelling urine. These properties of detrusor smooth muscle may change in various disease conditions (3). Interstitial cystitis is a syndrome characterized with chronic inflammation which causes overactive bladder (4). The physiological functions of the bladder may change in cystitis. Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite that plays a role in smooth muscle contraction via G-protein coupled receptors ($S1P_{1-3}$ receptors). $S1P_2$ and $S1P_3$ receptors are known to activate smooth muscle contraction by inducing RhoA/ROCK pathways. Besides some physiological events, S1P also takes part in some pathological situations as in overactive bladder syndrome (5). Moreover, it has been reported that S1P signaling and Rho kinase pathways were upregulated in partial urethral obstruction where in accordingly, $S1P_2$ and $S1P_3$ receptors together with Rho A and ROCKα expressions were elevated resulting in increased contraction by S1P (6). Effects of S1P in animal models with interstitial cystitis have not been studied yet.

Study of the intracellular mechanism of signal transduction and the role of intracellular organelles in smooth muscle contraction can be evaluated by permeabilizing the muscle membrane with various types of chemicals. As the regulatory and contractile proteins remain unaffected in permeabilized muscle cell,

chemical permeabilization helps to control intracellular ion concentration. The most commonly used chemicals for cell permeabilization include β-escin, saponin, triton X-100 and α-toxin (7, 8). Permeabilization of muscle cell also helps to study the mechanism of calcium sensitization involved in muscle contraction by an agonist. Maximum calcium sensitization activated by one agonist may differ by another agonist (9).

The present thesis focuses on the intracellular mechanisms of the change in S1P-induced contractile response in β-escin permeabilized detrusor smooth muscle of rats having cyclophosphamide (CYP)-induced interstitial cystitis. Thus, the effects of S1P on detrusor smooth muscle, the determination of receptor subtypes involved, the role of intracellular contractile proteins and intracellular calcium stores, and the calcium sensitization mechanisms have been investigated both under physiological and pathological conditions, i.e. interstitial cystitis.

2. GENERAL INFORMATION

2.1. Physiology of the Urinary Bladder

The lower urinary tract has three main parts, bladder, urethra and urethral sphincter complex. Bladder consists of the bladder body and the base. Bladder body lies above the ureteric orifices while the base is the part that lies between the ureteric orifices and the urethrovesical junction (10). The wall of urinary bladder consists of mucosa, muscularis propria and adventitia or serosa. Mucosa also contains three layers, the innermost layer is urothelium that faces the urine directly, basal lamina that separates the urothelium from other connective tissues and lamina propria that contains extracellular matrix, fibroblasts, interstitial cells, immune cells, efferent and afferent neurons and lymphatic and blood vessels. Urothelium acts as a blood-urine barrier to various types of pathogens, ions and molecules present in urine. Urothelium contains different types of receptors and ion channels. It also receives, amplifies and transmits information to other tissues of the bladder such as sensory nerve fibers, myofibroblast and smooth muscle cells. Autonomic nervous system coordinates normal bladder functioning by the effect of mediators released from urothelium. Muscularis propria consists of detrusor smooth muscle which are single nucleated spindle shaped cells. The smooth muscles are arranged in three layers in muscularis propria, in the inner most and the outer most layer, the muscles are arranged longitudinally while middle layer has circularly arranged detrusor smooth muscle fibers (11, 12). Urine is continuously produced by the kidneys but voiding of urine is not a continuous process. It occurs eight times a day in healthy person. The urinary bladder in humans is able to adjust its volume from 0 ml to more than 500 ml without permitting rise in intravesical pressure (pressure within bladder), so that the bladder can store a large amount of urine. During bladder filling, the rise in the intravesical pressure does not exceed the filteration pressure, otherwise filteration process will stop leading to renal failure (3, 13). The filling phase is accompanished with a relaxation of the bladder along with high outflow resistance offered by the internal and external urethral sphincters. The voiding phase compromises of synchronous contraction of the bladder coupled with low outflow resistance. The autonomic and somatic nervous system coordinate smooth

and skeletal muscle contractile activity of the bladder and the urethra to govern both the filling and the voiding phases of the bladder (14).

2.2. Innervation of the Urinary Bladder

Three groups of nerves (parasympathetic, sympathetic and somatic) exitting from the sacral and thoracolumbar regions of the spinal cord, coordinate the filling and voiding phases of urinary bladder. The stimulation of sacral parasympathetic (pelvic) nerves lead to excitatory input (cholinergic and purinergic) to the bladder and inhibitory input (nitrergic) to the urethra. The stimulation of thoracolumbar sympathetic (hypogastric) nerves (nonadrenergic) leads to inhibitory input to the bladder, excitatory input to the urethra and bladder neck and inhibitory and facilitatory inputs to the parasympathetic ganglia that is presented in the bladder. The stimulation of sacral somatic (pudendal) nerves leads to activation of nicotinic receptors which causes the contraction of striated urethral sphincter (2, 15, 16).

Three group of nerves are responsible for conveying the sensory activity from bladder and urethra to central nervous system. Afferent fibers passing in the pelvic nerve to spinal cord initiate the micturition reflex. These include small myelinated (A_γ) and large unmyelinated (C) fibers which carry sensory impulses from stretch, volume and pain receptors present in the wall of urinary bladder. Both types of fibers are widely distributed in the urothelium, muscularis propria and serosa of bladder and urethra. The afferent fibers present in urothelium are sensitive to various chemicals present in urine and various neuropeptides such as nitric oxide (NO), adenosine triphosphate (ATP) and prostaglandins released by urothelium cells (2, 15, 16). $A\gamma$ afferent fibers carry impulses due to presence of harmful stimuli or distention of bladder. Large C afferent fibers specifically respond to chemical irritation of mucosa and to different types of neurotoxins, tachykinins, endothelins and neurotrophic factors released by urothelial and inflammatory cells. These fibers are not sensitive to distention of the bladder (17-19).

The bladder contracts via both cholinergic and nonadrenergic, noncholinergic (NANC) pathways in most animals. Acetylcholine is released by postganglionic parasympathetic nerves as well as by urothelium. The two muscarinic receptor

subtypes M_2 and M_3 have been found in detrusor smooth muscle of many animal species. Although M_2 receptor subtype is found more predominantly, M_3 receptors play a major role in bladder contraction. The activation of M_3 receptors lead to formation of inositol triphosphate (IP_3) and diaceglycerol (DAG). IP_3 causes a release of calcium from intracellular calcium stores while DAG activates protein kinase C (PKC) that plays a major role in calcium sensitization. Activation of M_2 receptors inhibit the sympathetically mediated detrusor smooth muscle depression by blocking adenylate cyclase, thus helping the M_3 receptor function. Muscarinic receptors have also been found prejunctionally on bladder cholinergic nerve terminals. Stimulation of M_1 prejunctional receptors promote acetylcholine release while stimulation of M_2/M_4 prejunctional receptors inhibit acetylcholine release (1, 2, 13, 14).

NANC pathways involve the release of ATP from parasympathetic nerves of the bladder and also from urothelial cells. Urothelial cells release ATP in response to distention of bladder to initiate voiding. ATP stimulates the bladder contraction by acting on ligand gated cation channel receptors P2X, located in the bladder wall (20, 21).

Lower urinary tract also contains G-protein coupled α and β adrenergic receptors. The activation of α_1 adrenergic receptors causes a contraction in the bladder urethral sphincter while the stimulation of β_2 and β_3 receptors relax the bladder. Thus, these receptors help bladder to store urine effectively (2, 22).

Central nervous system controls the functions of bladder by a number of neurotransmittors including acetylcholine, norepinephrine, dopamine, serotonin, glutamic acid, gamma aminobutyric acid, glycine, ATP, NO, substance P, neurokinin A, calcitonin gene-related peptide, vasoactive intestinal polypeptide (VIP), and enkephalins. Dopamine inhibits the micturition reflex by acting on D_1 receptor while facilitates voiding by stimulating D_5 receptors in the brain. Serotonin stimulates the bladder contraction. Glutamate stimulates where as GABA and glycine both inhibit the micturition reflex pathway. Substance P, neurokinin A, calcitonin gene- related peptide and VIP are released from bladder afferent neurons and play role in inflammation. Enkephalins suppress the micturition reflex (2, 23).

2.3. Interstitial Cystitis

Abnormal changes in bladder function cause increased urgency, frequency and incontinence of voiding (11). Overactive bladder (OAB) is a complex chronic disease represented by involuntary detrusor smooth muscle overactivity during storage of urine (24). It has been found that detrusor smooth muscle overactivity is also increased in interstitial cystitis. However, pelvic pain is generally absent in OAB patients (25). Interstitial cystitis is a syndrome of bladder hypersensitivity which is manifested by bladder sensation and fullness, i.e. urgency for urination even at low volume of urine (26).

Interstitial cystitis or bladder pain syndrome is a condition that is defined by lower abdominal pain and some non specific symptoms such as increased frequency and urgency of urine, often of unknown etiology (27). It may be characterized pathologically by infection, urothelial dysfunction, autoimmunity and inflammation and mast cell activation. One or more observation of these mechanisms ultimately cause cystitis (28). Viral or bacterial infection of the bladder often leads to prevalence of interstitial cystitis symptoms (29). Urothelial dysfunction occurs due to loss of tight junctions between glycosaminoglycans which break down the urothelial permeability barrier. Increased passage of solutes and toxins to the bladder wall takes place through broken urothelium (30). Autoimmune mechanisms involve an increased number of antinuclear antibodies which then bind to the bladder epithelial surface (31). Upregulation of the sensory nerves and the proliferative factors cause lower abdominal pain and then a dysregulation of voiding. Besides, an over stimulation of the mast cells releases substance P which ultimately increases infilteration of lymphocytes (32, 33).

A number of chemicals have been used to induce interstitial cystitis in animals for research purposes. Among these, cyclophosphamide (CYP) and iphosphamide are preferred to induce interstitial cystitis in animal models. Mostly commonly, interstitial cystitis models in mice and rats have been produced by a systemic injection of CYP (34). Studies showed that one of the metabolic end products of CYP, acrolein, is a causative agent of inducing interstitial cystitis. Acrolein is thought to be precipitated massively in urinary bladder. It then crosses the urothelium and enters the epithelial cells where it generates free radicals such as reactive oxygen and nitric oxide species.

Peroxynitrites are produced by nitric oxide species. Increased levels of peroxynitrite damages DNA, lipids and proteins causing necrotic cell death (35). Thus, acrolein produces edema, haemorrhage and ulceration of urinary bladder wall (36, 37). Injection of cyclophosphamide also produces urinary bladder epithelial damage, haemorrhage, edema and inflammatory cells infilteration (35, 38, 39).

2.4. Sphingosine 1-Phosphate

Mammalian cell membrane contains sphingolipids as one of the common component of lipid bilayer. Sphingolipids are metabolized to ceramide by sphingomyelinases. Ceramide, in the presence of ceramidase, is converted to sphingosine which is then phosphorylated to S1P by sphingosine kinase-1 (SK1) and sphingosine kinase-2 (SK2). S1P is then degraded by S1P phosphatases and S1P lyase. The level of S1P in cells is controlled by S1P synthetic enzymes. S1P phosphatases dephosphorylate S1P back to sphingosine which then may be converted to ceramide by ceramide synthase. S1P lyase catabolizes S1P into ethanolamine 1-phosphate and hexadecanal (40-42). S1P, a bioactive lipid, supports cell growth and discourages programmed cell death while ceramide and sphingosine act inversely (43). SK1 and SK2 are located within the cell as well as extracellularly. SK1 is found in cell cytoplasm whereas SK2 is found in endoplasmic reticulum, nucleus and cytoplasm as well. SKs can be released from cells either constitutively or under abnormal conditions. SK1 can be released from fibroblasts to outside of the cell under cellular stress. Macrophages are sensitive to oxidized low density lipoprotein immune complex and they release SK1. Once released from cells, SK acts on sphingosine that is present on the plasma membrane, converting it into S1P. SK1 and SK2 act on same substrate and yield same product but activation of them produce entirely different functions. SK1 activation leads to cell growth promotion and inhibition of apoptosis while SK2 helps in apoptosis and inhibits cell growth. Various agonists such as GPCR ligands, cytokines, growth factors and lipopolysaccharides activate sphingosine kinase 1 gene (SphK1) to produce SK1 (44-46). S1P may act intracellularly or extracellularly by stimulating GPCRs. Calcium mobilization and cell proliferation and survival is induced by S1P intracellularly while extracellular functions involve cell migration, development and maturation of blood vessels (47).
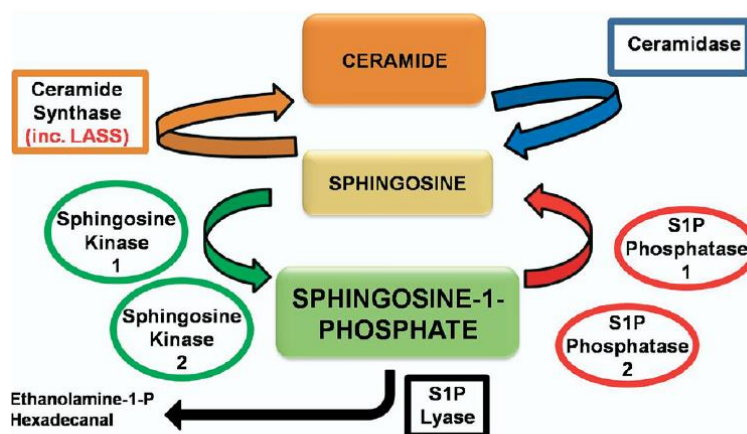
Figure 2.4 a Synthesis and degradation of Sphingosine 1-phosphate (48).

S1P is widely distributed in serum and plasma under physiological conditions. S1P stimulates specific five G-protein coupled receptors (S1PR$_{1-5}$) which belong to endothelial differentiation (EDG) gene receptors family. S1P$_1$ receptors are coupled to G$_i$ proteins while S1P$_4$ and S1P$_5$ receptors are G$_{i/o}$ proteins coupled receptors. Both S1P$_2$ and S1P$_3$ receptors are G$_{i/o}$, G$_q$, and G$_{12/13}$ proteins coupled receptors. Stimulation of these receptors initiate intracellular signalling pathways depending upon the type of G-protein. S1P$_1$ receptors are expressed on endothelial cells and are responsible for cell migration and development and maturation of blood vessels. Activation of S1P$_1$ receptors stimulate G$_i$ proteins which are responsible for extracellular signal regulated kinase activation, adenyl cyclase inhibition and activation of endothelial NO synthase. S1P$_2$ and S1P$_3$ receptors have been found in heart, lung, kidney, urinary bladder and brain tissues. Both are responsible for vasoconstriction and airway and visceral smooth muscle contraction. S1P$_2$ and S1P$_3$ receptors are G$_q$ proteins coupled, leading to activation of phospholipase C which produces IP$_3$ and DAG. Activation of G$_{12}$ and G$_{13}$ proteins cause Rho activation by S1P$_2$ and S1P$_3$ receptors. S1P$_4$ receptors are located in lymphoid and hematopoietic tissues while S1P$_5$ are expressed in spleen and brain white matter. S1P$_4$ and S1P$_5$ receptors have been recently identified so much less is known about them (5, 49-53).

S1P$_1$, S1P$_2$ and S1P$_3$ receptors have been widely distributed in vascular and nonvascular smooth muscles of the body. They are now well known for regulating the myogenic tone. Depending upon the diversity of these receptors, S1P produces a variable role in smooth muscle activity. Calcium is a secondary messenger required

for muscle contraction. Studies have shown that S1P plays a role in calcium homeostasis in smooth muscle cells. It induces contraction of gastrointestinal, airway, vascular and urinary bladder smooth muscle (5, 54). S1P induces esophageal smooth muscle contraction by stimulating $S1P_2$ receptors. PLC and PKC has been involved in contraction mechanism (55). S1P increases intracellular calcium by activating voltage gated calcium channels, IP_3 dependent intracellular calcium stores and Rho kinase pathway in airway smooth muscle. The airway smooth muscle contraction induced by S1P is independent of PKC and ERK1/2 which usually control airway smooth muscle tone. Airway hyperreactivity is induced by S1P as it enhances airway smooth muscle contractility initiated by agonists. Activation of RhoA/Rho-Kinase pathway by S1P in airway smooth muscle causes calcium sensitization by inhibiting myosin light chain phosphatase (MLCP). S1P may play a role in the etiology of asthma (56, 57).

S1P acts on $S1P_1$ receptors that present in the endothelial lining of blood vessels generating PLC and IP_3. IP_3 then releases calcium from IP_3 sensitive intracellular stores found on the endoplasmic reticulum, which in turn cause calcium induced calcium release from ryanodine sensitive stores (58), but mitochondrial calcium stores remain insensitive to cytosolic calcium and do not release calcium (59). By activating $S1P_2$ receptors in lymph vessels, S1P increases intracellular calcium by IP_3 formation. This effect does not depend upon generation of NO and prostaglandins (60). S1P controls vascular smooth muscle tone by activating calcium sensitization via Rho-associated protein kinase (ROCK) and NO in α-toxin permeabilized maternal arteries during pregnancy (61), by RhoA/Rho-kinase in gracilic muscle resistance arteries (62), basilar arteries (63) but calcium sensitization is not involved in vasoconstriction by S1P in β-permeabilized coronary arteries. S1P releases calcium from intracellular calcium stores only (64). When coronary artery smooth muscles are exposed to radiolabelled sphingosine, it is converted into S1P intracellularly and cause vasoconstriction by Rho pathway. Exposure to exogenous radiolabelled S1P does not produce vasoconstriction as it is rapidly degraded by phosphatases that are found on the membrane (65). S1P cause vascular smooth muscle contraction of retinal arteries by activating $S1P_2$ receptors, involving calcium sensitization mainly by Rho kinase and partially PKC pathways (66). By stimulating $S1P_3$ receptors, S1P produces PLC

and IP$_3$ leading to increase in intracellular calcium and vasoconstriction of cerebral arteries (67, 68), aorta (54) and coronary arteries (69).

S1P activates S1P$_3$ receptors leading an increase in intracellular calcium by an influx of calcium from outside the cell and by a release from ER stores in keratinocytes (70), where by IP$_3$ independent stores in saponin-permeabilized swiss T3 cells (71) and in permeabilized DDT1MF-2 smooth muscle cell line (72). When intact HEK-293, SKNMC and HepG2 cells have been exposed to caged S1P, photolysis of caged S1P produces S1P with in the cell that regulate calcium homeostais but S1P exposed to same cells, exogenously, does not play a role in calcium mobilization. Thus, S1P may control calcium within cell independent of GPCRs (73). Myoblastic cell contraction takes place in calcium independent but Rho kinase dependent pathway by S1P (74, 75). S1P induced ER released calcium activates transcription factor NF-kB in U937 cells (75). S1P may act as calcium influx factor. It has been found that depletion of intracellular calcium stores trigger formation of S1P within cell that helps in influx of calcium via store operated calcium channels (SOCC) in neutrophils. S1P activates some SOCC by unknown mechanism (76). Acetylcholine, tyrosine kinase receptor and antigen receptor activation stimulate SK1 producing intracellular S1P. Similarly, exogenously applied S1P itself, by activating intracellular Gi coupled receptors triggers S1P production that regulates calcium levels in HEK-293 cells (77).

S1P induces detrusor smooth muscle contraction by fast release of calcium from internal stores by stimulating S1P$_2$ receptors. Voltage-dependent L-type calcium channels play a role in S1P-induced detrusor smooth muscle contraction (5). S1P$_1$, S1P$_2$ and S1P$_3$ receptors have been found throughout the lower urinary tract of female rat but the ratio of expression of these receptors varies. S1P-induced detrusor smooth muscle contraction is mediated by both S1P$_2$ and S1P$_3$ receptors involving PLC and Rho-kinase pathways (48). S1P signalling pathway is significantly upregulated in partial urethral obstruction in male rats with the activation of RhoA/ROK pathway (6). S1P induces rabbit bladder smooth muscle contraction by activating S1P$_2$ receptors. S1P$_2$ receptors activate PKC and Rho-kinase to increase calcium sensitivity by phosphorylating CPI-17 and inhibiting MLCP (78). The level of SK-1 expression has been increased in patients with neurogenic detrusor over activity (79).
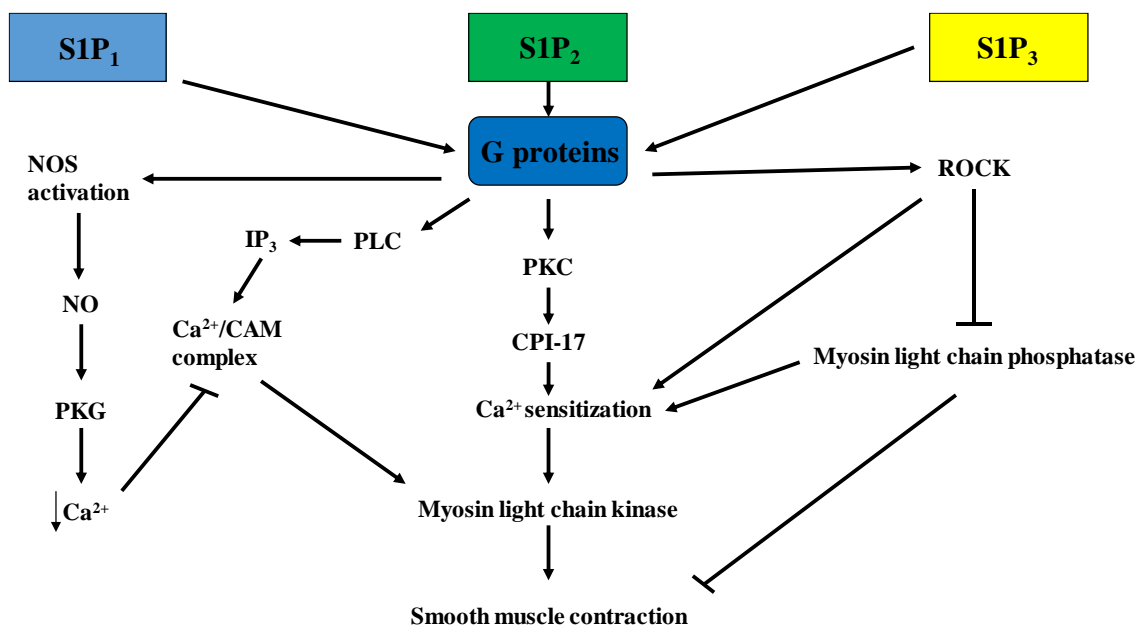
Figure 2.4. b Three main subtypes of sphingosine 1-phosphate receptors and their role in smooth muscle contractility.

2.5. Mechanism of Smooth Muscle Contraction and Calcium Sensitization

Smooth muscle cells line the walls of various hollow organs or tubes in the body. Smooth muscle cell layers are found in stomach, blood vessels, airway, urinary bladder, uterus and penile and clitoral cavernosal sinuses. A number of hormones, autocrine/paracrine agents and local chemical signals regulate the smooth muscle contraction. Variations in length or load produce tonic or phasic smooth muscle contraction (80). Pharmacomechanical and electromechanical coupling control the smooth muscle contractile activity. Pharmacomechanical coupling functions by various receptors mediated intracellular signalling by production of IP_3 and by changing MLC phosphorylation. Electromechanical coupling functions by changing membrane potential of smooth muscle cells. Activation of both pathways ultimately lead in increase intracellular free calcium. The increase of intracellular free calcium is a major secondary messenger responsible for smooth muscle contraction (81). There are two sources of rise in intracellular calcium either by influx of calcium from extracellular space or by release of calcium from intracellular stores. Influx of calcium

from extracellular space involves voltage operated calcium channels (VOCCs) or non-voltage gated channels. Non-voltage gated channels include receptor operated calcium channels (ROCCs) and store operated calcium channels (SOCCs). Pharmacomechanical coupling triggers rise in intracellular calcium by release of calcium from intracellular stores and by influx of calcium via ROCCs and SOCCs. Agonists which act on GPCRs activate ROCCs while depletion of intracellular stores (sarcoplasmic reticulum) activates SOCCs inducing smooth muscle contraction. Electromechanical coupling mediates a rise in intracellular calcium by membrane depolarization leading to opening of VOCCs. Various hormones and neurotransmitters depolarize muscle membrane to open VOCCs and cause contraction of smooth muscles (82). Intracellular calcium stores include sarcoplasmic reticulum (SR) and lysosome related organelles. SR contains IP_3 and ryanodine receptors. IP_3 receptors are activated by IP_3 formed by a number of agonists acting on GPCRs while ryanodine receptors are stimulated by rise in cytosolic calcium (calcium induced calcium release). Recent studies have shown that there are two other intracellular calcium mobilizing messengers nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine diphosphate ribose (cADPR) which take place in smooth muscle contraction. NAADP is formed by a reaction between nicotinic acid and NADP by ADP-ribosyl cyclases which belong to a family of enzymes that include CD38 and CD157. A number of hormones, vasoconstrictors and endothelin-1 triggers NAADP production. NAADP acts on two pore channels (TPCs) that present on lysosome related organelles and release calcium. cADPR acts on ryanodine receptors on SR and releases calcium. This intracellular free calcium forms calcium-calmodulin complex by binding with acidic calmodulin. Calcium-calmodulin complex activates the myosin light chain kinase (MLCK) that phosphorylates and activates the myosin light chain (MLC) which on activation, binds with actin to initiate smooth muscle contraction. Myosin light chain phosphatase (MLCP) dephosphorylates and inactivates the MLC. Thus, smooth muscle contraction is determined by the balance between MLCK and MLCP (83).

Intracellular calcium and corresponding contractile force does not go parallel always. When GPCR agonists activate IP_3 formation and subsequent rise in intracellular calcium, the ratio of force/calcium during contraction is greater than that

triggered by high potassium. It may be associated with G protein mediated change of calcium sensitivity of regulatory and contractile proteins or MLCP may play role in calcium sensitization or desensitization of contractile apparatus (84). A physiological phenomenon that inhibits MLCP causing leftward shift of the calcium-force response curve at constant calcium and MLCK level is known as calcium sensitization (85).

The major component of physiological calcium sensitization is RhoA. RhoA is a small monomeric G-protein that belongs to Rho subfamily of the Ras family of G-proteins. It may be GDP-bound (inactive state) or GTP-bound (active state). Inactive form of RhoA is bound with a protein guanine nucleotide dissociation inhibitor (GDI) by its lipid tail in the cytoplasm. In the presence of guanine nucleotide exchange factors (GEFs), activation of RhoA takes place by exchanging GDP with GTP. Active RhoA (GTP-bound) binds with plasma membrane by its lipid tail and activates cytoplasmic Rho kinase. Activated Rho kinase inhibits MLCP and induces calcium sensitization. GTPase activity of RhoA converts active RhoA back to inactive RhoA, a process also enhanced by GTPase-activating proteins (GAPs) (86).

MLCP is a trimeric enzyme, consisting of a catalytic 37-38 kDa PP1c, an associated 110-130 kDa regulatory targeting subunit (MYPT1), and a tightly bound 20-kDa subunit of unknown function. Phosphorylation of MYPTI or PPIc play role in inducing calcium sensitization. Rho kinase inhibits MLCP by phosphorylating the MYPT1 component. Thus, Rho kinase leads to increased phosphorylated form of MLC which increases the contraction of smooth muscle at constant calcium level is called calcium sensitization. (86).

The other mediator involved in physiological calcium sensitization is CPI-17. CPI-17 is a 17 kDa endogenous peptide. Different types of kinases such as PKC phosphorylates CPI-17 to active form which then phosphorylates the PPIc component of MLCP to inhibit MLCP activity. Thus, PKC acts through CPI-17 leading to increased MLC phosphorylated form, ultimately increased muscle contraction by calcium sensitization mechanism (9).
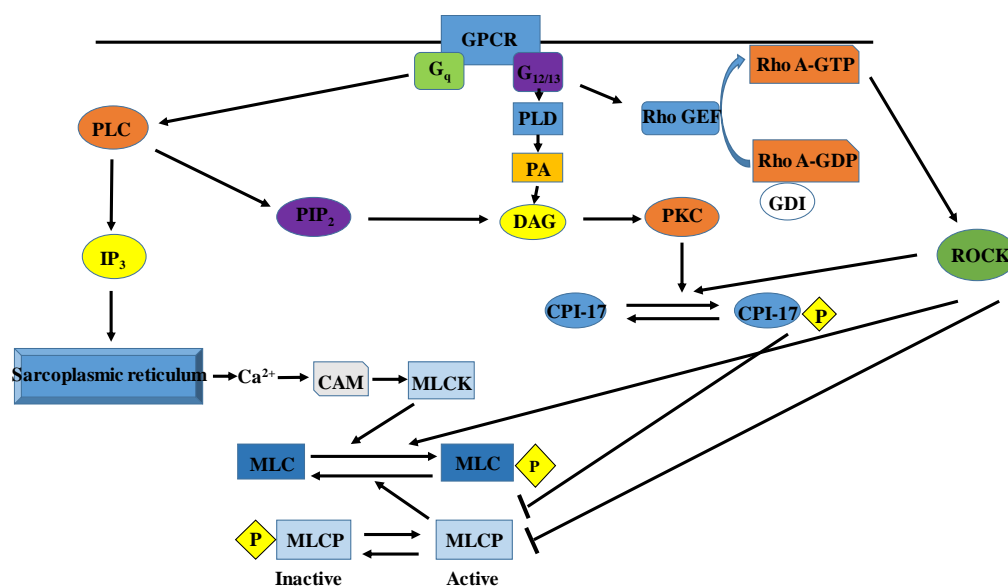
Figure 2.5. Mechanism of smooth muscle contraction and calcium sensitization.

2.6. Cyclic Adenosine Diphosphate Ribose (cADPR) and Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP)

Recent studies have shown that in addition to IP$_3$, the two other intracellular calcium mobilizing secondary messengers are cADPR and NAADP (87). cADPR and NAADP mediated calcium release was first found in sea urchin eggs (88) followed by in mammalian cells and smooth muscles (89, 90) later. cADPR and NAADP both are metabolic products of nicotinamide adenine dinucleotide (NAD). NAD acts as a cofactor (an acceptor or donor of hydrogen atoms) during a variety of reactions catalyzed by oxidoreductases (91). ADP-ribosyl cyclase removes nicotinamide from β-NAD to form cADPR (92-95). Soluble ADP-ribosyl cyclase was first isolated from the ovotestis of Aplysia californica. Later CD38, a lymphocyte antigen, has been found as a mammalian analogue of Aplysia cyclase (96, 97). Aplysia cyclase and CD38 also synthesize NAADP by replacing nicotinic acid with nicotinamide from β-NADP$^+$, a base-exchange reaction that takes place at acidic pH only. cADPR and NAADP are broken down by hydrolytic enzymes ultimately (98, 99).

It has been found that cADPR and NAADP secondary messengers are present in a number of body systems where they facilitate contraction of vascular and non-vascular smooth muscle. cADPR is involved in cardiac smooth muscle contraction under physiological conditions. It increases sensitivity of calcium induce calcium

release (CICR) to calcium (100). Ryanodine receptors (RyRs) release calcium by cADPR activated by muscarinic receptor agonist in coronary arteries and by CICR caused by NAADP in arterial smooth muscle cells (101, 102). α-adrenoceptor induced vascular smooth muscle contraction of aorta has been reduced in $CD38^{-/-}$ gene mice. cADPR enhances agonist induced aortic smooth muscle contraction (103). There is co-localization between lysosomes and SR region of RyR in pulmonary arteries as calcium released by NAADP from lysosomes cause CICR from RyR only (104).

Muscarinic receptor stimulated tracheal smooth muscle contraction also involves cADPR and NAADP pathways (105, 106). Similarly, oxytocin mediated uterine smooth muscle contraction depends on the activation of cADPR and NAADP messengers (107, 108).

Postaganglionic nerve stimulation of urinary bladder releases β-NAD, cADPR and ADPR indicating the presence of cADPR system (109). β-NAD has inhibitory effect on detrusor smooth muscle (110). Spontaneous calcium oscillations are related to influx of calcium from T- and L-type of calcium channels which are controlled by intracellular calcium stores including acidic stores in urinary bladder (111). FKBP12.6 protein is compulsory for the action of cADPR to release calcium from RyR_2. As it can not bind to RyR_2 directly so it binds with FKBP12.6 which is associated with RyR_2 in urinary bladder smooth muscle (112). NAADP mediated contractions are blocked by bafilomycin in permeabilized detrusor smooth muscle from wild type mice but with same preparations from $Tpcn^{-/-}$ mice, NAADP was unable to mediate contractions, however IP_3 mediated contractions remain unaffected. Muscarinic receptor activated contractions are dependent on SR and acidic stores in wild type mice but contractions induced by muscarinic receptor activation depends on SR stores only in $Tpcn^{-/-}$ mice bladder (113). Exogenously applied cADPR increases spontaneous release of ATP which synergizes the contractile response of cADPR in detrusor smooth muscles (114).
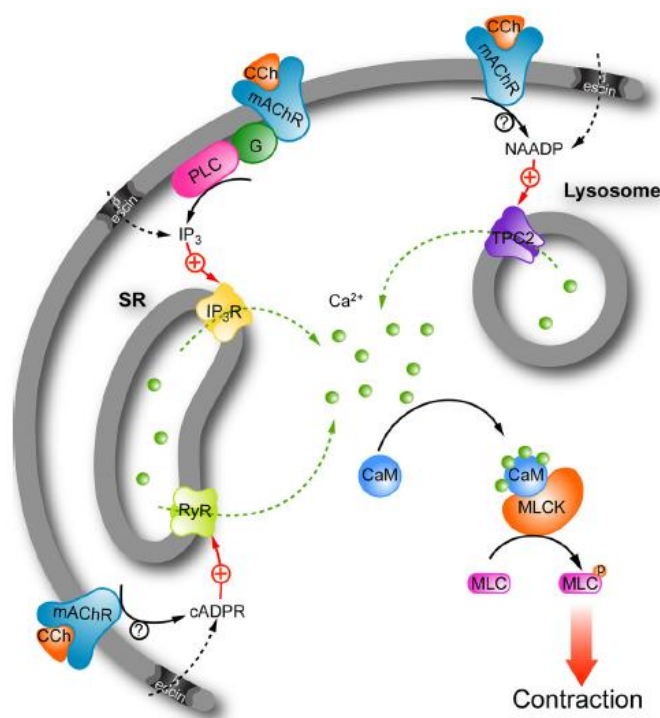
Figure 2.6. Receptor-mediated Ca^{2+} signaling pathways in detrusor smooth muscle. Muscarinic receptor activation causes release of Ca^{2+} from both sarcoplasmic reticulum and lysosomal stores (113).

2.7. Chemical Permeabilization of Smooth Muscles

Chemical permeabilization of smooth muscles helps to determine the importance of many intracellular elements which are involved in smooth muscle contractile activity. The role of calcium sensitization to regulatory and contractile proteins, calmodulin and possible sites of action of drugs can be evaluated by using chemical permeabilization technique. This technique also assists to differentiate intracellular from extracellular calcium sources, which take part in smooth muscle contraction stimulated by different agonists. Chemically permeabilized smooth muscle preparation enables to control intracellular calcium at a constant level. This technique is practicable to study the mechanism of calcium sensitization in a number of diseases (8).

A number of chemicals have been used for the permeabilization of smooth muscles. These include saponin, α-toxin, triton X-100 and β-escin. The size of plasma membrane pores, effect on intracellular calcium depots and receptor-effector coupling vary with all these permeabilizing chemicals (7).

Saponin makes complex with cell membrane cholesterol and cholesterol-micelles disturb the plasma membrane lipid bilayer. Intracellular calcium homeostasis and mobilization can be studied as saponin does not affect sarcoplasmic reticulum and mitochondrial membrane. The drawback of saponin is uncoupling of receptors from their effector proteins (7, 115, 116).

α-toxin is a cytolytic protein produced by Staphylococcus aureus. It forms hexamers with other toxins substances on the cell surface and inserts into plasma membrane making pores of 2-3 nm diameter. There is no loss of necessary contractile and regulatory proteins and receptor-effector coupling remains unaffected by using α-toxin (117, 118).

Triton X-100 damages the whole cell membrane functions and uncouples receptors from their respective effector proteins (119, 120).

β-escin forms larger pores than any other permeabilizing agent. Upto 150 kDa of proteins can easily cross pores formed by β-escin. It does not affect receptor-effector coupling. There is loss of guanosine triphosphate (GTP) and calmodulin from the cell so these should be provided exogenously. The advantages include the passage of IP3 and high molecular weight heparin through these pores to inside the cell (7, 121).

3. MATERIALS and METHODS

3.1. Animal Welfare and Ethical Statement

The study protocols were approved by the Hacettepe University Animal Ethics Committee (2014/34-6) and (2016/03-04). Female Sprague Dawley rats (200-250 g), kept under 12-h light/dark period with food and water ad libitum, were used.

3.2. Experimental model

Cyclophosphamide (CYP, 150 mg/kg, dissolved in saline) was injected into rats intraperitoneal once a day on days 1, 4 and 7 to induce interstitial cystitis. Control groups were injected with saline (0.9% NaCl), in respectively. On the 8th day, rats were used in studies.

Day 1	**Day 4**	**Day 7**	**Day 8**
			Detrusor smooth muscle
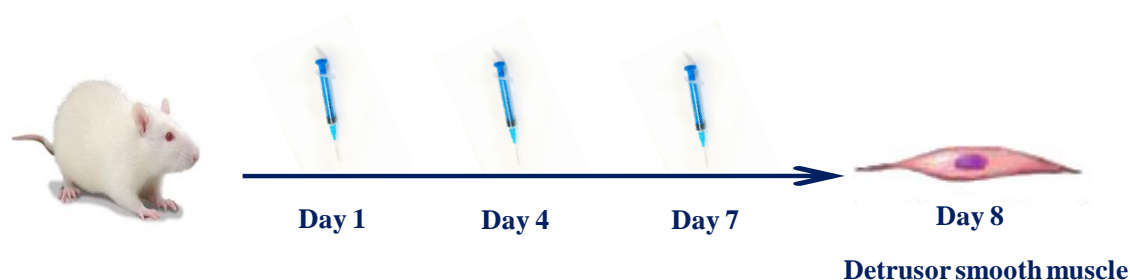
Figure 3.2. Demonstration of intraperitoneal injection once a day on first, 4th and 7th day to induce interstitial cystitis.

3.3. Histopathological analysis

Rats were euthanized by carbon dioxide inhalation and were killed by carotid artery bleeding. The urinary bladders were carefully removed on 8th day after CYP injection to assess bladder histopathology. Tissue samples were fixed in 10% formaldehyde for at least 24 h at room temperature. Following fixation, tissue samples were embedded in paraffin, sectioned (5 μm slices) and stained with hematoxylin and eosin. Histopathological damage was evaluated based on epithelial damage, hemorrhage, inflammatory cell infiltration and edema.

3.4. Tissue preparation

Rats were euthanized by carbon dioxide inhalation and were killed by carotid artery bleeding. The urinary bladder was isolated and placed in Hepes buffered modified Krebs' solution. The mucosa and connective tissues were removed from the bladder under a dissecting microscope. Small strips (150–250 μm in diameter, 3–4 mm in length) of smooth muscle were dissected from the urinary bladder. A small hook was tied to one end of a strip to attach it to the transducer, and a snare of 5/0 surgical silk captured the other end and was used to mount the strip in a fixed position in 1 ml chamber in one of a series of small chambers in a Perspex block. The chamber was filled with Hepes buffered modified Krebs' solution at room temperature and the strips were equilibrated for 30 min under a resting tension of 100 mg. Solution changes were made by moving the Perspex block. The contractile force was measured by a sensitive force transducer (Swema, Stocholm, Sweden) connected to a computer using Biopac Student Lab Pro 3.7.3 (Commat Ltd, Turkey) software.

Detrusor smooth muscle

150-250 µm diameter, 3-4 mm long

Hepes buffered Krebs' solution at room temperature
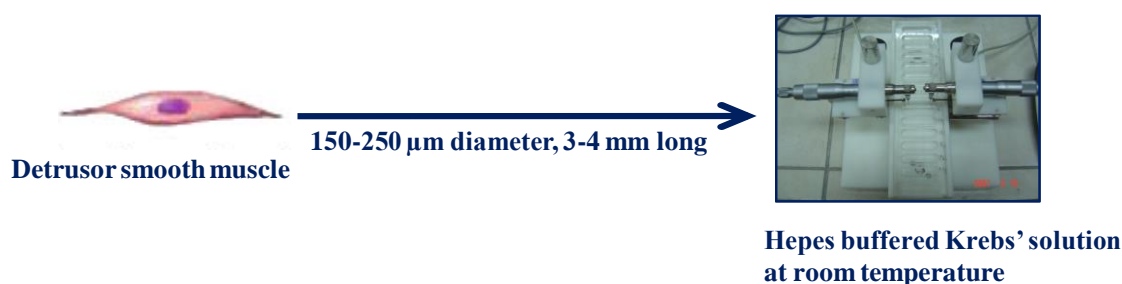
Figure 3.4. Small detrusor smooth muscle strips were hanged in myograph under 100 mg basal tension.

3.5. Experimental Protocols

Stable responses were achieved to 80 mM K^+ and 50 μM carbachol in intact strips. Strips were then moved into relaxing solution containing 4 mM EGTA with 1 minute interval for 2 times. Then the strips were permeabilized with 40 μM β-escin in relaxing solution for 30 minutes at pH 6.8. After that the strips were washed in relaxing solution containing 4 mM EGTA for 1 minute and in relaxing solution containing 0.05 mM EGTA solution for 3 times with 1 minute interval. Strips were accepted as permeabilized if the maximum tension obtained by 100 μM calcium after

permeabilization was found to be greater than the tension produced by 80 mM K^+ in same strip before permeabilization (122).

After permeabilization, the strips were incubated with 1 μM calcium (prepared in 0.05 mM EGTA) and calmodulin (1 μM) for 10 minutes to load the intracellular stores with calcium. GTP (100 μM) was added to bath and waited for 3 minutes. Then, S1P (50 μM) response was obtained. After permeabilization, strips were incubated with $S1P_2$ receptor inhibitor JTE-013 (10 μM), $S1P_3$ receptor inhibitor suramin (50 μM), Rho kinase inhibitor Y-27632 (1 μM), protein kinase C inhibitor GF-109203X (5 μM), ryanodine (10 μM), IP_3 inhibitor heparin (1mg/ml), nicotinic acid adenine dinucleotide phosphate (NAADP) inhibitor bafilomycin (100 nM), NAADP (100 μM), sarcoplasmic reticulum calcium-ATPase pump inhibitor cyclopiazonic acid (CPA) (1 μM), CPA (1 μM) + ryanodine (10 μM), bafilomycin (100 nM) + NAADP (100 μM) for 15 minutes in 0.05 mM EGTA and same procedure was repeated.

After intracellular calcium stores were loaded by activating solution by 1 μM calcium in the presence of both CPA (1 μM) and mitochondrial proton pump inhibitor carbonyl cyanide p-trifluromethoxyphenylhydrazone (FCCP; 1 μM) for 10 minutes to induce calcium sensitization, a contractile response was elicited by S1P (50 μM) in the presence of GTP (100 μM). The same procedure was repeated with Rho kinase inhibitor Y-27632 (1 μM), protein kinase C inhibitor GF-109203X (5 μM) and GTP-β-S (1 μM) after 15 minutes incubation.

3.6. Western Blotting

Freshly isolated rat bladders were immersed in liquid nitrogen to snap freeze. ~ 50 mg solid tissue was pulverized under liquid nitrogen and the powder homogenized with an electric homogenizer in 15 ml of ice cold lysis buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris pH 8.0). Samples were centrifuged for 20 min at 12,000 rpm at 4°C in a micro centrifuge. Total amount of proteins (~20 μl) were resolved by SDS-PAGE and transferred to a PVDF membrane and detected using rabbit polyclonal antibodies raised against EDG-5 (Santa Cruz/sc-25491, 1/200) and EDG-3 (Santa Cruz/sc-30024, 1/200). Additionally, EDG-5 immunoblots were stripped off (1 ml 20 % SDS + 9 ml TBST + 70 μl β-mercaptoethanol) for 30 min at

50ºC and then reprobed to visualize β-actin using mouse monoclonal antibody (Santa Cruz/sc-47778, 1/1000). ECL was used to analyse protein expression levels.

3.7. Solutions and drugs used in experiments

All solutions and drugs were prepared by using 18.2 MΩ-cm deionized water except FCCP, S1P, CPA, ryanodine and GF-109203X. FCCP was dissolved in ethanol, S1P and ryanodine were dissolved in methanol and CPA and GF-109203X were dissolved in DMSO but none of the vehicle affected the contractile response when tested alone.

3.8. Solution used for intact tissues

Hepes buffered modified Krebs' solution contained (mM) NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14 and HEPES 10.5. The pH was adjusted to 7.2 with NaOH. 80 mM K^+ Krebs' solution was prepared by replacing NaCl with an equivalent amount of KCl.

3.9. Solutions used for permeabilized tissues

Relaxing solution contained (mM) K propionate 130; $MgCl_2$ 4; Na_2ATP 4; tris-maleate 20; creatine phosphate 10; EGTA 4 and creatine phosphokinase 3.3 units/ml; protease inhibitor leupeptin (1 μM). The pH of this solution was adjusted to 6.8 with KOH. Activating solutions were the same as relaxing solution except that EGTA was lowered to 0.05 mM, free Ca^{2+} concentration was adjusted to the desired value and calmodulin (1 μM) added as specified. GTP (100 μM) was also added when S1P was used to activate the S1P receptors. The free Ca^{2+} concentration was calculated using a computer programme (123). When drugs were added to an organ chamber, they were made up in relaxing solution containing 0.05 mM EGTA, and the concentration given was the estimated final concentration.

3.10. Drugs used

Drugs were β-escin, carbamylcholine chloride (carbachol), sphingosine 1-phosphate (S1P), JTE-013, suramin, NAADP, ryanodine, heparin sodium salt, creatine phosphokinase, leupeptin, ethylene glycol-bis(β-aminoethylether)-N ,N ,N ′,N′-

tetraacetic acid (EGTA), adenosine 5′triphosphate (Na_2ATP), GTP, CPA, calmodulin, FCCP, dimethylsulphoxide (DMSO) from Sigma (St. Louis, Missouri) and creatine phosphate disodium salt, bafilomycin A1, (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxyamide 2HCl (Y-27632) and bisindolylmaleimide 1 HCl (GF-109203X) from Calbiochem (Nottingham, UK), and Endoxan (Cyclophosphamide, 1g) from Pfizer.

3.11. Data Analysis

Contractions were expressed as % of the response to 80 mM K^+ elicited in intact tissues before permeabilization. Data were given as mean ± s.e.m. of n experiments. Statistical analyses were carried out by using unpaired *Student's t* test for comparing two groups. *P*<0.05 was accepted as statistically significant.

4. RESULTS

4.1. Histopathological Changes in Rat Bladder having Cyclophosphamide-Induced Cystitis

Haemorrhage in mucosa and muscle (muscularis propria) layer, and inflammatory cell infiltration in subepithelial layer were observed in bladder isolated from rats in CYP-induced cystitis group as compared to control group (Figure 4.1).
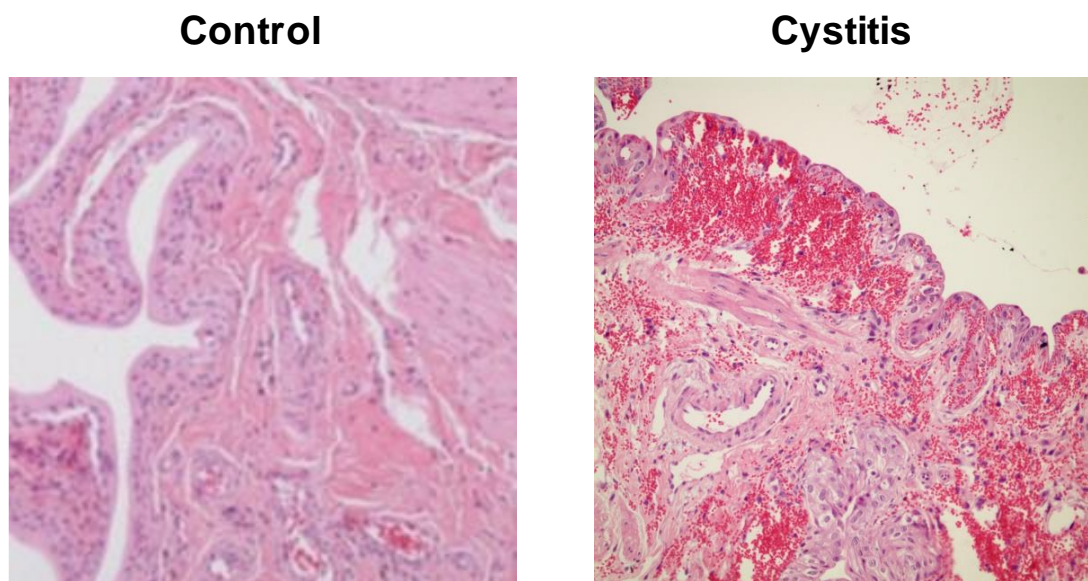
Control   **Cystitis**

Figure 4.1. Histopathological images of bladder isolated from rats of control and cystitis group after hematoxylin and eosin staining.

4.2. S1P-Induced Contraction

Rat bladder smooth muscle strips were mounted for isometric force recording under the basal tension of 100 mg and were subjected to S1P (50 μM) or solvent alone (methanol) after permeabilization. S1P produced an initial fast phasic contraction that is followed by a very slow but a sustained contraction yielding tonic response in rat detrusor smooth muscles (Figure 4.2A). S1P-induced contraction was increased significantly (42.90 ± 5.98, n=8) in bladder smooth muscle strips isolated from CYP-treated group of rats as compared to control group (20.07 ± 1.51, n=11) as shown in figure 4.2B.

A
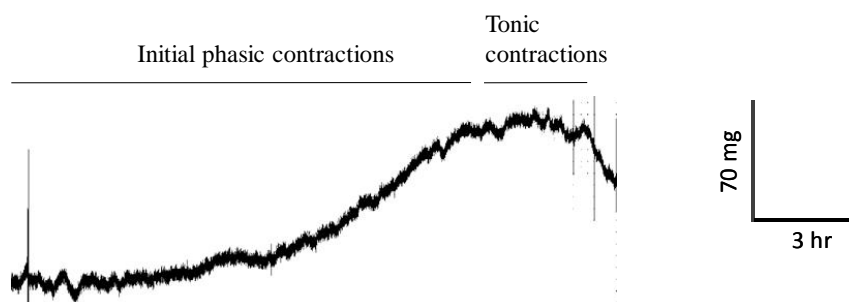
B

Figure 4.2. Contraction of permeabilized detrusor smooth muscle by S1P (50 μM) (A). The contractile response elicited with S1P (50 μM) in permeabilized detrusor smooth muscle isolated from control and CYP- treated group of rats (B) (*$P<0.05$ compared to control group; n=8-11).

4.3. The Role of S1P Receptor Subtypes in S1P-Induced Detrusor Smooth Muscle Contraction

S1P produces its effects by activating its GPCRs. In order to investigate the receptor subtypes involved in S1P-induced detrusor muscle contraction, $S1P_2$ selective receptor antagonist JTE-013 and $S1P_3$ selective receptor antagonist suramin were used. After permeabilization, detrusor smooth muscle strips were incubated with JTE-013 (10 μM) and suramin (50 μM) for 15 minutes and then strips were subjected to S1P (50 μM). S1P- induced detrusor contraction was significantly reduced in control (13.01 ± 1.39, n=7) and CYP-treated group of rats (17.38 ± 4.92 n=6) by $S1P_2$ selective receptor antagonist JTE-013. S1P-induced detrusor contraction was also reduced in both control (12.10 ± 2.43, n=6) and cystitis group of rats (13.47 ± 3.65, n=6) by $S1P_3$ selective receptor antagonist suramin as shown in figure 4.3A and 4.3B.

A

B
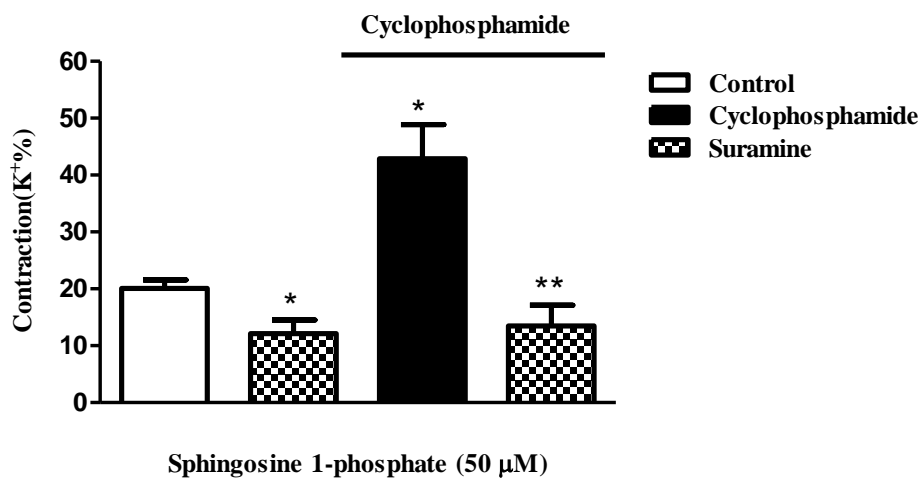
Figure 4.3. The contractile response elicited with S1P (50 µM) in the absence and presence of JTE-013 (10 µM) (A) and suramin (50 µM) (B) in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*$P<0.05$ compared to control response, **$P<0.05$ compared to CYP group; n=6-7).

4.4. Expressions of $S1P_2$ and $S1P_3$ Receptor Proteins

Densitometric quantification of western blots indicated that $S1P_2$ (EDG-5) and $S1P_3$ (EDG-3) protein expression were significantly increased in CYP-treated group of rat bladders when compared to controls (Figure 4.4A and 4.4B).

A   B
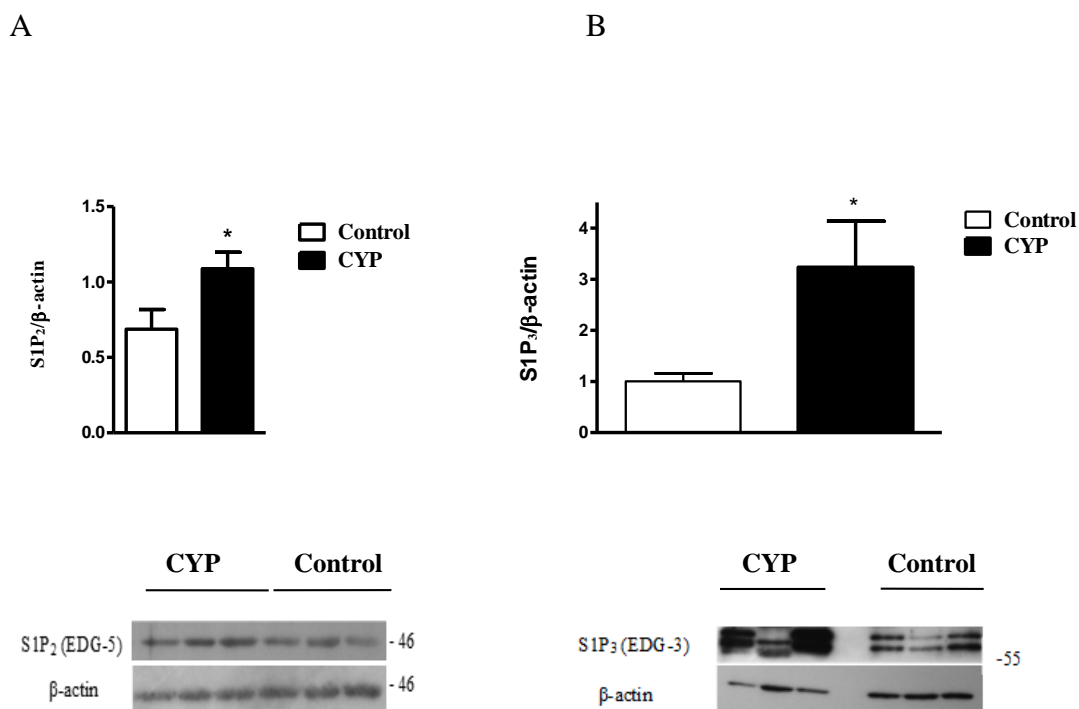
Figure 4.4. Western blots for $S1P_2$ (EDG-5) (A) and $S1P_3$ (EDG-3) (B) in control and CYP-treated group rat bladders detected using rabbit polyclonal antibodies. EDG-5 and EDG-3 protein levels were normalized to β-actin protein levels detected by the mouse monoclonal antibody (*$P<0.05$ compared to control group; n=3-4).

4.5. Effect of Y-27632 or GF-109203X on S1P-Induced Contraction

S1P-induced detrusor smooth muscle contraction was also obtained in the presence of ROCK inhibitor Y-27632 (1 µM) and PKC inhibitor GF-109203X (5 µM). S1P-induced contraction was significantly decreased in control (13.44 ± 2.39, n=6) and CYP-treated group of rats (12.51 ± 1.39, n=6) by ROCK inhibitor. S1P-induced contractile response was also inhibited in both control (12.18 ± 1.32, n=6) and cystitis group of rats (8.82 ± 1.66, n=6) by PKC inhibitor as shown in figure 4.5A and 4.5B.

A
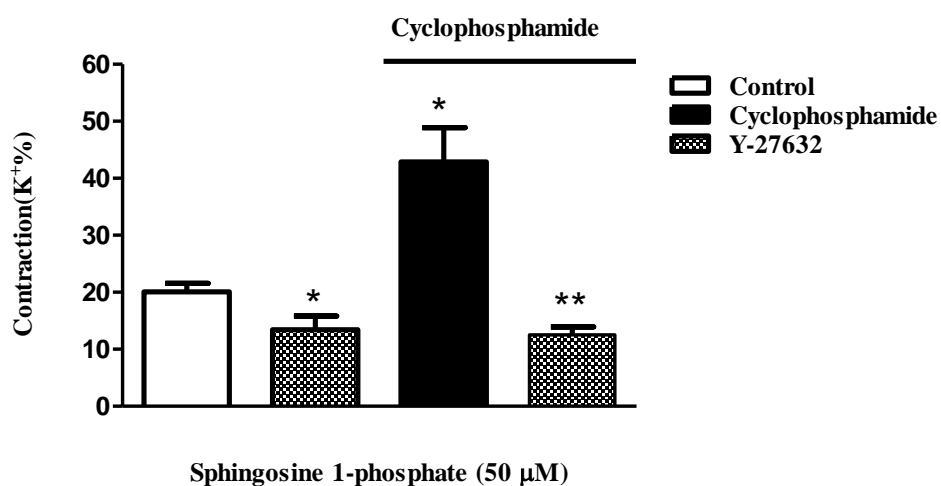
B
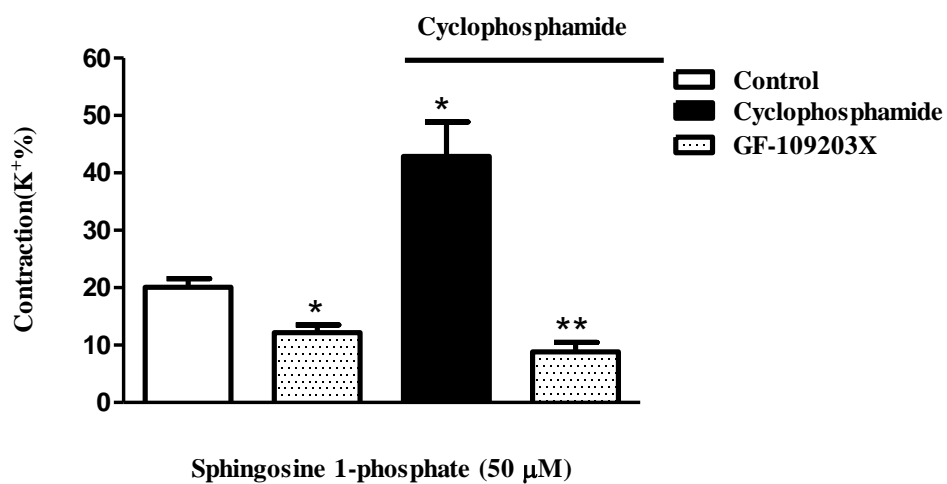
Figure 4.5. The contractile response elicited with S1P (50 μM) in the absence and presence of Y-27632 (1μM) (A) and (B) GF-109203X (5 μM) in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*P<0.05 compared to control response, **P<0.05 compare to CYP group; n=6-8).

4.6. S1P-Induced Calcium Sensitization

S1P-induced calcium sensitization response elicited in the presence of sarcoplasmic reticulum calcium-ATPase pump inhibitor CPA (1 μM) and mitochondrial blocker FCCP (1 μM) was increased (16.52 ± 2.64, n=6) in permeabilized detrusor smooth muscle isolated from CYP-treated group of rats compared to control group (8.37 ± 1.01, n=6) as shown in figure 4.6.
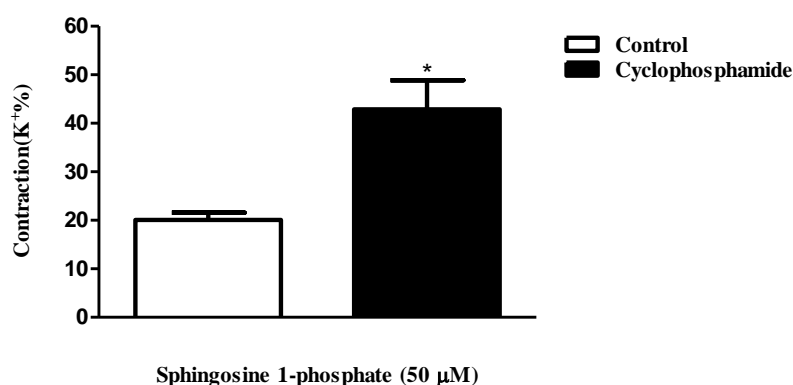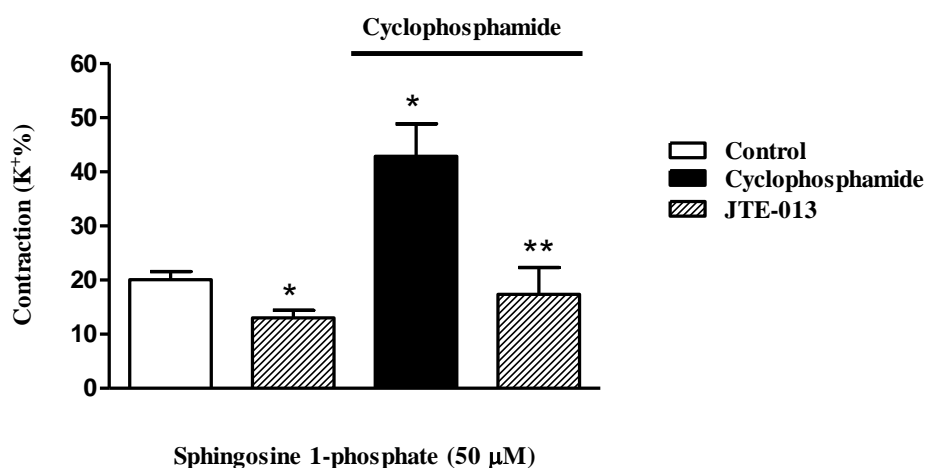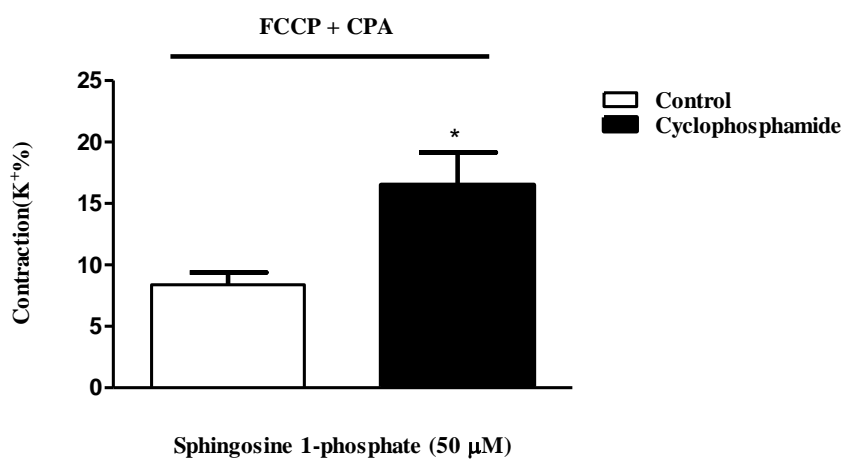

Figure 4.6. S1P (50 μM)-induced calcium sensitization response in permeabilized detrusor muscle isolated from control and CYP-treated group of rats (*P<0.05 compared to control group; n=6).

4.7. Effect of Y-27632 or GF-109203X or GTP-β-S on S1P-Induced Calcium Sensitization

The calcium sensitization response in control group was increased significantly with ROCK inhibitor Y-27632 (14.62 ± 2.063, n=6) and PKC inhibitor GF-109203X (13.54 ± 1.245, n=6) and G-protein inhibitor GTP-β-S (17.61 ± 2.02, n=8) but were inhibited in CYP-treated group of rats (11.53 ± 1.702, n=6), (8.23 ± 1.29, n=6), (9.83 ± 1.40, n=6) in respectively as shown in figure 4.7A, 4.7B and 4.7C reaching back to control group response.

A

Sphingosine 1-phosphate (50 μM)

B

FCCP + CPA

C

FCCP+CPA
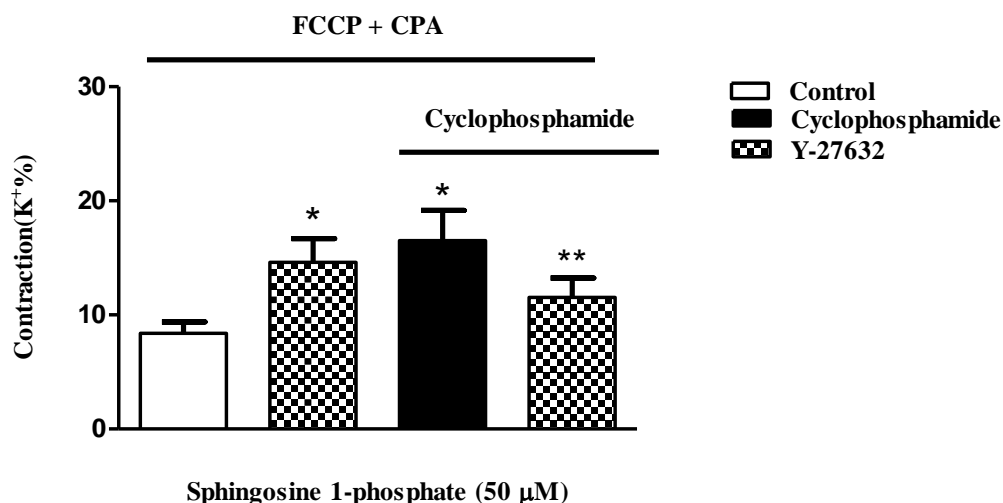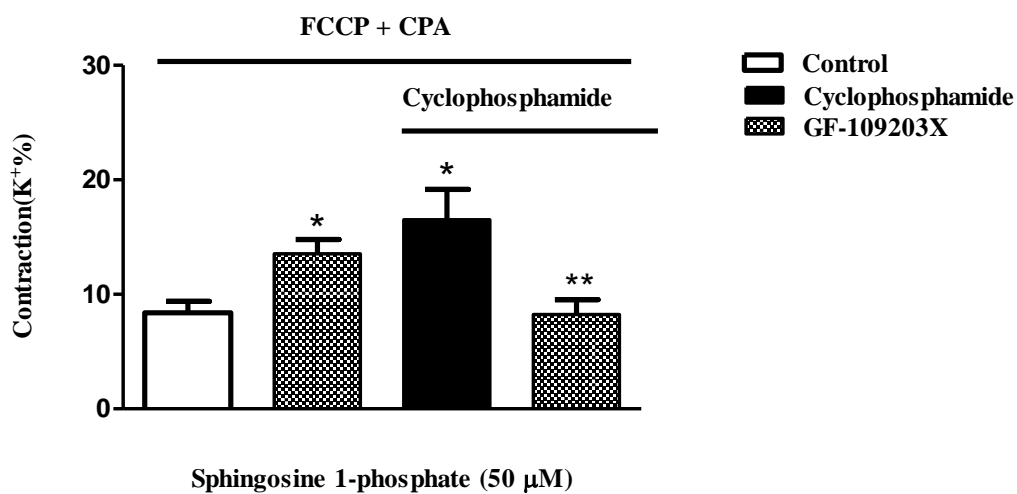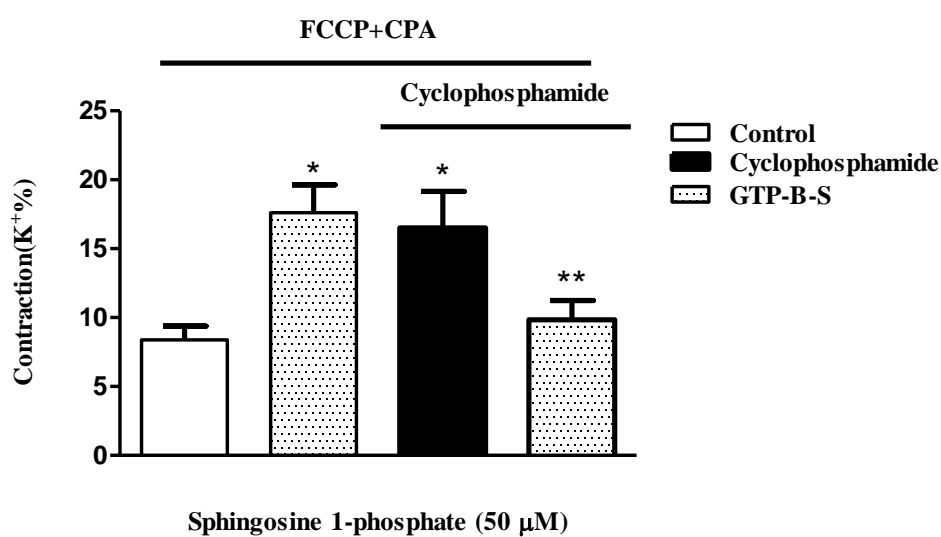
Figure 4.7. The calcium sensitization response elicited with S1P (50 μM) in the presence of Y-27632 (1 μM) (A) or GF-109203X (5 μM) (B) or GTP-β-S (1 μM) (C) in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*$P<0.05$ compared to control response, **$P<0.05$ compare to CYP group; n=6).

4.8. Effect of Ryanodine and CPA on S1P-Induced Detrusor Smooth Muscle Contraction

S1P-induced detrusor smooth muscle contraction was obtained in the presence of sarcoplasmic reticulum calcium-ATPase pump inhibitor CPA (1 µM) or sarcoplasmic reticulum channel inhibitor, ryanodine (10 µM) and combination of both inhibitors. S1P-induced contraction was decreased significantly in both control (9.578 ± 1.533, 11.13 ± 1.665, n=4-7) and CYP-treated group of rats (11.59 ± 2.507, 11.76 ± 2.308, n=4-5) by CPA and ryanodine respectively (Figure 4.8).
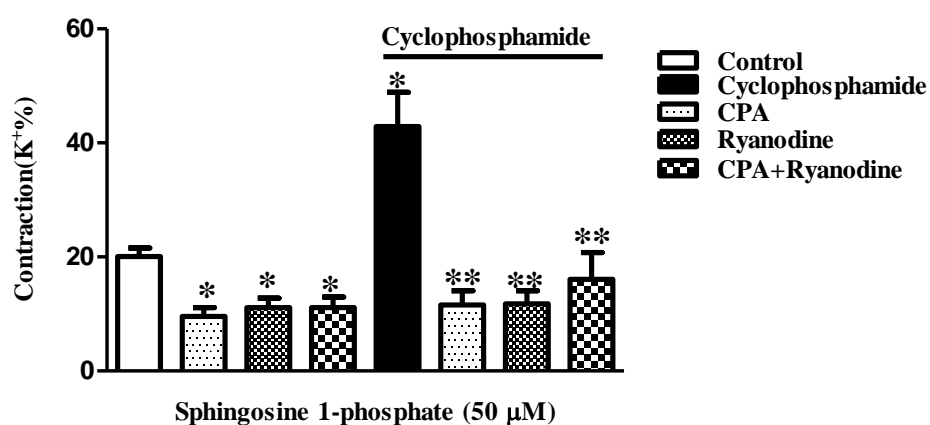
Figure 4.8. The contractile response elicited with S1P (50 µM) in the absence and presence of CPA(1 µM) or ryanodine (10 µM) and in combination of both in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*$P<0.05$ compared to control response, **$P<0.05$ compared to CYP group; n=4-11).

4.9. Effect of Heparin on S1P-Induced Detrusor Smooth Muscle Contraction

S1P-induced detrusor smooth muscle contraction was obtained in the presence of IP$_3$ receptor blocker heparin (1mg/ml). S1P-induced contraction was significantly decreased in both control (10.68 ± 1.048, n=6) and CYP-treated group of rats (11.90 ± 2.519, n=6) by heparin as shown in figure 4.9.
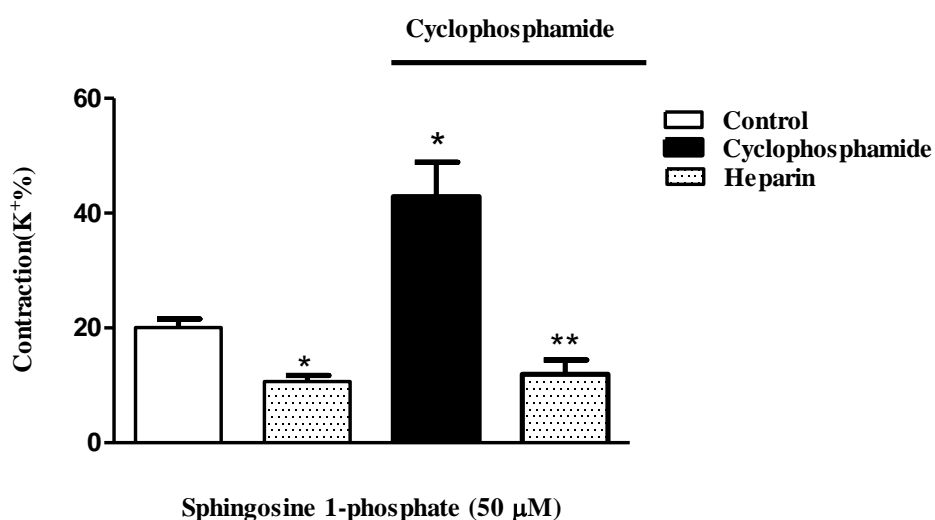
Figure 4.9. The contractile response elicited with S1P (50 µM) in the absence and presence of heparin (1mg/ml) in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*P<0.05 compared to control response, **P<0.05 compared to CYP group; n=6-11).

4.10. Effect of Bafilomycin and NAADP on S1P-Induced Detrusor Smooth Muscle Contraction

Bafilomycin, a vacuolar H^+-ATPase inhibitor, which abolishes calcium storage in acidic stores such as lysosomes. At higher concentration, NAADP (1-100 μM) desensitizes NAADP receptors failing to initiate Ca^{2+} release (124). Bafilomycin (100 nM) or NAADP (100 μM) and combination of both of these were used to elucidate the role of calcium storing lysosome related organelles in S1P-induced contractile response. S1P-induced contraction was significantly decreased in both control (8.878 ± 1.99, 12.08 ± 1.991, n=5-6) and CYP-treated group of rats (12.06 ± 3.92, 11.83 ± 2.346, n=6) by bafilomycin and NAADP respectively as shown in figure 4.10.
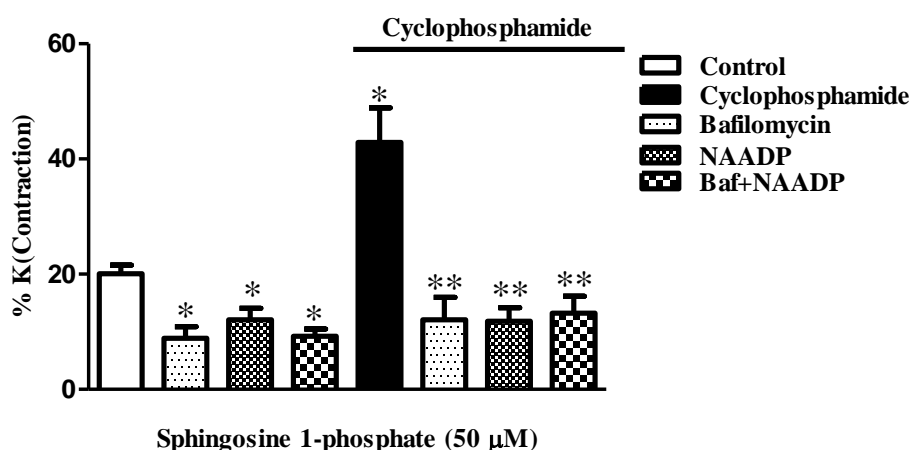
Figure 4.10. The contractile response elicited with S1P (50 μM) in the absence and presence of bafilomycin (100 nM) or NAADP (100 μM) and combination of both in permeabilized detrusor smooth muscle isolated from control and CYP-treated group of rats (*P<0.05 compared to control response, **P<0.05 compared to CYP group; n=5-11).

5. DISCUSSION

The contractile activity of detrusor smooth muscle is predominantly regulated by acetylcholine but nonadrenergic, noncholinergic (NANC) mechanisms also have small contributions (125). Recently, it has been shown that a new mediator of NANC pathway, S1P is also involved in detrusor smooth muscle contraction (5). In the present study, S1P-induced detrusor smooth muscle contractile response in female Sprague Dawley rats was evaluated. This response was also observed in male Sprague Dawley rats (6) although some researchers could not get S1P contractile response in other species of rat (78). Our finding that S1P-induced contractile force in female rats was lower than that of carbachol is also consistent with those previous studies in rabbits (5, 78).

The role of calcium sensitization pathways, contractile proteins and intracellular stores in action of agonists can be evaluated by using chemical permeabilization technique. Chemically permeabilized smooth muscle preparation enables to control intracellular calcium at a constant level (8). Among the permeabilization chemicals, β-escin, while not affecting receptor-effector coupling, forms large pores so that up to 150 kDa of proteins such as IP_3 and heparin can easily cross membrane permeabilized by this agent (7, 118, 121). There is a loss of GTP and calmodulin from the cells during permeabilization with β-escin so these should be provided exogenously.

Abnormal changes in bladder function cause increased urgency, frequency and incontinence of urine (1). Overactive bladder (OAB) is a complex chronic disease represented by involuntary detrusor smooth muscle overactivity during the storage of urine (24). It has been found that detrusor smooth muscle overactivity is also increased in interstitial cystitis. However, pelvic pain is generally absent in OAB patients. Interstitial cystitis is a syndrome of bladder hypersensitivity that is characterized by inflammation, urgency, frequency, painful urination and fullness (26). It is caused either by bacterial infection or by noninfectious conditions, mostly of unknown etiology (11). A number of animal models of interstitial cystitis have been used over a long period of time. Interstitial cystitis can be induced in animals by intravesical instillation of chemicals or lipopolysaccharides obtained from bacterial origin (126).

Systemic injection of CYP in rats and mice has been widely used to induce interstitial cystitis. The metabolic product of CYP, acrolein enters into epithelial cells of urinary bladder where it activates production of reactive oxygen species and nitric oxide species. Nitric oxide species lead to peroxynitrite production. Increased level of peroxynitrite damages DNA, lipids and proteins causing necrotic cell death (35). Thus, acrolein produces edema, hemorrhage and ulceration of urinary bladder wall (36, 37). In this study, epithelial damage, edema, mucosal and submucosal hemorrhage and subepithelial inflammatory cells infiltration after hematoxylin and eosin staining were observed in isolated urinary bladder of rats treated with cyclophosphamide (shown in figure 4.1), confirming histopathologically that our injection protocols induced cystitis. These observations were in parallel with previous literature findings (35, 38, 39).

It is widely known that interstitial cystitis changes detrusor smooth muscle contractile function and disturbs the normal functioning of urinary bladder. S1P produced an initial phasic contraction and this was followed by a consistent tonic contraction of permeabilized detrusor smooth muscle. Tonic contractions were observed over a long period as being lipid, S1P may be difficult to wash out. This result is in parallel with the previous studies (5). As given in figure 4.2, S1P-mediated contractile response was increased more than two folds in cystitis group compared with control. The increased contraction elicited by S1P in interstitial cystitis group showed that detrusor smooth muscle was more responsive to S1P, in accordance with that observed in partial urethral obstructive rat model (6). Both interstitial cystitis and partial urethral obstruction are manifested by overactive bladder tone. It has been demonstrated that detrusor smooth muscle contractile response to carbachol was also increased in *Escherichia coli*-induced cystitis (127). Moreover, previous literature has shown an increase in urination reflex in male rat models of interstitial cystitis induced by cyclophosphamide (128).

S1P produces its effects by stimulating its receptors. There are 5 subtypes of receptors in S1P receptor family but $S1P_1$, $S1P_2$ and $S1P_3$ receptors have been widely distributed in liver, lung, kidney, urinary bladder and brain tissues (48, 129). $S1P_1$ receptors are coupled to G_i protein and activation leads to an increase in intracellular

calcium in endothelial cells (129) but not in other tissues (130-132). $S1P_2$ and $S1P_3$ receptors are coupled to G_q protein and activation leads to an increase in intracellular calcium via release from intracellular calcium stores (69, 130, 131). All three main receptors $S1P_1$, $S1P_2$ and $S1P_3$ have been found in lower urinary tract of female Sprague Dawley rats (48). Higher $S1P_1$ receptors have been found in urethra, and are thought to modulate NO activity. NO facilitates micturition by relaxing urethral smooth muscles (133). We have investigated the S1P receptor subtypes in S1P-mediated contraction response in permeabilized smooth muscle strips by incubation with either $S1P_2$ receptor-specific antagonist, JTE-013 or $S1P_3$ receptor-specific antagonist, suramin. Reduction in S1P-mediated contraction by both $S1P_2$ receptor antagonist and $S1P_3$ receptor antagonist (35% and 40%, respectively) showed that both receptors are involved in S1P-mediated detrusor smooth muscle contraction. Moreover, inhibition of S1P-induced contractile response by these antagonists was more pronounced in cystitis group when compared to that of control group; i.e. $S1P_2$ receptor was blocked by 60% and $S1P_3$ receptor was inhibited by 69%. Previous literature has shown that both $S1P_2$ and $S1P_3$ receptors are responsible for urinary bladder contraction induced by S1P (4, 48, 78). S1P mediates contraction of smooth muscles of airway (57), gastric (134) and a vascular cells preparation (135) by $S1P_2$ receptors and vasoconstriction of cerebral arteries (67, 68), aorta (54), and coronary arteries (69) by activating $S1P_3$ receptors. S1P activates $S1P_3$ receptors leading to increase in intracellular calcium by influx of calcium from outside the cell and by release from ER stores in keratinocytes (70, 71), by IP_3 independent stores in saponin-permeabilized swiss 3T3 cells and in permeabilized DDT1MF-2 smooth muscle cell line (72). Upregulation of proteinase activated receptors, tryptase, β-nerve growth factor, nitric oxide synthase, nuclear transcription factor, phosphodiesterase 1C and cAMP-dependent protein kinase were found in animal models of interstitial cystitis (26). Therefore, protein expression of both $S1P_2$ and $S1P_3$ receptors was also studied by Western blotting and found to be increased at the molecular level, supporting our functional data with $S1P_2$ and $S1P_3$ receptor antagonists. Moreover, increased expression of $S1P_2$ and $S1P_3$ receptors showed that cystitis upregulates S1P signaling pathway. These results are in parallel with other studies showing both $S1P_2$ and $S1P_3$ receptors are responsible for bladder contraction induced by S1P (5, 48, 78).

We also studied the intracellular mechanism of contractile response to S1P in permeabilized detrusor of rats with interstitial cystitis. The effects of ROCK and PKC inhibitors were evaluated both in control and cystitis groups. ROCK inhibitor Y-27632 inhibited S1P contraction by 33% where PKC inhibitor GF-109203X inhibited this response by 39% in control group, as shown in figure 4.5. Moreover, these inhibitors blocked the increased S1P-induced contraction in interstitial cystitis by 71% and 79%, respectively, showing that both pathways are included under our experimental conditions. It was shown that different methods of induction of inflammation in bladder might lead to different mechanism of dysfunction. Detrusor smooth muscle contraction is dependent on PKC pathway in rats having lipopolysaccharides-induced cystitis (127). On the other hand, ROCK pathway is involved in dysfunction and histopathological changes in rats with hydrochloric acid-induced cystitis (136). S1P receptors are coupled to $G_{i/o}$, G_q, G_{12} and G_{13} proteins but PLC-PKC pathway is activated by $S1P_3$ receptors and ROCK pathway is activated by mainly $S1P_2$ receptors (137, 138). The role of PKC and ROCK in S1P-induced contractions has been studied in vascular smooth muscles (135), airway smooth muscles (57), gastric smooth muscles (134) and bladder smooth muscles (5). Both PKC and ROCK pathways have been involved in S1P-induced detrusor smooth muscle contraction in rabbits but S1P-induced contractile force is mainly dependent on ROCK pathway (78). Inhibition by both PKC and ROCK may suggest a relationship between these two enzymes, as cross talk (139-141), which may also be a further investigation focus in our study.

The level of intracellular calcium and the contraction of smooth muscle does not coordinate in parallel always (84). It has been shown that the contraction of smooth muscle has also been related to calcium sensitization that does not depend on the level of intracellular calcium (142, 143). The physiological phenomenon that is responsible for the increase in smooth muscle contractile force by inhibition of MLCP at constant level of calcium and MLCK is known as *calcium sensitization* (85). Inhibition of MLCP takes place either by ROCK or CPI-17. ROCK phosphorylates the MYPT1 component while CPI-17, activated by PKC, phosphorylates the PPIc component of MLCP to inhibit MLCP activity (86). Under our experimental conditions in the presence of sarcoplasmic reticulum calcium-ATPase pump inhibitor CPA and mitochondrial blocker FCCP at constant calcium level, S1P-induced calcium

sensitization response was increased significantly in the cystitis group (97%, as shown in figure 4.6). The inhibition of this increased calcium sensitization response in cystitis by ROCK and PKC inhibitors showed the involvement of both pathways. However, S1P-induced calcium sensitization response in control group of rats was increased significantly by ROCK and PKC inhibitors. This increase in contractile response may either involve activation of some other contractile proteins or an increase in detrusor smooth muscle sensitivity to calcium. Whether GPCRs are involved in S1P-induced calcium sensitization response, G-protein inhibitor GTP-β-S has been used. Inhibition of S1P-induced response in cystitis shows the involvement of GPCRs in S1P-induced calcium sensitization response.

In recent years, two other major intracellular Ca^{2+} mobilizing messengers cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) were revealed to take place in the process of inducing contractile responses in muscle tissues, the former acting on the sarcoplasmic reticulum, the latter on lysosome-related organelles. Sarcoplasmic reticulum is the major source of calcium in detrusor smooth muscle, having ryanodine and IP_3 receptors. cADPR acts on the sarcoplasmic reticulum, likely by activating the ryanodine receptors. Ryanodine receptors are also activated by an increase in free calcium concentration (calcium induced calcium release) (144-146). In order to investigate the mechanism of calcium release from sarcoplasmic reticulum, the depletors of this store were applied. Thus, sarcoplasmic reticulum calcium-ATPase pump inhibitor CPA (1 μM) or calcium channel inhibitor ryanodine (10 μM) on S1P-induced contraction response were evaluated separately and together. S1P-induced contractile response was significantly reduced in both control (52%) as well as in cystitis group (73%) of rats by CPA. Furthermore, inhibition of S1P-induced contractile response was observed in control (45%) and cystitis group (73%) by ryanodine also. Inhibition was seen more in cystitis group in both cases. This finding not only shows the role of sarcoplasmic reticulum calcium source in S1P-induced contractile response but also emphasizes the involvement of cADPR in this contraction, both in control and cystitis group of rats. It has been previously shown that S1P mobilizes Ca^{2+} from intracellular stores primarily by Ca^{2+} induced and IP_3 induced Ca^{2+} release (58, 59). However, combination of CPA and ryanodine did not cause a further inhibition of S1P-induced contractile response,

perhaps suggesting a possible cross-talk between sarcoplasmic reticulum calcium release pathways, i.e. IP_3 and cADPR.

Activation of GPCRs activate IP_3 production in bladder and ultimately release calcium from IP_3 sensitive sarcoplasmic reticulum intracellular store, which also plays a role to induce smooth muscle contraction (145, 146). The role of IP_3 sensitive sarcoplasmic reticulum intracellular stores was determined by blocking IP_3 receptors with heparin (1 mg/ml). Heparin has a very high molecular weight but it passes through β-escin permeabilized smooth muscle membrane easily (121). S1P-induced contractile force was reduced by heparin in both control (46.9%) and CYP-treated group (72%) of rats as shown in figure 4.9. Our finding confirms the participation of IP_3 sensitive sarcoplasmic reticulum intracellular stores in S1P-induced contractile response in parallel with previous studies (56-58).

In order to reveal the importance of acidic lysosome-related organelles in S1P-induced contractile response that are also a source of intracellular calcium, effect of bafilomycin and high concentration of NAADP on S1P response were studied. Bafilomycin A1, a macrolide antibiotic, is a potent and specific vacuolar H^+-ATPase pump inhibitor of acidic lysosomes (147). It prevents calcium storage in lysosomal related organelles by inhibiting proton pump and by increasing lysosomal pH (148). NAADP acts on acidic Ca^{2+} storage organelles to induce Ca^{2+} mobilization in different types of cells (149). However, at higher concentrations, NAADP desensitizes NAADP receptors failing to initiate Ca^{2+} release (124). Bafilomycin (100 nM) and high concentration of NAADP (100 μM) reduced S1P-induced contractile response significantly in control (56%, 40%) and cystitis group (72%, 73%) of rats respectively as shown in figure 4.10. This finding supports the idea that acidic lysosome-related organelles also play a role in S1P-induced contractile response and NAADP is involved in S1P-induced contractile response in rat bladders with cystitis. It has been reported previously that bafilomycin inhibits NAADP-induced contractile response in mice detrusor smooth muscle (113). Moreover, failure of potentiation effect of combination of bafilomycin and NAADP on inhibition might be due to cross-talk between lysosome-related organelles and sarcoplasmic reticulum calcium release mechanisms, including IP_3-, cADPR- and NAADP-induced Ca^{2+} release.

6. CONCLUSION

Our findings provide the first evidence that S1P induces a contractile response in bladder detrusor smooth muscle permeabilized with β-escin in rats having interstitial cystitis induced by cyclophosphamide. S1P enhanced isometric contractile force and calcium sensitization response in our rat model of interstitial cystitis. Both of these responses induced by S1P were reduced by the inhibition of ROCK and PKC pathways, confirming the involvement of these two pathways under cystitis situations. S1P-induced contractile force was inhibited by $S1P_2$ and $S1P_3$ selective receptor antagonist proving that $S1P_2$ and $S1P_3$ are the primary receptors involved in S1P-induced contractile force in rat bladder smooth muscle. $S1P_2$ and $S1P_3$ receptor protein expression was increased in cystitis. It indicates S1P signalling pathway is significantly upregulated in CYP-induced cystitis. Furthermore, reduction of S1P-induced contractile response by sarcoplasmic reticulum calcium store depletors and calcium storing lysosome-related organelles inhibitors showed the involvement of these both intracellular stores in S1P-induced contraction.

In conclusion, the findings of this study may help enlighten the molecular mechanisms underlying the pathology of interstitial cystitis with different causes, i.e. cyclophosphamide treatment in oncology. Therefore, the investigation of intracellular mechanisms of mediators which have a role in detrusor innervation under pathologic conditions may be of importance in developing new drugs for the treatment of bladder dysfunction.

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8. ATTACHMENTS

ATTACHMENT .1.ARTICLE and PRESENTATIONS RELATED TO THESIS RESEARCH

National Presentations:

I.Anjum, M. Denizaltı, N. T. Durlu-Kandilci, İ. Şahin-Erdemli. Siklofosfamid ile sistit oluşturulmuş sıçanların detrusor düz kasında sfingosin-1-fosfat (S1P) ile indüklenen kasılma yanıtlarının incelenmesi. Türk Farmakoloji Derneği 23. Ulusal Farmakoloji Kongresi, 7-9 Eylül, 2015, Ankara (Poster presentation).

International Presentations:

I.Anjum, M. Denizalti, N. T. Durlu-Kandilci, I. Sahin-Erdemli. Sphingosine 1-phosphate (S1P)-induced contractile responses in detrusor smooth muscle of rats having cyclophosphamide-induced cystitis. British Pharmacological Society, 15-17 December, 2015, London, UK. (Poster presentation).

I.Anjum, M. Denizalti, N. T. Durlu-Kandilci, I. Sahin-Erdemli. Sphingosine-1-phosphate (S1P)-induced contractile response in detrusor smooth muscle of rats having cyclophosphamide induced cystitis. EPHAR 2016, 26-30 June, Istanbul, Turkey (Oral presentation).

I. Anjum, M. Denizalti, H. B. Kandilci, N. T. Durlu-Kandilci, I. Sahin-Erdemli. Enhancement of S1P-induced contractile response in detrusor smooth muscle of rats having cystitis. British Pharmacological Society, 13-15 December, 2016, London, UK. (Poster presentation).

Article:

I. Anjum, M. Denizalti, H. B. Kandilci, N. T. Durlu-Kandilci, I. Sahin-Erdemli. Enhancement of S1P-induced contractile response in detrusor smooth muscle of rats having cystitis. European Journal of Pharmacology, Submitted March 3, 2017 (Under Revision).

ATTACHMENT 2.ETHICS PERMISSIONS RELATED TO THESIS RESEARCH

T.C.
HACETTEPE ÜNİVERSİTESİ
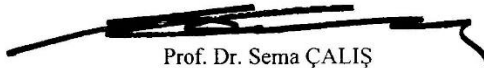Hayvan Deneyleri Yerel Etik Kurulu

Sayı : 52338575 – 56

25.04.2017

Doç. Dr. N. Tuğba KANDİLCİ
Eczacılık Fakültesi
Farmakoloji Anabilim Dalı
Öğretim Üyesi

Sayın Doç. Dr. KANDİLCİ

Kurulumuzun 10.06.2014 tarihli toplantısında 2014/34 kayıt numarası ile Etik Kurul onayı almış olan *"Siklofosfamid ile Sistik Oluşturulan Sıçanların Detrusor Düz Kasında Sfingosin-1-Fosfat (S1P) ile İndüklenen Kasılmaların Hücre İçi Mekanizmalarının İncelenmesi"* başlıklı proje için vermiş olduğunuz 25.04.2017 tarihli araştırmacı ve protokol revizyonu dilekçeniz Kurulumuzun 25.04.2017 tarihli toplantısında değerlendirilmiş ve araştırma ekibine Sağlık Bilimleri Enstitüsü Farmakoloji Programı Doktora öğrencisi Ecz. İrfan ANJUM' un dahil edilmesi **uygun bulunmuştur**.

Bilgilerinize rica ederim.

Prof. Dr. Sema ÇALIŞ
Başkan

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HACETTEPE ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU

Sayı: 52338575 -76

13 Haziran 2014

HAYVAN DENEYLERİ YEREL ETİK KURUL KARARI

TOPLANTI TARİHİ	: 10.06.2014 (SALI)
TOPLANTI SAYISI	: 2014/05
DOSYA KAYIT NUMARASI	: 2014/34
KARAR NUMARASI	: 2014/34-6
ARAŞTIRMA YÜRÜTÜCÜSÜ	: Doç.Dr.N. Tuğba KANDİLCİ
HAYVAN DENEYLERİNDEN SORUMLU ARAŞTIRMACI	: Uzm.Ecz.Merve DENİZALTI
YARDIMCI ARAŞTIRMACILAR	: Prof.Dr.İnci ERDEMLİ, Doç.Dr.N.Tuğba KANDİLCİ, Uzm.Ecz.Merve DENİZALTI
ONAYLANAN HAYVAN TÜRÜ ve SAYISI	: 84 Adet Dişi Sprague-Dawley Sıçan

Üniversitemiz Eczacılık Fakültesi Farmakoloji Anabilim Dalı öğretim üyelerinden Doç.Dr.N. Tuğba KANDİLCİ'nin araştırma yürütücüsü olduğu 2014/34 kayıt numaralı *"Siklofosfamid ile Sistik Oluşturulan Sıçanların Detrusor Düz Kasında Sfingosin-1-Fosfat (S1P) ile İndüklenen Kasılmaların Hücre İçi Mekanizmalarının İncelenmesi"* isimli çalışma Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak oy birliği ile onaylanmasına karar verilmiştir.

Sorumlu araştırmacı deneylere başlangıç tarihini Etik Kurula bildirmekle yükümlüdür.

Prof. Dr. Sema ÇALIŞ
Başkan

T.C.
HACETTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu

Sayı : 52338575— /5

HAYVAN DENEYLERİ YEREL ETİK KURUL KARARI

TOPLANTI TARİHİ	: 09.02.2016 (SALI)
TOPLANTI SAYISI	: 2016/01
DOSYA KAYIT NUMARASI	: 2016/03
KARAR NUMARASI	: 2016/03 – 04
ARAŞTIRMA YÜRÜTÜCÜSÜ	: Doç. Dr. N. Tuğba KANDİLCİ
HAYVAN DENEYLERİNDEN SORUMLU ARAŞTIRMACI	: Ecz. İrfan ANJUM, Doç. Dr. N. Tuğba KANDİLCİ Prof. Dr. İnci ERDEMLİ ve Dr. Ecz. Merve DENİZALTI
YARDIMCI ARAŞTIRMACILAR	: -
ONAYLANAN HAYVAN TÜRÜ ve SAYISI	: 84 adet dişi Sprague Dawley Sıçan

Üniversitemiz Eczacılık Fakültesi Farmakoloji Anabilim Dalı öğretim üyelerinden Doç. Dr. N. Tuğba KANDİLCİ'nin araştırma yürütücüsü olduğu 2016/03 kayıt numaralı *"Siklofosfamid ile Sistit Oluşturulan Sıçanların Detrusor Düz Kasında Sfingosin-1-Fosfat (S1P) ile İndüklenen Kasılma Yanıtlarında Siklik Adenozin Difosfat Riboz (cADPR) ve Nikotinik Asit Adenin Dinükleotid Fosfat (NAADP)'ın Etkisinin İncelenmesi"* isimli çalışma Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre uygun bulunarak oy birliği ile onaylanmasına karar verilmiştir.

Sorumlu araştırmacı deneylere başlangıç tarihini Etik Kurula bildirmekle yükümlüdür

Prof. Dr. Sema ÇALIŞ
Etik Kurul Başkanı
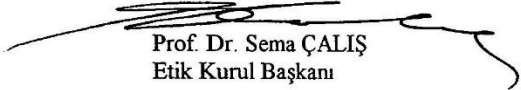
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Date of Birth : 23rd Oct 1984

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Nationality : Pakistani

Marital Status : Single

Languages : English, Turkish and Urdu

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Qualifications

Sr. No.	Name of Degree	Year	Marks /cGPA	Subjects
1	PhD	2017	3.67/4	Pharmacology
2	Pharm-D	2009	2955/4550	Pharmacy
3	H.S.S.C	2003	890/1100	Pre-med.
4	S.S.C	2001	736/850	Science

PhD Thesis Topic

Research work on 'Investigation of intracellular mechanisms of sphingosine-1-phosphate (S1P)-induced contractions in detrusor smooth muscle of rats having cyclophosphamide-induced cystitis'.

Experience

a) **Hospital Experience**

Worked as trainee clinical/retail pharmacist at Children's Hospital and Mayo Hospital Lahore during pharm-D graduation studies.

b)   Industrial Experience

Worked as Quality control analyst at Werrick Pharmaceutical, Islamabad. Responsibilities include in process and finished products testing, specifications and final proof reading of packing materials. (Sept. 2009 - April 2011).

Worked as internee at Ipram Pharmaceuticals, Rawat industrial area, Islamabad. (May 2009 - July 2009).

c)   Retail Experience

Worked as retail pharmacist at Olive Pharmacy, Summunabad, Lahore (June 2011 - Oct. 2011).

Member/Fellow of the Professional Societies

1. Registered as Pharmacist A in the Punjab Pharmacy Council, Lahore, Pakistan.

2. Member of Turkish Pharmacological Society

Conferences/Workshops/Courses Attended

1. Attended 6 months 'Experimental Use of Laboratory Animals Course' Hacettepe University, Ankara, Turkey (Oct. 2013-Feb. 2014).

2. Attended 4 weeks 'Laboratory Safety Education' workshop, Hacettepe University, Ankara, Turkey (April 2015).

3. Attended 23rd Turkish Pharmacological Society Congress, Ankara, Turkey (7-9 Sept. 2015) (Poster presentation).

4. Attended 1st National Pharmacy Education and Accreditation Congress, Hacettepe University, Ankara (9-10 May 2016).

5. Attended 7th European Congress of Pharmacology, Istanbul, Turkey (26-30 June 2016) (Oral Presentation).

Projects Completed

1. Investigation of intracellular mechanisms of sphingosine-1-phosphate (S1P)-induced contractions in detrusor smooth muscle of rats having cyclophosphamide-induced cystitis, 014D08301002 (15/08/ 2014).

2. Investigation of the role of protease activated receptors in detrusor smooth muscle contraction in interstitial cystitis model, THD-2016-10225 (24/08/2016).

3. Investigation of the effect of cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) on sphingosine 1-phosphate-induced contractile response in detrusor smooth muscle of rats having cyclophosphamaide-induced cystitis, THD201713342 (13/03/2017).