TRANSIENT RECEPTOR POTENTIAL FUNCTION IN BLADDER FROM CONTROL AND STREPTOZOTOCIN TREATED RATS

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Abstract

Diabetic cystopathy is a chronic and common complication of diabetes with a classical triad of symptoms; decreased bladder sensation, increased bladder capacity and impaired detrusor muscle contractility (Hunter and Moore, 2003). In animal models of diabetes such as streptozotocin-induced diabetes in the rat, abnormalities of bladder function have been reported (Longhurst and Belis, 1986). The prototypic TRPV channel, TRPV1, is activated by capsaicin, which has been shown to cause contraction of the rat bladder (Saitoh *et al*., 2007), and this is reduced in STZ-diabetic rat bladder (Pinna *et al*., 1994). Therefore we hypothesize that TRPV1 function will be reduced in the diabetic bladder.

The aim of this study are the following: Firstly, to investigate the effect of the streptozotocin (STZ) model of diabetes on a range of TRP channel functions in the urinary bladder smooth muscle preparation using TRP channel agonists and antagonists and to study the neurotransmitters involved in the contractile or relaxant responses. Some studies were also performed on colon tissues. Secondly, to explore the involvement of cholesterol modudation in TRP channel signalling. Thirdly, to study the change in TRP channel response with time following the treatment with streptozotocin.

The results showed that the contractile responses to the TRPV1 agonist capsaicin, TRPV4 agonist 4-α-PDD, and TRPA1 agonist allyl isothiocyanate were significantly reduced in diabetic bladder. The selective TRPV1 antagonist, SB-366791, inhibited the contractile responses to capsaicin confirming the involvement of TRPV1 channels. The effect of diabetes is unlikely to be at the level of contractile machinery since the contractile responses to muscarinic receptor agonist carbachol were not significantly reduced in diabetic tissues. It is reported for the first time that the combination of neurokinin 1 and 2 antagonists GR-205171 and SB-207164 inhibited the contractile responses to capsaicin suggesting that a neurokinin may be the neurotransmitter involved in the capsaicin responses. In addition, the reduction of the responses to capsaicin in STZ-induced diabetic tissues occurred not only in urinary bladder but also in colon.

Cholesterol-PEG significantly lowered the maximal contractile responses to capsaicin of rat bladder strips. Methyl-β-cyclodextrin, α-cyclodextrin and β-cyclodextrin at the same concentrations enhanced the contractile responses to capsaicin in the control and diabetic rat bladder strips. These effects of cyclodextrin are specific to

capsaicin activated contractions and not seen with TRPA1 activation, suggesting that the effects are not mediated downstream of channel activation. Since α-cyclodextrin does not sequester cholesterol, the enhanced responses to cyclodextrins may not be due to the cholesterol modulations. Instead, theses novel findings may possibly occur by changing the local membrane lipid environment of the TRPV1 channel.

As early as 36 hours after induction of diabetes by STZ, the contractile responses to capsaicin were significantly reduced in comparison to those of the controls and this reduction persisted until the eight weeks time point. In contrast, responses to the TRPA1 agonist allyl isothiocyanate were not affected at early time points but were reduced one week after STZ treatment. This detailed time course analysis suggests that there are novel mechanisms of modulation of the TRPV1 channels in this STZ model.

In conclusion, in the rat urinary bladder or colon preparations, diabetes mellitus using STZ animal model caused 1) the impairment of a number of TRP channel subfamily functions, TRPV1, TRPV4 and TRPA1 but not TRPM8. The combination of NK_1 and NK_2 antagonists significantly inhibited the responses to capsaicin. This may suggest the involvement of neurokinin in postsynaptic transmission in rat bladder following the activation of TRPV1 channel, 2) the impairment caused by STZ-induced diabetes occurred very early (within 36 hours after diabetes induction) in TRPV1 channel but not TRPA1 channel. There are specific early effects of STZ treatment on TRPV1 channel function at a time when other afferent nerve terminal channels (TRPA1) are functioning normally, suggesting that early onset of dysfunction in TRPV1 signalling may not merely be the consequence of nerve damage, 3) the mechanism of this impairment may not be the effect of neuropathy on neurotransmitter release or nerve damage. Improving the responsiveness of nerves of bladder in diabetic patients might be of therapeutic benefit. The present studies suggest that it is possible to enhance function using indirect modulators such as bradykinin which potentiated the TRPV1 channel function in diabetic rat bladders.

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Publications

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Table of Contents

Table of Contents (continued)

Table of Contents (continued)

Table of Contents (continued)

Content **Page**

Table of Contents (continued)

List of Figures

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Tables

Abbreviations

TRPV1	Transient receptor potential vanilloid subfamily 1
TRPV4	Transient receptor potential vanilloid subfamily 4
TRPA1	Transient receptor potential ankyrin subfamily 1
TRPM8	Transient receptor potential melastatin subfamily 8
STZ	Streptozotocin
CZP	Capsazepine
RR	Ruthenium red
NK1	Neurokinin 1
NK2	Neurokinin 2
SP	Substance P
Ach	Acetylcholine
CCH	Carbachol
CAP	Capsaicin
DW	Distilled water
MCD	Methyl-β-cyclodextrin
BCD	β-cyclodextrin
GCD	γ-cyclodextrin
ACD	α-cyclodextrin
AITC	Allyl isothiocyanate

Chapter 1

General Introduction

1.1 Diabetes mellitus

1.1.1 Definition

Diabetes mellitus belongs to a class of metabolic disorders characterized by hyperglycemia, which is caused by the deficiency in insulin secretion, insulin action, or both. Chronic hyperglycemia is associated with the development of damage to various organs in the body including the eyes, kidneys, nervous system, and cardiovascular system (Llorente and Malphurs, 2007).

Diabetes mellitus can cause long-term damage, dysfunction and failure of various organs. The characteristic symptoms of diabetes mellitus include thirst, polyuria, blurring of vision, and weight loss. In its most severe forms, ketoacedosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death. The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and neuropathy with risk of foot ulcers, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at risk of cardiovascular, peripheral vascular and cerebrovascular disease (World Health Organization, 1999).

Type 1 diabetes mellitus in man is characterized by a specific destruction of the pancreatic β-cells. Type 1 diabetes mellitus is caused by destructive processes targeting the β-cell of the pancreas, resulting in insulin deficiency (American Diabetes Association Position Statement, 2006). It results from T-cell-mediated destruction of insulin-secreting pancreatic islet β cells (Tisch and McDevitt, 1996). It becomes clinically apparent when 80-90% of the insulin-secreting cells are destroyed. However, the great proportion of people with diabetes mellitus (90-95%) have type 2 diabetes mellitus (Harris *et al*., 1998).

Type 2 diabetes mellitus is a consequence of insulin resistance when cells do not respond to insulin properly even if it is present at normal levels, and is closely associated with obesity and sedentary lifestyle (Suri and Szallasi, 2007). It is characterized by insulin resistance and impaired insulin secretion. It is observed by a raised fasting blood glucose. However, for most patients with diabetes, several genetic and environmental factors contribute to the causation and progression of the disease and complications (Rees and Alcolado, 2005).

1.1.2 Pathophysiology

In type 1 diabetes mellitus, T cells which act in islet β-cell recognition, are stimulated in the lymph nodes. The islet cells in the pancreas are infiltrated by a leukocyte subset which causes the death of β cells. There are two mechanisms underlying the pathophysiology of type 2 diabetes. One mechanism relates to the insulin resistance (when the tissues in the body do not respond to insulin). Another mechanism is thought to be due to the insufficient secretion of insulin from β-cell in the pancreas. When tissues are resistant to insulin, there are abnormalities in glucose metabolism including decreased glucose uptake, increased hepatic glucose production, enhanced lipolysis and increased free fatty acid which stimulate fat oxidation (Gagnerault *et al*., 2002; Hoglund *et al*., 1999; Levisetti *et al*., 2004; Shoda *et al*., 2005).

One of the possible mechanisms of diabetic complications may relate to oxidative stress, which is clearly observed in the cardiovascular complication of diabetes. In addition, hyperglycaemia may impair the antioxidant defense mechanism. Oxidative stress, the overproduction of oxidants, occurs when free radical production is increased or the antioxidant defense mechanism is decreased. It leads to the complications of many diseases including diabetes mellitus. Free radicals may easily diffuse into cells and damage DNA as well asl exert other effects such as lipid peroxidation which may contribute to atherosclerosis (Llorente and Malphurs, 2007).The reactive oxygen species (ROS) is increased in diabetes because of the abnormalities in catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)

antioxidants. The ROS produced may react with nitric oxide to form peroxynitrite, a highly reactive radical species (Green *et al*., 2004). Nitric oxide is produced by nitric oxide synthase (NOS) enzymes, which are present in endothelial cells, neurons and other cell types. Inducible nitric oxide synthase (iNOS) is present in smooth muscle cells and islet cells. It is Ca^{2+} independent and it produces high amounts of nitric oxide which has been implicated in cytotoxicity on target cells (Goycheva *et al*., 2006).

It is reported that diabetes mellitus is related to oxidative stress and hydroperoxide levels increase in diabetes mellitus. It is believed that auto-oxidation and protein glycosylation generate free radicals, resulting in oxidative stress in diabetes mellitus (Lipinski, 2001; Bennefont *et al*., 2000). There are abnormalities in lipid metabolism caused by diabetes such as an increased susceptibility of lipid peroxidation causing atherosclerosis (Llorente and Malphurs, 2007).

1.1.3 Genetic influence

Diabetes mellitus is influenced by both genetic and environmental factors. In very rare cases, diabetes can be inherited. Therefore, genetics may be involved in mature onset diabetes or diabetes due to mutations in mitochondrial DNA (Kahn *et al*., 1996).

A genetic influence is also evident in twin studies. It is reported that monozygotic twins (twins from the same fertilized eggs) have 100% concordance while it is 20% for dizygotic twins, indicating that environment in the uterus may affect the incidence of diabetes mellitus (Zimmet, 1997; Hales and Barker, 1992).

According to the study by Diamond (2003), it is found that the populations of Native Americans, Pacific Islander, Aboriginal Australians, East Asians and South Asian of Indian continent have higher prevalence of diabetes mellitus than those of European population.

Environmental factors, particularly obesity and a sedentary lifestyle, are important contributors to the development of diabetes mellitus, because of their

effects on insulin sensitivity (van Dam, 2003; Shaw and Chisholm, 2003). The tremendous increase in rates of type 2 diabetes in recent years has been attributed to the dramatic rise in obesity worldwide (Zimmet *et al*., 2001). It is reported that 80% of new cases of type 2 diabetes are due to obesity (Lean, 2000).

1.1.4 Epidemiology

The incidence of diabetes mellitus has increased throughout the world. The numbers are expected to continue to rise. Worldwide there were 194 million adults with diabetes mellitus in 2003, and this number is expected to reach 333 million by the year 2025 (Llorente and Malphurs, 2007).

The risk of developing diabetes mellitus rises not only with obesity and lack of physical activity but also with increasing age and a family history. Specific population subgroups have a higher prevalence of diabetes mellitus than the population as a whole (American Diabetes Association Position Statement, 2006). Risk factors for the development of diabetes mellitus include hypertension, dyslipidemia, vascular disease, impaired glucose tolerance or impaired fasting glucose (Llorente and Malphurs, 2007).

1.1.5 Diagnosis

There are now three criteria by which to diagnose diabetes mellitus: (1) a patient has a fasting plasma glucose level of 126 mg/dl or higher; (2) a symptomatic patient has a casual plasma glucose level of 200 mg/dl or higher; or (3) a patient has a 2-hour plasma glucose level of 200 mg/dl or higher during a 75-g oral glucose tolerance test (OGTT). The diagnosis must be confirmed by any of the three methods on a subsequent day (Table 1) (Palumbo, 2001).

Table 1 Criteria for the diagnosis of diabetes mellitus (Palumbo, 2001)

Fasting plasma glucose level ≤ 126 mg/dl or
Symptoms (polyuria, polydipsia, unexplained weight loss) plus casual plasma glucose level ≥ 200 mg/dl or
2-h plasma glucose level ≥ 200 mg/dl during a 75-g oral glucose tolerance test

The fasting plasma glucose (FPG) is the preferred diagnostic test because of simplicity of use, acceptability to patients, and low cost. In the presence of symptoms of diabetes mellitus (polyuria, polydipsia, weight loss, etc.), a plasma glucose level of > 200 mg/dl is diagnostic (Llorente and Malphurs, 2007).

In the absence of unequivocal hyperglycemia, any test used to diagnose diabetes mellitus must be confirmed on a subsequent day by a plasma glucose measured either in the fasting state or two hours after an oral glucose load. The use of the hemoglobulin A_{1c} (HbA_{1c}) test for the diagnosis of diabetes mellitus is not recommended, as it is less specific and assays are not completely standardized throughout the world (Llorente and Malphurs, 2007).

Hyperglycemia insufficient to meet the diagnostic criteria for diabetes is categorized as either impaired fasting glucose or impaired glucose tolerance, depending on whether it is identified by fasting plasma glucose or an oral glucose tolerance test. Impaired fasting glucose is diagnosed when the fasting plasma glucose is ≥ 100 mg/dl. Impaired glucose tolerance exists when the plasma glucose level 2 hours after a 75 g oral glucose load is ≥ 140 mg/dl but < 200 mg/dl. These are considered to be pre-diabetes mellitus states (Llorente and Malphurs, 2007).

1.1.6 Treatments

Dietary managements and increased exercises are the starting point of diabetes mellitus treatments. When there is very high blood glucose level and nutritional and physical activity fails to improve the diabetic conditions, pharmacological tools are essential for the treatment of diabetes mellitus.

The drugs for treatments of diabetes mellitus are usually in form of oral hypoglycaemic drugs, which are divided into two main groups; sulfonylureas and biguanides. Functionally, sulfonylureas enhance insulin production from β-cells in the pancreas and promote other action around the pancreas while biguanides reduce the production of glucose in the liver and increase the use of glucose in the body (Table 2) (Modi, 2007).

Sulfonylureas are believed to close potassium channels in pancreatic cells, resulting in an increase of insulin release (Stumvoll *et al*., 2005) from β-cells of pancreas. This is confirmed by the reduced fasting plasma glucose. Hypoglycaemia and weight gain are the side effect of sulfonylurea derivatives.

Maglitinides consist of nateglinide and repaglinide. Nateglinide binds to the same site of sulfonylurea receptor 1 as those of the sulfonylurea derivatives. Repaglinide binds to a nearby site of the receptor, stimulating the release of insulin from pancreatic β-cells (Bloomgarden, 1997; Phillips and Dunning, 2003).

Metformin lowers the glucose production in the liver and stimulates the transport of glucose into muscle (Stomvoll *et al*., 2005; Modi, 2007). The side effects that may be found from using metformin include bloating, flatulence, diarrhea and abdominal discomfort and pain (Modi, 2007).

Thiazolidinediones (TZDs) stimulate the function of insulin in muscle, fat and other tissues. They are selective and potent agonists for the peroxisome proliferator-activated receptor γ (PPAR-γ) nuclear receptors (Stomvoll *et al*., 2005; Modi, 2007). When they are activated, these receptors regulate the transcription of insulin responsive genes which are contributed to the control of production, transport, and utilization of glucose. The side effects for TZDs are edema and weight gain.

The α-glucosidase inhibitors consist of acarbose and miglitol. Acarbose lowers glycaemic excursions and prevent the development of diabetes and cardiovascular disease (Chiasson *et al*., 1998). They inactivate the rate of carbohydrate absorption in the small intestine, leading to a reduction in plasma glucose level. The α-glucosidase inhibitors inhibit the conversion of dietary starch and sucrose into glucose. The side effects caused by these drugs are bloating, flatulence, diarrhea and abdominal discomfort and pain (Modi, 2007).

Table 2 Drugs for treatment of diabetes mellitus with their mechanism of action and side effects (adapted from Modi, (2007)).

Oral antidiabetics	Mechanism of action	Side effects
Sulfonylureas Glimiperide (Amaryl) Glipiside (Glucotrol) Glipiside-gits (Glucotrol-XL) Glyburide (Diabeta, Micronase) Glyburide micronized (Glynase) Tolbutamide (Orinase) Chlorpropamide (Diabinese) Tolazamide (Tolinase) Acetoheximide (Dymelor)	Stimulate first-phase insulin secretion by blocking K^+ channel in β-cells	Late hyperinsulinemia and hypoglycaemia Weight gain
Meglitinides Repaglinide (Prandin) Nateglinide (Starlix)	Stimulate first-phase insulin secretion by blocking K^+ channel in β-cells	Hypoglycaemia Weight gain
Biguanides Meformin (Glucophage, Riomet) Metformin-XR (Glucophage-XR)	Decrease hepatic glucose production Increase muscle glucose uptake and utilization	Nausea, Diarrhea Anorexia, Lactic acidosis
Thiazolidinediones Rosiglitazone (Avandia) Pioglitazone (Actos)	Increase insulin sensitivity via activation of PPAR-γ receptors	Fluid retention and weight gain
α-Glucoside Inhibitors Acarbose (Precose) Miglitol (Glyset)	Decrease hepatic glucose production Delay glucose absorption	Flatulence Abdominal bloating

1.1.7 Complications of diabetes

1.1.7.1 Acute complications

The acute metabolic complications of diabetes consist of diabetic ketoacidosis, hyperosmolar non-ketotic coma, lactic acidosis, and hypoglycaemia. Diabetic ketoacidosis and hyperosmolar non-ketotic coma are related to insulin deficiency. Hypoglycaemia results from the treatment of diabetes, either with oral agents or insulin. Although hypoglycaemia may occur in conjunction with oral hypoglycaemic therapy, it is more common in patients treated with insulin. Lactic acidosis is usually associated with other factors that may be related to diabetes, such as cardiovascular disease (acute myocardial infarction) associated with hypoxia and excess lactic acid production (Siperstein, 1992).

Diabetic ketoacidosis is clinically defined by absolute insulin deficiency with hyperglycaemia (glucose levels usually > 200 mg/dl) with increased lipolysis, increased ketone production, hyperketonemia (ketone levels positive at 1:4 dilution of serum or greater or beta hydroxybutyrate > 0.5 mmol/L), and acidosis (pH ≤ 7.3 or bicarbonate ≤ 15 mEq/L) (Siperstein, 1992).

Hyperosmolar non-ketotic coma is the presence of relative insulin deficiency and hyperglycaemia, usually > 1,000 mg/dl with associated elevated serum osmolarity (> 300 mosm/kg), dehydration, and stupor, progressing to coma if uncorrected, without the presence of ketosis or acidosis. These patients have sufficient circulating insulin to prevent lipolysis and ketosis (Siperstein, 1992).

Lactic acidosis consists of elevated lactic acid (lactic acidemia, ≥ 2.0 mmol/L) with acidosis (pH ≤ 7.3) and without ketoacidosis. There may be low levels of ketones present (≤ 1:4 on serum dilution, or beta hydroxybutyrate > 0.4 but < 0.6 mmol/L). Approximate half of the reported cases of lactic acidosis have occurred in patients with diabetes (Kreisberg, 1980).

Hypoglycaemia is common in insulin-treated diabetic patients. Hypoglycaemia may range from low levels of glycaemia (60-70 mg/dl) with

minimal or no symptoms, to severe hypoglycaemia with very low levels of glucose (< 40 mg/dl) and neurologic impairment.

Experimental studies of acute hyperglycaemia have demonstrated effects on renal and nerve function, retinal perfusion, vasodilation, coagulation factors, and atherogenic vascular disease. Hyperglycaemia may be implicated in glomerular hyper-infiltration, which precedes diabetic renal disease. Acutely induced hyperglycaemia impaired nerve transduction velocities in diabetic patients and in nondiabetic subjects. Acute hyperglycaemia can lower pain thresholds in animals and in patients with diabetes mellitus and thereby contributes to neuropathic symptoms (Palumbo, 2001).

Acute hyperglycaemia impairs gastrointestinal motility in diabetic patients and in normal subjects. Gastric emptying is delayed. This delay may be related to neuropathic changes. Acute hyperglycaemia may produce gastroparesis by a direct effect. It also has adverse effect on oesophageal motility and gall bladder contractility (Palumbo, 2001).

Acetylcholine-induced vasodilation in vitro is impaired by exposure of blood vessel wall samples to acute hyperglycaemia. This impairment of vasodilation is glucose dependent. In vivo studies with acute hyperglycaemia have shown an increase in blood pressure in both diabetic patients and nondiabetic subjects. Acute hyperglycaemia with myocardial infarction and stroke is associated with an unfavorable prognosis in diabetic and nondiabetic patients and in animal studies. Hyperglycaemia with stroke aggravates neuronal damage. It is also accompanied by adverse changes in coagulation factors in diabetic patients and control subjects (Palumbo, 2001).

Thus, acute hyperglycaemia in patients with type 1 and type 2 diabetes mellitus is associated with metabolic and biochemical abnormalities that are sustained with persistent hyperglycaemia and lead to progression of microvascular and macrovascular disease. Two possible mechanisms for the progression to these diseases are enzymatic glycation and free radical formation (oxidative stress) (Palumbo, 2001).

1.1.7.2 Chronic complications

Diabetic neuropathy is a common complication of diabetes. It is one of the most prevalent diabetic complications with an incidence of 21% in diabetic patients. Diabetes may affect the autonomic, sensory and motor nerves and the central nervous system (Ozturk *et al*., 1998). Diabetic neuropathy progress gradually and involves small and large sensory fibers. Its symptoms include loss of ability to sense pain, loss of temperature sensation and developing neuropathic pain. The primary cause of diabetic neuropathy is thought to be hyperglycaemia (Wong *et al*., 2008).

In long-term diabetic patients, cardiomyopathy and congestive heart failure may develop as a result of the impaired left ventricular function. The function of the coronary arteries in diabetic patients is also impaired depending on the calcification of the arterial wall. Diabetic gastroenteropathy is one of the primary autonomic syndromes related to diabetes. Asymptomatic dilatation of the stomach, and impaired gastric acid secretion in diabetic patients have been reported. Nephropathy is one of the most significant complications seen in diabetes mellitus. Proteinuria, albuminuria, and glomerulopathy have been observed in animal model of diabetes. Urinary retention in the urinary bladder is another significant complication in diabetic patients. Reproductive complications are seen in both male and female patients suffering from diabetes mellitus. Impotence, retrograde ejaculation and lower fertility have been reported in male diabetic patients (Ozturk *et al*., 1998).

1.1.8 Animal models of diabetes

Animal models of diabetes are used to investigate the pathogenesis of diabetes and long-term diabetic complications seen in clinical studies. Streptozotocin (STZ) and alloxan (ALX) are two chemicals used to induce experimental diabetes, mostly in rodents (Ozturk *et al*., 1998).

Alloxan (ALX) is a uric acid derivative and is highly unstable in water at neutral pH, but reasonably stable at pH 3. ALX acts by selectively destroying

the pancreatic beta islets leading to insulin deficiency, hyperglycaemia and ketosis (Srinivasan and Ramarao, 2007).

Streptozotocin (STZ) is isolated from *Streptomyces achromogenes*. It is an alkalating agent that has been shown to interfere with glucose transport, glucokinase function and to induce multiple DNA strand breaks. It causes hyperglycaemia by cytotoxic action on the pancreatic beta cells (Srinivasan and Ramarao, 2007). A single large dose of streptozotocin can produce diabetes in rodents, probably as a result of direct toxic effects on the β cells. Alternatively, multiple small doses of streptozotocin can be used (e.g.40 mg/kg on five consecutive days). In susceptible rodents, this induces the insulinopenic diabetes in which immune destruction plays a role, as in human type 1 diabetes. The multiple low-dose streptozotocin model has been used extensively to study the immunological pathways that lead to insulitis and β cell death (Rees and Alcolado, 2005).

1.1.9 The effect of streptozotocin on rodent bladder function

The streptozotocin-induced diabetic rats showed impaired bladder function characterized by increased bladder capacity, decreased bladder contractility (voiding efficiency), and an increase in residual urine (Jiang *et al*., 2008).

In Spraque-Dawley rats, streptozotocin-induced diabetes decreased average body weight and increased bladder weight, capacity and compliance. Peak detrusor leak pressure increased gradually from week 3 to 9 in diabetic rats. However, at 12 and 20 weeks diabetic rats deviated strongly from this trend with peak detrusor leak pressure decreasing versus controls and post-void resting pressure increasing from 9-week levels versus controls. In contractility studies, increased contractile force response of diabetic animals to carbachol, potassium chloride, adenosine 5'-triphosphate and electrical field stimulation peaked at 6 or 9 weeks but 12 to 20 weeks they generally reverted toward those of controls (Daneshgari *et al*., 2006).

In rat treated with 60 mg/kg single dose of streptozotocin, there was a decreased in the number of urothelium layers, loss of dome shaped cells which are replaced with the intermediate layer cells (Kizilay and Uygun, 2005). The epithelium from diabetic rat urinary bladders was thicker and heavier and absolute amount of endogenous prostaglandin E_2 and $F_{2\alpha}$ was higher than for control animals.

In addition, there is the impairment of neurotransmitter release from both bladder sympathetic and parasympathetic efferent nerve endings in early (two weeks) streptozotocin-induced diabetes (Tong *et al*., 1996).

Treatment of rats with streptozotocin induces a diabetic state in which the bladder muscle is overactive and also supersensitive to muscarinic agonists. Isolated detrusor strips from diabetic animals showed an increased spontaneous activity. Carbachol produced contractile responses in tissues from both control and diabetic rats, but the diabetic tissues were more sensitive to this agonist (Stevens *et al*., 2006).

In streptozotocin treated F-334 rats, there were insulin-reversible increases in bladder weight, bladder capacity, micturition volume, residual volume, micturition pressure and spontaneous activity (Christ *et al*., 2006).

1.2 Urinary bladder

1.2.1 Anatomy and physiology

The lower urinary tract is composed of the bladder and urethra, the functional units for storage (the bladder body, or reservoir) and elimination (the bladder neck and urethra, or outlet) of urine. The main functions of the lower urinary tract are to store urine without leakage for longer period of time and to rapidly expel urine during micturition (Anderson and Arner, 2004).

The urethra contains both smooth and striated muscles. The bladder can be divided into two main components; the bladder body, which is located above the ureteral orifice, and the base, consisting of the trigone, urethrovesical junction, deep detrusor, and the anterior bladder wall (figure 1.1).

The wall of the bladder body is lined with bundles of intertwining smooth muscle fibers, forming the detrusor muscle. The smooth muscles lining the bladder neck and the urethra form the internal sphincter, which is surrounded by striated muscle, the rhabdosphincter. The periurethral striated muscle-striated muscle fibers surrounding the urethra-and the rhabdosphincter together constitute the external urethral sphincter (Yoshimara et al., 2007). The bladder is a hollow smooth muscle organ lined by a mucous membrane and covered on its outer aspect partly by peritoneal serosa and partly by fascia. Its muscular wall is formed by smooth muscle cells, which comprise the detrusor muscle. The detrusor is structurally and functionally different from trigonal and urethral smooth muscle (Anderson and Arner, 2004).

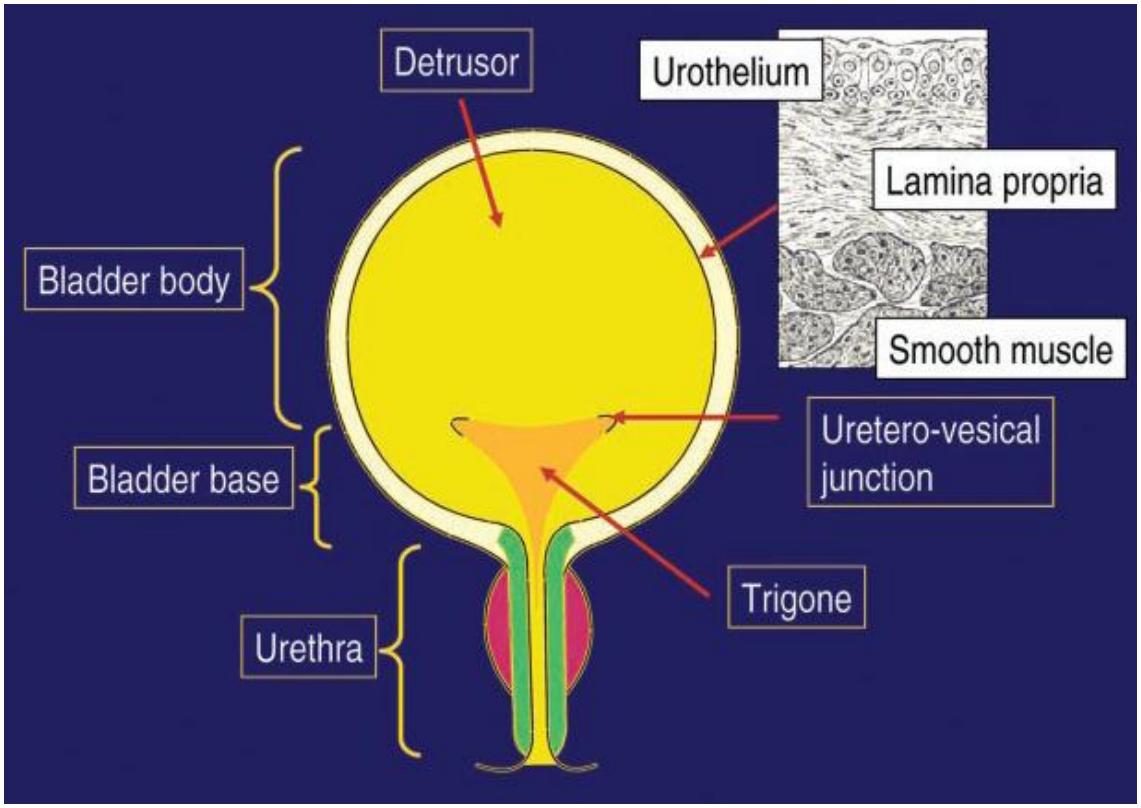


Figure 1.1 Schematic drawing of the urinary bladder (adapted from Anderson and Arner, 2004)

1.2.3 Neurophysiology

The lower urinary tract is innervated by parasympathetic, sympathetic, and somatic peripheral nerves that are components of intricate efferent and afferent circuitry derived from the spinal cord. The neural circuits act as an integrated complex of reflexes that regulate micturition, allowing the lower urinary tract to be in either a storage or elimination mode (Yoshimura *et al*., 2008) (Figure 1.2).

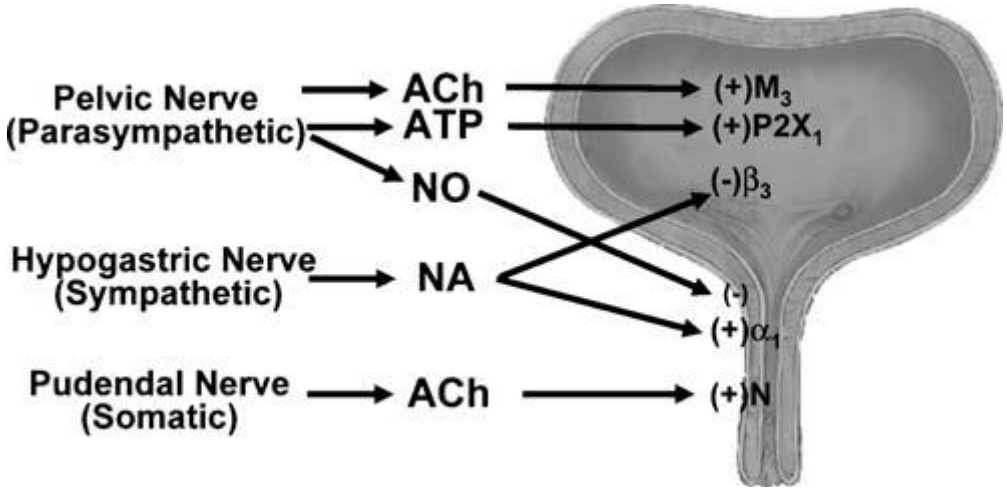


Figure 1.2 Innervations of bladder
(adapted from Yoshimura et al., 2008)

1.2.3.1 Parasympathetic nerves

The efferent parasympathetic pathway provides the major excitatory innervation of the bladder detrusor (Yoshimura and Chancellor, 2007; Andersson and Wein, 2004). Preganglionic axons emerge, as the pelvic nerve, from the sacral parasympathetic nucleus in the intermediolateral column of sacral spinal segments S2-S4 and synapse in the pelvic ganglia as well as in small ganglia on the bladder wall, releasing acetylcholine (ACh). ACh excitation of postsynaptic neurons is mediated by nicotinic receptors. Postganglionic axons continue for a short distance in the pelvic nerve and terminate in the detrusor layer, where they transmit ACh to the smooth muscle fibers, with consequent contraction of the bladder. This stimulatory effect of ACh at the postganglionic axon terminal is mediated by muscarinic receptors in detrusor cells (Chapple *et al*., 2002; de Groat and Yoshimura, 2001; Andersson and Wein, 2004).

While ACh is the principal excitatory transmitter at the parasympathetic nerve terminals, adenosine triphosphate (ATP)-induced stimulation of bladder smooth muscle contractions has been demonstrated in many mammalian species. This purine nucleotide is considered to be a parasympathetic cotransmitter responsible for the atropine-resistant detrusor activity mediated by stimulation of one or more members of the P2X family of purinoceptors (de Groat and Yoshimura, 2001; Andersson and Wein, 2004). Although purinergic stimulation is considered to be only a minor contributor to normal bladder function in humans (Husted et al., 1983; O'Reilly et al., 2001), upregulation of purinergic activity through P2X purinoceptors (predominantly the $P2X_1$ subtype) have been demonstrated in the overactive bladder (OAB) under pathologic conditions such as outlet obstruction (O'Reilly *et al*., 2001).

1.2.3.2 Sympathetic nerves

Sympathetic nerves stimulate smooth muscle contraction in the urethra and bladder neck and cause relaxation of the detrusor. Preganglionic

sympathetic neurons are located in the intermediolateral column of the thoracolumbar cord segments T11-L2 (de Groat and Yoshimura, 2001; Yoshimura and Chancellor, 2007). Most of the preganglionic fibers synapse with postganglionic neurons in the inferior mesenteric ganglia. The preganglionic neurotransmitter is Ach, acting via nicotinic receptors in the postganglionic neurons. The postganglionic axons travel in the hypogastric nerve and transmit norepinephrine at their terminals. The major terminals are in the urethra and bladder neck as well as in the bladder body and postganglionic parasympathetic neurons in the pelvic ganglia. Norepinephrine stimulates contraction of the urethral and bladder neck smooth muscle, via α_1-adrenoceptors, and causes relaxation of detrusor, via β_2-adrenoceptors (Nomiya and Yamaguchi, 2003).

1.2.3.3 Somatic nerves

Somatic nerves provide excitatory innervation of the striated muscles of the external urethral sphincter and of the pelvic floor. The motor neuron axons are carried in the pudendal nerve, and they release Ach at their terminals. The Ach acts on nicotinic receptors in the striated muscles, inducing muscle contraction to maintain closure of the external urethral sphincter during urine storage (Blaivas, 1982; Thor *et al*., 1989; Yoshimura and Chancellor, 2007) as well as stress conditions (Chancellor and Yoshimura, 2004; Chancellor *et al*., 2005).

1.2.3.4 Afferent pathways

The pelvic, hypogastric, and pudendal nerves carry sensory information in afferent fibers from the lower urinary tract to the lumbosacral spinal cord (Morgan *et al*., 1981, 1986; Thor *et al*., 1989; Andersson and Wein, 2004; Yoshimura and Chancellor, 2007). The afferent pelvic nerve monitors the bladder volume during the storage phase and the amplitude of bladder contractions during urination. Thus, this sensory nerve serves to initiate the mucturition reflex as well as to reinforce the drive that maintains bladder

contractions. It is composed of myelinated Aδ-fibers and unmyelinated C-fibers. Aδ-fibers, which are located primarily within the detrusor smooth muscle layer and respond primarily to detrusor stretching during the phase of bladder filling and convey sensations of fullness. Unmyelinated sensory C-fibers are more wide-spread and reside in the muscle, close to the urothelial cells themselves (Andersson, 2002; Ouslander, 2004; Yoshimura and Chancellor, 2007). C-fibers usually have a higher mechanical threshold than Aδ-fibers (Habler *et al*., 1990; Dmitrieva and McMahon, 1996) and can be activated by a variety of neurotransmitters and chemical mediators released by the detrusor and urothelium, including ATP, neurokinin, nerve growth factor, and others (Ouslandder, 2004; Yoshimura and Chancellor, 2002).

1.3 Diabetes and urinary bladder functions

Type 1 diabetes mellitus is associated with various pathologies, including thrombosis, hypertension, atherosclerosis, hyperlipidimia, impotence, abnormalities in autonomic nerves, retinopathy, nephropathy and neuropathy (Mauer *et al*., 1984; Cohen, 1993; Richard *et al*., 1993). Urological diseases are found as common health problems with increase in prevalence in diabetes (Brown *et al*., 2005). Urinary bladder disturbances are found in long-term diabetes mellitus (Buck *et al*, 1976; Ellenberg, 1980). The most common characteristics of bladder dysfunction in animal models of diabetes are increases in distension and bladder capacity (Andersson *et al*, 1988; Santicioli *et al*, 1987). The presence of residual urine is also a manifestation of the condition especially in the later stages (Buck *et al*, 1974; Ellenberg, 1980), which suggests that there may be some compromise of smooth muscle contraction.

Neuropathy plays a role in urinary bladder impairment (Steer *et al*, 1994). There are reports to suggest that alterations may also exist at the level of the bladder smooth muscle itself (Longhurst and Balis, 1986). These include changes in postsynaptic muscarinic receptor function (Kolta *et al*, 1985; Malmgren *et al*., 1989) and possible changes in intracellular messenger

systems (Tammela *et al*., 1994). The possibility that there may be a myogenic component to diabetic bladder dysfunction led to several studies of bladder smooth muscle contractility in various animal models of diabetes. The results of these studies are often conflicting. Some report that contractile responses of diabetic bladder, especially to muscarinic agonists, are enhanced (Eika *et al*., 1994; Kolta *et al*., 1985; Tammela *et al*., 1994; Steer *et al*., 1990), while others suggest that the responses to a variety of contractile stimuli are diminished (Santicoli *et al*, 1987; Longhurst and Balis, 1986). These apparent discrepancies may be due to the different animal models and duration of diabetes employed as well as the data normalization procedures applied. Despite the variability in the results of these studies, it seems likely that contractile function of urinary bladder smooth muscle is altered in diabetes. Little is known of the possible mechanisms that might underlie these alterations.

A high incidence of neurogenic bladder dysfunction (diabetic cystopathy) in diabetics has been demonstrated in many studies (Buck *et al*., 1976; Frimodt-Moller, 1980). The major clinical feature of this disorder is a gradual loss of bladder sensation and motor function resulting in a large bladder with a chronic residual urine volume. Much evidence suggests that diabetic cystopathy is a manifestation of the peripheral neuropathy which is a common complication of diabetes. There is a very good correlation between the incidence of cystopathy and of peripheral neuropathies in individual patients (Bartley *et al*., 1966; Andersen and Bradley, 1976). Histological damage was found in diabetic bladders, with an absence of or decrease in acetylcholinesterase activity (Faerman *et al*., 1973).

1.4 Diabetes and colon functions

Gastrointestinal disorders in diabetes may due to many reasons. The autonomic nervous supply to the gut is affected by long-standing diabetes. Segmental demyelination, reduction in size of neurons, altered vasoactive intestinal polypeptide, somatostatin and substance P in these neurons supplying the gut and axonal degeneration in the Meissneri plexus are

abnormalities found in diabetes. The autonomic neuropathy in diabetes seems to be a key pathogenetic event in the evolution of the disorders of the gastrointestinal tract. Most complications of motility are secondary to neuropathy and patients usually have peripheral neuropathy or other features of autonomic neuropathy (Sharma, 1989).

In the ileum from 8-week streptozotocin-induced diabetic rats, contractile responses to carbachol, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), the calcium ionophore A23184 and to EFS were increased in diabetic tissues compared to controls. The inhibitory effects of a potent and selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) on electrical field stimulation-evoked contractions were decreased in diabetic tissues compared to control but its ability to relax carbachol-contracted tissues were unaltered (Talubmook *et al*., 2003).

1.5 Transient receptor potential (TRP) channels

The transient receptor potential (TRP) superfamily consists of a diverse set of proteins whose primary function is to regulate the plasma membrane permeability of animal cells to a variety of ions. They are among the largest family of ion channels known, with representative members in many species from yeast to humans. The first member of the family to be identified, *Drosophila melanogaster* TRP, was discovered in the analysis of a mutant fly whose photoreceptors failed to maintain a sustained response to a prolonged stimulus of light (Montell and Rubin, 1989).

1.5.1 Structure of TRP channels

The TRP ion channels are a large class of channel subunits united by a common primary structure and permeability to monovalent cations and calcium ions (Ca^{2+}). TRP channels bring Ca^{2+} into cells at hyperpolarized membrane potentials (Clapham et al., 2001). Each TRP channel subunit consists of six putative transmembrane spanning segments (S1-6), a pore-forming loop

between S5 and S6, and intracellularly located located NH2 and COOH termini. Assembly of channel subunits as homo- or heterotetramers results in the formation of cation-selective channels (Figure 1.3) (Nilius and Voets, 2005).

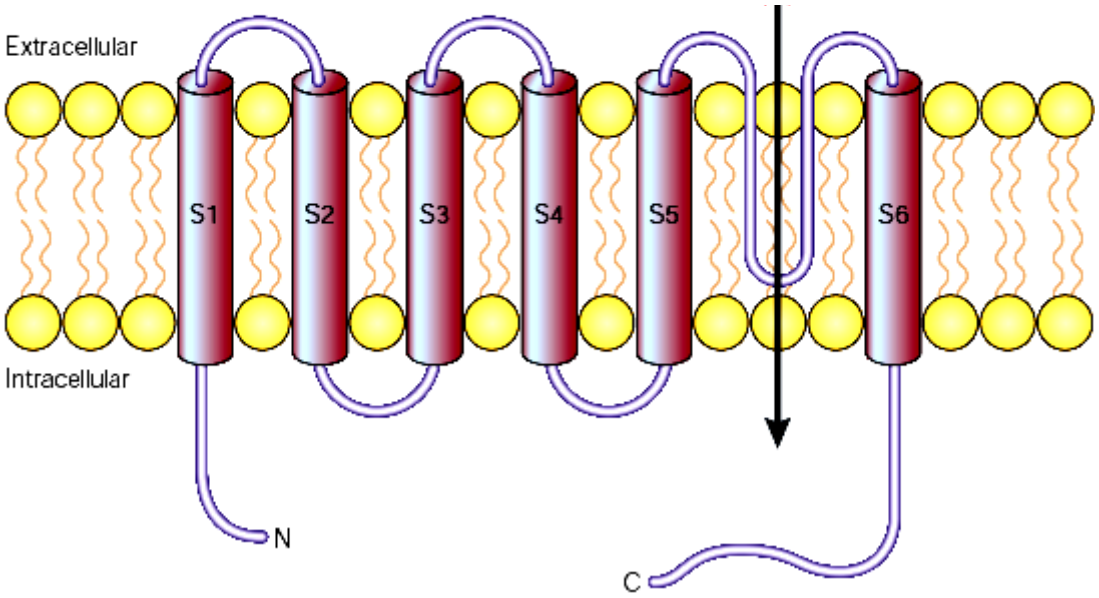


Figure 1.3 Structure of TRP channels (adapted from Clapham *et al*., 2001)

1.5.2 TRP subfamilies

There are more than 100 TRP sequences reported. They include members from *Saccharomyces cerevisiae*, *Dictostelium discoideum*, *Caenorhabditis elegans*, *Drosophila* and mammals. Based on amino acid homology, the TRP subfamily can be divided into seven subfamilies: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, TRPN (Nilius and Voets, 2005; Pedersen *et al*., 2005) (Figure 1.4).

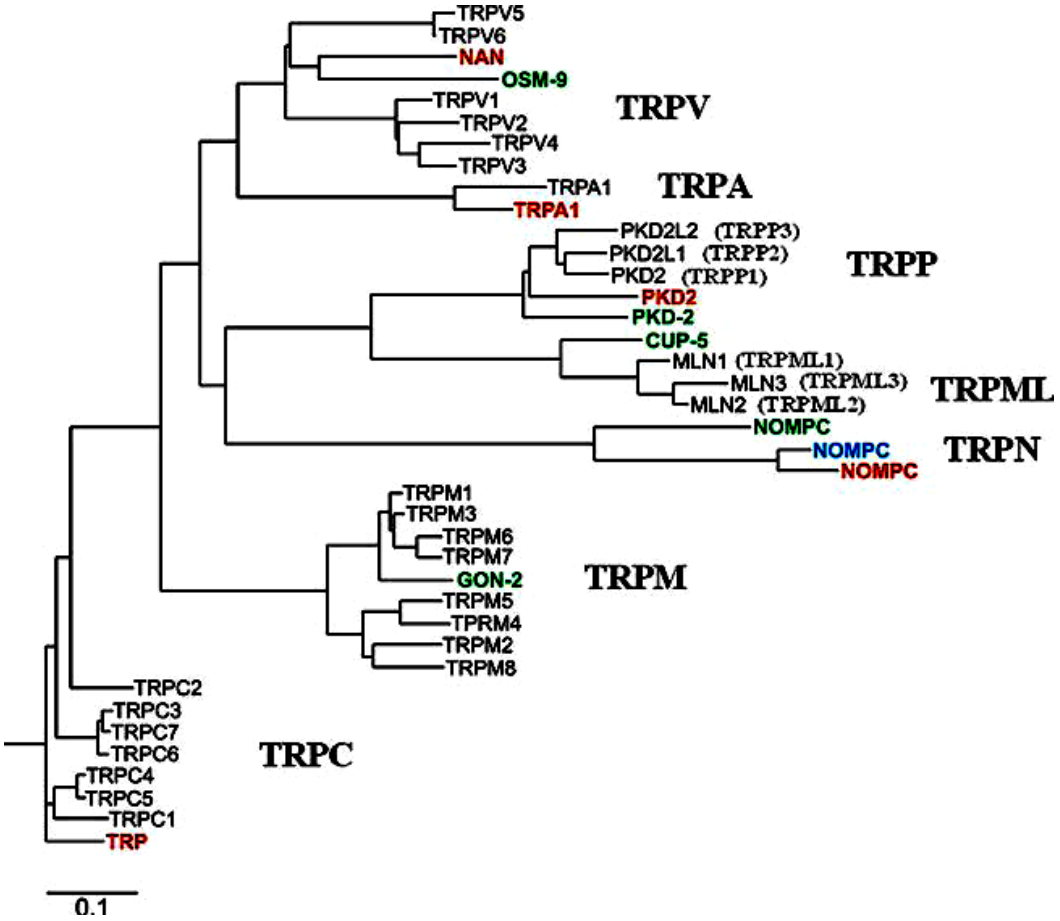


Figure 1.4 Phylogenetic analysis of representative channels of the TRP subfamily. The distance scale represents the evolutionary distance expressed in the number of substitutions per amino acid (Pedersen *et al*., 2005).

1.5.2.1 TRPC

TRPC channels are non-selective Ca^{2+} permeable cation channels (Ramsey *et al*., 2006). The TRPC subfamily can be subdivided into four subfamilies: TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5. Channels of the TRPC family are activated subsequent to stimulation of receptors that activate different

isoforms of phospholipase C (PLC). All TRPC channels have been proposed to act as stored-operated channels (SOCs), which are assumed to be activated by depletion of intracellular calcium stores (Nilius and Voets, 2005; Alexander *et al*., 2007).

1.5.2.2 TRPV

The TRPV channel subfamily has six members divided into four groups on the basis of structure and function: TRPV1/2, TRPV3, TRPV4 and TRPV5/6 (Gunthorpe *et al*., 2002). TRPV1-4 are thermosensitive, non-selective cation channels that can be activated by numerous stimuli (Benham *et al*., 2003). TRPV5 and TRPV6 are calcium-selective channels involved in the absorption and reabsorption of calcium across intestinal and kidney tubule epithelia (Alexander *et al*., 2007).

TRPV1 is selective for Ca^{2+} and Mg^{2+}. It is activated by capsaicin, resiniferatoxin, olvanil, moderate heat (≥ 43 $^{\circ}$C), low pH (≤ 5.9), the cannabinoid-receptor ligand anandamide, the eicosanoids 12-(*S*)-HPETE, 15-(*S*)-HPETE, 5-(*S*)-HPETE, leukotrine B_4, *N*-arachidononoyl-dopamine, adenosine, and 2-APB (2-amino ethoxyphenylborate) (Nillius and Voets, 2005). Blockade of TRPV1 by capsazepine, 6-iodo-*nor*dihydrocapsaicin, BCTC, JYL1421, and SB366791 is competitive. All other antagonists act by non-competitive antagonism (Alexander *et al*., 2007). TRPV1 is widely expressed, but its function has been most thoroughly studied in sensory neurons, in which it was first identified (Caterina *et al*., 1997). TRPV1 was identified in dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons, and is also highly expressed in spinal and peripheral nerve terminals, as well as in a multiple non-neuronal cell types (Cases *et al*., 2005). TRPV1 is involved in nociception, and analysis of vanilloid receptor gene knock-out mice confirmed that the channel contributes to the detection and integration of painful chemicals and thermal stimuli (Pedersen *et al*., 2005). TRPV1 is also required for evoked purinergic signaling in the bladder urothelium (Birder *et al*., 2002). This channel is now

recognized as being involved in several diseases including inflammation, asthma and pancreatitis (Liedtke and Simon, 2004; Nathan *et al*., 2002).

Phosphorylation and dephosphorylation signals are the key factors that influence the activation of TRPV1. Phosphorylation will sensitize the receptor for activation while dephosphorylation will inhibit or make the receptor to be non-responsive to stimuli (heat, acid, capsaicin, etc.). Various agonists which activate $G_{\alpha q}$-protein coupled receptor sensitize TRPV1. Protease can activate TRPV1 through protease-activated receptors. In addition, it can be activated by ATP (through purinergic receptor), bradykinin (through B_1 and B_2 receptors), and endothelin (through ET-AR). These receptors activate PLCβ, which cleaves phosphatidylinositol bisphosphate (PIP_2) to produce inositol trisphosphate and DAG, leading to the activation of protein kinase C (PKC). It has been reported that the isoform of PKC that is activated has been demonstrated to be PKC-ε, which requires DAG but not Ca^{2+} for activation. This kinase has been shown to phosphorylate TRPV1 on Ser^{502} and Ser^{801} (Premkumar and Ahern, 2000).

In addition, some other agonists can potentiate signaling through TRPV1 by protein kinase A (PKA) activation. These include $G\alpha_s$-protein coupled receptors, which enhance the synthesis of cAMP by adenylyl cyclase, with cAMP activating PKA. Agonists that have been reported to show this potential include PGE_2, which increases cAMP through EP_2 and EP_4, prostacyclin, serotonin and 5-hydroxytryptamine. Seven residues of TRPV1 are phosphorylated by PKA *in vitro*. The residues that are phosphorylated include Ser^{117}, Thr^{145}, Thr^{371}, and Ser^{502}. Finally, atrial natriuretic peptide, which uses receptors that increase cGMP to signal through protein kinase G (PKG), potentiate TRPV1 (Premkumar and Sikand, 2008).

PKCε and PKCα, calcium-independent and calcium dependent isoforms of PKC, respectively, have been shown to phosphorylate TRPV1 (Premkumar and Ahern, 2000). Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) has been reported to modulate TRPV1 function and capsaicin binding (Jung *et al*., 2004; Rosenbaum *et al*., 2004). In contrast, calcineurin-mediated dephosphorylation causes TRPV1 desensitization (Docherty *et al*., 1996).

Mutation of CaMKII phosphorylation on TRPV1 (S502 and T704) failed to elicit currents in response to application of capsaicin or RTX (Jung *et al*., 2004).

TRPV4 is an active Ca^{2+} permeable cation channel that responds to heating, hypotonic challenge, or the phorbol ester 4α-PDD (Ramsey *et al*., 2006). TRPV4 can be activated by stimuli including moderate heat (> 24 $^{\circ}$C) and 4α-phorbol 12,13-didecanoate (4α-PDD) (Nilius and Voets, 2005). TRPV4 is widely expressed in brain, dorsal root ganglion neurons, and multiple excitable and non-excitable peripheral cell types (Pedersen *et al*., 2005). The effect of hypotonicity on TRPV4 is attributable to swelling-induced production of the 5', 6'-epoxyeicosatrienoic acid, which directly activates TRPV4 channels. TRPV4 also alter voltage-dependent block by ruthenium red. TRPV4 knockout mice exhibit reduced pressure and osmotic sensitivity, altered thermal selection, and hearing loss (Ramsey *et al*., 2006).

1.5.2.3 TRPM

Members of the TRPM ("Melastatin") subfamily, on the basis of sequence homology, fall into four groups: TRPM1/3, TRPM2/8, TRPM4/5 and TRPM6/7 (Pedersen et al., 2005). TRPM channels exhibit highly varying permeability to Ca^{2+} and Mg^{2+}, from Ca^{2+} impermeable (TRPM4 and 5) to highly Ca^{2+} and Mg^{2+} permeable (TRPM6 and 7) (Pedersen *et al*., 2005).

TRPM play a role in a range of functions, for examples, a cellular redox sensor in TRPM2, microglial and choroid plexus function in TRPM3, contribution to myogenic vasoconstriction of cerebral arteries and taste transduction in TRPM4-5, and Mg^{2+} homeostasis in intestine and kidney in TRPM6-7 (Ramsey *et al*., 2006).

TRPM8 was originally detected in the prostate gland, but is widely expressed in sensory neurons, in which it may function as a cold thermosensor (Peier *et al*., 2002; McKemy *et al*., 2002). TRPM8 is a channel activated by cold temperature (8-28 $^{\circ}$C) and pharmacological agents evoking a 'cool' sensation such as menthol and icilin (Patapoutian *et al*., 2003). However, Anderson *et al*. (2004) suggested that intracellular pH modulates activation of TRPM8 by cold

and icilin, but not menthol. The main physiological role of TRPM8 is serving as a thermosensor in sensory neurons (Patapoutian *et al*., 2003). TRPM8 is upregulated in a variety of primary tumors (e.g. prostate, breast, colon, lung, skin) (Alexander *et al*., 2007). However, TRPM8 is also regulated by androgens and has been proposed to be important for Ca^{2+}-homeostasis in prostate epithelial cells (Zhang *et al*., 2004).

1.5.2.4 TRPA

The TRPA family comprises one mammalian member, TRPA1, which is likely to be activated by noxious cold and pungent substances including isothiocyanates (the pungent compounds in mustard oil, wasabi, horse radish, garlic and onions), Δ^9-tetrahydrocannabinol (THC, the main psychoactive compound in marijuana), and cinnamaldehyde, but it is insensitive to menthol and capsaicin. It is also activated by allicin, the active component in raw garlic and onions, members of the Allium family. TRPA1 has been thought to gate a transduction channel required for the auditory responses in mammals (Corey *et al*., 2004). The protein is present in the tips of stereocilia in hair cells and decreasing the TRPA1 expression causes defects in hearing. However, a clear mechano-activation of TRPA1 has not yet been demonstrated (Ramsey *et al*., 2006).

1.5.2.5 TRPML

The TRPML family contains three mammalian members (TRPML1-3) (Nillius and Voets, 2005; Pedersen *et al*., 2005). The TRPML channels are probably localized to intracellular vesicles and excluded from the plasma membrane (Ramsey *et al*., 2006).

TRPML1 is reported to be a nonselective channel that is inhibited by lowering pH. It may play a role in endosomal acidification (Raychowdhury *et al*., 2004). TRPML1 is important for sorting or transport of endosomes (Alexander *et al*., 2007). TRPML1 are the cause of the neurodegenerative disorder

mucolipidosis type IV in man. TRPML3 is expressed in hair cells and stereocilia and its function is implicated in hearing (Di Palma *et al*., 2002; Alexander *et al*., 2007). The function of TRPML2 is unknown (Ramsey *et al*., 2006).

1.5.2.6 TRPP

The TRPP family subsumes the polycystins that are structurally divided into two groups, polycystic kidney disease 1-like (PKD1-like) and polycystic kidney disease 2-like (PKD2-like) (Delmas, 2004). Members of PKD1-like group are PKD1 (now TRPP1), PKDREJ, PKD1L1, PKD1L2 and PKD1L3. The PKD2-like members comprise PKD2, PKD2L1 and PKD2L2, which have been renamed as TRPP2, TRPP3 and TRPP5, respectively (Moran *et al*., 2004).

TRPP1 and TRPP2 act as a signaling complex. The association of TRPP1 and TRPP2 suppresses the G-protein-stimulating activity of TRPP1 and also the constitutive channel activity of TRPP2. TRPP2 is important for cilia movement, development of the heart, skeletal muscle and kidney. TRPP2 is also likely to act as an intracellular Ca^{2+} -release channel. TRPP3 plays role in retinal development (Alexander *et al*., 2007).

1.5.2.7 TRPN

These channels are characterized by 29 ankyrin repeats in their N-terminal cystolic loop (Sidi *et al*., 2003; Walker *et al*., 2000). They probably act as mechano-transduction channel and are involved in hearing. Mammals lack the TRPN gene (Corey *et al*., 2004).

1.5.3 Physiological functions of TRP channels

TRP channels are cation channels. These ion channels are contributed to Ca^{2+} signalling. Ca^{2+} influx from the extracellular compartment affects cell surface receptor activation. This influx is a feature in most non-neuronal cell types and is referred to as capacitative calcium entry (CCE) or store-operated calcium entry (SOCE) (Putney and McKay, 1999).

1.5.4 Pathological function of TRP channels

TRPV1 is involved in pain reception especially neuropathic pain (Nilius, 2007). The impaired inflammatory thermal hyperalgesia is reported to be present in the TRPV1 knock-out mice (Caterina *et al*., 2000; Davis *et al*., 2000). It has been reported that TRPV1 is expressed in sensory neurones and urothelium of urinary bladder. Intravesical administration of TRPV1 agonist resiniferatoxin (RTX) provided improvement of voiding control by patients with incontinence (phase II trials) (Szallasi and Fowler, 2002).

There are reports about the expression of TRPV1 in sensory neurones innervating gastrointestinal tract. Visceral pain, irritable bowel syndrome and irritable bowel disease may be related to sensitization or upregulation of TRPV1 (Chan *et al*., 2003; Yiangou *et al*., 2001).

In addition, TRPV1 is expressed in the bronchial airways suggesting that TRPV1 is involved in the aetiology of asthma. Stimulation or inhibition of TRPV1 may cause the contraction of bronchi and neurogenic inflammation (Carr *et al*., 2003; Ji *et al*., 2002; Karlsson, 1996). Capsaicin-sensitive afferents are found in pulmonary fibres (Paintal, 1973). The activation of these fibres leads to cough, increased mucosal secretion and bronchoconstriction (Coleridge and Coleridge, 1984).

For cardiovascular pathophysiology, neurones expressing TRPV1 caused cardiogenic sympathoexcitatory reflex in myocardial ischemia. Therefore, TRPV1 antagonists may relieve the cardiac pain (Zahner *et al*., 2003). It is reported that TRPV1 agonist anandamide stimulates calcitonin

gene-related peptide (CGRP) release and cause vasodilation. It is therefore possible that TRPV1 antagonist may be used in migraine treatments (Akerman *et al*., 2004).

1.6 TRP channels and urinary bladder functions

Capsaicin-sensitive C type bladder fibers play a role in micturition. Capsaicin sensitive nerves exhibit both a sensory and efferent function which is determined by release of peptides including tachykinins such as substance P and calcitonin gene related peptides. Sensory function includes the regulation of the micturition threshold and the perception of pain from the urinary bladder, while the efferent function controls nerve excitability, smooth muscle contractility and plasma protein extravasation (Maggi *et al*., 1990; Szallasi and Bloomberg, 1999; Birder, 2007) (Figure 1.5).

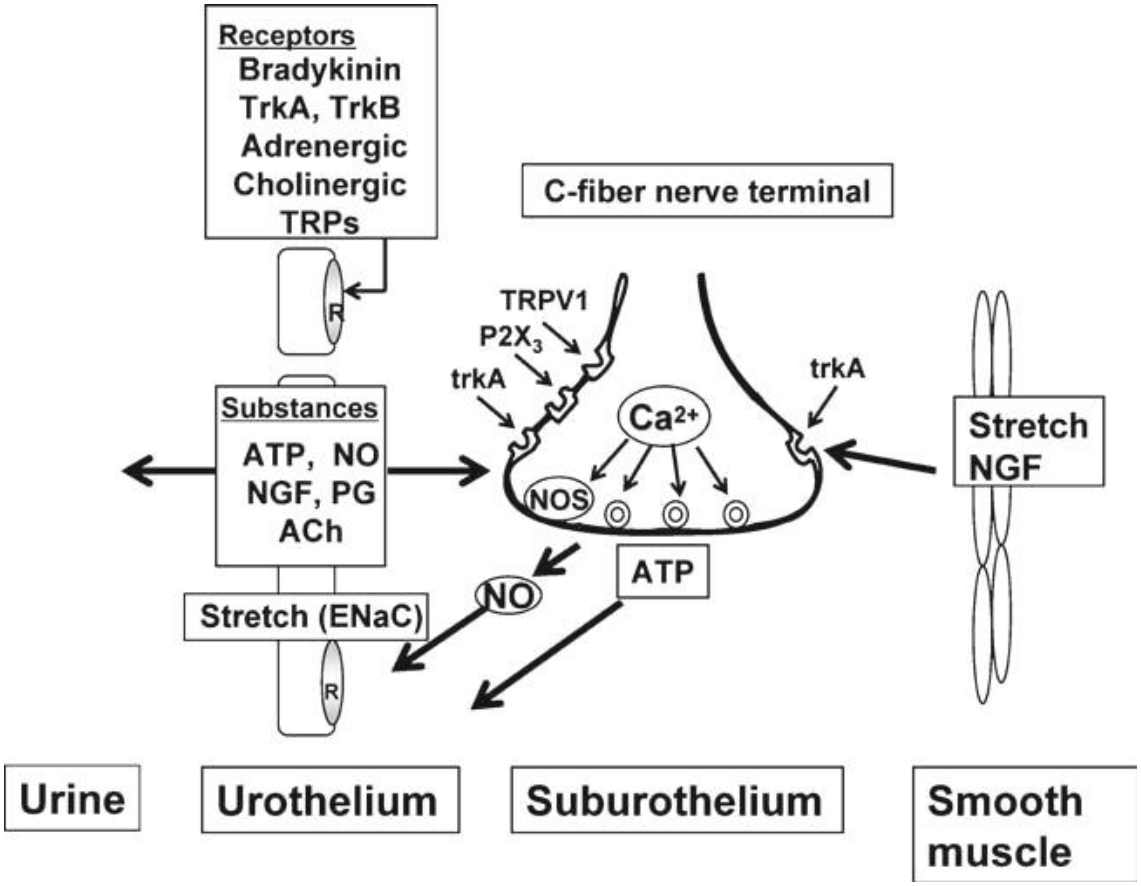


Figure 1.5 Diagram illustrating the involvement of TRP channel in bladder physiology (adapted from Yoshimura *et al*., 2008)

The use of antibodies to TRPV1 revealed TRPV1-immunoreactive nerve fibers in subpopulations of bladder nerves including unmyelined (C-fiber) nerves that detect bladder distension or the presence of irritant chemicals. Within the muscular layer, these nerve fibers appear close to the smooth muscle cells, suggesting that TRPV1 bladder nerves may modulate urothelial function and smooth muscle contractility via the release of sensory peptides contained in TRPV1 bladder fibers. Other findings suggested that TRPV1 is also expressed in non-neuronal cells including urothelial cells and myofibroblasts (Avelino and Cruz, 2006).

TRPV1 knockout mice do not develop bladder overactivity during acute bladder inflammation, suggesting that TRPV1 is involved in bladder hyperreflexia in inflammatory states. In addition, patients suffering from neurogenic detrusor overactivity exhibit significant increases in the number of

TRPV1-expressing nerves and TRPV1 expression within the urothelium (Birder, 2007).

The emergence of the capsaicin-sensitive, C fiber mediated spinal micturition reflex is thought to be due to reorganization of synaptic connections in the spinal cord and changes in the properties of the afferents. Systemic administration of capsaicin is effective in animal models to block the hyperreflexia associated with neurogenic bladder dysfunctions following spinal cord injury. This effect of capsaicin is attributable to the induction of a long lasting refractory state of primary afferent neurons termed “capsaicin desensitization” (Birder, 2007).

Intravesical treatments with vanilloid compounds are beneficial in bladder disorders such as neurogenic bladder in patients with multiple sclerosis or following spinal cord injury or hypersensitivity disorders such as interstitial cystitis (IC). Intravesical vanilloids have also been shown to reduce the number of bladder sensory fibers immunoreactive for TRPV1, substance P or CGRP in patients with painful bladder symptoms. This is due to either a depletion of afferent transmitters or degeneration of peripheral nerve endings in the wall of the urinary bladder (Birder, 2007).

In the patients with neurogenic detrusor overactivity exhibiting increased TRPV1 expression in both bladder nerves and the urothelium, intravesical treatment with resiniferatoxin (RTX) reduced TRPV1 immunoreactivity in both suburothelial afferent nerve and urothelial cells. However, RTX was ineffective in a clinical trial and additional studies would not be pursued (Birder, 2007).

Intravesical application of capsaicin or resiniferatoxin induces reflex activation of the bladder smooth muscle and neurogenic inflammation in the bladder wall (Maggi *et al*., 1989; Maggi *et al*., 1984). Capsaicin or resiniferatoxin directly activates capsaicin sensory neurons in the subepithelial layer of bladder, which, in turn releases substance P. Substance P then sensitizes smooth muscle cells resulting in increased contractions (Quartara and Maggi, 1998). TRPV1 is also expressed by epithelial cells of the transitional epithelium, and activation of these TRPV1-expressing cells results in ATP release, which then activates $P2X_3$ receptors expressed by bladder afferents (Birder *et al*.,

2001; Ferguson *et al*., 1997). Stretching of bladder wall during bladder filling activates TRPV1-expressing afferents either directly or through the action of a mechanotransducer. Co-assembly of TRPV1 with mechanosensitive proteins might underlay the mechanosensitivity of the capsaicin receptor in the bladder (Caterina, 2003).

TRPV1-expressing unmyelinated bladder afferents are insensitive to mechanical stimuli. It is therefore believed that TRPV1 may not be involve in micturition in naive conditions (De Groat and Yoshimura, 2001). However, TRPV1 knock-out mice have higher frequency of low amplitude, non-voiding bladder contractions and reduced reflex voiding during bladder filling (Birder *et al*., 2002) indicating that TRPV1 is involved in the bladder activity and not only in inflammatory conditions. Following sensitization, for example in inflammation, the activity of capsaicin-sensitive fibres plays a significant role in the pathological micturition reflex, which is characterized by frequent involuntary voiding (urge incontinence), decreased bladder capacity and occasional ureteral reflex (De Groat *et al*., 1990; Fowler, 2002). Selective sensory denervation of the bladder elicited by intravesical application of capsaicin or resiniferatoxin disrupts this overactive spinal reflex, resulting in decreased voiding frequency and increased bladder capacity (Cruz *et al*., 1997a, b; De Ridder *et al*., 1997; Silva *et al*., 2000).

The mechanism involved in the sensitization and activation of capsaicin-sensitive bladder afferents has not been elucidated. Inflammatory mediators, such as nerve growth factor released from activated inflammatory cells, have been implicated in the sensitization (Chuang *et al*., 2001; Vizzard, 2000). Inflammatory mediators inducing post-translational changes in TRPV1 can reduce the heat threshold of the receptor and contribute to the sensitization of TRPV1. However, Avelino *et al*. (2003) have reported that cyclophosphamide-induced cystitis, similar to toxin A-evoked ileitis (McVey *et al*., 2003) is accompanied by increased anandamide levels in the bladder. Both exo- and endogenous anandamide enhance bladder reflex activity in a pattern similar to that observed in cyclophosphamide-induced cystitis (Avelino *et al*., 2003).

These findings suggest that anandamide may be a major activator of TRPV1 in cystitis.

A definitive role of TRPV4 in bladder function has not been established. However, in other systems TRPV4 seems to play a role in hypo-osmotic hyperalgesia and in the development of neuropathic pain (Birder, 2007).

The installation of cold solutions (the ice water test) elicits involuntary detrusor contractions in patients with either chronic spinal cord lesions or following bladder outlet obstruction. This reflex is believed to be mediated by activation of C-type afferent fibers within the pelvic nerve sensitive to cold temperatures. The finding that intravesical installation of menthol facilitates the bladder cooling reflex in both the cat and human suggests that TRPM8 may be involved in triggering the reflex. In the urinary bladder, TRPM8-positive immunoreactivity has been demonstrated within bladder nerves and urothelium (Birder, 2007).

TRPA1 is expressed in sensory nerves that innervate the urinary bladder and mediates a contractile effect on bladder smooth muscle, due to release of tachykinins and cyclooxygenase metabolites. The effect on smooth muscle contractility of agents capable of stimulating TRPA1 was comparable in potency to capsaicin, supporting the speculation that this channel may play a role in bladder function (Birder, 2007).

1.7 TRP channels and colon functions

A number of cell types are thought to express TRPV1 in the gastrointestinal tract. They include peripheral terminals of primary and vagal sensory neurons, intrinsic enteric neurons in the myenteric plexi and gastric epithelial cells. Several motor effects of capsaicin on the guinea pig distal colon longitudinal muscle have been described (Maggi, 1987a). A specific contractile action involves myenteric cholinergic neurons. A more sustained, apparently specific inhibitory effect is partly inhibited by tetrodotoxin, probably indicating an involvement of intrinsic enteric neurons or “axon reflex” arrangement in capsaicin sensitive extrinsic nerves. The circular muscle of the guinea pig

proximal colon also shows relaxation in response to capsaicin, which seems partly mediated by CGRP (Maggi, 1996).

TRPV1 expressed by primary sensory neurons in the gastrointestinal tract has been implicated in the development of inflammation and hyper-motility/hyperreflexia. It has been shown that substance P is a major player mediating inflammation in the intestines (Pothoulakis *et al*., 1994). The finding that TRPV1 antagonist capsazepine prevents the development of Toxin A-induced inflammation indicated that the capsaicin receptor is involved in the process, and capsaicin-sensitive primary sensory fibres are the major source of substance P (McVey and Vigna, 2001). Recent findings suggest that the endogenous substance activating TRPV1 during ileitis is anandamide (McVey *et al*., 2003). Anandamide concentration in the inflamed tissues is increased and this endogenous TRPV1 ligand exacerbates ileitis. However, Mang *et al*. (2001) mentioned that anandamide induces acetylcholine release from intrinsic enteric neurons expressing TRPV1 receptors, suggesting that the capsaicin receptor expressed by neurons in the myenteric plexi might contribute to the development of enhanced intestinal motility or secretion. However, it should be noted that other groups using different chemicals to induce experimental colitis or enteritis (Evangelista and Tramontana, 1993; McCafferty *et al*., 1997; Reinshagen *et al*., 1996) reported a rather accentuated inflammation following ablation of capsaicin-sensitive nerve fibres with systemic capsaicin treatment of adult animals. This effect was explained by the possible protective actions of the sensory neuropeptide, CGRP on the mucous membrane.

Activity of TRPV1 has been implicated in the development of abdominal pain that occurs in irritable bowel syndrome, which is the most common form of the pathological condition termed functional bowel disorders. Since the pain experienced by the patients is not matched with any detectable structural abnormality by conventional diagnostic methods, the concept of an altered nociceptive function as the main etiological factor of irritable bowel syndrome-associated pain has been developed (Collins *et al*., 2001; Hunt and Tougas, 2002). According to the proposed mechanism, sensitization of TRPV1 by a variety of ligands including the protease activated receptor 2 expressed by

primary sensory neurons (Coelho *et al*., 2002; Kawao *et al*., 2004) and by paracrine/endocrine substances produced by the enterochromaffin (e.g. serotonin) or enteroendocrine (e.g. cholecystokinin) cells underlay the development of abdominal pain in irritable bowel syndrome (Hillsley and Grundy, 1998). Although the involvement of TRPV1 in the development of visceral hyperalgesia has been demonstrated in human (Drewes et al., 2003), the contribution of this mechanism to pathogenesis of the irritable bowel syndrome is stilled debated.

1.8 Diabetes mellitus and TRP channel functions

Stimulation of the TRPV1 channel is used in the treatment of neurogenic bladder dysfunction (Fowler *et al*, 1992). TRPV1 levels are reduced in skin biopsies from patients with diabetic neuropathy (Facer et al, 2007) and insulin has been shown to cause sensitization and translocation of TRPV1 receptors (Van Buren et al, 2005).

1.9 Cholesterol and TRP channel functions

The plasma membrane of eukaryotic cells contains more lipid species than other biological membrane, were need to form a simple bilayer. Specific lipids could serve to organize membranes into discrete domains with different properties. The microdomain is enriched in cholesterol and sphingolipids. These microdomains are usually called lipid rafts, and many studies suggested that rafts play a role in a wide range of important biological processes, including numerous signal transduction pathways, apoptosis, cell adhesion, and migration, synaptic transmission, organization of cytoskeleton, and protein sorting during both exocytosis and endocytosis (Hao *et al*., 2001).

Lipid rafts, microdomains rich in sphingolipids and cholesterol in the plasma membrane are involved in protein trafficking, regulation of cytoskeleton and formation of signalling complexes. Ca^{2+} signals are generated across wide

spatial and temporal ranges through various channels that can last from microsecond to several minutes. This broad range of Ca^{2+} signals can be efficiently coordinated through organization of specific Ca^{2+} channels, pumps, buffers, exchangers and protein scaffolds into common microdomains. Members of rafts provide such a microdomain wherein highly specific signalling events can be efficiently executed (Pani and Singh, 2009). Members of the TRP family such as TRPC1 are found to form signalling complexes in lipid rafts (Lockwich *et al*., 2000). Liu *et al*. (2007) suggested that TRPV1 might be concentrated in lipid rafts to facilitate interaction with specific lipid metabolites and by-products of inflammatory mediators. Cholesterol is an essential component of lipid rafts (Zajchowski and Robbins, 2002). It can be extracted from the plasma membrane of various cell types by methyl-β-cyclodextrin (MCD) (Kilsdonk *et al*., 1995).

1.10 Objectives

The purpose of the present study was to investigate the influence of the streptozotocin (STZ) model of diabetes, cholesterol modulation and time course of the streptozotocin (STZ) model of diabetes on the responses of rat urinary bladder to transient receptor potential (TRP) channel agonists and antagonists using conventional organ bath techniques. Some studies were also performed on colon tissues. In addition, the hypothesis that channel dysfunction is a consequence of hyperglycaemia that will be experienced by all tissues in the body, will be tested. In order to investigate the mechanism of action, the fluorimetry studies were conducted in TRPV1-expressing Human Embryonic Kidney (HEK) 293 cell line to explore the calcium ion influx modulated by hyperglycaemic state and cyclodextrin molecules.

The experimental designs have been conducted according to the three main proposed aims. Firstly, to investigate the effect of the streptozotocin (STZ) model of diabetes on a range of TRP channel function in urinary bladder smooth muscle preparations using TRP channel agonists and antagonists and to study the neurotransmitters involved in the contractile or relaxant responses

of the smooth muscle preparations. Secondly, to explore the involvement of cholesterol modudation in TRP channel signalling. Thirdly, to study the change in TRP channel response with time following the treatment with streptozotocin.

The objectives of these investigations are the following:

1.10.1 To examine the TRPV1, TRPV4, TRPA1, and TRPM8 channel function in bladder strips from STZ-diabetic and control rats using conventional organ bath techniques and a range of appropriate agonists and antagonists which are available

1.10.2 To check the downstream mechanisms of these pathways by the use of neurokinin ligands

1.10.3 To examine the effect of STZ-induced diabetes on TRPV1 channel function in the rat colon

1.10.4 To examine the effect of STZ-induced diabetes on ion secretory function in the rat colon

1.10.5 To study the effect of cholesterol and cyclodextrins on muscarinic receptor function in rat bladder

1.10.6 To study the effect of cholesterol and cyclodextrins on muscarinic receptor function in HEK 293 cells

1.10.7 To study the effect of cholesterol and cyclodextrins on TRP channel function in rat bladder

1.10.8 To study the effect of cholesterol and cyclodextrins on TRP channel function in HEK 293 cells

1.10.9 To study the effect of time frame of STZ injection on TRP channel function

1.10.10 To study the effect of elevated glucose level on TRP channel function in rat bladder

1.10.11 To study the effect of elevated glucose on TRP channel function in HEK 293 cells

Chapter 2

General Materials and Methods

2.1 Animals

In the present study, male wistar rats with body weight of 300-400 g were used. These animals were supplied by the Biological Science Unit (BSU), School of Life Sciences, Faculty of Health and Human Science, University of Hertfordshire. Animals were divided into two groups: control and diabetic.

The animal use and welfare were in accordance with the recommendations of the Animals (Scientific Procedures) Act, 1986 under Project License No. PPL70 5855.

2.2 Maintenance of animals

All rats were kept in separate cages. One control and one diabetic rat were kept in the same cage. They were provided with feed and water daily for up to 8 weeks until used in the study. All groups were kept in a temperature-controlled room (22 ± 2 ºC), artificially lit from 6.00 to 18.00 hours daily. The initial weights and blood glucose levels of the rats were recorded and again at sacrifice.

2.3 Induction of diabetes mellitus

The initial blood glucose level of rats was measured using an Accu-Check active testing kit. The blood was taken from the tail vein. The initial body weight was also measured to quantify the change in body weight over the eight weeks. In order to induce diabetes, streptozotocin (STZ) at dose of 65 mg/kg bodyweight was injected intraperitoneally to the rats with a single injection. Streptozotocin was freshly dissolved in 20 mM citrate buffer at pH 4.5. The control rats were injected with 20 mM citrate buffer (pH 4.5) at an equal volume

to the diabetic group. To avoid the initial hypoglycemia, 2% sucrose was added to drinking water for the streptozotocin-induced diabetic rats for 48 hours. The weight of the rats and their blood glucose levels were measured immediately after sacrifice. The blood glucose level in control rats should be approximately 100 mg/dl. For diabetic groups, the blood glucose level of 400 mg/dl or more is confirmed as diabetic.

In the present study, the high single dose (65 mg/kg bodyweight) of streptozotocin was chosen as diabetes model in rat in order to establish diabetes in animal model. The reasons behind this decision is that diabetes induced by a single intravenous or intraperitoneal injection of streptozotocin is probably the most widely used experimental model of insulin-dependent diabetes mellitus or type 1 diabetes mellitus. Streptozotocin is efficacious after intraperitoneal administration of 40-60 mg/kg body weight, but a single dose below 40 mg/kg body weight may be ineffective (Katsumata *et al*., 1992). Streptozotocin can induce severe insulin-deficient diabetes in rats and other rodents, either when given as a single high dose (50-100 mg/kg in rats) or as multiple low doses. In addition, the high single dose model is simple and less time-consuming. It also provides a reproducible diabetes model. In the multiple low dose model, diabetes develops more gradually and appears to have an autoimmune, rather than toxic, basis (Islas-Andrade *et al*., 2000). This multiple low dose is used predominantly in the mouse (Szkudelski, 2001).

2.4 Tissue preparations

2.4.1 Urinary bladder tissues

The bladder was immediately removed by opening the lower abdomen. The bladder was cut at the bladder neck to obtain the whole bladder. Then the removed bladder was kept in Krebs solution gassed with 95%O_2 and 5% CO_2.

The bladder was cut longitudinally along the neck to the body. Then four strips were taken from the whole bladder. The strips were approximately 1 cm long and 0.5 cm wide. The strip was tied with tread at one end to be attached to the force transducer. The other end was tied to a hook to be attached to organ bath (Figure 2.1).

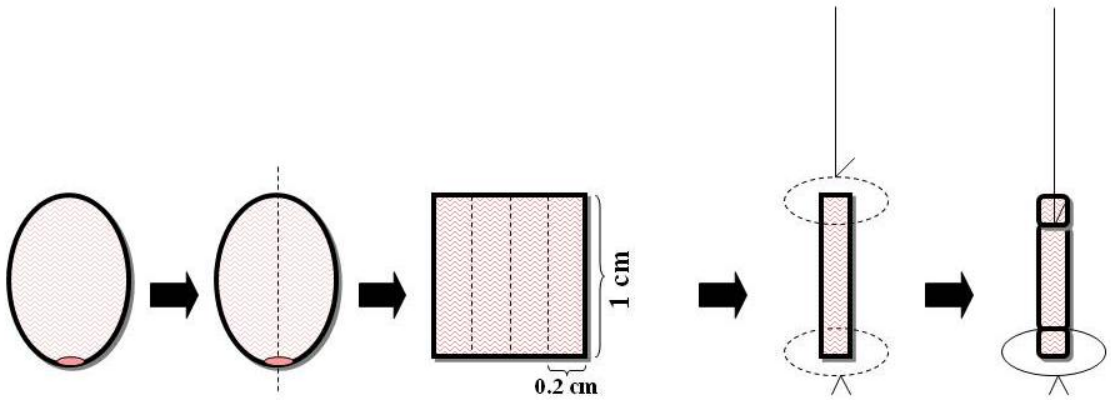


Figure 2.1 Diagram illustrating urinary bladder strip preparations

2.4.2 Colon tissues

Eight weeks after injection, the diabetic and control rats were sacrificed by asphyxiation with a CO_2 overdose. A 3 cm segment of the proximal and distal colon was removed from control and STZ-induced diabetic rats and immersed in Krebs-Henseleit solution of the composition (in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, and gassed with 95% O_2 and 5% CO_2 at room temperature (21 ± 4 $^{\circ}$C).

The colonic segment was opened longitudinally along the mesenteric border after trimming the mesentery and rinsing the faecal contents away with Krebs-Henseleit solution. The tissue was then pinned as flat sheet with the basolateral (serosal) side up on a cork board lined with a piece of paper towel soaked with Krebs-Henseleit solution. The smooth muscle layers with the attached mesenteric plexus were then gently stripped away by blunt dissection leaving a submucosal plexus-mucosal sheet (Figure 2.2). A maximum of two submucosal plexus-mucosal sheets were prepared from the 3 cm segment.

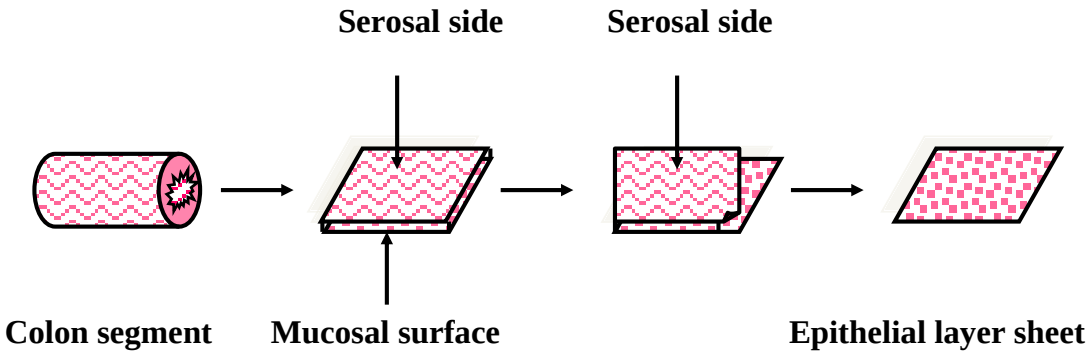


Figure 2.2 Diagram illustrating the preparation of a submucosal plexus-mucosal sheet from the colon

2.5 Apparatus settings

2.5.1 Organ bath

The bridge amplifier (Power Lab; AD Instrument, U.K.) was connected to the force transducer and Chart 5 software. The force transducer was connected to the organ bath apparatus to determine the change in tension caused by either contraction or relaxation of the muscle strips (Figure 2.3). The calibration of the apparatus was begun by opening the Chart 5 software on the computer screen. Two channels were selected in accordance with the two organ baths which were available. The range was selected to 2 m/s. For the bridge amplifier settings, the low pass was 20 mV and zeroing was set. When the baseline was at zero, a one gram weight was attached to the force transducer to obtain the initial tension at one gram. To convert the unit from mV to gram, the trace of one gram weight was highlighted. The baseline was labeled as zero mV and the peak from 1 g weight was labeled as one mV. The unit was then changed from mV to grams.

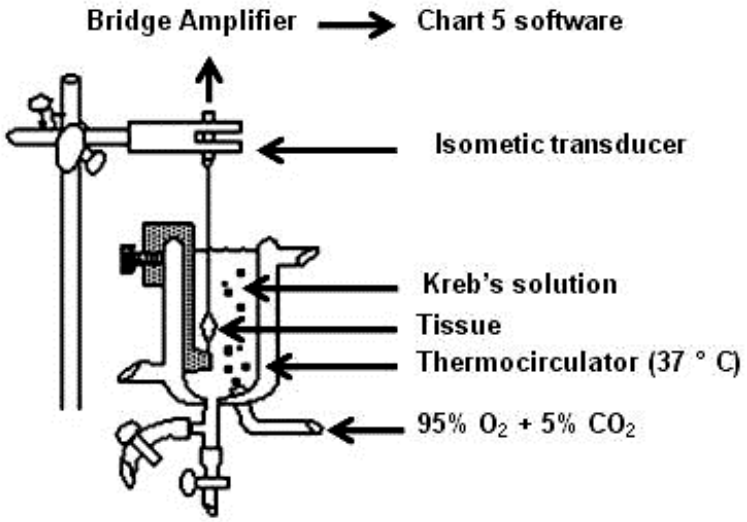


Figure 2.3 Diagram of the organ bath and apparatus setting (adapted from Kenakin, 2001)

The 10 ml organ bath was rinsed with distilled water. The Krebs' solution was then added. The bath was aerated by 95%O_2 and 5% CO_2 and warmed at 37 ºC by thermocirculator.

When the calibrations were complete, the longitudinal strips of control and streptozotocin-induced diabetic rat bladders were placed into the bath. The top end of the bladder strip with thread was attached to the force transducer. The bottom end with the hook was attached to the organ bath (Figure 2.3). Recording was started after the resting tension on the strip was set to 1 g. The bladder strips were equilibrated for at least 30 minutes before any drug applications.

2.5.2 Ussing chamber

Each submucosal plexus-mucosal sheet (Figure 2.2) was gently clamped between the Ussing Chambers (WP Instruments, U.S.A) with a circular window surface area of 0.63 cm^2. Both apical and basolateral surfaces of the submucosal plexus-mucosal sheet were bathed with 5 ml of Krebs-Henseleit solution maintained at 37 $^{\circ}C$ and circulated with a stream of 95% O_2 and 5% CO_2 (Figure 2.4).

The chamber was connected through 3M KCl agar electrodes to a voltage clamp apparatus (DVC 1000, WP Instruments, U.S.A) and the preparations automatically short-circuited by voltage clamping the tissue at a holding potential of 0 mV. The short circuit current (I_{sc}) was continuously recorded in units of $\mu A.cm^{-2}$ on a MacLab data-acquisition recording system running the Chart version 3.5 software (AD Instrument, U.K.) on an Apple Macintosh computer (Apple Macintosh, U.K.).

At the end of every experiment, the chambers were washed thoroughly with 70% ethanol followed by 2 M hydrochloric acid and distilled water to ensure the complete removal of residual drugs used in the experiments. The 3 M KCl-agar electrolyte in the electrodes was also renewed regularly.

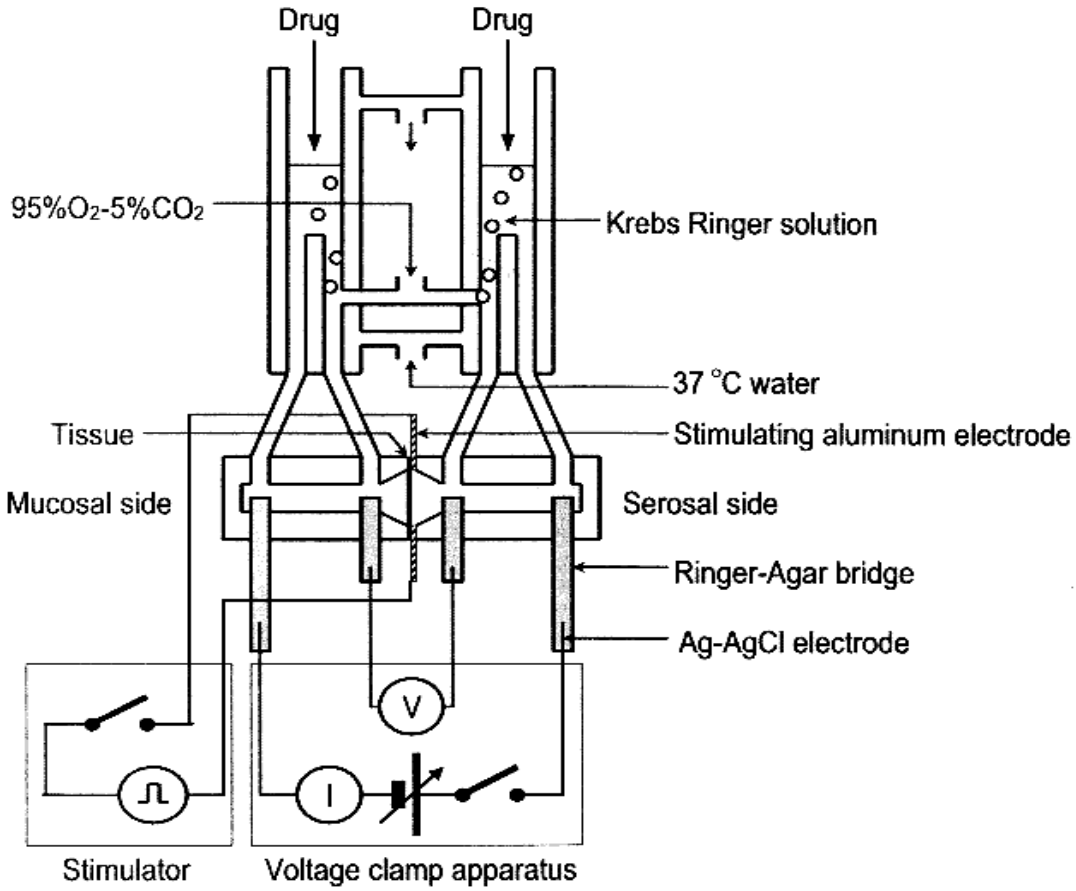


Figure 2.4 Diagram of apparatus settings of Ussing Chamber (adapted from Karaki and Kuwahara, 2004)

2.6 Measurement of cholesterol contents from rat bladder tissues

Cholesterol contents from rat bladder tissues treated with and without cyclodextrins were measured by application of the procedure described by Maraschiello et al. (1996). The methods are summarized as the follows.

2.6.1 Extraction of cholesterol from rat bladder tissues

After treated with or without cyclodextrins, 100 mg of rat bladder tissues were directly saponified with 2 ml of 0.5 N KOH in methanol for 1 hour at 80 °C. After cooling, 2 ml of distilled water saturated with NaCl was added. The tubes

were vortexed for 30 seconds followed by addition of 3 ml ether/hexane (1:1, v/v) and centrifuged for 10 minutes at 300 g. The upper phase was recovered and the hexane/ether extraction step was repeated twice. The extracts were combined and evaporated to dryness under a stream of nitrogen and redissolved in 1 ml of acetonitrile/isopropanol for HPLC analysis. Cholesterol was used as external standard for HPLC analysis. Injection volumes were 20 µl.

2.6.2 High Performance Liquid Chromatography (HPLC) Analysis

HPLC is a chromatographic technique that can separate a mixture of compounds and is used to identify, quantify, and purify the individual components of the mixture. HPLC utilizes different types of stationary phase, a pump that moves the mobile phases and analyte through the column, and a detector that provide a characteristic retention time for the analyte. Analyte retention time varies depending on the strength of its interaction with the stationary phase, the ratio/composition of solvent used, and the flow rate of the mobile phase.

The HPLC system consisted of a 600 multi-solvent delivery system, an octadecylsilica reverse-phase column (150 x 4 mm) with 5 µl particle diameter operated at room temperature and diode array detector 990. Detection was performed at 210 nm. The HPLC mobile phase consisted of acetonitrile : 2-propanol (55:45 v/v) at a flow rate of 1.2 ml/minute. All solvents were HPLC grade.

The response linearity was studied for HPLC. 10, 20, 40, 60 and 90 µg/ml of cholesterol was injected (Figure 2.5). Linear plot of concentration versus peak areas were calculated. Consequently, 20 µl samples from rat bladder tissues treated with and without cyclodextrins were injected

The results show that the peak of cholesterol contents of standard cholesterol solution are in concentration dependent manner (Figure 2.6) with the retention time of 4.0 minutes.

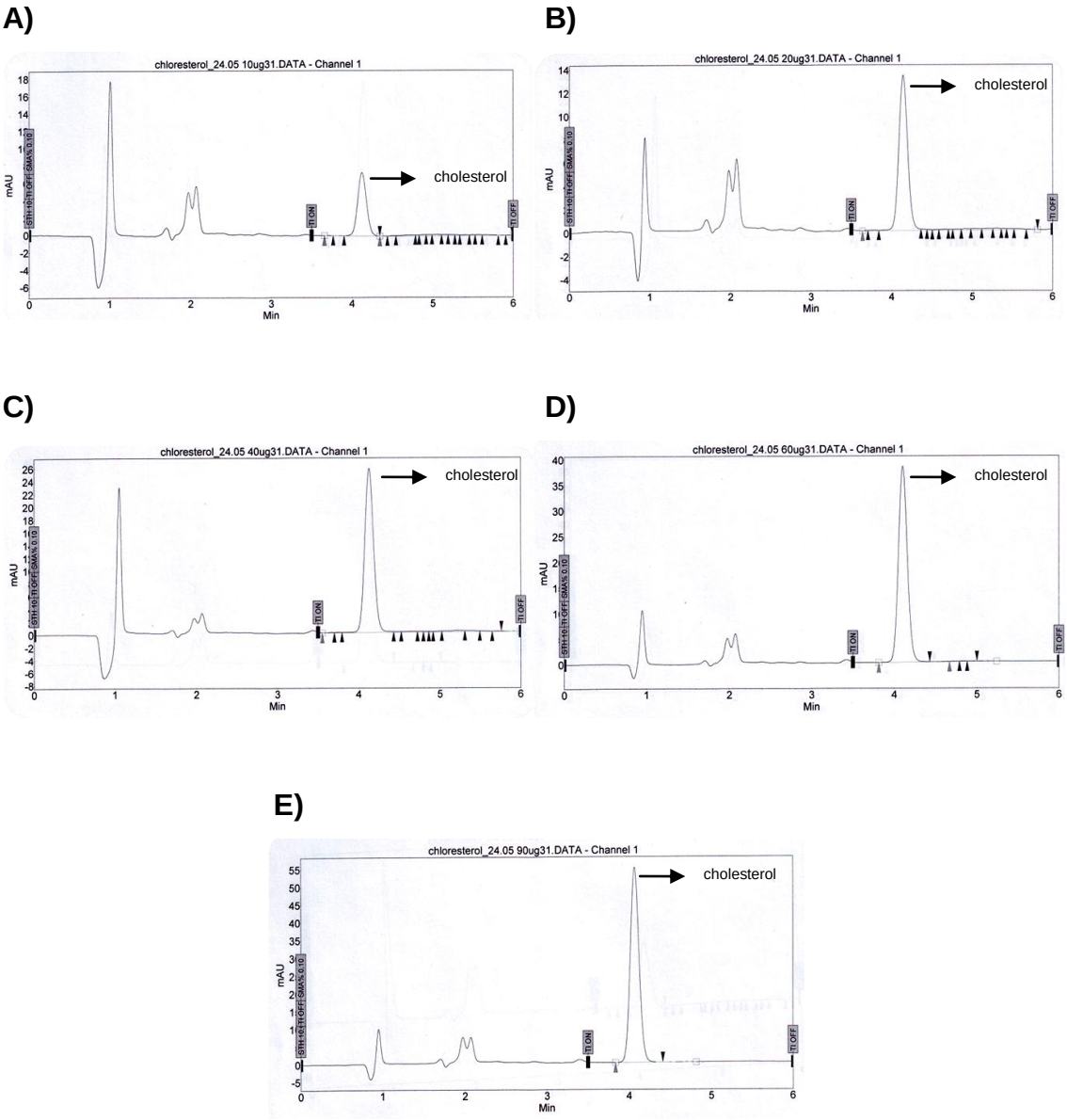


Figure 2.5 Chromatogram illustrating HPLC analysis of standard cholesterol samples at concentrations of 10 (A), 20 (B), 40 (C), 60 (D) and 90 µg/ml (E). The retension time for standard cholesterol samples is 4-5 minutes so that the peak of standard cholesterol samples appeared at about 4 minutes.

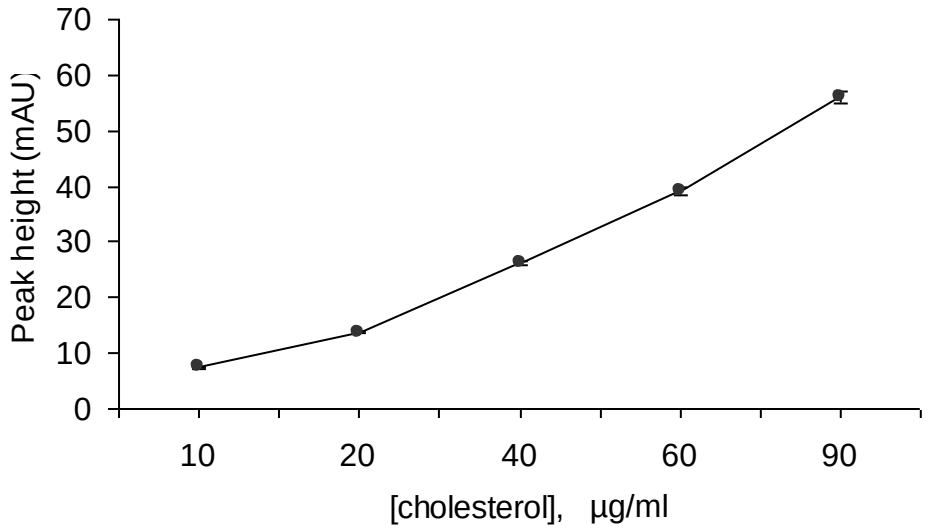
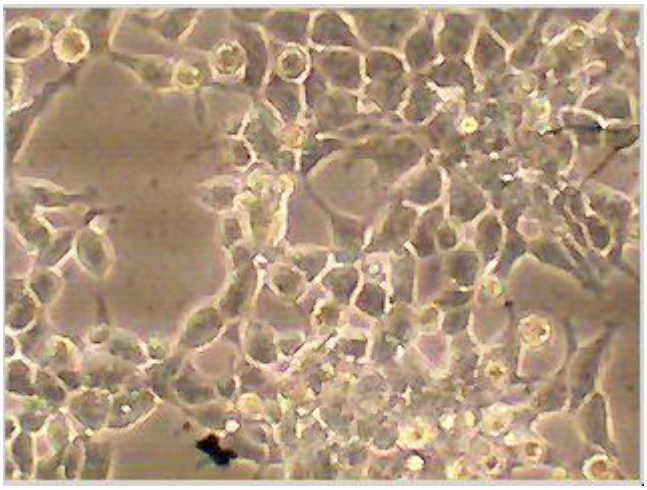


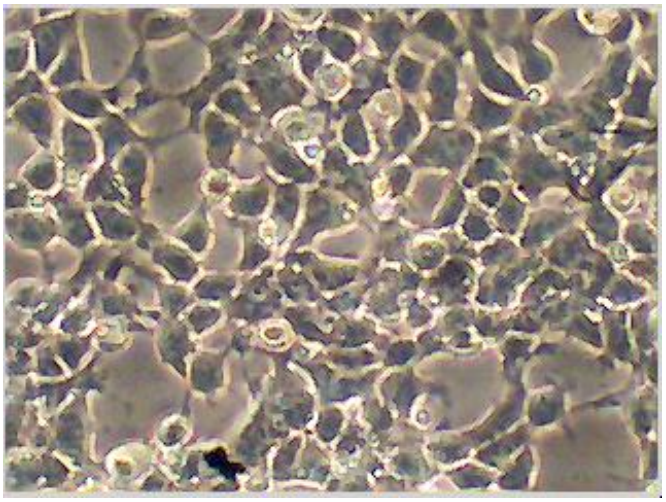
Figure 2.6 Standard curve of cholesterol contents in different concentrations of cholesterol standard solution.

2.7 Cell culture

Human Embryonic Kidney (HEK) 293 cell stably expressing rat TRPV1 were used in the present study (Figure 2.7). Cells were maintained in an incubator (37 °C, 5% CO_2) in Dulbecco's modified Eagle's medium (invitrogen, UK) containing 110 mg/l pyruvate supplemented with 10% FBS, L-Glutamine, Penicillin-Streptomycin, Non-essential amino acid, and 500 μg/ml Geneticin as a selection agent. Cells were removed from their culture flasks by treatment with Accutase (Sigma Aldrich, UK), then plated onto poly-D-lysine-coated coverslips and incubated with high and low glucose media at 37 °C for 24, 48 and 72 hours (Figure 2.8) before experimentation.



A)



B)

Figure 2.7 Morphology of TRPV1 expressing-Human Embryonic Kidney (HEK) 293 cells incubated in low (A) and high (B) glucose media for 72 hours.

2.8 Calcium imaging

The coverslips with HEK 293 cells were loaded with Fura 2 acetyloxymethyl ester (Fura-2 AM) (Molecular Probes, Carlsbad, CA) in extracellular solution (containing in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$ 5 ml, 1 $CaCl_2$, 10 HEPES 10 D-glucose, pH 7.3 with NaOH) and incubated for 40 minutes at 37 °C.

Fura-2 is UV light-excitable, ratiometric Ca^{2+} indicators. It is one of the most popular Ca^{2+}-indicators used in measurement of intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$). It is more practical to change excitation wavelengths than emission wavelengths. When binding Ca^{2+}, fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at 500 nm (Figure 2.8). Fluorescence of fura-2 is classically studied using two excitation wavelengths, 340 and 380 nm (Gillis and Gailly, 1994).

Unlike the salt forms, the acetoxymethyl (AM) esters of fura-2 can passively diffuse across cell membranes, enabling researchers to avoid the use of invasive loading techniques. Once inside the cell, these esters are cleaved by intracellular esterases to yield cell-impermeant fluorescent indicators. Cells can be loaded simply by immersion in a solution of the permeant acetoxymethyl ester form (Fura-2 AM), which is fluorescent but Ca^{2+}-insensitive. Subsequent cleavage by intracellular esterases liberates fura-2, which is then trapped into the cell (Gillis and Gailly, 1994).

For imaging, the coverslip with HEK-293 rTRPV1 cells was placed in a custom-built chamber (bath volume of 600 μl) and superfused with extracellular solution 10 minutes before each experiment. Images were acquired with a CCD camera through a 40x oil immersion objective lens of an inverted Nikon Diaphot TMD microscope. After application of immersion oil, cells were selected as region of interest by marking the whole single cell using cursor. In addition, background was selected by marking on the surface of coverslip where there are no the cells. Excitation wavelengths of 350 and 380 nm were used with an

emission wavelength of 510 nm. Calcium levels were reported as F350/F380 versus time.

Fluorescent readings were made over a 3 minutes period at 1-5 seconds intervals following addition of agonist to the cells. Capsaicin (1 μM) (Sigma Aldrich, UK) was used as a TRPV1 agonist. Stock solutions for all stimuli were made in absolute ethanol and diluted in extracellular solution. The maximal responses (F*max*) were achieved by using 5 μM ionomycin and the minimal responses (F*min*) were obtained from treatment with 10 mM EGTA (Figure 2.9).

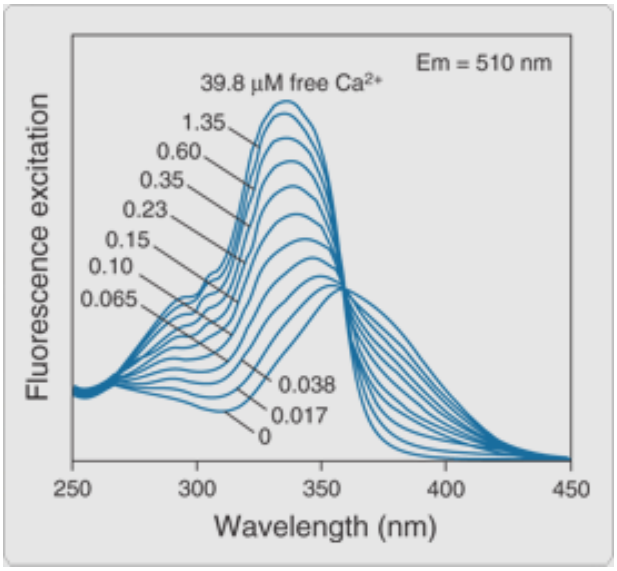


Figure 2.8 Fluorescence excitation spectra of fura-2 in solutions containing 0 – 39.8 μl free calcium (adapted from Molecular Probes, 2010).

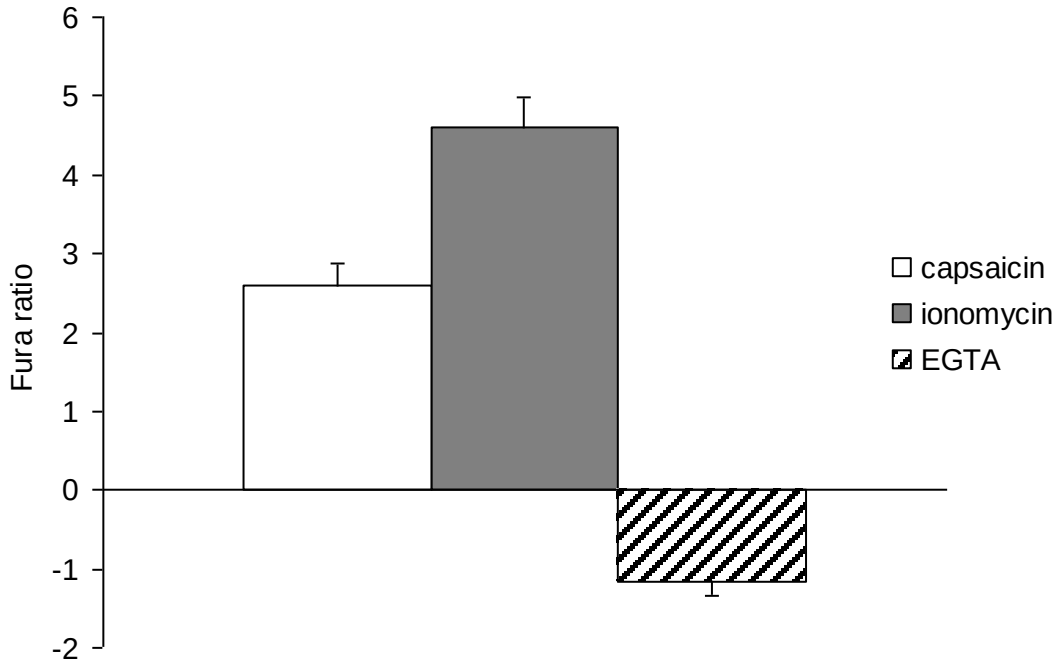


Figure 2.9 Change in Fura 2 ratio in HEK 293 rTRPV1 cells in response to capsaicin stimulation and to treatment with 5 μM ionomycin and 10 mM EGTA. Values represent the mean ± S.E.M for 6 coverslips.

2.9 Drugs

The drugs with their supplier and solvents used in the present study are shown in Table 3.

Table 3 Drugs used in the present study

Drugs	Mode of action	Supplier	Solvent
Carbachol	Muscarinic receptor agonist	Sigma	Distilled water
Capsaicin	TRPV1 agonist	Sigma	Absolute ethanol
Allyl isothiocyanate	TRPA1 agonist	Sigma	Absolute ethanol
Veratridine	Voltage dependent Na^{2+} channel activator	Sigma	Absolute ethanol
Cholesterol-PEG	Membrane lipid enhancer	Sigma	Distilled water
Methyl-β-cyclodextrin	Cholesterol extractor	Sigma	Distilled water
β-cyclodextrin	Cholesterol extractor	Sigma	Distilled water
α-cyclodextrin	Cholesterol extractor	Sigma	Distilled water
Isoprenaline	Adrenoceptor agonist	Sigma	Distilled water
Capsazepine	TRPV1 antagonist	Sigma	Absolute ethanol
Ruthenium Red	TRPV1 antagonist	Sigma	Absolute ethanol
SB-366791	TRPV1 antagonist	GSK	Dimethyl sulfoxide
Spantide	Substance P antagonist	Sigma	Dimethyl sulfoxide
Substrance P	Neurotransmitter	Sigma	Distilled water
Allyl isothiocyanate	TRPA1 agonist	Sigma	Absolute ethanol
Menthol	TRPM8 agonist	Sigma	Dimethyl sulfoxide
Icilin	TRPM8 agonist	Sigma	Dimethyl sulfoxide
Neurokinin alpha	Neurotransmitter	Cambridge Research Biochemical	Distilled water
Bombesin	Neurotransmitter	Tocris	Distilled water
Resiniferatoxin	TRPV1 agonist	Tocris	Absolute ethanol
4-α-PDD	TRPV4 agonist	Sigma	Absolute ethanol
Citral	TRPV4 antagonist	Sigma	Absolute ethanol
CGRP	Neurotransmitter	Tocris	Distilled water
GSK-1016790A	TRPV4 agonist	GSK	Dimethyl sulfoxide

Drugs	Mode of action	Supplier	Solvent
Chlopromazine	Dopamine receptor antagonist	Sigma	Distilled water
CP55,940	Cannabinoid receptor agonist	Tocris	Distilled water
Bradykinin	Pro-inflammatory mediator	Sigma	0.1 M acetic acid
SB207164A	NK_2 antagonist	GSK	Distilled water
GR205171A	NK_1 antagonist	GSK	Distilled water
γ-cyclodextrin	Cholesterol modulator	Sigma	Distilled water

2.10 Data analysis

2.10.1 Motility responses

For the motility studies, the values were expressed as gram of contraction or relaxation versus gram of strip's wet weight. The wet weight of strips can vary and this could influence the level of contraction or relaxation. Tissue wet weight thus was taken into account. The initial tension at the baseline was subtracted and the amplitude of contraction or relaxation after drug application was calculated. This difference was then divided by the tissue wet weight to obtain the amplitude contraction (g)/wet weight (g). Relaxant responses were expressed as a percentage (%) of relaxation with regardless to tissue wet weight. Contractile responses were expressed as a g tension/g wet weight. All data values were expressed as mean ± S.E.M. Statistical significance was determined by using student's unpaired *t*-test and two-way ANOVA as appropriate. *P*-values less than 0.05 were taken to be significant.

2.10.2 Ion secretory responses

The I_{sc} was taken as a measure of active ion transport. A positive change in the I_{sc} in response to stimulation of ion transport by a neurotransmitter or exogenous agonist indicated a net basolateral to apical anion flux or a cation flux in opposite direction, and this increase was recorded as an upward

deflection on the computer. The amplitude of the response was taken a measure of the intensity of the secretory response.

The change in the I_{sc} were measured as units of $\mu A.cm^{-2}$ and presented as the mean ± S.E.M. Comparisons between the data were performed using a Student's unpaired *t*-test and the probability of $P<0.05$ was taken to signify statistical difference between groups.

Chapter 3

The effect of streptozotocin-induced diabetes on TRP channel function in rat urinary bladder

3.1 Introduction

Lower urinary tract complications are commonly found in diabetic patients (Daneshgari and Moore, 2006). Bladder dysfunction is the most common lower urinary tract complication in diabetes. Urine storage and dispersal are two main functions of urinary bladder. Diabetic bladder dysfunction is characterized by a triad of decreased sensation, increased capacity and poor emptying with a prevalence estimated to be between 32% and 45% (Hunter and Moore, 2003). In asymptomatic diabetic patients, increased bladder volume at first voiding sensation and decreased detrusor contractility are the main problems (Ueda *et al*., 2000). Therefore, diabetic bladder dysfunction manifestations are a combination of storage and voiding problems. It has been proposed that there are two main mechanisms of diabetic bladder dysfunction (Daneshgari *et al*., 2009). In the early diabetic state, there is osmotic polyuria causing bladder hypertrophy. However, in the late phase of diabetic state, there is decomposition of bladder tissue and function. Moreover, diabetic bladder dysfunction induces the alterations in the function of bladder detrusor, urethra, autonomic nerves and urothelium (Yoshimura *et al*., 2005).

The study in animal models of diabetes especially in streptozotocin induced- diabetes in rats indicated that diabetes both decreased (Longhurst and Belis, 1986) and increased detrusor contractility (Warning and Wrendt, 2000). Abnormalities of bladder function such as reduced contractile responses to nerve stimulation and applied acetylcholine have been reported (Longhurst and Belis, 1986). In addition, there are abnormalities in afferent nerve signaling in bladder from streptozotocin-induced diabetic rats (Steer *et al*., 1994). A decrease in nerve growth factor in bladder from rats with 12 weeks streptozotocin-induced diabetes has also been reported (Sasaki *et al*., 2002).

Transient Receptor Potential (TRP) channels are a recently identified large group of calcium permeable ion channels that allow calcium entry without requiring cell depolarization. It has been documented that TRP channels are associated with nociception and mechanosensation in various organs. TRP channels that are expressed in bladder include TRPV1, TRPV2, TRPV4, TRPM8 and TRPA1 (Andersson *et al*., 2010).

The prototypic TRPV channel, TRPV1 is the most investigated channel compared to other TRP subfamilies. TRPV1 channel is activated by capsaicin, which has been shown to cause contraction of the rat bladder (Saitoh *et al*., 2007) and has been used in the treatment of neurogenic bladder dysfunction (Fowler *et al*., 1992). Pinna *et al*. (1994) have shown decreased capsaicin responses in STZ-diabetic rat bladder. TRPV1 levels are reduced in skin biopsies from patients with diabetic neuropathy (Facer *et al*., 2007) and insulin has been shown to cause sensitization and translocation of TRPV1 receptors (Van Buren *et al*., 2005). Therefore we hypothesize that TRPV1 function will be reduced in the diabetic bladder.

Three other TRP channels, TRPV4, TRPM8 and TRPA1 are also emerging as playing a significant role in bladder function. TRPV4 is a Ca^{2+} - permeable stretch-activated cation channel. TRPV4 is expressed in bladder urothelium where it may play a role in transducing myogenic tone in the bladder wall (Birder *et al*., 2007). It is suggested to be involved in stretch-induced ATP release. The study in TRPV4-deficient mice showed an abnormality in frequencies of voiding and non-voiding contraction in bladder. TRPM8 is activated at low temperatures and is reported to be expressed in the urothelium and suburothelial sensory fibers. Increased TRPM8 expression has been demonstrated in nerve fibers of overactive and painful bladder (Mukerji *et al*., 2006). TRPV1 is co-expressed with TRPA1 and the later is reported to be expressed on capsaicin-sensitive primary sensory neurons.

This study is aimed to investigate the responses to TRPV1, TRPV4, TRPA1, and TRPM8 agonists and antagonists of streptozotocin-induced diabetic and control rat bladders. The downstream mechanisms of these pathways were checked by the use of, for example, neurokinin ligands. Bladder

strips from STZ-diabetic and vehicle treated control rats were mounted in organ baths for tension measurement.

3.2 Methods

To study whether diabetes had any effects on the functions of TRP channel function, TRPV1, TRPV4, TRPA1 and TRPM8 agonists and TRPV1 antagonists were used. For example, the TRPV1 agonist capsaicin at concentrations of 10^{-10} M to 10^{-6} M was used. The tissues were equilibrated for 30 minutes before adding capsaicin or other TRP channel ligand. Capsaicin was added to the organ bath in a cumulative manner (Figure 3.3).

3.2.1 TRPV1 agonist

To study whether diabetes had any effects on the functions of TRPV1 channel, capsaicin at concentrations of 10^{-10} M to 10^{-6} M was used. The tissues were equilibrated for 30 minutes before adding capsaicin. Capsaicin was added to the organ bath in a cumulative manner. Contractile responses to capsaicin of the tissues from control and STZ-diabetic rat bladders were measured and compared.

3.2.2 TRPV1 antagonists

To check the selectivity of capsaicin for the TRPV1 in the rat bladder strips, the TRPV1 antagonists, capsazepine and ruthenium red, were used. Capsazepine at the concentration of 10^{-5} M was added to the organ bath to expose the bladder strips to the antagonist. After incubation for 20 minutes, capsaicin was added cumulatively at the concentration of 10^{-10} M to 10^{-6} M to obtain the contractile responses in the presence of TRPV1 antagonist. Another TRPV1 antagonist, ruthenium red, was used at the concentration of 10^{-5} M. In addition, another TRPV1 antagonist SB-366791, was used at the concentration of 10^{-6} M. The solvent (vehicle) was added to organ bath with an equal volume

as those used for the antagonists. The same experiments were performed in STZ-diabetic rat bladder strips.

3.2.3 TRPV4 agonist

To study another TRPV subfamily member, the TRPV4 agonist 4α-PDD was used to activate the TRPV4 channel. 4α-PDD was added to the organ bath non-cumulatively. Concentrations of 10^{-8} M to 10^{-5} M of 4α-PDD were used throughout the study.

3.2.4 TRPV4 antagonist

The TRPV4 antagonist citral was used to study the inhibitory effect on the TRPV4 channel activation. Before adding the antagonist, the tissues were contracted with potassium chloride (KCl) ($3x10^{-2}$ M). When the contractile response of potassium chloride reached the plateau, citral was added to the organ bath in cumulative manner at the concentrations of 10^{-8} M to 10^{-4} M.

3.2.5 TRPA1 agonist

The TRPA1 agonist, allyl isothiocyanate, was used to determine the function of another TRP channel in diabetes. The concentration range of allyl isothiocyanate used in the present study was between 10^{-5} M to 10^{-4} M. This agonist was added in non-cumulative manner. This procedure was done according to the study of Andrede *et al*. (2006). They found that when non-curves were plotted, a marked increase in the efficacies in comparison to cumulative concentration-response was observed. This is evidenced by the observations that the contractions of rat urinary bladder to capsaicin, allyl isothiocyanate, and cinnamaldehyde was greater in non-cumulative curves that those of cumulative ones. After the first dose of drug was applied, the organ bath was washed out. The tissues were equilibrated for 30 minutes before application of the next concentration.

3.2.6 TRPM8 agonists

The TRPM8 channel is activated by cooling agents. Thus, the TRPM8 agonist, menthol was used. Menthol produced relaxations in the urinary bladder. The relaxant responses to menthol of control and STZ-induced diabetic bladder were studied. To study relaxant responses, tissues were pre-contracted with carbachol (10^{-6} M). When the contractile responses to carbachol reached the plateau, cumulative concentrations of menthol were applied. The concentration range of menthol used in the present study was between 10^{-4} M and 10^{-3} M.

Another TRPM8 agonist, icilin was used to study its potency compared to menthol. The effect of icilin on the relaxant responses of control and STZ-induced diabetic rat bladder were studied using the same protocol as menthol.

3.2.7 Muscarinic receptor agonist

To determine the effect of muscarinic receptor agonist on the contraction of bladder smooth muscle, carbamylcholine chloride (carbachol) was used. The cumulative concentration response curve was conducted on the control and STZ-induced diabetic rat bladder smooth muscle. The concentration ranges of carbachol were between 10^{-10} M and 10^{-6} M.

3.2.8 Substance P

To determine if the diabetic state had a direct effect on smooth muscle, substance P was studied. Substance P at the concentrations of 10^{-10} M to 10^{-7} M was added cumulatively to the organ bath. The contractile responses to substance P of control and STZ-induced diabetic rat bladder were then compared.

3.2.9 Neurokinin alpha

The neurokinin receptor agonist, neurokinin alpha was used to study whether diabetes had effects on the receptor in smooth muscle isolated from control and diabetic rat urinary bladder. The concentration range of neurokinin alpha used in the present study was between 10^{-9} M to 10^{-7} M. Neurokinin alpha was added in non-cumulative manner with washing out after each application.

3.2.10 NK1-receptor agonist

The NK1-receptor agonist, bombesin was used to study whether diabetes had effects on smooth muscle or on the nerve terminal. The concentration range of bombesin used in the present study was between 10^{-9} M to 10^{-6} M. Bombesin was added in non-cumulative manner with washing out after each application.

3.2.11 Substance P antagonist

The substance P antagonist spantide was used to study the inhibitory effect of substance P on the responses to capsaicin. Spantide at the concentration of 10^{-5} M was added to the organ bath to expose the bladder strips to the antagonist. After incubation for 20 minutes, capsaicin was added cumulatively at the concentration of 10^{-10} M to 10^{-6} M to obtain the contractile responses in the presence of substance P antagonist.

3.2.12 Neurokinin 1 and 2 antagonists

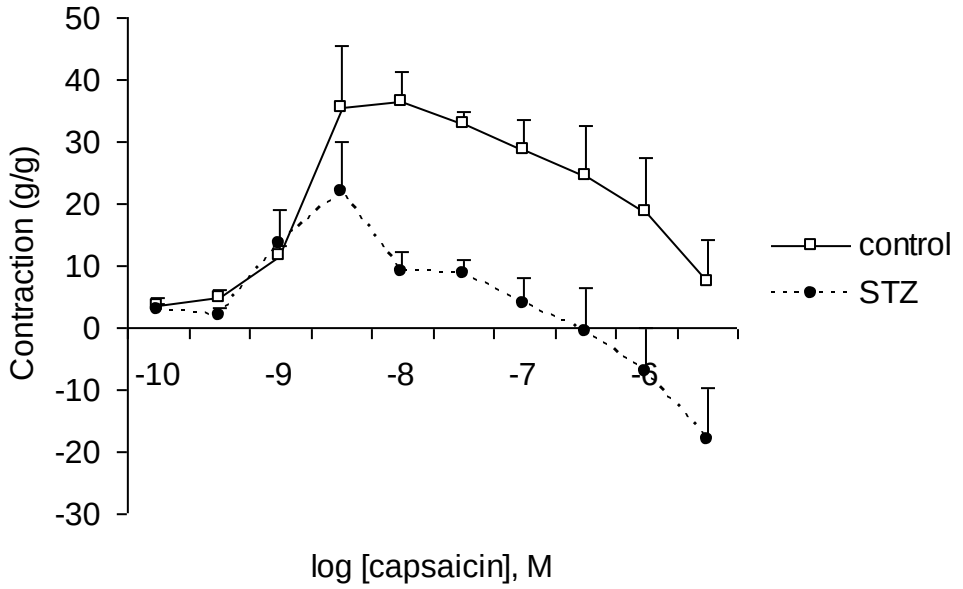
The combination of neurokinin 1 and 2 antagonist respectively was used to study the inhibitory effect of neurokinins on the responses to capsaicin. The combination of GR-205171A and SB-207164A at the concentration of 10^{-7} M each was added to the organ bath to expose bladder strips to the antagonists.

After incubation for 20 minutes, capsaicin was added cumulatively at concentration of 10^{-7} M to 10^{-5} M to obtain the contractile responses in the presence of the combination of neurokinin 1 and 2 antagonist. The experiment was conducted in parallel with the absence of antagonists by using vehicles.

3.2.13 Normalization of data

For the motility studies, the values were expressed as gram of contraction or relaxation versus gram of tissue wet weight, measured at the end of each experiment. The wet weight of strips can vary and this could influence the level of contraction or relaxation. Bladders from STZ-treated animals tended to be heavier. Tissue wet weight thus was taken into account. The initial tension at the baseline was subtracted and the amplitude of contraction or relaxation after drug application was calculated. This difference was then divided by the tissue wet weight to obtain the amplitude contraction (g)/wet weight (g). Figure 3.1 compares data normalized in this way with untransformed data. The trend of the results was similar between normal (no wet weight normalization) and normalized (g tension divided by tissue wet weight) data (Figure 3.1).Therefore, although it was logical to make this transformation, the impact on the data was small in most experiments.

A)



B)

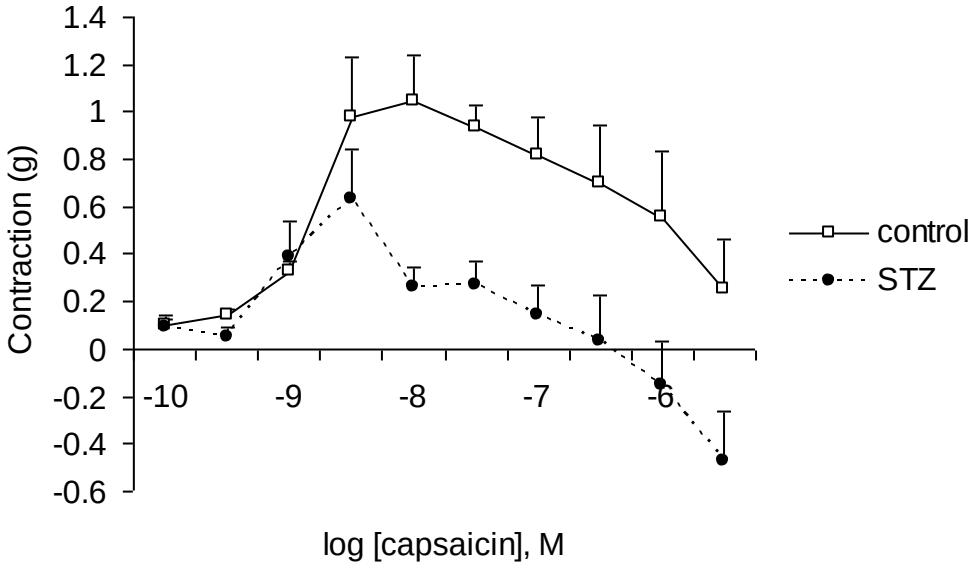


Figure 3.1 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls and 36 hours STZ-induced diabetic rats. A: normalized (g tension divided by tissue wet weight) and B: the same data unnormalized (no wet weight normalization).

3.3 Results

3.3.1 Characterization of STZ-induced diabetes model in rat

3.3.1.1 The effect of STZ-induced diabetes on blood glucose level

8 weeks after induction of diabetes by STZ in rats, the blood glucose was elevated four-folds compared to the controls (treated with citrate buffer) confirming a diabetic state (Figure 3.2).

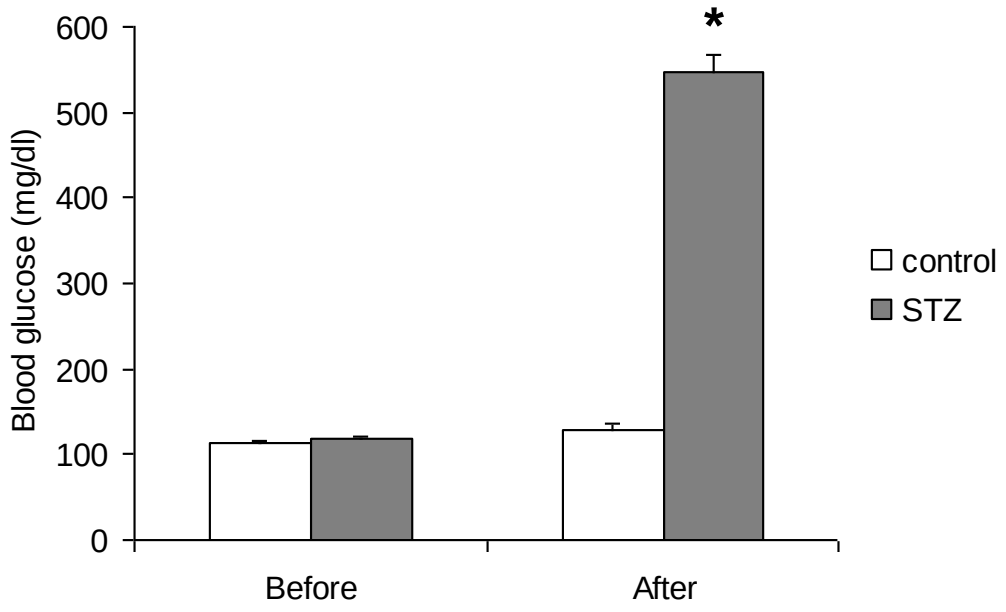


Figure 3.2 Blood glucose level of age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

3.3.1.2 The effect of STZ-induced diabetes on body weight

In contrast, body weight of rats treated with STZ was stable while it was increased in the controls which were treated with citrate buffer. These suggest that in diabetes, there is no weight gain while the control gained weight over time (Figure 3.3).

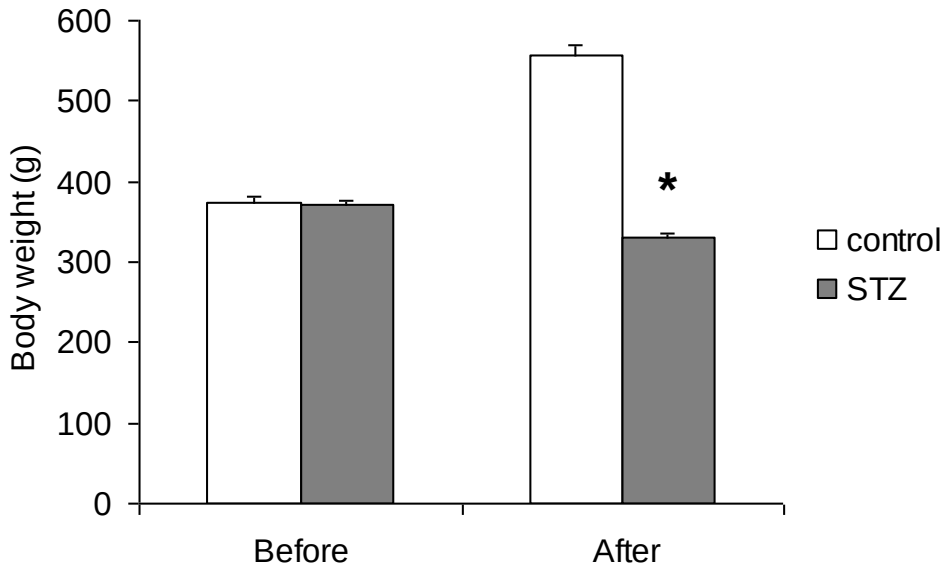


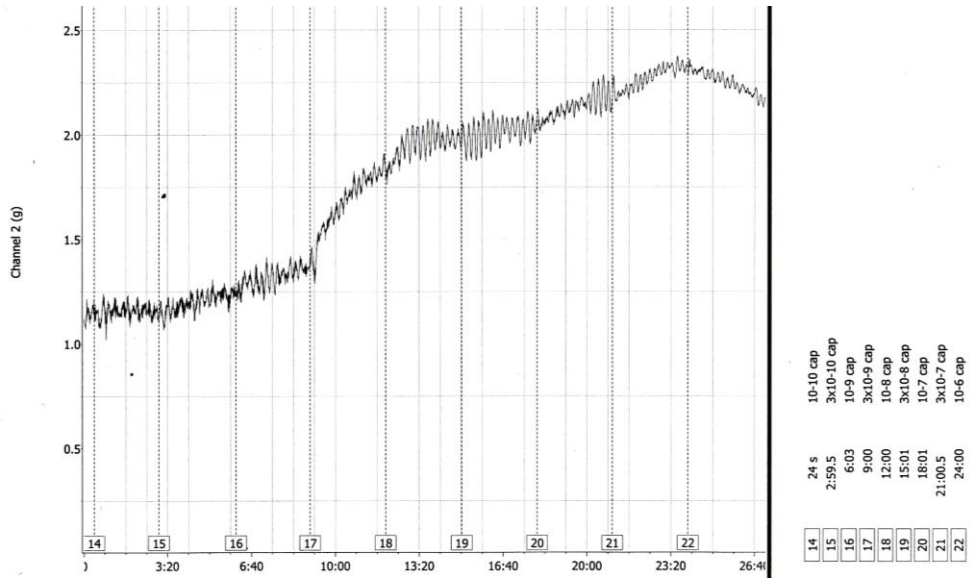
Figure 3.3 Body weight of age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

3.3.2 Motility studies

The longitudinal muscle of rat urinary bladder taken from both control and diabetic rats exhibited spontaneous activity (Figure 3.4). This spontaneous activity was greater in the bladder tissues from diabetic rats, characterized by the higher amplitudes of the responses than the controls.

A similar finding has been mentioned by Forrest and Parsons (2003) that spontaneous activity was observed in both control and diabetic colon tissues, but this activity was almost doubled in colon tissues taken from diabetic rats. In addition, Talubmook *et al*. (2003) reported that spontaneous activity was significantly greater in ileum tissues from diabetic animals than controls. The significant increase in spontaneous activity in diabetic tissues may reflect the hyperactivity of bladder in diseased state.

A)



B)

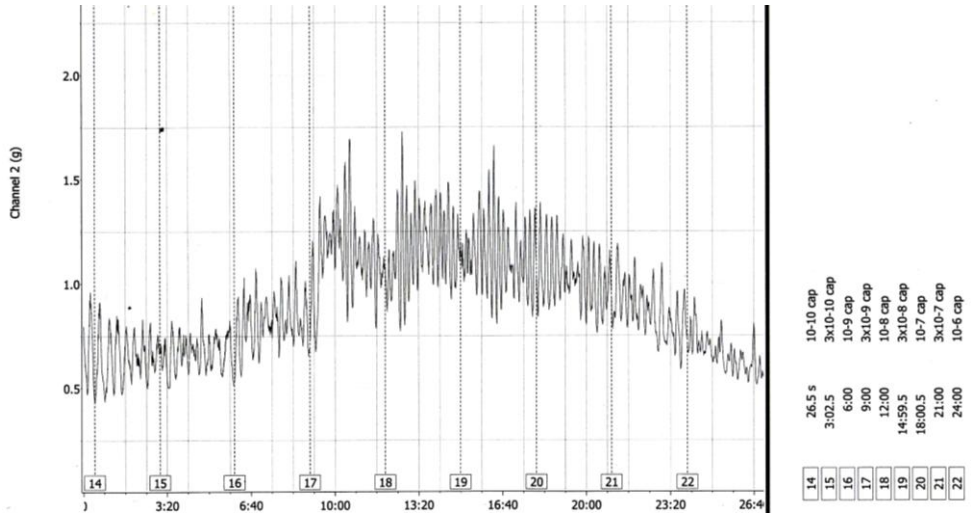


Figure 3.4 Original traces illustrating contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls (A) and STZ-induced diabetic rats (B), 8 weeks after the administration of STZ.

3.3.2.1 TRPV1 agonist capsaicin

It has been previously reported that capsaicin produced rapidly developing phasic contractions within 1 minute in a concentration-dependent manner (10^{-8} – 10^{-6} M) in rat urinary bladder muscle strips (Saitoh *et al*., 2007). It is also mentioned that EC_{50} for capsaicin in rat is 10^{-8} – 10^{-6} M (Caterina *et al*., 1997). The present study decided to use capsaicin at the concentration range of 10^{-10} to 3 x 10^{-6} M to see the whole effect of this agonist on bladder tissues. Capsaicin at the concentrations of 10^{-10} M to 3 x 10^{-6} M produced contractile response in control and STZ-induced diabetic rat bladder strips in a concentration-dependent manner. The responses to capsaicin were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls (Figure 3.5).

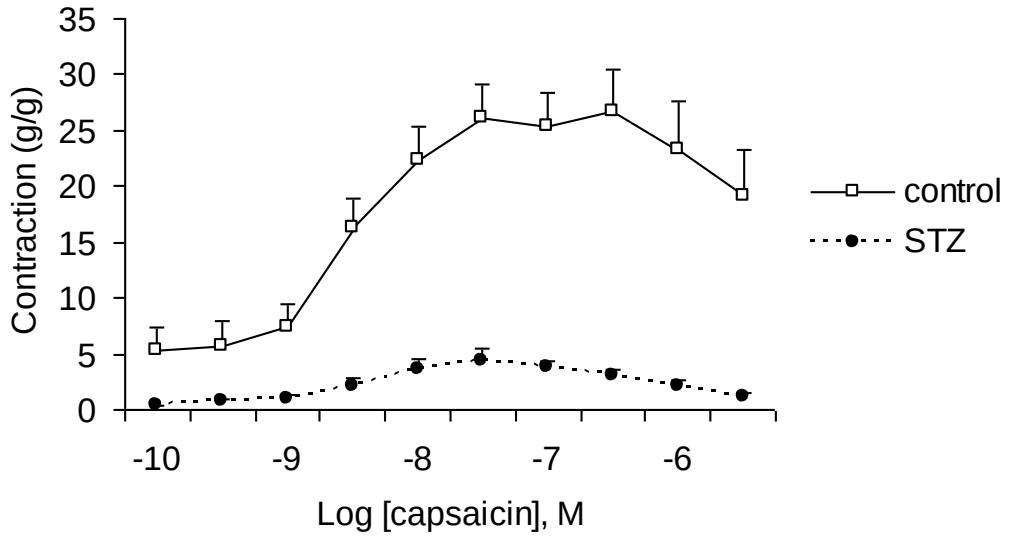


Figure 3.5 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.2 TRPV1 antagonist capsazepine

In order to determine whether the contractile responses in rat urinary bladder strips were due to the activation of TRPV1 by capsaicin, TRPV1 antagonists were used. Caterina *et al*. (1997) reported that IC_{50} for capsazepine in rodent is 10^{-7} – 10^{-6} M. To make sure that the concentration of antagonist is sufficient to block the response due to the agonist, capsazepine at the concentration of 10^{-5} M was selected for use in the present study. From the previous study, capsazepine (3 – 30 µM) produced a concentration-dependent rightward shift of the curve to capsaicin in the rat bladder (Maggi *et al*., 1993).

In the present study, the contractile responses to capsaicin at the concentrations of 10^{-10} M to 10^{-6} M in the presence of capsazepine (10^{-5} M) were less than the responses to capsaicin in absence of capsazepine. Responses to capsaicin in STZ-induced diabetic rat bladder were very small (Figure 3.6).

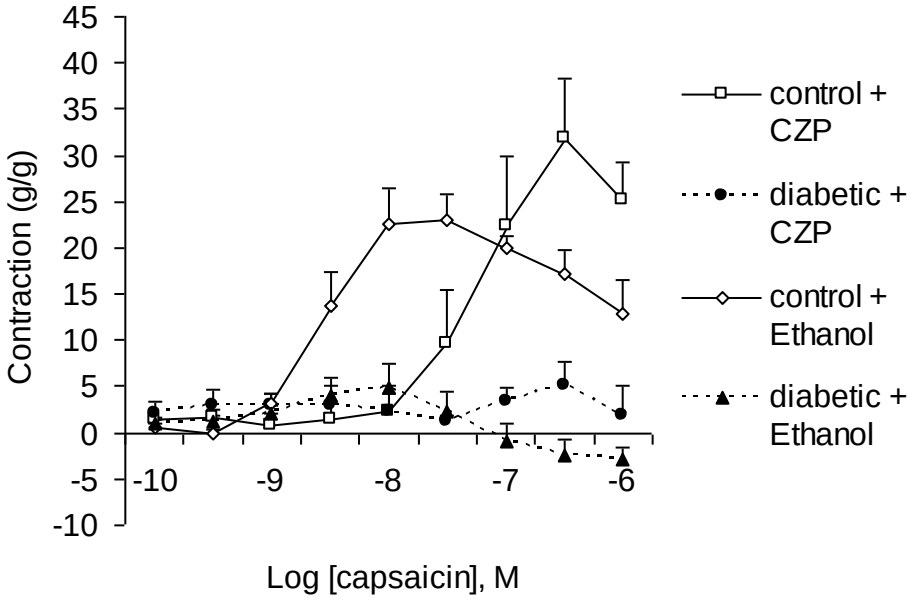


Figure 3.6 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M in the presence and absence of capsazepine (10^{-5} M) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

3.3.2.3 TRPV1 antagonist ruthenium red

Ruthenium red (10 – 30 μM) produces a non-competitive type of antagonism of TRPV1, characterized by marked depression of the maximal attainable tension (Maggi *et al*., 1993). It is reported that IC_{50} for ruthenium red in the rat tissues is 10^{-7} M (Garcia-Martinez *et al*., 2000). In addition, ruthenium red (30 μM) significantly reduced capsaicin induced contraction in rat isolated urinary bladder (Patacchini *et al*., 2005).

The contractile responses to capsaicin at the concentrations of 10^{-10} M to 10^{-6} M in the presence of ruthenium red (10^{-5} M) were less compared to the responses to capsaicin in absence of ruthenium red. This pattern was found in both control and STZ-induced diabetic rat bladder strips. However, the responses to capsaicin in presence of ruthenium red cannot reach the same responses as those of the absence of ruthenium red (Figure 3.7).

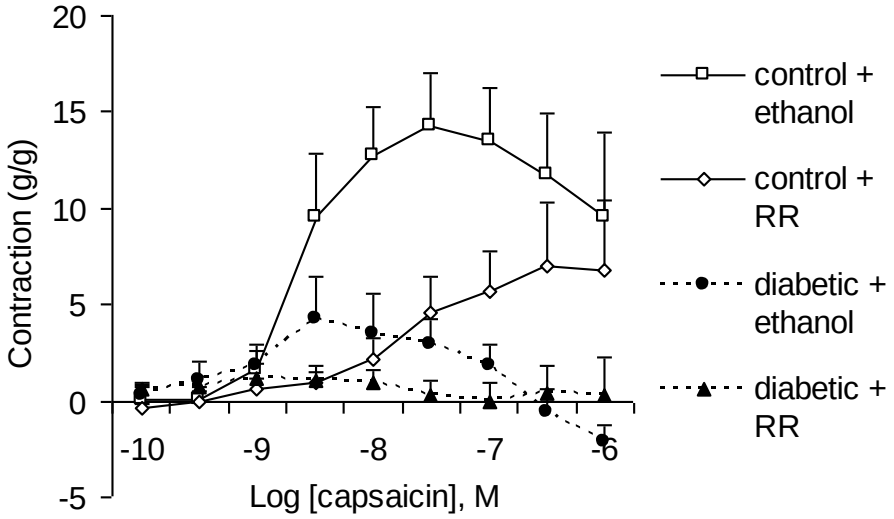


Figure 3.7 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M in the presence and absence of ruthenium red (10^{-5} M) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

3.3.2.4 TRPV1 antagonist SB-366791

The selective TRPV1 receptor antagonist SB-366791, (10 µM) blocks capsaicin-induced contraction in rat urinary bladder (Andrade *et al*., 2006). The contractile response curve (percentage of twitch contraction elicited by electrical field stimulation) to capsaicin in the presence of SB-366791 (10 µM) was shifted rightward (Patacchini *et al*., 2005). In addition, IC_{50} for SB-366791 in human and rat is 10^{-9} M (Patwardhan *et al*., 2006).

The contractile responses to capsaicin at the concentrations of 10^{-10} M to 10^{-6} M in the presence of SB-366791 (10^{-6} M) were less compared to the responses to capsaicin in absence of SB-366791 (Figure 3.8).

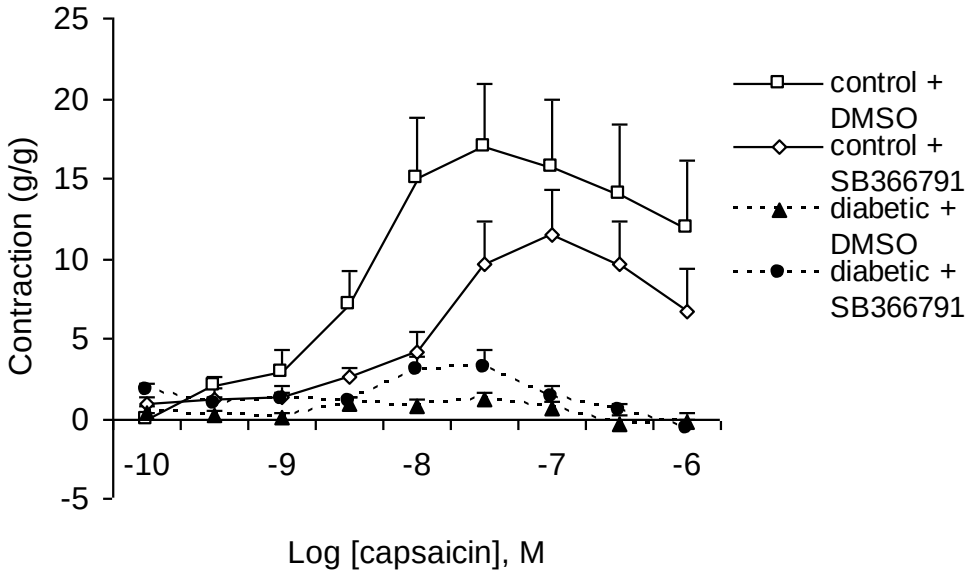


Figure 3.8 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M in the presence and absence of SB-366791 (10^{-6} M) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

3.3.2.5 TRPV4 agonist 4-α-PDD

Phorbol esters, like 4-α-PDD, bind to TRPV4 in a very specific and membrane delimited manner (Vriens *et al*., 2005; Watanabe *et al*., 2002). In addition, it is reported that EC_{50} for 4-α-PDD in mouse is 10^{-6} M (Watanabe *et al*., 2002).

In a preliminary study, the non-cumulative application of the TRPV4 agonist 4-α-PDD at the concentration of 10^{-8} M to 10^{-6} M to organ bath caused very small contractions in rat bladder strips. A high concentration of 4-α-PDD (10^{-5} M) produced clear contractile responses in control and STZ-induced diabetic rat bladder strips. The responses to 4-α-PDD at this concentration were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls (Figure 3.9).

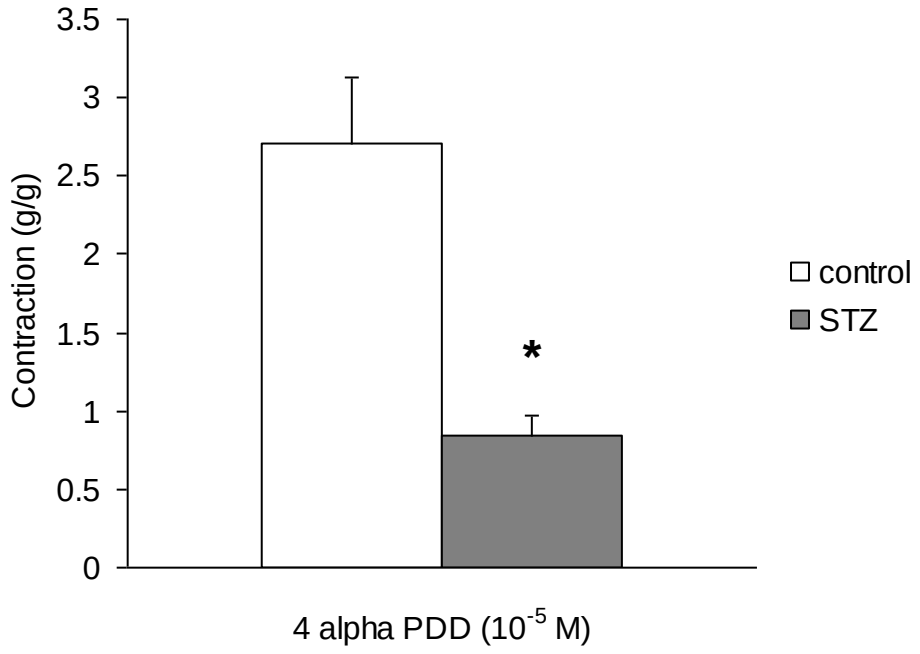


Figure 3.9 Contractile responses of bladder to TRPV4 agonist 4-α-PDD at the concentration of 10^{-5} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

3.3.2.6 TRPV4 antagonist citral

Citral from lemon grass oil, a compound commonly used to repel insects, inhibits TRPV4 activity with IC_{50} about 32 µM (Stotz *et al*., 2008). However, citral was found to both activate and inhibit TRP channel function (Stotz *et al*., 2008). Therefore preliminary experiments were performed using potassium chloride induced contractions to assess the specificity of citral action in the bladder.

After contraction by potassium chloride (KCl), citral at the concentrations of 10^{-8} M to 10^{-4} M was added to the organ bath. Citral produced relaxant responses in control rat bladder strips, suggesting that it inhibits voltage gated calcium channel activity. The relaxant responses to citral in diabetic rat bladder strips were significantly reduced or abolished in comparison to those of control tissues (Figure 3.10). These non-specific effects of citral suggest that it is not suitable to use as a TRPV4 antagonist in this preparation.

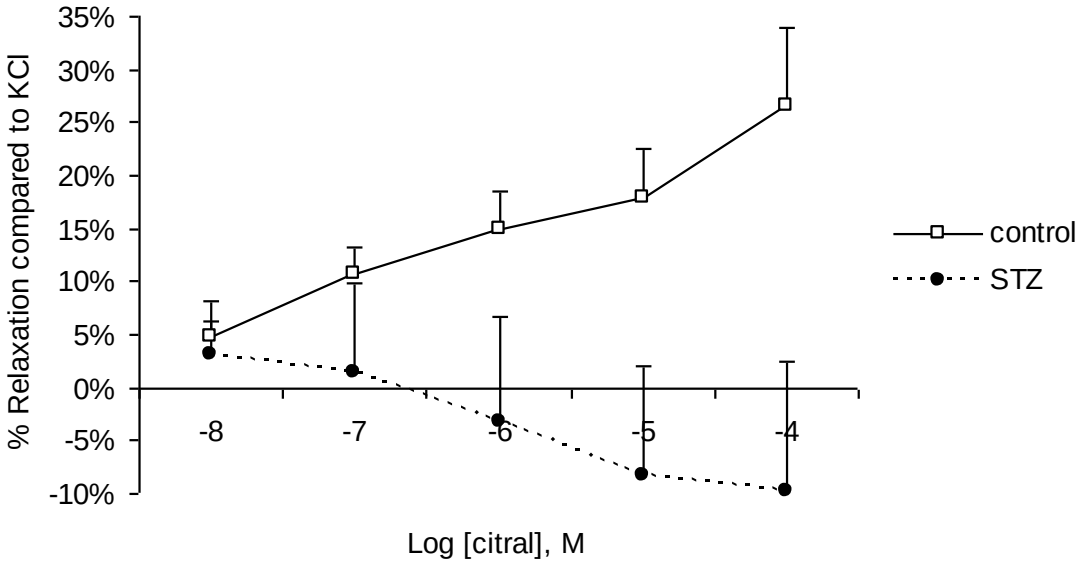


Figure 3.10 Effects of citral on control and diabetic rat bladder preparations. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.7 TRPA1 agonist allyl isothiocyanate

Activation of TRPA1 with allyl isothiocyanate caused a graded contraction of the rat urinary bladder in vitro. Addition of allyl isothiocyanate (0.001 – 3000 µM) to the bath solution caused a concentration-dependent contraction of rat urinary bladder. When the non-cumulative concentration-response curves were plotted, a marked increase in the efficacies of allyl isothiocyanate was observed (Andrade *et al*., 2006). For this reason allyl isothiocyanate was applied non-cumulatively.

The non-cumulative application of Allyl isothiocyanate at the concentrations of 10^{-5} M to 10^{-4} M to organ bath produced contractile responses in control and STZ-induced diabetic rat bladder strips in a concentration-dependent manner. The responses to allyl isothiocyanate were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls at $3x10^{-5}$ M to 10^{-4} M except 10^{-5} M (Figure 3.11).

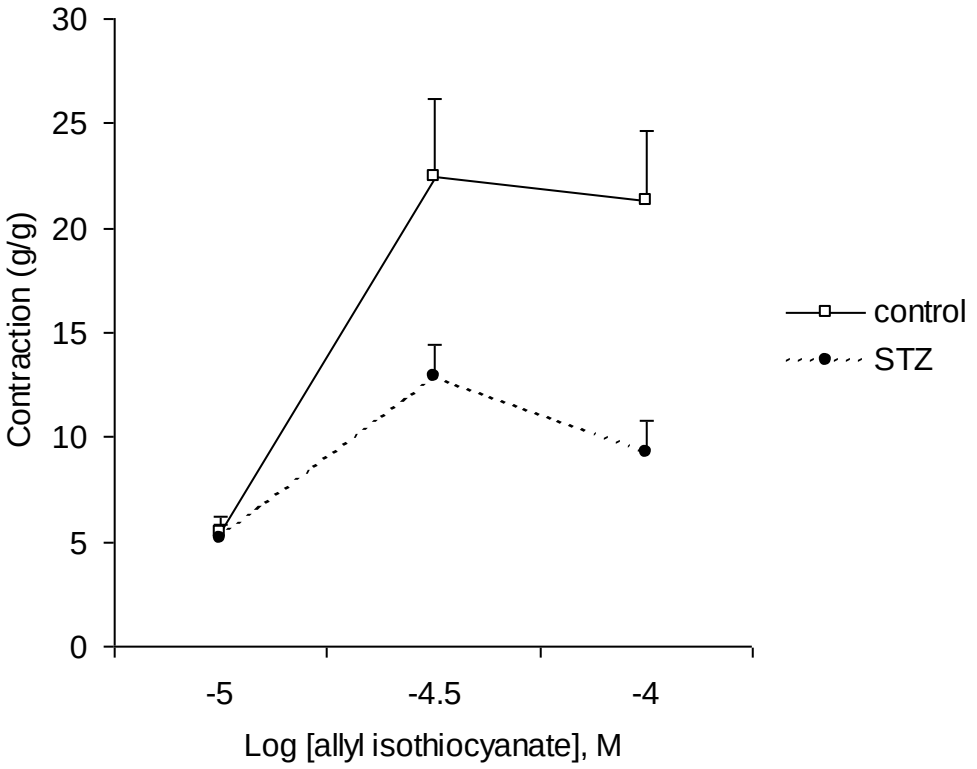


Figure 3.11 Contractile responses of bladder to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-5} M - 10^{-4} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.8 TRPM8 Agonist menthol

TRPM8 is thought to be activated by cooling agents. Menthol produces sensation of cooling and this is associated with the activation of TRPM8. It is reported that EC_{50} for menthol in *Xenopus* oocyte is 196 µM (Sherkheli *et al*., 2010). The contractile response of detrusor strips in organ bath to 0.001 mM carbachol was inhibited by menthol in a concentration-dependent manner. At 0.1, 0.3 and 1 mM, menthol inhibited carbachol-induced contraction by 10.7%, 36.7% and 97.3%, respectively (Nomoto *et al*., 2008). After pre-contraction with carbachol, menthol at the concentrations of 10^{-4} M to 10^{-3} M was added when the contractile responses to carbachol reached a plateau. Menthol produced relaxant responses in control and diabetic rat bladder strips. However, the relaxations were obtained from adding the high concentrations of menthol (10^{-4} M to 10^{-3} M). When the relaxant responses between control and diabetic rat bladder strips were compared, there was no significant difference (Figure 3.12).

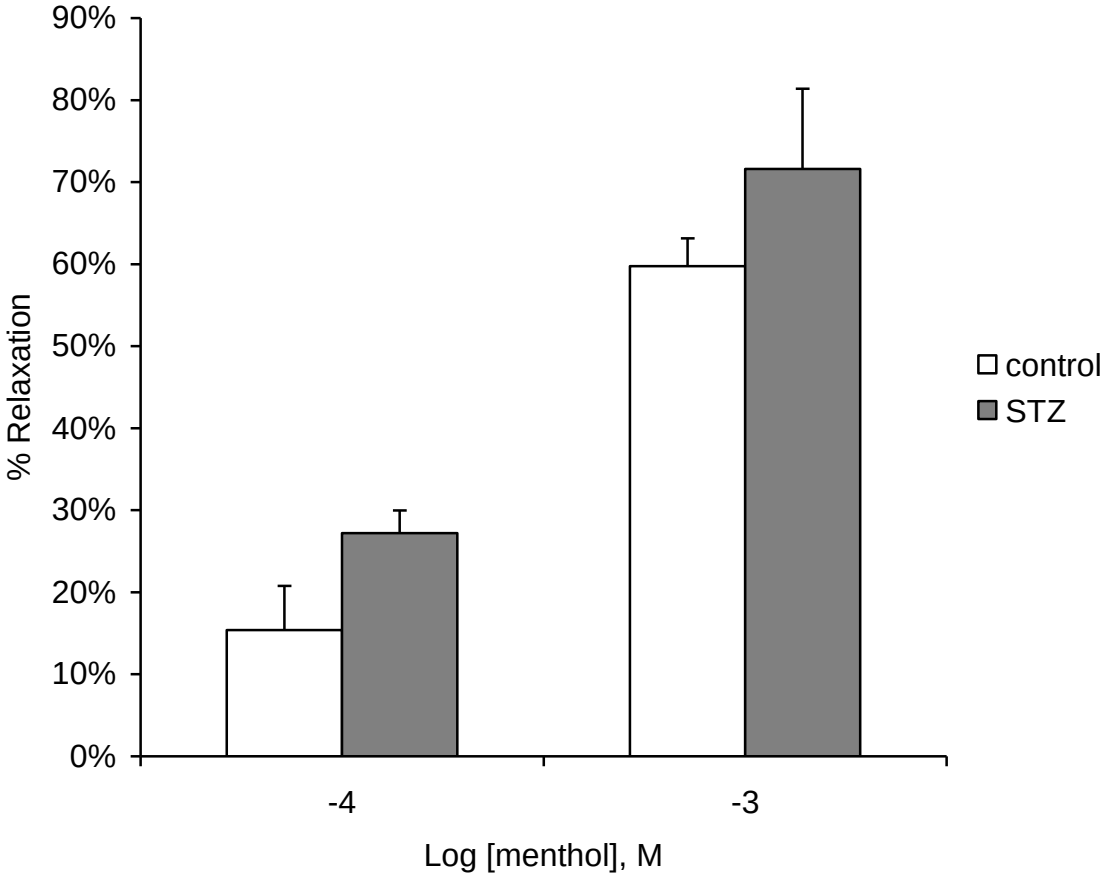


Figure 3.12 Relaxant responses of bladder to TRPM8 agonist menthol at the concentration of 10^{-4} M and 10^{-3} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats (Student's *t* test for unpaired observations).

3.3.2.9 TRPM8 agonist icilin

Another TRPM8 activator is icilin, which is a more potent agonist than menthol, and may have more specific activity on TRPM8. This was used to provide further evidence of functional TRPM8 channels in bladder. It is reported that EC_{50} for icilin on TRPM8 channels expressed in *Xenopus* oocyte is 7 µM (Sherkheli *et al*., 2010).

After pre-contraction with carbachol, icilin at the concentrations of 10^{-6} M to 10^{-5} M were added when the contractile responses to carbachol reached a plateau. Icilin produced relaxant responses which were small and variable but greater in diabetic than control tissues. However, the relaxations were obtained from adding the high concentrations of icilin (10^{-6} M to 10^{-5} M). When the relaxant responses between control and diabetic rat bladder strips were compared, there was no significant difference probably because of the variability (Figure 3.13).

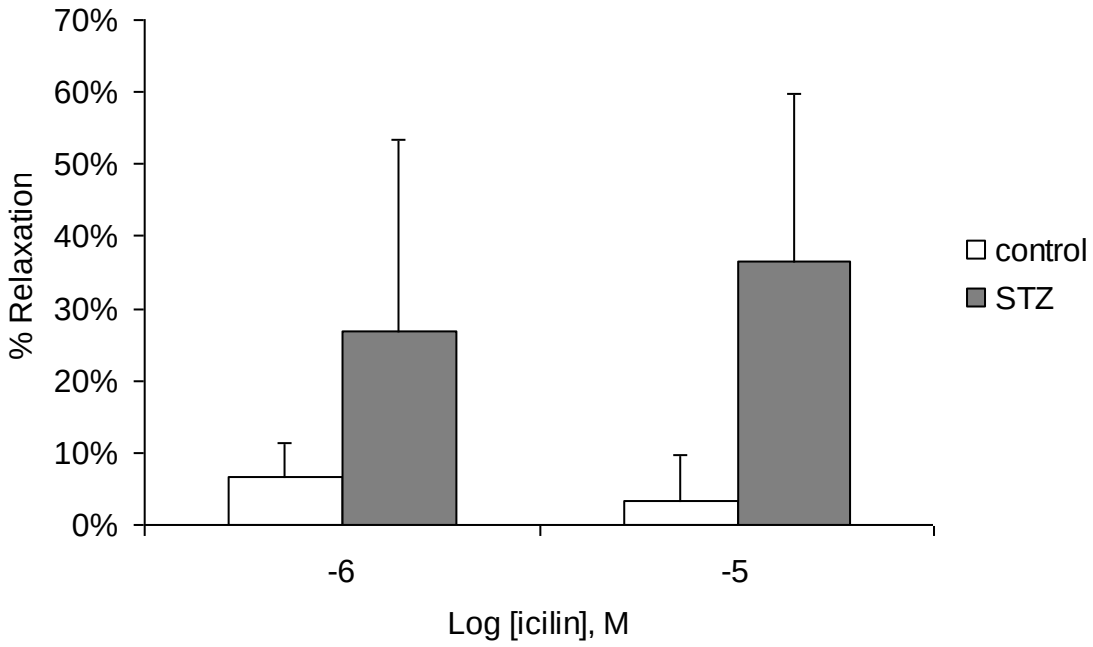


Figure 3.13 Relaxant responses of bladder to TRPM8 agonist icilin at the concentration of 10^{-4} M and 10^{-3} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats (Student's *t* test for unpaired observations).

3.3.2.10 Muscarinic receptor agonist carbachol

It has been reported that carbachol (10^{-9} M - 10^{-5} M) produced concentration-related contractile responses of ileum (Talubmook *et al*., 2003). In addition, carbachol induced contraction in isolated bladder tissues taken from control and diabetic rats (Stevens *et al*., 2006).

In the present study, carbachol at the concentrations of 10^{-8} M to 10^{-4} M produced the contractile responses in control and STZ-induced diabetic rat bladder strips in a concentration-dependent manner. However, no significant difference in the responses to carbachol of control and STZ-induced diabetic rat bladder strips was found (Figure 3.14).

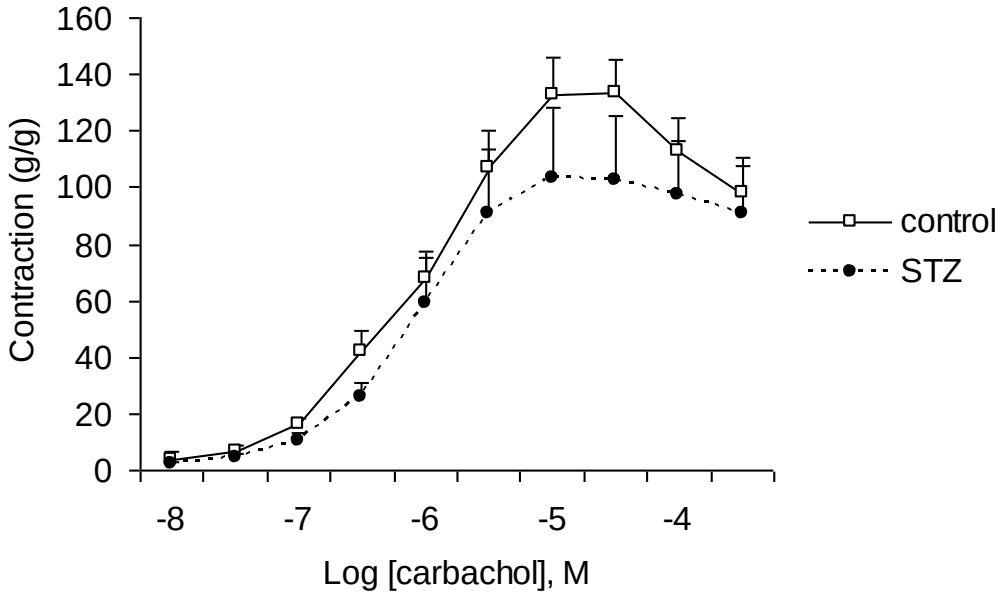


Figure 3.14 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} M and 10^{-4} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.11 Substance P

Substance P is a neuropeptide that can be released from presynaptic terminals. It is reported that a low concentration of substance P (30 nM) produced a rapid contraction which faded to baseline within 10 minutes in isolated rat urinary bladder (Maggi *et al*., 1991).

In the present study, it was found that substance P at the concentration of 10^{-10} M to 10^{-7} M produced contractile responses in control and diabetic rat bladder strips. The contraction was concentration-dependent. The contractile responses were similar between control and diabetic strips without any significant difference (Figure 3.15).

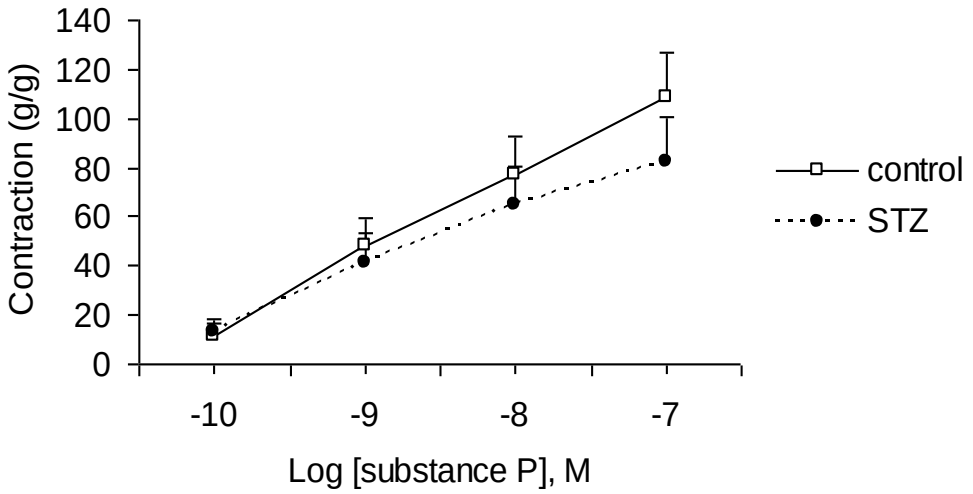


Figure 3.15 Contractile responses of bladder to substance P at the concentration of 10^{-10} M and 10^{-7} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.12 Neurokinin receptor agonist neurokinin alpha

It is reported that a low concentration of neurokinin A (10 nM) produced a slow developing contraction which was still evident at 10 minutes in isolated rat urinary bladder (Maggi *et al*., 1991).

Neurokinin alpha at the concentration of 10^{-9} M to 10^{-7} M produced contractile responses in control and diabetic rat bladder strips. The contraction was concentration-dependent. The contractile responses to neurokinin alpha in diabetic tissues were less than those of the controls, but no significant difference was found (Figure 3.16).

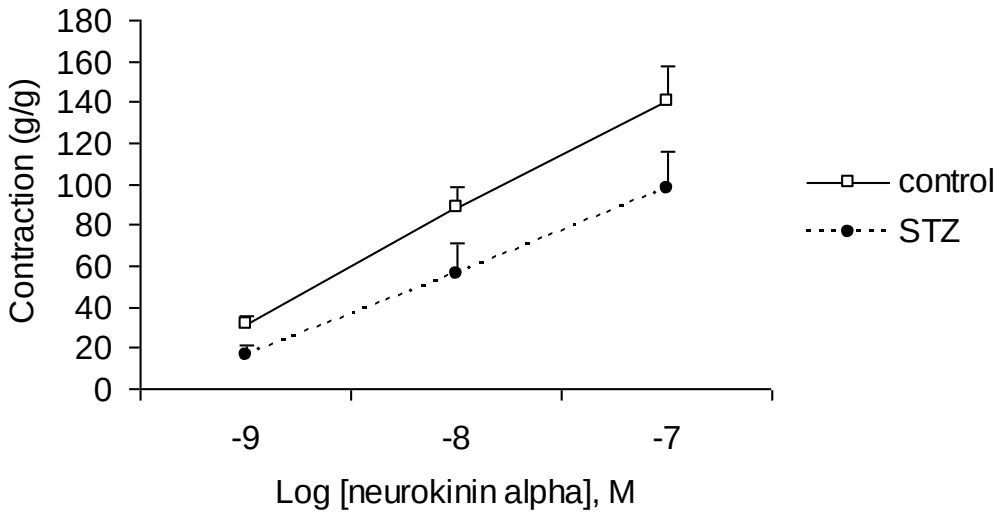


Figure 3.16 Contractile responses of bladder to neurokinin alpha at the concentration of 10^{-9} M and 10^{-7} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.13 Bombesin

Bombesin (10 nM) produced a tonic contraction in isolated rat urinary bladder similar to that induced by neurokinin A (Maggi *et al*., 1991). In addition, rat bladder body and bladder neck contracted to bioactive peptide bombesin (Watt and Cohen, 1991).

When non-cumulative applications of bombesin at the concentration of 10^{-9} M to 10^{-6} M were performed, bombesin produced contractile responses in control and diabetic rat bladder strips, and contractions were concentration-dependent. The contractile responses to bombesin in diabetic tissues tended to be less than those of the controls, but no significant difference was found (Figure 3.17).

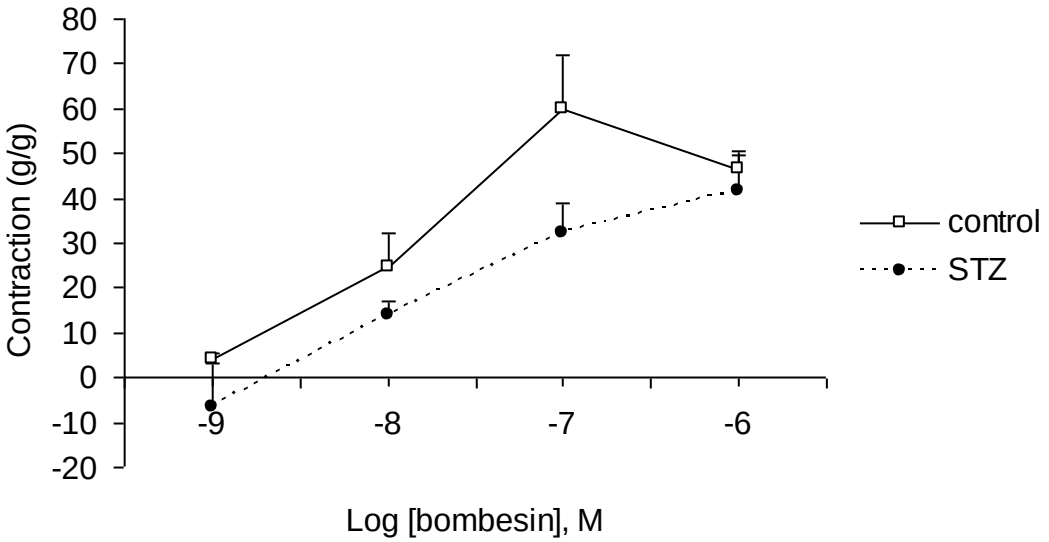


Figure 3.17 Contractile responses of bladder to bombesin at the concentration of 10^{-10} M and 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are not different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.3.2.14 Substance P antagonist spantide

Spantide is one of the most potent tachykinin antagonists (Yanagisawa and Otsuka, 1990). Spantide (3 µM) selectively antagonized substance P-induced contraction. Spantide (3 µM) also significantly reduced (43% inhibition) the peak response to 1 µM capsaicin in isolated rat urinary bladder while leaving the late response unaffected (Maggi *et al*., 1991).

The contractile responses to capsaicin at the concentrations of 10^{-10} M to 10^{-6} M in the presence of spantide (10^{-5} M) were similar to the responses to capsaicin in absence of spantide in both control and diabetic tissues. No significant difference was found. This suggests that spantide can not inhibit the responses to capsaicin (Figure 3.18).

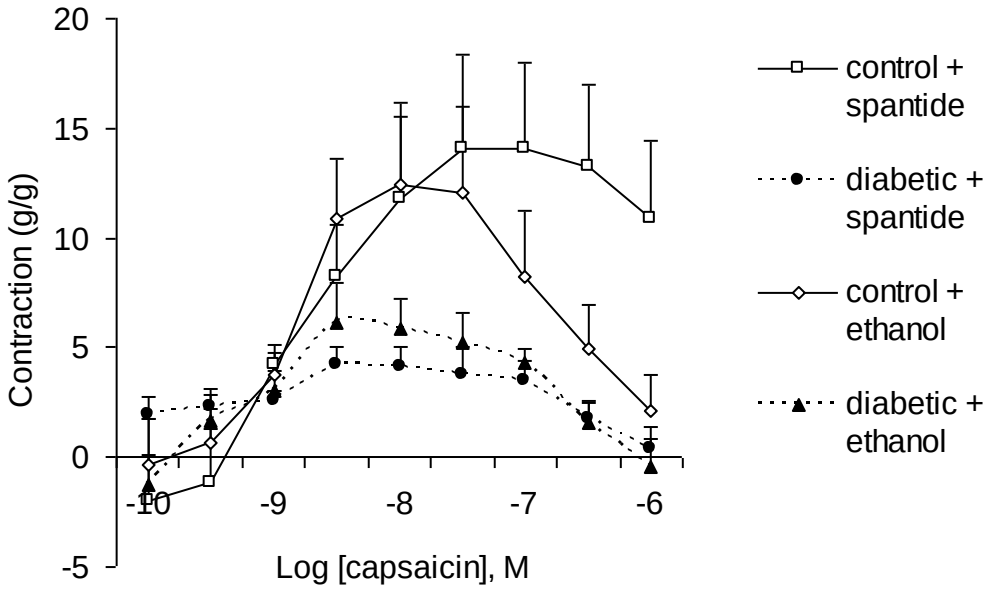


Figure 3.18 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M in the presence and absence of substance P antagonist spantide (10^{-5} M) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

3.3.2.15 Neurokinin 1 and 2 antagonists

Since GR-205171A and SB-207164A alone produced very small inhibitory effect on the contractile responses of rat urinary bladder tissues to capsaicin, the combination of these neurokinin antagonists was used.

The contractile responses to capsaicin at the concentrations of 10^{-7} M to $3x10^{-5}$ M in the presence of the combination of GR-205171A and SB-207164A at the concentration of 10^{-7} M were less than those in the absence of these antagonists. It is clear that the combination between NK_1 and NK_2 antagonists inhibited the contractile responses to capsaicin in rat bladder strips (Figure 3.19).

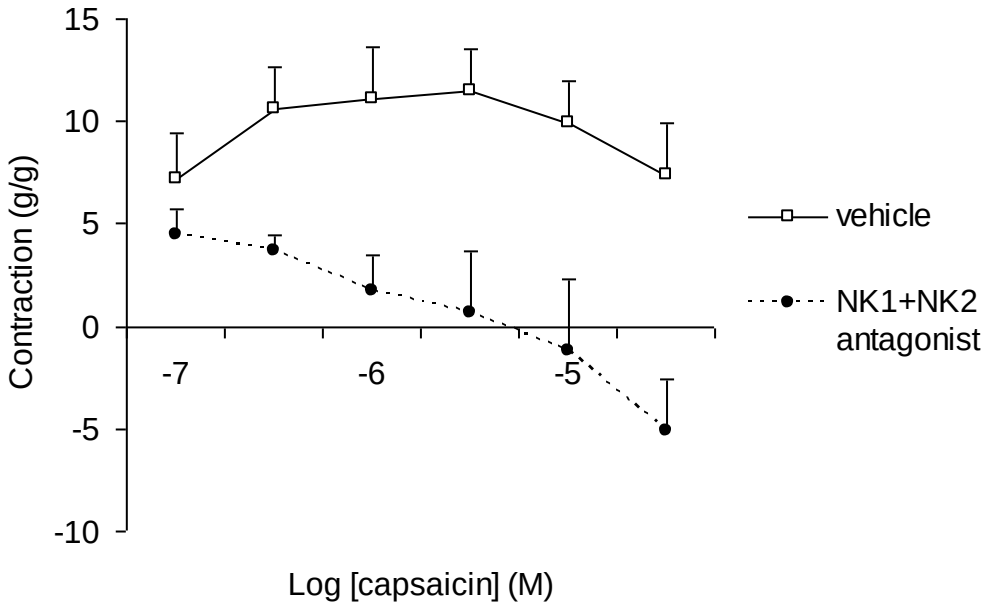


Figure 3.19 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M in the presence and absence of combination of NK_1 and NK_2 antagonist (10^{-7} M) from age-matched controls rats. Values represent the mean±S.E.M for 6 animals. Note that responses to capsaicin in this group of experiments were smaller than normal. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

3.4 Discussion

In the present study, the contractile responses to capsaicin were investigated in muscle strips isolated from the control and STZ-induced diabetic rat urinary bladder. The result showed that capsaicin produced contraction in control rat bladder strips in the concentration-dependent manner. These findings are similar to those observed in the previous studies (Saitoh *et al*., 2007). It is found that capsaicin and anandamide produced concentration-dependent contractions of the muscle strips isolated from the rat urinary bladder. Moreover, Saitoh *et al*. (2007) proposed that capsaicin produces muscle contractions by stimulating the TRPV1 receptor, followed by release of neuropeptides that can activate tachykinin NK_1 and/or NK_2 receptors in the urinary bladder. This finding explains the possible contractile mechanism in normal rat urinary bladder mediated by capsaicin.

In addition, the responses to capsaicin were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls in the present study. The result obtained from the present study is similar to Pinna *et al*. (1994) who found that the bladder response to capsaicin gradually decreased with the progression of streptozotocin-induced diabetes in rat. In rat urinary bladder, diabetes provokes impairment of capsaicin-sensitive sensory fibers but not of the cholinergic system even at early stage (4 week) of the disease (Pinna *et al*., 1994). Although there is no report involving the effect of diabetes on TRPV1 function in rat urinary bladder directly, our results are consistent with those of Facer *et al*. (2007) who found that TRPV1 levels are reduced in skin biopsies from patients with diabetic neuropathy. In additions, Rosta *et al*. (2007) reported that capsaicin reduced neurogenic sensory vasodilation, due to impairment of meningeal TRPV1 channel, in STZ-treated rats. From the present study, it could be inferred that TRPV1 receptor reduced during the diabetic state and consequently decreased the neurotransmitter released. The possible mechanism to explain why and where diabetes affect the function of TRPV1 channel in rat bladder needs further study of the neurotransmitter release mediated by capsaicin in STZ-induced diabetic tissue.

In the previous studies, there are reports suggesting that capsazepine and ruthenium red are the antagonists of TRPV1 channels receptors (Maggi *et al*., 1989; Chahl, 1989; Maggi *et al*., 1993; Nocerino *et al*., 2002). To check if the contractile response found in rat bladder is due to TRPV1 activation, TRPV1 antagonists, capsazepine and ruthenium red, were used in the present study.

The contractile responses to capsaicin in the presence of capsazepine were less than the responses to capsaicin in absence of capsazepine in both control and STZ-induced diabetic rat bladder strips. Interestingly, the contractile responses to capsaicin the presence of capsazepine were increased with the high concentration of capsaicin. The change was not statistically significant. This finding demonstrates the competitive antagonism of capsazepine on the response to capsaicin. The competitively antagonistic responses in rat urinary bladder are similar to those found in the study of Alexander *et al*. (2007) who mentioned that blockade of TRPV1 by capsazepine is competitive. In the rat bladder, capsazepine (3-30 µM) produced a concentration-dependent rightward shift of the curve to capsaicin without any depression of the maximal response to the agonist (Maggi *et al*., 1993). Similar findings were obtained in the rat isolated vas deferens in which capsazepine (10 µM) produced a rightward shift of the curve to capsaicin (Maggi *et al*., 1993). The antagonism of the action of capsaicin by capsazepine is entirely consistent with the proposed interaction of this substance with a vanilloid (TRPV1) receptor located on primary afferents (Maggi *et al*., 1993).

Like capsazepine, the contractile responses to capsaicin in the presence of ruthenium red were less compared to the responses to capsaicin in absence of ruthenium red in both control and STZ-induced diabetic rat bladder strips. However, the responses to capsaicin in presence of ruthenium red did not reach the same maximum as those in the absence of ruthenium red. Ruthenium red totally inhibited the contractile responses to capsaicin. This shows the non-competitive antagonism of ruthenium red. This result is similar to those observed by Maggi *et al*. (1989) who found that ruthenium red (10-100 µM) prevented the motor response of the urinary bladder to topical administration of capsaicin. Maggi *et al*. (1989) proposed that ruthenium red acts quite selectively

as a capsaicin antagonist preventing both reflex and efferent responses activated by peripherally administered capsaicin. In addition, Alexander *et al*. (2007) suggested that blockade of TRPV1 by capsazepine, 6-iodo-*nor*dihydrocapsaicin, BCTC, JYL1421, and SB366791 is competitive. All other antagonists act by non-competitive antagonism.

However, in the guinea-pig ileum, ruthenium red abolished responses to capsaicin. Ruthenium red binds irreversibly to the calcium channel part of the complex but reversibly to some other site which prevents the action or binding of capsaicin at its specific receptor (Chahl, 1982). It is clear that ruthenium red is not a selective antagonist for TRPV1.

In this study, the contractile responses to capsaicin in the presence of SB-366791 were less compared to the responses to capsaicin in absence of SB-366791 in both control and STZ-induced diabetic rat bladder strips. These results reflect the antagonism of SB-366791 on the contractile responses to capsaicin in the muscle strips isolated from rat urinary bladders. The study on the effect of SB-366791 on capsaicin-evoked or electrical stimulation-induced release of the sensory neuropeptide substance P from isolated rat tracheae suggested that SB-366791 is a more selective and potent in vitro TRPV1 receptor antagonist than capsazepine in the rat (Varga *et al*., 2005). In additions, in cultured sensory neurons, SB-366791 is a TRPV1 antagonist with high potency and an improved selectivity profile in comparisons to other commonly used TRPV1 antagonists (Gunthorpe *et al*., 2004). In the present, it is found that SB-366791 is a selective antagonist for TRPV1.

A high concentration of 4α-PDD (10^{-5} M) produced clear contractile responses in control and STZ-induced diabetic rat bladder strips. This result corresponds to the study of Birder et al. (2007). Birder *et al*. (2007) found that functional TRPV4 protein is expressed in urothelium of renal pelvis, ureters, urinary bladder, and urethra. Exposure of cultured rat urothelial cells from the urinary bladder to the TRPV4-selective agonist 4α-PDD promoted Ca^{2+} influx, evoked ATP release, and augmented ATP release evoked by hypo-osmolarity. Activation of urothelial TRPV4 by 4α-PDD and release of mediators such as ATP trigger a novel neural mechanism that regulates the late phase of detrusor

muscle contraction after micturition. However, 4α-PDD (1-100 μM) did not alter the contractility to electrical stimulation of excised bladder strips (Birder *et al*., 2007) which is consistent with the contractile responses to 4α-PDD in high concentration of the present study. In additions, Thorneloe *et al*. (2008) showed the weak ability of 4α-PDD to evoke TRPV4 currents in TRPV4 HEK cells and 4α-PDD demonstrated a poor ability to contract mouse bladder halves eliciting only small contractile responses at 10 and 100 μM. This observation also support the result obtained in the present study.

In the present study, the responses to 4α-PDD at this concentration (10^{-5} M) were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls. It is possible that TRPV4 protein is less expressed in diabetic urinary bladder in comparison to those of normal bladder. Although there is no report involving the effect of diabetic state on TRPV4 channel function, it is known that long-term hyperglycaemia from diabetes lowers the function of nerve terminals in many organs seen in diabetic neuropathy. Activation of TRPV4 in urothelial cell by 4α-PDD and ATP release triggers a neural mechanism regulate the detrusor muscle contraction (Birder *et al*., 2007). Thus, it is possible that the diabetic state may obstruct mediator release or other neural mechanisms and consequently disturb the function of TRPV4 channel in urothelium.

In normal rat urinary bladder, it was found that activation of TRPA1 with allyl isothiocyanate or cinnamaldehyde cause a graded contraction of the rat urinary bladder in vitro (Andrade *et al*., 2006). This finding is confirmed by the result from the present study which was found that allyl isothiocyanate produced the contractile responses in control and STZ-induced diabetic rat bladder strips in a concentration-dependent manner. For the contractile mechanism mediated by TRPA1 agonists, Andrade *et al*. (2006) mentioned that TRPA1 agonists contract rat urinary bladder through sensory fiber stimulation, depending on extracellular Ca^{2+} influx and release of tachykinins and cyclooxygenase metabolites, probably prostaglandin E_2. TRPA1 is expressed in sensory nerves that innervate the urinary bladder and mediates a contractile effect on bladder smooth muscle, due to release of tachykinins and cyclo-oxygenase metabolites.

The effect on smooth muscle contractility of agents capable of stimulating TRPA1 was comparable in potency to capsaicin, supporting the speculation that this channel may play a role in bladder function (Birder, 2007).

However, this study found that the responses to allyl isothiocyanate were significantly reduced in the STZ-induced diabetic rat bladder strips compared to the controls. This result demonstrates that diabetes had a negative effect on TRPA1 function in rat bladder, since TRPA1 is expressed in sensory nerves that innervate the urinary bladder and mediates a contractile effect on bladder smooth muscle. Diabetic state or long-term hyperglycaemia may destroy the nerve terminals or lead to a reduction in release of neurotransmitters.

There is a report suggesting that menthol is TRPM8 agonist (Thebault *et al*., 2005). In present study, responses to menthol in control and STZ-induced diabetic rat bladder strips were measured. Menthol produced the relaxant responses in control and diabetic rat bladder strips. The relaxations were obtained from high concentrations of menthol. However, it is interesting that the relaxant responses to menthol were greater in diabetic rat bladder strips in comparison to the controls. This demonstrates that diabetes may affect the function of TRPM8 in rat urinary bladder. TRPM8 is increased in nerve fibers of overactive and painful bladder (Mukerji *et al*., 2006). In addition, there is a report suggesting that intravesical installation of menthol facilitates the bladder cooling reflex in both the cat and human suggests that TRPM8 function may be involved in triggering the reflex. In the urinary bladder, TRPM8-positive immunoreactivity has been demonstrated within bladder nerves and urothelium (Birder, 2007).

In this study, there is a very small response of rat bladder strips to menthol. Potency of menthol expressed as EC_{50} is 4.1 ± 1.3 µM in HEK293 cells (De Petrocellis *et al*., 2007). In the present study, menthol has relaxant effect on rat bladder strips and there is no significant difference between control and diabetic tissues. The relaxant effect occurs at mM concentrations, which suggests that menthol could act at other targets than the TRPM8 channel, for example menthol was shown to block voltage-gated calcium channels.

Icilin produced relaxant responses which were small and variable but greater in diabetic than control tissues, although, there is no significant difference, probably because of the variability. However, the relaxations were obtained from the high concentrations of icilin. The response to icilin in urinary bladder strip is less than those of menthol. This is probably because of the preparation technique of tissues. In this study, bladder strip preparations were used. As TRPM8 is expressed in bladder nerve and urothelium, the whole isolated intact bladder preparation should provide the obvious response to icilin or menthol in the rat urinary bladder.

From the findings mentioned above, it is clear that there is a reduction in the responses to TRP channel agonists in STZ-induced diabetic rat bladder tissues. To explain the reasons behind the impairment of TRP channel function caused by the diabetic state, there are some possibilities. Firstly, it may due to the effect of STZ-induced diabetes on smooth muscle function. Muscarinic receptor agonist were therefore used. In the present study, carbachol produced contractile responses in control and STZ-induced diabetic rat bladder strips in a concentration-dependent manner. This finding is similar to those observed by Abdel-Hakim *et al*. (1981) in which carbachol was found to produce dose-dependent increase of the basal tone of the rat bladder detrusor muscle and the maximal contraction produced by carbachol was about four times greater than that elicited by bombesin or substance P. The contraction in control and diabetic bladder tissue is similar without significant difference. This suggests that muscarinic receptors on smooth muscle are not affected by diabetes or hyperglcaemia. Thus, the contraction produced by muscarinic receptor agonist carbachol is the result of its effect on bladder smooth muscle directly.

In the present study, it is assumed that the effect of STZ-induced diabetes may be at the level of the sensory endings. However, there may also be other sites of action. Previous studies have shown that TRPV1 is expressed not only by afferent nerves that form close contacts with bladder epithelial (urothelial) cells but also by the urothelial cells themselves (Birder *et al*., 2001). This leads to the possibility that the deleterious effect of STZ-induced diabetes may also be at the level of the urothelium.

Secondly, it may be due to the disturbances or alterations of postsynaptic receptors caused by STZ-induced diabetes. Therefore the exogenous neurotransmitters including substance P, Neurokinin alpha and bombesin that are thought to be released following TRPV1 activation, were added.

In the present study, substance P produced the concentration-dependent contractions in control and diabetic rat bladder strips. This result is supported by previous studies (Uckert *et al*., 2002; Pinna *et al*., 1994). Uckert *et al*. (2002) found that endothelin and substance P elicited dose-dependent contractions of human bladder detrusor muscle.

The contractile responses to substance P were similar between control and diabetic bladder strips without significant difference. This result demonstrates that the diabetic state did not have any effect on bladder smooth muscle cells. The result from the present study is consistent with those observed by Pinna *et al*. (1994). It was found that the bladder contractile response to exogenous substance P was similar in both control and STZ-induced diabetic groups at all stage (1-26 weeks) studied and was proposed that diabetes had no effect on the sensitivity of smooth muscle cells to substance P.

Neurokinin alpha produced concentration-dependent contraction in control and diabetic rat bladder strips. This finding is supported by the observations from the previous studies (Guiliani *et al*., 2001; Tramontana *et al*., 2000; Maggi *et al*., 1991), which a low concentration of neurokinin A (10 nM) produced a slowly developing contraction in the rat isolated urinary bladder (Maggi *et al*., 1991). In isolated strips of the hamster urinary bladder the selective tachykinin NK_2 receptor agonist [βAla^8] NKA (4-10) induced a concentration-dependent contraction associated with significant release of prostaglandin E_2 (Tramontana *et al*., 2000). In an electrical field stimulation study, stimulation by neurokinin A (10 nM) increased the amplitude of twitches and produced a concentration-dependent tonic contraction in the hamster isolated urinary bladder (Giuliani *et al*., 2001).

The contractile responses to neurokinin alpha in diabetic tissues were less than those of the controls, but no significant difference was found. This also supports the idea that diabetes had no effect on rat bladder smooth muscle.

Bombesin is another neurotransmitter which is thought to be involved in synaptic transmission. This study showed that bombesin produced contractile responses in control and diabetic rat bladder strips and contractions were concentration-dependent. This result corresponds with previous studies (Watts and Cohen, 1991; Rouisii *et al*., 1991; Abdel-Hakim *et al*., 1981). Prostate, bladder body, and bladder neck tissues contracted to bombesin (Watts and Cohen, 1991). Guinea pig and rat urinary bladder, rat stomach, and guinea pig gall bladder responded to bombesin and related peptide with concentration-dependent contractions (Rouisii *et al*., 1991). Bombesin was found to produce dose-dependent increases of the basal tone of the rat bladder detrusor muscle and the contractile effect of bombesin on the rat isolated urinary bladder is likely to be the result of a direct effect on the smooth muscle cell (Abdel-Hakim *et al*., 1981).

The contractile responses to bombesin in diabetic tissues were less than those of the controls, but no significant difference was found. This also support that diabetes had no effect on the sensitivity of bladder smooth muscle to NK_1 receptor agonist bombesin which is similar to those of substance P, although there is no supported study on the effect of bombesin on diabetic bladder smooth muscle.

The contractile responses to capsaicin in the presence of spantide were similar to the responses to capsaicin in absence of spantide in both control and diabetic tissues without significant difference. This suggests that spantide can not inhibit the responses to capsaicin. This result is different from the observation by Maggi *et al*. (1991). They found that spantide (3 μM) selectively antagonized the SP-induced contraction. Spantide selectively reduced the peak response to capsaicin, while leaving the late response unaffected.

The NK_1 receptor antagonists GR205171 (100 μM) and SDZ NKT 376 (1 mM) also reduced the response to capsaicin, indicating that capsaicin acts via TRPV1 in series with NK_1 (Hu *et al*., 2005). The results from the present

study showed that the combination between the two neurokinin receptor (NK_1 and NK_2) antagonist GR205171A and SB207164A totally inhibits the contractile responses to TRPV1 agonist capsaicin. From this finding, it would be concluded that a neurokinin receptor agonist may be the neurotransmitter mediated by the activation of TRPV1 channel.

Chapter 4

The effect of streptozotocin-induced diabetes on TRP channels function in rat colon

4.1 Introduction

Gastrointestinal alterations are reported to occur in diabetic patients. Patients with diabetes mellitus suffer from gastrointestinal abnormalities including gastroparesis, constipation and diarrhoea (Verne and Sninsky, 1998). The mechanisms underlying the gastrointestinal disorders in diabetes are believed to be due to autonomic neuropathy or hyperglycaemia (El-Sally, 2002). These are believed to be the consequence of altered innervation in the gastrointestinal tract in diabetes (Anjaneyulu and Ramarao, 2002).

In animal models of diabetes, it is reported that there were alterations in presynaptic and postsynaptic function in ileum taken from the eight week streptozotocin induced diabetic rats (Talubmook *et al*., 2003). In addition, abnormalities in the adrenergic, cholinergic and peptidergic innervations in the gastrointestinal tract of streptozotocin-treated rats are also reported. There is a report mentioned about the increase in basal spontaneous contraction in colon tissues taken from streptozotocin-induced diabetic rats (Forrest *et al*., 2003). Although the alterations due to diabetes are reported in colon, the mechanism underlying these circumstances has been little studied. The gastrointestinal absorption and secretion changes in diabetes are interesting and little known. Study of these aspects may help clarifying the pathophysiology of diabetes in colon. Using the Ussing chamber technique, Forrest *et al*. (2006) found that short circuit current of colon is not altered in the streptozotocin diabetic rat while it is altered in ileal tissues, which was due to a change in cellular glucose transport.

Enteric nervous system in the gastrointestinal tract modulates function of secretory and absorptive processes through the excitatory submucosal secretory motor neurons. TRPV1-expressing nerves (capsaicin sensitive

sensory nerves) are important in modulating gastrointestinal function (Bomstein and Furness, 1988). Release of neuropeptide from these nerve terminals is reported to provide “local effector” role in the intestine (Takaki and Nakayama, 1989).

In the previous chapter, it was found that there was the reduction in the contractile responses to TRPV1 agonist capsaicin at the concentrations range from 10^{-8} M to 10^{-6} M in STZ-induced diabetic rat urinary bladder strips. In addition, the effect of STZ-induced diabetes on the function of TRP channels in another tissue was examined to see how widespread the effects were. Responses were thus examined in rat colon.

4.2 Methods

4.2.1 The effect of diabetes on TRP channel functions in rat colon

To study whether diabetes had any effects on the functions of TRPV1 channel in rat colon, the TRPV1 agonist capsaicin at concentrations of 10^{-5} M was used. Before adding the agonist, the tissues were contracted with muscarinic receptor agonist, carbachol (10^{-6} M). When the contractile response of carbachol reached a plateau, capsaicin at a concentration of 10^{-5} M was added to the organ bath. Relaxant responses to capsaicin of the carbachol contracted tissues from control and STZ-diabetic rat bladders were compared.

4.2.2 The effect of diabetes on ion secretory function in rat colon

A minimal 45 minutes equilibration period was established before addition of any drug to the tissues. During this time the basal I_{sc} had stabilized. Only one concentration of carbachol, veratridine and capsaicin was used per tissue because drugs could not be washed out because of the design of the chambers.

Drugs were only added to the basolateral side of the colon tissue. The TRPV1 agonist capsaicin was added 30 minutes prior to the addition of

carbachol or veratridine. All experiments were performed in parallel with control and diabetic colonic tissues.

4.3 Results

4.3.1 Motility Studies

4.3.2.1 TRPV1 agonist capsaicin

The results showed that capsaicin at the concentration of 10^{-5} M produced relaxant responses in rat colon both from STZ-treated rats and age-matched vehicle controls. The relaxant responses in the STZ-treated rats were significantly reduced in comparison to the controls (Figure 4.1).

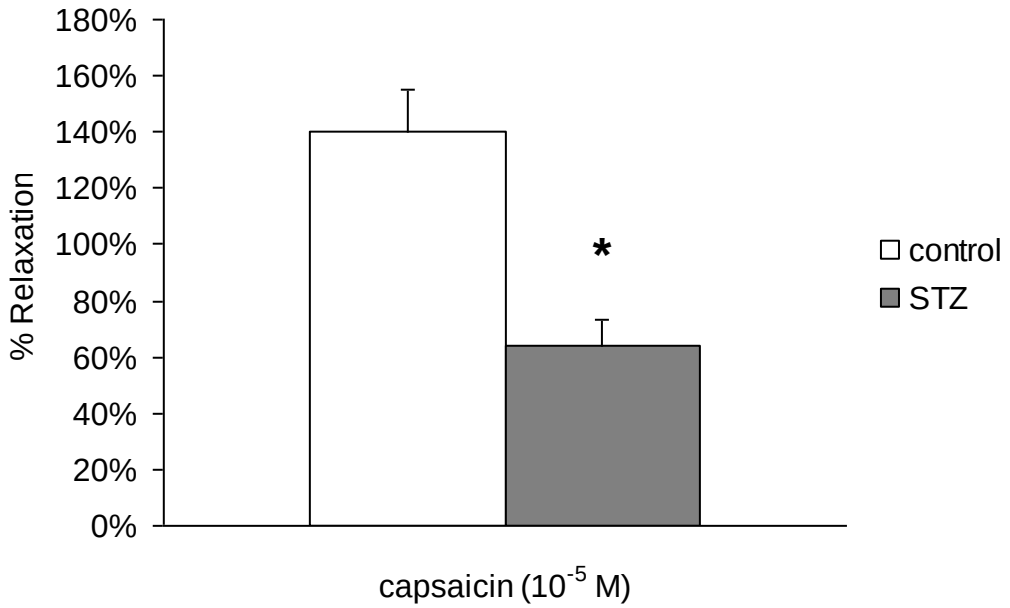


Figure 4.1 Relaxant responses of rat colon to TRPV1 agonist capsaicin at the concentration of 10^{-5} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

4.3.2 Secretory studies

4.3.2.1 Ion secretory responses to capsaicin

TRPV1 agonist capsaicin produced a reduction in ion secretion (short circuit current) both in control and diabetic proximal and distal colon tissues. This reduction can be found at concentrations of capsaicin at 10^{-6} M and 10^{-5} M. The reduction in ion secretory responses to capsaicin in diabetic rat colon tissues were greater than those of control tissues but did not reach statistical significance (Figure 4.2, 4.3).

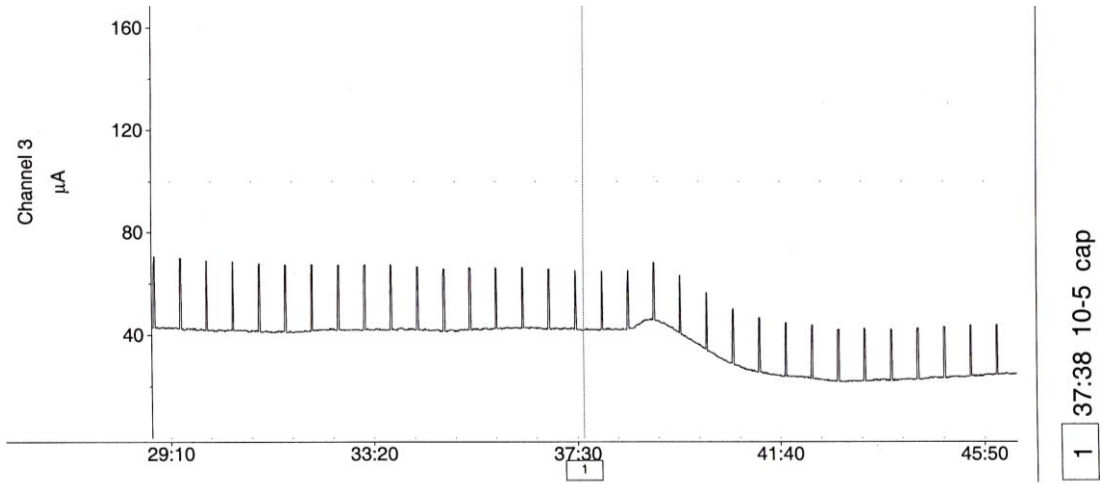
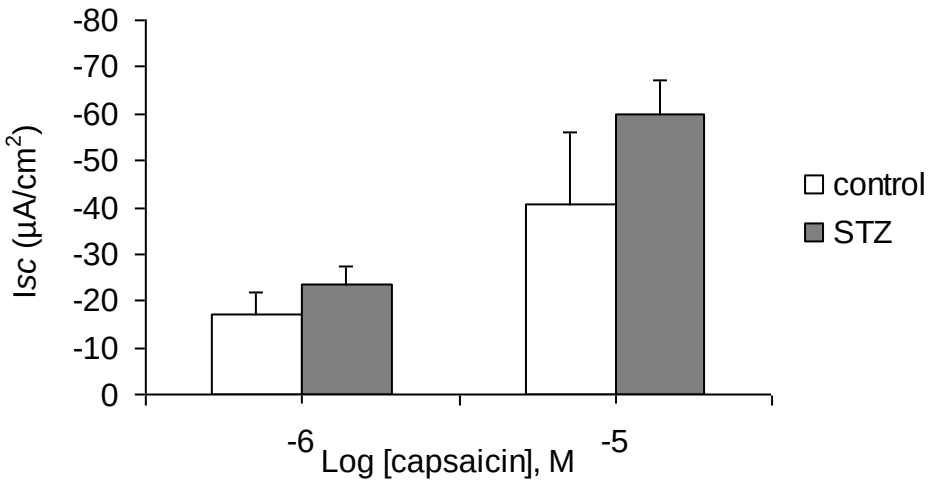


Figure 4.2 Original trace illustrating ion secretory responses of control rat proximal colon to TRPV1 agonist capsaicin (10^{-5} M). When capsaicin (10^{-5} M) was added to the basolateral side of tissues, it produced an initial transient increase in short circuit current, followed by a sustained reduction for 20 minutes.

A)



B)

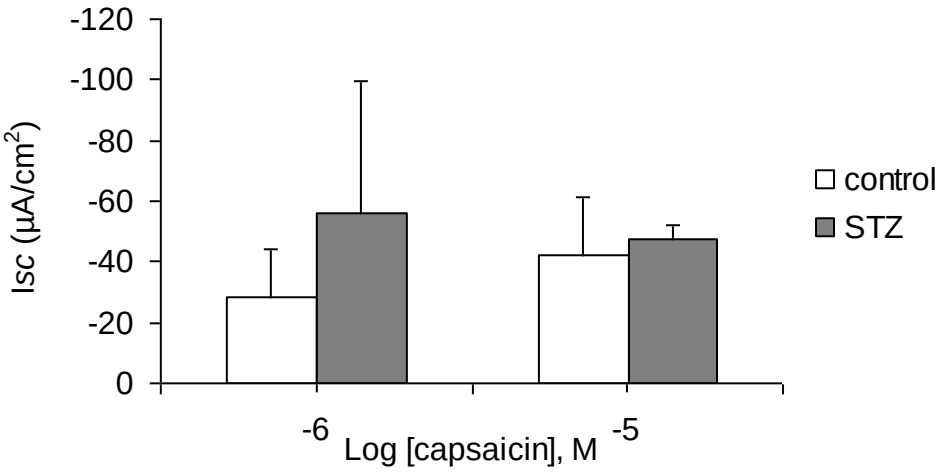


Figure 4.3 Ion secretory responses of proximal colon (A) and distal colon (B) to TRPV1 agonist capsaicin at the concentration of 10^{-6} M and 10^{-5} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 5 animals.

4.3.2.2 Ion secretory responses to carbachol

Muscarinic receptor agonist carbachol produced an increase in ion secretion (short circuit current) both in control and diabetic colon tissues. The ion secretory responses to carbachol were similar between proximal and distal colon but the increase in ion secretory responses to carbachol in diabetic tissues were greater than those of control tissues. The responses were significantly different between control and diabetes in distal colon (Figure 4.4, 4.5).

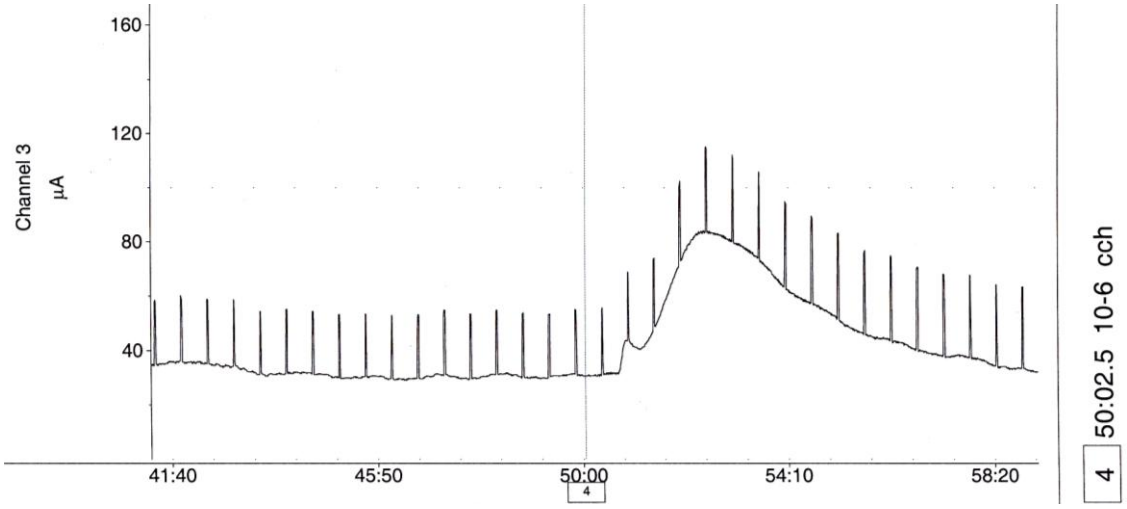


Figure 4.4 Original trace illustrating ion secretory responses of control rat proximal colon to muscarinic receptor agonist carbachol (10^{-6} M). The administration of muscarinic receptor agonist carbachol (10^{-6} M) produced the sustained increase in short circuit current followed by a slightly reduction, indicating a biphasic responses.

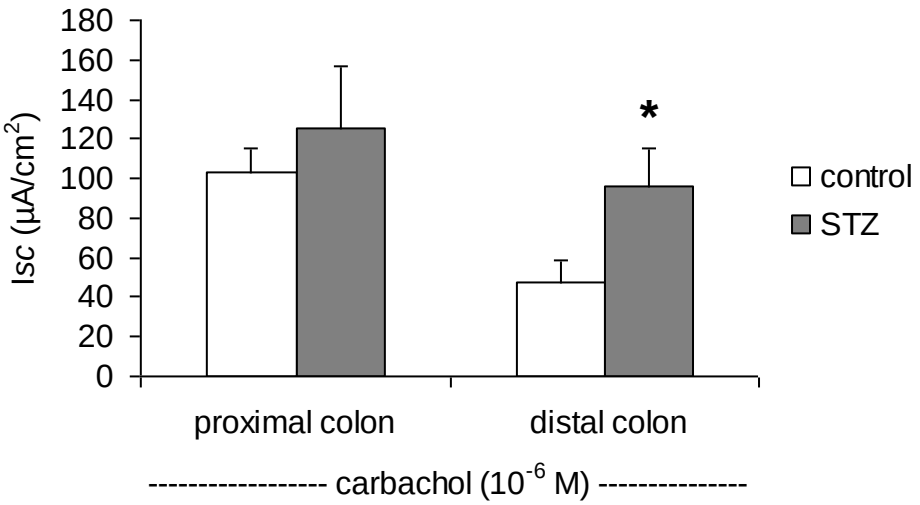


Figure 4.5 Ion secretory responses of proximal colon and distal colon to muscarinic receptor agonist carbachol at the concentration of 10^{-6} M in age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 5 animals. *P<0.05 is significantly different from age-matched controls (Student's t test for unpaired observations).

4.3.2.3 Ion secretory responses to veratridine

The sodium channel activator veratridine produced an increase in ion secretion (short circuit current) both in control and diabetic colon tissues. This finding is similar to the ion secretory responses to muscarinic receptor agonist carbachol. The ion secretory responses to veratridine were similar between control and diabetic tissues in proximal colon. However, in the distal colon, it was found that the ion secretory responses to carbachol in diabetic tissues tended to be greater than those of control tissues but were not statistically different (Figure 4.6, 4.7).

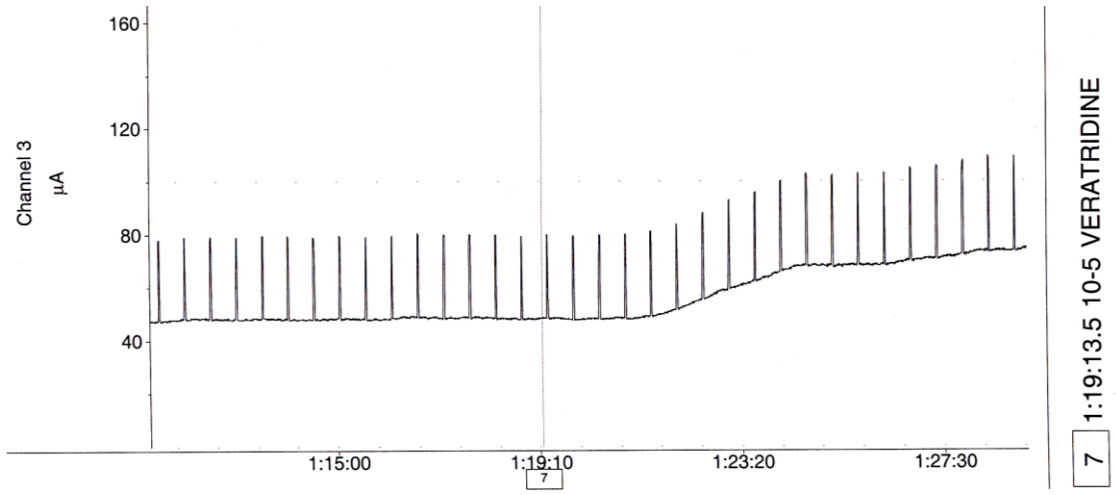


Figure 4.6 Original trace illustrating ion secretory responses of STZ-induced diabetic rat proximal colon to sodium channel activator veratridine (10^{-5} M). When veratridine (10^{-5} M) was added to the basolateral side of tissue preparations, it produced a sustained increase in short citcuit current and this increase was constant for 20 minutes.

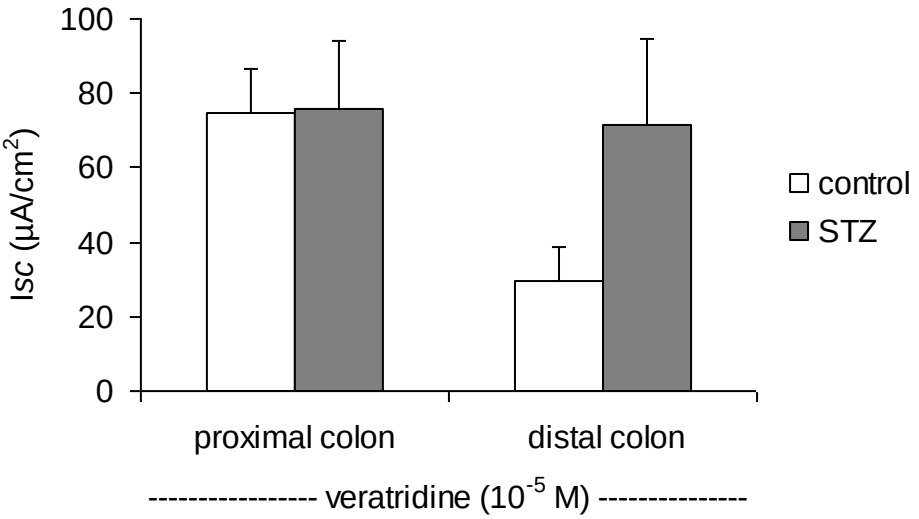


Figure 4.7 Ion secretory responses of proximal colon and distal colon to veratridine at the concentration of 10^{-5} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 5 animals.

4.4 Discussion

In the present study, it was found that the responses to capsaicin in rat colon tissues were different from those observed in rat urinary bladder tissues. Capsaicin produced relaxant responses in colon tissues but it produced contractile responses in urinary bladder tissues. This may due to different neurotransmitters released in these tissues. The contractile responses in bladder may be caused by an excitatory neurotransmitter release, which is induced by capsaicin (Saitoh *et al*., 2007). In contrast, the relaxant responses in colon may be caused by an inhibitory neurotransmitter release induced by capsaicin.

Although there were different responses to capsaicin between urinary bladder and colon tissues, significant reduction of the responses to capsaicin in both STZ-induced diabetic urinary bladder and colon were observed. This confirms that the reduction of the responses to capsaicin in STZ-induced diabetic tissues occurred not only in urinary bladder but also in colon.

In previous studies, it was found that capsaicin was shown to induce contraction and or relaxation in different gastrointestinal preparations (Maggi, 1990). Capsaicin induced relaxation followed by a contraction in stomach fundus strips of control rats on the resting tone. Only a contraction was evoked in diabetic state (Pinna *et al*., 1995). Capsaicin evoked relaxation in precontracted colonic longitudinal and circular muscle (Smith and Smid, 2005). These findings are similar to the results obtained from the present study in which capsaicin produced relaxations in colon tissues.

However, it was found that the relaxations evoked by capsaicin in diabetic colonic tissues were less than those of control tissues. Smith and Smid (2005) found that capsaicin evoked relaxation was significantly diminished in the inflammatory bowel disease-affected colon. This result is similar to those of the diabetic colonic tissues, suggesting the deleterious effect of both diseases (inflammatory bowel disease and diabetes) on motility in colon.

In this study, capsaicin produced the reduction in ion secretion (short circuit current) both in control and diabetic colon tissues. The reduction in ion

secretory responses to capsaicin in diabetic rat colon tissues was greater than those of control tissues.

In a previous study, Yarrow *et al*. (1991) found that capsaicin caused an initial transient increase in short circuit current in the rat descending colon mucosa, followed by a more prolonged reduction that lasted for 20-30 minutes. Repeated applications of 3μM capsaicin caused desensitization of the secretory response. The antisecretory effects (i.e. reduction in short circuit current from original baseline) remained, although they were significantly reduced. In some preparations described as “non-responders”, 3μM capsaicin did not elicit a secretory response. No desensitization of the remaining antisecretory responses was observed in these tissues. In fact, these reductions in short circuit current were consistently larger than those from tissues which responded with a secretory response.

These findings are similar to the results from the present study in which capsaicin produced a reduction in ion secretion (short circuit current) both in control and diabetic colon tissues.

Forrest *et al*. (2006) found that the basal short circuit current of the colonic epithelium did not significantly differ between control and diabetic tissues. The calculated resistance of the diabetic epithelium was not significantly different to that of the control tissues. The ion secretory response to carbachol did not significantly differ between control and diabetic tissue at any concentration used. The increase in the short circuit current from basal values that was produced by carbachol (1μmol/L) was significant in both control and diabetic tissues. In summary, no differences were observed between control and diabetic colonic muscosal short-circuit current under basal or carbachol (100 nmol/L – 1 μmol/L)-stimulated conditions.

The results from the present study showed that carbachol produced an increase in ion secretion (short circuit current) both in control and diabetic colon tissues, similar results to those observed by Forrest *et al*. (2006). The results suggest that diabetes did not have any effect on ion secretion evoked by carbachol in rat colon.

Veratridine is a Na^+ channel opener. It acts at the neurotoxin receptor site 2 and preferentially binds to activated Na^+ channels causing a persistent activation and causes depolarization of enteric neurones, resulting in increased chloride secretion secretion across the colonic mucosa (Fichna *et al*., 2009; Hyland and Cox, 2005). Veratridine induces a fast, monophasic positive change in Isc (measured as maximal increase in Isc above baseline) within 3 minutes after veratridine administration, and a secondary stabile increase in Isc (measured as increase in Isc over baseline within 25 minutes after veratridine administration (Hagl *et al*., 2008).

In the present study, veratridine produced an increase in ion secretion (short circuit current) both in control and diabetic colon tissues. The ion secretory responses to veratridine was similar between control and diabetic tissues in proximal colon. This suggests that STZ-induced diabetes had no effect on sodium channel function.

In a prevoius study, Hagl *et al*. (2008) found that the basic Isc, representing mainly chloride ion secretion, was reduced in the FGF-2 knockout animals; an effect reaching statistical significance. The stimulating of anion secretion by the neurotoxin veratridine lead to a decrease only in the caecum and distal colon while the duodenal secretion was not altered. The latter being consistent with the morphological findings in the duodenum.

Chapter 5

The effect of cholesterol and cyclodextrins on TRPV1 channel function

5.1 Introduction

The TRPV1 ion channel is localized on sensory nerve endings and is activated by heat, acid and by lipid ligands including capsaicin. The TRPV1 channel may be associated with lipid rafts to facilitate interaction with specific lipid metabolites that activate the receptor. Cholesterol is an important component of lipid rafts. It has been reported that cholesterol depletion caused a significant reduction of capsaicin- and proton- induced responses and that TRPV1 is regulated by the level of cholesterol in sensory neurons in dorsal root ganglia in rats (Liu *et al*., 2006). TRPV1 is localized in cholesterol rich microdomains, regulating the function and membrane expression of TRPV1. Many previous studies have mentioned the significant role of lipid rafts in the regulations of ion channel functions in different cells or tissues (Bari *et al*., 2005; Hering *et al*., 2003).

In diabetes, cholesterol levels in plasma membrane and tissues are changed (Torii, 1954). So it was of interest to see if this might potentially affect TRPV1 responses in bladder tissues.

Cyclodextrins are reported to remove cholesterol from membranes, affecting their function and also the ion channel located in the membranes. Study on the effect of cyclodextrins on human erythrocytes suggested that the factor affecting membrane function is not only cholesterol but also phospholipids (Ohtani *et al*., 1989). Liu *et al*. (2006) showed that the depletion of cholesterol from primary cultures of sensory neurons with methyl-β-cyclodextrin reduced capsaicin-activated currents. Manipulating cholesterol and other lipid levels in rat bladder tissues by using cyclodextrin molecules may alter the membrane composition of sensory neurons (in bladder) where TRPV1 is believed to be expressed.

In streptozotocin (STZ) treated rats, capsaicin evoked contractions in bladder are reduced compared to controls (Pinna *et al*., 1994). We have examined the effects of cholesterol depletion with α-cyclodextrin and and β-cyclodextrin on capsaicin responses in bladder from control and STZ treated rats to see if modulation of cholesterol levels might explain the changes in response. In addition, the other cyclodextrin molecules, α-cyclodextrin and γ-cyclodextrin were used. Since α-cyclodextrin and γ-cyclodextrin seems not to remove cholesterol from the membrane, they are therefore used as negative control.

In a preliminary study, it was found that contractile responses to the muscarinic receptor agonist carbachol were similar between control and diabetic rat bladder tissues. It is interesting to investigate whether cholesterol and cyclodextrins have any effect on the muscarinic receptor function. These experiments were designed to investigate the effect of STZ-induced diabetes on the responses to the muscarinic receptor agonist carbachol in the presence and absence of cholesterol-PEG.

We examined effects of adding cholesterol by incubation in cholesterol-PEG and removing with methyl-β-cyclodextrin. α-cyclodextrin and γ-cyclodextrin do not remove cholesterol so they were used as negative control.

In addition, it is interesting to investigate that modulation by cyclodextrin would affect the cholesterol contents in rat bladder smooth muscle since there are changes caused by cyclodextrin molecules in rat bladder in vitro. Therefore, after treatment with or without cyclodextrin molecules, the tissues were extracted to get cholesterol samples. These samples were then identified and quantified for cholesterol using High Performance Liquid Chromatography (HPLC).

5.2 Methods

5.2.1 The effect of cholesterol and cyclodextrins on muscarinic receptor function

The bladder strips were equilibrated for 30 minutes before addition of carbachol at cumulative concentrations of 10^{-8} M to 10^{-6} M. The tissues were then washed out and left for 30 minutes. Cholesterol-PEG (5 mg/ml), methyl-β-cyclodextrin (0.005 mg/ml), β-cyclodextrin (10^{-5} M), and α-cyclodextrin (10^{-5} M) were added to the organ bath. The tissue were exposed to these drugs for 1 hour before the second addition of carbachol at concentrations of 10^{-8} M to 10^{-6} M.

5.2.2 The effect of cholesterol and cyclodextrins on TRPV1 channel function

The bladder strips were equilibrated for 30 minutes before addition of cholesterol-PEG (5 mg/ml), methyl-β-cyclodextrin (0.005 mg/ml), β-cyclodextrin (10^{-5} M), α-cyclodextrin (10^{-5} M), and γ-cyclodextrin (10^{-5} M). The tissure were exposed to these drugs for 1 hour before the addition of capsaicin at concentration of 10^{-10} M to 10^{-6} M. All experiments were performed in parallel in the presence and absence of cholesterol-PEG and these three cyclodextrins in control and STZ-induced diabetic tissues.

5.2.3 The effect of cyclodextrins on TRPA1 channel function

Methyl-β-cyclodextrin was used for studying the effect of cholesterol modulation on another TRP channel, TRPA1 function. After equilibartion for 30 minutes, the control and STZ-induced diabetic rat bladder strips were exposed to methyl-β cyclodextrin (0.005 mg/ml) for 1 hour. Then the contractile responses to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-5} M to 10^{-4} M were performed. Allyl isothiocyanate was added to the organ bath in non-cumulative manner.

5.2.4 The effect of chlorpromazine on muscarinic receptor, potassium ion channel and TRP channel function

To study the disturbance of lipid bilayer, the experiments were designed by using dopamine receptor antagonist chlorpromazine to see the inhibitory effect in bladder tissue.

The bladder tissues were equilibrated for 30 minutes prior to be exposed to chlorpromazine at the concentration of 10^{-5} M for 30 minutes. Then the tissues were treated cumulatively with TRPV1 agonist capsaicin (10^{-10} M to 10^{-6} M), and potassium chloride (50 mM) in the presence or absence of chlorpromazine. However, the tissues were treated non-cumulatively with TRPA1 agonist allyl isothiocyanate after exposure to chlorpromazine or vehicle (distilled water).

5.3 Results

5.3.1 The effect of cholesterol and cyclodextrins on muscarinic receptor function

5.3.1.1 The effect of cholesterol-PEG

The results indicated that carbachol in the concentration range of 10^{-8} M to 10^{-6} M produced concentration dependent contractions of urinary bladder strips from STZ-treated animals and age-matched vehicle controls. The contractile responses to carbachol in the presence and absence of cholesterol-PEG (5 mg/ml, 1 h) were similar in both control and diabetic tissues. The responses were slightly smaller in STZ-treated tissues than in control tissues, but these differences did not reach statistical significance (Figure 5.1).

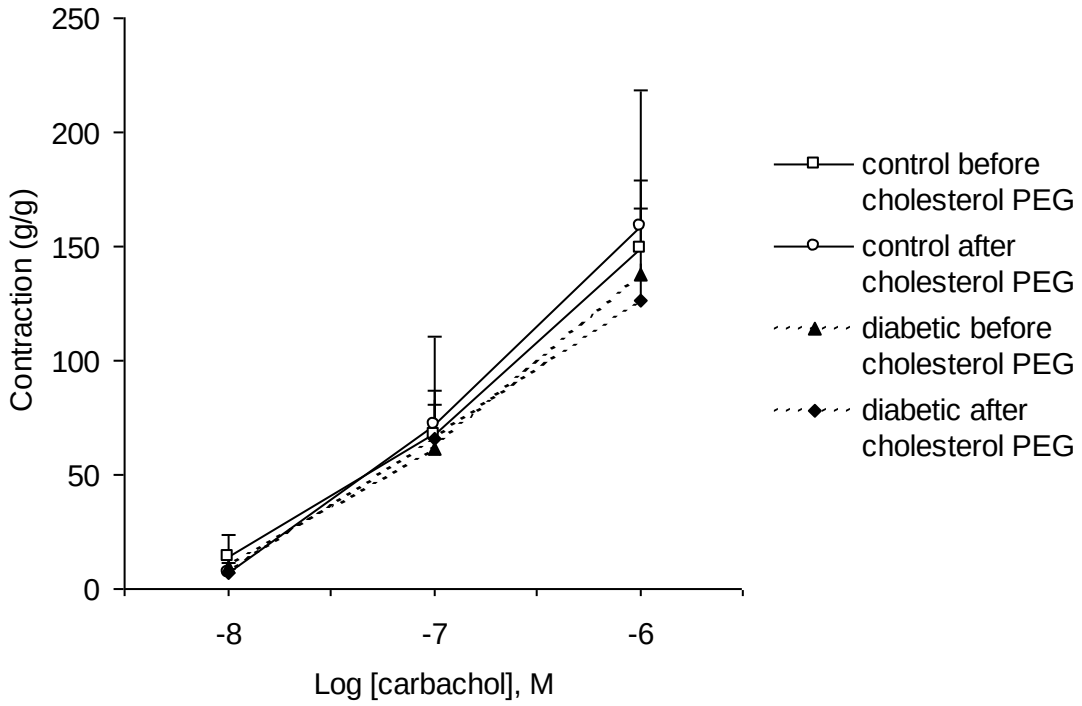


Figure 5.1 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} M - 10^{-6} M in the presence and absence of cholesterol-PEG (5 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.1.2 The effect of methyl-β cyclodextrin

The results indicated that carbachol in the concentration range of 10^{-8} M to 10^{-6} M produced a concentration dependent contraction of urinary bladder strips from STZ-treated animals and age-matched vehicle controls. The contractile responses to carbachol in the presence and absence of methyl-β cyclodextrin (0.005 mg/ml, 1 h) were similar (Figure 5.2).

In this experiment, there was a significant reduction in responses to carbachol at the concentrations of 10^{-7} - 10^{-6} M in STZ-diabetic rat bladder tissues. However, this reduction is not consistent with previous experiments.

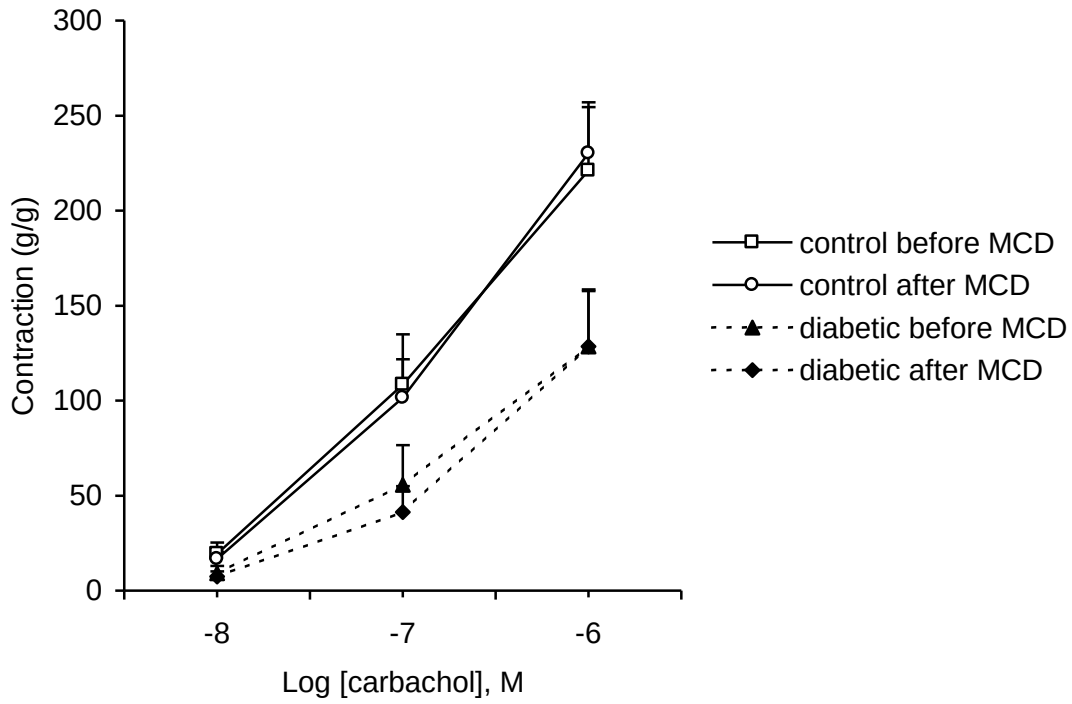


Figure 5.2 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} M - 10^{-6} M in the presence and absence of methyl-β cyclodextrin (MCD) (0.005 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.1.3 The effect of β-cyclodextrin

The results indicated that carbachol in the concentration range of 10^{-8} M to 10^{-6} M produced the concentration dependent contractions of urinary bladder strips from STZ-treated animals and age-matched vehicle controls. The contractile responses to carbachol in the presence and absence of β-cyclodextrin (10^{-5} M, 1 h) were similar in both control and diabetic tissues (Figure 5.3).

In this experiment, there was a significant reduction in responses to carbachol at the concentrations of 10^{-7} - 10^{-6} M in STZ-diabetic rat bladder tissues. However, this reduction is not consistent with previous experiments.

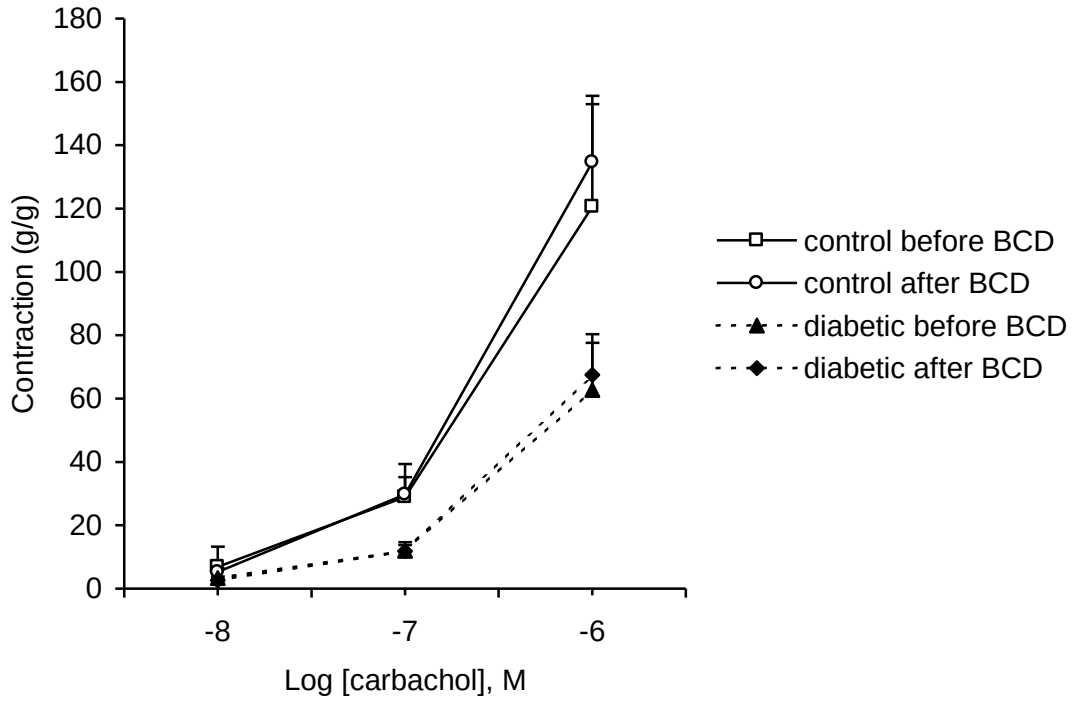


Figure 5.3 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} M - 10^{-6} M in the presence and absence of β-cyclodextrin (BCD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals.

5.3.1.4 The effect of α-cyclodextrin

The results indicated that carbachol in the concentration range of 10^{-8} M to 10^{-6} M produced concentration dependent contractions of urinary bladder strips from STZ-treated animals and age-matched vehicle controls. The contractile responses to carbachol in the presence and absence of α-cyclodextrin (10^{-5} M, 1 h) were similar in both control and diabetic tissues (Figure 5.4). The responses were slightly smaller in STZ-treated tissues than those of control tissues. However, these differences did not reach a statisatical significance.

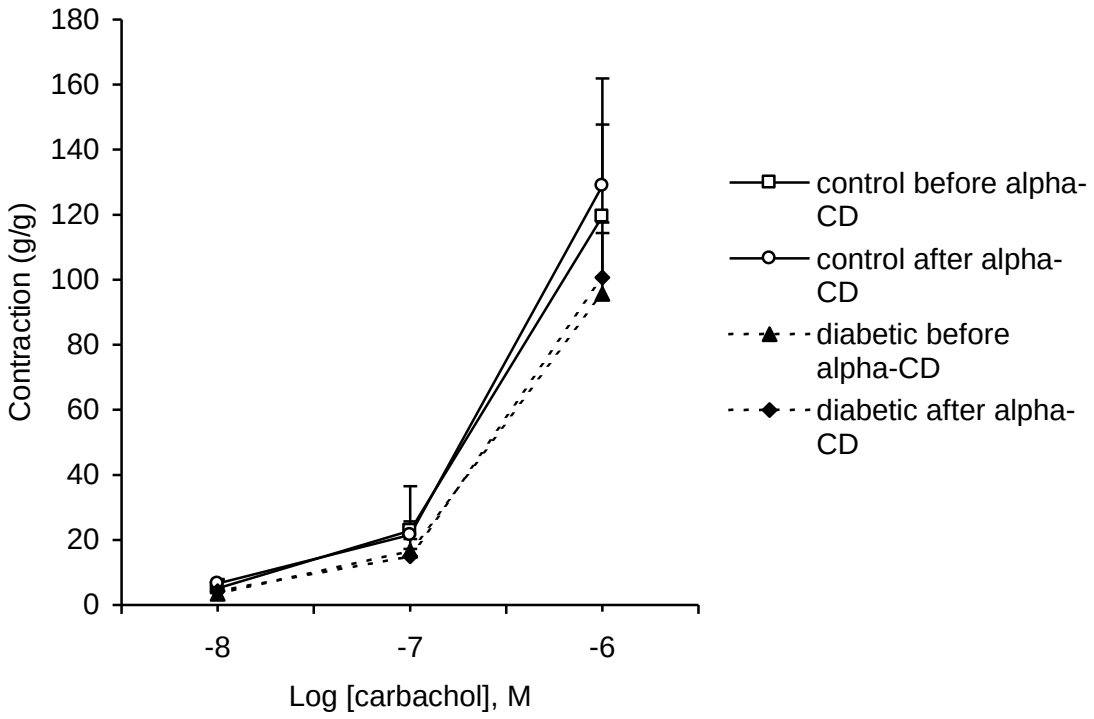


Figure 5.4 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} M - 10^{-6} M in the presence and absence of α-cyclodextrin (alpha-CD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals.

5.3.2 The effect of cholesterol and cyclodextrins on TRPV1 channel function

5.3.2.1 The effect of cholesterol-PEG

The presence of cholesterol-PEG (5 mg/ml, 1 h) produced the significantly decreased contractile responses to capsaicin in comparison to those in the absence of cholesterol-PEG in both age-matched-controls and STZ-treated tissues. The reductions of the contractile responses to capsaicin in the presence of cholesterol-PEG were seen at both the concentrations of 10^{-6} M and 10^{-5} M of capsaicin. The contractile responses to capsaicin at the concentration of 10^{-6} M to 10^{-5} M in the presence and absence of cholesterol-PEG in STZ-treated bladder tissues were significantly smaller than those of age-matched controls (Figure 5.5, 5.6).

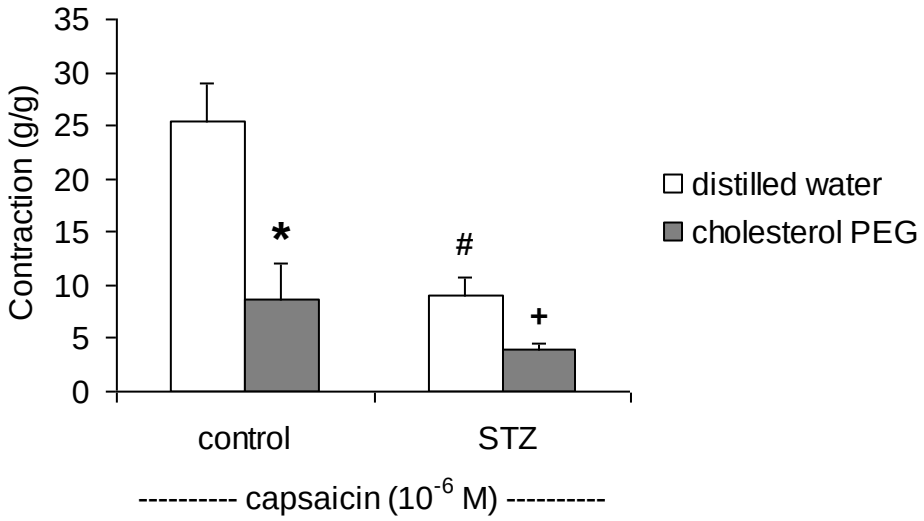


Figure 5.5 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-6} M in the presence and absence of cholesterol-PEG (5 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals.$^{*, \#, +}$ $P<0.05$ is significantly different (Student's *t* test for unpaired observations). * = significant difference between control + distilled water and control + cholesterol PEG. # = significant difference between control + distilled water and diabetic + distilled water. + = significant difference between diabetic + distilled water and diabetic + cholesterol PEG.

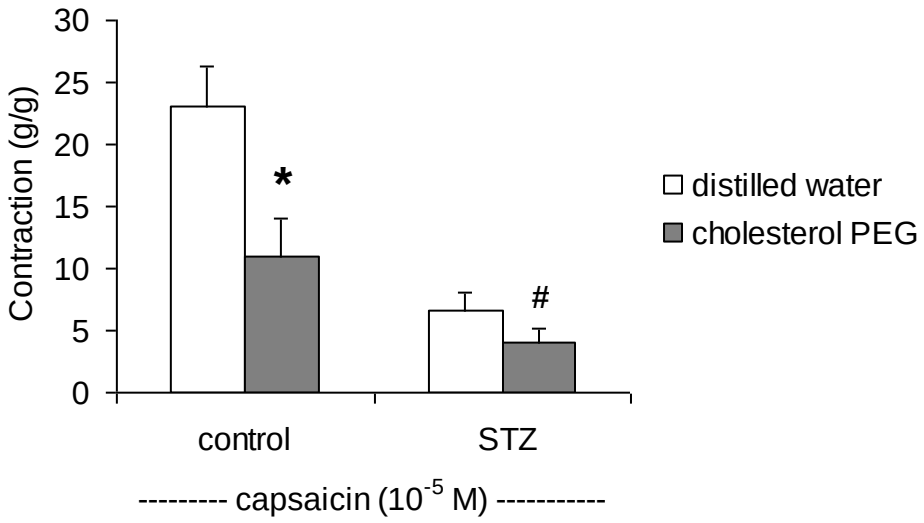


Figure 5.6 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-5} M in the presence and absence of cholesterol-PEG (5 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean ± S.E.M for 6 animals. $^{*,\#}$ $P<0.05$ is significantly different (Student's t test for unpaired observations). * = significant difference between control + distilled water and control + cholesterol PEG. # = significant difference between control + distilled water and diabetic + distilled water.

5.3.2.2 The effect of methyl-β-cyclodextrin

The contractile responses to capsaicin at concentrations of 10^{-6} M to 10^{-5} M in the presence and absence of methyl-β- cyclodextrin in STZ-treated bladder tissues were smaller than those of age-matched controls.

The presence of methyl-β cyclodextrin (0.005 mg/ml, 1 h) produced the increased contractile responses to capsaicin in comparison to those in the absence of methyl-β-cyclodextrin in both age-matched-controls and STZ-treated tissues. The increase of the contractile responses to capsaicin in the presence of methyl-β-cyclodextrin was seen at both concentrations of 10^{-6} M and 10^{-5} M of capsaicin. However, the changes were not statistical significant from control (Figure 5.7, 5.8).

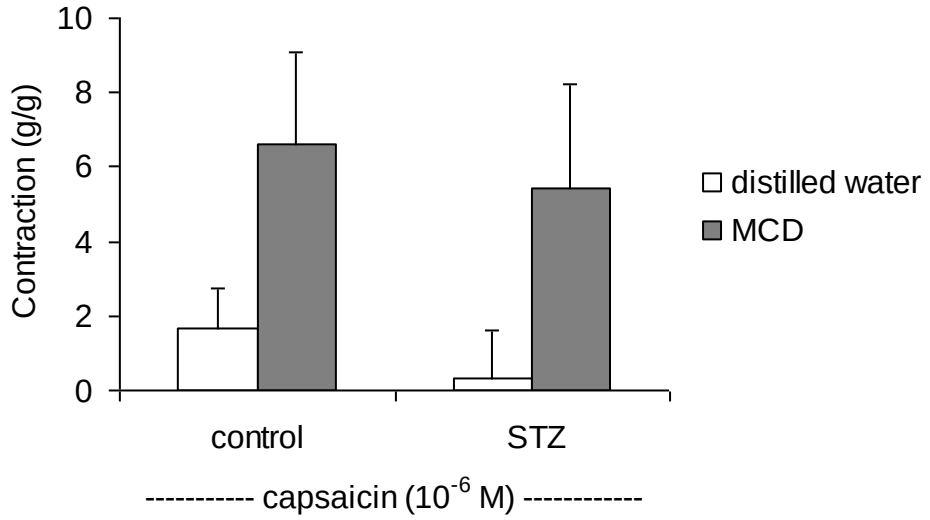


Figure 5.7 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-6} M in the presence and absence of methyl-β-cyclodextrin (MCD) (0.005 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

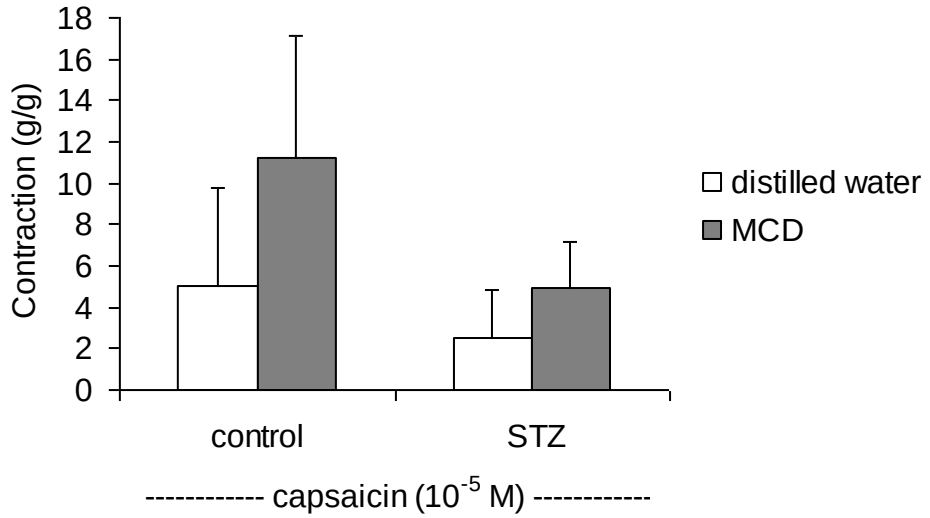


Figure 5.8 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-5} M in the presence and absence of methyl-β-cyclodextrin (MCD) (0.005 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.2.3 The effect of β-cyclodextrin

The contractile responses to capsaicin in the absence of β-cyclodextrin at concentrations of 10^{-6} M in STZ-treated bladder tissues were significantly smaller than those of age-matched controls.

The presence of β-cyclodextrin (10^{-5} M, 1 h) produced increased contractile responses to 10^{-6} M capsaicin when compared to those in the absence of β-cyclodextrin in both age-matched-controls and STZ-treated tissues. However, there was no change in capsaicin responses in STZ tissues in the presence of 10^{-5} M β-cyclodextrin (Figure 5.9, 5.10).

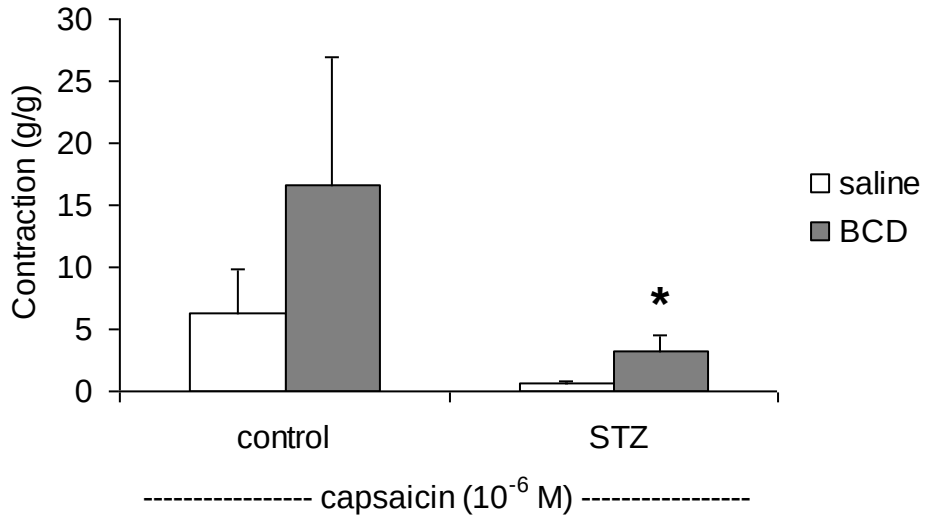


Figure 5.9 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-6} M in the presence and absence of β-cyclodextrin (BCD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. *P<0.05 is significantly different (Student's *t* test for unpaired observations) between diabetic + saline and diabetic + BCD. Values represent the mean±S.E.M for 6 animals.

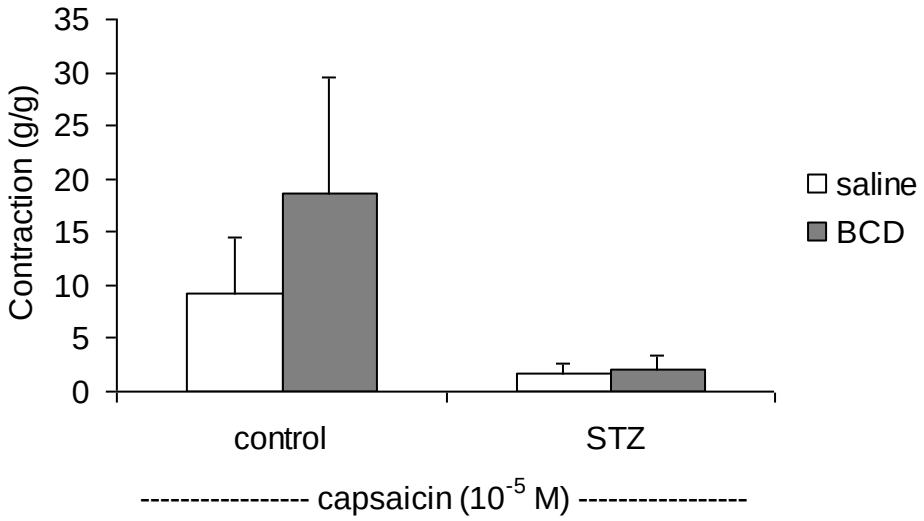


Figure 5.10 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-5} M in the presence and absence of β-cyclodextrin (BCD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.2.4 The effect of α-cyclodextrin

The contractile responses to capsaicin in the presence of α-cyclodextrin at the concentrations of 10^{-6} M to 10^{-5} M in STZ-treated bladder tissues were significantly greater than those of age-matched controls.

The presence of α -cyclodextrin (10^{-5} M, 1 h) produced significantly increased contractile responses to 10-6 M capsaicin in comparison to those in the absence of α-cyclodextrin in both age-matched-controls and STZ-treated tissues. However, there was no change in capsaicin responses in STZ tissue in the presence of 10^{-5} M α-cyclodextrin (Figure 5.11, 5.12).

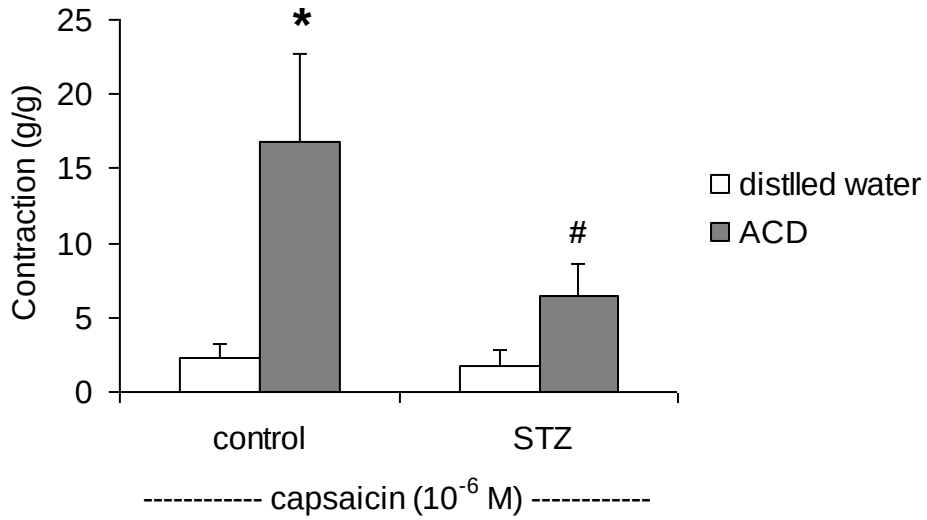


Figure 5.11 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-6} M in the presence and absence of α-cyclodextrin (ACD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. $^{*, \#}$ $P<0.05$ is significantly different (Student's t test for unpaired observations). * = significant difference between control + distilled water and control + ACD. # = significant difference between diabetic + distilled water and diabetic + ACD.

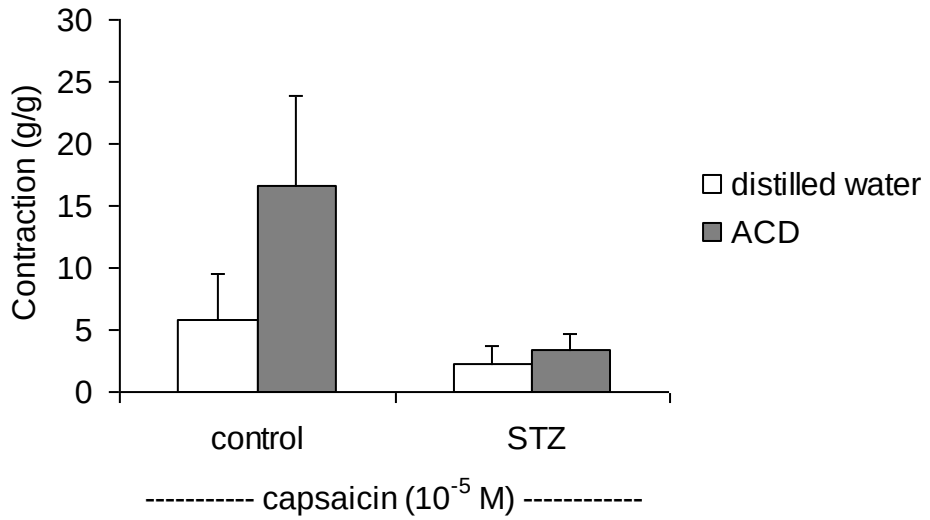


Figure 5.12 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-5} M in the presence and absence of α-cyclodextrin (ACD) (10^{-5} M, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.2.5 The effect of γ-cyclodextrin

The contractile responses to capsaicin (10^{-6} M) in the presence and absence of γ-cyclodextrin (10^{-5} M, 1 h) in normal bladder tissues were similar (Figure 5.13). This indicates that of γ-cyclodextrin did not have any effect on the contractile responses to capsaicin. Therefore γ-cyclodextrin did not have any effect on the TRPV1 channel function.

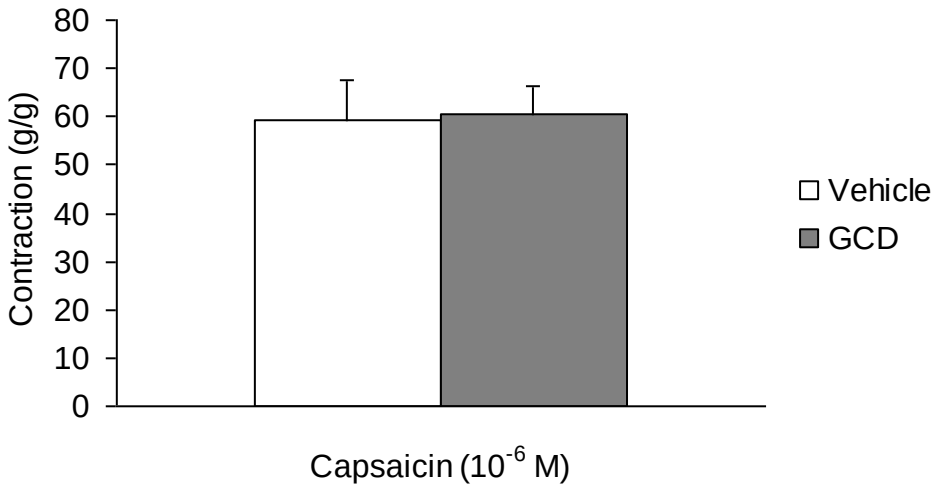


Figure 5.13 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-6} M in the presence and absence of γ-cyclodextrin (GCD) (10^{-5} M, 1 h) from age-matched controls. Values represent the mean±S.E.M for 6 animals.

5.3.2.6 The effect of cyclodextrin on TRPA1 channel function

The contractile responses to allyl isothiocyanate (10^{-4} M) in the presence and absence of methyl-β-cyclodextrin (0.005 mg/ml, 1 h) in STZ-treated bladder tissues were significantly smaller than those of age-matched controls. However, contractile responses to allyl isothiocyanate in the presence and absence of methyl-β-cyclodextrin were similar (Figure 5.14).

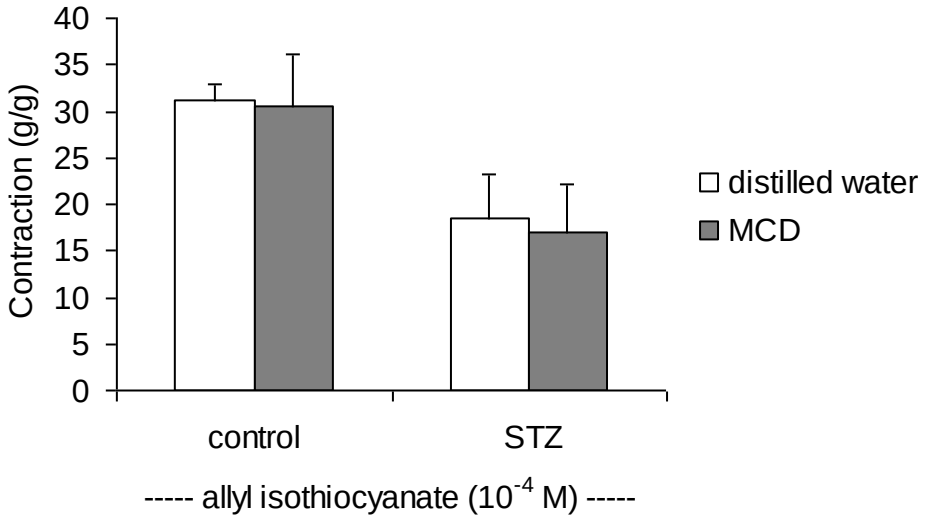


Figure 5.14 Contractile responses of bladder to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-4} M in the presence and absence of methyl-β cyclodextrin (MCD) (0.005 mg/ml, 1 h) from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

5.3.3 The effect of chlorpromazine on muscarinic receptor function

The contractile responses of normal rat bladder tissues to all concentrations (10^{-8} M – 10^{-6} M) of carbachol were significantly reduced in the tissues treated with chlorpromazine at the concentration of 10^{-5} M. While the responses were significantly greater in tissues treated with vehicle (distilled water). This indicated that chlorpromazine inhibited the contractile responses to carbachol (Figure 5.15).

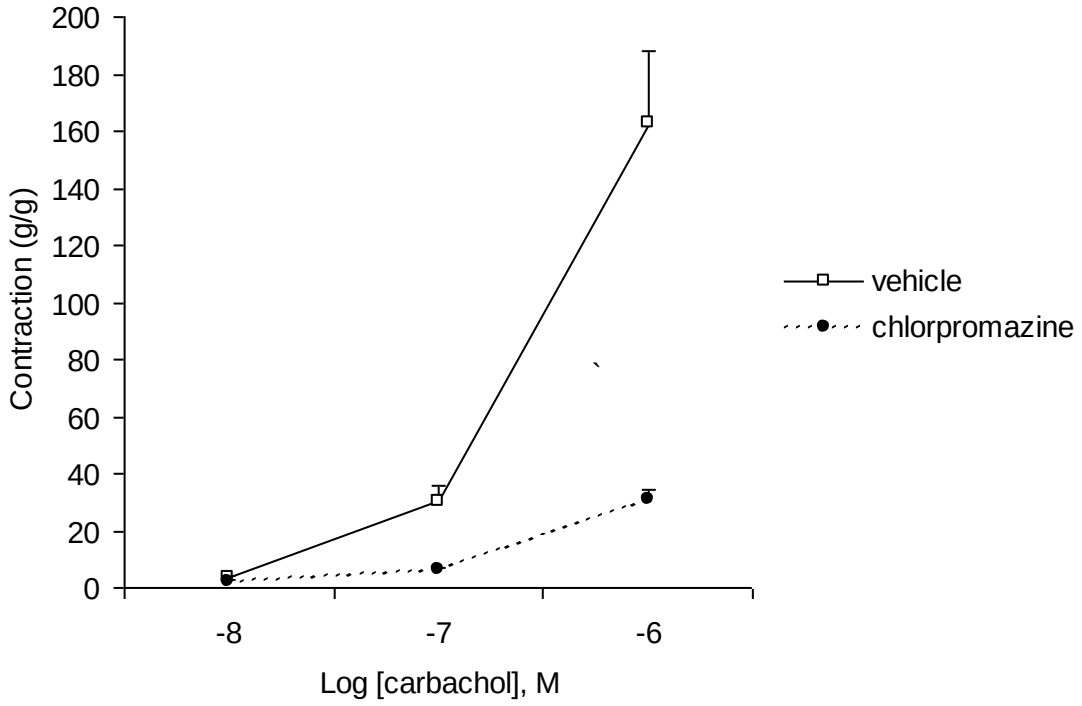


Figure 5.15 Contractile responses of bladder to muscarinic receptor agonist carbachol at the concentration of 10^{-8} - 10^{-6} M in the presence and absence of chlorpromazine (10^{-5} M) from age-matched controls. Values represent the mean±S.E.M for 6 animals. Means are different between in the absence and presence of chlorpromazine ($P<0.05$, two-way ANOVA).

5.3.4 The effect of chlorpromazine on the response to potassium chloride

Potassium chloride (KCl) caused contraction in normal rat bladder tissues. The contractile responses of normal rat bladder tissues to potassium chloride at the concentration of 50 mM in the presence of chlorpromazine at the concentration of 10^{-5} M were significantly reduced in comparison to those in the absence of chlorpromazine. It is clear that chlorpromazine inhibited the contractile responses to potassium chloride (Figure 5.16).

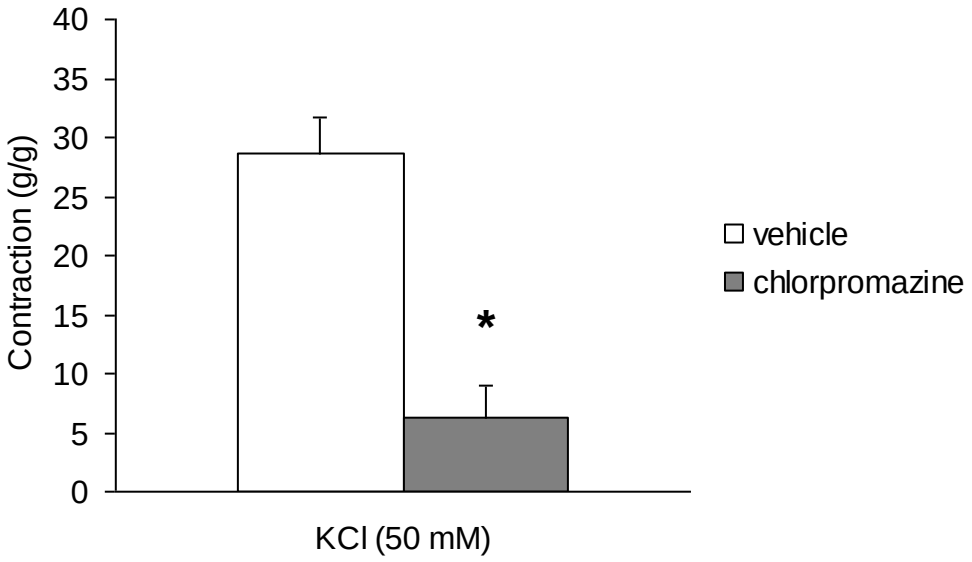


Figure 5.16 Contractile responses of bladder to potassium chloride at the concentration of 50 mM in the presence and absence of chlorpromazine (10^{-5} M) from normal rats. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from the vehicle (Student's *t* test for unpaired observations).

5.3.5 The effect of chlorpromazine on TRPV1 channel function

Chlorpromazine blocked the contractile responses to capsaicin in normal rat bladder tissues. The contractile responses to capsaicin in the presence of chlorpromazine in normal rat bladder tissues were significantly reduced in the presence of chlorpromazine in comparison to those in the absence of chlorpromazine. It is clear that chlorpromazine inhibited the responses to all concentrations of capsaicin (Figure 5.17).

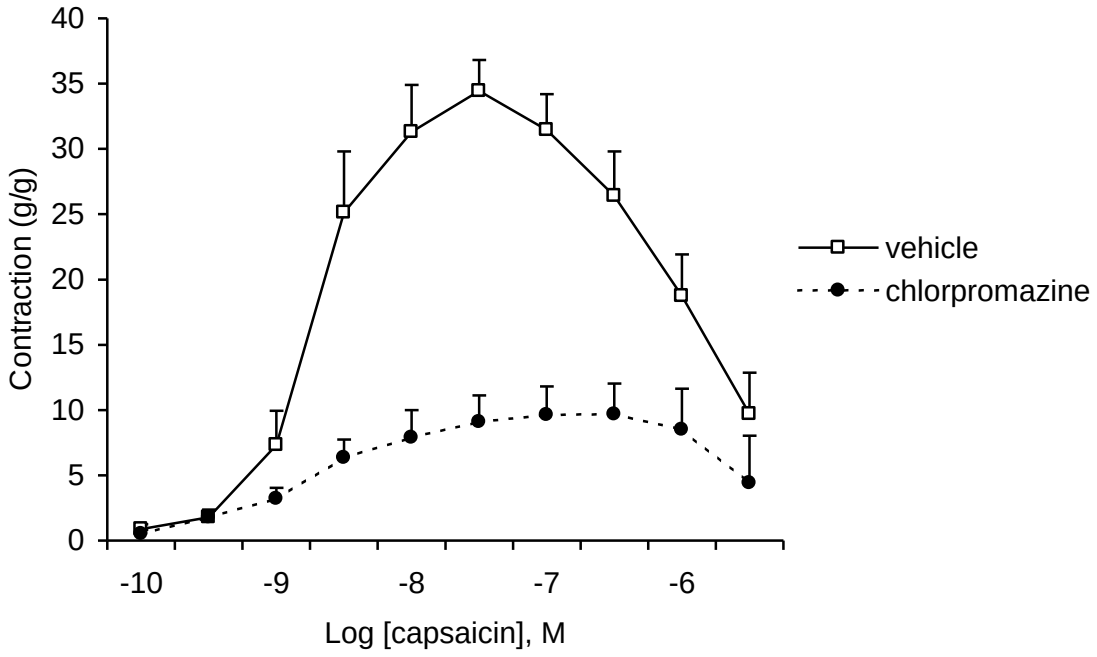


Figure 5.17 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls in the presence and absence of chlorpromazine (10^{-5} M). Values represent the mean±S.E.M for 6 animals. Means are different between in the absence and presence of chlorpromazine ($P<0.05$, two-way ANOVA).

5.3.6 The effect of chlorpromazine on TRPA1 channel function

The contractile responses to allyl isothiocyanate in the presence of chlorpromazine in normal rat bladder tissues were significantly reduced in comparison to those in the absence of chlorpromazine. The reductions are more obvious in higher concentrations of allyl isothiocyanate (Figure 5.18).

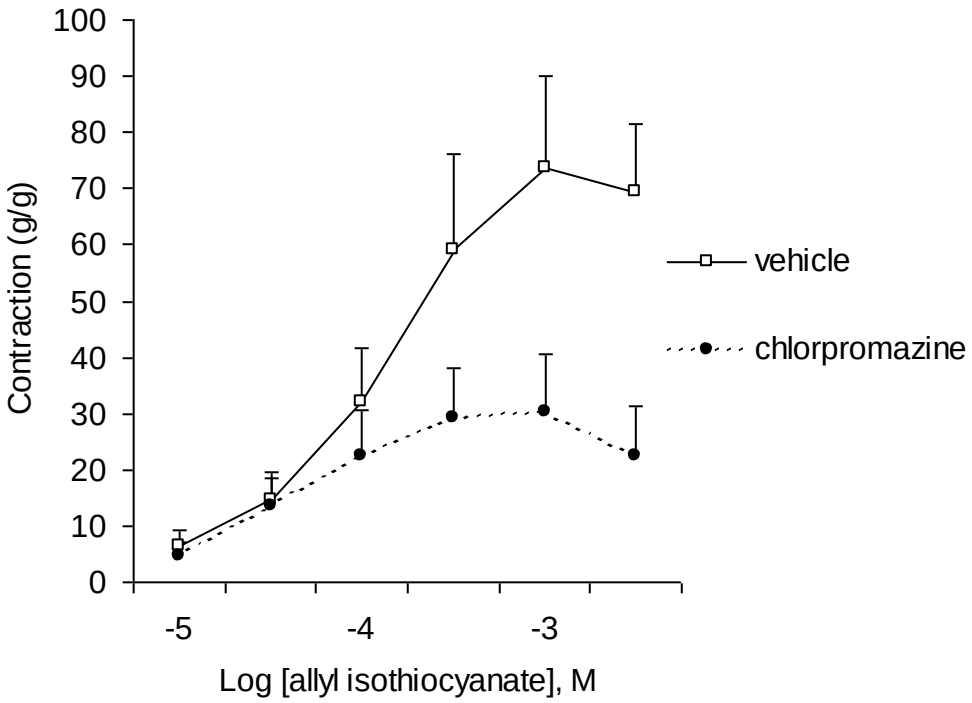


Figure 5.18 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-5} – 10^{-3} M from age-matched controls in the presence and absence of chlorpromazine (10^{-5} M). Values represent the mean±S.E.M for 6 animals. . Means are different between in the absence and presence of chlorpromazine ($P<0.05$, two-way ANOVA).

5.3.7 The effect of cyclodextrins on cholesterol contents in rat urinary bladder tissues

In these experiments, we try to measure the cholesterol contents in rat bladder tissues after treatment with methyl-β-cyclodextrin. The aim is to see the correlation between cholesterol, cyclodextrins, and the responses to TRPV1 agonist capsaicin. With the limitation of size of the rat bladder, the High Performance Liquid Chromatography (HPLC) was used according to methods presented by Maraschiello et al (1996). The response linearity was studied for HPLC. 10, 20, 40, 60 and 90 µg/ml of cholesterol was injected. Linear plot of concentration versus peak areas were calculated. Consequently, 20 µl samples from rat bladder tissues treated with and without cyclodextrins were injected (Figure 5.19).

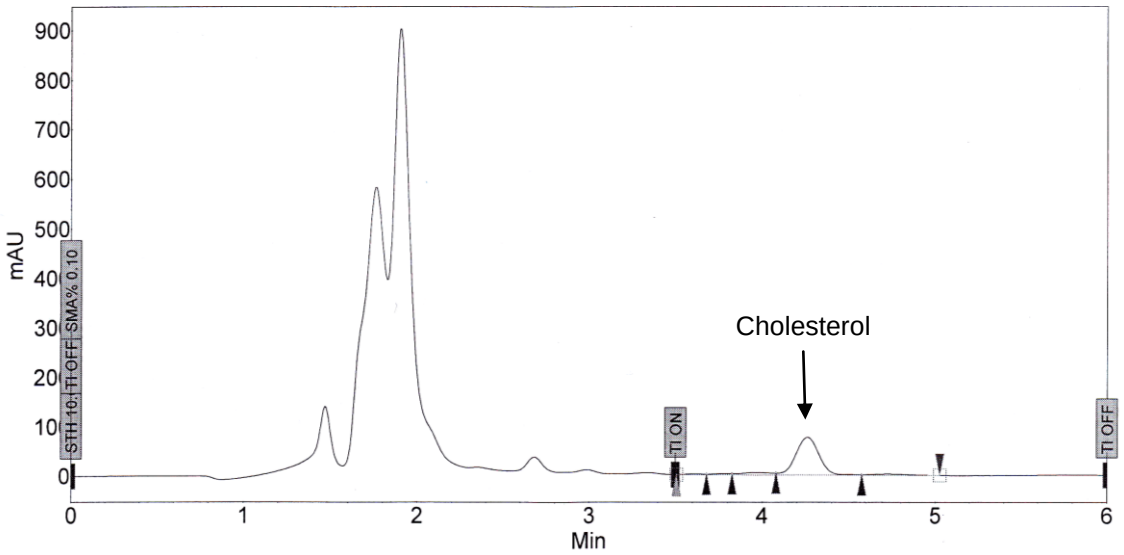


Figure 5.19 Chromatogram illustrating HPLC analysis of cholesterol samples extracted from whole rat urinary bladder at 20 µl injection.

When the cholesterol contents from different sizes of rat bladder tissues (whole bladder and quarter bladder) were compared, it was found that the cholesterol contents from whole bladder tissues are higher than those from the quarter bladder tissues (Figure 5.20). This is unlikely to happen because when the wet weights of the tissues were compared, the cholesterol contents from all difference size should be similar. In the present sudy, it is indicated that there is loss of cholesterol during the extraction process.

Rat bladder tissues treated with 0.005 mg/ml (0.5%) or 0.001 mg/ml (1 %) methyl-β-cyclodextrin for 1 hours seem to provide the highest cholestreol contents than those of the controls (Figure 5.21). However, the difference did not reach statistical significance. These results indicate that methyl-β-cyclodextrin is not involved in cholesterol extraction from rat smooth muscle tissues. On the other hand, cholesterol may not be involved in the modulation of TRPV1 channel function by this concentration of methyl-β-cyclodextrin.

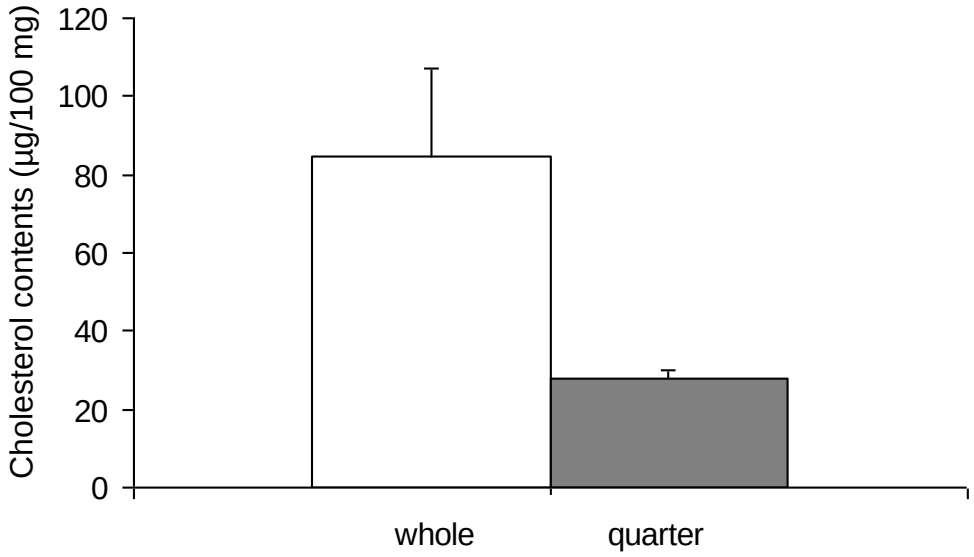


Figure 5.20 Cholesterol contents from whole and quarter rat urinary bladder. Values represent the mean ± S.E.M for 6 animals.

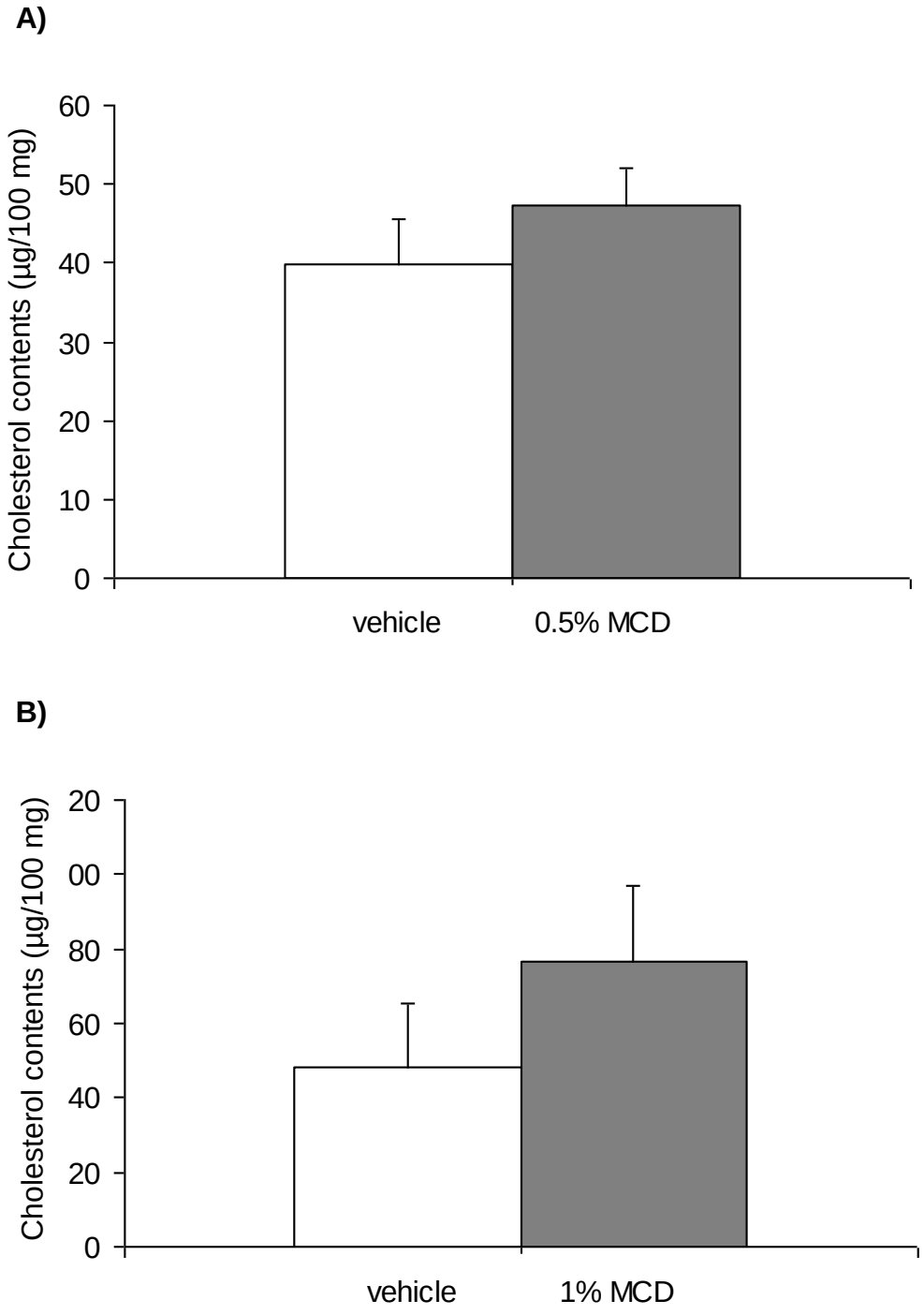


Figure 5.21 Cholesterol contents from half rat urinary bladder in the presence and absence of 0.5 % (A) and 1.0 % (B) methyl-β-cyclodextrin. Values represent the mean ± S.E.M for 6 animals.

5.3.8 The effect of cyclodextrins on TRPV1 channel function in HEK 293 cell

The TRPV1-expressing HEK 293 cells were incubated with and without methyl-β-cyclodextrin and γ-cyclodextrin for 1 hour and then activated by capsaicin. Changes in intracellular Ca^{2+} in response to capsaicin were examined using fura-2 calcium imaging.

The results show that methyl-β-cyclodextrin and γ-cyclodextrin had no effect on calcium signalling mediated by capsaicin since the fura ratio from the cells treated with methyl-β-cyclodextrin and γ-cyclodextrin or vehicle were similar (Figure 5.22, 5.23).

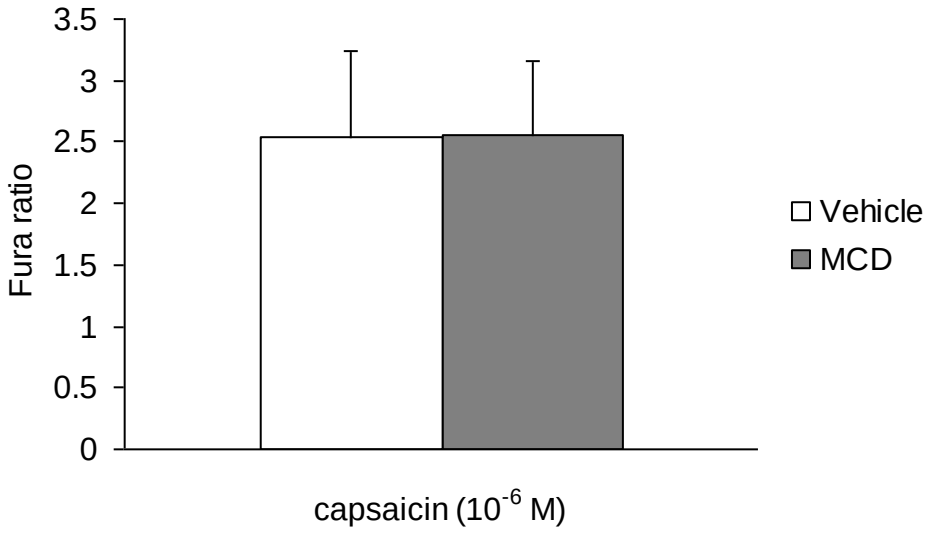


Figure 5.21 Peak change in fura-2 ratio mediated by 1 μM capsaicin in TRPV1 expressed rat HEK293 cells exposed to methyl-β-cyclodextrin (0.005 mg/ml) and vehicle for 1 hour. Values represent the mean ± S.E.M. for 6 animals.

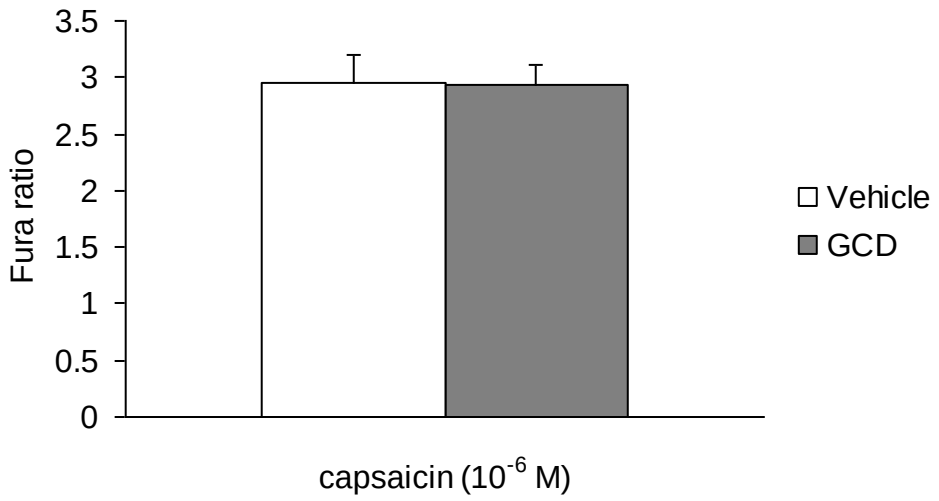


Figure 5.22 Peak change in fura-2 ratio mediated by 1 μM capsaicin in TRPV1 expressed rat HEK293 cells exposed to γ-cyclodextrin (10^{-5} M) and vehicle for 1 hour. Values represent the mean ± S.E.M. for 6 animals.

5.4 Discussion

In the preliminary study, cholesterol (water-soluble) and methyl-β-cyclodextrin did not have any effect on the responses of control and diabetic rat bladder strips to carbachol. Cholesterol and methyl-β-cyclodextrin enhanced the contractile responses to TRPV1 agonist capsaicin in control and diabetic rat bladder strips. However, the contractile responses to TRPV1 agonist capsaicin in diabetic tissues are reduced compared to controls. The contractile responses to capsaicin in the presence of cholesterol and methyl-β-cyclodextrin in the rat bladder strip are similar.

This may caused by the presence of methyl-β-cyclodextrin in the water-soluble cholesterol used in the first study. The 1 g of water-soluble cholesterol contains 48 mg cholesterol and 952 mg methyl-β-cyclodextrin (to be soluble in water). Thus, the contractile responses to capsaicin both in the presence of cholesterol and methyl-β-cyclodextrin may mainly be due to the effect of methyl-β-cyclodextrin. The pure cholesterol (cholesterol-PEG) was then used to investigate the effect of cholesterol on the activity and amount of membrane TRPV1.

Cholesterol is an essential component of lipid rafts (Zajchowski and Robbins, 2002). It has been shown that elevation of cholesterol decreases uterine activity. Both LDLs and cholesterol inhibited spontaneous uterine force production and associated Ca^{2+} transients; frequency, amplitude, and duration of contraction were all significantly reduced compared with preceding control contractions (Smith *et al*., 2005).

Decreased levels of membrane cholesterol are accompanied by a highly specific inhibition of phasic, but not tonic contractions in rat uterus smooth muscle. In additions, the electrically evoked phasic mechanical activity of guinea pig ureter and the spontaneous contractions of rat portal vein were severely decreased after 40 minutes of cholesterol extraction (Babiychuk *et al*., 2004).

The present study showed that the contractile responses to carbachol were unaffected by cholesterol-PEG and cyclodextrins (methyl-β-cyclodextrin,

β-cyclodextrin, and α-cyclodextrin) at the concentrations tested. Carbachol caused contraction in both control and diabetic tissues. The presence of cholesterol-PEG produced the significantly decreased contractile responses to capsaicin in both age-matched-controls and STZ-treated tissues.

Methyl-β-cyclodextrin (MCD) significantly diminished the intracellular Ca^{2+} influx induced by capsaicin, but had no significant effect on the Ca^{2+} influx induced by resiniferatoxin. In contrast, MCD caused significant inhibition in TRPV1 receptor activation evoked by capsaicin and resiniferatoxin on cultured trigerminal neurons (Szoke *et al*., 2009).

In the in vitro studies, it was found that uterine activity (force) was significantly increased by cholesterol extraction with MCD or cholesterol oxidase treatment (Smith *et al*., 2005). Similarly, extraction of cholesterol resulted in inhbition of both force and intracellular Ca^{2+} signals (Babiychuk *et al*., 2004). The depletion of cholesterol with methyl-β-cyclodextrin (MCD) disrupts caveolar microdomains. The changes in membrane excitability produced by MCD underlies the changes found in Ca^{2+} signalling and uterine contractility (Shmygol and Wray, 2007).

However, some previous studies have suggested that MCD significantly reduced TRPV1-mediated capsaicin- and proton-activated currents. Immunoreactivity for TRPV1, but not $P2X_3$, in the plasma membrane was markedly reduced by MCD. A reduction of TRPV1 protein in membrane fractions was also found following cholesterol depletion (Liu *et al*., 2006).

β-cyclodextrins, cyclic oligosaccharides consisting of 7 β(1-4)-glucopyranose units, are water-soluble compounds with a hydrophobic cavity capable of dissolving hydrophobic compounds and thus enhancing their solubility in aqueous solution (Kilsdonk *et al*., 1995).

In vitro studies have demonstrated that exposure of fibroblasts to cyclodextrins can produce cell toxicity, and that the extent of this toxicity is reduced by the presence of serum. Exposure of erythrocytes to cyclodextrins results in hemolysis in order of β > α > γ. This hemolysis may attributed to the removal of erythrocyte membrane components, particularly cholesterol (Kilsdonk *et al*., 1995).

Vial and Evans (2005) demonstrated that recombinant and smooth muscle (rat tail artery, vas deferens, and bladder) $P2X_1$ receptors are present in cholesterol-rich lipids rafts and co-localize with lipid raft markers flotillin-1 and -2. Addition of the cholesterol-depleting agent methyl-β-cyclodextrin (10 mM for 1 h) led to a redistribution of the $P2X_1$ receptor throughout the sucrose gradient and reduced $P2X_1$ receptor-mediated (α, β-methylene ATP, 10 μM) currents in HEK293 cells and contractions of the rat tail artery. Contractions evoked by potassium chloride were unaffected by methyl-β-cyclodextrin and the inactive analoque α-cyclodextrin had no efect on $P2X_1$ receptor-mediated currents or contractions. The cholesterol-depleting agents MCD and β-CD (both 10 mM for 1 h) reduced the amplitude of the current with no obvious effect on the time course of responses. The inactive analoque α-CD had no effect on $P2X_1$ receptor current.

In the present study, methyl-β-cyclodextrin enhanced the maximal contractile responses to the TRPV1 agonist capsaicin in control and diabetic rat bladder strips. Cholesterol-PEG (pure cholesterol) significantly lowered the maximal contractile responses to capsaicin of rat bladder strips in both control and STZ treated strips. α-cyclodextrin and β-cyclodextrin are not thought to sequester cholesterol (Vial and Evans, 2005) and so these two compounds were used as negative controls. Surprisingly, α-cyclodextrin and β-cyclodextrin at the same concentrations enhanced the contractile responses to capsaicin in the control and diabetic rat bladder strips, an effect similar to that of methyl-β-cyclodextrin. However, γ-cyclodextrin did not have any effect on the contractile responses to capsaicin. In addition, adding cholesterol as cholesterol-PEG, or methyl-β-cyclodextrin did not have any effect on the responses of control and diabetic rat bladder strips to carbachol or to the TRPA1 agonist allyl isothiocyanate.

These effects of cyclodextrin are specific to capsaicin activated contractions and not seen with TRPA1 activation, suggesting that the effects are not mediated downstream of channel activation. The results show that all three cyclodextrin molecules produce the same effect on the response to

capsaicin in rat bladder possibly by disturbing the local environment of the TRPV1 channel.

Chlorpromazine acts as a membrane perturbing compound. It is an antagonist on different postsynaptic receptors, including dopamine receptors (subtype D_1, D_2, D_3 and D_4), serotonin receptors (5-HT_1 and 5-HT_2), α_1- and α_2-adrenergic receptors, and M_1 and M_2 muscarinic acetylcholine receptor (Peroutka and Synder, 1980). In the present study, it was found that chlorpromazine inhibited the contractile responses to carbachol, potassium chloride, capsaicin, and allyl isothiocyanate. This indicates that chlorpromazine antagonized muscarinic receptor, potassium ion channel, TRPV1, and TRPA1 channel mediated responses. Therefore, this antagonist is not selective to TRPV1 or TRPA1 channels suggesting that it is not suitable for studying the alterations of TRP channel function in rat bladder smooth muscle preparations.

In the present study, it was found that treatment of 0.005 mg/ml (0.5%) or 0.01 mg/ml (1%) methyl-β-cyclodextrin for 1 hour to rat bladder tissue did not affect the cholesterol contents. However, from *in vitro* study, it was found that methyl-β-cyclodextrin enhanced the maximal contractile responses to TRPV1 agonist capsaicin in control and diabetic rat bladder strips. Cholesterol significantly lowered the maximal contractile responses to capsaicin of rat bladder strips. The mismatch between the functional data and biochemical data may be due to the fact that measurement of cholesterol contents in the whole rat bladder tissues may not reflect cholesterol level where the ion channels are localized on nerve endings.

Chapter 6

Time course studies on changes in TRPV1 channel function following streptozotocin-induced diabetes

6.1 Introduction

Peripheral neuropathy, one of the consequences of diabetes, is reproduced in the streptozotocin (STZ) model of diabetes in rats (Bestetti *et al*., 1981; Filho and Fazan, 2006). In preliminary observations we confirmed the reduction in contractile responses to the TRPV1 agonist capsaicin in diabetic rat bladder eight weeks after STZ treatment. It was interesting to examine the time course of onset of this dysfunction.

Hyperglycaemia (elevated blood glucose concentration) is the major alteration that occurs in diabetes mellitus. The previous studies suggested that hyperglycaemia was directly or indirectly related to the development or progression, or both, of diabetic complications including retinopathy, nephropathy and neuropathy (Talubmook, 2002).

The aims of these experiments were as follows: firstly, to investigate the time cause of onset of TRPV1 channel dysfunction in STZ model of diabetes in rat bladder, and secondly, to examine the effect of acute hyperglycaemia to see if this was sufficient to produce TRPV1 dysfunction.

6.2 Methods

6.2.1 The effect of time frame of streptozotocin-induced diabetes on TRP channel function

In order to examine the effect of the time frame for the induction of STZ diabetes on TRPV1 channels, the rats were used at eight, two, one week, and 36, and 24 hours after administration of STZ or citrate buffer. The tissues from different time frames after STZ injection were equilibrated for 30 minutes before adding capsaicin. Capsaicin at concentrations of 10^{-10} M to 10^{-6} M was added to the organ bath in cumulative manner. The comparisons of contractile responses to capsaicin of the carbachol contracted tissues of control and STZ-treated rat bladders were measured.

To study the effect of time frame of STZ-diabetes induction on another TRP ion channel, TRPA1, which is reported to be expressed on the sensory nerve terminal similar to the TRPV1 channel, the contractile responses to the TRPA1 agonist allyl isothiocyanate at concentrations of 10^{-5} M to 10^{-4} M were measured. Allyl isothiocyanate was added to the organ bath in a non-cumulative manner. The dose response studies to allyl isothiocyanate were performed using the tissues from the rat treated with STZ or citrate buffer (control) for 8, 2, 1 week, and 36 hours to see the changes in the responses according to the time courses of STZ treatments.

6.2.2 The effect of elevated glucose concentrations on TRPV1 channel function

In the study on the influence of elevated glucose on TRPV1 channel function, 4 bladder strips were obtained from the same rat. Two of them were incubated in normal glucose concentration media for 30 minutes, 2 hours, and 24 hours, while the other two strip preparations were incubated in parallel in elevated glucose concentration media. The bladder strip preparations were mounted in organ bath contained a Krebs solution of the following composition

(in mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5, and gassed with 95% O_2 and 5% CO_2 at room temperature (21 ± 4 °C).

For elevated glucose incubation, 33.3 mM glucose was added into the solution to produce 44.4 mM glucose. The preparations were allowed to equilibrate in Krebs's solution with normal and elevated glucose under the resting tension of 1 g for 30 minutes and 2 hours before application of capsaicin. For the 24 hours glucose incubation experiment, the bladder tissues were kept in Krebs's solution with normal and elevated glucose in the refrigerator at 4 °C for 24 hours before conducting the experiments.

6.2.3 The effect of elevated glucose concentration on TRPV1 channel function in HEK293 cell lines

After loading with Fura-2 AM for 45 minutes, TRPV1-expressing HEK 293 cells cultured in high and low glucose media were washed out with extracellular solution. Then the cells were placed under the microscope with the objective lens of 40X for calcium imaging. The cells were checked and marked the target area. Capsaicin at the concentration of 1 μM was administered to the cells in extracellular solution. Fluorescence readings were made over a 3 minutes period at 1-5 seconds intervals following addition of agonist to the cells. Excitation wavelengths of 350 and 380 nm were used with an emission wavelength of 510 nm. Intracellular calcium levels were measured as a ratio of fluorescence F350/F380.

6.2.4 The effect of diabetes on sodium ion channel function

In order to check if the diabetic state has effect on other receptors or ion channels present in sensory nerve terminal other than TRP ion channels, veratridine (sodium ion channel activator) was used to study the effect of diabetes on sodium ion channel function. The contractile responses of control and STZ-induced diabetic rat bladder tissues to veratridine at the concentration

of 10^{-5} M were measured. The contractile responses to veratridine in control and STZ-induced diabetic tissues were compared.

6.2.5 The direct effect of streptozotocin on TRPV1 channel function

It is possible that the reduction of contractile responses to a range of TRP channel agonists in diabetic rat bladder preparations seen in these studies may be due to the direct effect of STZ on the channels. STZ at the concentration of 10^{-5} M was added to the organ bath. The normal rat bladder tissues were exposed to STZ or vehicle (distilled water) for 30 minutes. Subsequently, dose responses to TRPV1 agonist capsaicin were conducted to see if STZ affects the contractile responses to capsaicin.

6.2.6 The effect of cannabinoid receptor agonist on TRPV1 channel function

The phosphorylation process may be involved in the TRPV1 channel function. The cannabinoid receptor agonist CP55,940 was used to study the alteration in TRPV1 responses. After equilibration for 30 minutes, the control rat bladder tissues were incubated with CP55,940 at the concentration of 10^{-5} M for 30 minutes. Afterwards, cumulative dose responses to capsaicin at the concentration of 10^{-10} M to 10^{-6} M were performed in the presence or absence (distilled water) of CP55,940. The similar methods were also used in STZ-diabetic rat bladder tissues.

6.2.7 The effect of pro-inflammatory agent on TRPV1 channel function

After equilibration for 30 minutes, the control rat bladder tissues were incubated with bradykinin at the concentration of 10^{-5} M for 30 minutes. Afterwards, cumulative dose responses to capsaicin at concentrations of 10^{-10} M to 10^{-6} M were performed in the presence or absence (distilled water) of

bradykinin. The similar methods were also used in STZ-diabetic rat bladder tissues.

6.3 Results

6.3.1 The effect of time frame of STZ-induced diabetes on TRPV1 channel function

6.3.1.1 Two weeks after STZ-injection

For 2 weeks after STZ injection, it was found that the blood glucose level of STZ-treated rats was significantly elevated, reaching a diabetic state (Figure 6.1). In addtion, the contractile responses to capsaicin at the concentrations of 10^{-10} M - 10^{-6} M in STZ-induced diabetic tissues tended to be reduced although the values did not reach the statistical differences when the control and diabetic groups were compared (Figure 6.2).

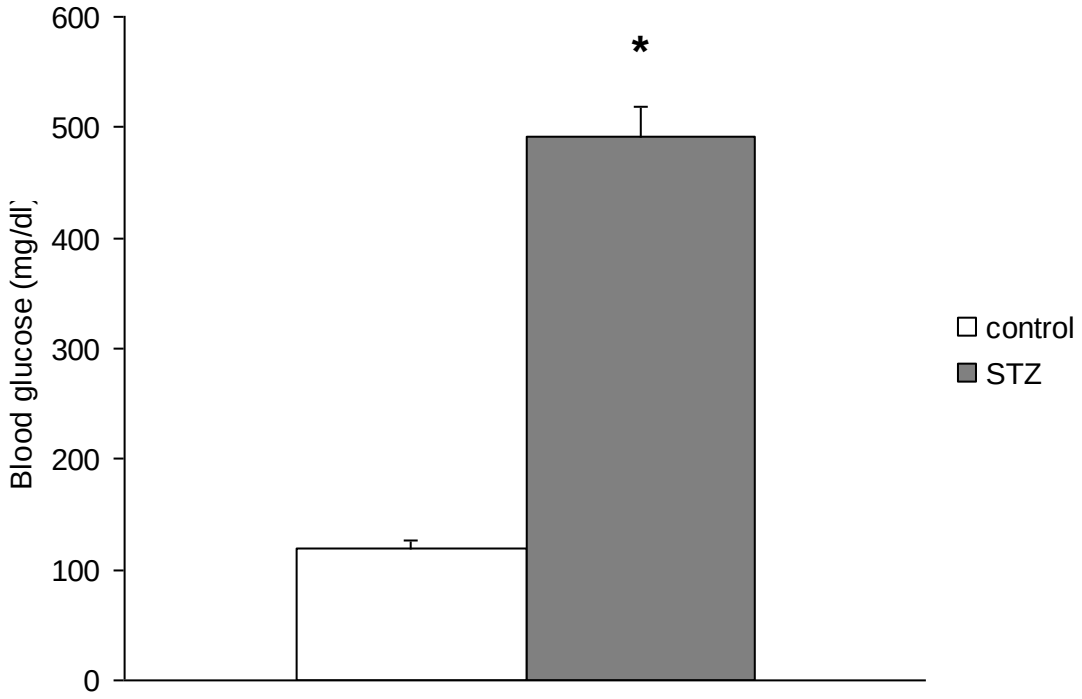


Figure 6.1 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 2 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

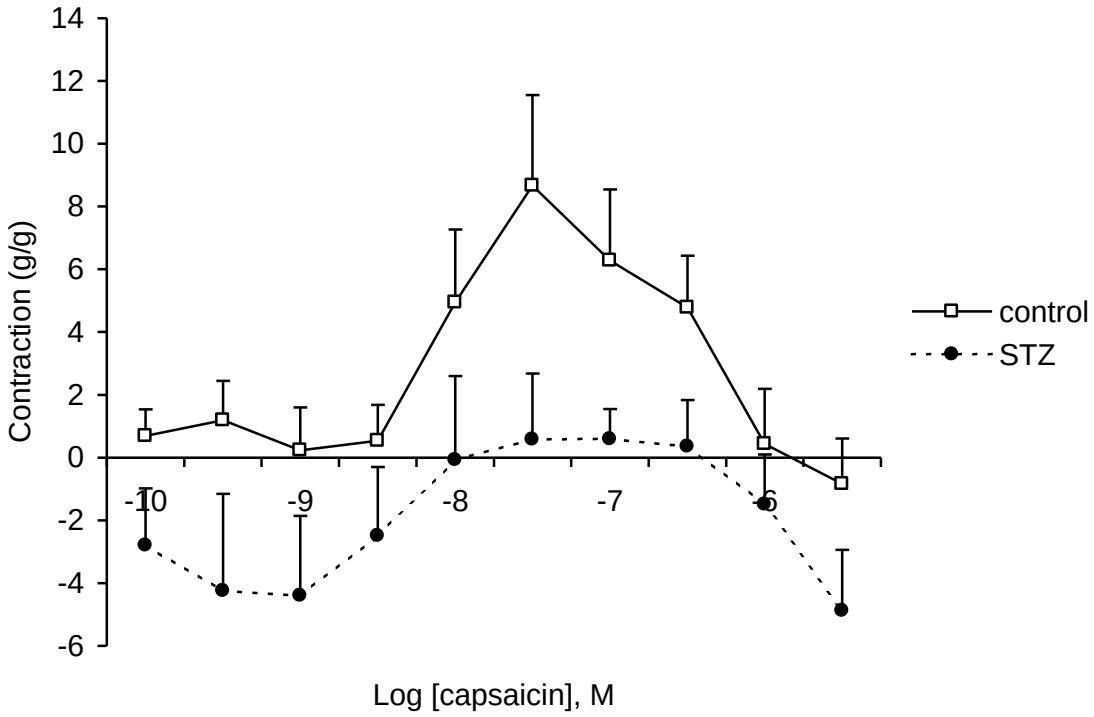


Figure 6.2 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 2 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

6.3.1.2 One week after STZ-injection

One week after STZ-injection, the blood glucose level in the STZ-treated animals were significantly elevated and similar to 8 weeks after STZ-injection, confirming the diabetic state in this animal model (Figure 6.3). The contractile responses to capsaicin at the concentration of 10^{-10} M - 10^{-6} M in one week STZ-treated animals were significantly reduced in comparisons to the citrate buffer-treated controls (Figure 6.4).

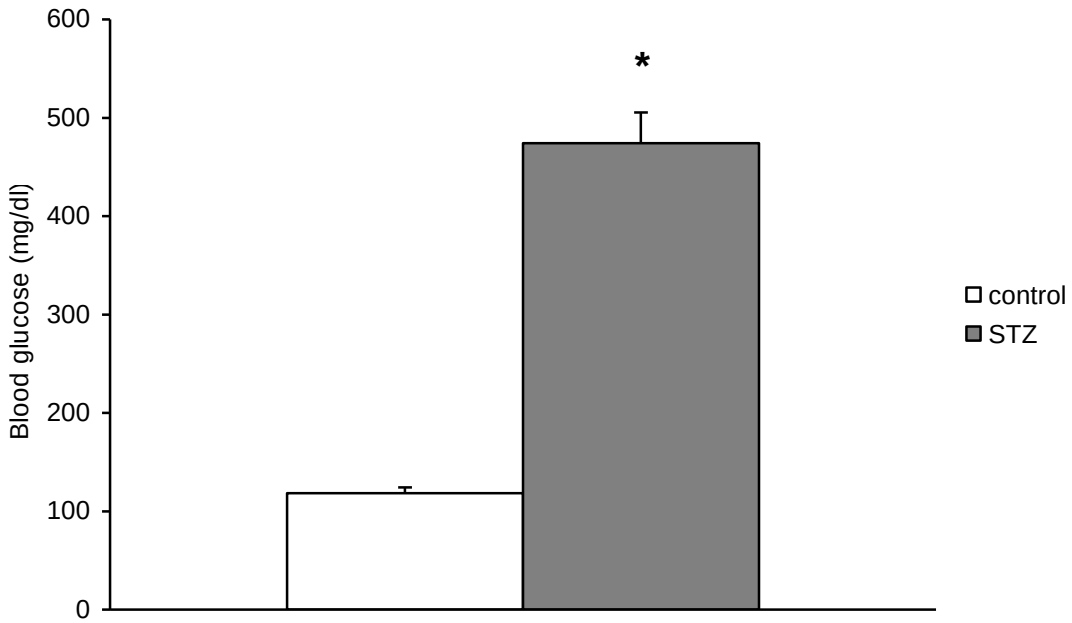


Figure 6.3 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 1 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

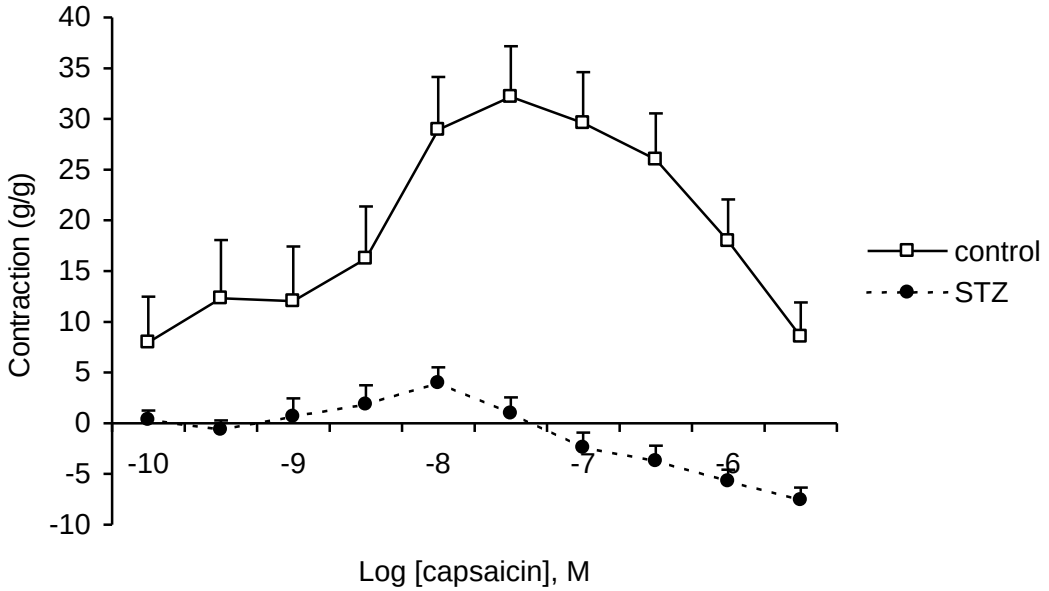


Figure 6.4 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 1 week after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

6.3.1.3 Thirty six hours after STZ-injection

As described above, it was found that eight weeks, two weeks, and one week STZ-treated animals had a significant reduction of the contractile responses to capsaicin. It is interesting to investigate the more acute time frame of STZ-induction to see its effect on contractile responses to TRPV1 agonist capsaicin.

36 hours after STZ-injection, bladder tissues were used for studying the contraction to capsaicin. The results showed that the blood glucose level of 36 hours STZ-treated rats was significantly elevated, reaching a diabetic state (Figure 6.5). In addtions, the contractile responses to capsaicin at the concentration of 10^{-10} M - 10^{-6} M in STZ-induced diabetic tissues were significantly reduced compared to the controls (Figure 6.6).

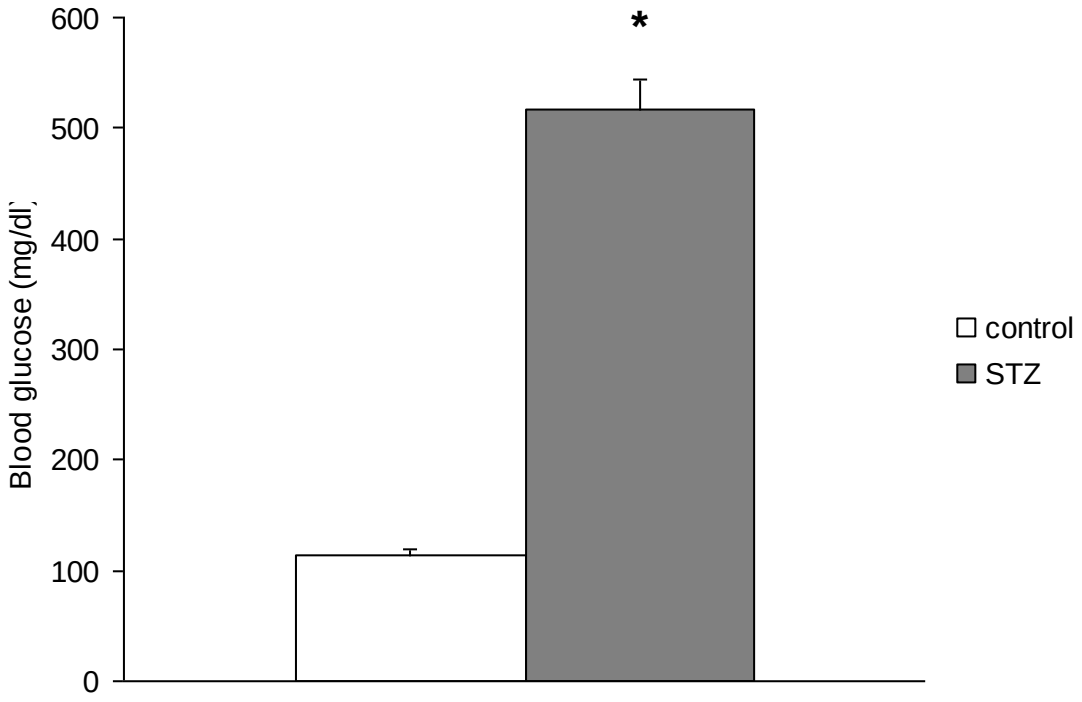


Figure 6.5 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 36 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

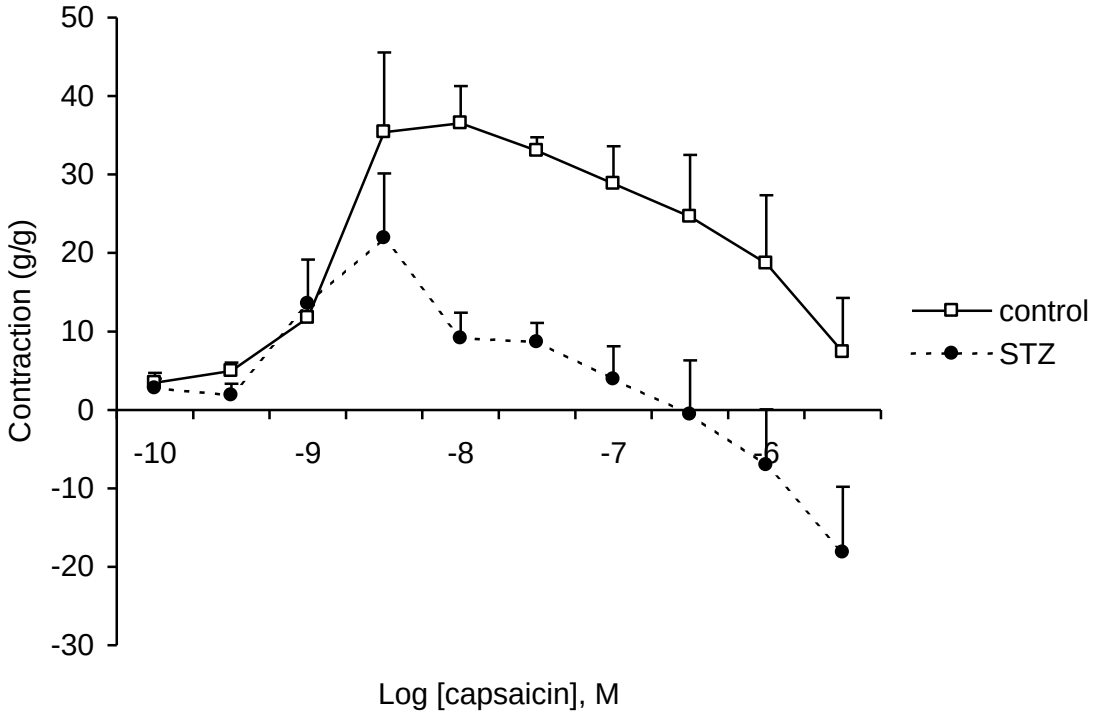


Figure 6.6 Contractile responses of bladder to the TRPV1 agonist capsaicin at concentrations of 10^{-10} M - 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 36 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

6.3.1.4 Twenty four hours after STZ-injection

24 hours after STZ injection, it was found that the blood glucose level of STZ-treated rats was significantly elevated but did not reach that in the diabetic state, only a hyperglycamic state (Figure 6.7). In addtion, the contractile responses to capsaicin at concentrations of 10^{-10} M - 10^{-6} M in STZ-induced diabetic and control tissues were similar. The values did not reach the statistical difference when the control and diabetic groups were compared. Capsaicin produced the dose-dependent contraction in both control and STZ-treated tissues (Figure 6.8).

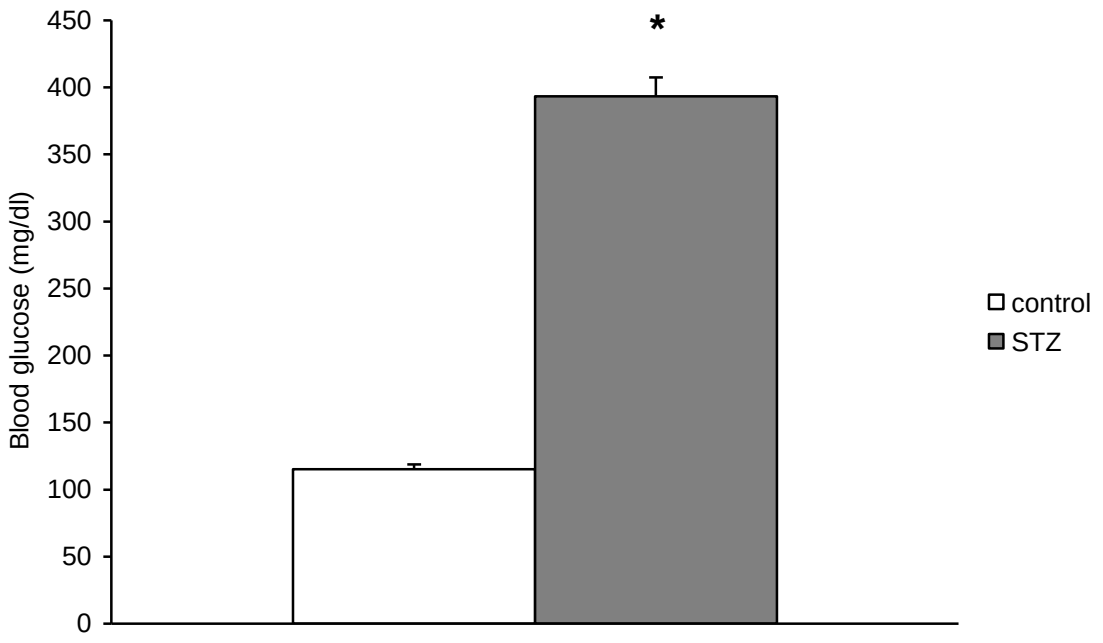


Figure 6.7 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 24 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

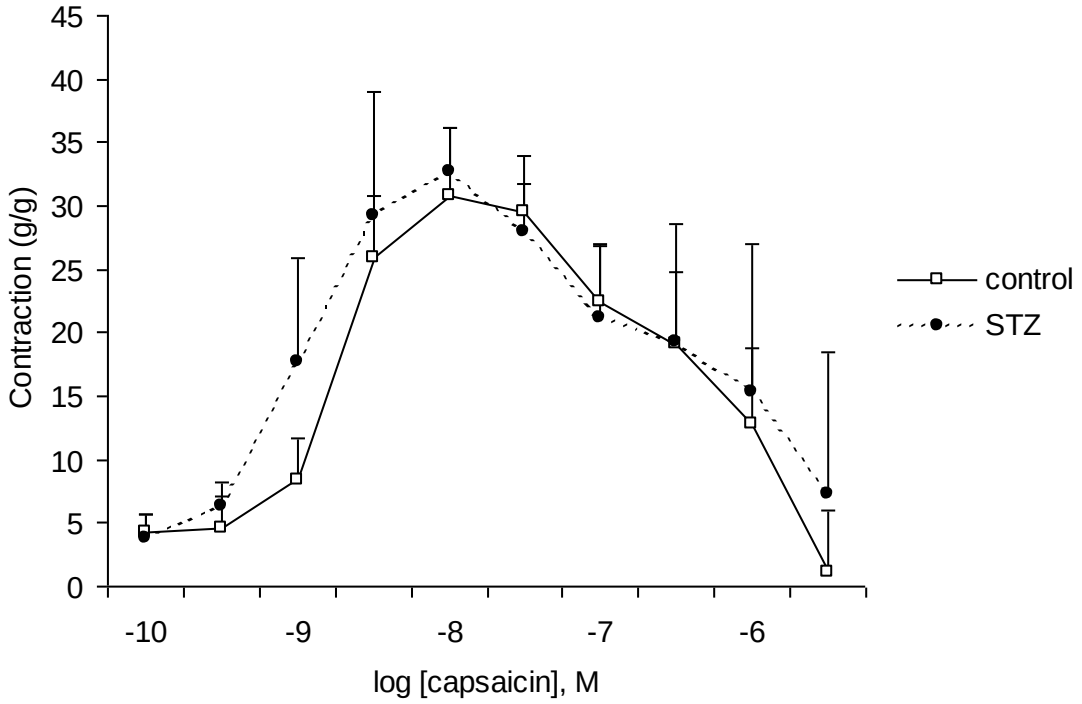


Figure 6.8 Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M from age-matched controls and STZ-induced diabetic rats, 24 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

To emphasize the changes in blood glucose and TRPV1 induced contractions over time the data for 10^{-8} M capsaicin is summarised in Figures 6.9 and 6.10 respectively. It is clear that in the STZ model of diabetes in rat, blood glucose levels were markedly increased from 24 hours after induction with STZ and constant for up to 8 weeks thereafter (Figure 6.9).

In contrast, changes in capsaicin-induced contractility became evident 36 hours after induction of diabetes by STZ and remained depressed over the 8 weeks period. Surprisingly, contractile responses in controls declined at 2 weeks but appear to partially recover at 8 weeks (Figure 6.10). The reasons for these unexpected changes in controls are unclear but are also seen in some of the experiments with cyclodextrins (Chapter 5).

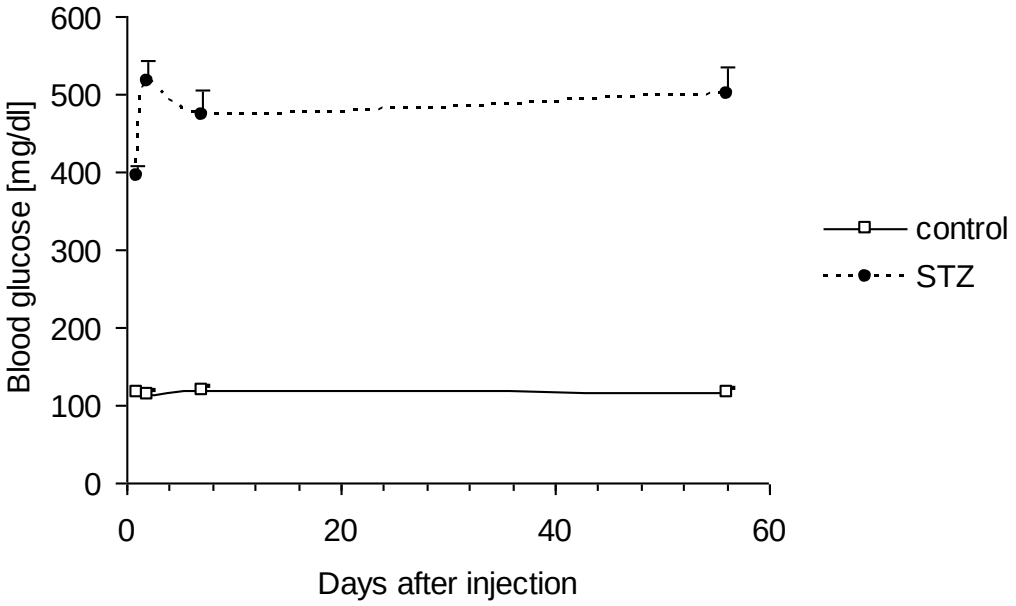
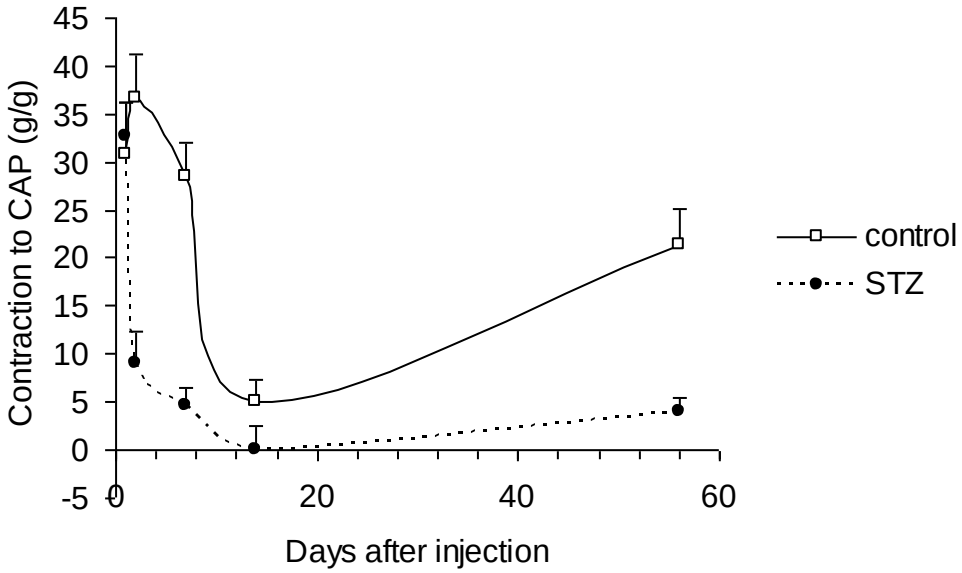


Figure 6.9 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 8, 2, 1 week, and 36 and 24 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

A)



B)

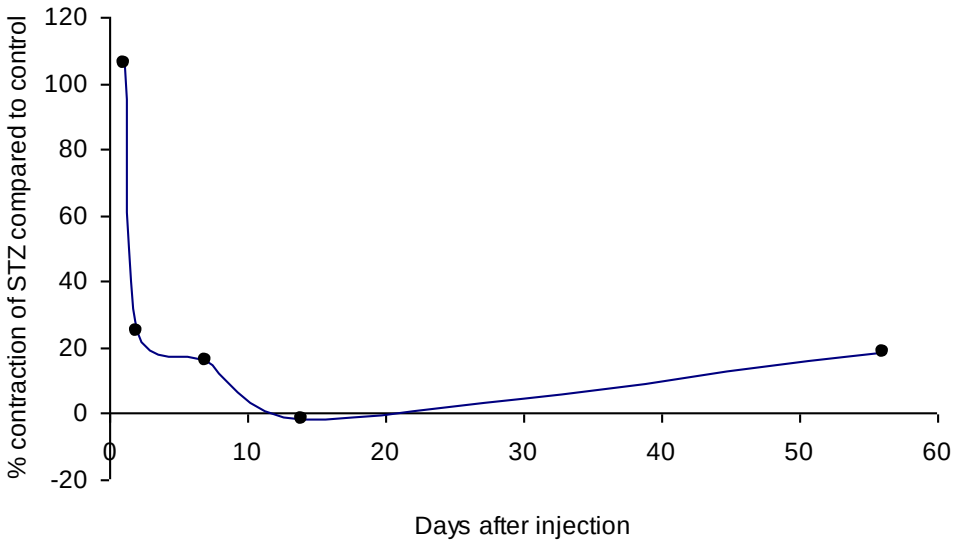


Figure 6.10 A : Contractile responses of bladder to TRPV1 agonist capsaicin at the concentration of 10^{-8} M from age-matched controls and STZ-induced diabetic rats, 8, 2, 1 week, and 36 and 24 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA). B : Percentage contraction of STZ- diabetic tissues compared to the controls.

6.3.2 The effect of time frame of STZ-induced diabetes on TRPA1 channel function

6.3.2.1 Two weeks after STZ-injection

For 2 weeks after STZ injection, it was found that the blood glucose level of STZ-treated rats was significantly elevated, reaching a diabetic state (Figure 6.11). In addtion, the contractile responses to allyl isothiocyanate at the concentration of 10^{-5} M - 10^{-4} M in STZ-induced diabetic tissues were reduced although the values did not reach the statistical differences when the control and diabetic groups were compared (Figure 6.12).

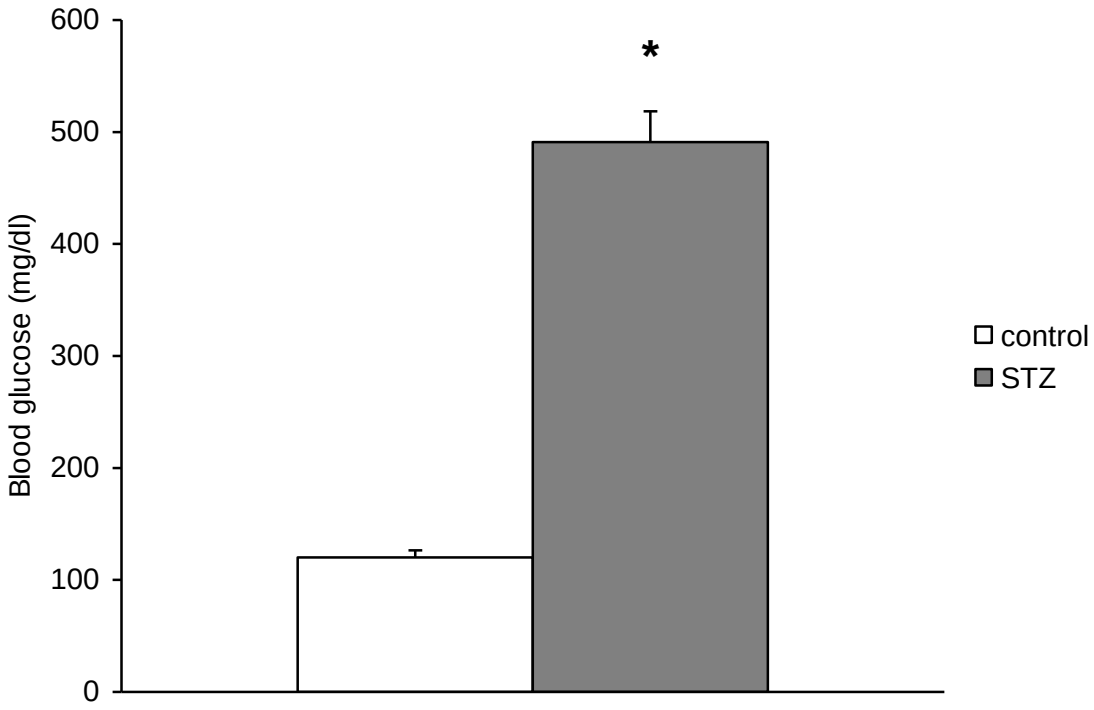


Figure 6.11 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 2 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

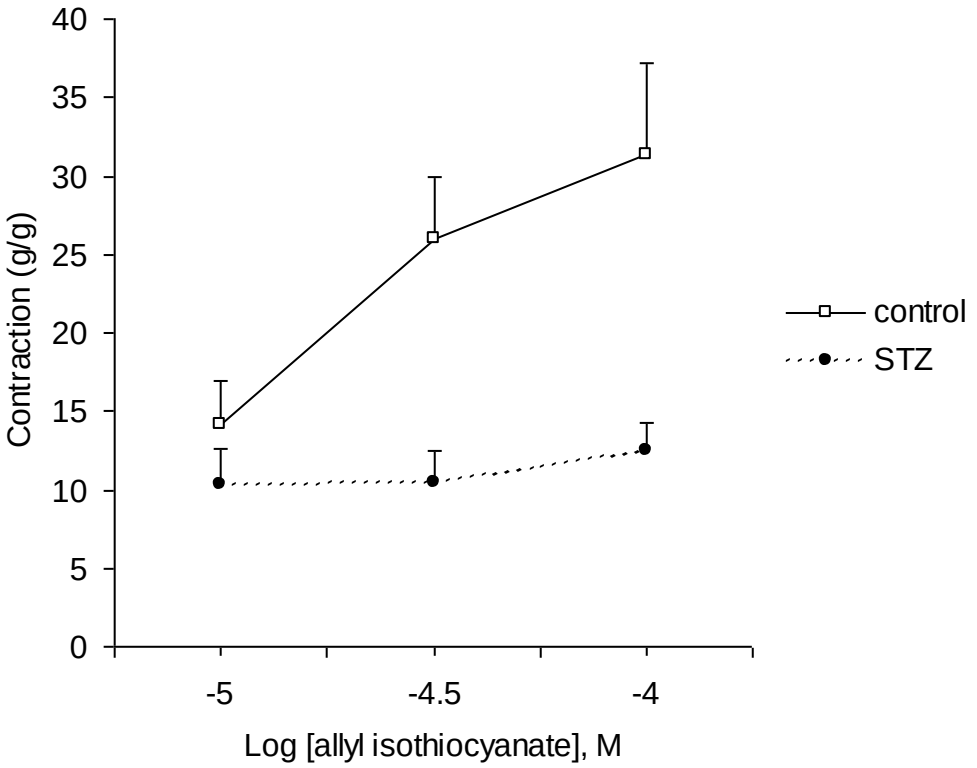


Figure 6.12 Contractile responses of bladder to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-5} M - 10^{-4} M from age-matched controls and STZ-induced diabetic rats, 2 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

6.3.2.2 One weeks after STZ-injection

For 1 weeks after STZ injection, it was found that the blood glucose level of STZ-treated rats was significantly elevated, reaching a diabetic state (Figure 6.13). In addtion, the contractile responses to allyl isothiocyanate at the concentration of 10^{-5} M - 10^{-4} M in STZ-induced diabetic tissues were reduced although the values did not reach the statistical differences when the control and diabetic groups were compared (Figure 6.14).

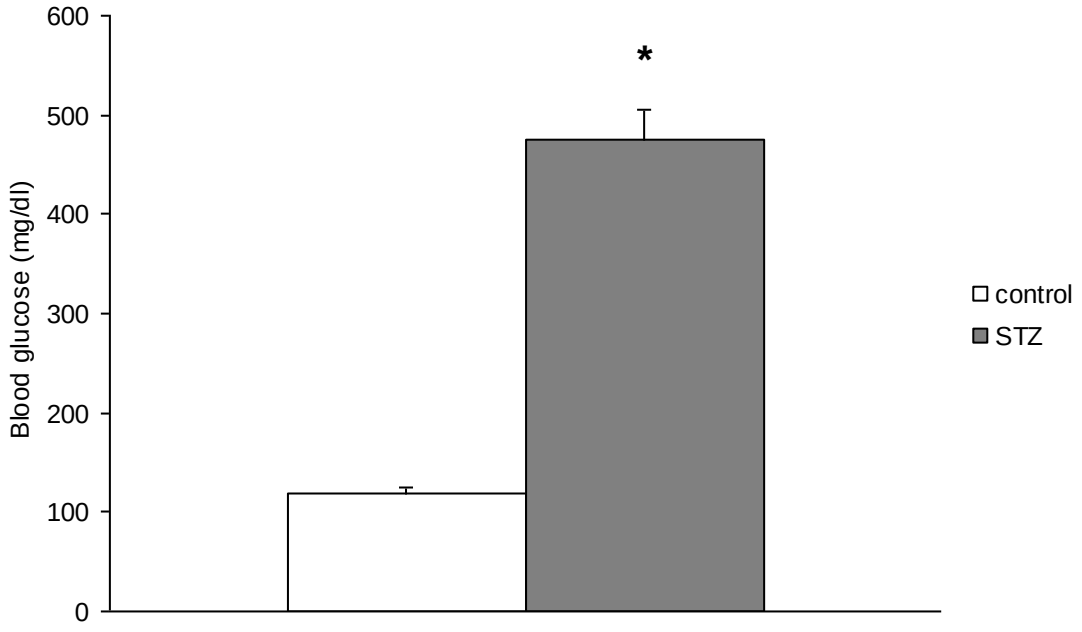


Figure 6.13 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 1 week after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

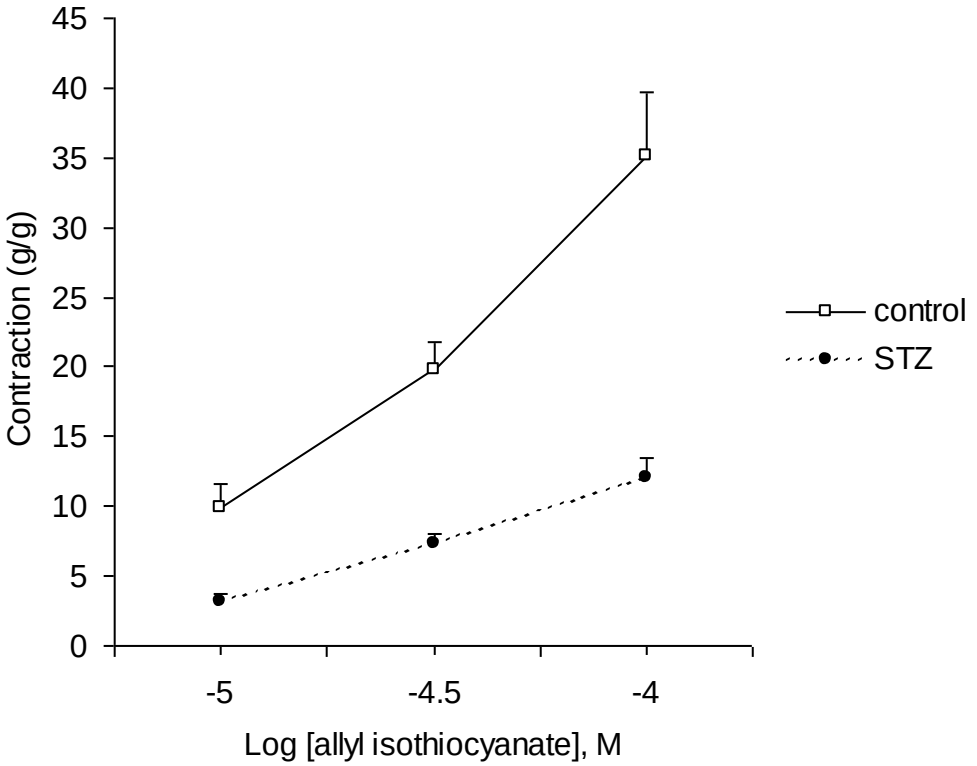


Figure 6.14 Contractile responses of bladder to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-5} M - 10^{-4} M from age-matched controls and STZ-induced diabetic rats, 1 week after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

6.3.2.3 Thirty six hours after STZ-injection

The results showed that the blood glucose level of 36 hours STZ-treated rats was significantly elevated, reaching a diabetic state (Figure 6.15). However, the contractile responses to allyl isothiocyanate at the concentration of 10^{-10} M - 10^{-6} M in STZ-induced diabetic tissues were not significantly different compared to the controls. In addition, the contractile responses of diabetic rat bladder tened to be greater than those of the controls. (Figure 6.16).

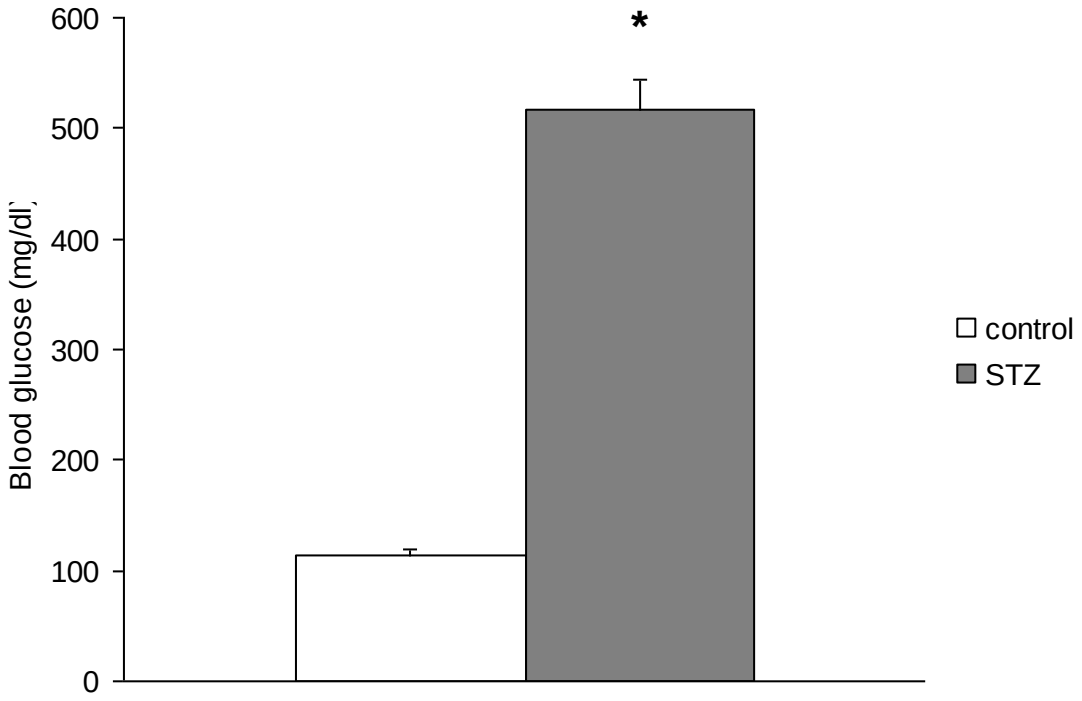


Figure 6.15 Blood glucose levels of age-matched controls and STZ-induced diabetic rats, 36 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. *P<0.05 is significantly different from age-matched controls (Student's *t* test for unpaired observations).

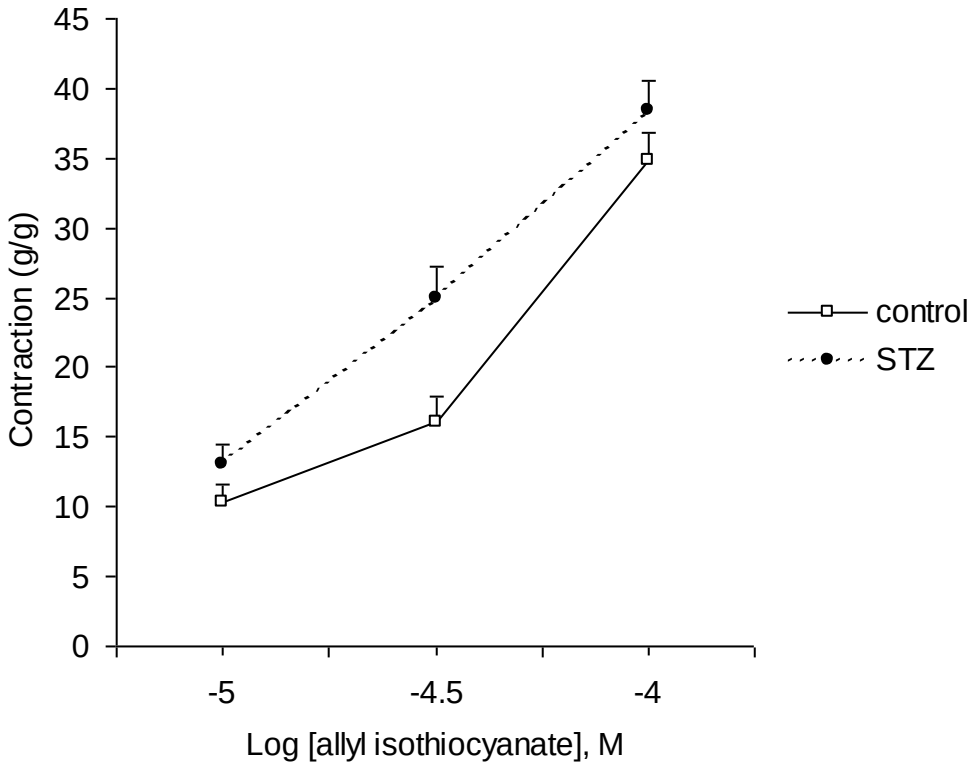


Figure 6.16 Contractile responses of bladder to the TRPA1 agonist allyl isothiocyanate at concentrations of 10^{-5} M - 10^{-4} M from age-matched controls and STZ-induced diabetic rats, 36 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

In contrast to TRPV1, responses to the TRPA1 agonist allyl isothiocyanate were not affected at 36 hours but were reduced one week after STZ treatment (Figure 6.17).

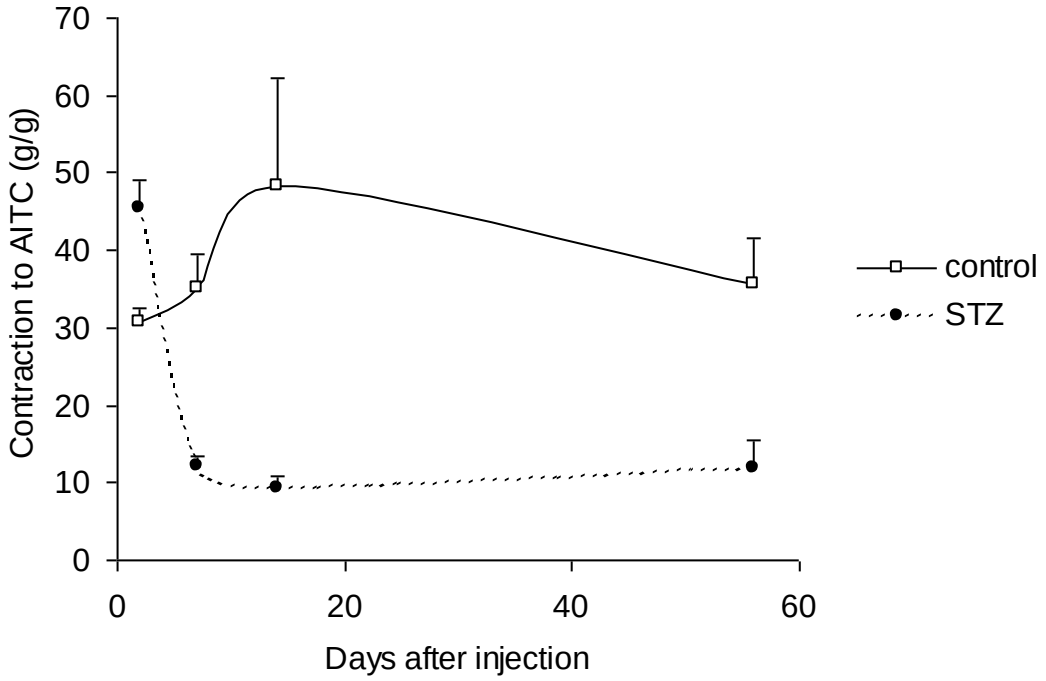


Figure 6.17 Contractile responses of bladder to TRPA1 agonist allyl isothiocyanate at the concentration of 10^{-8} M from age-matched controls and STZ-induced diabetic rats, 8, 2, 1 week, and 36 hours after the administration of STZ. Values represent the mean±S.E.M for 6 animals. Means are different between age-matched controls and STZ-induced diabetic rats ($P<0.05$, two-way ANOVA).

6.3.3 The effect of acute elevation of glucose concentrations on TRPV1 channel functions

As STZ treatment rapidly induced changes in TRPV1 function, we wondered whether directly adding high glucose concentrations to bladder tissues may cause some changes in TRPV1 responses.

6.3.3.1 Thirty minutes glucose incubation

A four fold increase in glucose (44.4 mM) was added to Krebs's solution which is similar to that seen in STZ-diabetic animals. The bladder tissues were exposed to the elevated glucose in the bath for 30 minutes before the application of capsaicin. After 30 minutes glucose incubation, it was found that capsaicin at concentrations of 10^{-10} M - 10^{-6} M produced contractile responses in tissues exposed to elevated (44.4 mM) and normal (11.1 mM) glucose. The contractile responses to capsaicin in tissues exposed to normal glucose were slightly greater than those of elevated glucose. However, the differences did not reach the statistical significance (Figure 6.18).

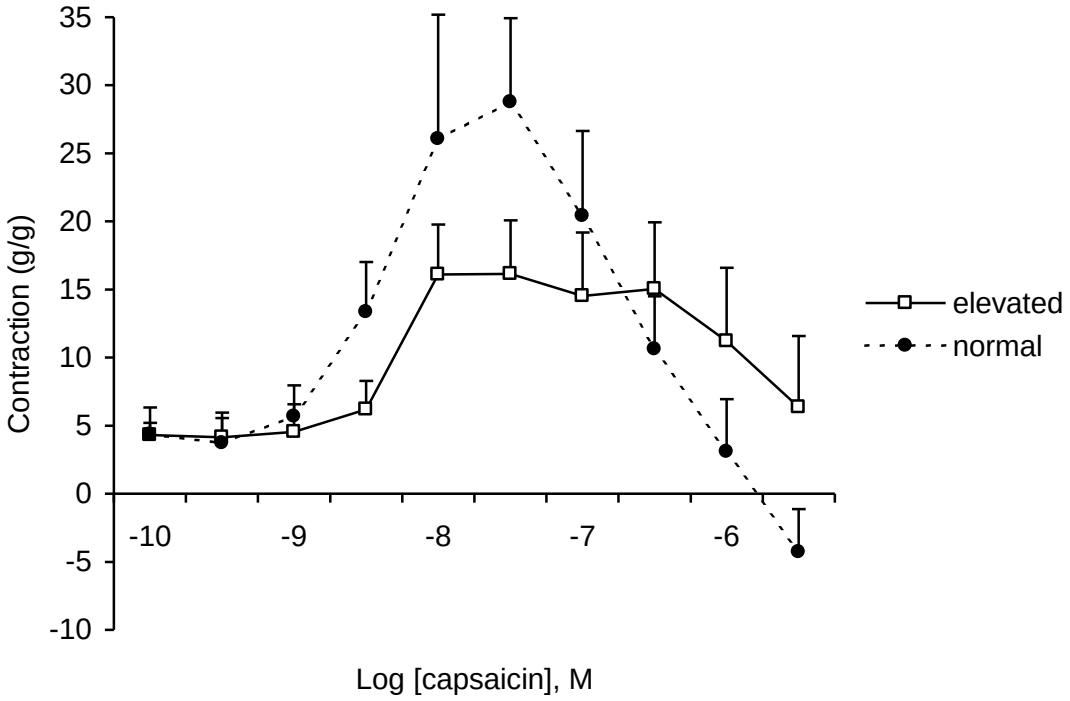


Figure 6.18 Contractile responses of bladder in tissues exposed to normal (11.1 mM) or elevated (44.4 mM) glucose concentration for 30 minutes to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M. Values represent the mean±S.E.M for 6 animals.

6.3.3.2 Two hours glucose incubation

A four fold increase glucose (44.4 mM) was added to Krebs's solution. The bladder tissues were exposed to the elevated glucose in the bath for 2 hours before the application of capsaicin. After 2 hours glucose incubation, it was found that capsaicin at concentrations of 10^{-10} M - 10^{-6} M produced contractile responses in tissues exposed to elevated (44.4 mM) and normal (11.1 mM) glucose. However, the contractile responses to capsaicin in tissues exposed to elevated glucose were slightly greater than those of normal glucose. However, the differences did not reach statistical significance (Figure 6.19).

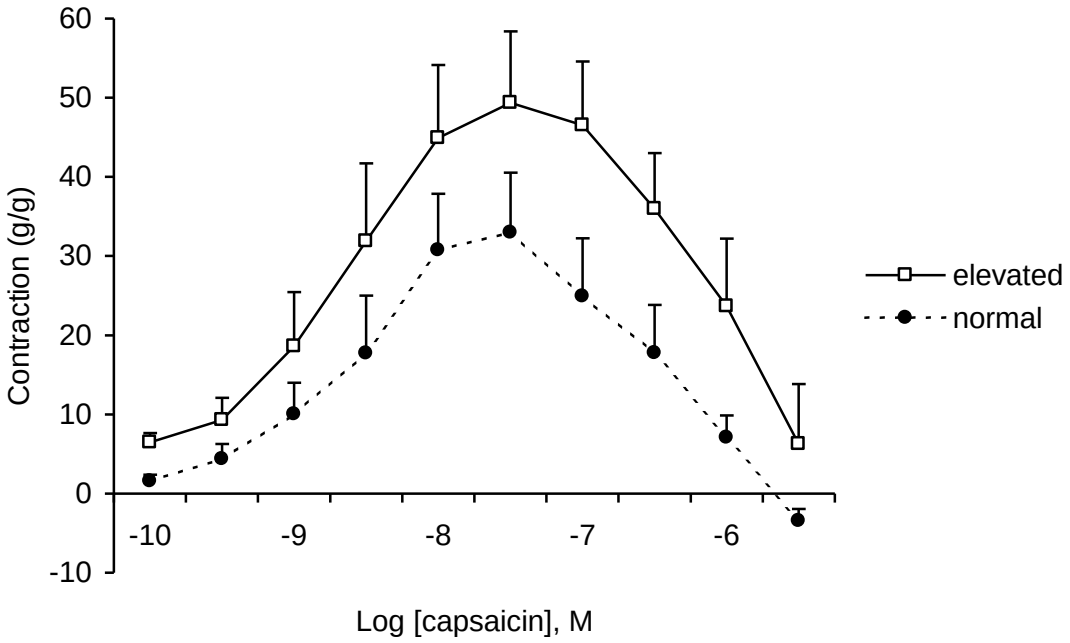


Figure 6.19 Contractile responses of bladder tissues exposed to normal (11.1 mM) or elevated (44.4 mM) glucose concentration for 2 hours to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M. Values represent the mean ± S.E.M for 6 animals.

6.3.3.3 Twenty four hours glucose incubation

A four fold glucose (44.4 mM) was added to Krebs's solution. The bladder tissues were kept in Krebs's solution with the elevated glucose in the fridge at 4 ^{o}C for 24 hours before the experiments in organ bath. After 24 hours elevated glucose incubation in fridge at the 4 ^{o}C, it was found that capsaicin at concentrations of 10^{-10} M - 10^{-6} M produced the contractile responses in tissues exposed to elevated (44.4 mM) and normal (11.1 mM) glucose. The contractile responses to capsaicin in tissues exposed to normal glucose were slightly greater than those of elevated glucose. However, the differences did not reach statistical significance (Figure 6.20).

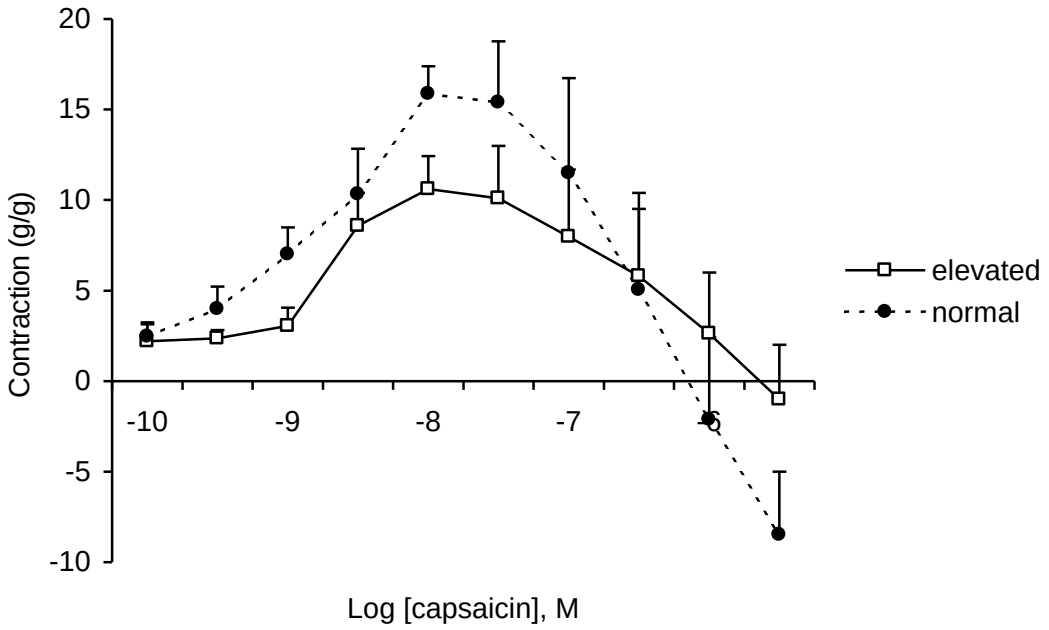


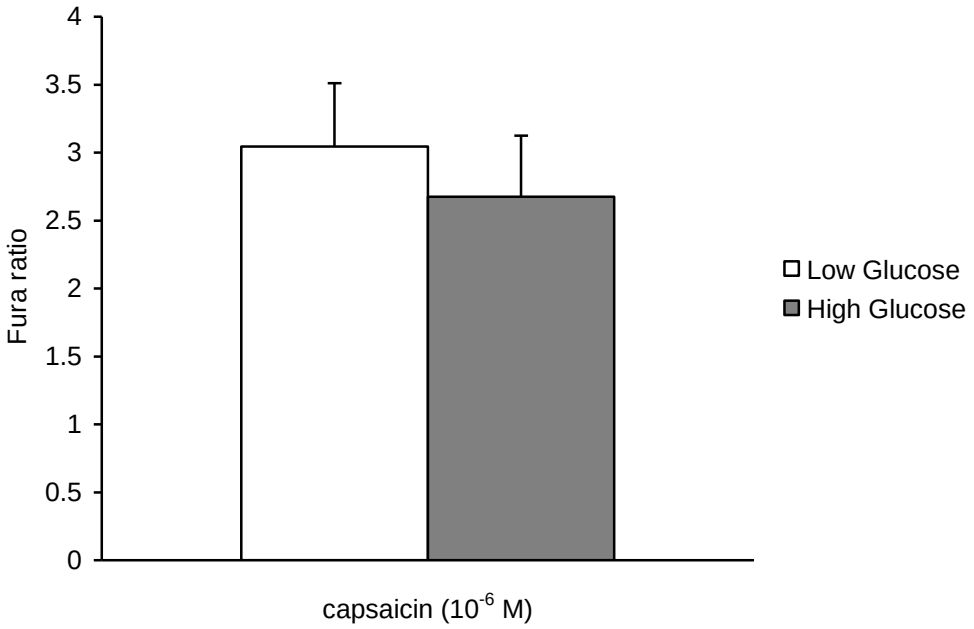
Figure 6.20 Contractile responses of bladder tissues exposed to normal (11.1 mM) or elevated (44.4 mM) glucose concentration for 24 hours to TRPV1 agonist capsaicin at the concentration of 10^{-10} M - 10^{-6} M. Values represent the mean±S.E.M for 4 animals.

6.2.4 The effect of hyperglycaemia on TRPV1 channel function in HEK293 cell lines

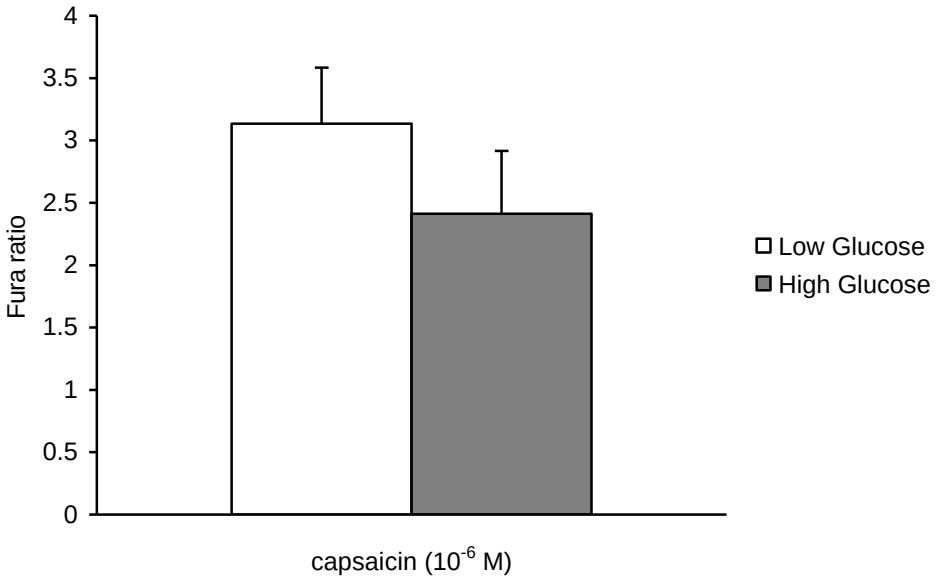
When activated by 1 μM capsaicin, the calcium ion influx in TRPV1-expressed HEK293 cells were decreased in cells exposed to high glucose (45 mM) media in comparison to those exposed to low glucose (11 mM) media. When the incubation periods were taken into account, it was found that the longer period of exposure (48 hours) the cells to high glucose media caused more reduction of calcium influx in comparison to those of shorter period of exposure (24 hours). However, there is no significant difference in calcium ion influx between the cells treated with high and low glucose media

The TRPV1-expressing HEK293 cell exposed to high glucose concentration for 72 hours showed the significant reduction in calcium influx in comparison to those exposed to low glucose concentration (Figure 6.21).

A)


4
3.5
3
2.5
2
1.5
1
0.5
0
Fura ratio
capsaicin (10-6 M)
Low Glucose
High Glucose

B)


4
3.5
3
2.5
2
1.5
1
0.5
0
Fura ratio
capsaicin (10-6 M)
Low Glucose
High Glucose

C)

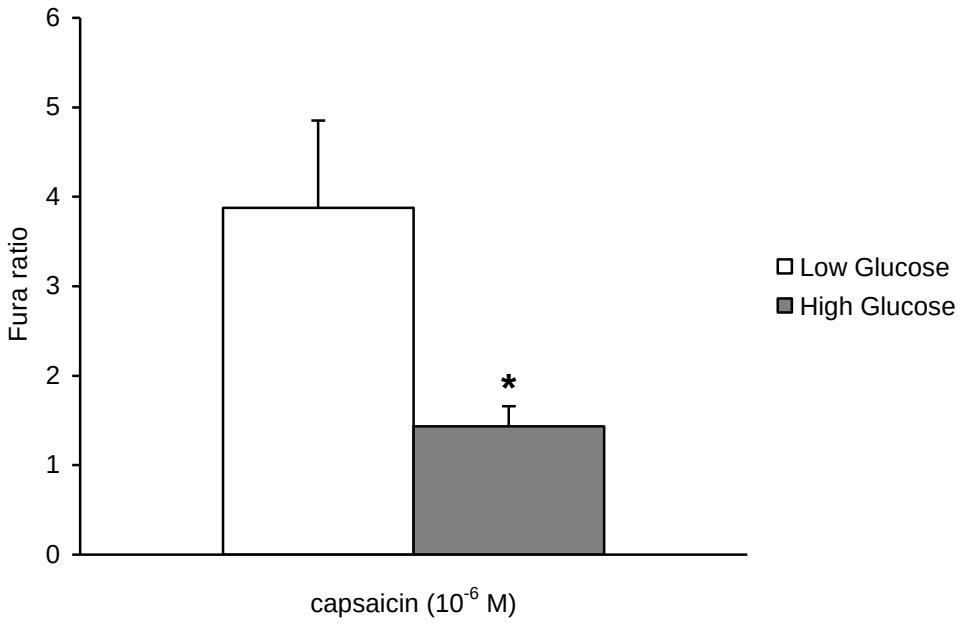


Figure 6.21 Change in fura-2 ratio mediated by 1 μM capsaicin in TRPV1 expressing rat HEK293 cells exposed to high (45 mM) and low (11 mM) glucose media for 24 (a), 48 hours (B) and 72 hours (C). Values represent the mean ± S.E.M for 6 animals. *P<0.05 is significantly different from low glucose (Student's *t* test for unpaired observations).

6.3.5 The effect of diabetes on sodium ion channel function

In order to check whether diabetic state has any effect on another ion channel function, and to check whether diabetic state has effect at the depolarization process, sodium ion channel activator veratridine was chosen to study the responses in normal rat bladder in comparison to the diabetic tissues. Voltage gated sodium channels are present in all nerve terminals so this will also provide evidence of any degeneration of nerve terminals.

Veratridine at the concentration of 10^{-5} M caused contraction in both control and STZ-induced diabetic rat bladder tissues. The contractile responses of control and STZ-induced diabetic rat bladder to veratridine were similar (Figure 6.22).

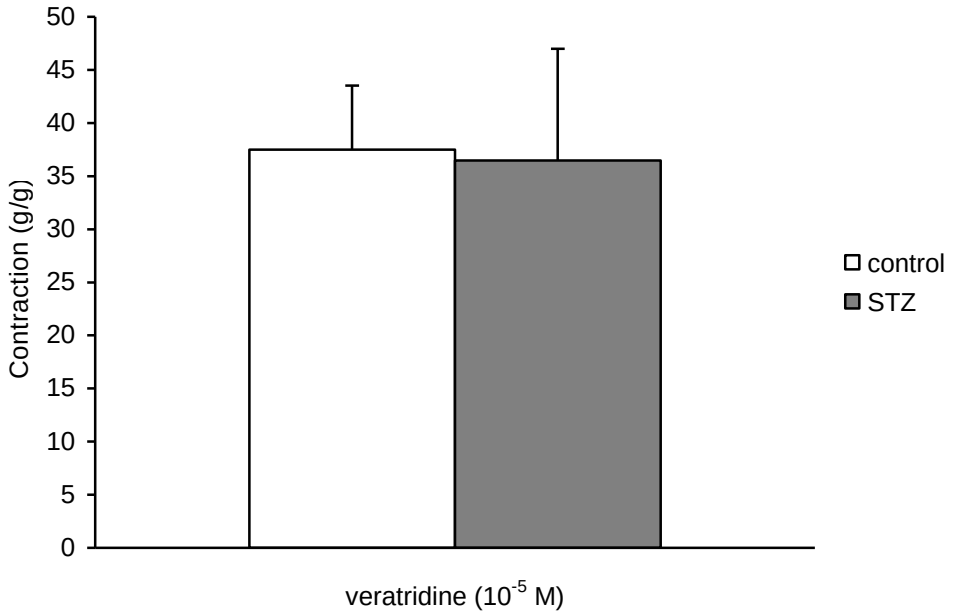


Figure 6.22 Contractile responses of rat bladder to sodium ion channel activator veratridine at the concentration of 10^{-5} M from age-matched controls and STZ-induced diabetic rats, 8 weeks after the administration of STZ. Values represent the mean±S.E.M for 6 animals.

6.3.6 The direct effect of streptozotocin on TRPV1 channel function

It was questioned whether the reduction in contractile responses of STZ-induced diabetic rat bladder tissues to capsaicin is due to the diabetic state (hyperglycaemia) directly or caused by the direct effect of STZ itself on the TRPV1 channel since direct effect of STZ on TRPV1 have been reported in dorsal root ganglion and TRPV1 expressing human HEK 293 cell (Pabbidi et al., 2007).

The experiments were thus designed by directly adding STZ at the concentration of 10^{-5} M to the organ bath with normal tissues. The tissues were exposed to STZ or vehicle (distilled water) for 30 minutes and the contractile responses to capsaicin were evoked.

The results showed that the contractile responses of normal rat bladder tissues to capsaicin following 30 minutes treatment with STZ were similar to controls (Figure 6.23).

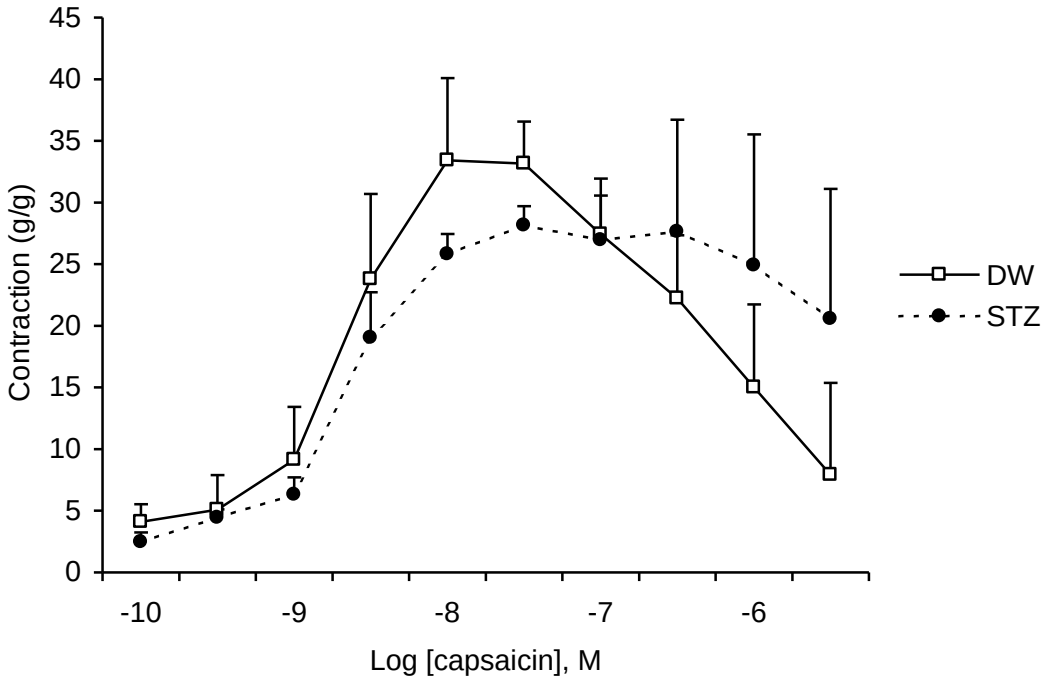


Figure 6.23 Contractile responses of rat bladder to capsaicin following 30 minutes treatment with STZ (10^{-5} M) from controls. Values represent the mean ± S.E.M for 6 animals.

6.3.7 The effect of cannabinoid receptor agonist on TRPV1 channel function

The contractile responses to capsaicin in the presence and absence of CP55,940 were similar. This indicated that cannabinoid receptor agonist did not have any effect on TRPV1 channel function in normal condition (Figure 6.24).

However, in diabetic rat bladder tissues, the contractile responses to capsaicin in the presence of CP55,940 were greater than those of the absence of CP55,940, suggesting that there is a sensitization of TRPV1 responses by cannabinoid receptor agonist in diabetic condition. However, the effects were not statistically significant (Figure 6.25).

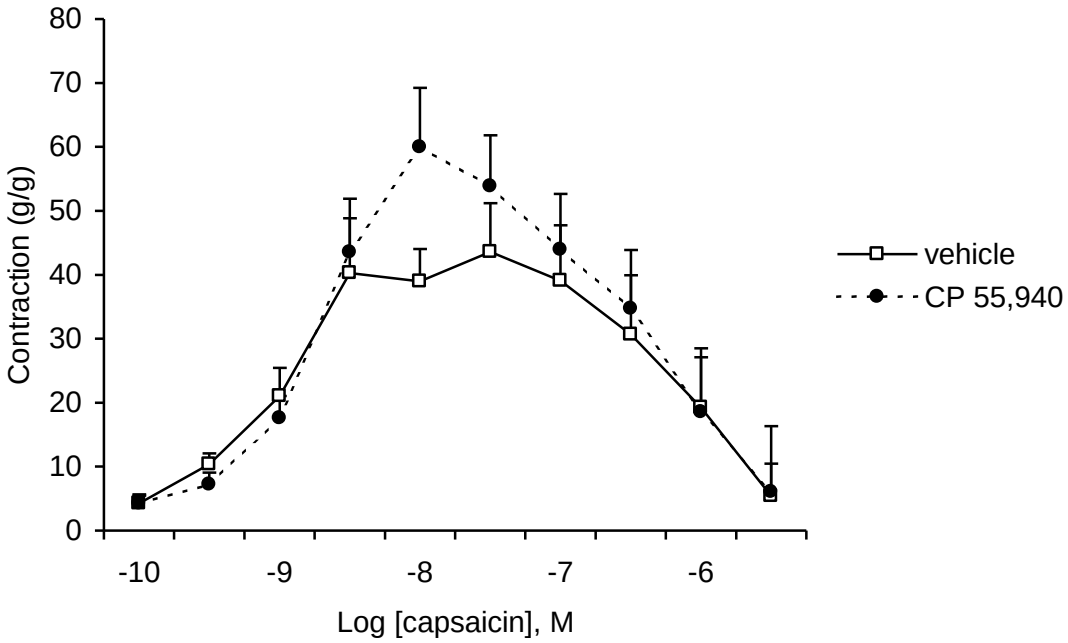


Figure 6.24 Contractile responses of rat bladder to capsaicin following 30 minutes treatment with CP55,940 (10^{-7} M) from age-matched controls. Values represent the mean±S.E.M for 6 animals.

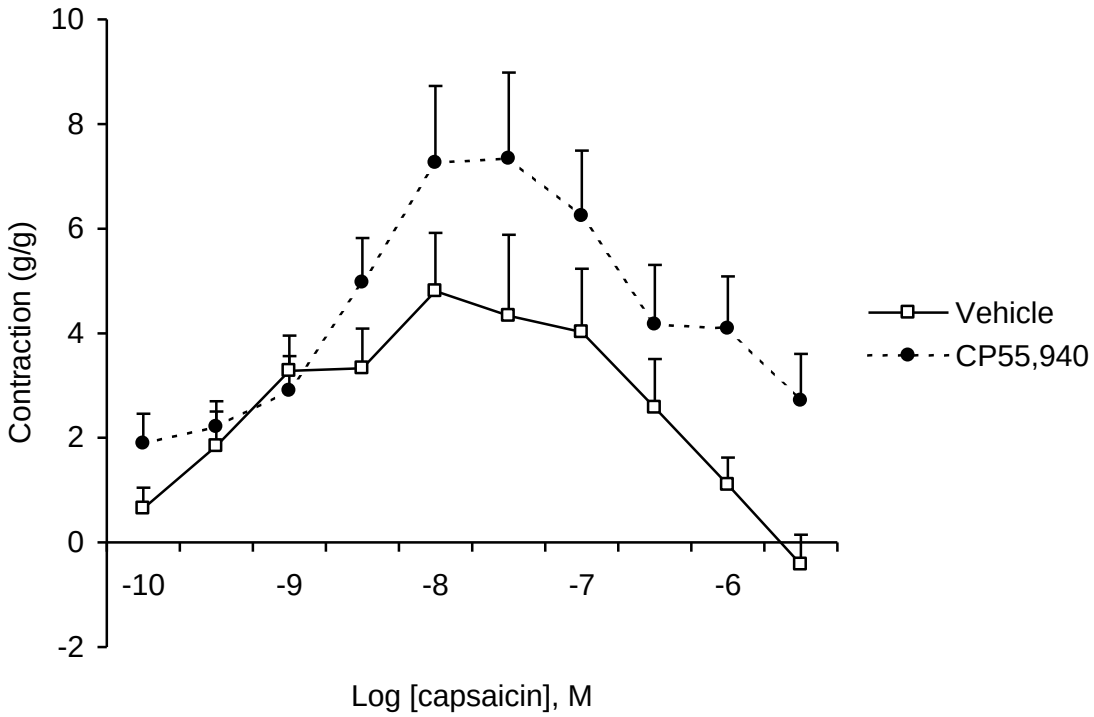


Figure 6.25 Contractile responses of rat bladder to capsaicin in the presence and absence of CP55,940 at the concentration of 10^{-7} M from STZ-induced diabetic rats. Values represent the mean±S.E.M for 6 animals. Means are different between vehicle and CP55,940 ($P<0.05$, two-way ANOVA).

6.3.8 The effect of pro-inflammatory agent on TRPV1 channel function

Bradykinin acting through bradykinin receptor is known to potentiate TRPV1 function through PKC mediated phosphorylation (Premkumar and Sikand, 2008). Thus, it was of interest to examine the effect of bradykinin. The results show that the contractile responses of control rat bladder tissues to capsaicin in the presence and absence of bradykinin were similar reponses (Figure 6.26).

In STZ-induced diabetic rat bladder tissues, it was found that the contractile responses to capsaicin in the presence of bradykinin were higher than those of the absence of bradykinin, suggesting that bradykinin potentiates the TRPV1 channel responses (Figure 6.27).

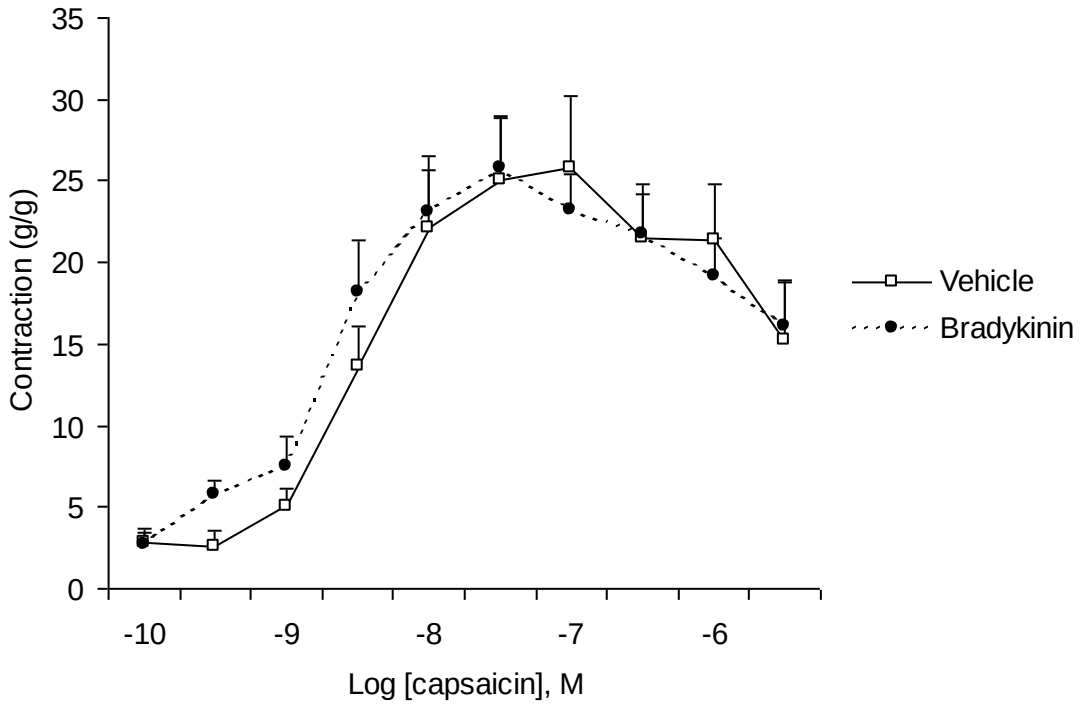


Figure 6.26 Contractile responses of rat bladder to capsaicin in the presence and absence of bradykinin at the concentration of 10^{-5} M from age-matched controls. Values represent the mean±S.E.M for 6 animals.

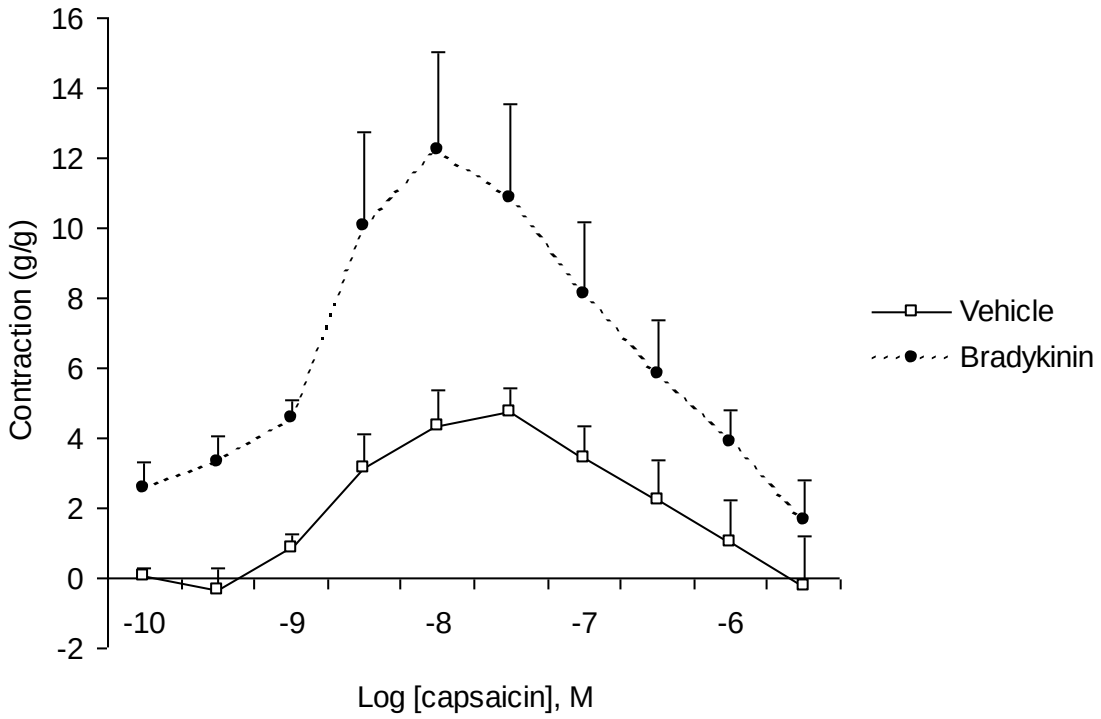


Figure 6.27 Contractile responses of rat bladder to capsaicin in the presence and absence of bradykinin at the concentration of 10^{-5} M from STZ-induced diabetic rats. Values represent the mean±S.E.M for 6 animals. Means are different between vehicle and bradykinin ($P<0.05$, two-way ANOVA).

6.4 Discussion

6.4.1 The time course of changes in transient receptor potential channel function following streptozotocin-induced diabetes

The results indicated that eight, two, and one weeks after induction of diabetes by STZ, the contractile responses to TRPV1 agonist capsaicin were significantly reduced in comparison to those of the controls. Only one week after STZ-injection, the blood glucose level in the STZ-treated animals were significantly high and similar to the 8 weeks after STZ-injection, confirming the diabetic state in this animal models. As mentioned above, it was found that eight weeks, two weeks, and one week STZ-treated animals had significant reduction of the contractile responses to capsaicin. It was of interest to investigate the acute effect of STZ on the contractile responses to capsaicin.

Streptozotocin (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by *Streptomyces achromogenes* and used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (Szkudelski, 2001). It is an antimicrobial agent and has also used as chemotherapeutic alkylating agent. The insulinopenia syndrome, called “streptozotocin diabetes”, is caused by the specific necrosis of the pancreatic beta cells and streptozotocin has been the agent choice for the induction of diabetes mellitus in animals (Lenzen, 2008). STZ is efficacious after intraperitoneal administration of 40-60 mg/kg b.w. or higher, but single dose below 40 mg/kg b.w. may be ineffective (Katsumata *et al*., 1992).

Lenzen (2008) proposed that there are triphasic blood glucose responses induced by streptozotozin when injected. The first phase starts with an increase in blood glucose concentration, 1 hour after administration of the toxins, and a decrease in plasma insulin. This first hyperglycaemic phase, which usually lasts 2-4 hours, is caused by inhibition of insulin secretion leading to hypoinsulinaemia. The second phase, the hyperglycaemic phase, typically occurs 4-8 hours after the injection of the toxins and lasts several hours. The

third phase is the permanent diabetic hyperglycaemic phase. Morphologically, complete degranulation and loss of beta cell is seen within 12-48 hours.

The results showed that blood glucose level in 36 hours STZ-treated rats was significantly high, reaching those in the diabetic state and contractile responses to capsaicin were significantly reduced. However, 24 hours after STZ injection, the blood glucose level of STZ-treated rats was significantly raised but did not reach diabetic state, only a hyperglycamic state. Under these conditions, the contractile responses to capsaicin at 24 hours STZ-induced diabetic and control tissues were not different. In addition, the contractile responses to capsaicin were not affected by the exposure (for 30 minutes, 2 hours, and 24 hours) of urinary bladder tissues to elevated (44.4 mM) and normal (11.1 mM) glucose.

This may suggest that the impaired TRPV1-mediating sensory nerves in urinary bladder occurred from 36 hours after the STZ injection. The hyperglycaemia has effect on the signalling of the TRPV1 channel. The deleterious effect of diabetes on bladder tissue responses to capsaicin may be due to the damage of beta cell in pancreas, by streptozotocin, resulting in the decrease of insulin production, high blood glucose level and diabetes mellitus but not simply by the elevated glucose in the tissues.

As early as 36 hours after induction of diabetes by STZ, the contractile responses to capsaicin were significantly reduced in comparison to those of the controls and this reduction persisted until the eight weeks time point. In contrast, responses to the TRPA1 agonist allyl isothiocyanate were not affected by early time points but were reduced eight weeks after STZ treatment. The contractile responses of bladder strips to TRPV1 agonist capsaicin were not affected by exposure to elevated glucose. There are specific early effects of STZ treatment on TRPV1 channel function at a time when other afferent nerve terminal channels (TRPA1) are functioning normally, suggesting that early onset of dysfunction in TRPV1 signalling may not merely be the consequence of nerve damage.

6.4.2 The effect of inflammation on transient receptor potential channel function following streptozotocin-induced diabetes

The results from this study suggested that bradykinin sensitized TRPV1 responses in diabetes mellitus, which may cause inflammation. Some previous studies have reported that activation of calcium permeant nociceptive ion channels on the peripheral and central terminals of sensory neurons leads to the synthesis and/or release of a variety of proinflammatory agents and neuropeptides such as ATP, BK, PGs, CGRP, SP and vasoactive intestinal peptide (VIP). TRPV1 play a significant role in inflammatory thermal hyperalgesia. The inflammatory mediators activate their respective G-protein coupled receptors to initiate secondary messenger pathways resulting in activation of either PKA, PKC, MAPK, extracellular calcium/CaM-dependent kinase II (CaMKII) or Src kinase, which phosphorylate TRPV1 (Premkumar and Sikand, 2008).

Activation of CB1 or CB2 receptors has been shown to increase or decrease adenylate cyclase levels, which will modulate the phosphorylation state of TRPV1. Activation of the CB1 receptors decreases Ca^{2+} and increases K^{+} conductance in the presynaptic terminals that can interfere with the action of TRPV1 distributed at the central terminals of sensory neurons. Phosphoryation at S116 in the amino terminus of TRPV1 is vital in PKA mediated regulation of TRPV1 desensitization (Bhave *et al*., 2002).

Phosphorylation by PKC has been shown to sensitize TRPV1. Various algesic agents like BK, ATP, trypsin and PGs are known to sensitize TRPV1 by activating PKC downstream of their G-protein coupled receptors in sensory neurons and in expressed system (Cesare and McNaughton, 1996).

Chapter 7

General Discussion

7.1 Discussion

In preliminary experiments, a reduced contractile response to capsaicin in bladder strips from STZ-induced diabetic rats was observed. The aim of the present study was to investigate the effect of diabetes on the function of TRP channels. The study examined TRPV1, TRPV4, TRPA1 and TRPM8 channel function in bladder strips from STZ-diabetic and control rats using conventional organ bath techniques and a range of appropriate agonists and antagonists available. Downstream functions of these pathways were checked by the use of, for example, neurokinin ligands. These observations were extended to isolated cells such as HEK 293 cells using calcium imaging and electrophysiological techniques. These techniques enable us to find out if the signaling pathways of TRP channels are disturbed. The time course of diabetes induction on TRP channel function was studied in order to explain the changes in these channels during the onset or development of the disease. This study may help in our understanding of bladder dysfunction in diabetic patients and may suggest novel therapeutic strategies.

7.1.1 The effect of streptozotocin treatment on transient receptor potential channel signalling

According to the findings expressed in Chapter 3, it is quite obvious that STZ-induced diabetes impair the responses of a range of TRP channel subfamilies including TRPV1, TRPV4 and TRPA1. However, its effect is not clear in the response of TRPM8 since the results are variable. The possible pathway by which TRPV1 and TRPA1 agonists cause bladder muscle contraction is expressed in Figure 7.1.

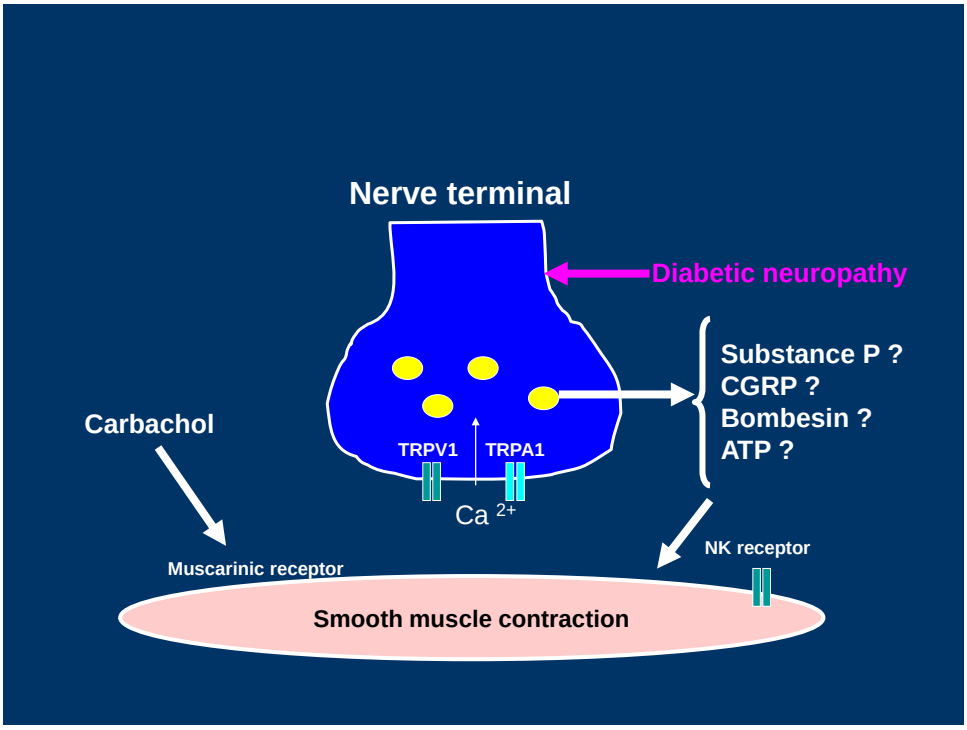


Figure 7.1 The possible pathway by which TRPV1 and TRPA1 agonists cause bladder muscle contraction

This possible contractile mechanism in normal rat urinary bladder mediated by capsaicin has been proposed by the previous study that capsaicin produces muscle contractions by stimulating the TRPV1 receptor, followed by release of neuropeptides that can activate tachykinin NK_1 and/or NK_2 receptors in the urinary bladder (Saitoh *et al*., 2007).

For the contractile mechanism mediated by TRPA1 agonist allyl isothiocyanate, it has been reported that TRPA1 agonists contract rat urinary bladder through sensory fiber stimulation, depending on extracellular Ca^{2+} influx and release of tachykinins and cyclooxygenase metabolites, probably prostaglandin E_2. TRPA1 is expressed in sensory nerves that innervate the urinary bladder and mediates a contractile effect on bladder smooth muscle, due to release of tachykinins and cyclooxygenase metabolites (Andrade *et al*., 2006).

With regards to the findings in the present study, the results are in agreement with Pinna *et al*. (1994) who found that the bladder response to capsaicin gradually decreased with the progression of diabetes. In rat urinary bladder, diabetes provokes impairment of capsaicin-sensitive sensory fibers but not of the cholinergic system even at an early stage (4 week) of the disease. Although there is no report involving the effect of diabetes on TRPV1 function in rat urinary bladder directly, the results from the present study correspond to the study of Facer *et al*. (2007) who found that TRPV1 levels are reduced in skin biopsies from patients with diabetic neuropathy. In addition, Rosta *et al*. (2007) reported that capsaicin reduced neurogenic sensory vasodilation, due to impairment of meningeal TRPV1 channel, in STZ-treated rats.

To check if the contractile or relaxant responses of bladder smooth muscle is mediated by activation of TRPV1 channel, TRPV1 antagonists were used. It is found that all antagonists (capsazepine, ruthenium red and SB366791) antagonize the contractile responses to TRPV1 agonist capsaicin. It has been previously reported that blockade of TRPV1 by capsazepine is competitive (Alexander *et al*., 2007). In the rat bladder, capsazepine produced a concentration-dependent rightward shift of the curve to capsaicin without any depression of the maximal response to the agonist (Maggi *et al*., 1993). Similar findings were obtained in the rat isolated vas deferens in which capsazepine (10 µM) produced a rightward shift of the curve to capsaicin (Maggi *et al*., 1993). The antagonism of the action of capsaicin by capsazepine is entirely consistent with the proposed interaction of this substance with a vanilloid (TRPV1) receptor located on primary afferents (Maggi *et al*., 1993). In addition,

Alexander *et al*. (2007) suggested that blockade of TRPV1 by capsazepine, 6-iodo-*nor*dihydrocapsaicin, BCTC, JYL1421, and SB366791 is competitive. In the present study, the results suggest that ruthenium red acts quite non-competitive as a capsaicin antagonist. The study on the effect of SB-366791 on capsaicin-evoked or electrical stimulation-induced release of the sensory neuropeptide substance P from isolated rat tracheae suggested that SB-366791 is a more selective and potent in vivo TRPV1 receptor antagonist than capsazepine in the rat (Varga *et al*., 2005). In addition, in cultured sensory neurons, SB-366791 is TRPV1 antagonist with high potency and an improve selectivity profile in comparisons to other commonly used TRPV1 antagonists (Gunthorpe *et al*., 2004). Therefore, the potency of the antagonists are consistent with TRPV1 blockade. This has been confirmed by the results in the present study. These findings confirm that the contractile responses are due to the activation of TRPV1 channel by capsaicin.

To explain the reasons behind the impairment of TRP channel function caused by the diabetic state, there are some possibilities. Firstly, it may due to the effect of STZ-induced diabetes on smooth muscle function. It has been reported that muscarinic receptor agonist carbachol was found to produce dose-dependent increase of the basal tone of the rat bladder detrusor muscle and the maximal contraction produced by carbachol was about four times greater than that elicited by bombesin or substance P (Abdel-Hakim *et al*., 1981). However, carbachol produced the hypersensitivity in diabetic gastrointestinal tissues in comparison to the controls (Talubmook *et al*., 2002). In the present study, since the contractile responses to muscarinic receptor agonist were not affected by STZ-induced diabetes, it can be inferred that diabetes affects at the presynaptic level, not postsynaptic level. Therefore, diabetes may impair the neurotransmitter transmission function or the TRP channel signalling or gating itself.

Secondly, it may due to the disturbances or alterations of postsynaptic receptors caused by STZ-induced diabetes. Therefore the exogenous neurotransmitters that are thought to be released following TRPV1 activation, were added. It was found that the contractile responses of control and diabetic

bladder tissues to neurotransmitters such as substance P, neurokinin alpha or bombesin were similar. The result from the present study is consistent with those observed by Pinna *et al*. (1994). It was found that the bladder contractile response to exogenous substance P was similar in both control and STZ-induced diabetic groups at all stage (1-26 weeks) studied and is proposed that diabetes had no effect on the sensitivity of smooth muscle cells to substance P. This suggests that these neurotransmitter were not released by TRP channel agonists or that the receptors of these neurotransmitters were not affected by diabetic state.

The selective tachykinin NK_2 receptor agonist [βAla^8] NKA (4-10) induced a concentration-dependent contraction associated with significant release of prostaglandin E_2 in isolated strips of the hamster urinary bladder (Tramontana *et al*., 2000). For electrical field stimulation study, stimulation of neurokinin A increased the amplitude of twitches and produced a concentration-dependent tonic contraction in the hamster isolated urinary bladder (Giuliani *et al*., 2001). The NK_1 receptor antagonists GR205171 (100 μM) and SDZ NKT 376 (1 mM) reduced the response to capsaicin, indicating that capsaicin acts via TRPV1 in series with NK_1 (Hu *et al*., 2005). However, when the combination of neurokinin 1 and 2 antagonists (GR205171A and SB207164A) was used in the present study, it can inhibit the responses of bladder smooth muscle to TRPV1 agonist capsaicin at all concentrations used. This suggests that the neurokinin may be one of the neurotransmitter involved in the contractile responses of bladder tissue to TRPV1 agonist. Since the neurokinin receptor is on the postsynaptic position, the results confirm that a neurokinin receptor agonist may be important in synaptic transmission.

A number of experiments have focussed a TRPV1 function in the nerve terminal and the possibility that responses are affected by diabetic neuropathy. The impairment of contractile responses in STZ-treated bladder tissues was found not only in TRPV1 activation but also in TRPA1 activation. This confirm that STZ model of diabetes caused impairment in both subfamily of TRP channel, which are reported to be expressed in the same nerve terminal.

In addition, using the quantitative real time PCR assay, it was found that TRPV1 gene in periaqueductal gray of diabetic rats has been down-regulated by the fold change expression ratio in comparison to those of non-diabetic rats (Mohammadi-Farani *et al*., 2010). Similarly, quantitative PCR confirmed that TRPV1 mRNA expression in diabetic hearts was decreased compared to that in control heart. CGRP and SP levels in diabetic hearts were also significantly decreased (Song *et al*., 2008). Therefore, it would be inferred that the reduction of the contractile responses to TRPV1 activation in STZ-diabetic bladder tissues may be due to the decrease in TRPV1 gene and TRPV1 mRNA expression. However, polymerase chain reaction (PCR) and western blot technique was not carried out in the present study to investigate the TRPV1 expression in control and STZ-induced diabetic rat because of the problem with the specificity of antibody to TRPV1 receptor and the little quantity of TRPV1 protein in the bladder tissues.

Diabetes is one of the most common causes of neuropathy. Diabetic neuropathy may present as severe pain, burning or tingling sensation and even loss of pain sensation (Freynhagen and Bennett, 2009). Animals show different responses in experimental models of diabetic neuropathy. These include hyperalgesia (increased sensitivity to noxious stimuli), hypoalgesia (decreased sensitivity to painful stimuli), and allodynia (getting pain from a previously non-painful stimuli) (Ohsawa and Kamei, 1999a,b; Pabbidi *et al*., 2008). In the present study, decreased contractile responses to TRPV1 agonist capsaicin were found. These should belong to hypoalgesia which is the decreased sensitivity to painful stimuli caused by diabetic neuropathy.

However, diabetic hyperalgesia with respect to TRPV1 receptors is reported. It was found that painful diabetic neuropathy is associated with enhanced function of TRPV1 receptors in neurons of the dorsal root ganglion (Hong and Wiley, 2005).

7.1.2 The effect of streptozotocin-induced diabetes on TRP channels function in rat colon

The hypothesis that channel dysfunction is a consequence of hyperglycaemia that is experienced by all tissues in the body was tested. In addition to urinary bladder smooth muscle, the impairment of TRPV1 function caused by diabetes was investigated in colon smooth muscle. The relaxant responses of diabetic colon tissues to TRPV1 agonist capsaicin were reduced in comparison to the controls confirming an impairment of TRPV1 channel due to diabetes in another organ, the gastrointestinal colon.

According to the motility studies, there was a reduction in TRP channel responses in diabetic smooth muscle tissues. It is worthwhile to confirm this finding using other parameters. The ion secretory studies were therefore performed. In the present study, small changes have been found in ion secretory responses evoked by TRPV1 agonist capsaicin in normal and STZ-induced diabetic colon tissues but there were the clear changes in those activated by other activators (carbachol and veratridine). In addition, the differences between ion secretory responses in distal colon are clearer than those in proximal colon, indicating the different responses along the intestine. Taken together, it would be indicated that ion transport parameter shows less sensitivity to any changes in TRPV1 channel function in diabetic state.

7.1.3 The effect of cholesterol modulation by cyclodextrins on transient receptor potential channel function

Diabetic complications are associated with cholesterol regulation. In chronic hyperglycaemic condition, HDL cholesterol level increases. In addition, it is associated with obesity, since the obesity affects insulin sensitivity caused type 2 diabetes mellitus (Suri and Szallasi, 2007). Cholesterol plays a significant role in membrane signalling including ion channel function (Zajchowski and Robbins, 2002). Lipid rafts play an important role in maintaining and regulating functions of ion channel in various cell types. For example, the properties of

type-1 cannabinoid receptor signalling are markedly altered following cholesterol depletion (Bari *et al*., 2005).

It is interesting to study the modulation of cholesterol at the cell membrane using cholesterol modulators, cyclodextrin molecules on TRP channel function. It is previously reported that using methyl-β-cyclodextrin as cholesterol extractor from cultured sensory neurons reduced capsaicin-activated currents (Liu *et al*., 2006). However, cholesterol removal increased the contractile responses in rat myometrium (Smith *et al*., 2005; Babiychuk *et al*., 2004). It is clear that cholesterol has varied actions on cell signaling.

The most common mean of modifying the cholesterol content of cell membranes with cyclodextrins, a family of compounds, which due to the presence of relatively hydrophobic cavity, can be used to extract cholesterol from cell membranes. However, the mechanism of this activity of cyclodextrins is not completely established. Moreover, under conditions commonly used for cholesterol extraction, cyclodextrins may be removed from both raft and non-raft domains of membranes. In addition, other hydrophobic molecules such as phospholipid may also be extracted from the membranes by cyclodextrins (Zidovetski and Levitan, 2007).

Cyclodextrins are cyclic oligosaccharide consisting of α-(1-4)-linked D-glycopyranose units, which are primary degradation products of starch. These compounds have been long recognized as potent carriers for hydrophobic drugs. Cyclodextrins typically exist as hexamers (αCDs), heptamer (βCDs) or octomer (γCDs). β-cyclodextrins have the highest affinity for inclusion of cholesterol and are the most efficient in extracting cholesterol from erythrocyte and model membrane. On the other hand, αCDs are the most efficient in extracting phospholipid (Zidovetski and Levitan, 2007).

It is previously reported that the cholesterol removal or enhancement properties of cyclodextrins depend upon the concentration of cyclodextrins, duration of exposure to cyclodextrins and the types of cells or tissues used (Zidovetski and Levitan, 2007). In the present study, it is found that cholesterol lowered the contractile responses of TRPV1 channel in rat bladder preparation. In contrast, cyclodextrin molecules (methyl-β-cyclodextrin, β-cyclodextrin and α-

cyclodextrin) enhanced the contractile responses of TRPV1 channel in bladder smooth muscle preparation but not in TRPV1-expressing HEK 293 cells. However, γ-cyclodextrin did not have any effect on the contractile responses of rat bladder strips activated by TRPV1 agonist capsaicin. In addition, methyl-β-cyclodextrin did not have any effect on the contractile responses of rat bladder strips activated by TRPA1 agonist allyl isothiocyanate suggesting the specific effect of methyl-β-cyclodextrin on TRPV1 channel function.

According to the results, it can be inferred that the different concentration of cyclodextrins and exposure time used in the present study may cause the different effects on rat bladder smooth muscle preparations and therefore TRPV1 channel function in bladder. In addition, cyclodextrins may remove phospholipid as methyl-β-cyclodextrin, β-cyclodextrin and α-cyclodextrin) enhanced the contractile responses of TRPV1 channel in bladder smooth muscle preparation while γ-cyclodextrin did not have any effect on the contractile responses of rat bladder strips activated by TRPV1 agonist capsaicin.

Another possibility is that the different results obtain from the present study may be possibly due to the different membrane model used. It is shown that there was an impairment in TRPV1 function following cholesterol depletion caused by methyl-β-cyclodextrin in dorsal root ganglia (Liu *et al*., 2006) while the increase in TRPV1 responses was found in rat bladder smooth muscle tissues treated with methyl-β-cyclodextrin in the present study.

The study using high performance liquid chromatography (HPLC) to measure the cholesterol contents in rat bladder tissues after treatment with or without cyclodextrin molecules was carried out. Our hypothesis for this experiment is that, if it is correspond with in vitro pharmacology results, cholesterol contents of bladder tissues treated with methyl-β-cyclodextrin will be reduced in comparison to those of untreated. The results are varied since some cholesterol contents from rat bladder tissues have been lost during the cholesterol extraction processes. Moreover, the cholesterol amounts in the whole tissue may not accurately reflect the cholesterol levels in the nerve endings. Thus, we decided not to pursue these experiments further.

7.1.4 The time course of changes in transient receptor potential channel function following streptozotocin-induced diabetes

As the results showed that TRP channel function is impaired by the diabetic state, it is interesting to investigate how rapidly the changes in TRPV1 function occur following administration of STZ. It is found that impairment of TRPV1 channel function occurred very early (36 hours) after STZ-diabetes induction and this impairment persisted until eight weeks of the disease. However, for another TRP subfamily member, the TRPA1 channel, the impairment appeared at the longer time point (1 week) than those of TRPV1 channel and persisted until the eight weeks time point. These findings were confirmed by the study on the effect of hyperglycaemia on the calcium signalling in TRPV1-expressing HEK 293 cell. It was found that exposure of the cells to high glucose media for 3 days significantly reduced calcium signalling.

According to the time course of STZ-induced diabetes on changes in blood glucose level, it has been proposed that there are triphasic blood glucose responses induced by streptozotozin when injected. The first phase starts with an increase in blood glucose concentration, 1 hour after administration of the toxins, and a decrease in plasma insulin. This first hyperglycaemic phase, which usually lasts 2-4 hours, is caused by inhibition of insulin secretion leading to hypoinsulinaemia. The second phase, the hyperglycaemic phase, typically occurs 4-8 hours after the injection of the toxins and lasts several hours. The third phase is the permanent diabetic hyperglycaemic phase. Morphologically, complete degranulation and loss of beta cell intergrity is seen within 12-48 hours. This mechanism is clearly at variance with that which underlies autoimmune type 1 diabetes, where beta cell demise is the result of apoptotic cell death without leakage of insulin from ruptured secretory granules (Lenzen, 2008).

Although there is no direct study on the time course of STZ-induced diabetes on the TRP channel function in rat urinary bladder, it was previously reported that 8-week streptozotocin-induced diabetes clearly leads to a number of significant alterations in the functional responses of the rat ileum (Talubmook,

2003). There is the presence of neuropathy in Schwann cell 4 months after induction of diabetes by STZ (Bestetti *et al*., 1981a) and there are neuropathy and myopathy in the diaphragm of rat after 12 months of STZ-induced diabetes (Bestetti *et al*., 1981b)

In addition, STZ-induced diabetes provokes impairment of capsaicin-sensitive sensory fibers but not of the cholinergic system even at early stage (4 week) of the disease in rat urinary bladder and the bladder response to capsaicin gradually decreased with the progression of diabetes (Pinna *et al*., 1994).

From the result obtained in the present study, it suggests that at the early time point of the onset, diabetes mellitus affected TRPV1 but not TRPA1 which are reported to be expressed in the same nerve terminals and that the early impairment of TRPV1 channel function may not due to the diabetic neuropathy.

7.1.5 The effect of inflammation on transient receptor potential channel function following streptozotocin-induced diabetes

To explain what mechanisms might affect TRPV1 function in STZ-treated animals, we looked at inflammatory agents as inflammation is known to occur in diabetes. In the present study, using the proinflammatory agents, bradykinin or cannabinoid ligands, caused the potentiation of TRPV1 channel function in diabetic tissues but not control tissues. This suggests that STZ treatment may cause inflammation and therefore alter the function of TRPV1.

Activation of calcium permeant nociceptive ion channels on the peripheral and central terminals of sensory neurons leads to the synthesis and/or release of a variety of proinflammatory agents and neuropeptides such as ATP, BK, PGs, CGRP, SP and vasoactive intestinal peptide (VIP). TRPV1 play a significant role in inflammatory thermal hyperalgesia. The inflammatory mediators activate their respective G-protein coupled receptors to initiate secondary messenger pathways resulting in activation of either PKA, PKC,

MAPK, extracellular calcium/CaM-dependent kinase II (CaMKII) or Src kinase, which phosphorylate TRPV1 (Premkumar and Sikand, 2008).

Activation of CB1 or CB2 receptors has been shown to increase or decrease adenylate cyclase levels, which will modulate the phosphorylation state of TRPV1. Activation of the CB1 receptors decreases Ca^{2+} and increases K^{+} conductance in the presynaptic terminals that can interfere with the action of TRPV1 distributed at the central terminals of sensory neurons. Phosphorylation at S116 in the amino terminus of TRPV1 is vital in PKA mediated regulation of TRPV1 desensitization (Bhave *et al*., 2002).

Phosphorylation by PKC has been shown to sensitize TRPV1. Various algesic agents like BK, ATP, trypsin and PGs are known to sensitize TRPV1 by activating PKC downstream of their G-protein coupled receptors in sensory neurons and in recombinant expression systems (Cesare and McNaughton, 1996).

TRPV1 is involved in both afferent (sensation of pain) and efferent (neurotransmitter release) function. TRPV1 can mediate both inflammation and pain. Important properties of the TRPV1 receptor include sensitization, desensitization (a reduction in response to continued exposure to an agonist) and tachyphylaxis (a reduction in response to repeated application of an agonist). Both desensitization and tachyphylaxis require extracellular Ca^{2+}, suggesting the involvement of Ca^{2+} -dependent intracellular signalling mechanism. Tachyphylaxis can be abolished by inhibitors of Ca^{2+} -dependent phosphatases, suggesting the requirement for phosphorylation in channel activation. Thus, ca^{2+} -mediated dephosphorylation may render the channel inactive leading to desensitization or tachyphylaxis. Prolonged TRPV1 activation by capsaicin may lead to its desensitization. PKA-, but not PKC-mediated phosphorylation is able to reverse tachyphylaxis, suggesting distinct actions for these kinases. Desensitization may result from Ca^{2+} -induced modulation of TRPV1 sensitivity, and/or Ca^{2+} toxicity; a large and sustained Ca^{2+} influx via TRPV1 has been linked to neurodegeneration of the peripheral nerve terminals (Premkumar and Sikand, 2008).

TRPV1 plays an important role in enhancing bladder reflex contractions in the chronically inflamed bladder and its responses can be significantly potentiated by the activation of PKC. Application of capsazepine resulted in decreased contractions in bladder inflamed by cyclophosphamide. In acetic acid or lipopolysaccharide-induced inflammation of the bladder, the frequency of urinary bladder contraction was significantly enhanced in wild type TRPV1 mice but not in TRPV1 knock-out mice. The frequency of bladder reflex contractions were reported to be similar in both $TRPV1^{+/+}$ and $TRPV1^{-/-}$ mice and it was shown that high concentration of capsazepine had no effect on the bladder reflex activity of normal bladders (Premkumar and Sikand, 2008). These reports may be different from the findings in this study that adding inflammatory mediators acutely is quite different from possible prolonged inflammation in the STZ model. The STZ treatment might produce desensitization due to persistent release of inflammatory mediators. This might give opposite effect to acute addition of bradykinin.

7.1.6 Possible clinical interpretations

Diabetic cystopathy is a common symptom found in diabetic patients. There is more urination in order to excrete the high levels of blood glucose. In this study, the down regulation of TRPV1 in STZ-diabetic rats was found. This might suggest a hypoalgesic effect in reducing pain sensation in the diabetic rats. TRPV1 is a nociceptive receptor therefore pain receptors will be less activated in diabetic rats. In the present study, the reduction in the responses to TRP channel activations in the nerves of diabetic rat bladder was found. The findings from the present study are consistent with the long term effects of diabetes impairing the contractile responses in bladder. This is consistent with the observation that diabetic patients suffer from difficulty to empty the bladder. Improving the responsiveness of nerves of bladder in diabetic patients may improve their bladder function. These studies suggest that indirect modulation of TRPV1 through bradykinin receptor activation has potential since it potentiated the TRPV1 channel in diabetic rat bladders.

7.2 Conclusion

In conclusion, in the rat urinary bladder or colon preparations, diabetes mellitus using STZ animal model caused 1) the impairment of a number of TRP channel subfamily function, TRPV1, TRPV4 and TRPA1 but not TRPM8. The combination of NK_1 and NK_2 antagonists significantly inhibited the responses of TRPV1 channel. This may suggest the involvement of neurokinin in postsynaptic transmission in rat bladder following the activation of TRPV1 channel, 2) the impairment caused by STZ-induced diabetes occurred very early (within 36 hours after diabetes induction) in TRPV1 channel but not TRPA1 channel. There are specific early effects of STZ treatment on TRPV1 channel function at a time when other afferent nerve terminal channels (TRPA1) are functioning normally, suggesting that early onset of dysfunction in TRPV1 signalling may not merely be the consequence of nerve damage, 3) the factors influence in the mechanism of this impairment may not be the neuropathy effect on neurotransmitter release or nerve damage.

7.3 Future work

Since the mechanism involved in the impairment of TRP channel function in STZ-diabetic animal model is still unclear, the following issues are waiting to be completed in order to explain the whole phenomena.

7.3.1) To study the localization of TRP channels in control and diabetic rat urinary bladder smooth muscle preparations using immunohistochemistry

7.3.2) To study the neurotransmitter release mediated by TRP channel agonists in control and diabetic rat urinary bladder smooth muscle preparation using neurotransmitter release measurement. However, it is quite difficult to achieve this experiment since there are very few nerve ending in bladder tissues.

7.3.3) To compare the TRPV1 receptor expression in control and diabetic tissues using western blotting technique. However, the problem with this technique is that it is difficult to find the specific antibody. Therefore, polymerase chain reaction (PCR) technique may be useful to study the TRPV1 expression. As there is very little TRPV1 in the bladder tissues, it is unlikely to determine the TRPV1 receptor expression using western blot analysis.

7.3.4) To study the TRPV1 channel function in wild type and knockout mice using TRPV1 labeling technique

7.3.5) To further compare the cholesterol contents from the rat bladder tissues treated with or without cyclodextrin molecules by increasing the concentration of cyclodextrins

References

Abdel-Hakim, A.M., Rioux, F. and Elhilali, M. (1981). The contractile effect of bombesin on the rat urinary bladder. *Eur. J. Pharmacol*. 70(2):167-173.

Akerman, S., Kaube, H. and Goadsby, P.J. (2004). Anandamide acts as a vasodilator of dural blood vessels in vivo by activating TRPV1 receptors. *Br. J. Pharmacol*. 142:1354-1360.

Alexander, S.P.H., Mathie, A. and Peter, J.A. (2007). Guide to receptor and channels (GRAC), 2nd edition (2007 revision). *Br. J. Pharmacol*. 150 (1):S1-S168.

American Diabetes Association Position Statement. (2006). Standards of medical care for diabetes. *Diabetes Care.* 29(Suppl. 1):S4-S42.

Andersen, J.T. and Bradley, W.H. (1976). Abnormalities of bladder innervation in diabetes mellitus. *Urology*. 7:442-448.

Andersson, D.A., Chase, H.W.N. and Bevan, S. (2004). TRPM8 activation by menthol, icilin, and cold is differentially modulated by intracellular pH. *The Journal of Neuroscience*. 24(23):5364-5369.

Andersson, K.E. (2002). Bladder activation : afferent mechanisms. *Urology*. 59(5 Suppl.1):43-50.

Andersson, K.E. and Arner, A. (2004). Urinary bladder contraction and relaxation: physiology and pathophysiology. *Physiol. Rev*. 84:935-986.

Andersson, K.E. and Wein, A.J. (2004). Pharmacology of the lower urinary tract : basis for current and future treatments urinary incontinence. *Pharmacol. Rev.* 56:581-631.

Andersson, K.E., Gratzke, C., Hedlund, P. (2010). The role of the transient receptor potential (TRP) superfamily of cation-selective channels in the management of the overactive bladder. *BJU International*. 106(8):1114-1127.

Andersson, P.O., Malmgren, A. and Uvelius, B. (1988). Cystometrical and in vitro evaluation of urinary bladder function in rats with streptozotocin-induced diabetes. *J. Urol.* 139:1359.

Andrade, E.L., Ferreira, J., Andre, E. and Calixto, J.B. (2006). Contractile mechanisms coupled to TRPA1 receptor activation in rat urinary bladder. *Biochemical Pharmacology*. 72:104-114.

Anjaneyulu, M. and Ramarao, P. (2002). Studies on gastrointestinal tract functional changes in diabetic animals. *Methods Find. Exp. Clin. Pharmacol.* 24(2):71.

Avelino, A. and Cruz, F. (2006). TRPV1 (vanilloid receptor) in the urinary tract : expression, function and clinical applications. *Naunyn-Schmiedeberg's Ach Pharmacol*. 373:287-299.

Avelino, A., Dinis, P., Charrua, A., Nagy, I., Yaqoob, M. and Cruz, F. (2003). The endogenous TRPV1 ligand anandamide increases in the rat inflamed urinary bladder and may contribute to inflammatory pain. *Soc. Neurosci.* 33:608.3.

Babiychuk, E.B., Smith, R.D., Burdyga, T., Babiychuk, V.S., Wray, S., Draeger, A. (2004). Membrane cholesterol regulates smooth muscle phasic contraction. *J. Membrane Biol.* 198:95-101.

Bari, M., Battista, N., Fezza, F., Finazzi-Agro, A. And Maccarrone, M. (2005). Lipid rafts control signaling of type-1 cannabinoid receptors in neuronal cells. *The Journal of Biological chemistry.* 280(13):12212-12220.

Bari, M., Paradisi, A., Pasquariello, N., Maccarrone, M. (2005). Cholesterol-dependent modulation of type 1 cannabinoid receptors in nerve cells. *Journal of Neuroscience*. 81:275-283.

Bartley, O., Brolin, I., Fagerberg, S.E. and Wilhelmsen, L. (1966). Neurogenic disorders of the bladder in diabetes mellitus : a clinical-roentgenological investigation. *Acta Med. Scand.* 180:187-198.

Benham, C.D., Gunthorpe, M.J. and Davis, J.B. (2003). TRPV channel as temperature sensors. *Cell Calcium*. 33:479-487.

Benko, R., Lazar, Z., Porszasz, R., Somogyi, G.T. and Bartho, L. (2003). Effect of experimental diabetes on cholinergic, purinergic and peptidergic motor responses of the isolated rat bladder to electrical field stimulation or capsaicin. *European Journal of Pharmacology.* 478:73-80.

Bestetti, G., Rossi, G.L. and Zemp, C. (1981). Changes in peripheral nerves of rats four months after induction of streptozotocin diabetes. *Acta Neuropathologica (Berl)*. 54:129-134.

Bestetti, G., Zemp, C., Probst, D. and Rossi, G.L. (1981). Neuropathy and myopathy in the diaphragm of rats after 12 months of streptozotocin-induced diabetes mellitus. *Acta Neuropathologica (Berl)*. 55:11-20.

Bhave, G., Zhu, W., Wang, H., Brasier, D.J., Oxford, G.S. and Gereau, R.W. (2002). cAMP-dependent protein kinase regulates desensitization of capsaicin receptor (VR1) by direct phosphorylation. *Neuron*. 35:721-731.

Birder, L.A. (2007). TRPs in bladder diseases. *Biochimica et Biophysica Acta*. 1772:879-884.

Birder, L.A. (2004). More than just a barrier: urothelium as a drug target for urinary bladder pain. *Am.J.Physiol*. 289:F489-F495.

Birder, L.A., Kullmann, F.A., Lee, H., Barrick, S., de Groat, W., Kanai, A. and Caterina, M. (2007). Activation of urothelial transient receptor potential vanilloid 4 by 4α-Phorbol 12,13-Didecanoate contributes to altered bladder reflexes in the rat. *The Journal of Pharmacology and Experimental Therapeutics*. 323(1):227-235.

Birder, L.A., Nakamura, Y., Kiss, S. and Vass, Z. (2002). Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1. *Nature Neuroscience.* 5: 856-860.

Birder, L.A., Kanai, A.J., de Groat, W.C., Kiss, S., Nealen, M.L., Burke, N.E., Dineley, K.E., Watkins, S., Reynolds, I.J. and Caterina, M.J. (2001). Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. *Proc. Natl. Acad. Sci. U S A.* 98(23):13396-13401.

Blaivas, J.G. (1982). The neurophysiology of micturition : a clinical study of 550 patients. *J. Urol.* 127:958-963.

Bloomgarden, Z.T. (1997). New therapeutic approaches to non-insulin-dependent diabetes mellitus. *Endocrine Practice*. 5(3):307-312.

Bornstein, J.C. and Furness, J.B. (1988). Correlated electrophysiological and histochemical studies of submucous neurons and their contribution to understanding enteric neural circuits. *J. Auton. Nerv. Syst.* 25(1):1-13.

Brown, J.S., Wessels, H., Chancellor, M.B., Howards, S.S., Stamm, W.E., Stapleton, A.E., Steers, W.D. Van Den Eeden, S.K. and McVary, K.T. (2005). Urologic complications of diabetes. *Diabetes Care*. 28(1):177-185.

Buck, A.C., McRae, C.U., Reed, P.I. and Chisholm, G.D. (1974). The diabetic bladder. *Proc. R. Soc. Med.* 67:81.

Buck, A.C., Reed, P.I., Siddiq, Y.K., Chisolm, G.D. and Russel, F.T. (1976). Bladder dysfunction and neuropathy in diabetes. *Diabetologia*. 12:251-258.

Cardozo, A.H.M., Carbrini, D.A., Campos, M.M., Rae, G.A., Huidobro-Toro, J.P. and Calixto, J.B. (2002). Diabetes-induced changes in responsiveness of rat bladder and vas deferens to peptides in vitro : susceptibility to reversal by insulin. *Can. J. Physiol. Pharmacol*. 80:334-340.

Carr, M.J., Kollarik, M., Meeker, S.N. and Undem, B.J. (2003). A role for TRPV1 in bradykinin-induced excitation of vagal airway afferent nerve terminals. *J Pharmacol Exp Ther.* 304(3):1275-9.

Cases, R.P., Sanz, N.G., Paloa, C.M. and Montiel. A.F. (2005). Functional aspects and mechanisms of TRPV1 involvement in neurogenic inflammation that leads to thermal hyperalgesia. *Pflugers Archiv*. 451(1):151-159.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Pétersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I. and Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science.* 288:306–313.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in pain pathway. *Nature*. 89: 816-824.

Cesare P. and McNaughton, P. (1996). A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc Natl. Acad Sci USA*. 93:15435-15439.

Chahl, L.A. (1982). Evidence that the contractile response of the guinea-pig ileum to capsaicin is due to substance P release. *Arch. Pharmacol*. 319:212.

Chan, C.L., Facer, P., Davis, J.B., Smith, G.D., Egerton, J., Bountra, C., Williams, N.S. and Anand, P. (2003). Sensory fibres expressing capsaicin receptor TRPV1 in patients with rectal hypersensitivity and faecal urgency. *Lancet.* 361:385-91.

Chancellor, M.B., Perkin, H. and Yoshimura, N. (2005). Recent advances in the neurophysiology of stress urinary incontinence. *Scand. J. Urol. Nephrol.* 39:21.

Chancellor, M.B. and Yoshimura, N. (2004). Neurophysiology of stress urinary incontinence. *Rev. Urol.* 6(Supp.1):S19-S28.

Chapple, C.R., Yamanichi, T., Chess-Willium, R. (2002). Muscarinic receptor subtypes and management of the overactive bladder. *Urology*. 60(Suppl.1):82-88.

Chiasson, J.L., Gomis, R., Hanefeld, M., Josses, R.G., Karasik, A., Laakso, M. (1998). The STOP-NIDDM Trial: an international study on the efficacy of an alpha-glucosidase inhibitor to prevent type 2 diabetes in a population with impaired glucose tolerance: rationale, design, and preliminary screening data. Study to prevent non-insulin-depejndent diabetes mellitus. *Diabetes Care*. 21:1720-1725.

Christ, G.J., Hsieh, Y., Zhao, W., Schenk, G., Venkateswarlu, K., Wang, H.Z., Tar, M.T., and Melman, A. (2006). Effects of streptozotocin-induced diabetes on bladder and erectile (dys)function in the same rat *in vivo*. *BJU Int.* 97(5):1076-1082.

Chuang, H.H., Prescott, E.D., Kong, H., Shields, S., Jordt, S.E., Basbaum, A.I., Chao, M.V. and Julius, D. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature*. 411: 957-962

Clapham, D.E., Runnels, L.W. and Strubing, C. (2001). The TRP ion channel family. *Nature Reviews Neuroscience*. 2:387-396.

Coelho, M., Vergnolle, N., Guiard, B., Fioramonti, J. and Bueno, L. (2002). Proteinases and proteinase-activated receptor 2: a possible role to promote visceral hyperalgesia in rats. *Gastroenterology*. 122:1035–1047.

Cohen, R.A. (1993). Dysfunction of vascular endothelium in diabetes mellitus. *Circulation.* 87:V67-V76.

Coleridge, J.C. and Coleridge, H.M. (1984). Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Rev. Physiol., Biochem. Pharmacol.* 99:1–110.

Collins, S.M., Piche, T. and Rampal, P. (2001). The putative role of inflammation in the irritable bowel syndrome. *Gut*. 49:743–745.

Corey, D.P., Gacia-Anoveros, J., Holt, J.R., Kwan, K.Y., Lin, S.Y., Vollrath, M.A., Amalfitano, A., Cheung, E.L., Derfler, B.H., Dugan, A., Geleoc, G.S., Gray, P.A., Hoffman, M.P., Rehm, H.L., Tamasauskas, D. and Zhang, D.S. (2004). TRPA1 is a candidate for mechanosensitive transduction channel of vertebrate hair cell. *Nature*. 432:723-730.

Cruz, F., Avelino, A. and Coimbra, A., 1997. Intravesical resiniferatoxin desensitizes bladder sensory fibers in a dose-dependent manner. *J. Urol.* 157, p. 79 suppl. 79.

Cruz, F., Guimaraes, M., Silva, C. and Reis, M., 1997. Suppression of bladder hyperreflexia by intravesical resiniferatoxin. *Lancet* 350, pp. 640–641

Daneshgari, F. and Moore, C. (2006). Diabetic uropathy. *Seminar in Nephrology.* 26(2):182-185.

Daneshgari, F., Liu, G. and Imrey, P.B. (2006). Time dependent changes in diabetic cystopathy in rats include compensated and decompensated bladder function. *J Urol.* 176: 380-386.

Daneshgari, F., Liu, G., Birder, L., Hanna-Mitchell, A.T. and Chacko, S. (2009). Diabetic bladder dysfunction: Current translational knowledge. *The Journal of Urology*. 182:S18-S26.

De Groat, W.C. and Yoshimura, N. (2001). Pharmacology of the lower urinary tract. *Annu. Rev. Pharmacol. Toxicol.* 41:691-721.

De Groat, W.C., Kawatani, M., Hisamitsu, T., Cheng, C.L., Ma, C.P., Thor, K., Steers, W. And Roppolo, J.R. (1990). Mechanisms underlying the recovery of urinary bladder function following spinal cord injury. *J Auton Nerv Syst*. 30(Suppl):S71–S77.

De Petrocellis, L., Stariwicz, K., Moriello, A.S., Vivese, M., Orlando, P. and Di Marzo, V. (2007). Regulation of transient receptor potential channels of melastatin type 8 (TRPM8) : effect of cAMP, cannabinoid CB_1 receptors and endovanilloids. *Experimental Cell Research*. 313:1911-1920.

De Ridder, D., Chandiramani, V., Dasgupta, P., Van Poppel, H., Baert, L. and Fowler, C.J. (1997). Intravesical capsaicin as a treatment for refractory detrusor hyperreflexia: a dual center study with long-term follow up. *J Urol* 158 (6): 2087-2092.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A. and Sheardown, S.A. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature.* 405:183-187.

Diamond, J. (2003). The double puzzle of diabetes. *Nature*. 423:599-602.

Di Palma, F., Belyantseva, I., Kim, H., Vogt, T., Kachar, B. and Noben-Trauth, K. (2002). Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. *Proc. Natl. Acad. Sci. USA*. 99:14994-14999.

Dmietrieva, N. and McMahom, S.B. (1996). Sensitization of visceral afferents by nerve growth factor in the adult rats. *Pain*. 66:87-97.

Docherty, R.J., Yeats, J.C., Bevan, S. And Boddeke, H.W. (1996). Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. *Pflugers Arch.* 431, 828–837.

Drewes, A.M., Schipper, K.P., Dimcevski, G., Petersen, P., Gregersen, H. Funch-Jensen, P. and Arendt-Nielsen, L. (2003). Gut pain and hyperalgesia induced by capsaicin: a human experimental model. *Pain* 104:333–341.

Dux, M., Rosta, J., Pinter, S., Santha, P. and Jancso, G. (2007). Loss of capsaicin-induced meningeal neurogenic sensory vasodilatation in diabetic rats. *Neuroscience*. 150:194-201.

Eika, B., Levin, R.M. and Longhurst, P.A. (1994). Comparison of urinary bladder function in rats with hereditary diabetes insipidus, streptozotocin-induced diabetes mellitus, and nondiabetic osmotic diuresis. *J. Urol.* 151:496.

Ellenberg, M. (1980). Development of urinary bladder dysfunction in diabetes mellitus. *Ann. Int. Med.* 92:321.

El-Salhy, M. (2002). The possible role of the gut neuroendocrine system in diabetes gastroenteropathy. *Histology and Histopathology*. 17:1153-1161.

Evangelista, S. and Tramontana, M. (1993). Involvement of calcitonin gene-related peptide in rat experimental colitis. *J. Physiol.* 87:277–280.

Facer, P., Casula, M.A., Smith, G.D., Benham, C.D., Chessell, I.P., Bountra, C., Sinisi, M., Birch, R. and Anand, P. (2007). Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy. *BMC Neurology*. 7(11):1-12.

Faerman, I., Glocer, L., Celener, D., Jadzinsky, M., Fox, D., Maler, M. and Alvarez, E. (1973). Autonomic nervous system and diabetes : histological and histochemical study of autonomic nerve fibers of the urinary bladder in diabetic patients. *Diabetes*. 22:225-273.

Fichna, J., Schicho, R., Andrew, C.N., Bashashati, M., Klompus, M., Mckay, D.M., Sharkey, J.K., Janecka, A. and Storr, M.A. (2009). Salvinorin A inhibits colonic transit and neurogenic ion transport in mice by activating *k*-opiod and cannabinoid receptors. *Neurogastroenterol. Motil.* 21:1326–e128.

Filho O.A.R. and Fazan, V.P. (2006). Streptozotocin induced diabetes as a model of phrenic nerve neuropathy in rats. *J. Neurosci. Methods*. 151(2):131-138.

Forrest, A. and Parsons, M. (2003). The enhanced spontaneous activity of the diabetic colon is not the consequence of impaired inhibitory control mechanisms. *Autonomic and Autocoid Pharmacology*. 23:149-158.

Forrest, A., Makwana, R., Parsons, M. (2006). The short-circuit current of the ileum, but not the colon, is altered in the streptozotocin diabetic rat. *Can. J. Physiol. Pharmacol.* 84:173-179.

Fowler, C.J. (2002). Bladder afferents and their role in the overactive bladder. *Urology*. 59(suppl. 1):37-42.

Fowler, C.J., Jewkes, D., Mcdonald, W.I., Lynn, B. and de Groat, W.C. (1992). Intravesical capsaicin for neurogenic bladder dysfunction. *Lancet*. 339:1239.

Freynhagen, R. and Bennett, M.I. (2009). Diagnosis and management of neuropathic pain. *BMJ*. 339:b3002.

Frimodt-Moller, C. (1980). Diabetic cystopathy : epidemiology and related disorders. *Ann. Int. Med.* 92:318-321.

Ferguson, D.R., Kennedy, I. and Burton, T.J. 1997. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes – a possible sensory mechanism?. *J. Physiol.* 505:503–511.

Gagnerault, M.C., Luan, J.J., Lotton, C. and Lepault, F. (2002). Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. *J. Exp. Med.* 196:369–377.

Garcia-Martinez, C., Morenilla-Palao, C., Planells-Cases, R., Merino, J.M. and Ferrer-Montiel, A. (2000). Identicication of an aspartic residue in the P-loop of the vanilloid receptor that modulates pore properties. *Journal of Biological Chemistry*. 275:32552-32558.

Gillis, J.M. and Gailly, P. (1994). Measurement of $[Ca^{2+}]_i$ with the diffusible Fura-2 AM: can some potential pitfalls be evaluate?. *Biophysical Journal*. 67:476-477.

Giuliani, S., Santicioli, P., Lippi, A., Lecci, A., Tramontana, C. and Maggi, C.A. (2001). The role of sensory neuropeptides in motor innervation of the hamster isolated urinary bladder. *Naunyn-Schmiedeberg's Ach Pharmacol*. 364:242-248.

Goycheva, P., Gadjeva, V. And Popov, B. (2006). Oxidative stress and their complications in diabetes mellitus. *Trakia Journal of Sciences*. 4(1):1-8.

Green, K., Brand, M.D. and Murphy, M.P. (2004). Prevention of Mitochondrial Oxidative Damage as a Therapeutic Strategy in Diabetes. *Diabetes*. 53(1):S110-S118.

Gunthorpe, M.J., Benham, C.D., Randall, A. and Davis, J.D. (2002). The diversity in the vanilloid (TRPV) receptor family of ion channels. *Trends. Pharmacol. Sci.* 23:183-191.

Habler, H.J., Janig, W. and Koltzenberg, M. (1990). Activation of unmyelinated afferent fibers by mechanical stimuli and inflammation of the urinary bladder in the cat. *J. Physiol (Lond).* 425:545-562.

Hagl, C.I., Klotz, M., Wink, E., Kranzle, K., Holland-Cunz, S., Gretz, N., Diener, M. and Schafer, K.H. (2007). Temporal and regional morphological differences as a consequence of FGF-2 deficiency are mirrored in the myenteric proteome. *Pediatr. Surg. Int.* 24:49-60.

Hales, C.N. and Barker, D.J. (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia.* 35(7):595-601.

Hao, M., Mukherjee, S. and Maxfield, F.R. (2001). Cholesterol depletion induces large scale domain segregation in living cell membrane. *Proc. Natl. Acad. Sci. USA*. 98:13072-13077.

Harris, M.I., Flegal, K.M. and Cowie, C.C. (1998). Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988-1994. *Diabetes Care.* 21:518-524.

Hering, H., Lin, C.C. and Sheng, M. (2003). Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *J. Neurosci.* 23:3262–3271.

Hillsley K. and Grundy, D. (1998). Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents. *Neurosci. Lett.* 255:63–66.

Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C. and Mathis, D. (1999). Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J. Exp. Med.* 189:331–339.

Hong., S. and Wiley, J.W. (2005). Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J. Biol. Chem.* 280:618-627.

Hu, D.E., Easton, A.S. and Fraser, P.A. (2005). TRPV1 activation results in disruption of the blood-brain barrier in the rat. *Br. J. Pharmacol*. 146(4):576-584.

Hunt, R.H. and Tougas, G. (2002). Evolving concepts in functional gastrointestinal disorders: promising directions for novel pharmaceutical treatments. *Best Pract. Res. Clin. Gastroenterol*. 16 869–883.

Hunter, K.F. and Moore, K.N. (2003). Diabetes-associated bladder dysfunction in the older adult. *Geriatric Nursing*. 24(3):138-147.

Husted, S., Sjogren, C. and Andersson, K.E. (1983). Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J. Urol.* 130:392-398.ion transport in mouse colon by neuropeptide Y (NPY), Y_1 and Y_2 receptors. *British Journal of Pharmacology*. 146:712-722.

Hyland, N.P. and Cox, H.M. (2005). The regulation of veratridine-stimulated electrogenic ion transport in mouse colon by neuropeptide Y (NPY), Y_1 and Y_2 receptors. *British Journal of Pharmacology*. 146, 712–722.

Islas-Andrade, S., Monsalve, M.C.R., de la Peria, J.E., Polanco, A.C., Palomino, M.A. and Velasco, A.F. (2000). Streptozotocin and alloxan in experimental diabetes: comparison of the two models in rats. *Acta Histochemica et Cytochemica*. 33(3):201-208.

Ji, R.R., Samad, T.A., Jin, S.X., Schmoll, R. and Woolf, C.J. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron.* 36(1):57-68.

Jiang, Y.J., Gong, D.X., Liu, H.B., Yang, C.M., Sun, Z.X. and Kong, C.Z. (2008). Ability of alpha-lipoic acid to reverse the diabetic cystopathy in a rat model. *Acta Pharmacol Sin.* 29(6):713-9.

Jung, J., Shin, J.S., Lee, S.Y., Hwang. S.W., Koo, J., Cho, H. and Oh, U. (2004). Phosphorylation of vanilloid receptor 1 by Ca^{2+}/calmodulin-dependent kinase II regulates its vanilloid binding. *J. Biol. Chem.* 279, 7048–7054.

Karlsson, J.A. (1996). The role of capsaicin-sensitive C-fibre afferent nerves in the cough reflex. *Pulm Pharmacol.* 9:315-21.

Katsumata, K., Katsumata, K.Jr. and Katsumata, K. (1992). Protective effect of diltiazem hydrochloride on the occurrence of alloxan- or streptozotocin-induced diabetes in rats. *Horm. Metab. Res.* 24(11):508-510.

Kenakin, T. (2001). Isolated Cardiac Muscle Assays. *Current Protocols in Pharmacology*. DOI: 10.1002/0471141755.ph0403s00.

Kizilay, G. and Uygun, M. (2005). Structural Changes in Tunica Mucosa Cells of Bladder in Rats with Experimental Diabetes Mellitus. *Pakistan Journal of Biological Sciences*. 8(2):181-185.

Jerreat, L. (1999). Diabetes for nurses. London : Whurr Publishers Ltd. 1-247 pp.

Kahn, C.R., Vicent, D. And Doria, A. (1996). Genetic of non-insulin-dependent (Type-II) diabetes mellitus. *Annu. Rev. Med*. 47:509–531

Kamata, K., Inoue, K. and Kasuya, Y. (1993). Changes in contractile responses of the urinary bladder to substance P in streptozotocin-induced diabetic rats. *Gen. Pharmacol*. 24(3):547-553.

Karaki, S. and Kuwahara, A. (2004). Electrophysiological measurement of transepithelial ion transport: short-circuit current (Ussing chamber) technique. *Folia Pharmacol. Jpn*. 123:211-218.

Kawao, N. Ikeda, H., Kitano, T., Kuroda, R., Sekiguchi, F., Kataoka, K., Kamanaka Y. and Kawabata, A. (2004). Modulation of capsaicin-evoked visceral pain and referred hyperalgesia by protease-activated receptors 1 and 2. *J. Pharmacol. Sci.* 94:277–285.

Kilsdonk, E.P.C., Yancey, P.G., Stoudt, G.W., Bangerter, F.W., Johnson, W.J., Phillips, M.C. and Rothblat, G.H. (1995). Cellular cholesterol efflux mediated by cyclodextrins. *The Journal of Biological Chemistry.* 270(29):17250-17256.

Kolta, M.G., Wallace, L.J. and Gerald, M.C. (1985). Streptozotocin-induced diabetes affects rat urinary bladder response to autonomic agents. *Diabetes*. 34:917.

Kreisberg, R.A. (1980). Lactate homeostasis and lactic acidosis. *Ann. Int. Med.* 92:227-237.

Lean, M.E. (2000). Obesity: burdens of illness and strategies of prevention or management. *Drugs Today (Barc)*. 36:773-784.

Lenzen, S. (2008). The mechanism of alloxan- and streptozotocin-induced diabetes. *Diabetologia.* 51:216-226.

Levisetti, M.G. Suri, A., Frederick, K. and Unanue, E.R. (2004). Absence of lymph nodes in NOD mice treated with lymphotoxin-beta receptor immunoglobulin protects from diabetes. *Diabetes*. 53:3115–3119.

Liedtke, W. and Simon, S.A. (2004). A possible role for TRPV4 receptors in asthma. *Am. J. Physiol. Lung. Cell. Mol. Physiol*. 287:L269-L271.

Lipinski, B. (2001). Pathophysiology of oxidative stress in diabetes mellitus. *J Diabetes Complications.* 15(4):203-210.

Liu, M., Huang, W., Wu, D. and Priestley, J.V. (2006). TRPV1, but not $P2X_3$, requires cholesterol for its function and membrane expression in rat nociceptors. *European Journal of Neuroscience.* 24:1-6.

Liu, Y., Pathak, N., Zucker, A.K. and Drummond, I.A. (2007). Notching signaling controls of the differentiation of transporting epithelia and multiciliated cells in the zebrafish pronephros. *Development*. 134:1111-1122.

Llorente, M.D. and Malphurs, J.E. (2007). Psychiatric disorders and diabetes mellitus. London : Informa UK Ltd. 1-249.

Lockwich, T.P., Liu, X., Singh, B.B., Jadlowiec, J., Weiland, S. and Ambudkar, I.S. Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *The Journal of Biological Chemistry*. 275(16):11934-11942.

Lomax, A.E., O'Hara, J.R., Hyland, N.P., Mawe, G.M. and Sharkey, K. (2007). Persistent alterations to enteric neural signaling in the guinea pig colon following the resolution of colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292:G482-G491.

Longhurst, P.A. and Belis, J.A. (1986). Abnormalities of rat bladder contractility in streptozotocin-induced diabetes mellitus. *The Journal of Pharmacology and Experimental Therapeutics*. 238(3):773-777.

Maggi, C.A. (1990). The dual function of capsaicin-sensitive sensory nerves in the bladder and urethra. *Ciba Foundation Symposium*. 151:77-83.

Maggi, C.A., Bevan, S., Walpole, C.S.J., Rang, H.P. and Giuliani, S. (1993). A comparison of capsazepine and ruthenium red as capsaicin antagonists in the rat isolated urinary bladder and vas deferens. *British Journal of Pharmacology*. 108:801-805.

Maggi, C.A., Giuliani, S. and Meli, A. (1989). Effect of ruthenium red on responses mediated by capsaicin-sensitive nerves of the rat urinary bladder. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 340(5):541-546.

Maggi, C.A., Giuliani, S. and Zagorodynuk, V.P. (1996). Calcitonin gene related peptide (CGRP) in the circular muscle of guinea pig colon: role as inhibitory transmitter and mechanisms of relaxation. *Regul. Pept*. 61:27-36.

Maggi, C.A., Patacchini, R,. Santicioli, P. and Giuliani, S. (1991). Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary baldder : eveidence for tachykinin-mediated cotransmission. *Br. J. Pharmacol*. 103:1535-1541.

Maggi, C.A., Geppetti, P., Santicioli, P., Spillantini, M.G., Frilli, S. and Meli, A. (1987a). The correlation between sensory efferent functions mediated by capsaicin-sensitive neurons and substance P content of the rat urinary bladder. *Neurosci. Lett*. 76:351-356.

Maggi, C.A., Lippe, I.T., Giuliani, S., Abelli, L., Somma, V., Geppetti, P., Jancsó, G., Santicioli, P. and Meli, A. (1989). Topical versus systemic capsaicin desensitization: specific and unspecific effects as indicated by modification or reflex micturition in rats. *Neuroscience*. 31:745–756.

Maggi, C.A. Santicioli, P. and Meli, A. (1984). The effects of topical capsaicin on rat urinary bladder motility in vivo. *Eur. J. Pharmacol.* 103:41–50.

Malmgren, A., Andersson, P.O. and Uvelius, B. (1989). Bladder function in rats with short and long-term diabetes : effect of age and muscarinic blockade. *J. Urol.* 142:1608.

Mang, C.F., Erbelding, D. and Kilbinger, H. (2001). Differential effects of anandamide on acetylcholine release in the guinea-pig ileum mediated via vanilloid and non-CB1 cannabinoid receptors, *Br. J. Pharmacol.* 134:161–167.

Maraschiello, C., Diaz, I. and Regueiro, J.A.G. (1996). Determination of cholesterol in fat and muscle of pig by HPLC and capillary gas chromatography with solvent venting injection. *J. High Resol. Chromatogr*. 19:165-168.

Mauer, S.M., Steffes, M.W., Ellis, E.N., Sutherland, D.E., Brown, D.M. and Goetz, F.C. (1984). Structural-functional relationships in diabetic nephropathy. *J. Clin. Invest.* 74:1143-1155.

McCafferty, M., Wallace, J.L. and Sharkey, K.A. (1997). Effects of chemical sympathectomy and sensory nerve ablation on experimental colitis in the rat. *Am. J. Physiol.* 272:G272–G280.

McKemy, D.D., Neuhausser, W.M. and Julius, D. (2002). Identification of a cold receptors reveals a general role for TRP channels in thermosensation. *Nature*. 416:52-58.

McVey, D.C. and Vigna, S.R. (2001). The capsaicin VR1 receptor mediates substance P release in toxin A-induced enteritis in rats. *Peptides* 22:1439–1446.

McVey, D.C., Schmid, P.C., Schmid, H.H. and Vigna, S.R. (2003). Endocannabinoids induce ileitis in rats via the capsaicin receptor (VR1). *J. Pharmacol. Exp. Ther.* 304:713–722.

Modi, P. (2007). Beyond Insulin: review of new drugs for treatment of diabetes mellitus. *Current Drug Discovery Technologies*. 4:39-47.

Mohammadi-Farani, A., Sahebgharani, M., Sepehrizadeh, Z., Jaberi, E. and Ghazi-Khansari, M. (2010). Diabetic thermal hyperalgesia: role of TRPV1 and CB1 receptors of periaqueductal gray. *Brain Research*. 1328:49-56.

Molecular Probes. (2010). Fluorescence Ca^{2+}indicators excited with UV light – section 19.2. http://www.invitrogen.com/site/us/en/home/references/Molecular-Probes-The-Handbook/Indicator-for-Ca2-Mg2-Zn2-and-Other-Metal-Ions/Fluorescent-Ca2.

Montell, C. and Rubin, G.M. (1989). Molecular characterization of the *Drosophila* trp locus – a pupative integral membrane-protein require for phototransduction. *Neuron*. 2:1313-1323.

Morgan, C., Nadelhaft, I., de Groat, W.C. (1981). The distribution of visceral primary afferents from the pelvic nerve to Lissauer's tract and the spinal gray matter and its relationship to the sacral parasympathetic nucleus. *J. Comp. Neurol.* 201:415-440.

Moran, M.M., Xu, H. and Clapham, D.E. (2004). TRP ion channels in the nervous system. *Curr. Opin. Neurobiol.* 14:362-369.

Mukerji, G., Yiangou, Y., Corcoran, S.L., Selmer, I.S., Smith, G.D., Benham, C.D., Bountra, C., Agarwal, S.K. and Anand, P. (2006). Cool and menthol receptor TRPM8 in human urinary bladder disorders and clinical correlations. *BMC Urology*. 6(6):1-11.

Munro, S. (2003). Lipid rafts: Elusive or illusive?. *Cell.* 115:377-388.

Nathan, J.D., Peng, R.Y., Wang, Y., McVey, D.C., Vigna, S.R. and Liddle, R.A. (2002). Primary sensory neurons : a common final pathway for inflammation in experimental pancreatitis in rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283:G938-946.

Nilius, B. and Voets, T. (2005). TRP channels : a TR(I)P through a world of multifunctional cation channels. *Pfluger Archiv European Journal of Physiology*. 451:1-10.

Nocerino, E., Izzo, A.A., Borrelli, F., Capasso, F., Capasso, R., Pinto, A., Sautebin, L. and Mascolo, N. (2002). Relaxant effect of capsazepine in the isolated rat ileum. *Naunyn-Schmiedeberg's Ach Pharmacol*. 365:187-192.

Nomiya, M. and Yamaguchi, O. (2003). A quantitative analysis of mRNA expression of alpha 1 and beta adrenoceptor subtypes and their functional roles in human normal and obstructed bladders. *J. Urol.* 170(2):649-653.

Nomoto, Y., Yoshida, A., Ikeda, S., Kamikawa, Y., Harada, K., Ohwatashi, A. and Kawahira, K. (2008). Effect of menthol on detrusor smooth-muscle contraction and the micturition reflex in rats. *Basic and Translational Science*. 72(3):701-705.

O'Hara, J.R., Lomax, A.E., Mawe, G.M. and Sharkey, K.A. (2006). Ileitis alters neuronal and enteroendocrine signaling in guinea pig distal colon.

Ohsawa, M. and Kamei, J. (1999a). Possible involvement of spinal protein kinase C in thermal allodynia and hyperalgesia in diabetic mice. *Eur. J. Pharmacol.* 372:221-228.

Ohsawa, M. and Kamei, J. (1999b). Role of intracellular calcium in thermal allodynia and hyperalgesia in diabetic mice. *Brain Res.* 833:278-281.

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K. and Pitha, J. (1989). Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. *European Journal of Biochemistry*. 186:17-22.

O'Reilly, B.A., Kosaka, A.H., Chang, T.K., Ford, A.O., Popert, R. and McMahom, S.B. (2001). A quantitative analysis of purinoceptor expression in the bladder of patients with symptomatic outlet obstruction. *BJU. Int.* 87:617-622.

Ouslander, J.G. (2004). Management of overactive bladder. *N. Engl. J. Med.* 350:786-799.

Ozturk, Y., Altan, V.M. and Ari, N. (1998). Diabetic complications in experimental models. *Tr. J. of Medical Sciences.* 22:331-341.

Pabbidi, R.M., Yu, S.Q., Peng, S., Khardori, R., Pauza, M.E., Premkumar, L.S. (2008). Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. *Mol. Pain.* 4:9.

Paintal, A.S. (1973). Vagal sensory receptors and their reflex effects. *Physiol. Rev.* 53:159–227.

Palumbo, P.J. (2001). Glycemic control, mealtime glucose excursions, and diabetic complications in Type 2 diabetes mellitus. *Mayo Clin. Proc.* 76:609-618.

Pani, B. and Singh, B.B. (2009). Lipid rafts/caveolae as microdomains of calcium signaling. *Cell Calcium*. 45:625-633.

Patacchini, R., Santicioli, P., Giuliani, S. and Maggi, C.A. (2005). Pharmacological investigation of hydrogen sulfide (H_2S) contractile activity in rat detrusor muscle. *Eur. J. Pharmacol.* 509:171-177.

Patapoutian, A., Peier, A.M., Story, G.M. and Visvanath, V. (2003). Thermo TRP channels and beyond : mechanisms of temperature sensation. *Nat. Rev. Neurosci.* 4:529-539.

Patwardhan, A.M., Jeske, N.A., Price, T.J., Gamper, N., Akopian, A.N. and Hargreaves, K.M. (2006). The cannabinoid WIN 55,212–2 inhibits transient receptor potential vanilloid 1 (TRPV1) and evokes peripheral antihyperalgesia via calcineurin. *Proc. Natl. Acad. Sci. USA.* 103:11393-11398.

Pedersen, S.F., Owsianik, G. and Nilius, B. (2005). TRP channels: an overview. *Cell Calcium*. 38:233-252.

Peier, A.M., Reeve, A.J., Andersson, D.A., Moqrich, A., Earley, T.J., Hergarden, A.C., Story, G.M., Colley, S., Hogenesch, J.B., McIntyre, P., Bevan, S. and Patapoutian, A. (2002). A heat-sensitive TRP channel expressed in keratinocytes. *Science*. 296:2046-2049.

Philips, L.S. and Dunning, B.E. (2003). Nateglinide (Starlix^{TM}), update on a new antihyperglycemic agent. *International Journal of Clinical Practice*. 57:535-541.

Pinna, C., Bolego, C. and Puglisi, L. (1994). Effect of substance P and capsaicin on urinary bladder of diabetic rats and the role of epithelium. *Eur. J. Pharmacol*. 271(1):151-158.

Pinna, C., Bolego, C. and Puglisi, L. (1995). Effect of substance P and capsaicin on stomach fundus and ileum of streptozotocin-diabetic rats. *European Journal of Pharmcology.* 276:61-69.

Pothoulakis, C., Castagliuolo, I., LaMont, J.T., Jaffer, A., O'Keane, J.C., Snider, R.M. and Leeman, S.E. (1994). CP-96,345, a substance P antagonist, inhibits rat intestinal responses to *Clostridium* difficile toxin A but not cholera toxin, *Proc. Natl. Acad. Sci. U. S. A.* 91:947–951.

Premkumar, L.S., Ahern, G.P. (2000). Induction of vanilloid receptor channel activity by protein kinase C. *Nature*. 408:985–990.

Premkumar, L.S. and Sikand, P. (2008). TRPV1: a target for next generation analgesics. *Current Neuropharmacology*. 6:151-163.

Putney, J.W.Jr. and McKay, R.R. (1999). Capacitative calcium entry channels. *Bioessays*. 21:38-46.

Quartara, L. and Maggi, C.A. (1998). The tachykinin NK_1 receptor. Part II: distribution and pathophysiological roles. *Neuropeptides*. 32:1–49

Ralevic, V., Belai, A. and Burnstock, G. (1993). Impaired sensory-motor nerve function in the isolated mesenteric aterial bed of streptozotocin-diabetic and ganglioside-treated streptozotocin-diabetic rats. *British Journal of Pharmacology*. 110:1105-1111.

Rami, H.K., Tompson, M., Wyman, P., Jerman, J.C., Egerton, J., Brough, S., Stevens, A.J., Randall, A.D., Smart, D. and Gunthorpe, M.J. (2004). Discovery of small molecule antagonists of TRPV1. *Bioorg. Med. Chem. Lett.* 14:3631-3634.

Ramsey, I.S., Delling, M. and Clapham, D.E. (2006). An introduction to TRP channels. *Annu. Rev. Physiol*. 68: 619-647.

Raychowdhury, M.K., Gonzalez-Perrett, S., Montalbetti, N., Timpanaro, G.A., Chasan, B., Goldmann, W.H., Stahl, S., Cooney, A., Goldin, E.and Cantiello, H.F. (2004). Molecular pathophysiology of mucolipidosis type IV : pH dysregulation of the mucolipin-1 cation channel. *Hum. Mol. Genet.* 13:617-627.

Rees, D.A. and Alcolado, J.C. (2005). Animal models of diabetes mellitus. *Diabetic medicine*. 22:359-370.

Reichard, P., Nilsson, B.Y. and Rosenqvist, U. (1993). The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus. *N. Engl. J. Med.* 329:304-309.

Reinshagen, M., Patel, A., Sottili, M., French, S., Sternini, C. and Eysselein, V.E. (1996). Action of sensory neurons in an experimental at colitis model of injury and repair. *Am. J. Physiol.* 270:G79–G86.

Rosenbaum, T., Gordon-Shaag, A., Munari, M. and Gordon, S.E. (2004). Ca^{2+}/calmodulin modulates TRPV1 activation by capsaicin. *J. Gen. Physiol.* 123, 53–62.

Rouissi, N., Rhaleb, N.E., Nantel, F., Dion, S., Drapeau, G. and Regoli, D. (1991). Characterization of bombesin receptors in peripheral contractile organs. *Br. J. Pharmacol*. 103:1141-1147.

Saitoh, C., Kitada, C., Uchida, W., Chancellor, M.B., De Groat, W.C. and Yoshimaru, N. (2007). The differential contractile responses to capsaicin and anandamide in muscle strips isolated from the rat urinary bladder. *European Journal of Pharmacology*. 570:182-187.

Santicioli, P., Gamse, R., Maggi, C.A. and Meli, A. (1987). Cystometric changes in the early phase of streptozotocin-induced diabetes in rats : evidence for sensory changes not correlated to diabetic neuropathy. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 335:541-580.

Sasaki, K., Chancellor, M.B., Phelan, M.W., Yokoyama, T., Fraser, M.O., Seki, S., Kubo, K., Kumon, H., Groat, W.C. and Yoshimura, N. (2002). Diabetic cystopathy correlates with a long-term decrease in nerve growth factor levels in the bladder and lumbosacral dorsal root Ganglia. *The Journal of Urology*. 1259-1264.

Sidi, S., Friedrich, R.W. and Nicolson, T. (2003). NompC TRP channel required for vertebrate sensory hair cell mechanotransduction. *Science*. 301:96-99.

Sharma, M.D. (1989). Diabetes and the gastrointestinal tract. Department of Gastroenterology, India Institute of Medical Sciences. 63-68.

Shaw, J. and Chisholm, D. (2003). Epidemiology and prevention of type 2 diabetes and the metabolic syndrome. *MJA*. 179:379-383.

Sherkheli, M.A., Vogt-Eisele, A.K., Bura, D., Marques, L.R.B., Gisselmann, G. and Hatt, H. (2010). Characterization of selective TRPM8 ligands and their structure activity response (S.A.R.) relationship. *J. Pharm. Pharmaceut. Sci*. 13(2):242-253.

Shoda, L.K., Young, D.L., Ramanujan, S., Whiting, C.C., Atkinson, M.A., Bluestone, J.A., Eisenbarth, G.S., Mathis, D., Rossini, A.A., Campbell, S.E., Kahn, R. and Kreuwel, H.T. (2005). A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity*. 23:115–126.

Shmygol, A., Noble, K. and Wray, S. (2007). Depletion of membrane cholesterol eliminates the Ca^{2+} -activated component of outward potassium current and decreases membrane capacitance in rat uterine myocytes. *J. Physiol.* 581(2):445-456.

Silva, C., Rio, M.E. and Cruz, F. (2000). Desensitization of bladder sensory fibers by intravesical resiniferatoxin, a capsaicin analog: long-term results for the treatment of detrusor hyperreflexia. Eur Urol 38: 444-452.

Siperstein, M.D. (1992). Diabetic ketoacidosis and hyperosmolar coma. *Endocrinal. Metab. Clin. North. Am.* 21:415-432.

Smith, A.S. and Smid, S.D. (2005). Impaired capsaicin and neurokinin-evoked colonic motility in inflammatory bowel disease. *Journal of Gastroenterology and Hepatology*. 20:697-704.

Smith, R.D., Babiychuck, E.B., Noble, K., Draeger, A. and Wray, S. (2005). Increased cholesterol decreased uterine activity: functional effects of cholesterol alteration in pregnant rat myometrium. *Am. J. Physiol. Cell Physiol.* 288-C982-C988.

Song, J.X., Wang, L.H., Yao, L., Xu, C., Wei, Z.H. and Zheng, L.R. (2008). Impaired transient receptor potential vanilloid 1 in streptozotocin-induced diabetic hearts. *Elsevier Ireland Ltd, Letters to the Editor*. 290-292.

Srinivasan, K. and Ramarao, P. (2007). Animal models in type 2 diabetes research: an overview. *Indian J. Med. Res*. 125:451-472.

Steers, W.D., Mackway-Gerardi, A.M., Ciambotti, J. and de Groat, W.C. (1994). Alterations in neural pathways to the urinary bladder of the rat in responses to streptozotocin-induced diabetes. *J. Auton Nervous Sys.* 47:83-94.

Steers, W.D., Mackway-Gerardi, A.M., Ciambotti, J. and de Groat, W.C. (1990). Effects of streptozotocin-induced diabetes on bladder function in the rat. *J. Urol.* 143:1032.

Stevens, L.A., Sellers, D.J., McKay, N.G., Chapple, C.R., and Chess-Williams, R. (2006). Muscarinic receptor function, density and G-protein coupling in the overactive diabetic rat bladder. *Auton. Autacoid. Pharmacol.* 26:303-309.

Stotz, S.C., Vriens, J., Martyn, D., Clardy, J. and Clapham, D.E. (2008). Citral sensing by transient receptor potential channels in dorsal root ganglion neurons. *Plosone*. 3(5):1-14.

Stumvoll, M., Goldstein, B.J. and van Haeften, T.W. (2005). Type 2 diabetes: principles of pathogenesis and therapy. *Lancet*. 365:1333-1346.

Suri, A. and szallasi, A. (2007). The emerging role of TRPV1 in diabetes and obesity. *Trends in pharmacological sciences*. 29(1):29-36.

Szallasi, A. and Blumberg, P.M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacological Reviews*. 51:150-221.

Szallasi, A. and Fowler, C.J. (2002). After a decade of intravesical vanilloid therapy: still more questions than answers. *Lancet Neurol.* 1(3):167-72.

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cell of rat panceas. *Physiol. Res.* 50:536-546.

Szoke, E., Borzsei, R., Toth, D.M., Lenhl, O., Helyes, Z., Sandor, Z. and Szolcsanyi, J. (2009). Effect of lipid raft disruption on TRPV1 receptor activation of trigerminal sensory neurons and transfected cell line. *Frontiers in System Neuroscience. Conference Abstract. 12th Meeting of the Hungarian Neuroscience Society.* Doi10.3389/conf.neuro.2009.04.241.

Takaki, M. and Nakayama, S. (1989). Effects of capsaicin on myenteric neurons of the guinea pig ileum. *Neuroscience Letters*.105:125–130.

Talubmook, C. (2002). The influence of elevated glucose levels and the diabetic state on neuromuscular function in the gut. *Ph.D. Dissertation. University of Hertfordshire*. 1-229.

Talubmook, C., Forrest, A. and Parsons, M. (2003). Streptozotocin-induced diabetes modulates presynaptic and postsynaptic function in the rat ileum. *European Journal of Pharmacology*. 469:153-158.

Tammela, T.L.J., Briscoe, J.A.K., Levin, R.M. and Longhurst, P.A. (1994). Factors underlying the increased sensitivity to field stimulation of urinary bladder strips from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.* 113:195.

Thebault, S., Lemonnier, L., Bidauxm G., Flourakis, M., Bavencoffe, A., Gordienko, D., Roudbaraki, M., Delcourt, P., Panchin, Y., Shuba, Y., Skryma, R. and Prevarskaya. (2005). Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. *Journal of Biological Chemistry*. 280(47):39423-39435.

Thor, K.B., Morgan, C., Nadelhaft, I., Houston, M. and de Groat, W.C., (1989). Organization of afferent pathways in the pudendal nerve of the female cat. *J. Comp. Neurol.* 288:263-279.

Thorneloe, K.S., Sulpizio, A.C., Lin, Z., Figueroa, D.J., Clouse, A.K., McCafferty, G.P., Chendrimada, T.P., Lashinger, E.S.R., Gordon, E., Evans, L., Misajet, B.A., DeMarini, D.J., Nation, J.H., Casillas, L.N., Marquis, R.W., Votta, B.J., Sheardown, S.A., Xu, X., Brooks, D.P., Laping, N.J. and Westfall, T.D. (2008). *N*-((1*S*)-1-{[4-((2*S*)-2-{[(2,4-Dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperrazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK 1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induced urinary bladder contraction and hyperactivity: part I. *The Journal of Pharmacology and Experimental Therapeutics*. 326(2):432-442.

Tisch, R, McDevitt, H .(1996). Insulin-dependent diabetes mellitus. *Cell*. 85: 291–297.

Tominaga, M. (2010). Activation and regulation of nociceptive transient receptor potential (TRP) channels, TRPV1 and TRPA1. *The pharmaceutical society of Japan*. 130(3):289-294.

Tong, Y.C., Hung, Y.C., Lin, S.N. and Cheng, J.T. (1996). Alterations in urinary bladder synaptosomal neurotransmitter concentrations in two-week streptozotocin-induced diabetic rats. *Neurosci Lett.* 15:165-168.

Torii, T. (1954). The cholesterol content of blood and tissues in alloxan diabetes. *The Journal of Biochemistry*. 41(4):451-456.

Tramontana, M., Catalioto, R.M., Lecci, A., Maggi, C.A. (2000). Role of prostanoids in the contraction induced by a tachykinin NK_2 receptor agonist in the hamster urinary bladder. *Naunyn-Schmiedeberg's Ach Pharmacol*. 361:452-459.

Turner, W.H. and Brading, A.F. (1997). Smooth muscle of the bladder in the normal and the diseased state : pathophysiology, diagnosis and treatment. *Pharmacol. Ther.* 75(2):77-110.

Uckert, S., Stief, C.G., Lietz, B., Burmester, M., Jonas, U. and Machtens, S.A. (2002). Possible role of bioactive peptides in the regulation of human detrusor smooth muscle-functional effects in vitro and immunohistochemical presence. *World. J. Urol*. 20:244-249.

Ueda, T., Takami, K., Kageyama, S., Yoshimura, N. and Yoshida, O. (2000). Urinary incontinence among community-dwelling people aged 40 years or older in Japan: prevalence, risk factors, knowledge and self-perception. *International Journal of Urology*. 7:95-103.

Van Buren, J.J., Bhat, S., Rotello, R., Pauza, M.E. and Premkumar, L.S. (2005). Sensitization and translocation of TRPV1 by insulin and IGF-I. *Molecular Pain*. 1(17):1-11.

Van Dam, R.M. (2003). The epidemiology of Lifestyle and risk for type 2 diabetes. *European Journal of Epidemiology*. 18:1115-1125.

Vanner, S. and Macnaughton, W.K. (1995). Capsaicin-sensitive afferent nerves activate submucosal secretory neurons in guinea pig ileum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 269(32): G203-G209.

Varga, A., Nemeth, J., Szabo, A., McDougall, J.J., Zhang, C., Elekes, K., Pinter, E., Szolcsanyi, J. and Helyes, Z. (2005). Effect of novel TRPV1 receptor antagonist SB366791 in vitro and in vivo in the rat. *Neuroscience Letters*. 385:137-142.9.

Verne, G.N. and Sninsky, C.A. (1998). Diabetes and the gastrointestinal tract, *Gastroenterol. Clin. North. Am.* 27(4):861–874.

Vial, C. and Evans, R.J. (2005). Disruption of Lipid rafts inhibits $P2X_1$ receptor-mediated currents and arterial vasoconstriction. *The Journal of Biological chemistry.* 280(35):30705-30711.

Vizzard, M.A. (2000). Changes in urinary bladder neurotrophic factor mRNA and NGF protein following urinary bladder dysfunction. *Exp. Neurol.* 161**:** 273–284.

Vriens, J., Appendino, G. and Nilius, B. (2009). Pharmacology of vanilloid receptor potential cation channels. *Molecular Pharmacology*. 75(6):1262-1279.

Vriens, J., Owsianik, G., Fisslthaler, B., Suzuki, M., Janssens, A., Voets, T., Morisseau, C., Hammock, B.D., Fleming, I., Busse, R. and Nilius, B. (2005) Modulation of the Ca^{2+} permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ. Res.* 97: 908-915

Walker, R.G., Willingham, A.T. and Zuker, C.S. (2000). A *Drosophila* mechanosensory transduction channel. *Science*. 287:2229-2234.

Warning, J.V. and Wendt, I.R. (2000). Effect of streptozotocin-induced diabetes mellitus on intracellular calcium and contraction of longitudinal smooth muscle from rat urinary bladder. *The Journal of Urology*. 163:323-330.

Watanabe, H., Davis, J.B., Smart, D., Jerman, J.C., Smith, J.D., Hayes, P., Vriens, J., Cairns. W., Wisswnbach, U. and Prenen, J. (2002). Activation of TRPV4 channels (hVRL-1/mTRP12) by phorbol derivatives. *Journal of Biological chemistry*. 277:12569-13577.

Watts, S.W. and Cohen, M.L. (1991). Effect of bombesin, bradykinin, substance P and CGRP, in prostate, bladder body and neck. *Peptides*. 12(5):1057-1062.

Wong, M.C., Chung, J.W.Y. and Wong, T.K.S. (2007). Effect of treatments for symptoms of painful neuropathy : systematic review. *BMJ online.* 335(87):1-11.

World Health Organization, Department of Noncommunicable Disease Surveillance. (1999). Definition, diagnosis and classification of diabetes mellitus and its complications. *Report of a WHO Consultation, Part 1:Diagnosis and Classification of Diabetes Mellitus*. 1-59.

Yanagisawa, M. and Otsuka, M. (1990). Pharmacological profile of a tachykinin antagonist, spantide, as examined on rat spinal motoneurones. *British Journal of Pharmacology*.

Yarrow, S., Ferrar, J.A. and Cox, H.M. (1991). The effect of capsaicin upon electrogenic ion transport in rat descending clolon. *Naunyn-Schmiedeberg's Ach. Pharmacol.* 344:557-563.

Yiangou, Y., Facer, P., Dyer, N.H., Chan, C.L., Knowles, C., Williams, N.S. and Anand, P. (2001). Vanilloid receptor 1 immunoreactivity in inflamed human bowel. *Lancet.* 357:1338-9.

Yoshimura, N. and Chancellor, M.B. (2002). Current and future pharmacological treatment of overactive bladder. *J. Urol.* 168:1897-1913.

Yoshimura, N. and Chancellor, M.B. (2007). Physiology and pharmacology of the bladder and urethra (Section XIV, Chapter 56). *In* : Wien, A.J. (ed). *Campbell-Walsh Urology.* Vol. 3, 9th edn., Saunders, Philadelphia, PA., pp. 1922-1972.

Yoshimura, N., Chancellor, M.B., Andersson, K.E. and Christ, G.J. (2005). Recent advances in understanding the biology of diabetes-associated bladder complications and novel therapy. *BJU International*. 95:733-738.

Yoshimura, N., Kaiho, Y., Miyazato, M., Yunoki, T., Tai, C., Chancellor, M.B. and Tyaki, P. (2008). Therapeutic receptor targets for lower urinary tract dysfunction. *Naunyn-Schmiedeberg's Ach Pharmacol*. 377:473-448.

Zahner, M.R., Li, D.P., Chen, S.R. and Pan, H.L. (2003). Cardiac vanilloid receptor 1-expressing afferent nerves and their role in the cardiogenic sympathetic reflex in rats. *J. Physiol.* 551:515–523.

Zajchowski, L.D. and Robbins, S.M. (2002). Lipid rafts and little caves: compartmentalized signaling in membrane microdomains. *Eur. J. Biochem.* 269:737-752.

Zhang, J., Kendrick, A., Quenby, S. and Wray, S. (2007). Contractility and calcium signaling of human myometrium are profoundly affected by cholesterol manipulation: implications for labor?. *Reproductive Sciences.* 14(5):456-466.

Zhang, L. and Barritt, G.J. (2004). Evidence that TRPM8 is an androgen-dependent Ca^{2+} channel required for the survival of prostate cancer cells. *Cancer Res.* 64:8365-8373.

Zidovetzki, R. and Levitan, I. (2007). Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta*. 1768(6):1311-1324.

Zimmet, P. (1997). in *The Medical Challenge: Complex Traits* (eds Fischer, E. & Moller, G.) 55-110 (Piper, Munich,).

Zimmet P, Alberti KG, Shaw J. (2001). Global and societal implications of the diabetes epidemic. *Nature.* 414:782-787.