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Investigation of the physiology and pathophysiology of streptozotocin-induced diabetic rat bladder

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A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy

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"I want to dedicate this thesis to my wonderful parents for their love, endless support and encouragement."

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Abstract

In recent decades one of the major forces driving lower urinary tract research has been the aim of discovering the origins of bladder dysfunction and developing new therapeutic agents for treatment of conditions such as detrusor overactivity (DO). It is now becoming clear that the simplistic view of the control of detrusor function is far more complicated and involves complex interactions between various components of the bladder. With limited access to human tissue, animal models have been used for investigating the underlying mechanisms that control the detrusor function under pathological states.

The aim of this project was to investigate the physiology and pathophysiology of bladder function in the streptozotocin (STZ)-induced diabetic rat model, 1, 4, 8 and 12-weeks post STZ injection. This was achieved by a combination of pharmacological, molecular biology and immunostaining techniques.

Antimuscarinic drugs are the current pharmacological agents available for treatment of DO; however, there is much controversy surrounding the muscarinic mediated responses of the bladders from animal models of DO. Therefore, the muscarinic receptor mediated smooth muscle contractions were investigated in detrusor strips from the diabetic rat. An increased agonist mediated response was detected in detrusor strips from 1-week diabetic rats, which was mediated by M3-muscarinic receptor subtype.

Basal spontaneous activity (SA) was detected in detrusor strips from control (non-diabetic) and diabetic but not in 12-week diabetic tissues. 1-week diabetic tissues showed increased amplitude of basal SA compared to all other groups. Since increased amplitude of SA of the bladder is thought to be associated with DO, the mechanisms that may be regulating these contractions were explored. SA could be induced/modulated by low concentrations of muscarinic receptor agonist carbachol (CCH). Once again, the amplitude of CCH-induced SA was significantly bigger in 1-week diabetic tissues compared to all other groups. No role for the mucosa in mediating the CCH-induced SA was demonstrated; however, the mucosa may have a role in 12-week diabetic tissues.

The role of various potassium (K^+) channels in modulating the SA was also investigated. Results demonstrated that opening of large conductance calcium activated potassium channels (BK_{Ca}) reduced the amplitude of CCH-induced SA in both control (non-diabetic) and diabetic groups, whilst blocking these channels resulted in an increased basal SA in all groups except 12-week diabetic tissues. A decreased expression of BK_{Ca} channel subunits' mRNA was detected in all diabetic bladders. Opening of ATP sensitive K^+ channels (K_{ATP}) only decreased the frequency of CCH-induced SA. Blocking K_{ATP} channels had no effect on basal SA in all tissues. A reduced expression of K_{ATP} channel subunits' mRNA was also detected in diabetic rat bladders.

Recently a specialised type of cell, termed interstitial cells (ICs) has become the focus of research. It is postulated that they are involved in mediating the mechanosensory function of this organ. In this study, ICs were also identified in the rat urinary bladder using molecular biology and immunostaining. ICs were shown to play a role in mediating the CCH-induced SA of detrusors from rats, since their inhibition resulted in reduced SA in both control (non-diabetic) and diabetic tissues.

Marked differences were seen between 1-week and 12-week diabetic rat bladders and it was concluded that 1-week diabetic rats can be used as a model of DO.

In conclusion, the data presented in this thesis, indicates that complex mechanisms and physiological processes are present in the urinary bladder. It is clear that little is known about the detailed integrated physiology of the bladder wall and the structures involved in mediating the detrusor contractility.

Publications

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Chapter 1: General Introduction

1.1 The structure and function of the urinary bladder

The urinary bladder is a hollow distensible muscular organ which has two important functions: to store urine and to void urine in a controlled way. The storage and periodic elimination of urine is dependent on the activity of the reservoir, the urinary bladder, and an outlet consisting of the bladder neck, the urethra, and the striated muscles of the urethral sphincter.

1.1.1 Anatomy of the urinary bladder

The bladder is situated in the pelvic cavity posterior to the pubic symphysis, which in males is directly anterior to the rectum and in females is anterior to the vagina and inferior to the uterus. The shape of the urinary bladder alters according to the amount of urine that it contains. The empty bladder is collapsed and lies entirely within the pelvis. As it fills it takes on a spherical or pear shape and its superior wall rises into the hypogastric region (Tortora & Grabowski, 2003; Snell, 2004). The position of the bladder is stabilised by ligaments which connect the inferior and anterior surface of the bladder to the pubic and pelvic bones.

The urinary bladder can be divided into two pharmacologically and physiologically distinct regions. The first region is the bladder body or dome which is a hollow smooth muscle region lined by a mucous membrane and covered on its outer aspect by peritoneal serosa and partly by fascia (Gosling, 1979). The second region of the bladder is a much smaller region extending from the ureters to the urethra called the bladder base or neck and consists of the trigone, urethrovesical junction, deep detrusor and the anterior bladder wall. A schematic drawing of the bladder can be seen in Figure 1.

1.1.2 The bladder wall

The wall of the urinary bladder comprises of three layers. The deepest is the mucosa which is a mucous membrane composed of urothelium and the underlying lamina propria. Below the mucosa is the intermediate muscularis or the detrusor muscle. The most superficial coat of the urinary bladder, on the

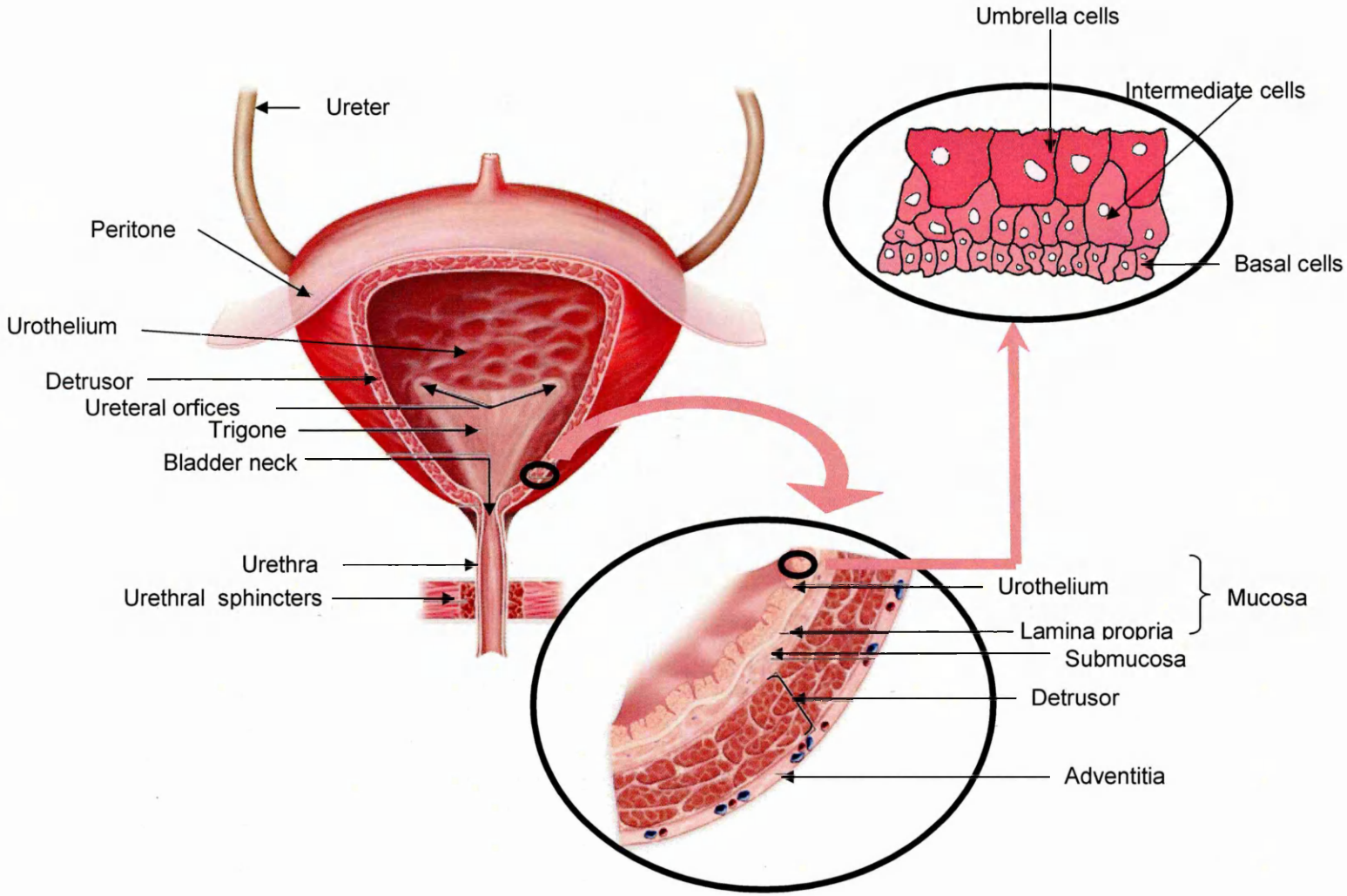


Figure 1) Schematic drawing of the structure of the bladder and its urothelium. Adapted from Lewis (2000) & McCinley & O'Loughlin (2006)

posterior and inferior surfaces, is the adventitia or serosa, a layer of areolar connective tissue.

1.1.2.1 The detrusor

The main component of the wall of the bladder is the smooth muscle or the detrusor. The detrusor smooth muscle cells are arranged into fascicles or bundles which are organised in groups around the bladder. The orientation of the detrusor muscle fibres is neither circular nor longitudinal, rather the muscle bundles cross each other forming a meshwork, particularly at the sides of the bladder. The muscle bundles may interconnect and furthermore individual smooth muscle cells themselves are interconnected via gap junctions. The individual smooth muscle cells in the detrusor are typically long spindle shaped cells with a central nucleus. Interspersed between smooth muscle bundles is connective tissue, composed primarily of collagen and elastic fibres (reviewed by Andersson & Arner, 2004).

During the filling of the bladder, the smooth muscle cells have to relax and be able to stretch and rearrange themselves over a very large area to accommodate the increase in volume without a significant rise in pressure. In other words, the bladder wall must be extremely compliant. Thus, there must be a continuous contractile activity in the smooth muscle cells to adjust their length during filling (reviewed in Turner & Brading, 1997; Andersson & Arner, 2004). The bladder of many species, including man, is rhythmically active during the filling phase. It is believed that this activity, also called spontaneous activity (SA), autonomous activity or micromotions, is generated within the bladder wall and may be important in sensation of bladder fullness and maintaining bladder compliance. Recent work has shown that the SA involves waves of contraction and localised stretches of the bladder wall. The mechanisms generating these complex events are still unclear and they may involve a network of specialised cells known as interstitial cells (ICs) driven by excitatory and inhibitory neural inputs (Gillespie, 2004), which are discussed in more detail in Chapter 6.

During voiding, force generation and shortening of smooth muscle cells must be initiated relatively quickly to allow the shape of the bladder to conform to the minimum surface area/volume ratio possible. In the normal bladder, the smooth

muscle possesses a unique structure and characteristics that allows these requirements to be fulfilled. This structure is composed of contractile proteins and filaments such as actin and myosin that are not arranged into distinct sarcomeres. Cyclic attachment and detachment of the globular portion of the myosin molecules to the actin filaments results in the sliding of the filaments past each other and smooth muscle contraction (reviewed by Adelstein & Eisenberg, 1980 & Andersson & Arner, 2004).

1.1.2.2 The urothelium

The urothelium, or transitional epithelium, is a specialised lining of the urinary tract, extending from the renal pelvis to the urethra. The urothelium is composed of three layers; a basal cell layer attached to a basement membrane which is germinal in nature; an intermediate layer; and a superficial apical layer with large hexagonal cells with diameters of 25–250 µm, which are also termed "umbrella cells" (Figure 1) (reviewed in Lewis, 2000). The apical membrane of umbrella cells is covered with scalloped shaped plaques which are separated by plasma membrane domains called the "hinge". High resistance tight junctions are found between the umbrella cells and effectively divide the cell surface into apical and basolateral membrane domains. These cells function as a barrier against most substances found in the urine thus protecting the underlying tissue. The apical membrane of the umbrella cells also has a unique lipid and protein composition that contributes to the low permeability of these cells (reviewed in Lewis, 2000 & Apodaca, 2004).

Historically, the bladder urothelium has been considered only as a passive barrier that prevents urine leakage. However, recent studies have shown that the urothelium is a highly specialised structure, involved in antigen presentation, physical protection, the micturition reflex, metabolic secretion, inflammatory regulation and mechano-sensory signal transduction (reviewed in Birder, 2004 & Moore & Goldman, 2006). Studies over the last few years indicate that urothelial cells display a number of properties similar to sensory neurons. It is believed that the urothelium, when exposed to physiological and mechanical stimuli, receives and transmits signals to submucosal neurons. Specialised sensory and signalling properties that allow the urothelial cells to respond to different stimuli include the expression of nicotinic, muscarinic, tachykinin,

adrenergic, bradykinin and transient potential receptors, including the vanilloid receptors, TRPV1, TRPV2 and TRPV4 etc. (Birder *et al*, 2001; Birder, 2004; Beckel *et al*, 2006). The sensory role of the urothelium is also suggested in its responsiveness to transmitters released from sensory nerves, close association with suburothelial afferent nerves and release of chemical mediators such as ATP (Ferguson *et al*, 1997), tachykinins (Lecci & Maggi, 2001); ACH (Yoshida *et al*, 2008) and nitric oxide (NO) (De Groat, 2004) allowing reciprocal communication with neighbouring urothelial cells as well as underlying components such as myofibroblasts.

The urothelium can also modulate the underlying smooth muscle tone and inhibit detrusor contractility *in vitro*. Substances such as NO released from the urothelium are able to alter smooth muscle tone by activating guanyl cyclase which increases cyclic guanosine monophosphate (cGMP) levels and causes smooth muscle relaxation (Persson *et al*, 1993). Endogenous prostaglandins are also released from the urothelium which are important in increasing detrusor tone (Pinna *et al*, 2000). The presence of an unidentified urothelium derived inhibitory factor (UDIF), which is released from the urothelium and can inhibit detrusor contraction *in vitro,* has also been reported by several groups (Hawthorn *et al*, 2000; Templeman *et al*, 2002; Chaiyaprasithi *et al*, 2003).

The mechanisms by which the urothelium modifies detrusor contractile responses may be important for normal bladder function and thus dysfunction of any of these mechanisms may well be involved in bladder disorders such as detrusor overactivity (DO).

1.1.2.3 Interstitial cells (ICs) / myofibroblasts

A recently identified population of cells, known as myofibroblasts or ICs, has been identified in the bladder of many species (McCloskey & Gurney, 2002; Shafik *et al*, 2004; Van der AA *et al*, 2004; Metzger *et al*, 2008). These cells are morphologically similar to interstitial cells of Cajal (ICCs) in the gut and are thought to be found immediately beneath the urothelium as well as on the outer detrusor layers and on the boundary of smooth muscle bundles, where they seem to play an important role in processing of sensory information, pacemaking and modulating detrusor contractility (Sanders, 1996; McCloskey & Gurney, 2002; Hirst & Ward, 2003; Shafik *et al*, 2004; Van der AA *et al*, 2004;

Metzger *et al*, 2008). Further analysis of the function and the location of these cells in the urinary bladder will be discussed in Chapter 6.

1.2 Innervation of the bladder and control of voiding

1.2.1 The micturition reflex

Storage of urine and voiding comprise the two phases of the micturition cycle. The bladder and urethra function reciprocally to maintain the cycle. As the bladder fills during the storage phase, the detrusor remains relaxed with little change in the intravesical pressure. The neural pathways that stimulate the bladder are quiescent during this phase, and inhibitor pathways are active. The urethral outlet stays closed, with progressively increasing external urethral sphincter contractions, which act as a guarding reflex. When the bladder volume reaches a critical threshold, the urethral sphincter relaxes and the detrusor engages in a series of contractions which result in opening of the bladder neck and elimination of urine (Hanno *et al*, 2001).

1.2.2 Autonomic Pathways

The control of the micturition cycle is dependent upon neural circuits located in the brain, spinal cord and peripheral ganglia. The bladder has dense innervation of autonomic parasympathetic (pelvic), thoracolumbar sympathetic (hypogastric) and somatic (pudendal) nerves (Reviewed in De Groat, 1997 & Andersson and Arner, 2004), which often have opposing functions (Figure 2).

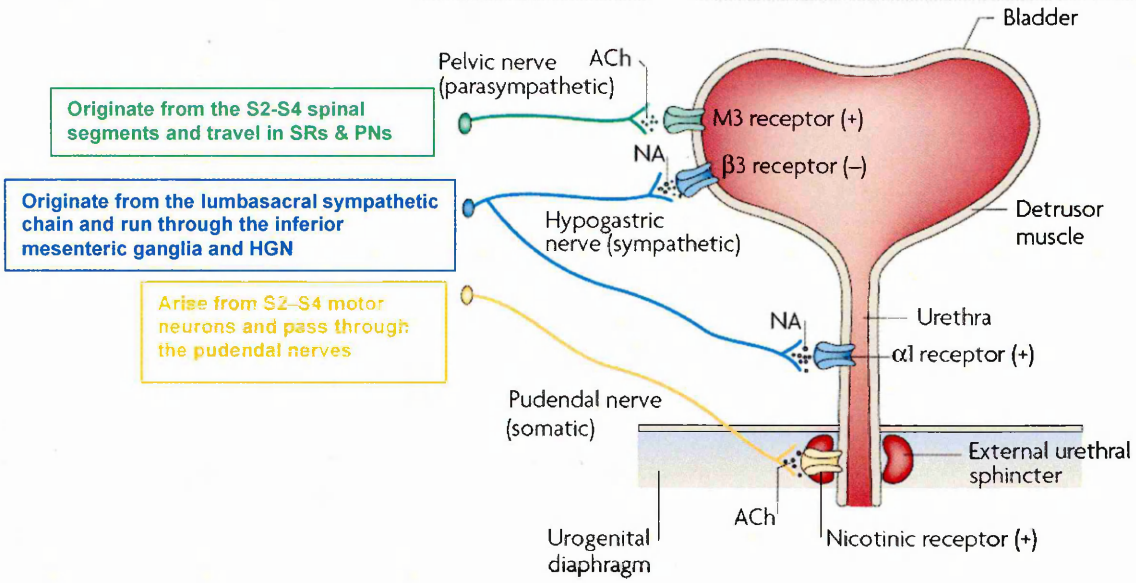


Figure 2) Efferent pathways of the lower urinary tract. Efferent pathways and neurotransmitter mechanisms that regulate the lower urinary tract. Parasympathetic postganglionic axons which arise from the S2-S4 spinal segments and travel in sacral roots and pelvic nerves (PNs) release acetylcholine (ACH), which produces bladder contraction by stimulating muscarinic receptors (M3-subtype) in the bladder smooth muscle. Sympathetic postganglionic neurons originating from the T11-L2 segments in the spinal cord and travelling through the inferior mesenteric ganglia and the hypogastric nerve (HGN), release noradrenaline (NA), which activates β3 adrenergic receptors to relax bladder smooth muscle and activates α1 adrenergic receptors to contract urethral smooth muscle. Somatic axons which arise from the S2-S4 spinal segments and pass through the pudendal nerves, also release ACH, which produces a contraction of the external sphincter striated muscle by activating nicotinic cholinergic receptors. Adapted from Fowler et al, 2008.

1.2.2.1 Parasympathetic pathways

The parasympathetic outflow is the main provider of excitatory input to the bladder. It densely innervates the bladder, in a way that nerve fibres are intimately related to individual muscle cells. Parasympathetic postganglionic nerves arise from the S2-S4 spinal segments through the pelvic nerves where they release both ACH and non-adrenergic non-cholinergic (NANC) transmitters (Figure 2). Cholinergic transmission is the major excitatory mechanism in human bladder and results in detrusor contraction and micturition (reviewed by Bissada *et al*, 1977, Andersson, 1993, Andersson & Arner, 2004 & De Groat, 2006). ACH, the primary neurotransmitter responsible for initiation of contraction in the detrusor, acts on the muscarinic receptors on the smooth muscle and

results in subsequent activation of the contractile machinery leading to micturition. Muscarinic receptors are also present on parasympathetic nerve terminals at the neuromuscular junction and in the parasympathetic ganglia. Activation of these receptors on the nerve terminals can inhibit (through M4-muscarinic receptors) or enhance (through M1-muscarinic receptors) ACH release, depending on the intensity of neural firing (Somogyi *et al*, 1994; Inadome *et al*, 1998; Shen & Mitchelson, 2001). There is a small contribution from other neurotransmitters (NANC) and receptors towards bladder contraction. This NANC component seems to vary with species and the frequency of neuronal stimulation of bladder detrusor (reviewed by Andersson & Arner, 2004 & Canda *et al*, 2006). It is believed that contribution of NANC component can increase in conditions associated with DO (reviewed by Canda *et al*, 2006). NANC pathways involve the purinergic system/ATP, nitric oxide (NO), vanilloide, prostanoids, neuropeptides and their receptors (reviewed by Andersson, 1993, Burnstock, 2001 & Andersson & Arner, 2004).

1.2.3 Sympathetic Pathways

Sympathetic efferent innervation of the urinary bladder results in relaxation of the detrusor body and contraction of the bladder neck and urethra during the filling phase of the micturition cycle. Sympathetic nerves originate in T11-L2 segments in the spinal cord and travel through the inferior mesenteric ganglia and the hypogastric nerve (HGN), and subsequently release noradrenaline (NA) (Figure 2). The bladder body contains a high density of β-adrenoreceptors (mainly of β2 and β3 subtypes), stimulation of which results in relaxation and urine storage. NA also activates the α-adrenergic excitatory receptors (mainly of α1 subtype) in the urethra, resulting in smooth muscle contraction and subsequent closure of the bladder neck during the filling phase of the micturition cycle reviewed in Andersson and Arner, 2004 & De Groat, 2006; Yamaguchi & Chapple, 2007).

1.2.4 Somatic pathways

Somatic innervation of the urethral sphincter results in contraction of the external striated muscle and subsequent inhibition of voiding. Somatic efferent pathways arise in S2-S4 motor neurons in Onuf's nucleus and reach the periphery through the pudendal nerves (Figure 2). The ACH released from

these nerves excites nicotinic receptors on the sphincter muscle and results in urethral contraction (reviewed in de Groat, 1993, 2006 & Yoshimura & Chancellor, 2003).

1.2.5 Afferent pathways

During storage of urine there is ongoing afferent signalling that allows continuous sensation of bladder fullness and stretch. Signals from the bladder body are transmitted to the spinal cord by the pelvic and HGNs, whereas sensory input from the bladder neck and urethra is carried in the pudendal and HGNs (reviewed by Fowler *et al*, 2008). The most important afferent components of these nerves are the small myelinated A-δ and non-myelinated C fibres. A-δ fibres, which are located primarily within the detrusor muscle layer, respond to passive distension and active contraction and thus convey information about bladder filling. On the other hand, C fibres reside in the muscle close to the urothelium and are insensitive to bladder filling under physiological conditions. It appears that C fibres are more important in signalling of inflammatory and noxious events in the bladder and are sensitive to many different substances including tachykinins, NO, ATP, prostaglandins, endothelins and other neurotrophic factors which are released in the bladder from the afferent nerves as well as urothelial cells (Habler *et al*, 1990 ; De Groat, 1993 ; Rong *et al*, 2002; De Groat *et al*, 2006).

Since the cholinergic neurotransmitter, ACH, is the main mediator responsible for detrusor and urethral smooth muscle contraction, it is therefore important to understand the structure and function of the muscarinic receptors involved in mediating the effects of ACH in urinary bladder smooth muscle (UBSM).

1.2.6 Muscarinic Receptors: characterisation of structure and function

The muscarinic ACH receptors are members of a family of plasma membrane receptors which mediate signal transduction by coupling with heterotrimeric guanine nucleotide binding proteins (G-proteins). There are five receptor subtypes (M1-M5) and they are known to share a similar three dimensional structure, consisting of a single protein with seven transmembrane domains (TM1-7) linked by three intracellular and three extracellular loops (Figure 3)

(Burstein *et al*, 1995; Wess *et al*, 1995; Felder, 1995). A schematic structure of these receptors is illustrated in Figure 3. It is believed that the seven transmembrane domains of muscarinic receptors assemble in a packed ring-like structure to form the ligand binding pocket. The N-terminus of the muscarinic receptor resides on the extracellular side of the membrane and contains glycosylation sites. The C-terminus is located on the cytoplasmic domain and contains a cysteine residue which is highly conserved across G-protein coupled receptors. There is a high degree of conservation between the 5 different muscarinic receptor subtypes in the sequence of their 7 transmembrane domains. Marked differences are seen in the hydrophilic cytoplasmic domains and extracellular loops and tails and it has been shown that cytoplasmic domains are important for G-protein coupling (Felder, 1995).

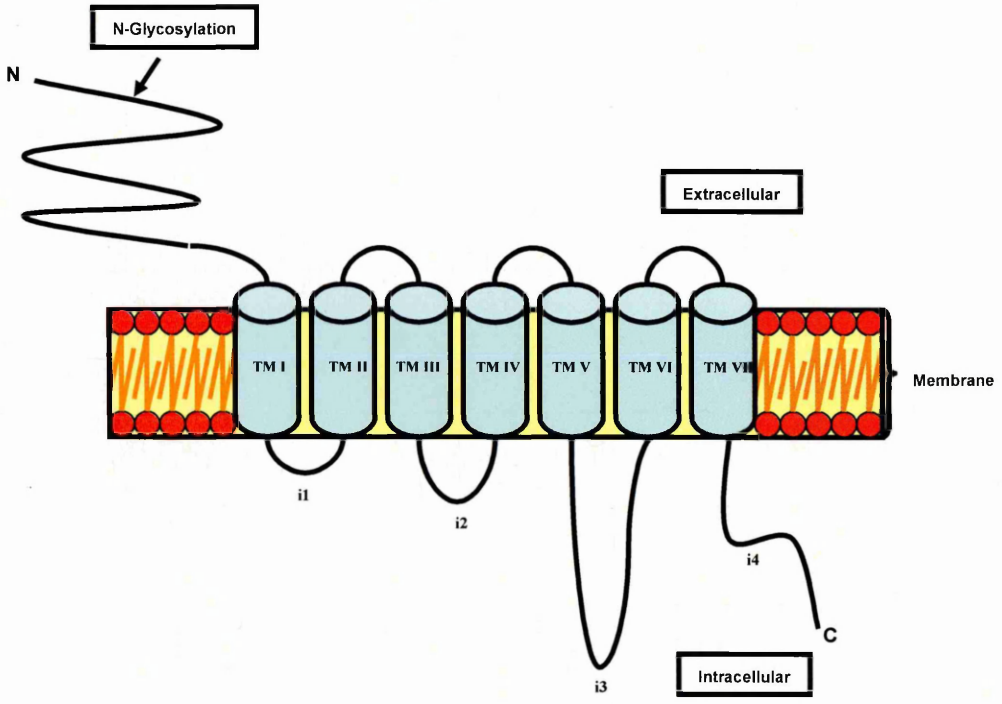


Figure 3) Topology of a muscarinic acetylcholine receptor. i: intracellular loop. TM: transmembrane domain. Diagram produced from information given by Felder *et al*, 1995.

By a mechanism that is still unknown, ACH binding to the transmembrane core of the muscarinic receptors triggers conformational changes in the receptor proteins which enable distinct cellular receptor domains to recognise and activate specific sets of G proteins. Muscarinic receptors stimulate signalling by binding to a heterotrimeric G-protein complex consisting of α, β and γ subunits which provide the specificity for coupling to an appropriate effector molecule.

Upon interaction with the muscarinic receptor, the α-subunit dissociates from the β and γ subunits and then interacts with an effector protein or ion channel to stimulate or inhibit a cascade of intracellular signalling pathways (Felder, 1995).

An important molecular distinction between the different muscarinic receptor subtypes is their specific coupling preferences to G-proteins. Muscarinic receptors can be classified into two major functional categories: pertusis toxin sensitive and insensitive receptors. M1, M3, and M5 are coupled to the pertussis toxin insensitive G_q/G_{11} protein family. These are a class of G-proteins that are not susceptible to ADP-ribosylation by pertussis toxin (Caulfield, 1993; Kostenis *et al*, 1997; Longhurst and Uvelius, 2001). Stimulation of these receptors mediates the activation of several isoforms of phospholipase C-β (PLCβ) resulting in the breakdown of phosphatidyl inositol lipids (PI hydrolysis). The second group includes M2 and M4 receptors, which are linked to pertussis toxin sensitive G (G_i) proteins (G proteins that are susceptible to ADP-ribosylation by pertussis toxin), activation of which results in inhibition of adenylate cyclase (Felder, 1995; Wess *et al*, 1995).

1.2.7 Muscarinic receptors in the bladder

Cholinergic muscarinic receptors are found throughout the bladder and are found both prejunctionally and postjunctionally. Prejunctionally, they have been identified on parasympathetic and sympathetic nerve endings, where they regulate ACH and NA release. It is believed that M1 receptors at prejunctional sites on cholinergic nerve terminals in the bladder are facilitatory with activation resulting in ACH release, especially during voiding. However M2- and M4-muscarinic receptors are inhibitory receptors and their activation reduces the release of ACH (Braverman *et al*, 1998; Igawa, 2000). Postjunctional muscarinic receptors can be found in two locations: on the detrusor smooth muscle, where they mediate contraction and on the urothelium at a density twice that of the detrusor, where they cause release of UDIF which inhibits detrusor contraction (Chess-Williams, 2002). The presence of M2 and M3, but not M1, M4 or M5, has been confirmed at the postjunctional level by immunoprecipitation studies using human, rabbit, guinea pig and rat urinary bladder. Several studies have

reported that the M2 receptor is the most abundant muscarinic subtype in the bladder, with a M2:M3 ratio of 9:1 in rat bladder and 3:1 in all other species studied. Despite the dominant presence of the M2 receptor, pharmacological *in vitro* characterisation of muscarinic receptors in rat (Wang *et al*, 1995; Longhurst *et al*, 1995), rabbit (Choppin *et al*, 1998), guinea pig (Noronha-Blob *et al*, 1989), pig (Sellers *et al*, 2000) and human (Chess Williams, 2001) bladders indicate that the direct contraction of detrusor is mediated by the M3-muscarinic receptor, with the role of the M2-muscarinic receptor remaining unclear.

1.2.7.1 M3 muscarinic receptor signalling pathways

M3-muscarinic receptors act via a variety of second messenger systems to cause direct smooth muscle contraction. In the bladder of several species, including human, the traditional signal transduction mechanism of the M3-muscarinic receptor is stimulation of phosphoinositide hydrolysis. It is generally accepted that the M3 muscarinic receptor activates phospholipase C (PLC) via pertusis toxin-insensitive G_q/G_{11} proteins. PLC in turn accelerates the rate of phosphatidyl inositol (4, 5) biphosphate (PIP2) hydrolysis. The breakdown of PIP2 results in formation of inositol triphosphate (IP3) and diacylglycerol (DAG) and these molecules act as the second messengers. IP3 mobilises calcium (Ca^{2+}) from intracellular stores and DAG activates protein kinase C (PKC). Ca^{2+} is believed to bind to calmodulin, a 16.8kD protein containing four Ca^{2+} binding sites, and the Ca^{2+}/calmodulin complex then activates myosin light chain kinase (MLCK) which catalyses transfer of phosphate from MgATP to a serine residue near the N-terminal of myosin regulatory light chains. Phosphorylation of myosin light chain allows cross-bridge cycling and smooth muscle contraction. The reverse reaction is catalysed by myosin light chain phosphatase type I (MLCP) (Figure 4) (reviewed by Gallagher *et al*, 1997; Sanders, 2001; Andersson & Arner, 2004;).

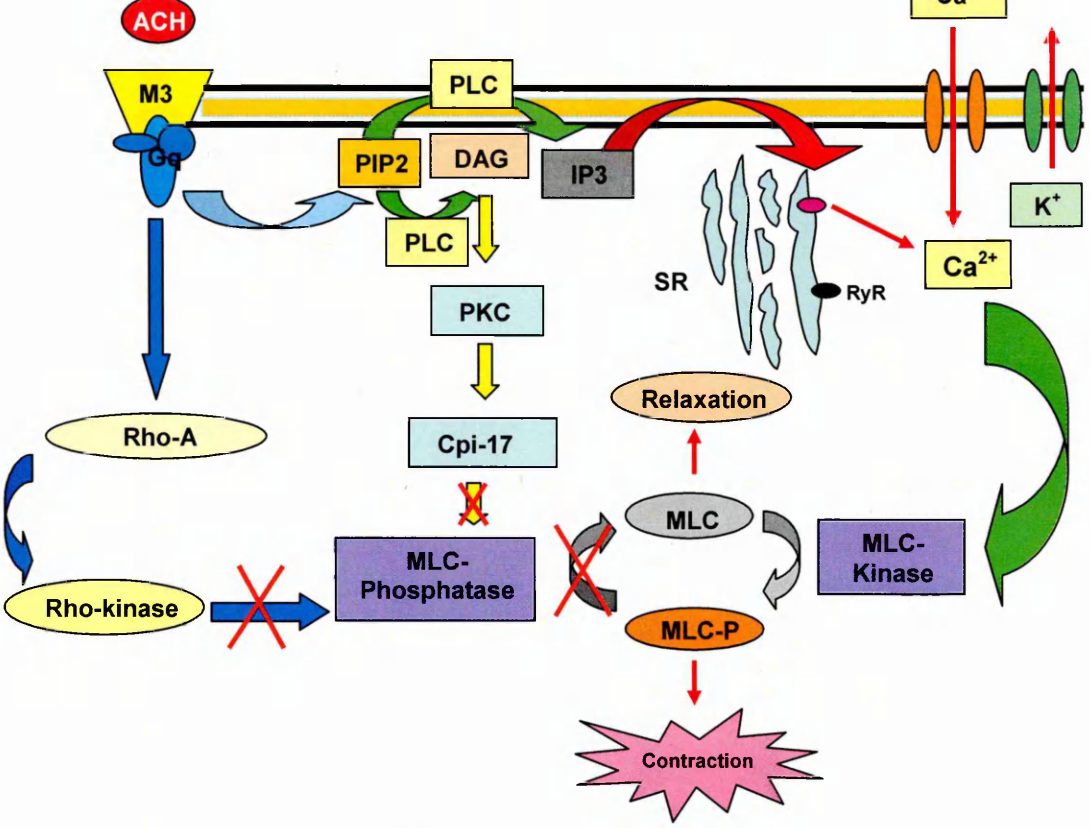


Figure 4) Regulation of smooth muscle contraction via M3-muscarinic receptor stimulation: M3-muscarinic receptor contributes to smooth muscle contraction via Ca^{2+} dependent and independent mechanisms. ACH, acetylcholine; PIP2, phosphatidyl inositol (4, 5) biphosphate; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; CPi-17 for PKC-potentiated inhibitory protein of 17 kDa; MLC, myosin light chain; IP3, inositol trisphosphate; SR, sarcoplasmic reticulum; RyR, ryanodine receptor. Adapted from Somlyo & Somlyo, 2000; Andersson & Arner, 2004.

Although elevation of intracellular Ca^{2+} concentration plays a central role in smooth muscle contraction, there is still much debate about the precise mechanism of intracellular Ca^{2+} release. It was originally thought that the M3-muscarinic receptor mediated contractile response in the bladder was mediated by intracellular Ca^{2+} mobilisation by IP3. This assumption has been re-examined by studies which have shown that the M3-mediated response in the bladder of various species is mediated by an increase in intracellular Ca^{2+} through L-type voltage dependent Ca^{2+} channels (VDCCs), especially at low concentrations of muscarinic agonists, since force development is diminished in Ca^{2+} channel (CAv1.2) knockout mice or in the presence of VDCCs blocker nifepidine (Wu *et al*, 1999; Fry *et al*, 2002; Schneider *et al*, 2004a, Wegner *et al*, 2004; Rivera & Brading, 2006; Wuest *et al*, 2007). Thus, M3-muscarinic receptor mediated detrusor contraction involves Ca^{2+} entry via VDCCs followed by uptake of Ca^{2+} into intracellular stores and subsequent release either via

IP3-dependent or Ca^{2+}-induced Ca^{2+} release processes via ryanodine receptors (Figure 4) (Wuest *et al*, 2008; Rivera & Brading, 2006; Christ & Hodge, 2006). It is believed that at high agonist concentration the release of Ca^{2+} from intracellular stores becomes increasingly important (Chess-Williams, 2002; Schneider *et al*, 2004a, Schneider *et al*, 2004b).

Although Ca^{2+}-dependent mechanisms are important in mediating M3-muscarinic receptor modulated smooth muscle contraction, recently secondary mechanisms have been identified that can modulate bladder contractility independently of Ca^{2+} (i.e. without necessarily changing the intracellular Ca^{2+}). This process is called Ca^{2+} sensitisation. The small GTPase Rho and one of its downstream effectors, Rho-associated kinase (ROCK), have been shown to play an important role in this mechanism (Wu *et al*, 1999; Fry *et al*, 2002; Schneider *et al*, 2004a, Peters *et al*, 2006). Activated ROCK phosphorylates the regulatory subunit of MLCP and inhibits phosphatase activity, which results in prevention of myosin light chain dephosphorylation, resulting in Ca^{2+} sensitisation of the detrusor smooth muscle and contraction (Figure 4) (Wibberley *et al*, 2003). Ca^{2+} sensitisation by the Rho/ROCK pathway is believed to be important in regulating the tonic phase of agonist-induced contraction in smooth muscle.

1.2.7.2 M2-muscarinic receptor signalling pathways

The signalling mechanisms for the M2-muscarinic receptors are less clear than those for the M3-muscarinic receptor subtype. The M2-muscarinic receptor is not involved in direct contraction of the bladder smooth muscle. It is generally believed that the M2-muscarinic receptors are coupled through the inhibitory G protein to adenylate cyclase (AC) and aid bladder contraction by preventing β-adrenoceptor-induced formation of cyclic adenosine mono phosphate (cAMP), thus reversing NA-induced relaxation. Therefore, activation of M2-muscarinic receptor during micturition may "switch off" the sympathetic inhibitory β-adrenoceptor-mediated relaxation and result in more efficient emptying (Figure 5) (Chess-Williams, 2002).

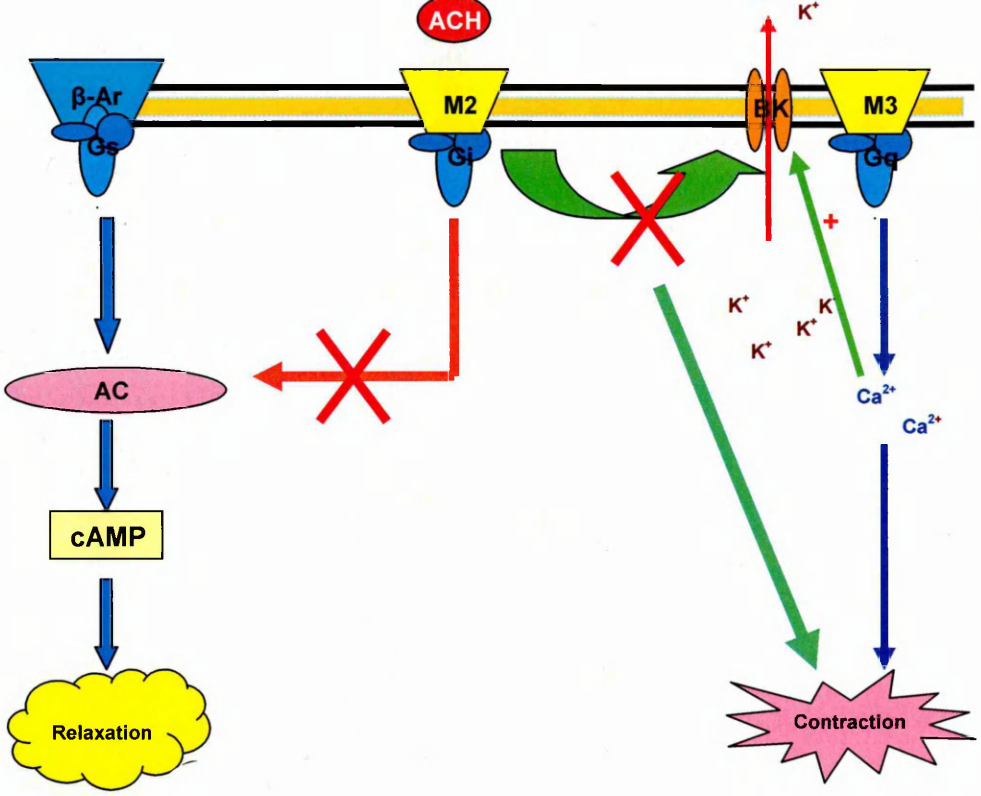


Figure 5) Regulation of smooth muscle contraction via M2-muscarinic receptor stimulation: M2-muscarinic receptor contributes to smooth muscle contraction by reversing the β-adrenoreceptor mediated relaxation via cAMP. M2-muscarinic receptor also inhibits the BK_{Ca} channels thus enhancing the contraction initiated by M3 muscarinic receptor stimulation. ACH, acetylcholine; β-Ar, β-adrenoreceptors; AC, adenylate cyclase; BK, large conductance Ca^{2+}-activated K^+ channels. Diagram was produced from information given in review by Andersson & Arner, 2004, Chess-Williams, 2002 & Nakamura *et al*, 2002.

M2-muscarinic receptors can also activate smooth muscle cells by decreasing the probability of potassium (K^+) channels opening. For example Nakamura *et al* (2002) have shown that M2-muscarinic receptor stimulation in rat bladder smooth muscle cells results in inhibition of large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) and subsequent contraction of UBSM cells. Since BK_{Ca} channels are important in modulation of UBSM excitability and restoration of the resting membrane potential, inhibition of these channels by M2-muscarinic receptors should result in increased action potential duration and prolonged contraction. However, M2-muscarinic receptor mediated smooth muscle contraction is contingent upon Ca^{2+} mobilisation by another receptor like the M3-muscarinic receptor (Nakamura *et al*, 2002). Thus, it has been suggested that the M2-muscarinic receptor may only enhance smooth muscle contraction

which is initiated by the M3-muscarinic receptor (Heppner *et al*, 1997; Ehlert, 2003).

It has also been demonstrated that under conditions where the M3-muscarinic receptor is inactivated and cAMP levels have been increased, the contractile responses of the bladder smooth muscle can be attributed to M2-muscarinic receptor activation (Yaminishi *et al*, 2000). For example, in pig bladder removal of the M3 receptor population with an alkylating agent e.g. 4-DAMP mustard (N-2-chloroethyl-4-piperidinyl diphenylacetate), a selective M3-muscarinic receptor antagonist, followed by elevation in cAMP levels can illicit a response to muscarinic receptor agonists that is at least partially mediated via the M2-muscarinic receptor subtype (Chess-Williams, 2002). The same type of mechanism has been demonstrated in rat urinary detrusor muscle (Hedge *et al*, 1997). Thus, it has been suggested that the function of this muscarinic receptor, subtype maybe more important in disease states or conditions where the M3-muscarinic receptor is dysfunctional.

Other postulated mechanisms by which the M2-muscarinic receptor may mediate contraction include opening of non-specific cation channels resulting in depolarisation and Ca^{2+} influx which results in stimulation of Rho and Ca^{2+} sensitisation (Bolton & Zholos, 1997).

Since the major signalling cascade of M2-muscarinic receptor is dependent on β-adrenoceptor mediated smooth muscle relaxation, then it is important to understand the role of adrenoceptors as part of sympathetic neurotransmitter pathway in mediating the UBSM contractility.

1.2.8 Adrenoceptors in the bladder

The urinary bladder has been shown to possess functional α- and β-adrenergic receptors that are important in modulating bladder smooth muscle tone. Adrenergic mechanisms are especially important during the filling phase of micturition cycle, where the bladder muscle needs to be relaxed and compliant to allow urine storage (Anderson & Marks, 1983; Michel & Vrydag, 2006). α-adrenoceptors are most prominent in the bladder base and neck and their stimulation results in smooth muscle contraction. However, the key adrenoreceptor involved in mediating detrusor relaxation is the β-subtype (β2

and β3) (reviewed in Andersson & Arner, 2004). β-adrenoceptors mediate detrusor relaxation via coupling of Gs protein to AC, which once activated, catalyses the conversion of ATP to cAMP. cAMP in turn activates PKA, or alternative effector molecules, to mediate smooth muscle relaxation. β-adrenoceptors can also activate BK_{Ca} channels in the detrusor via modulation of localised Ca^{2+} signals and increasing VDCC mediated Ca^{2+} influx (Petkov & Nelson, 2005; Ferro, 2006).

Since both cholinergic and adrenergic receptors, as part of their signalling pathways, interact with various ion channels on the UBSM cell membrane, it is important to understand the role of these channels in mediating the detrusor excitability and contractility in normal and pathological conditions.

1.3 Ion channels involved in mediating bladder smooth muscle function

In smooth muscle, ion conductance across the membrane provides the main trigger for the regulation of cell contraction and excitability. Ion channels provide the major access route to alter the cytoplasmic Ca^{2+} and K^+ concentrations. Various Ca^{2+} and K^+ channels have been identified in UBSM, that play key roles in modulating cellular excitability, signalling and the response to external stimuli.

1.3.1 Role of Ca^{2+} channels in bladder smooth muscle

As described earlier, to activate the contractile apparatus, an increase in intracellular Ca^{2+} concentration must be initiated. The Ca^{2+} utilised for activation of the contractile apparatus enters the cytoplasmic compartment of the smooth muscle cell via at least four types of Ca^{2+} channels: voltage dependent (L-type or VDCCs), store-operated, IP3 receptor and ryanodine receptor channels.

VDCCs and store operated channels are important in influx of Ca^{2+} from the extracellular space and are found abundantly in the plasma membrane of the cell. VDCCs activate upon membrane depolarisation and mediate Ca^{2+} influx in response to action potentials and membrane depolarisation (Catteral, 2000).

Ryanodine receptors (RyR) and IP3 receptors are important in mediating Ca^{2+} release from intracellular stores and are located in the membranes of the SR (reviewed by Sanders, 2001 & Fraizer *et al*, 2008). Ca^{2+} entry via VDCCs triggers Ca^{2+} induced Ca^{2+} release (CICR) from RyRs, resulting in localised Ca^{2+} release termed Ca^{2+} sparks (Collier *et al*, 2000; reviewed by Sanders, 2001 & Chalmers *et al*, 2007). RyR inhibition has been shown to inhibit bladder contraction in rabbits (Zderic *et al*, 1994), guinea-pig (Buckner *et al*, 2002) and human bladder (Visser & Van Mastrigt, 2000), although other investigators have not reported such inhibitions in mouse, pig and human bladders (Wuest *et al*, 2007).

The role of IP3 receptors (IP3-R) in muscarinic receptor mediated smooth muscle contraction was described earlier. IP3-R is the predominant Ca^{2+} release channel on the sarcoplasmic reticulum (SR). Ca^{2+} alone is not enough for IP3-R activation and both IP3 and Ca^{2+} are required for channel activation (Wray *et al*, 2005; Chalmers *et al*, 2007).

Figure 4 demonstrates the role of some of these channels in M3-muscarinic-receptor-mediated smooth muscle contraction.

1.3.2 Role of K^+ channels in bladder smooth muscle

K^+ channels belong to a diverse family of membrane proteins that play important roles in mediating smooth muscle cell excitability and tone. Various types of K^+ channels exist, which are not only activated under distinct conditions but also mediate different physiological functions. The main K^+ channels which have been investigated in the bladder include Ca^{2+}-activated K^+ channels (K_{ca}), ATP-sensitive (K_{ATP}) channels and voltage-gated (K_v) channels.

1.3.2.1 Ca^{2+}-activated potassium (K_{ca}) channels

K_{ca} channels are activated by a local or global increase in the intracellular Ca^{2+} concentration. UBSM exhibits spontaneous action potentials which are related to the phasic nature of spontaneous contractions in this tissue. These phasic contractions depend upon Ca^{2+} entry via VDCCs and production of an action potential. K_{ca} channels are important in restoration of the resting membrane

potential by hyperpolarising the plasma membrane to reduce or inhibit Ca^{2+} entry via VDCCs (Herrera *et al*, 2000). K_{ca} channels are classified by their conductance: small (SK_{Ca})(2-25 pS), intermediate (IK_{Ca})(25-100 pS) and large (BK_{Ca})(100-300pS) (Ghatta *et al*, 2006).

Large Conductance Ca^{2+}- activated potassium channels (BK_{Ca})

BK_{ca} channels are also known as BK, Slo and MaxiK channels. These channels are activated both by changes in intracellular Ca^{2+} and by membrane depolarisation and play a significant role in mediating UBSM tone and excitability. The activation of BK_{Ca} channels results in membrane hyperpolarisation and inhibition of action potential firing.

BK_{Ca} channels consist of two distinct subunits: α and β, arranged in 1:1 stoichometry. Each channel exists as a tetramer, composed of 4 α -subunits either alone or in association with β-subunit pairs. The α-subunit, also termed Slo-1, is encoded by the KCNMA1 gene and is the pore-forming subunit of BK_{ca} channels, whereas the β-subunit, also termed the Sloβ, is the regulatory part of these channels and is the protein product of the KCNMB gene.

The α-subunit is composed of 7 transmembrane spanning domains (S0-S6) at the N-terminus, the P-loop, which is the pore forming loop between S5 and S6 domains, and four hydrophobic segments (S7-10) at the large intracellular C-terminus (Figure 6). The extracellular N-terminus of the α-subunit is important for binding of the β-subunit. The centre of the four α subunits is the potassium selective pore. The pore forming motif of each subunit consists of the P-loop and the S5-S6 domains. The P-loop also contains the binding site for the pore blockers such as iberiotoxin (IBTX) and charybdotoxin (CHTX). The S2, S3 and S4 domains are important in voltage sensitivity of this channel. The Ca^{2+} binding site is believed to reside between the S9 and S10 domains (Ca^{2+} bowl). The C-terminal region also consists of a regulator of conductance of K^+ (RCK) (the S7 and S8 domains), which is important for the physiological regulation of BK_{Ca} channels by Ca^{2+}. However, further Ca^{2+} binding sites may also be present in other regions of this channel (Figure 6) (reviewed by Ghatta *et al*, 2006).

There are four putative β-subunit types : β1- β4. The β-subunit is composed of two transmembrane (TM) units which are connected via an extracellular loop. The residues within this extracellular domain are important in inducing the appropriate conformational change within the α-subunit to promote IBTX or CHTX binding. The β-subunit can also increase the sensitivity of the α-subunit to Ca^{2+}. However, the association of the α-subunit with different β-subunits can modify the pharmacological properties of these channels. The β1-subunit in bladder smooth muscle is important in modulating BK_{Ca} activity and plays a major role in regulation of phasic activity in the detrusor (reviewed by; Andersson & Arner, 2004 & Ghatta *et al*, 2006).

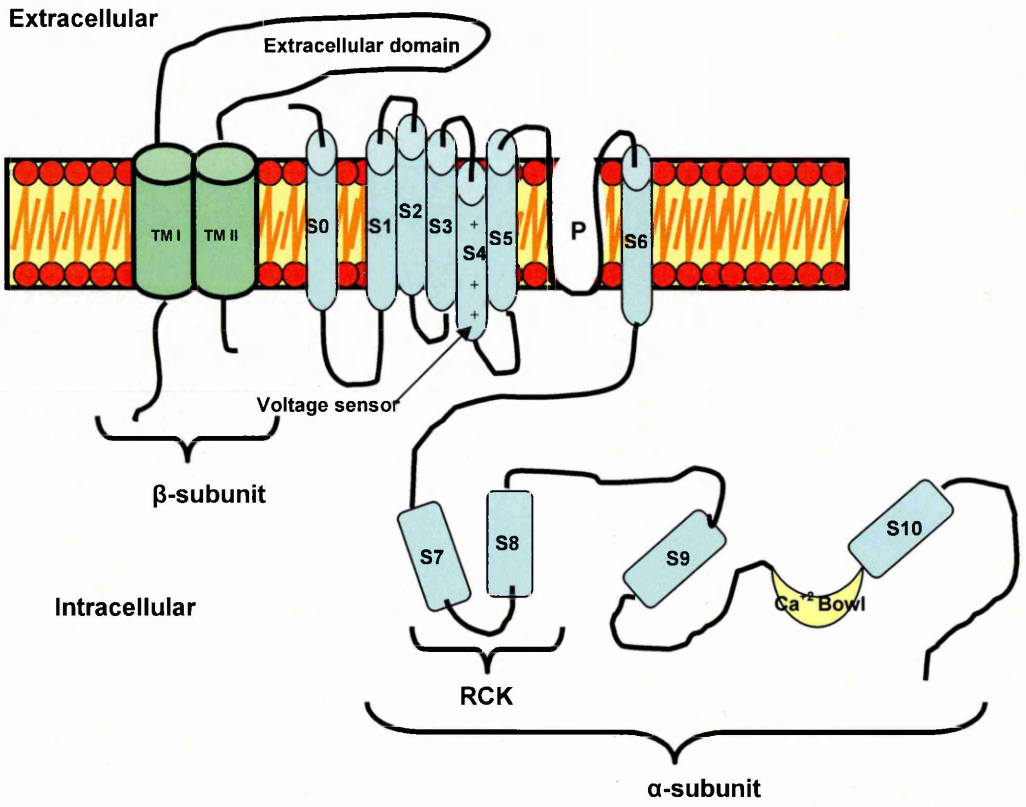


Figure 6) Schematic representation of the structure of BK_{Ca} channels. TM: transmembrane unit of β-subunit, P: pore-forming loop, S: transmembrane spanning domain of the α-subunit . Adapted from Ghatta *et al*, 2006.

Intermediate conductance Ca^{2+} activated potassium (IK_{Ca}) channels

IK_{Ca} channels are not voltage sensitive and are activated only by an elevation of intracellular Ca^{2+} concentration which results in hyperpolarisation of the plasma membrane. It is believed that IK_{Ca} channels in addition to BK_{Ca} channels play an important role in the regulation of membrane potential and bladder smooth muscle tone (Ohya *et al*, 2000).

IK_{Ca} channels are composed of four putative α-subunits only with no regulatory β-subunit associated with these channels. The KCNN4 gene, the protein product of which is termed SK4, encodes the α-subunit of IK_{Ca} channels and has been found in mouse and human urinary bladders (Ishii *et al*, 1997; Ohya *et al*, 2000). The exact function of these channels in UBSM requires further investigation.

Small conductance Ca^{2+}-activated potassium channels

SK_{Ca} channels are activated by an elevation of intracellular Ca^{2+} concentration, but are not voltage sensitive. These channels are important determinants of UBSM excitability and contractility. They underlie the action potential after hyperpolarisation in UBSM. Blockade of these channels results in a substantial increase in action potential frequency and subsequent detrusor contractility (Fujii *et al*, 1990; Herrera *et al*, 2000; Chalmers *et al*, 2007).

The α-subunit of SK_{Ca} channels in the UBSM is encoded by either the KCNN2 (the protein product of which is termed SK2) or KCNN3 (the protein product of which is termed SK3) genes (Wickenden, 2002) and it consists of six transmembrane segments (S1-S6) with the pore located between S5-S6. No regulatory β-subunit is associated with the function of these channels. The Ca^{2+} sensitivity of SK_{Ca} channels is derived from a calmodulin binding site on the intracellular C-terminal domain of the channel near the pore forming region (Stocker, 2004).

1.3.2.2 ATP sensitive K (K_{ATP}) channels

K_{ATP} channels are inhibited by a rise in intracellular ATP and are very important in the regulation of UBSM excitability. Their activity may confer a voltage-independent brake which limits UBSM depolarisation and controls myogenic activity (Bonev & Nelson, 1993; reviewed by Teramato, 2006). K_{ATP} channels are composed of four pore-forming, inwardly rectifying, channel subunits (Kir6.x) and four modulatory sulphonylurea receptor subunits (SUR.x) that are members of the ATP-binding cassette (ABC) super-family of proteins (reviewed by Aguilar-Bryan *et al*, 1998). So far, two Kir6.x isoforms, Kir6.1 (encoded by the KCNJ8 gene) and Kir6.2 (endcoded by the KCNJ11 gene), and two SUR isoforms, SUR1 (protein product of the ABCC8 gene) and SUR2 (protein products of the ABCC9 gene), have been identified in various tissues (reviewed by Aguilar-Bryan *et al.* 1998 & Wickenden *et al*, 2002). Alternative splicing of the last exon of ABCC9 gene gives rise to the isoforms SUR2A and SUR2B, which differ only in their carboxy-terminal 42 amino acid residues (reviewed by Aguilar-Bryan *et al.* 1998). Different combinations of Kir6.x and SUR.x isoforms/variants yield tissue-specific K_{ATP} channel subtypes. Each subtype possesses different features and distinct functional properties. From functional expression studies, it is accepted that Kir6.1-SUR2B and Kir6.2-SUR2B channels are the two main types of K_{ATP} channels expressed in smooth muscle cells (reviewed in Teramoto, 2006).

Kir6.x subunits have two transmembrane domains, M1 and M2, cytoplasmic N- and C-termini and a pore-forming loop (Figure 7). SUR subunits possess large cytoplasmic domains containing two conserved nucleotide binding folds (NBFs), NBF1 and NBF2, with Walker A and B motifs (Figure 7). Both Kir and SUR subunits are needed for cell surface expression of these channels (reviewed by Teramoto, 2006).

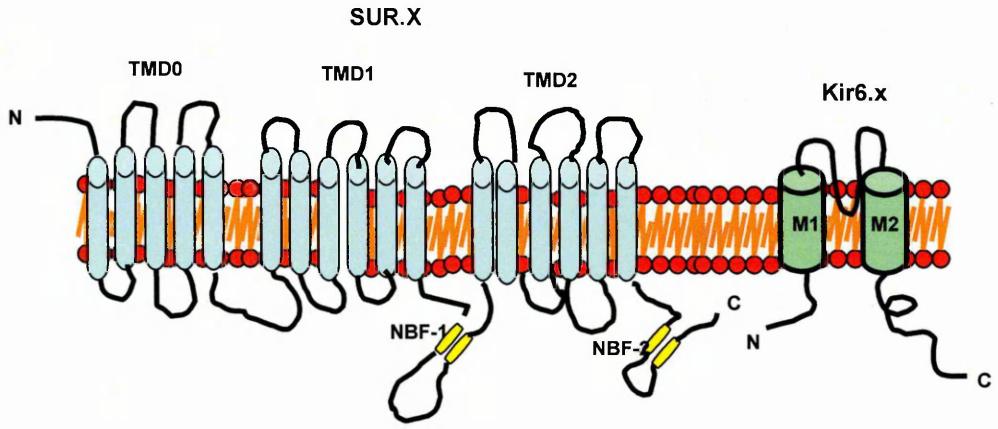


Figure 7) Schematic illustration of the predicted topologies of SUR.x and Kir6.x in K_{ATP} channels. TMD: transmembrane spanning domains of SUR.X, M: transmembrane segments of Kir6.x, NBF: nucleotide binding folds. Adapted from Teramato, 2006

1.3.2.3 Voltage gated K^+ channels

The UBSM also expresses voltage sensitive K^+ (K_v) channels, also known as delayed rectifier channels. These channels are not activated by intracellular Ca^{2+} (Davies *et al*, 2002). Instead they are activated by depolarisation in a voltage range near the resting membrane potential of detrusor smooth muscle. The UBSM is a rich source of $K_V2.1$ (Ohyo *et al*, 2000b; Gan *et al*, 2008). It is believed that these channels may be important in regulation of bladder SA (Gan *et al*, 2008).

The role of various K^+ channels in mediating bladder smooth muscle spontaneous contractions will be discussed further in the Chapter 5.

1.4 Pathophysiology of the urinary bladder

The structure and function of the urinary bladder is known to be altered by a number of disease states and pathological conditions. Urodynamic investigations can identify bladder disorders and provide information that allows them to be classified. Bladder disorders can lead to failure to store or empty urine properly. Failure to store urine, which is one of the most common complications of urinary bladder dysfunction, is associated with urinary incontinence and DO.

1.4.1 Urinary incontinence

Urinary incontinence is 'the involuntary leakage of urine' and can be classified as stress, overflow or urge incontinence (Abrams *et al*, 2002).

Stress urinary incontinence is associated with an increase in intra-abdominal pressure due to activities such as sneezing, coughing and physical exertion and is caused by various factors such as lack of pelvic floor support and weakness in the urethral sphincter (Chapple *et al*, 2000).

Overflow incontinence is caused by situations where incomplete bladder emptying and overdistension of the bladder result in overflow and involuntary leakage of urine.

Urge incontinence is the complaint of involuntary leakage accompanied by or immediately preceded by urgency (Abrams *et al*, 2001; Wagg *et al*, 2007). Urgency is a common symptom associated with detrusor overactivity (DO) or overactive bladder syndrome (OABS), which is characterised by a strong desire to void that is hard to defer.

1.4.2 Detrusor overactivity

DO is urodynamically characterised by involuntary contractions of the detrusor during the filling phase, which may be spontaneous or provoked (Abrams *et al*, 2002). It is associated with the troublesome symptoms of urgency, with or without urge incontinence, with increased daytime frequency and nocturia (Milsom *et al*, 2001; Abrams *et al*, 2002; Hashim & Abrams, 2007). Urgency is defined as a sudden compelling desire to pass urine that is difficult to defer

(Abrams *et al*, 2002), whilst nocturia is defined as waking up more than twice at night to urinate. Increased daytime frequency is the complaint by the patient who considers he/she voids too often by day (Abram *et al*, 2006b). This complex of symptoms is known as OABS, which is reported to have a prevalence of 12-17% in the adult population in Europe and the United States. The syndrome increases with age, with approximately 1 in 4 individuals older than 65 years experiencing OABS (Milsom *et al*, 2001; Andersson & Hedlund, 2002; Andersson & Yoshida, 2003; Abrams & Andersson, 2007). Although OABS and DO do not always coexist, it has been reported that 83% of patients with DO had symptoms of OABs, and 64% of patients with OABS had DO (Abrams & Andersson, 2007). However, DO is an urodynamic observation and should not be confused with OABS.

1.4.3 The aetiology of detrusor overactivity

The precise causes of DO have not yet been defined. Possible explanations of how detrusor becomes overactive are provided by the neurogenic or nerve related, myogenic and autonomous theories (Abrams & Andresson, 2007).

1.4.3.1 The neurogenic theory of overactivity

The dependence of bladder functions on complex CNS signalling pathways renders these functions susceptible to disruption due to a variety of neurological disorders (De Groat, 1997) such as dementia, stroke, multiple sclerosis, Alzheimer, spinal stenosis, spinal cord injury and diabetes (Turner & Brading, 1997; Mostwin, 2002). The neurogenic theory of DO suggests that damage to or reduction in peripheral or central inhibitory pathways in the brain or spinal cord, or enhancement of transmission of excitatory input and sensitisation of peripheral afferent terminals in the bladder, secondary to a neurologic disease can unmask primitive voiding reflexes that trigger DO (de Groat, 1997). Interruption of descending spinal pathways results in reorganisation of afferent pathways with unmyelinated capsaicin-sensitive C-fibres assuming prominence in the micturition reflex arc, sensitisation of which increases bladder excitability. The common sources of afferent information are likely to be from the urothelium, lamina propria, ICs and afferents that originate in the bladder wall (Fowler, 2002; Dmochowski, 2006).

1.4.3.2 The myogenic theory of overactivity

The myogenic or muscle related theory of DO suggests that changes in structure and function of the smooth muscle are necessary for the production of involuntary detrusor contractions, especially during the filling phase of the micturition cycle. The evidence for functional changes in smooth muscle properties come from experiments on strips of detrusor smooth muscle dissected from overactive bladders, which show abnormal spontaneous mechanical activity. The abnormal spontaneous activity contains elements of fused tetanic tension, which is never seen in normal bladders (Brading, 1997). One hypothesis is that changes in the properties of the detrusor in overactive bladder are caused by an overall reduction in the activity of the excitatory nerves to the smooth muscle. This triggers alterations in the smooth muscle, leading to increased excitability and electrical coupling between cells (Brading, 1997; Hashim & Abrams, 2007). Thus, a local contraction in any part of the detrusor will spread throughout the bladder wall, resulting in coordinated myogenic contractions of the entire bladder.

Although it is believed that DO requires structural changes in the smooth muscle, the sequence of events leading to this varies with different aetiologies of overactivity such as bladder outlet obstruction and aging (Elbadawi *et al*, 1993; Brading, 1997; Hashim & Abrams, 2007).

1.4.3.3 The autonomous theory of overactivity

The third theory, which is relatively new, is also myogenic in nature. In this theory, it is hypothesised that the bladder detrusor is modular i.e. it is made up of circumscribed areas of muscle which are supplied by an individual intramural bladder ganglia, or by a node of ICs (Drake *et al*, 2001; Hashim *et al*, 2007). It was suggested that a module can act as the smallest functional unit in the detrusor. Contraction of a module in isolation would generate localised activity, or micromotions. Synchronisation of activity between modules, which could propagate through the intramural nerve or IC networks, may result in macroscopic movements of the entire bladder (Drake *et al*, 2001). In the modular model, if the balance between excitation and inhibition is moved towards excitation, the module is predisposed to contraction. Therefore, exaggerated symptomatic expression of SA might result in DO (Drake *et al*,

2001; Gillespie, 2004). Furthermore, any factors that enhance communication between modules will predispose the bladder to DO.

It is possible that the true pathophysiology of DO may vary between individuals, and may involve one or more of the above mechanisms. A better understanding of the normal and pathophysiological function of the urinary bladder by use of different animal models may provide a better insight into the underlying mechanisms leading to DO.

1.4.4 Animal Models

Limited availability of human tissue due to ethical issues have led to the use of animal models for the study of bladder physiology and pathophysiology. Animal studies are useful for studying the underlying mechanisms of bladder function and can be used as models of human conditions. Use of animal models also allows for the comparison to normal age-matched controls, which is usually hard to achieve in human tissue (Turner & Brading, 1997). A wide variety of animal species have been utilised to investigate bladder function including non-human primates, dogs, pigs, cats, rabbits, rats, guinea-pigs, mice and hamsters (reviewed by McMurray *et al*, 2006). The majority of these species share a number of common anatomical, pharmacological and neurophysiological features with humans. However, some of these species are also known to have specific differences in normal urinary tract structure and function compared to humans, which may be a problem when extrapolating animal findings to the human situation (reviewed in Andersson & Arner, 2004 & McMurray *et al*, 2006). Several types of animal model have been used to study bladder dysfunction, and these include bladder outlet obstruction (BOO), neurogenic, and diabetic animal models.

1.4.4.1 Bladder outlet obstruction model

DO is commonly found secondary to conditions such as benign prostatic hyperplasia (BPH), bladder neck dysfunction and uncoordinated sphincter activity causing bladder outlet obstruction (BOO) (Mostwin, 2002). BOO causes many of the clinical, structural and neuropathic changes detected in DO. BPH

represents the most common clinical example in males. 80% of cases suffering from BPH also suffer from symptoms of DO (Goldberg & Sand, 2002).

To mimic DO secondary to BOO, models of bladder obstruction have been created in various animal species by partial obstruction of the urethra using some form of ligature that either immediately occludes the urethra or that increasingly occludes the urethra as the animal grows (reviewed by Levin *et al*, 2000). For instance, outflow obstruction is produced in rats by placing a ligature around the urethra or by testosterone-induced enlargement of the prostate leading to DO and muscle hypertrophy (Lluel *et al*, 1998; Pandita *et al*, 1998). Such models show many of the structural and physiological changes seen in human obstructed bladder including increased spontaneous myogenic activity, altered responsiveness to stimuli, patchy denervation of the smooth muscle and bladder hypertrophy (Reviewed by Turner & Brading, 1997 & Brading, 1997 & McMurry *et al*, 2006). However, induction of bladder outlet obstruction using surgical procedures is invasive to the animal and can vary in reproducibility.

1.4.4.2 The neurogenic overactive bladder model

Animal models of neurogenic DO are induced by cerebral infarction or chronic spinal cord injury (SCI) (reviewed by Turner & Brading, 1997 & McMurray *et al*, 2006). SCI is known to result in bladder dysfunction, including detrusor hyper-reflexia, in both humans and animal models (reviewed by Turner & Brading, 1997). Apart from SCI a number of central nervous system disorders are known to be associated with DO such as Parkinson's disease, stroke and multiple sclerosis, all of which are associated with some form of central lesion (reviewed by McMurray *et al*, 2006). Therefore, animal models in which central lesions are induced experimentally exhibit an abnormal bladder phenotype such as reduced bladder capacity and DO (reviewed by McMurray *et al*, 2006).

1.4.4.3 Diabetic Mellitus (DM) and urinary bladder dysfunction

Diabetes mellitus and urological complications are very common health problems that markedly increase in prevalence and incidence with advancing age (reviewed by Brown *et al*, 2005). Over 50% of the male and female population with diabetes have bladder dysfunction (Kaplan *et al*, 1995; Brown

et al, 2005). Current understanding of diabetic bladder dysfunction encompasses a broad spectrum of lower urinary tract symptoms including urinary urgency, frequency, nocturia, incontinence and an increased incidence of DO (Kaplan *et al*, 1995; Salinas Casado *et al.*, 1999; Brown *et al*, 2005). Previously diabetic-induced bladder dysfunction, termed bladder cystopathy, was described with common symptoms of diminished bladder sensation, poor bladder contractility and increased postvoid residual urine (Frimodt-Moller, 1978). However, bladder cystopathy most likely represents end-stage bladder failure, since symptoms of infrequent voiding, difficulty in initiation of voiding and postvoid fullness are relatively uncommon (Buck *et al*, 1973; Brown *et al*, 2005). Diminished bladder contractility or sensation has also been found less often in diabetic patients and an acontractile bladder appears to be quite uncommon (Brown *et al*, 2005). Therefore, DO may be associated with earlier stages of diabetes.

The aetiology of DM-associated bladder complications is multifactorial. The different effects of DM on detrusor muscle function are demonstrated in Figure 8. The underlying cause of the detrusor dysfunction may be altered physiology of the detrusor smooth muscle, altered innervation or dysfunction of the neuronal component or even urothelial dysfunction. Therefore it is important to know to what extent dysfunction of motor nerves and the detrusor itself contribute to bladder dysfunction in diabetes (reviewed by Turner & Brading, 1997; Yoshimura *et al*, 2005; Brown *et al*, 2005).

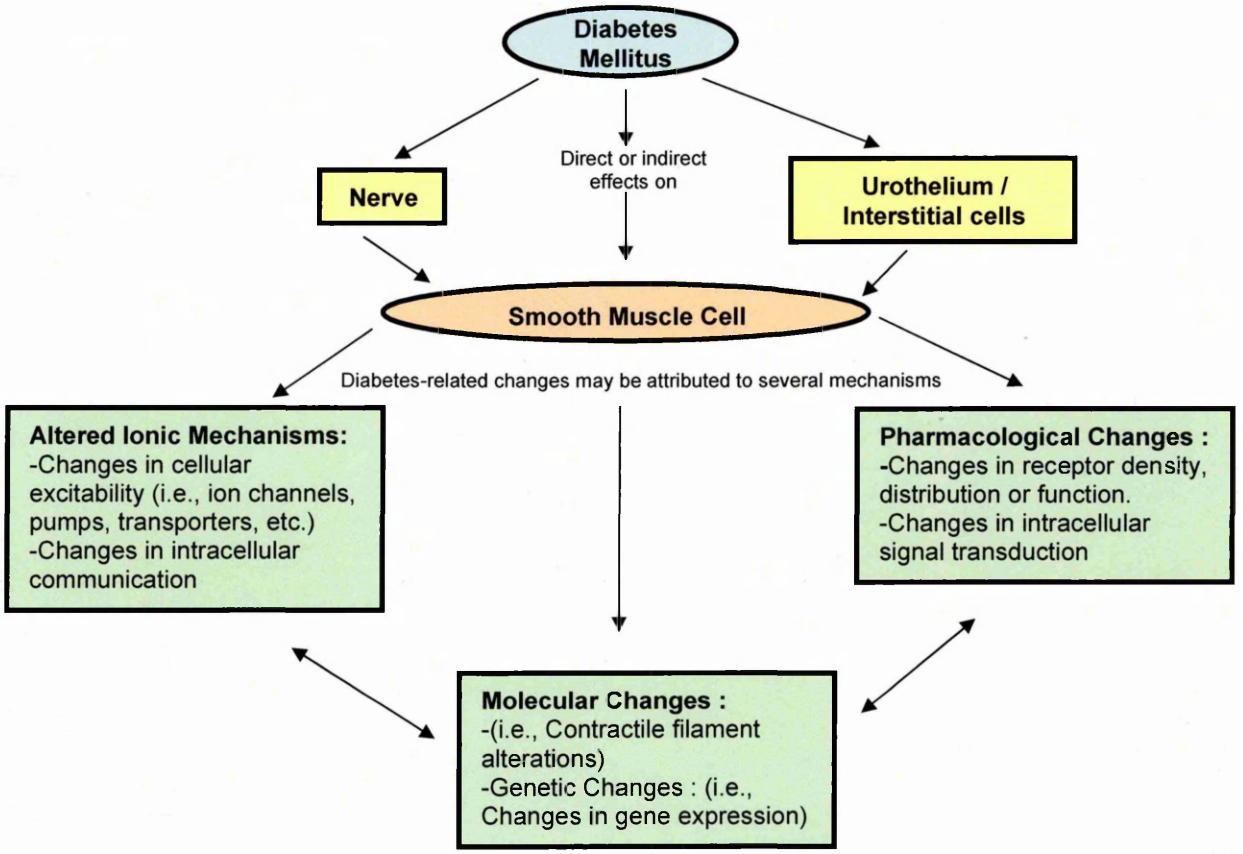


Figure 8) Effects of diabetes mellitus on detrusor smooth muscle. Adapted from Yoshimura et al, 2005.

Streptozotocin (STZ)-induced diabetic rat model

A number of diabetic animals have been used to investigate bladder and urethral dysfunction. The diabetic rat is one of these models and has been shown to mimic structural and functional changes seen in diabetic human bladders, including polyuria, urinary retention, increased bladder mass and capacity (Steers, 1994; Pitre *et al*, 2002). The induction of experimental diabetes in the rat, using chemicals which selectively destroy pancreatic β cells is a common and simple method, with the most widely used chemicals being alloxan and streptozotocin (STZ) (Szkudelski, 2001).

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyronose) is synthesised by *Streptomycetes Achromogenes* and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM and NIDDM, respectively). STZ impairs glucose oxidation and decreases insulin biosynthesis and secretion. It is believed that STZ is taken up by pancreatic β cells via the glucose transporter, GLUT2, where it alters the DNA of these cells either by fragmentation or alkylation (Morgan *et al*, 1994; Elsner *et al*, 2000). STZ is a nitric oxide (NO) donor and NO is liberated when STZ is metabolised inside the cells, and has been shown to be responsible for the destruction of pancreatic islet cells and DNA damage (Morgan *et al*, 1994). As well as releasing NO, STZ has been found to generate reactive oxygen species such as superoxide ions, which also contribute to DNA fragmentation and cell damage (Takasu *et al*, 1991).

Frequently a single intravenous dose of STZ is used in adult rats to induce IDDM. Intraperitoneal administration of a 40-60mg/kg body weight dose (b.w) is also efficacious, although single doses below 40mg/kg b.w may be ineffective.

STZ action in the β cell is accompanied by characteristic alterations in blood insulin and glucose concentration. Two hours following injection, hyperglycaemia is detected in rats, with a simultaneous drop in blood insulin levels. Six hours after STZ administration, hypoglycaemia occurs with high levels of blood insulin. Finally hyperglycaemia returns with a decrease in blood insulin levels to below normal average values. Thus, within six hours of

injection, STZ initiates pancreatic β-cell damage which leads to the development of diabetic mellitus (West *et al*, 1996).

Smooth muscle dysfunction in the STZ-diabetic rat bladder

Diabetes mellitus has been shown to alter detrusor smooth muscle function in the STZ-diabetic rat model (Longhurst *et al*, 1986; Malmgren *et al*, 1989; Kubota *et al*, 2003; Beshay & Carrier, 2004; Liu & Daneshgari, 2005). Some studies have concluded that the major changes observed in bladder function in the STZ-diabetic rat model are a physiological adaptation of the organ to increased mass and workload due to the diuresis and polyuria that accompanies diabetes (Uvelius, 1986; Kudlacz *et al*, 1988; Kudlacz *et al*, 1989; Pitre *et al*, 2002). However, others have shown that there are true changes at the mygenic level leading to bladder dysfunction in this model (Beshay & Carrier, 2004; Stevens et al, 2006). Of the studies conducted, alterations in cholinergic, adrenergic and nitric oxide-related functional responsiveness, as well as changes in K^+ channel function have been reported in UBSM cells from diabetic bladders (Longhurst & Belis, 1986; Malmgren *et al*, 1989; Steers *et al*, 1990; Kamata *et al*, 1992; Tammela *et al*, 1994; Kubota *et al*, 2003; Nakahara *et al*, 2004; Stevens *et al*, 2006).

Pharmacological studies of muscarinic receptors in isolated bladder strips from the STZ-rat model have generated much uncertainty, with some studies reporting a supersensitivity and an increased contractile response of the diabetic detrusor to muscarinic agonists (Steers *et al*, 1990; Kamata *et al*, 1992; Kubota *et al*, 2003; Stevens *et al*, 2006) whilst others have reported a decreased (Longhurst & Belis, 1986; Malmgren *et al*, 1989; Liu & Daneshgari, 2005) muscarinic component. An upregulation of M2- and M3-muscarinic receptors was also reported by Tong *et al* (1999, 2002) at the protein and mRNA level in the bladders from STZ-induced diabetic rats. This was associated with increased reactivity of detrusor strips to ACH and increased detrusor contractility. Recent research has also shown that muscarinic receptors are expressed on the urothelium and the suburothelium, and urothelial cells are capable of releasing ACH (Cheng *et al*, 2007; Tong *et al*, 2006; Yoshida et al, 2006). Increased expression of urothelial M2- and M3-muscarinic receptors has been detected in STZ-rat bladder. This abnormal

urothelial muscarinic receptor expression may be related to sensory disturbances of the bladder such as urgency, frequency and other symptoms of DO (Cheng *et al*, 2007; Tong *et al*, 2006).

Thus, changes in muscarinic receptor function of the STZ-diabetic rat bladder are still unclear and are further discussed in Chapter 3.

Alterations in ion channels have also been observed in detrusor smooth muscle from diabetic rats. The roles of BK_{Ca} and SK_{Ca} channels have been investigated and it is believed that the function of these channels is impaired in diabetic overactive detrusor and that this results in enhanced mechanical activity and spontaneous contractions (Nakahara *et al*, 2004). Changes in Ca^{2+} channel activity in STZ-diabetic rat detrusor have also been suggested to be associated with altered pharmacological responses of bladder smooth muscle strips from these animals (Belis *et al*, 1992). However the underlying mechanism of the signalling pathways of these channels requires further investigation.

Other changes, such as enhanced β1-adrenoreceptor relaxation (Kubota *et al*, 2003), increased responsiveness of isolated rat bladder strips to electrical field stimulation (Tammela *et al*, 1994), changes in nitric oxide synthase (NOS) and reactive nitrogen species formation during DM related bladder remodelling (Poladia & Bauer, 2003) and various other alterations have also been observed in bladders from diabetic animals (reviewed by Yoshimura *et al*, 2005). Mediators such as ATP, NO and prostanoids, which are released from the urothelial cells, may also influence afferent nerve activity and result in abnormalities detected in the diabetic bladder (Yoshimura et al, 2005; Pinna *et al*, 2000).

Since the biology of diabetic-associated bladder complications is multifactorial, alterations in the innervation or function of the neuronal component, i.e. diabetic neuropathy, may also play a role in UBSM dysfunction (reviewed by Yoshimura *et al*, 2005). The proposed mechanisms for neurogenic dysfunction in the diabetic bladder include altered metabolism of glucose, ischaemia and superoxide-induced free-radical formation as well as metabolic derangement of the Schwann cell and impairment of nerve conduction (Kudlacz *et al*, 1989; reviewed by Yoshimura, 2005). Defects in the sensory nerves of the bladder have also been implicated as a result of neuropathy in diabetes. A decrease in

production of nerve growth factor (NGF), which is important in afferent signalling, was detected in 12-week STZ-diabetic rats, showing a relationship between bladder function and NGF levels (Sasaki *et al*, 2002).

Since there are no longitudinal studies conducted under similar experimental conditions, there is still much confusion over the time course, magnitude and mechanism of diabetic-related changes in detrusor smooth muscle function.

1.4.5 Pharmacological treatments of overactive bladder

Pharmacologic therapy remains the cornerstone of management for the majority of patients with overactive bladder. The principal pharmacological treatment used to improve the symptoms of OABS is based on muscarinic receptor antagonism (antimuscarinics). The antimuscarinic drugs available include oxybutynin, tolterodine and trospium chloride (reviewed by, Sellers and McKay, 2007). The mechanism of action of antimuscarinic drugs was traditionally considered to be at muscarinic receptors within the detrusor smooth muscle. This concept has now become increasingly controversial. At prescribed doses, antimuscarinic drugs do not inhibit the normal voiding phase of the micturition cycle, whilst they do alter bladder sensation during filling, as evidenced by an improvement in filling symptoms associated with OABS and bladder capacity. This has led to a recent hypothesis suggesting that antimuscarinic drugs may act via other mechanisms related to the afferent mechanosensory system rather than the efferent system (Chapple *et al*, 2005).

Although antimuscarinic drugs remain the main treatment for OABS, their usage is limited due to significant side effects such as dry mouth, blurred vision, constipation and urinary retention. Thus, there is a need for more effective drug treatments. Recent research has highlighted several potential targets for treatment of the overactive bladder, particularly within the mechanosensory pathways. Compounds currently under research include beta-adrenoceptor agonists, tachykinins, K^+ channel openers, vanilloids and botulinum toxin (reviewed by Sellers & McKay, 2007).

1.5 Aims and Objectives of the thesis

The STZ-diabetic rat is used commonly as a model of urinary bladder dysfunction and DO. However, there is much controversy regarding the underlying causes of urinary bladder dysfunction in this model. Thus, the aim of this thesis was to use this model along with normal control (non-diabetic) animals, in order to investigate the underlying physiology and pathophysiology of urinary bladder function, using a combination of *in vitro* pharmacology, molecular biology and immunology.

Chapter 2: Validation and optimisation of the *in vitro* tissue bath technique for study of isolated bladder smooth muscle strips from the rat

2.1 Introduction

In order to investigate the functional pharmacological properties of bladders from STZ-diabetic rats, an *in vitro* tissue bath technique was used throughout this thesis. The tissue bath is a traditional experimental set-up that has been used extensively to investigate the physiology and pharmacology of *in vitro* preparations. Typically, contractions of isolated tissues in response to particular concentrations of a pharmacological agent are measured and studied. However, in order to be able to compare the functional data obtained for animals where the bladder mass has increased, it is important to optimise and validate the experimental parameters and to ensure that all strips are at optimal condition for tension measurements.

2.2 Length-tension properties of bladder smooth muscle

Contraction of smooth muscle tissue involves interactions between the active elements, the contractile cells and passive structures, the connective tissue and extracellular matrix in which the cells are embedded (Meiss & Pidaparti, 2005). More than 100 years ago it was demonstrated that the active tension of skeletal muscle was enhanced with increasing muscle length up to a certain point (Blix, 1891, 1893, 1894). Above this optimal length active tension starts to decrease, although passive tension increases exponentially with muscle length (Blix, 1891, 1893, 1894; Uvelius, 2001). When muscle is stretched beyond the length where maximum active tension is developed, the decrease in active tension is considered to be a result of decreasing overlap between the thick and thin filaments within the sarcomeres (Blix, 1891, 1893, 1894). A decreased active tension is also seen at shorter length intervals, which is harder to explain and was suggested to be due to inefficient coupling between membrane activation and force output at shorter lengths (Rudel & Taylor, 1971).

Smooth muscle has a length-tension property that in principle appears the same as that of skeletal muscle. The active force developed by striated muscle fibres is directly proportional to the extent of over-lap between actin and myosin filaments. However, in contrast to this, the myofilaments of smooth muscle fibres do not form organised sarcomeres and the interactions between myosin

and actin filaments differ (Cooke & Fay, 1972). In smooth muscle, passive tension becomes considerable at short lengths which are below the optimal length for active tension development. Also smooth muscle seems to develop maximal active force over a much greater range of passive tension, which could be due to the different arrangement of the contractile filaments (Uvelius, 2001). There are no distinct layers of musculature in the urinary bladder and the structural units of the muscle are the bundles which are extremely variable in cross section, length and orientation. Thus, this may explain the extraordinary stretchability of the wall and development of active tension over a greater range of passive tension. These properties of the urinary bladder have a prime importance in its physiological role of accommodation and storage of urine (Longhurst *et al*, 1995; Uvelius, 2001). The presence of collagen fibres in the mucosa and serosa, as well as between muscle bundles and smooth muscle cells could also explain the stretchability of the bladder tissue.

When using *in vitro* tissue baths, it is common practice to set the isolated strips of bladder at the optimal length, L_0 or optimal passive tension (loading tension), where a population of strips will, on average, generate their maximum active tension. In experimental animals, where bladder mass has increased, length tension experiments should especially be carried out to ensure that all strips are under optimal condition for active tension measurements. For example, Ekstrom & Uvelius (1981) demonstrated that length-tension properties of normal and denervated rat bladders differed and that denervated bladder strips had to be stretched to a considerably greater extent than those from normal bladders in order to reach optimum length for force tension development.

Changes in length-tension properties of bladders from diabetic animals have also been reported. Andersson *et al* (1988) demonstrated a rightward shift of length-passive and active tensions in bladders from 6-week diabetic female Sprague-Dawley rats. The same pattern was observed by Eika *et al* (1992) in 2 months diabetic male Sprague-Dawley rats. It was hypothesised that changes in the relative concentration of connective tissue and contractile proteins and/or the orientation of smooth muscle fibres in the bladders, as well as biochemical changes that might have taken place and have had an impact on energy

utilisation, are important in the functional changes observed in these animals (Longhurst *et al*, 1995).

2.3 Aim of the chapter

The aims of this chapter were to investigate the length-tension properties of detrusor strips from control (non-diabetic) and STZ-diabetic rats, in order to optimise the methodology for investigating the contractile function in subsequent tissue bath experiments and to measure the body weight and blood glucose levels of diabetic and control (non-diabetic) rats.

2.4 Materials and Methods

2.4.1 Induction of diabetes

In this study, male Wistar rats (Harlan, UK) (approx 200-350g) received a single intraperitoneal injection of streptozotocin (STZ) (65mg/kg body weight) (Sigma, Dorset, UK), dissolved in 0.01mM citrate buffer (pH=4) to induce diabetes. Solutions of STZ were prepared immediately before use due to the instability of this drug at room temperature.

Animals were used at 1, 4 and 8 weeks after injection. Non-diabetic rats were also used as controls. At termination blood glucose levels were measured using an ACCU-CHEK Advantage blood glucose monitoring system (Roche Diagnostics, Mannheim, Germany) and body weight was taken. Animals with glucose levels higher than 16mM were considered diabetic. Termination was via a schedule 1 method i.e. stunning followed by cervical dislocation. All procedures were carried out according to Home Office project licence 40/6774.

2.4.2 Tissue preparation

2.4.2.1 Dissection and removal of the bladder

The abdominal cavity of the rat was opened by a midline incision exposing the urinary bladder and urethra. The prostate was trimmed away using fine scissors taking care not to perforate the bladder wall. Figure 9 demonstrates the location of the urinary bladder from the ventral aspect. The bladder was removed and then placed in ice cold modified Krebs-bicarbonate solution (Krebs) (118.3mM NaCl, 11.7mM D-Glucose, 24.9mM $NaHCO_3$, 4.7mM KCl, 1.15mM $MgSO_4$, 1.15mM KH_2PO_4 and 1.9 mM $CaCl_2$).

2.4.2.2 Bladder strip preparation

The urinary bladder was placed on a dissection tray containing Krebs and the loose serosal connective tissue, any remains of the prostate and fat were removed. A longitudinal incision was performed through the bladder from the

base to the dome and the bladder was opened up to form a flat sheet. Using a razor blade, the base and the top of the dome were removed and 3-4 longitudinal strips (depending on bladder size) measuring 2-4 x 6-12mm were then cut from the bladder body.

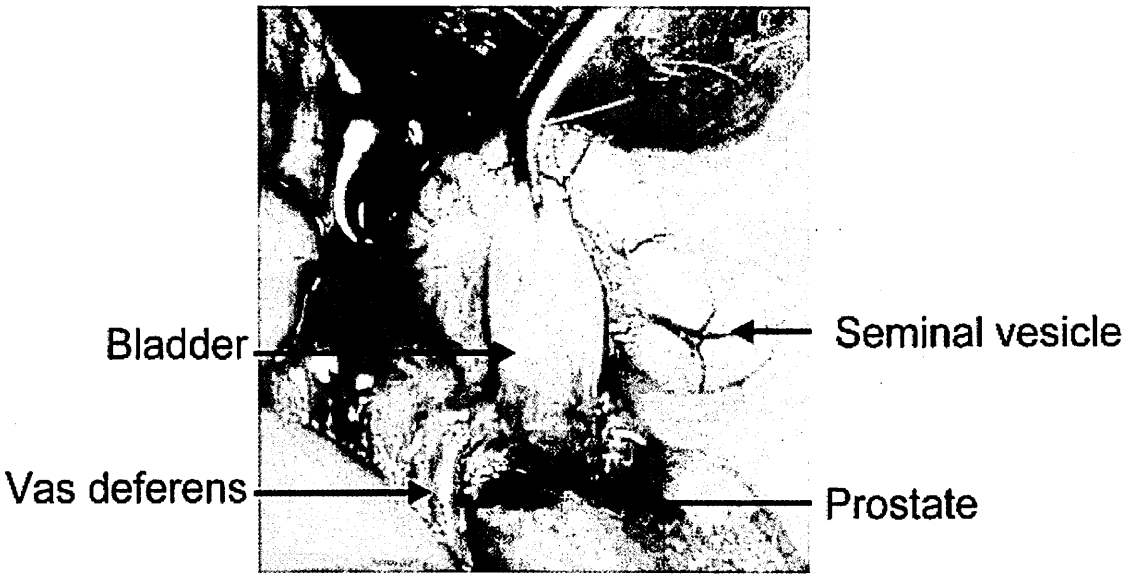


Figure 9) Location of the rat urinary bladder from the ventral aspect. Adapted form Longhurst and Uvelius, 2001.

2.4.3 *In vitro* tissue bath preparation

The detrusor strips cut from the bladder body were subsequently attached at one end to tissue holders and at the other to Dynamometer UF1 force transducers (Pioden Controls Ltd, UK) using a surgical thread (Figure 10). The detrusor strips were then mounted in 15ml organ baths containing Krebs solution maintained at 37°C and gassed with 95% O_2 and 5% CO_2. A pH of 7.4 was consistent under these conditions due to the buffering system established by the CO_2 and $NaHCO_3$. Figure 10 illustrates the tissue bath set up.

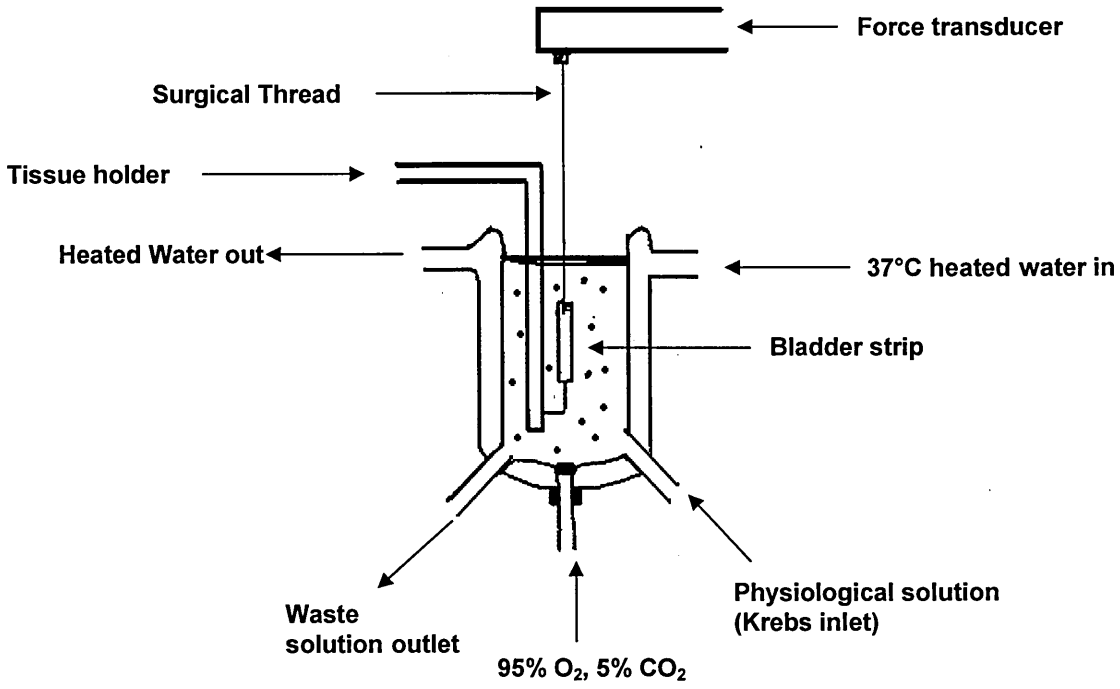


Figure 10) Double-walled glass tissue bath setup (adapted from Longhurst and Uvelius, 2001)

The tension developed by the tissue was measured by the isometric force transducer connected to a Powerlab data acquisition system (ADInstruments, UK), which converted the analogue signal to digital, and used 'Chart 4.0' software (ADInstruments, UK), to enable analysis of the acquired data.

2.4.4 Investigation of length-tension relationship of detrusor strips from control (non-diabetic) and diabetic rats

After mounting the detrusor strips in the tissue baths, the initial passive tension was adjusted to 1g. Tissues were equilibrated for 60 minutes, with a wash every 15 minutes with fresh Krebs buffer. Following equilibration the tissues were stimulated for 5 minutes with high (60mM) potassium (K^+)-Krebs buffer (63.71mM NaCl, 11.7mM D-Glucose, 24.9mM $NaHCO_3$, 60mM KCl, 1.15mM $MgSO_4$, 1.15mM KH_2PO_4 and 1.9 mM $CaCl_2$ (obtained by exchanging NaCl with equimolar amounts of KCl)) to produce an active tension. Tissues were then allowed to relax for 10 minutes in calcium (Ca^{2+})-free Krebs buffer (118.3mM NaCl, 11.7mM D-Glucose, 24.9mM $NaHCO_3$, 4.7mM KCl, 1.15mM $MgSO_4$,

1.15mM KH_2PO_4). This sequence was repeated twice, allowing a stable contractile response to develop. The tissues were then shortened to a length at which there was no response to stimulation with high- K^+ Krebs and then allowed to relax in Ca^{2+}-free Krebs.

The length-tension curve was then constructed by increasing the length of the tissues by 1mm increments using a micrometer. At each length, tissues were stimulated for 5mins with high-K^+ Krebs solution and then allowed to relax for 10mins in calcium-free Krebs. Immediately after each switch to Ca^{2+}-free Krebs, the length of the tissues was increased by 1mm (Figure 11). The strips were stretched in this stepwise manner until the responses to high-K^+ Krebs started to decline. At the end of experiments tissues were weighed.

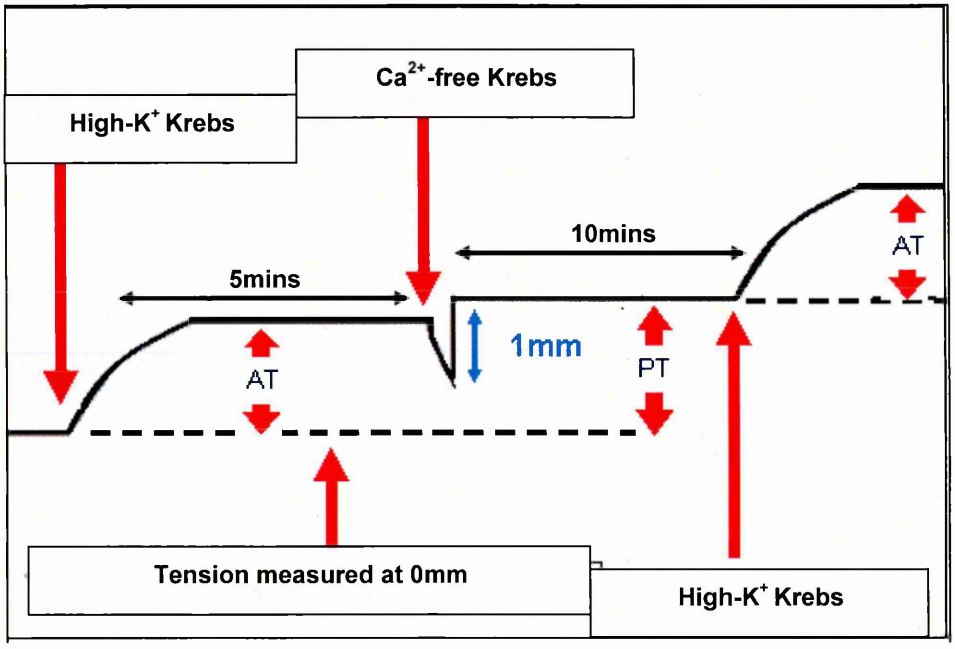


Figure 11) An illustration of the length-tension protocol. Note that as the muscle is stretched, the baseline of the force record is raised due to passive tension (PT) in the muscle and contributes more to overall force than the active tension (AT)

2.4.5 Data analysis

Active tension (maximal tension in high-K^+ Krebs minus passive tension) and passive tension (in Ca^{2+}-free Krebs) tensions (g) were determined at each length (mm) (as shown in Figure 11) and were normalised for tissue weight (mg). The maximum passive tension was considered to have been reached

when a subsequent increase in the passive tension produced no significant difference in the active tension developed to high-K^+ Krebs compared to the previous active response. Length-tension curves were constructed by plotting the increase in tissue length (mm) against the active and passive tensions (g/mg tissue) developed by the detrusor strips at each length. Data was presented as mean±SEM.

Passive-active tension curves for control (non-diabetic) and diabetic tissues were also constructed by plotting the passive tension (g) at each length against the active tension (g/mg tissue) at the same length to determine the optimum passive tension (g) resulting in maximum active tension development.

2.4.6 Statistical analysis

Differences in maximum active contractions (absolute (g) and normalised per mg tissue) between control and diabetic tissues were compared using one way *ANOVA* followed by Dunnetts multiple comparison post hoc test. $p<0.05$ was considered significant.

2.5 Results

2.5.1 STZ-diabetic rats

After 1-8 weeks of STZ-induced hyperglycaemia, significant differences in body weight were detected in the diabetic rats versus the control (non-diabetic) rats, such that at sacrifice STZ-diabetic rats had significantly decreased body weights compared to control (non diabetic) rats (Figure 12). In addition, the blood glucose concentrations of the STZ-diabetic rats were significantly higher than those of controls (Figure 12).

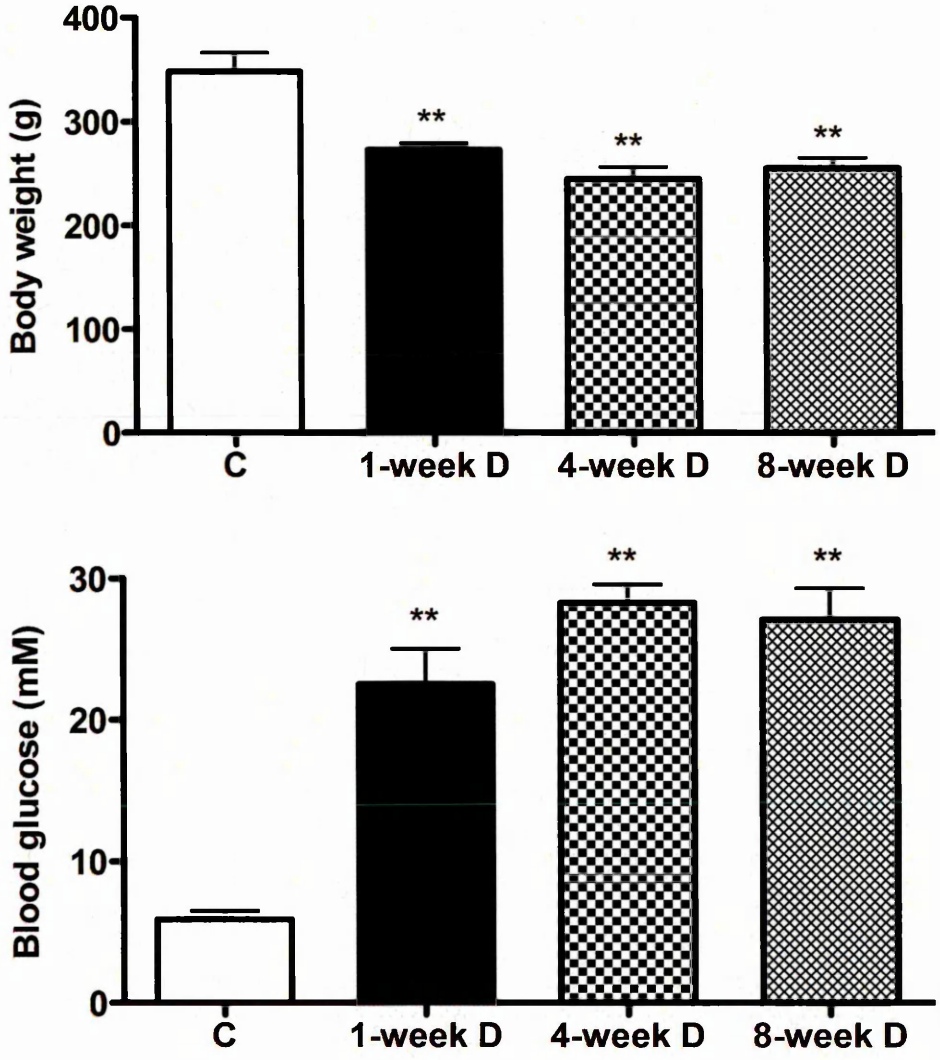


Figure 12) Body weights and blood glucose levels of control (non-diabetic) (C) and diabetic (D) rats. C: N=5, 1-week D: N=6, 4-week D: N=5 and 8-week D: N=5. **p<0.01 vs. control (non-diabetic) animals. Data is presented as mean±SEM. N=number of animals.

STZ-diabetic rats also demonstrated an increase in the micturition frequency and volume compared to control (non-diabetic) rats (observational data only).

Non-urological complications, such as development of cataracts were also detected in these animals after 8-weeks of diabetes.

2.5.2 Investigation of length-tension relationship of detrusor strips from control (non-diabetic) and diabetic rats

Length-tension curves for bladder strips from control (non-diabetic), 1-week, 4-week and 8-week STZ diabetic rats are presented in Figure 13. Passive tension increased with tissue length in control (non-diabetic) and diabetic bladder strips (Figure 13). Active tension (in response to high-K^+ Krebs) was increased up to a maximum in all groups before starting to decline as the tissue length continued to increase.

Detrusor strips from diabetic animals developed a significantly greater absolute active tension (g) than those from control (non-diabetic) rats (Table 1). However, when the active tension was expressed as tension g/mg tissue to normalise for differences in tissue weight, only the active tension (g/mg tissue) of 1-week diabetic tissues remained significantly greater than control (non-diabetic) tissues.

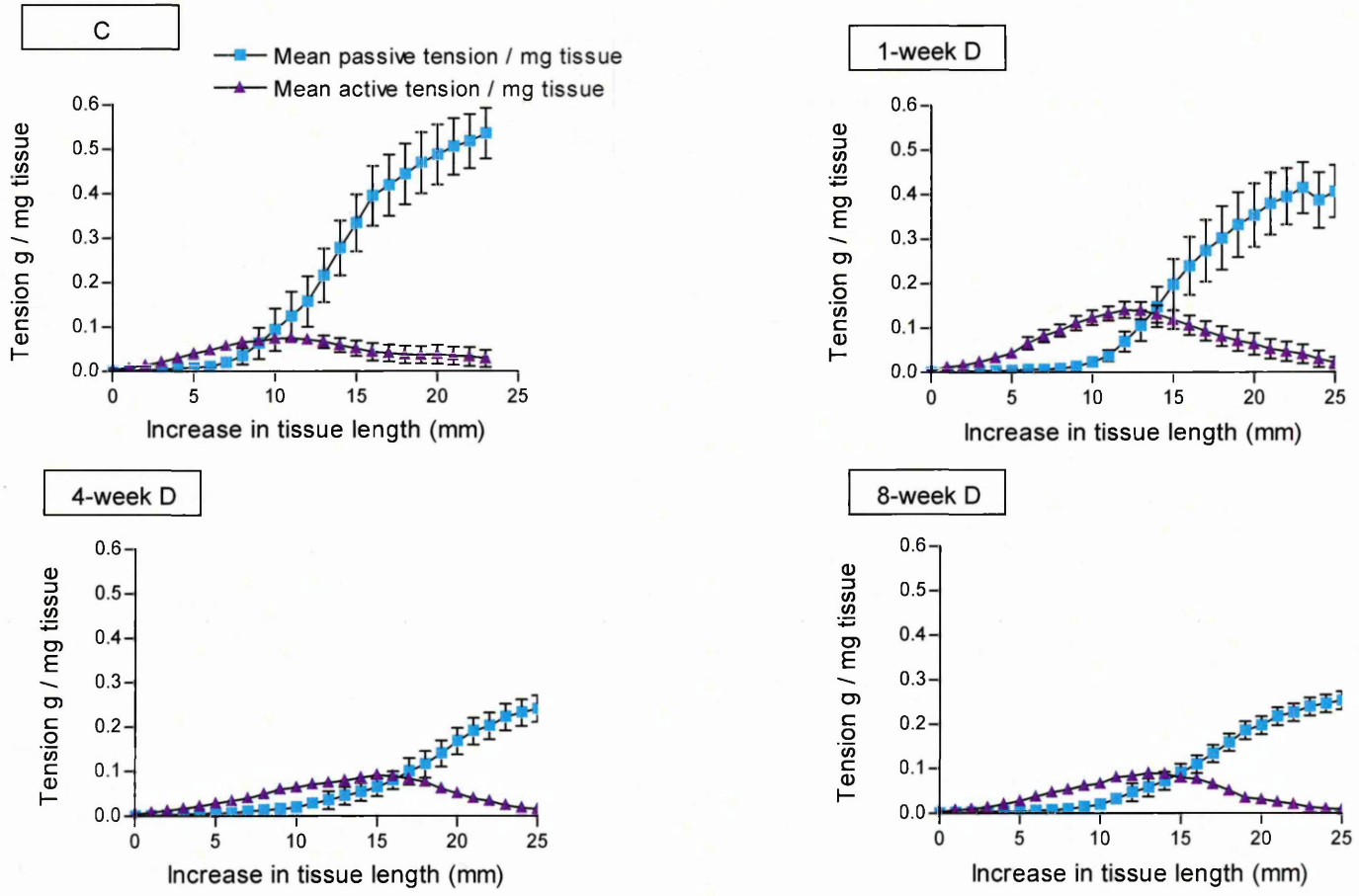


Figure 13) Length-tension curves for bladder strips from control (non-diabetic) (C) and diabetic (D) rats. C: n=12, 1-week D: n=11, 4-week D: n=11 and 8-week D: n=12. Data is presented as mean grams tension/mg tissue weight ± SEM. n=number of detrusor strips

	C (n=12)	1-week D (n=11)	4-week D (n=11)	8-week D (n=12)
Active tension (g)	1.68±0.26	3.32±0.43*	3.77±0.48**	3.84±0.39**
Active tension (g/mg tissue)	0.08±0.11	0.14±0.02**	0.09±0.01	0.09±0.01

Table 1) Maximum active tension developed in presence of high K+ Krebs in control (non-diabetic) (C) and diabetic (D) tissues. Data is expressed as mean grams or g/mg tissue ± SEM. n= number of detrusor strips. *p<0.05 & **p<0.01 vs. control (non-diabetic) tissues

To determine the effect of passive tension on the contractile response to high K^+ Krebs, the active tension was plotted against the passive tension. The optimum passive tension, at which maximum active tension was generated, was determined using these graphs (Figure 14).

The active tension developed by detrusor strips from control (non-diabetic) rats reached a maximal level at 2.2g of passive tension. Bladder strips from 1 week, 4 week and 8 week diabetic rats reached a maximum active tension at 2 g, 2.3g and 2.1g of passive tension respectively (Figure 14).

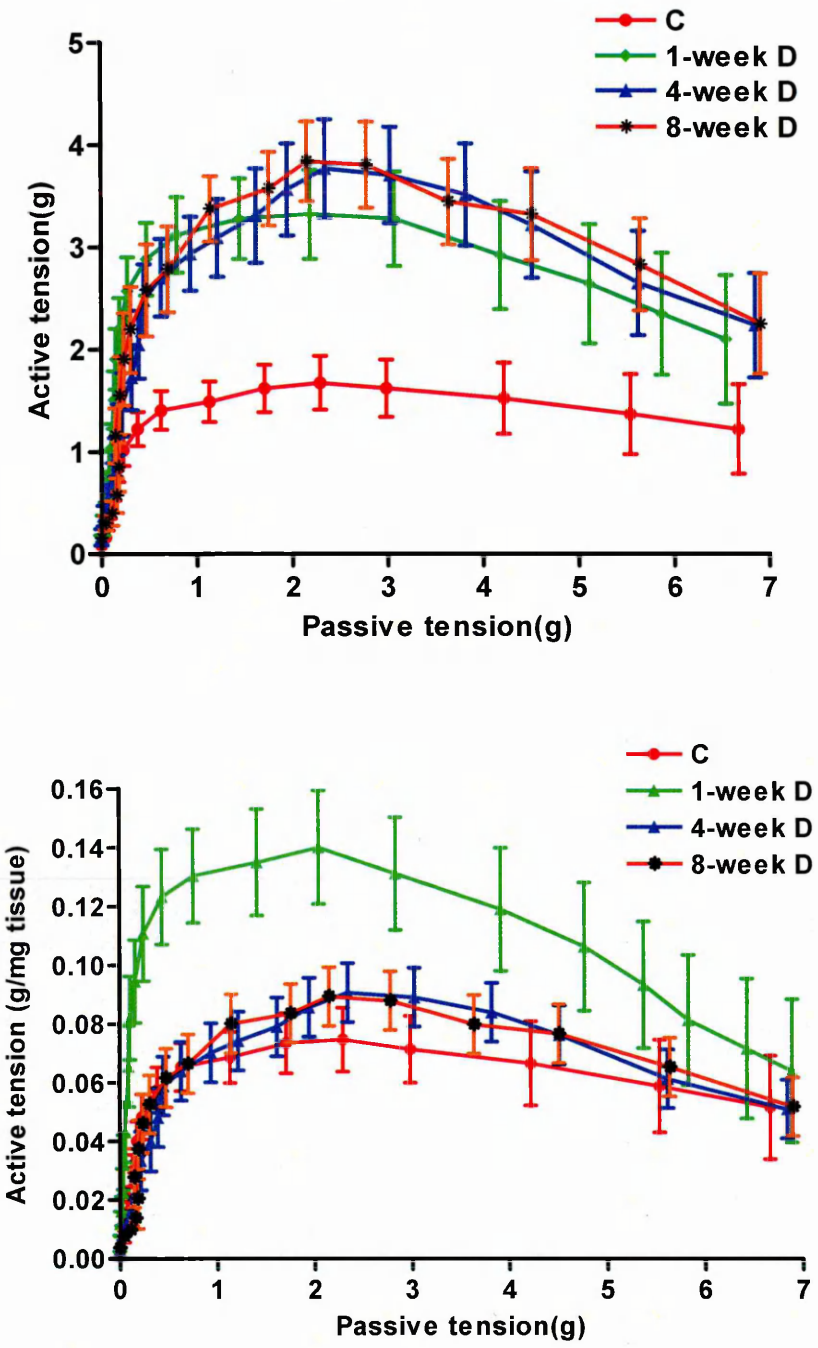


Figure 14) Passive tension-active tension curves for detrusor strips from control (non-diabetic) (C) and diabetic (D) rats . Top panel: absolute values (g). Bottom Panel: g/mg tissue. C: n=12, 1-week D: n=11, 4-week D: n=11 and 8-week D:n=12. Data is presented as mean± SEM.

2.6 Discussion

2.6.1 The STZ-diabetic rat

The chemical induction of diabetes using STZ, which specifically damages the β-cells in the pancreas, provides permanent diabetes that is suitable for long-term studies. Chemically-induced type 1 diabetes is the most commonly used animal model of diabetes. Organ dysfunctions, such as those affecting the cardiac, renal, retinal and reproductive and digestive systems, seen in human patients are also detected in the STZ-diabetic rat (reviewed by Ozturk *et al*, 1998 & McNeill, 1999). Although these complications were not the focus of this study, but cataracts, enlarged abdominal cavity and changes in the shape and size of the gastrointestinal tract were observed in these animals in the time of sacrifice. Also within one week following induction of diabetes, the STZ-diabetic rats demonstrated a loss in body weight and blood glucose concentrations increased significantly from normal levels of around 6mM to greater than 20mM. A decrease in body weight and an increase in blood glucose levels in STZ- diabetic rats at different durations of diabetes have also been noted by several other investigators (Steers *et al*, 1990; Stevens *et al*, 2006; Liu & Daneshgari, 2006). Rats in this study also consumed a large amount of fluid and produced correspondingly high urine volumes, which is consistent with the observations of other studies (Reviewed by McNeill, 1999).

Significant alterations in urinary bladder morphology, physiology and pharmacology have been reported after the induction of diabetes mellitus. For example, a time-dependent increase in total bladder tissue mass and cross sectional lumen area was reported in 5-week STZ-diabetic rats by Pitre *et al* (2002). Rizk *et al* (2006) reported collagen accumulation, degenerated nerve fibres and myeline bodies between smooth muscle cells of 8-16-week STZ-diabetic rats. Other alterations in the pharmacological and physiological properties of the urinary bladder from STZ-diabetic rats are discussed further in the relevant chapters.

Since it has been suggested that an increase in bladder size affects length-tension properties of this organ (Ekstrom & Uvelius, 1981; Andersson *et al*, 1988), it was necessary to perform length-tension experiments in order to

determine what initial passive tension detrusor strips from control (non-diabetic) and diabetic animals should be placed under to achieve the optimum contractile responses during *in vitro* tissue bath experiments.

2.6.2 Length-tension properties of detrusor strips from control (non-diabetic) and diabetic rats

In the present study, the maximum active tension generated in response to high K^+ Krebs was greater in all bladder strips from diabetic animals compared to those from control (Table 1). The reason for this is not clear, however, it could be the result of increased tissue mass in diabetic bladder due to polyuria. Indeed, after normalising data to account for tissue weight (g/mg tissue), there was no significant difference between the maximum active tensions of control, 4-week and 8-week diabetic bladder strips. However detrusor strips from 1-week diabetic rats still showed a significantly greater active tension compared to control (non-diabetic) tissues. It is possible that there are compensatory changes to the bladder shortly after induction of diabetes, which stabilises with longer duration, indicative of time dependent changes in stimulus-contraction coupling.

The length-tension curves generated in this study indicate that in all groups, active tension increased with increasing the passive tension and reached a maximum around 2g of passive tension. However, from the length-tension curves for control (non-diabetic) and diabetic tissues, it is clear that a plateau can be observed where, over a range of passive tensions (1.5-3g), similar maximum active tensions can be generated. These results are similar to findings by Longhurst *et al* (1990) who demonstrated that comparison of contractile responses of bladders from control and 8-week diabetic rats should be performed at an optimal passive tension of ≥2 g to ensure that maximal active tension was generated. The range of passive tensions over which maximal active responses were observed can probably be related to the nonlinear arrangement of smooth muscle fibres and high collagen and elastin contents of the bladder wall, which form a three dimensional network. This can result in different groups of smooth muscle fibres being at optimal length at different degrees of passive tension (Andersson and Arner, 2004; Longhurst *et*

al, 1990). Therefore, it appears that in these tissues the maximum active tension could be achieved over a range of passive tensions.

2.7 Summary

Overall, the results presented in this chapter illustrate that diabetic rats have significantly lower body weight and higher blood glucose levels compared to control (non-diabetic) rats. Also comparison of functional responses of urinary bladder strips between control and STZ-diabetic rat tissues should be performed at an optimal passive tension of ≥2g, to ensure that maximal active tension is generated. As such, in all subsequent experiments described, detrusor strips were mounted under 2g of passive tension.

Chapter 3: Investigation of muscarinic-receptor- mediated contraction of detrusor strips from the rat

3.1 Introduction

Muscarinic receptors mediate the inhibitory and excitory effects of acetylcholine (ACH) in the urinary bladder smooth muscle. M2- and M3-muscarinic receptors predominate in the detrusor, with the population of M2-muscarinic receptor subtype being greater than the M3-receptor subtype so that the M2:M3 ratio is 9:1 in the rat bladder and 3:1 in other species examined (reviewed by Eglen *et al*, 1994). In spite of this, it has been demonstrated that it is the minor population of M3-muscarinic receptors that mediate *in vitro* contraction of the detrusor in all species studied to date under normal physiological conditions (Wang et la, 1995; Choppin *et al*, 1998; Sellers *et al*, 2000; Chess-Williams, 2002).

Alterations in muscarinic receptor function have been associated with detrusor overactivity (DO) and the widespread use of antimuscarinic agents for the treatment of DO over the past three decades highlights the key role of these receptors in the pathophysiology of this condition (Braverman *et al*, 1998; Pontari *et al*, 2004; Hedge, 2006).

3.1.1 The role of muscarinic receptors in detrusor overactivity (DO)

Alterations in muscarinic receptor-mediated pathways have been implicated in DO/pathological conditions of the bladder. An increased sensitivity and responsiveness of detrusor smooth muscle to muscarinic receptor agonists has been observed in various species and in different conditions leading to DO, including neurogenic overactive bladders (Saito *et al*, 1993; Stevens *et al*, 2007), bladder outlet obstruction in humans (Harrison *et al*, 1987) and denervation, spinal cord-injury and diabetic-induced DO in rats (Gunasena *et al*, 1995; Braverman *et al*, 1998b; Stevens *et al*, 2006). Possible mechanisms for the enhanced detrusor sensitivity to muscarinic agonists in these studies include an increase in muscarinic receptor density and/or alterations in the receptor response coupling.

Although some reports have not supported the idea of supersensitivity of bladder smooth muscle to muscarinic agonists (Nilverbrant *et al*, 1986), the

balance of evidence supports the importance of these receptors in mediating the bladder function under pathological conditions.

Several studies have attempted to elucidate which muscarinic receptor subtypes may be involved in mediating detrusor contraction under these pathological conditions. It has been suggested that the M2-muscarinic receptor subtype may contribute to direct bladder smooth muscle contraction when the M3-muscarinic receptor subtype becomes pharmacologically inactivated or is dysfunctional. For example, it has been demonstrated that the pharmacological profile of the key muscarinic receptor antagonist inhibiting the detrusor contractions in neurogenic bladder dysfunction in humans is consistent with M2-muscarinic receptor mediated responses (Pontari *et al*, 2004; Braverman & Ruggieri, 2006). The same has been observed in detrusor strips from rat models of bladder outlet obstruction (Ruggieri & Braverman, 2006) and denervation (Braverman & Ruggieri, 2003; Braverman *et al*, 2006b). Immunoprecipitation studies in bladders from denervated and outlet obstructed rats demonstrated an increase in M2-muscarinic receptor protein density (Braverman & Ruggieri, 2003; Braverman & Ruggieri, 2006c) with a decrease (Braverman & Ruggieri, 2003) or no change (Ruggieri & Braverman, 2006c) in M3-muscarinic receptor density.

This concept of a role for the M2-muscarinic receptor in mediating bladder smooth muscle contraction in bladder dysfunction has been challenged by several studies. Stevens *et al* (2004a, 2004b, 2007) demonstrated that direct responses to muscarinic receptor stimulation of the detrusor muscle from patients with idiopathic and neurogenic overactive bladder are mediated via the M3-muscarinic receptor, with no change in the role of M2-muscarinic receptors. Krichevsky *et al* (1999) also demonstrated that in obstructed rat bladder, M3-muscarinic receptors have the predominant role in mediating detrusor contraction.

Thus, it is still unclear whether the M2-muscarinic receptor has a role in DO and bladder dysfunction.

3.1.2 Muscarinic receptors in STZ-diabetic rat bladder

Pharmacological studies of muscarinic receptors in isolated detrusor strips from the sterptozotocin (STZ)-diabetic rat have generated contradictory results. Functionally, various studies have documented a supersensitivity and an increased contractile response of detrusor strips from STZ-diabetic rats to muscarinic agonists (Steers *et al*, 1990; Kamata *et al*, 1992; Kubota *et al*, 2003; Stevens *et al*, 2006) whilst others have reported a decrease (Longhurst & Belis, 1986; Malmgren *et al*, 1989; Liu & Daneshgari, 2005) in the muscarinic mediated responses. An up-regulation of the M2- and M3-muscarinic receptors at the protein and mRNA levels has been reported by Tong *et al* (1999, 2002) in the bladders from STZ-diabetic rats. It was suggested that the up-regulation of M2-muscarinic receptors may upset detrusor stability by acting against the sympatho-inhibitory effect (mediated via β-adrenoceptors and increase in urinary bladder smooth muscle (UBSM) cAMP levels) during the filling phase of the micturition cycle. Also up-regulation of M3-muscarinic receptors was suggested to account for the augmented response to acetylcholine (ACH) and the increased detrusor contractility found in the bladder strips from diabetic rats (Tong *et al* 1999, 2002). An increase in density of muscarinic receptors in the STZ-diabetic rat bladder has also been observed in radioligand binding studies by Stevens *et al* (2006). However, the increased sensitivity of detrusor strips from diabetic rats in this study was only partially related to the increased muscarinic receptor density. It was suggested that other mechanisms must also be operating at a cellular level to cause the tissue supersensitivity (Stevens *et al*, 2006).

The contradictory results on muscarinic-receptor mediated function of bladders from diabetic animal models may be due to the species of animal used, the duration of diabetes employed, as well as data normalisation procedures applied in each study (Waring & Wendt, 2000; Yoshimura *et al*, 2005). For example, in studies by Chang *et al* (2005) and Saito *et al* (2008) a general smooth muscle hypersensitivity and increased contractility to muscarinic receptor stimulation was reported in the bladder detrusor from alloxan-induced diabetic rabbit and type 2 Goto-kakizaki diabetic rats respectively, whilst in a study by Longhurst & Belis (1986), using STZ-diabetic rats, a decreased sensitivity of bladder smooth muscle to muscarinic agonists was observed

indicating species-dependent differences. Duration dependent differences have been hypothesised in the reports of Stevens *et al* (2006), who demonstrated an increase in sensitivity of detrusor to muscarinic stimulation from 1-week STZ-induced diabetic rats, whilst Longhurst & Belis (1986) demonstrated a decrease in sensitivity of detrusor to muscarinic stimulation in 2-months STZ-induced diabetic rats.

Due to these conflicting reports, further pharmacological and molecular experiments are needed to characterise and understand the role of the M2- and M3-muscarinic receptor-subtypes involved in mediating the detrusor contractions in this model of bladder dysfunction.

3.1.3 Pharmacological characterisation of muscarinic receptors in the smooth muscle

Classically, muscarinic receptors were defined on the basis of selective agonism by muscarine and antagonism by atropine. However, pharmacological characterisation of muscarinic receptors has long been hindered by a lack of selective agonists and highly selective antagonists for any single receptor subtype. In addition, cells frequently co-express more than one subtype of these receptors, adding further difficulty to assigning a functional response to a single receptor population (Caulfield & Birdsall, 1998; Scarpero & Dmochowski, 2001). The presently available muscarinic antagonists, although not completely selective, are the best current tools for discriminating muscarinic receptor subtypes in a given tissue. Obtaining affinity constants (pK) or affinity estimates (pA_2) for the currently available muscarinic antagonists to generate an affinity profile of these drugs both at functional and cloned muscarinic receptors has been used extensively by pharmacologists, allowing for better characterisation of muscarinic receptors in a specific tissue (Caulfield & Birdsall, 1998; Scarpero & Dmochowski, 2003). Thus, a particular muscarinic receptor subtype is defined with affinity constants for a range of antagonists. Affinity constants of the key antagonists that can be used to achieve a pharmacological definition for M1-M3 muscarinic subtypes are given in Table 2.

Antagonist	Affinity constants(log dissociation constants of pKb values) for muscarinic receptors in mammalian smooth muscle		
	M1	M2	M3
Pirenzepine	7.8-8.5	6.3-6.7	6.7-7.1
AF-DX 116	6.4-6.7	7.1-7.2	5.9-6.6
Methoctramine	7.1-7.8	7.8-8.3	6.3-6.9
4-DAMP	8.6-9.2	7.8-8.4	8.9-9.3
p-FHHSiD	7.2-7.5	6.0-6.9	7.8-7.9
Himbacine	7.0-7.2	8.0-8.3	6.9-7.4

Table 2) Affinity ranges of key antagonists that can be used to achieve a pharmacological definition of M1-M3 muscarinic receptor subtypes. The values were estimated from studies on cloned receptors, radioligand- binding displacement studies as well as from functional studies of muscarinic receptors in various smooth muscle tissue in a variety of mammalian species. Collated from Caulfield, 1993; Eglen *et al*, 1994; Eglen *et al*, 1996; Caulfield & Birdsall, 1998.

According to the antagonist affinities listed in Table 2, M1-muscarinic receptors can be defined as those with a relatively high affinity towards pirenzepine and 4-DAMP and a low affinity towards compounds such as AF-DX 116, compared to M2- and M3-muscarinic receptors. M2-muscarinic receptors exhibit a relatively high affinity towards methoctramine, AF-DX 116 and a low affinity for pirenzepine, 4-DAMP, and p-FHHSiD, compared to M1- and M3-muscarinic receptor subtypes. M3-muscarinic receptors have a relatively high affinity towards 4-DAMP, a moderate affinity for *p*-FHHSiD and a low affinity for pirenzepine, AF-DX 116 and methoctramine when compared with M1- and M2-muscarinic receptors.

There are also a number of relatively new muscarinic receptor antagonists such as darifenacin and solifenacin that demonstrate a higher affinity for M3-

muscarinic receptors (Nilvebrandt *et al*, 1997; Gillberg *et al*, 1998; Wallis & Napier, 1999).

In order to obtain pKb and pA2 values for a specific muscarinic receptor antagonist in functional studies, cumulative concentration-response curves (CRCs) to a muscarinic receptor agonist in the absence and presence of the specific muscarinic receptor antagonist are obtained using a typical tissue bath set up. Postjunctional contractile responses of isolated smooth muscle tissue at each concentration of the muscarinic agonist are measured, up to a clearly defined sustained plateau. The correspondence between the concentration of the agonist and magnitude of the effect produced is an example of a concentration-response/dose-response relationship (Talarida & Jacob, 1979). The molar concentration of the agonist that produces 50% of the maximal possible effect is termed the EC_{50} value. The negative logarithm to base 10 of the EC_{50} value is termed pEC_{50}. The EC_{50} values of each CRC to the muscarinic receptor agonist in the absence and the presence of the antagonist are calculated. The presence of a competitive antagonist in a given concentration (B) produces a rightward displacement, or shift, of the agonist concentration-response curve. For a given concentration, the agonist dose-ratio or concentration-ratio (CR) can be determined. Affinity constants (pK_b) can then be calculated using the formula:

$$pK_b = log\ (CR\text{-}1) - log\ [B]$$

In practice the calculation of pK_b is made from not just one, but from several concentrations of the antagonist. By plotting the log (CR-1) values against log [B] values, a straight line with a slope of unity with an intercept with the abscissa is obtained. This method of plotting was used by Arunlakshana and Schild and is termed a Schild plot. The intercept of the straight line with the abscissa gives the pA_2 value (affinity estimate) (Talarido & Jacob, 1979, Arunlakshana & Schild, 1959). The Schild plot is based on the assumption that agonist and antagonist combine with the receptor in a freely reversible but mutually exclusive manner, that equilibrium has been reached and that the law of mass action can be applied. Schild analysis is a fundamental measure of the antagonist affinity for the specified receptor and usually correlates with the affinity constants obtained from functional *in vitro* and radio-ligand binding studies (Arunlakshana & Schild, 1959; Neubig *et al*, 2003).

If the slope of a Schild plot is similar to unity, this indicates that the responses of the tissue are mediated via a homogenous population of receptors. A slope different from unity implies a more complex interaction between the antagonist, the agonist and the receptor, including antagonism of the agonist action at more than one receptor, a stoichiometric difference from one antagonist molecule competing for the same receptor, a receptor with promiscuous contact with second messenger systems and others (Arunlakshana & Schild, 1959).

The functional analysis of muscarinic receptors in smooth muscle is not however, in itself, sufficient to fully assess their role in muscle function, since the approach does not yield any information on the receptor density or indeed the proportion of different muscarinic receptors expressed. Consequently, reverse transcription-polymerase chain reaction (RT-PCR), northern blot, western blot and other quantitative techniques should be used in concert with a functional approach to provide better characterisation (Eglen *et al*, 1996).

3.1.4 Molecular characterisation of muscarinic receptors in the smooth muscle

3.1.4.1 Real-time polymerase chain reaction (RT-PCR)

The fluorescence-based quantitative real-time reverse transcription-polymerase chain reaction (real time RT-PCR), first developed by Higuchi *et al* (1992), is a development of the established PCR technique and uses fluorescent labels to enable the continuous monitoring of amplicon formation throughout the reaction. Since many cellular processes concerning survival, growth, differentiation and function are reflected in altered patterns of gene expression, it has always been important in research to quantitate transcription levels of specific genes.

Real-time RT-PCR is now firmly established as a mainstream technology. Its high throughput, together with its use of novel chemistries, analysis of gene expression from very small amounts of sample, more reliable instrumentation and improved protocols have made it the method of choice for detection of mRNA. It allows quantification of relative differences in amounts of mRNA of the same gene between different samples e.g. control vs. treated.

Real-time RT-PCR adheres to the same basic principles of PCR (denaturation, primer annealing and polymerisation), but the monitoring of the accumulation of the amplicon has been made possible by utilisating fluorescence dyes. The simplest method uses a fluorescent dye that binds specifically to double-stranded DNA. SYBR-green is one of these fluorescent dyes and is used extensively. It binds to the minor groove of the PCR double stranded products as the reaction continues. This results in an increase in the fluorescence signal that is recorded during the annealing step, when double stranded products are formed. Fluorescence values are recorded during every cycle of a real-time PCR reaction and represent the amount of product amplified. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is significantly above the background. This point is defined as the threshold cycle or the Ct value. Data obtained from a real-time PCR run yields a sigmoidal curve when fluorescence intensity is plotted against the cycle number (reviewed in Wong and Medrano, 2005) (Figure 15). This curve can be broken down into four major phases: the linear ground phase, early exponential phase, log linear (exponential phase) and plateau phase. The Ct value is calculated at the early exponential phase. During the log linear phase, if the efficiency of the reaction is 100%, PCR reaches its optimal amplification period with amplified products doubling after every cycle. The plateau stage is reached when reaction components become limited and the PCR reaction is no longer useful for data calculation (reviewed in Wong and Medrano, 2005).

It must be noted that the DNA-binding fluorophore, SYBR green, may also bind to nonspecific products such as primer dimers. Primer-dimers can confuse interpretation of the results as false signals are generated. This problem can be solved using software capable of fluorescent melting curve analysis. Here the temperature at which the dsDNA amplicon is denatured is noted. The shorter primer-dimer can be discriminated by its reduced melting temperature compared with the full-length amplicon (Mackay et al, 2002).

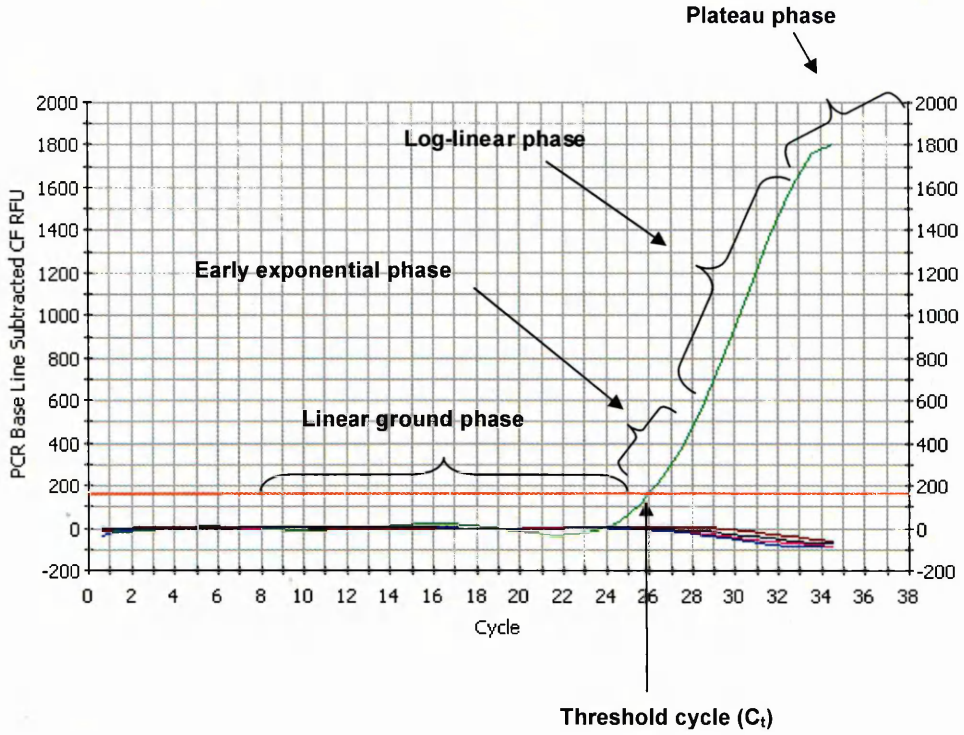


Figure 15) Phases of the PCR amplification curve. Adapted from Wong & Medrano, 2005.

3.1.4.2 Data analysis for real-time PCR

The Ct values obtained from a real-time RT-PCR reaction are then used in two types of calculations: absolute and relative quantitation.

Absolute quantitation of mRNA

In the absolute quantitation method, a standard curve is constructed from known standards and then is used to determine the concentration (copy number) of the starting templates according to their Ct values. This method assumes that all standards and samples have equal amplification efficiencies.

Relative quantitation

In relative quantitation, changes in sample gene expression are measured relative to an external standard or reference gene (house keeping gene). House keeping genes should have a stable expression in all cell types and their

expression should not be affected by different experimental treatments. Using a house keeping gene compensates for introducing varying amounts of mRNA in the total RT reaction and thus producing different concentrations of the starting template between different samples.

There are two methods of relative quantitation: the delta-delta Ct method described by Livak & Schmittgen (2001) and the method described by Pfaffl (2001). In the delta-delta Ct method, it is assumed that the efficiency of the target and the reference genes are within a close range. The delta–delta Ct method is only applicable for a quick estimation of the relative expression ratio and follows the formula:

$$ratio = 2^{[\Delta Ct\ control - \Delta Ct\ sample]}$$

where ΔCt control is the difference between the Ct values of the target and the reference gene in the control (non diabetic) samples, and the ΔCt sample is the difference between the Ct values of the target and the reference gene in the diabetic samples.

However, for a more reliable and accurate result, it is important to calculate the amplification efficiency of the target and the reference genes. The equation described by Pfaffl (2001) is then used to calculate the relative increase/decrease in expression ratio of the target gene versus a housekeeping gene and also incorporates the amplification efficiencies of the target and the reference gene (Pfaffl, 2001).

$$ratio = \frac{(E_{target})^{\Delta C_t\ target\ (control - treated)}}{(E_{ref})^{\Delta C_t\ ref\ (control - treated)}}$$

E= efficiency of the target (E_{target}) or the housekeeping (E_{ref}) gene primers. ΔCt= the difference between the Ct values of the control (non-diabetic) sample and the treated (diabetic) sample.

3.2 Aims of the chapter

The aim of this chapter was to characterise the muscarinic receptors mediating smooth muscle contraction in bladders from control (non-diabetic) and STZ-diabetic rats and to investigate of the expression of M2- and M3-muscarinic receptor subtypes in bladders from these animals.

3.3.1 Pharmacological characterisation of muscarinic receptors in the rat bladder smooth muscle

Diabetes was induced in male Wistar rats (weight range of 200-350g) by intraperitoneal injection of STZ as described in Chapter 2. Rats were sacrificed and the bladder was removed from control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic animals. 3-4 longitudinal intact detrusor strips were cut from the bladder wall and were mounted in tissue baths as described in Chapter 2.

Following the equilibration period (60mins) in Krebs solution containing 5μM indomethacin (cyclooxygenase inhibitor) (Sigma, Dorset, UK), cumulative CRCs (1nM-10mM) to carbachol (CCH) a muscarinic receptor agonist, (Sigma, Dorset, UK) were obtained.

Tissues were washed with Krebs for 20 min until baseline tension returned and were then equilibrated for 30-45 min with Krebs solution containing a selective muscarinic receptor subtype selective antagonist (Figure 1).

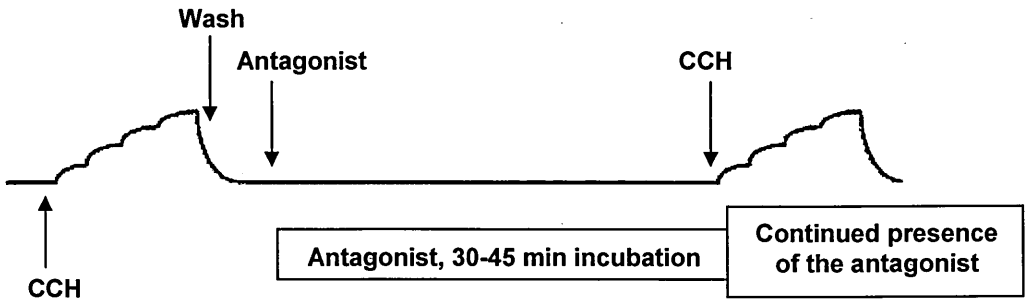


Figure 16) Illustration of the experimental protocol employed for *in vitro* functional studies of muscarinic-receptors in isolated detrusor strips from the rat bladder.

The antagonists used were 4-DAMP (relatively selective M3-muscarinic receptor antagonist) (Tocris, Bristol, UK), methoctramine (relatively selective

M2-muscarinic receptor antagonist) (Sigma, Dorset, UK) and pirenzepine (relatively selective M1-muscarinic receptor antagonist) (Sigma, Dorset, UK). Following incubation with the antagonist, a second concentration-response curve to CCH was constructed in the continued presence of the antagonist. Four consecutive concentration-response curves were obtained to CCH in each tissue, with washes between each; one control curve and three curves in the presence of increasing concentrations of pirenzepine (3-30 μM), 4-DAMP (3-30nM) or methoctramine (3-30 μM). Each tissue was subjected to only one antagonist throughout the experiment.

3.3.2 Data analysis

The changes in developed tension to CCH were plotted as a percentage of the maximum increase for each CRC or as absolute grams of tension. Individual EC_{50} values (molar concentrations producing half maximal response) were determined by non-linear regression curve fitting of CRCs using GraphPad 'Prism' (CA, San Diego, USA) software. Mean -log EC_{50} ($-pEC_{50}$) values with SEM were calculated. CRCs were also plotted where the responses were normalised per mg of tissue, in order to calculate the maximum tension developed and to ensure that any changes observed in the responses of the bladder strips to CCH were not due to changes in the size of the bladder strips. In order to eliminate any time dependent changes in the tissues or any vehicle dependent effect, time control experiments were performed, where one detrusor strip was set up in parallel to experimental tissues and was treated with the vehicle only. These experiments were used to obtain correction factors which were then applied to the experimental data to eliminate any non-experimental changes. Correction factors were calculated for the changes in maximum contractions and pEC_{50}s using the formula below:

$$CF = \frac{2^{nd}\ Max\ stimulation\ response\ or\ pEC_{50}\ value}{1^{st}\ Max\ stimulation\ response\ or\ pEC_{50}\ value}$$

Affinity constants (pK_B) for muscarinic antagonists were determined using the formula described in Section 3.1.3. pK_B values are presented as mean ±SEM.

Schild plots were also constructed and pA_2 values determined from the intercept on the abscissa (Arunlakshana & Schild, 1959).

3.3.3 Statistical Analysis

Data are expressed as mean ±SEM. Differences in pEC_{50} values and mean maximum responses to CCH between control (non-diabetic) and diabetic tissues were compared using one way *ANOVA* followed by Dunnett's multiple comparison post test. Differences between the mean maximum responses in the absence and the presence of the highest concentration of antagonist, was compared using Student's *t*-test (two-tailed).

Departure from unity of the slopes of the Schild plots was determined using 95% confidence intervals. Confidence intervals were calculated for the slopes of the Schild plots for each antagonist in control (non-diabetic) and diabetic tissues. If the confidence intervals overlapped with unity, then it was concluded that the slope is not significantly different from 1. If the range of confidence intervals did not span the number 1, then the slopes are significantly different from unity. If the confidence intervals of the Schild plots, for a specified antagonist, in diabetic tissues overlapped that of the control (non-diabetic) tissues, then it was concluded that the slopes are not significantly different from control (non-diabetic) tissues.

3.3.4 Molecular characterisation of muscarinic receptors in the rat bladder smooth muscle

3.3.4.1 RNA extraction

Freshly isolated whole bladders from control (non-diabetic) (n=5), 1-week (n=3), 4-week (n=5), 8-week (n=6) and 12-week (n=8) diabetic rats were cut open and the urothelium was carefully dissected off the underlying tissue. The bladders were then snap frozen in liquid nitrogen and ground to powder using a pestle and mortar. The powdered tissue was transferred into 1ml of Tri-reagent

(Ambion, Austin, TX, USA) and homogenised manually using a homogenisation vessel. The mixture of homogenised tissue in Tri-reagent was then transferred to a 1.5ml Eppendorf tube (Starlabs ltd, Milton Keynes, UK). Upon addition of 200µl chloroform (Sigma, Dorset, UK) to the homogenised tissue and centrifugation (13000rpm for 10mins at 4°C), the mixture was separated into three phases: 1) an aqueous phase 2) an interphase containing DNA and 3) an organic protein phase. 400µl of the aqueous phase containing RNA was transferred into a new RNAse free Eppendorf tube. RNA was then isolated using a Ribo Pure^{TM} kit (Ambion, Warrington, UK) according to the protocol provided by the manufacturer, which involved passing the RNA solution through a filter cartridge by centrifugation at 13000rpm for 1min. The RNA was subsequently bound to the resin of the cartridge and was then washed with high salt solutions provided in the kit. Finally the RNA was eluted using 50-100µl of water. All RNA preparations were treated with RNAse free DNAseI to ensure that no genomic DNA was present in the reactions.The DNAseI was then inactivated by heating the mixture for 15mins at 65ºC.

RNA integrity and concentration were analysed using an Experion automated electrophoresis system (Biorad laboratories, Hemel Hempstead, UK). In brief, 1µl of RNA was loaded onto the microfluidic chips in the priming station. The samples were then automatically subjected to gel-based electrophoresis, staining, destaining, band detection and imaging. RNA integrity was evaluated by examination of the electropherogram and simulated gel view.

3.3.4.2 Complementary DNA (cDNA) synthesis

RNA samples were then used as templates for synthesis of first-strand cDNA by reverse transcription (RT). 100µl of RT reaction was prepared by mixing 65µl of RNA (final concentration in the reaction: 65-325ng/ul) with 8µl of oligo dT_{18} primers (final concentration in the reaction: 0.04mg/ml) and 5µl dNTPs (final concentration in the reaction: 0.5mM) in a 1.5ml Eppendorf tube and incubating the mixture at 65ºC for 10min. The primed RNA was then incubated with 20µl reaction buffer (5x) (Invitrogen, UK) and 2µl superscript III enzyme (final concentration in the reaction: 4units/µl) (Invitrogen, UK) at 50ºC for 60 minutes

to initiate the cDNA synthesis reaction and then heated at 70°C for 10 min to inactivate the reaction.

3.3.4.3 Real time PCR and house-keeping gene validation

2µl of each RT reaction was diluted at 1:3 to 1:1000 in distilled RNAse/DNAse free water. These serial dilutions of cDNA were subsequently used in real-time PCR to determine the efficiency of the primers. A 20µl PCR reaction, consisting of the components described in Table 3, was prepared to the indicated end concentration.

Reagent	Stock concentration	Final concentration in the reaction	Volume added
SYBR Green iQ PCR mastermix (Biorad)	2x	1x	10µl
Forward primer	5µM	0.25µM	1µl
Reverse primer	5µM	0.25µM	1µl
cDNA		1:3-1:1000	2µl
H_2O			6µl
		Total	20µl

Table 3) The real-time PCR reaction components. Reagents used for amplification of serial dilutions of cDNA samples obtained from control (non-diabetic) and diabetic rat bladders with housekeeping gene and M2- and M3-muscarinic receptor primers.

Real-time PCR reactions were set up with either the housekeeping gene or the target gene (M2- or M3-muscarinic receptors) primers and were performed in 96 well plates. The amplification primer pairs used for housekeeping genes and M2- and M3-muscarinic receptors are listed in Table 5. Primers were previously designed and validated for qPCR by Dr Neil McKay.

Target gene	Accession no	Primer sequence	P.S
GAPDH	NM-008084	F: 5′ ACCCAGAAGACTGTGGATGG 3′ R: 5′ CACATTGGGGGTAGGAACAC 3′	171bp
Mouse β-actin	NM_007393	F: 5′ TGTTACCAACTGGGACGACA 3′ R: 5′ GGGGTGTTGAAGGTCTCAAA 3′	165bp
Collagen	NM_053304	F: 5′ CAACCTCAAGAAGTCCCTGC 3′ R: 5′ AGGTGAATCGACTGTTGCCT 3′	77bp
Albumin	NM_134326.	F: 5′ CATCCTGAACCGTCTGTGTG 3′ R: 5′ TTTCCACCAAGGACCCACTA 3′	86bp
HPRT	NM_012583	F: 5′ GCGAAAGTGGAAAAGCCAAGT 3′ R: 5′ GCCAGACTGGAAAGCCAACTA 3′	155bp
UBC	NM_017314	F:5′TCGTACCTTTCTCACCACAGTATCTAG 3′ R:5′GAAAACTAAGACACCTCCCGATCA 3′	82bp
γWHAZ	BC094305	F: 5′ CAAGCATACCAAGAAGCATTTGA 3′ R: 5′ GGGCCAGACCCAGTCTGA 3′	76bp

Table 4) House keeping genes primer sequences, gene accession numbers and product size. GAPDH=glyceraldehyde-3-phosphate dehydrogenase; HPRT=Hypoxanthine guanine phosphoribosyl transferase 1;UBC=ubiquitin; γWHAZ=tyrosine 3-monooxygenase/tryptophane 5-monooxygenase activation protein, zeta polypeptide. bp= base pairs; P.S=product size, F=forward R=reverse.

Target gene	Accession no	Primer sequence	P.S
M2-muscarinic receptor subtype	NM_031016	F: 5′ TTAAAGTCAGCCGCCACCTT 3′ R: 5′ CAGGTCCCAAAGCCCAGTAG 3′	138bp
M3-muscarinic receptor subtype	M62826	F: 5′ AAGACCTGGCTGGCCTACAG 3′ R: 5′ TCCTGGATGATCGTTTCACG 3′	129bp

Table 5) Muscarinic M2- and M3-receptor gene primer sequences, gene accession numbers and product size. bp=base pairs. P.S= product size. F=forward R=reverse.

Duplicate preparations of each dilution were used in the PCR reaction. Negative controls consisted of sterile distilled water instead of the cDNA template. Real-time PCR was performed in a Bio-Rad iCycler and the data was collected automatically by the computer using iCycler software. The amplification cycle protocol was 3 min denaturation at 95 ºC followed by 35 cycles at 95ºC for 15s, 60ºC for 15s and 72ºC for 30s, with a final step of 95ºC for 30s (Figure 17).

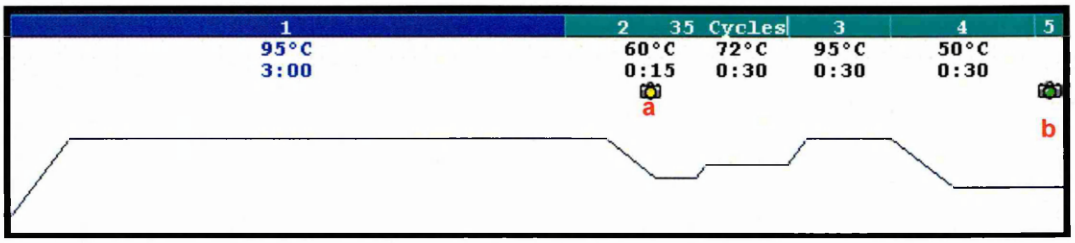


Figure 17) Illustration of the real-time PCR thermal programme in the iCycler. a: data collection stage b: melt curve analysis.

Following the PCR reaction, the amplified products were subjected to a melt curve test.

3.3.4.4 Data analysis

Amplification efficiencies were calculated automatically by iCycler software by plotting the Ct values for each of the dilutions against the concentration. This resulted in a linear graph and the slope of the graph was subsequently used in the equation stated below to calculate the efficiency of the primers:

$$E = 10^{[\frac{-1}{Slope}]}$$

Signals were considered to be indicative of correctly amplified products if the melt curve analysis exhibited only one sharp peak at high temperature and the negative control (no template) yielded no signal in real-time PCR.

Since it has been reported that the expression of some housekeeping genes can also vary in different experimental conditions it was important to validate the

expression stability of a housekeeping gene for the specific requirements of our experiment prior to its use in normalisation (Dheda *et al*, 2004). In order to do this, seven housekeeping genes primers listed in Table 4 were used in a real time PCR reaction with cDNA obtained from control (non-diabetic) and diabetic rat bladder tissues. The most stable housekeeping gene was then determined using Excel based geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/).

Real-time PCR reactions were then set up as described to determine the expression ratios of M2 and M3 muscarinic receptors in diabetic bladder compared to control bladder.

Analysis was performed by calculating the relative expression ratio of the target gene (M2- or M3-muscarinic receptors) based on its efficiency (E) and the difference in C_t value (delta C_t) of the treated sample (diabetic) against that of a control (non-diabetic) sample and expressed in comparison to a reference gene according to the method describe by Pfaffl (2001).

3.4 Results

3.4.1 Pharmacological characterisation of muscarinic receptors in rat bladder smooth muscle

The muscarinic receptor agonist CCH produced concentration-dependent contraction of the bladder detrusor strips from control (non-diabetic) and diabetic animals (Figure 18).

The -pEC_{50} values for control and diabetic tissues were not significantly different from each other, demonstrating no change in sensitivity of diabetic tissues to CCH (Table 6).

	C	1-week D	4-week D	8-week D	12-week D
-pEC_{50}	5.72±0.04	5.80±0.03	5.63±0.05	5.52±0.05	5.83±0.03
Max(g)	2.90±0.20	4.69±0.22***	5.16±0.30***	6.54±0.30***	6.48±0.20***
Max(g/mg tissue)	0.17±0.01	0.22±0.01*	0.14±0.01	0.19±0.02	0.15±0.01
n	30	31	24	20	20

Table 6) Responses of bladder strips from control (non-diabetic) (C) & diabetic (D) rats to CCH. pEC_{50} and maximum tension values are compared between control (non-diabetic) tissues versus diabetic tissues. ***p<0.001 & *p<0.05 vs. control (non-diabetic tissues) (C). n=number of detrusor strips.

Maximum responses to CCH in detrusor strips from control and diabetic rats are presented in Table 6. The maximum contractile responses (g) produced by CCH were significantly greater in bladder strips from diabetic rats compared to the controls (Table 6). However, since diabetic bladders showed an increased mass, the responses were normalised per mg tissue. Following normalisation of data to account for tissue weight, a significantly greater contractile response to CCH was observed only in bladder strips from 1-week diabetic rats compared to bladder strips from control rats (p=0.021). The normalised maximum responses of 4-week, 8-week and 12-week diabetic tissues did not differ significantly from the control (non-diabetic) tissues (Table 6) (Figure 18).

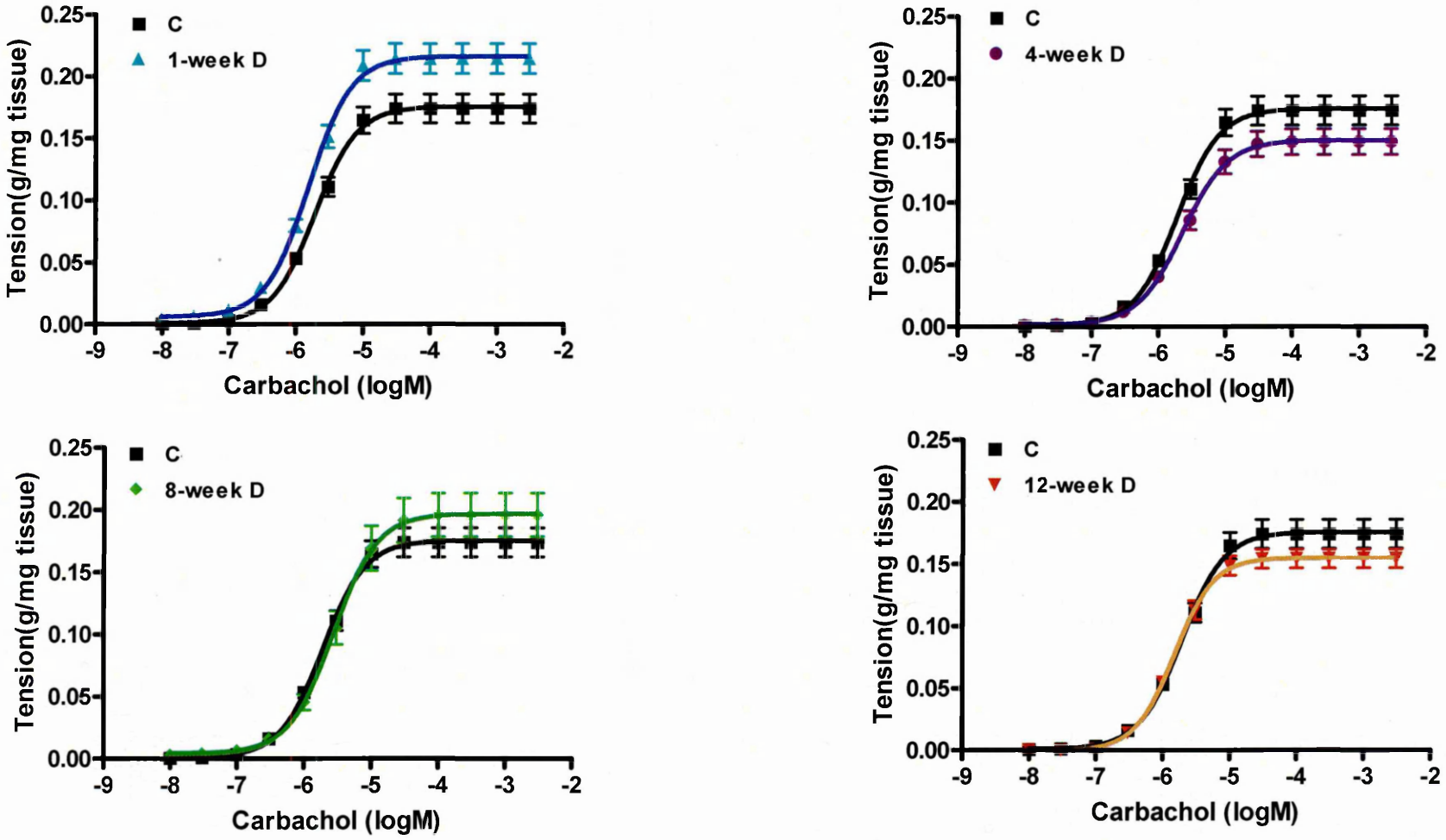


Figure 18) Responses of bladder strips from control (non diabetic) (C) & diabetic (D) animals to CCH. C: n=30, 1-week D: n=31, 4-week D: n=24, 8-week D: n=20 & 12-week D: n=20. Data is presented as mean grams tension/mg tissue weight ± SEM. n=number of detrusor strips. All data is expressed as mean±SEM. n=number of bladder strips.

4-DAMP, methoctramine and pirenzepine produced parallel, rightward displacements of the CRCs to CCH, without affecting maximum responses in bladder strips from control (non-diabetic) and diabetic rats (Figures 19-23; Tables 7-10). Mean affinity (pK_B) values calculated for each antagonist are also shown in Tables 7-9 for bladder strips from control and diabetic rats. 4-DAMP antagonised CCH responses with high affinity whilst methoctramine and pirenzepine antagonised CCH responses with lower affinity. Mean affinity (pK_B) constants for each antagonist were similar in control and diabetic tissues (Tables 7, 8 and 9).

Schild plots for each of the muscarinic receptor antagonists are demonstrated in Figures 19-23. The slope and pA_2 values obtained from these plots for each antagonist in each group are demonstrated in Tables 7-9. Schild plots for the antagonism of responses by 4-DAMP and pirenzepine had slopes that did not differ significantly from unity confirming a competitive mode of action of the antagonists. However, the slopes of schild plots for methoctramine in 1-week, 4-week and 8-week diabetic tissues were significantly less than 1 but not significantly different from control (non-diabetic) based on 95% confidence intervals (Table 11). The slope of Schild plots for methoctramine in 12-week diabetic tissues was not significantly different from 1 (based on 95% confidence intervals).

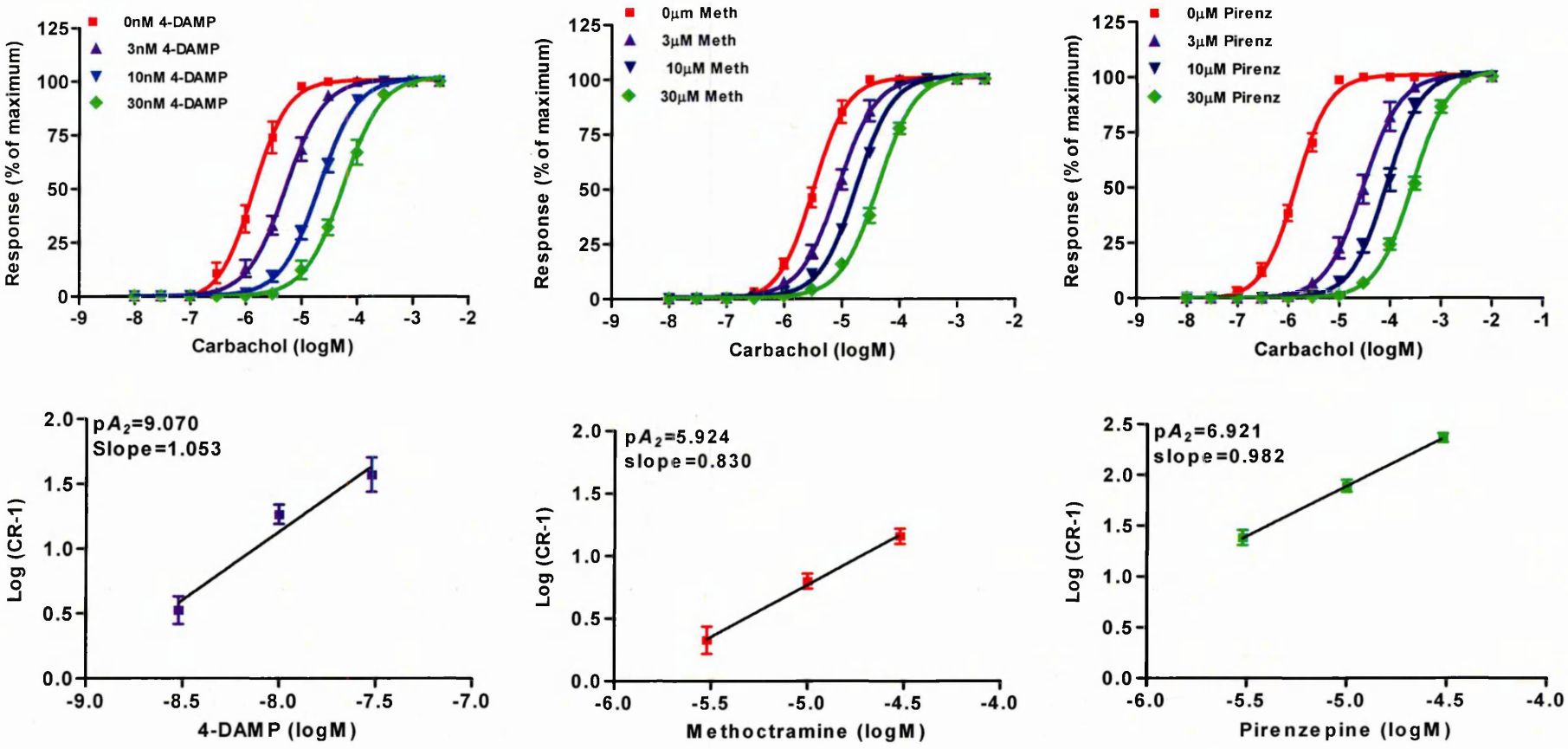


Figure 19) Antagonism of CCH-induced contractions of bladder strips from control (non-diabetic) rats by 4-DAMP, methoctramine and pirenzepine. Upper panel: cumulative concentration-response curves (uncorrected) to CCH in the absence and presence of 4-DAMP, methoctramine and pirenzepine respectively. Responses are expressed as percentage of the maximum response obtained in each curve. Lower panel: Schild plots for the antagonism of CCH responses by 4-DAMP, methoctramine and pirenzepine. All data is expressed as mean±SEM.

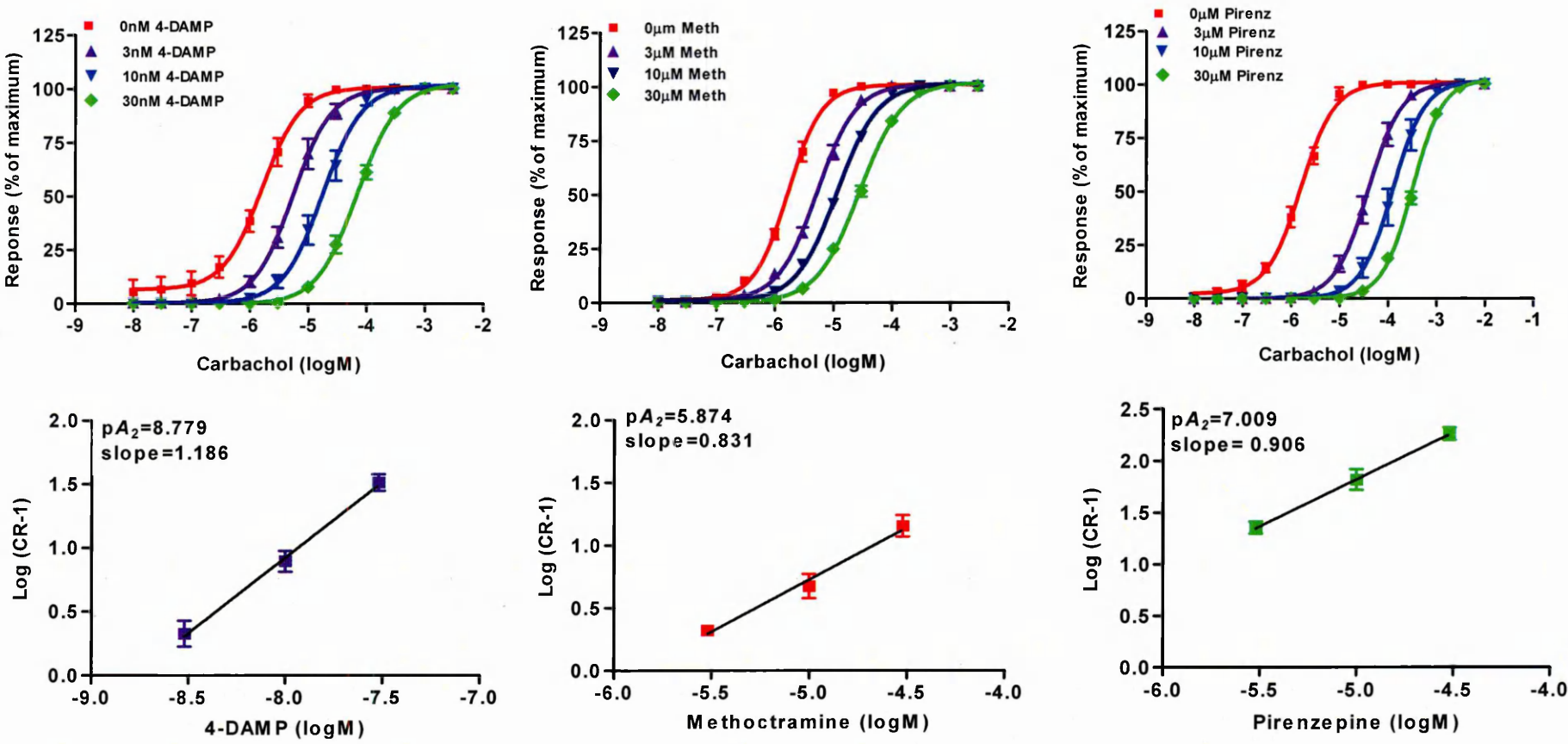


Figure 20) Antagonism of CCH-induced contractions of bladder strips from 1-week diabetic rats by 4-DAMP, methoctramine and pirenzepine. Upper panel: cumulative concentration-response curves (uncorrected) to CCH in the absence and presence of 4-DAMP, methoctramine and pirenzepine respectively. Responses are expressed as percentage of the maximum response obtained in each curve. Lower panel: Schild plots for the antagonism of CCH responses by 4-DAMP,methoctramine and pirenzepine. All data is expressed as mean±SEM.

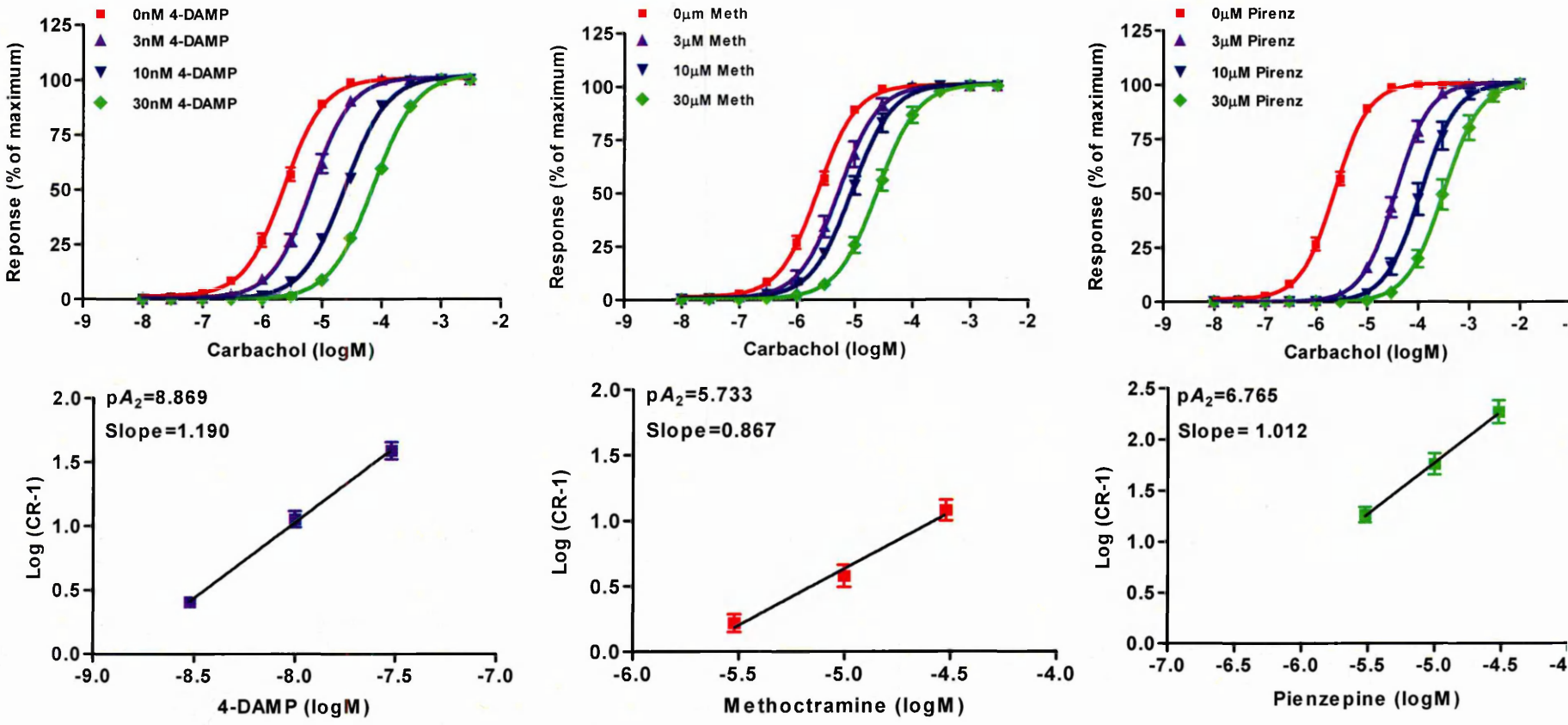


Figure 21) Antagonism of CCH-induced contractions of bladder strips from 4-week diabetic rats by 4-DAMP, methoctramine and pirenzepine. Upper panel: cumulative concentration-response curves (uncorrected) to CCH in the absence and presence of 4-DAMP, methoctramine and pirenzepine respectively. Responses are expressed as percentage of the maximum response obtained in each curve. Lower panel: Schild plots for the antagonism of CCH responses by 4-DAMP,methoctramine and pirenzepine. All data is expressed as mean±SEM

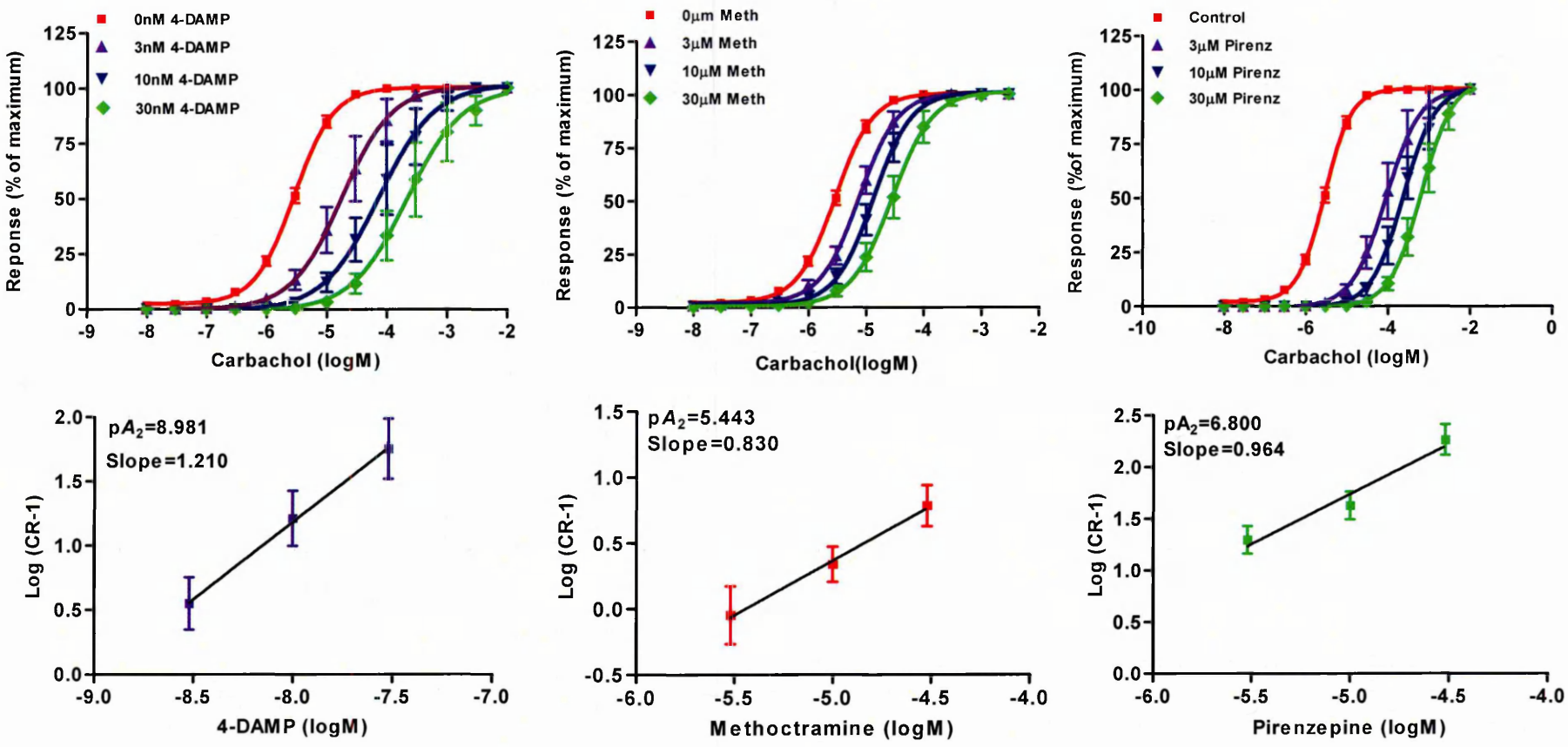


Figure 22) Antagonism of CCH-induced contractions of bladder strips from 8-week diabetic rats by 4-DAMP, methoctramine and pirenzepine. Upper panel: cumulative concentration-response curves (uncorrected) to CCH in the absence and presence of 4-DAMP, methoctramine and pirenzepine respectively. Responses are expressed as percentage of the maximum response obtained in each curve. Lower panel: Schild plots for the antagonism of CCH responses by 4-DAMP,methoctramine and pirenzepine. All data is expressed as mean±SEM.

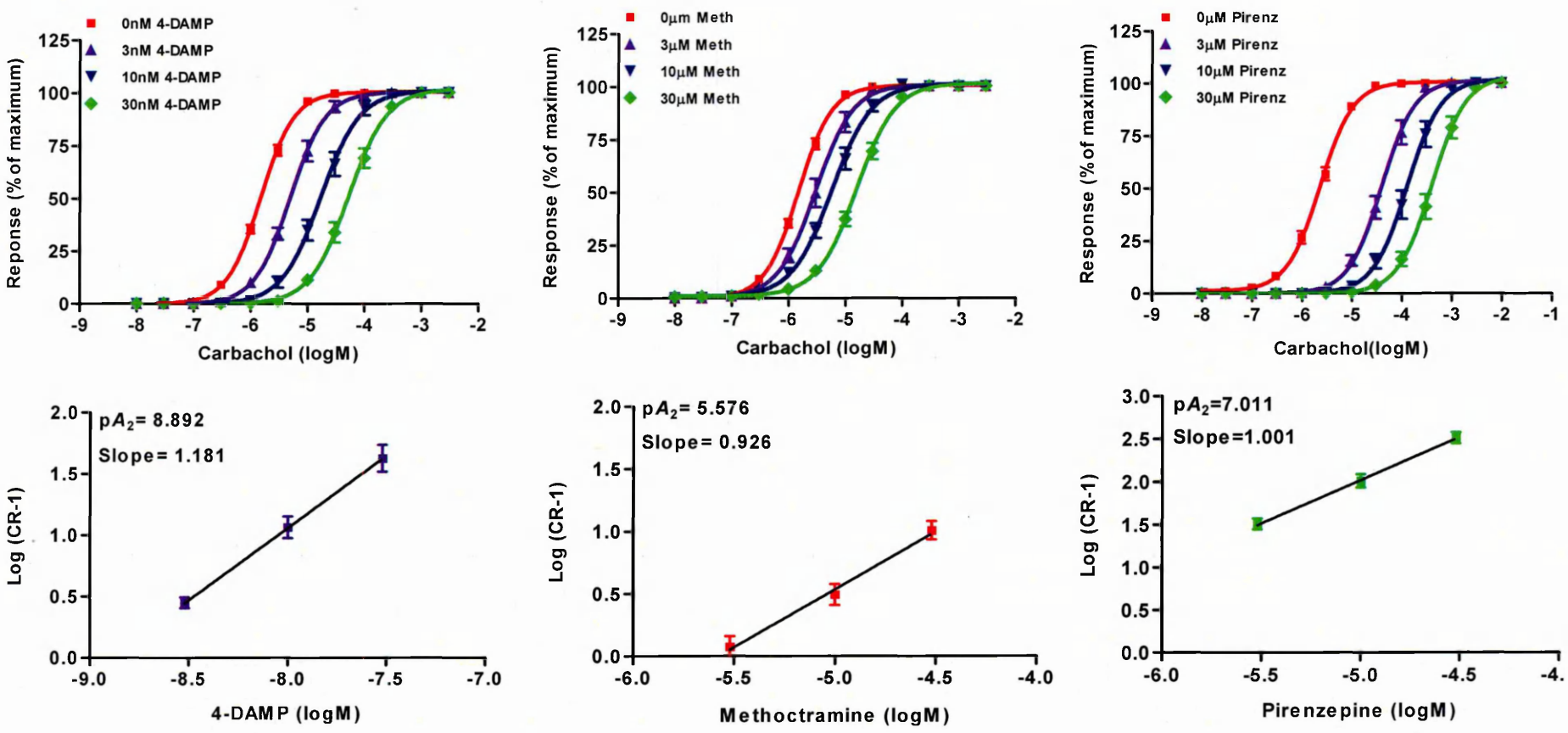


Figure 23) Antagonism of CCH-induced contractions of bladder strips from 12-week diabetic rats by 4-DAMP, methoctramine and pirenzepine. Upper panel: cumulative concentration-response curves (uncorrected) to CCH in the absence and presence of 4-DAMP, methoctramine and pirenzepine respectively. Responses are expressed as percentage of the maximum response obtained in each curve. Lower panel: Schild plots for the antagonism of CCH responses by 4-DAMP,methoctramine and pirenzepine. All data is expressed as mean±SEM.

4-DAMP	Control	1-week D	4-week D	8-week D	12-week D
pK_B	9.13±0.09	8.92±0.07	9.03±0.05	9.18±0.22	9.05±0.07
n	6	6	6	5	5
pA_2	9.07	8.78	8.87	8.98	8.89
slope	1.17±0.15	1.19±0.05	1.12±0.04	1.21±0.04	1.18±0.01

Table 7) Affinity estimates for 4-DAMP in control (non-diabetic) (C) & diabetic (D) detrusor strips. p*A*2 and slope values were derived from Schild plots. n=number of tissues. All data is expressed as mean±SEM

Meth	Control	1-week D	4-week D	8-week D	12-week D
pK_B	5.77±0.06	5.73±0.07	5.48±0.08	5.37±0.20	5.54±0.07
n	5	6	6	5	5
pA_2	5.92	5.87	5.73	5.44	5.58
slope	0.83±0.09	0.83±0.05	0.87±0.04	0.83±0.14	0.93±0.07

Table 8) Affinity estimates for methoctramine in control (non-diabetic) (C) & diabetic (D) detrusor strips. p*A*2 and slope values were derived from Schild plots. n=number of tissues. All data is expressed as mean±SEM.

Pirenz	C	1-week D	4-week D	8-week D	12-week D
pK_B	6.89±0.04	6.82±0.06	6.78±0.09	6.79±0.10	7.02±0.07
n	6	6	6	5	5
pA_2	6.92	7.01	6.77	6.80	7.01
slope	0.98±0.01	0.91±0.01	1.01±0.03	0.96±0.20	1.00±0.02

Table 9) Affinity estimates for pirenzepine in control (non-diabetic) (C) & diabetic (D) detrusor strips. p*A*2 and slope values were derived from Schild plots. n=number of tissues. All data is expressed as mean±SEM.

Group		4-DAMP (30nM)	Meth (30μM)	Pirenz (30μM)
C	1st curve (no antagonist)	2.94±0.5g	2.41±0.5g	3.78±0.3g
	Max (g) (antagonist present)	3.74±0.5g	3.37±0.5g	5.00±0.4g
1-week D	1st curve (no antagonist)	5.06±0.5g	4.62± 0.5g	4.31±0.6g
	Max (g) (antagonist present)	5.77±0.6g	4.79±0.5g	5.96±0.3g
4-week D	1st curve (no antagonist)	5.91±0.4g	4.66±0.6g	5.27±0.6g
	Max (g) (antagonist present)	6.19±0.4g	5.69±0.5g	6.11±0.4g
8-week D	1st curve (no antagonist)	7.17±0.3g	6.51±0.3g	5.63±0.9g
	Max (g) (antagonist present)	7.69±0.8g	7.45±0.4g	6.36±1.2g
12-week D	1st curve (no antagonist)	5.96±0.3g	5.95±0.4g	7.25±0.3g
	Max (g) (antagonist present)	6.60±0.3g	6.07±0.5g	7.80±0.4g

Table 10) Effect of muscarinic antagonists on maximum responses to CCH in detrusor strips from control (non-diabetic) (C) & diabetic (D) rats . Maximum responses are expressed as absolute tension (g) (n=5-6). All data is expressed as mean±SEM.

Group	Confidence intervals
C (n=5)	0.72-0.95
1-week D (n=6)	0.77-0.87
4-week D (n=6)	0.82-0.91
8-week D (n=5)	0.66-1.00
12-week D (n=5)	0.84-1.02

Table 11) Confidence intervals (95%) of slopes of Schild plots for methoctramine in control (non-diabetic) (C) and diabetic (D) tissues.

3.4.2 Molecular characterisation of M2- and M3-muscarinic receptor expression in rat bladder smooth muscle

3.4.2.1 Analysis of RNA integrity and quantity using Experion

The integrity of RNA samples from control (non-diabetic) and diabetic rat bladders were evaluated using Experion microfluidic electropherogram and subsequent simulated gel views. RNA integrity was confirmed by the presence of 28S and 18S rRNA peaks on the electropherogram and gel views produced by the Experion software. Figure 24 demonstrates an example of an electropherogram and stimulated gel view produced by the Experion software after the analysis of a control (non-diabetic) rat bladder sample. The presence of 28S and 18S ribosomal RNA bands confirms the high quality of this sample. Similar results were obtained for all diabetic rat bladder RNA samples (electropherogram not shown).

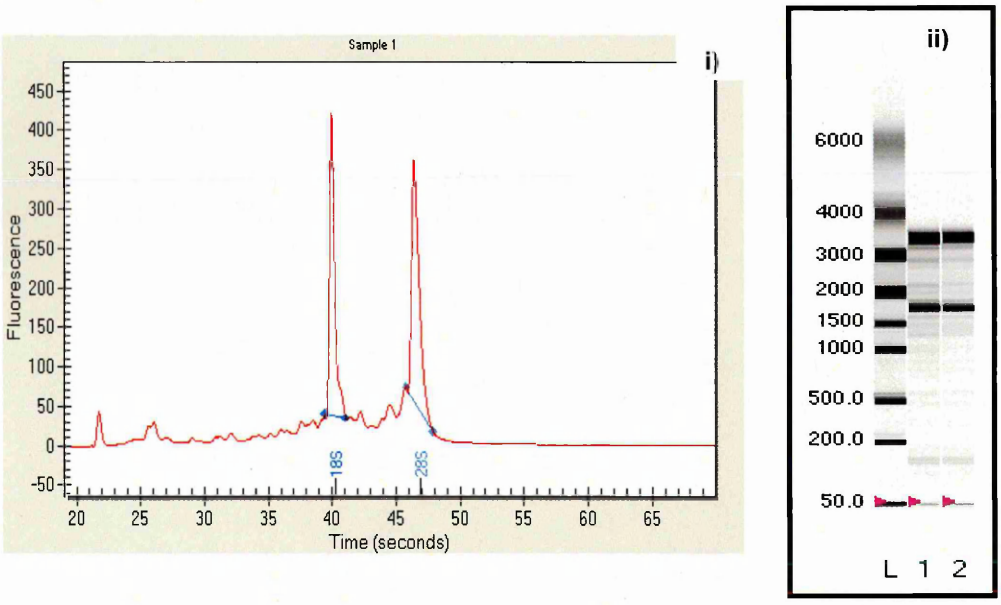


Figure 24) Electropherogram and stimulated gel view obtained from Experion software. i) Integrity of RNA was tested in a control (non-diabetic) sample by visualization of 18S and 28S rRNA peaks on the electropherogram. ii) Stimulated gel analysis: Lanes 1 and 2 represent RNA samples run in duplicate from a control (non-diabetic) bladder. No degradation can be seen in either lane.

3.4.2.2 Housekeeping gene validation

Primers designed for house keeping genes, listed in Table 4, were used in real-time RT-PCR reactions with both control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic bladder samples (n=6 for all groups) to determine the most stable housekeeping genes for the experimental conditions. The results of geNorm analysis are demonstrated in Figure 25. According to the results obtained from geNorm software, the most and the least stable housekeeping genes were HPRT and β-actin respectively. Therefore, HPRT was used as the internal reference gene for calculation of expression ratios of M2- and M3-muscarinic receptors in the diabetic tissues compared to the control (non-diabetic) tissues.

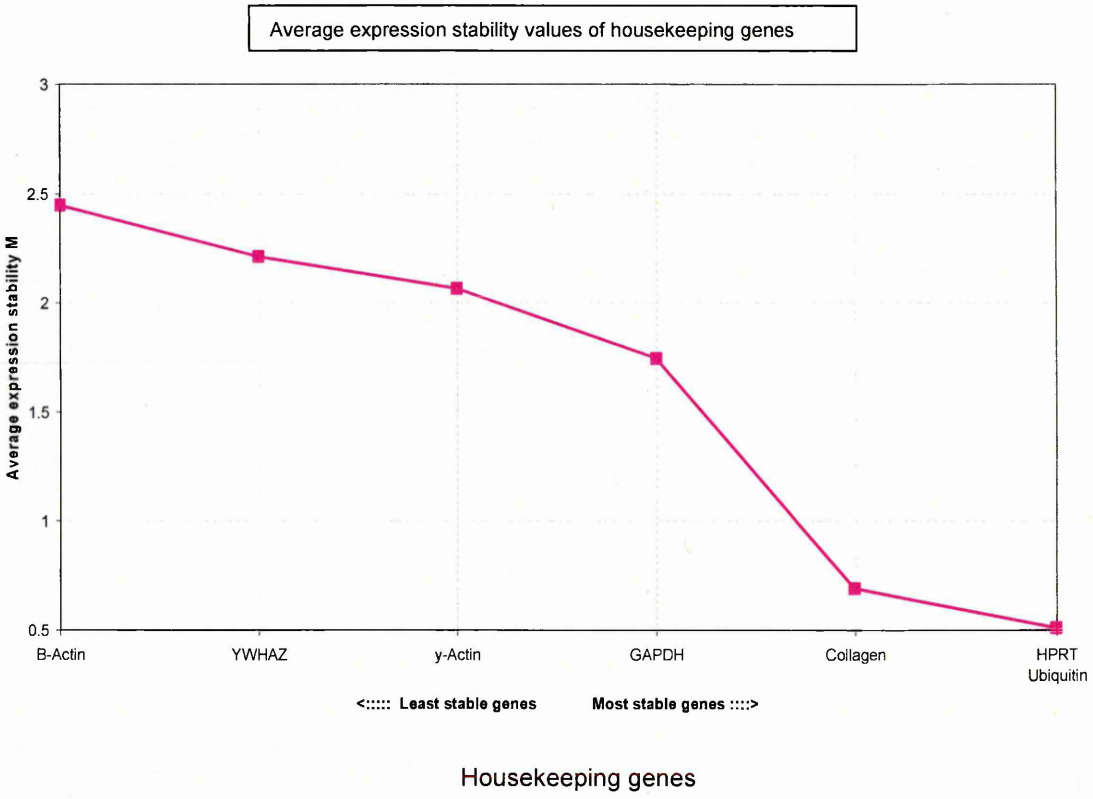


Figure 25) geNorm analysis demonstrating the most and the least stable housekeeping genes from control (non-diabetic) and diabetic samples.

3.4.2.3 Investigation of muscarinic receptor expression

In order to calculate the relative expression ratios of M2- and M3-muscarinic receptors in diabetic rat bladders vs. control (non-diabetic) rat bladders, amplification with house keeping and target gene primers was carried out in all dilutions (1:3, 1:10. 1:30, 1:100, 1:300, 1:1000) of each sample. The amplification curves for the serial dilutions of each sample (control (non-diabetic) and diabetic) for each set of primers were analysed and the Ct values for each dilution were recorded. The efficiencies of each primer set for each sample were determined.

Figures 26 demonstrates examples of a real-time PCR amplification curve, an efficiency plot and a melt curve analysis for serial dilutions of a control (non-diabetic) sample amplified with HPRT primers. The melt curve analysis demonstrates a clear sharp peak which is an indication of a specific amplified product. The Ct values of each dilution versus log10 concentration were plotted by iCycler, the result of which is a linear graph (Figure 26). The efficiency was then calculated by the software according to the formula described in section 3.3.4.4. (Figure 26). Figures 27 and 28 also demonstrate the amplification curves, efficiency plots and melt curve analysis for M3- and M2-muscarinic receptor primers with cDNA samples obtained from control (non-diabetic) rat bladders. Identical analysis was also performed for samples from diabetic rat bladder tissues.

Since there was no significant difference in the efficiency values between any primer pair for any sample group, the mean efficiency value obtained for each was calculated and used in the equation described by Pfaffl (2001) to determine the relative expression ratios. The average efficiency values of each primer set (HPRT, M2 & M3) and the average Ct values of each sample group (control, 1, 4, 8 and 12-week diabetic) for each primer set were subsequently used in the equation described by Pfaffl (2001) to obtain expression ratios.

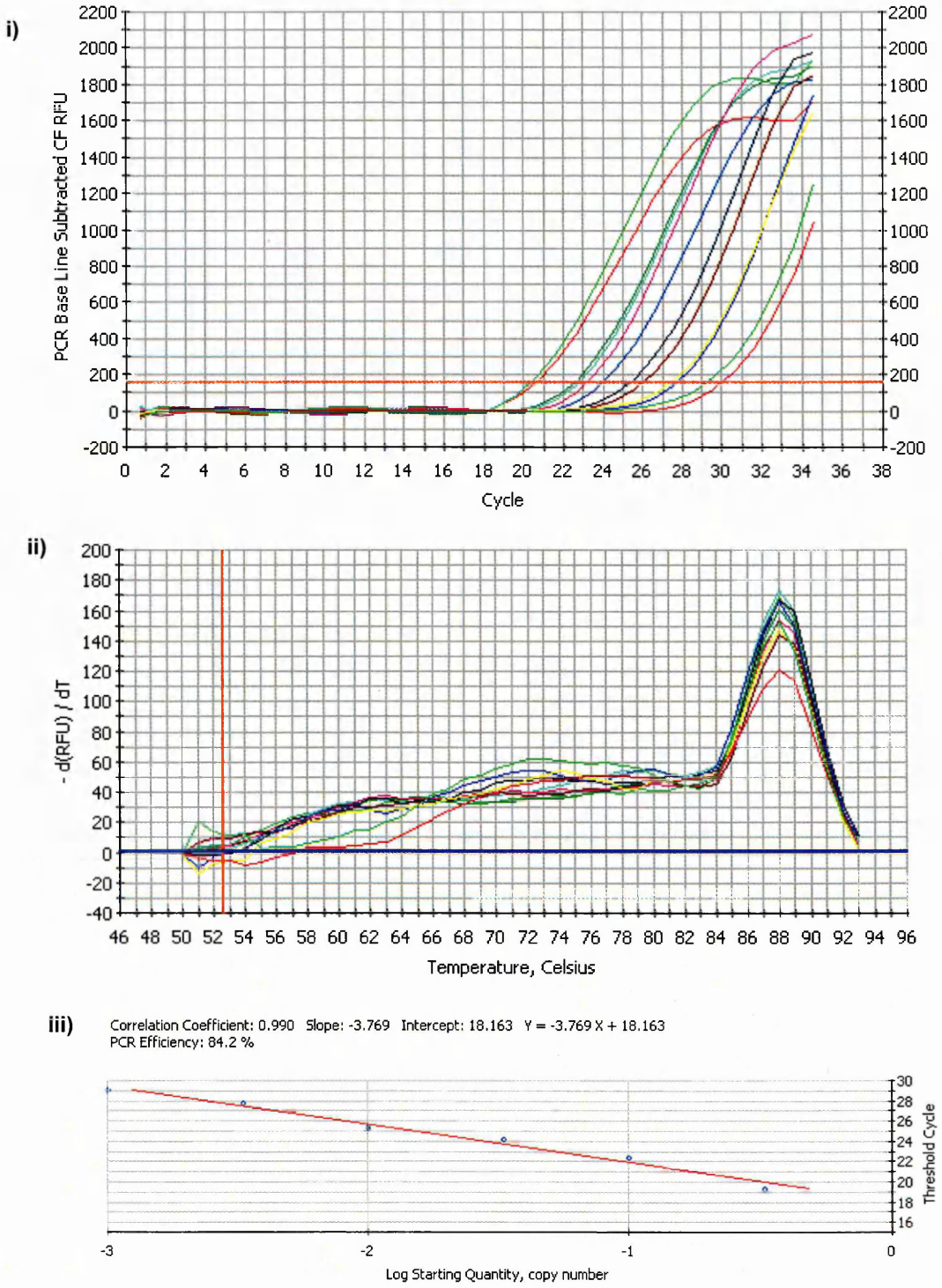


Figure 26) Real-time PCR amplification of HPRT primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1-1000), from left to right red & light green:1:3, light blue and dark green: 1:10 , pink and dark blue: 1:30, purple and brown:1:100, yellow and dark purple: 1:300, red and green:1:1000 dilutions respectively. ii) Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced iii) Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

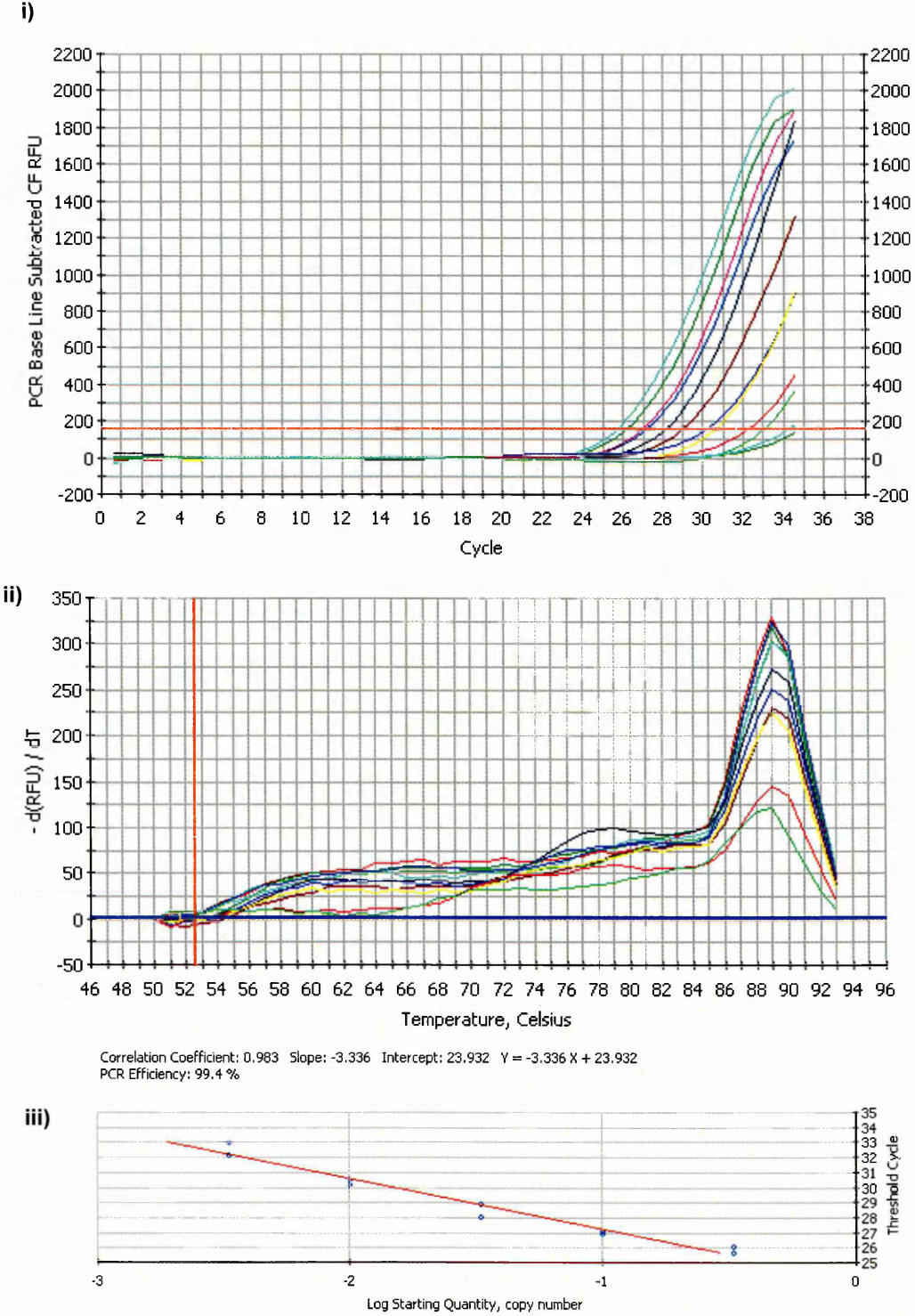


Figure 27) Real-time PCR amplification of M3 primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1-1000), from left to right: light blue & dark green: 1:3, pink & blue: 1:10, dark blue & brown: 1:30, yellow & purple: 1:100, red and light green: 1:300, light blue & dark green: 1:1000 dilutions. ii) Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced iii) Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

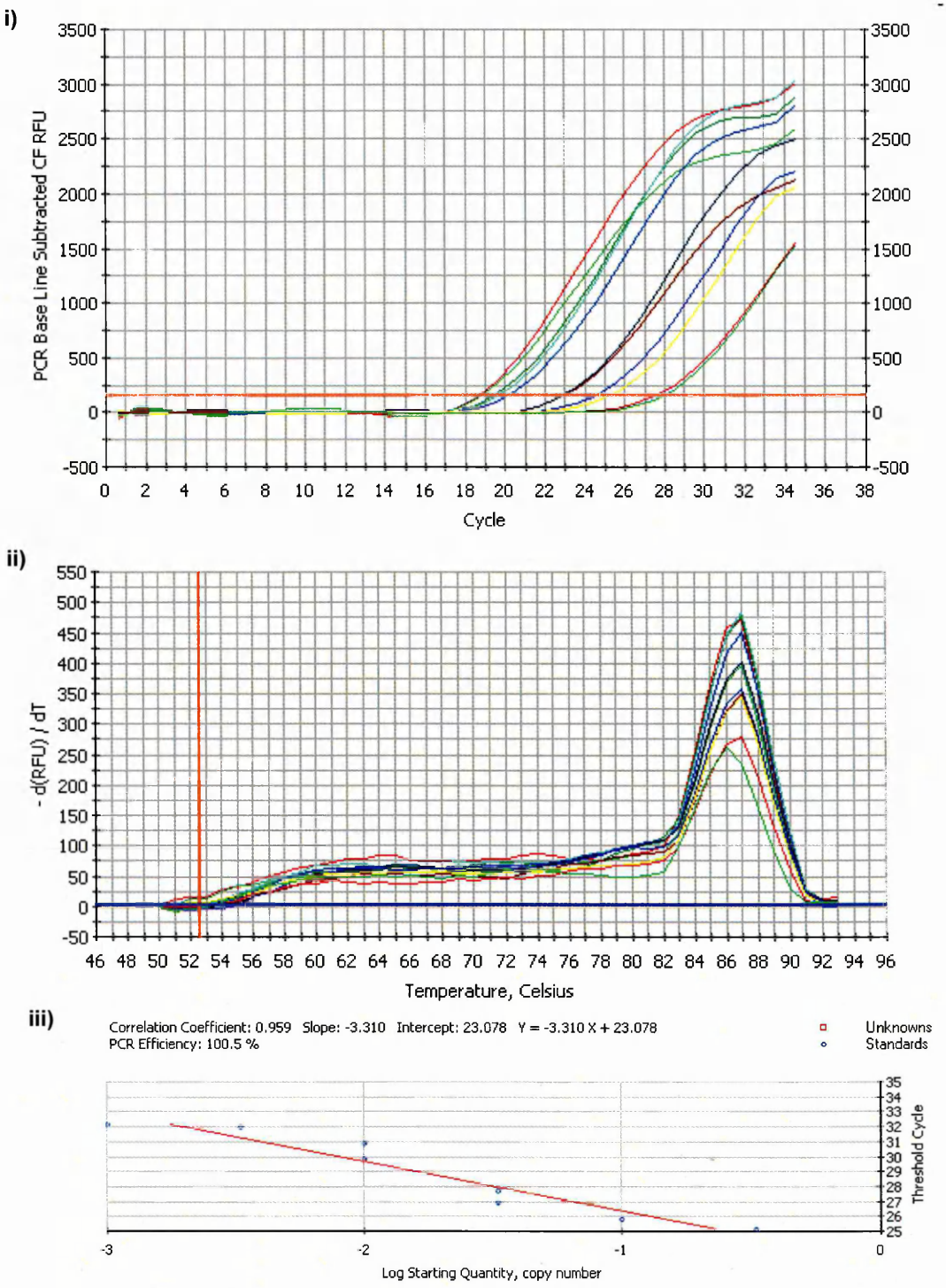


Figure 28) Real-time PCR amplification of M2 primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1-1000), from left to right: Red & light green: 1:3 (duplicate), light blue and dark green: 1:10, dark blue: 1:30, purple and brown: 1:100, yellow and dark purple: 1:300, red and green: 1:1000 dilutions respectively. ii) Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced iii) Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

The relative expression ratios of M2- and M3-muscarinic receptors for 1-week (n=3), 4-week (n=4), 8-week (n=6) and 12-week (n=8) diabetic denuded rat bladders compared to control (non-diabetic) tissues (n=5) (n=number of bladders) are shown in Table 12 .

Relative expression ratios	1-week diabetics	4-week diabetics	8-week diabetics	12-week diabetics
M2	2.20	1.14	1.19	1.01
M3	1.11	0.44	0.23	0.21

Table 12) Relative expression ratios of M2 and M3 muscarinic receptors in diabetic bladders compared to control (non-diabetic) bladders. Values <1 show a decrease in expression and values >1 demonstrate an increase in expression.

The relative expression ratio of the M2-muscarinic receptor was increased in 1-week, 4-week and 8-week diabetic tissues compared to control (non-diabetic) tissues, whilst it did not alter in 12-week diabetic rat bladders.

The relative expression ratio of the M3-muscarinic receptor was slightly increased in 1-week diabetic tissues, but was decreased in 4-week, 8-week and 12-week diabetic tissues compared to control (non-diabetic) tissues.

3.5 Discussion

Muscarinic antagonists are the mainstay of treatment for DO, yet little is known about the possible underlying changes in muscarinic-receptor-mediated pathways in this condition. Understanding the exact role of M2 and M3-muscarinic receptors in mediating the bladder detrusor contractions under normal and pathological conditions could help the development of better drug treatments in patients with voiding dysfunction and DO.

The sensitivity of the detrusor muscle to muscarinic agonists is altered in several disease states which lead to DO in humans, including denervation and bladder outflow obstruction (Harrison *et al*, 1987), with the animal models of these conditions demonstrating similar changes in detrusor function (Ruggieri & Braverman, 2006).

Diabetes has also been associated with DO (Salinas Casado *et al*. 1999) and in animal models of diabetes the detrusor can exhibit changes in the responses to cholinergic agonists similar to that observed in the human overactive detrusor (Mimata *et al*, 1995; Stevens *et al*, 2006).

On the basis of suggested altered biochemical and functional characteristics of muscarinic receptors in animal models of detrusor overactivity (DO) (Latifpour *et al*, 1989, Stevens *et al*, 2006), muscarinic-receptor-mediated function was investigated in the STZ-diabetic rat using muscarinic receptor agonists and receptor subtype selective antagonists

3.5.1 Responses of detrusor strips from control (non-diabetic) and diabetic rats to CCH.

In the present study, although the absolute contractile responses (g) to CCH were larger in diabetic tissues compared to control (non-diabetic) tissues, after normalising the contractile responses to account for change in tissue weight, an increased maximum response to the muscarinic agonist, CCH, was only detected in detrusor strips from 1-week diabetic rats compared to control (non-diabetic) tissues. The reason for this increased responsiveness of detrusor strips from 1-week diabetic animals is unknown but could be an initial compensatory response in 1-week diabetic tissues to diuresis and the metabolic

effects of diabetes, as suggested by Stevens *et al* (2006). Daneshgari *et al* (2006), who studied bladders from 3, 6, 9, 12 and 20-week STZ-diabetic rats, showed that diabetic bladders may undergo a contractile transition from a compensated to a decompensated state, with the decompensated state beginning 9-12 weeks after induction of diabetes. Liu & Daneshgari (2006), who also studied bladders from 4-day, 1-week, 2-week, 3-week and 9-week STZ-diabetic rats, showed that diabetic bladders undergo time dependent adaptive physical changes in the functional and structural components of the bladder wall. They believed that the significant increase in urine output in diabetic animals during the early stages of diabetes results in an increase in the bladder capacity and stimulation of hypertrophy and hyperplasia in the tissue. A major initial increase (from the normal levels) in the areas of the two main components of the bladder wall, the urothelium and smooth muscle, as a result of a vigorous growth response to polyuria, was detected in the bladders of 1-3-week diabetic rats. It was postulated that related alterations in bladder volume, rate of filling, and rate of stretch of the bladder results in changes in the shape of the smooth muscle cells and stimulation of DNA synthesis in these cells. This subsequently results in increased protein synthesis, cell mass and hyperplasia in the bladder which could account for the initial compensatory responses of the bladder smooth muscle in 1-3-week diabetic animals (Liu & Daneshgari, 2006). They suggested that the state of decompensation in the contractility of STZ-diabetic rat bladder at later stages of diabetes could be due to organ adaptation to the increased urine volume and increased bladder mass. Although Liu & Daneshgari (2006) concluded that the alterations detected in STZ-diabetic rat bladder are due to polyuria as the result of diabetes, Pitre *et al* (2002) noted that polyuria does not necessarily mimic the structural changes observed in bladders from STZ-diabetic animals. The results of the current study can not be associated with increase in tissue weight since the responses of detrusor strips from diabetic animals were normalised to the tissue weight.

An increased sensitivity to muscarinic agonists has been reported in the human overactive detrusor (Stevens *et al*, 2004a; 2004b). A similar increased sensitivity was also detected in the 1-week diabetic STZ-diabetic rat model of overactive bladder by Stevens *et al* (2006). However, conflicting results regarding the sensitivity of diabetic bladder detrusor to muscarinic agonists

have been reported, with some groups demonstrating increased (Kolta *et al*, 1985), decreased (Longhurst and Belis,1986) or no change in the contractile responses (Lincoln *et al*, 1984). In the present study, no change was seen in the sensitivity of diabetic tissues to muscarinic receptor stimulation compared to control (non-diabetic) tissues. Differences in the results obtained in this study compared to previous studies, such as Stevens *et al* (2006), could be due to differences in the experimental protocols. After conducting length-tension experiments (Chapter 2), the optimised initial load applied to the detrusor strips in the current study was 2g, whilst Stevens *et al* (2006) used an initial load of 1g during the equilibration period, which may have had an effect on the contractile responses. Differences in the time point at which diabetic animals have been used, or variations in the species of animals utilised in the experimental procedures, could also lead to discrepancies detected between various studies. For example, in the study by Malmgren *et al* (1989), who reported no change in muscarinic mediated contractions of detrusor strips, 6-week and 6-month female Sprague-Dawley diabetic rats were used (Malmgren *et al*, 1989), whilst in the study by Stevens *et al* (2006), an increase in sensitivity of CCH-induced contraction was detected in detrusor strips from 1-week diabetic male Wistar rats.

Although only 1-week diabetic tissues demonstrated a significant increase in their contractile responses to CCH, with no change in their sensitivity to this drug, changes in muscarinic-receptor subtype-mediated function could not be ruled out and were further investigated using muscarinic-receptor subtype selective antagonists.

3.5.2 Pharmacological characterisation of muscarinic receptors in the rat bladder smooth muscle

Muscarinic receptor subtype-mediated responses play a key role in bladder contractility and function. Thus, alterations in muscarinic receptor subtype mediated function may be important in the understanding of the disease state. Some groups (Braverman & Ruggieri, 2003) have suggested that muscarinic-receptor mediated detrusor contractions can alter from M3-mediated to M2-

mediated in pathological conditions such as bladder outlet obstruction and denervation in rats. Results of the present chapter demonstrated that the muscarinic-receptor mediating the direct contractile responses to CCH in bladder strips from control and diabetic rats displayed a high affinity for 4-DAMP (8.7-9.1), a low affinity for methoctramine (5.4-5.9) and an intermediate affinity for pirenzepine (6.8-7.0) and. In rat urinary bladder smooth muscle the affinity estimates of key antagonists pirenzepine (6.7-6.9) methoctramine (5.7-6.1) AF-DX 116 (6.2) and 4-DAMP (8.7-9.2) obtained from similar functional in vitro studies suggest the singular involvement of M3-muscarinic receptors in mediating the detrusor contraction (Longhurst *et al*, 1995b; Tong *et al*, 1997; Hedge *et al*, 1997).

The antagonist profile obtained in this chapter indicates that contractile responses of the detrusors from control (non-diabetic) and diabetic are mediated via M3-mucarinic receptor. In a recent similar study by Saito *et al* (2008), it was also demonstrated the detrusor contractions in young and old Type 2 Goto-Kakizaki diabetic rats are also mediated via the M3-muscarinic receptor only, with the role of M2-muscarinic receptor remaining unclear.

The antagonists used in this chapter had a competitive mode of action since the maximum contractions to CCH were unchanged in the presence of the highest concentration of the antagonists (30nM 4-DAMP, 30μM methoctramine and 30μM pirenzepine). The slopes of the Schild plots for 4-DAMP and pirenzepine were not significantly different from unity in all tissues. However, the slope of the Schild plots for methoctramine were significantly less than unity, but not significantly different from control (based on 95% confidence intervals), in all groups except in 12-week diabetics in which Schild plots for methoctramine demonstrated a slope not significantly different form unity. The reason for the low slope of the Schild plots for methoctramine in 1-week, 4-week and 8-week diabetic tissues is not clear. One possible explanation might be the antagonism of a heterogeneous receptor population by methoctramine (Kenakin, 1993). In a study by Braverman *et al* (1998), involving pharmacological characterisation of muscarinic receptors in denervated rat bladder, the slope of Schild plots obtained for methoctramine were also significantly different from unity, but not significantly different from control values. Braverman *et al* (1998) suggested that

a combination of M2- and M3-muscarinic receptors could be mediating the contractile responses in denervated rat bladder. However, methoctramine might have other non-specific effects. In the present chapter, involvement of M2- and M3-muscarinic receptors in mediating the STZ-diabetic rat bladder smooth muscle contraction can not be confirmed based on the profile of the muscarinic-receptor antagonist affinity values. However, the role of M2-muscarinic receptor in mediating the detrusor contraction in the STZ-diabetic rat bladder contraction can not be ruled out completely, especially since real-time PCR analysis demonstrated an increase in the expression levels of this muscarinic receptor-subtype.

3.5.3 Molecular characterisation of muscarinic receptors in the rat bladder smooth muscle.

Although no apparent change was detected in muscarinic receptor subtype-mediating direct contraction of detrusor in the present study, an increased expression of M2-muscarinic receptor mRNA was detected in 1-week, 4-week and 8-week diabetic bladders with no change in 12-week diabetic tissues. The expression of M3-muscarinic receptor was slightly increased in 1-week diabetic tissues, although 4-week, 8-week and 12-week diabetic bladders demonstrated a drastic decrease in the expression of M3-muscarinic receptor. The increase in expression of M2- and M3-muscarinic receptors in 1-week diabetic rat bladders may contribute to the significantly increased maximum responses to CCH seen in these tissues compared to control tissues. However, in 4-week and 8-week diabetic tissues, where M2-muscarinic receptor expression was increased and the M3-muscarinic receptor expression was decreased, the CCH mediated responses were unchanged compared to the controls (non-diabetics). Thus, it could be postulated that the M2-muscarinic receptor has a compensatory role in mediating the detrusor contraction in these tissues in the absence or reduced expression of M3-muscarinic receptors. However, this is not supported by the *in vitro* functional results described in section 3.5.2. since in 12-week diabetic rat bladders, the expression of M2-muscarinic receptor was unchanged, whilst there was a noticeable decrease in the expression of M3-muscarinic receptor. Functional data obtained for 12-week diabetic tissues, demonstrate no change

in the CCH mediated responses, and thus it can be assumed that expression levels of muscarinic receptors at the mRNA level do not necessarily relate to functional results obtained in this chapter.

Previous studies have demonstrated an up-regulation of both muscarinic receptor subtypes at both the RNA and protein level in the detrusor and the urothelium of diabetic bladders (Tong *et al*, 2002; Tong & Cheng, 2002; Cheng *et al*, 2007). An increased expression of M2-muscarinic receptors was also detected in denervated rat bladders by Braverman *et al* (1998), with no change in the expression levels of muscarinic M3-muscarinic receptor. In another study by Braverman *et al* (2007) it was demonstrated that the sub-population of muscarinic receptors within the urothelium are different between bladders with normal and pathological contraction and that the M2-muscarinic receptor mediated contractions of human bladder from organ donors is associated with an increase in urothelial muscarinic receptors (Braverman *et al*, 2007). In the present study, urothelium/mucosa was removed from the detrusor and the bladders were then used for RNA extraction and real-time PCR analysis. Thus, an alteration in the urothelial muscarinic receptor expression can not be ruled out. It must be noted that changes in the muscarinic receptor subtype expression detected by various studies in diabetic and denervated bladders (Tong *et al*, 2002, Tong & Cheng, 2002; Cheng *et al*, 2007; Braverman *et al*, 1998) could be due to changes in the expression levels of these receptors at the smooth muscle, nerve or urothelial levels. Thus, ideally, it would be informative to investigate the expression levels of muscarinic receptors in these cell populations before a conclusion can be made.

The change in the expression levels of M2- and M3-muscarinic receptors mRNA may also alter the M2:M3 ratio in the detrusors from diabetic rats. In this study, the M2:M3 ratio has increased in bladders from all diabetic rats compared to control (non-diabetic) tissues. However, it is important to consider that alterations in muscarinic receptor expression at the mRNA levels may not necessarily represent an upregulation or downregulation of functionally active muscarinic receptors, which may explain why in the present study no changes were observed in CCH-induced responses in 4-week, 8-week and 12-week diabetic tissues. Therefore, alterations in M2- and M3-muscarinic receptor

expression at the protein level should be confirmed using radioligand binding experiments, Western blotting or other techniques.

3.6 Summary

Muscarinic receptor-mediated contractions of control (non-diabetic) and diabetic rat bladder detrusor were analysed using classical pharmacological techniques.

In this study, the sensitivity of tissues from diabetic rats to CCH was not significantly different from control (non-diabetic) and only tissues from 1-week diabetic rats demonstrated a greater maximum response to CCH compared with controls.

The antagonism data demonstrated that the direct contractile response to CCH is mediated by the M3-muscarinic receptor in control (non-diabetic) rat bladder detrusor and no evidence could be found to indicate changes in the muscarinic receptor subtype mediating contraction of the diabetic overactive rat bladder detrusor.

Expression levels of the M2-muscarinic receptor subtype were increased in 1-week, 4-week, and 8-week diabetic detrusors, with no change in 12-week diabetic tissues. M3-muscarinic receptor expression was increased in 1-week diabetic rat bladder tissues compared to control (non-diabetic) tissues. However, in contrast the expression of the M3-muscarinic receptor was decreased in 4-week, 8-week and 12-week diabetic tissues compared to control (non-diabetic tissues. Changes in molecular expression of muscarinic receptors may not necessarily relate to changes seen at the functional receptor level.

Chapter 4: Investigation of spontaneous activity in detrusor strips from rats

4.1 Introduction

The myogenic theory of overactive detrusor suggests that as well as changes in structure and function of the smooth muscle, involuntary detrusor contractions, especially during the filling phase of the micturition cycle, are important in generation of the symptoms associated with urgency and frequency. In the STZ-diabetic rat bladder, increased involuntary detrusor contractions or SA have also been detected (Stevens et al, 2006) which may be associated with DO in these animals. However, in normal bladders, SA may also be important in mediating bladder tone and sensory afferent information from the detrusor.

4.1.1 The role of smooth muscle SA in normal urinary bladder function

Previously, the commonly accepted view of bladder function was that during the storage phase the bladder was not active and behaved as a compliant low pressure reservoir. It was thought that as the bladder filled, stretch receptors in the wall sent signals to the central nervous system (CNS) and at the "critical volume" the CNS coordinated a series of events which resulted in smooth muscle contraction and micturition. However, this view is now known to be simplistic. It is now believed that there are two distinct mechanisms generating contractile activity in the bladder: the autonomous or SA and the micturition contraction (Gillespie *et al*, 2003; Gillespie, 2003; Kanai *et al*, 2007).

Spontaneous contractions have been suggested to be myogenic in nature, since they occur in isolated *in vitro* bladder preparations (Liu *et al*, 1998; Hashitani & Brading, 2003). In the detrusor SA is associated with action potentials whose upstroke is carried by voltage dependent Ca^{2+} channel (VDCC) current and repolarised by large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) channel activity. Transmembrane Ca^{2+} fluxes may result in Ca^{2+}-induced Ca^{2+} release (CICR) from internal stores, which are replenished through feedback mechanisms employing BK_{Ca} and VDCCs (Hashitani *et al*, 2004; Reviewed by Brading, 2006).

In many species spontaneous bladder contractions are believed to be important in generating periodic sensation and allowing the individual muscle bundles to adjust their length in response to filling, which allows the bladder to maintain its

shape during this period (reviewed by Brading, 2006). At baseline, this activity is localised and can be multifocal, with separate areas of bladder wall contracting independently. In response to an increase in intravescical pressure or cholinergic stimulation by low concentrations of muscarinic agonists, propagating waves of contraction emerge (Drake *et al*, 2005). It is believed that these autonomous or spontaneous contractions have implications for the generation of additional afferent information from the bladder wall to the CNS and that without these contractions, the bladder would remain a floppy and relaxed organ (Drake *et al*, 2005).

Although the spontaneous contractions of the detrusor can occur in the absence of neural stimulation, they may be modulated by activation of various types of receptors (muscarinic, purinergic and adrenergic) (Ng *et al*, 2006). It has previously been shown that the amplitude and the frequency of spontaneous contractions can be augmented by addition of muscarinic receptor agonists such as CCH (Gillespie *et al*, 2003; Ng *et al*, 2006). The cells involved in mediating these spontaneous contractions must therefore express muscarinic receptors. It seems that at low concentrations (<300nM) of muscarinic agonists phasic contractions are activated, whilst at higher concentrations (>3μM), phasic activity and a tonic contracture are activated (Gillespie *et al*, 2003). The exact mechanisms of generation of spontaneous contractions in the bladder detrusor are still unclear, but may involve bladder smooth muscle cells, intra-mural nerves, urothelial and suburothelial cells, as well as a network of ICs (which are described in chapter 6).

4.1.2 Implications of bladder smooth muscle spontaneous contractions in pathological conditions

In bladders from humans with DO and animal models of DO, spontaneous contractions have been shown to be increased (German *et al*, 1995; Mills *et al*, 2000, Drake *et al*, 2006b) and these may underlie the abnormal rises in intravesical pressure which result in symptoms of urgency, frequency and urge incontinence (Brading, 2006). Intrinsic or spontaneous contractions have also been demonstrated to be more prominent in neonatal rats than adult rats (Ng *et al*, 2006). Whole rat bladders and isolated strips from neonatal rats exhibit high-amplitude, low frequency spontaneous contractions which may promote voiding

as neural control is still immature. Whilst every spontaneous contraction does not result in voiding, large-amplitude spontaneous contractions may enhance the likelihood of urine loss. The same high amplitude spontaneous contractions have also been demonstrated in the spinal cord transected rat, which is used as an animal model of DO (Drake *et al*, 2003b; Gevaert *et al*, 2006; Ikeda & Kanai, 2008). Bladders from neonatal rats have also been used in numerous studies as a pathological model in an attempt to understand the underlying mechanisms of overactive detrusor spontaneous contractions (Ng *et al*, 2006; Kanai *et al*, 2007).

As described in chapter 2, antimuscarinic agents are the preferred pharmacological treatment of DO and overactive bladder syndrome. Their mechanism of action, however, is not clear and it is believed that they may act during the filling phase when parasympathetic activity is low. These drugs are more effective in suppressing bladder activity in patients with DO than in normal subjects (reviewed by Yoshida & Andersson, 2003). It has been suggested that symptoms of DO may be caused by enhancement of spontaneous contractions due to leakage of small amounts of ACH from intramural nerves during bladder filling (Ng *et al*, 2006). The source of acetylcholine is still under debate, with some groups believing that urothelial cells are the main site of release (Yoshida *et al*, 2006), whilst others believe that intramural nerves are the main source (reviewed by Yoshida & Andersson, 2003). It has been demonstrated that both M2- and M3-muscarinic receptors are the targets of this ACH leakage and thus may be responsible for mediating bladder smooth muscle spontaneous contractions in pathological conditions. This may explain an effect of antimuscarinic agents in the filling phase of the micturition cycle (Ng *et al*, 2006; Mukerji *et al*, 2006). Many studies have tried to mimic these conditions by applying low concentrations of muscarinic agonists to bladder detrusor strips from normal and overactive bladders, in order to study the effect of muscarinic receptor stimulation on generation of spontaneous contractions (Gillespie *et al*, 2003; Gillespie, 2003; Ng *et al*, 2006; Kanai *et al*, 2007; Finney *et al*, 2007).

SA in the UBSM of the STZ-diabetic rat model has only been investigated in a few previous studies (Tammela *et al*, 1994; Nakahara *et al*, 2004; Stevens *et al*, 2006). Tammela *et al* (1994) demonstrated an increased SA in detrusor strips from STZ-diabetic rats, which was sensitive to the cyclooxygenase inhibitor,

indomethacin, demonstrating the importance of prostaglandins in mediating this activity. These spontaneous contractions were insensitive to inhibition by the neurogenic blocker tetrodotoxin, confirming a myogenic basis. In the report by Nakahara *et al* (2004) enhanced SA in detrusor strips from STZ-diabetic rats was demonstrated after modulation of UBSM excitability by various ion channel openers and blockers. In addition the study by Stevens *et al* (2006) demonstrated an increase in basal SA of detrusor strips from 1-week diabetic rats. Apart from these few reports, studies on the properties of spontaneous contractions in the detrusor of the STZ-diabetic rat are very limited, with no reports on the cholinergic modulation of this SA. Further characterisation of SA in the modulation of UBSM contractility in STZ-diabetic rat may aid a better understanding of the underlying mechanisms that could lead to DO.

4.2 Aims of the chapter

The aim of the current chapter was to characterise the basal and cholinergic stimulated spontaneous contractions of detrusor strips from control (non-diabetic) and STZ- diabetic rat bladders using *in vitro* pharmacology.

4.3 Materials and Methods

4.3.1 Investigation of basal SA in bladder strips from control and diabetic rats

Longitudinal mucosa-intact strips (2-4 x 6-12mm) were isolated from the body of bladders from control (non-diabetic) (weight range 200-350g), 1-week, 4-week, 8-week and 12-week diabetic Wistar rats (Harlan, UK), and suspended in 15ml organ baths containing Krebs bicarbonate solution (containing 5μM indomethacin) as described in chapter 2. They were allowed to equilibrate for 60mins and during the last 30mins of the equilibration period they were assessed for generation of basal SA. The frequency and the amplitude of basal SA were measured.

4.3.2 Induction of SA in bladder detrusor strips using low concentrations of carbachol (CCH)

In separate experiments, longitudinal mucosa-intact and denuded strips (2-4 x 6-12mm) were isolated from the bladder body of 1-week, 4-week, 8-week and 12-week diabetic Wistar-Hans rats (Charles River, UK) and their age- matched controls. Mucosa was removed by sharp dissection under the dissecting microscope. Tissues were suspended in 15ml organ baths as described in chapter 2 and allowed to equilibrate for 60mins before being stimulated with low cumulative concentrations of CCH (0.05, 0.1 and 0.5μM). The tissues were incubated with each concentration of CCH for 5-10mins. The last 5-min period with each concentration was used to calculate the mean amplitude and the frequency of the spontaneous contractions.

4.3.3 Data analysis

To calculate the amplitude and the frequency of SA, a slightly modified method to that proposed by Imai *et al* (2001) was used to define a single spontaneous contractile event. Firstly, maximum amplitude of contractile activity over a 5mins period was calculated in control (non-diabetic) tissues. Any contractions over and above the 30% of this mean were considered as single spontaneous contractions and counted for calculation of the frequency. The same 30% threshold line was also used for calculation of the frequency of SA in diabetic

tissues (Figure 29). The amplitude of SA was expressed as absolute values (g) or normalised for tissue weight (g/mg). Data was expressed as mean±SEM.

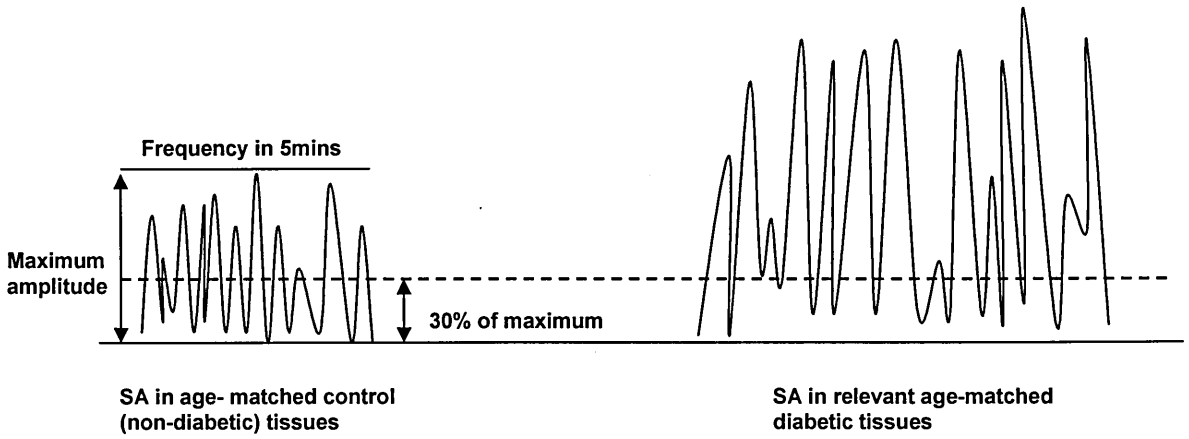


Figure 29) Calculation of the amplitude and the frequency of SA over a period of 5mins in control (non-diabetic) and diabetic tissues. Modified from Imai *et al* (2001).

4.3.4 Statistical analysis

Statistical analysis was performed using repeated measures *ANOVA* followed by Dunnett's post hoc test for intra-tissue variations and unpaired Student's *t*-test for inter-tissue variations. For multiple comparisons between different control (non-diabetic) and diabetic groups, one way *ANOVA* followed by Tukey's post hoc test was performed.

4.4 Results

4.4.1 Investigation of basal SA in detrusor strips from control and diabetic rats

Basal spontaneous contractions were detected in detrusor strips from control (non-diabetic) and diabetic rats. However, of all the control and diabetic tissues tested, only 30% of control, 55% of 1-week, 54% of 4-week, 55% of 8-week and 5% of 12-week diabetic tissues developed basal spontaneous contractions. Typical chart recordings of these basal spontaneous contractions are shown in Figure 30.

The mean amplitude and frequency of basal SA in control (non-diabetic) (n=10) tissues were 0.021±0.001 g/mg tissue and 27.7±2.7 events in 5mins respectively. Detrusor strips (n=16) from 1-week diabetic rats demonstrated a basal SA with an amplitude of 0.045±0.007 g/mg tissue and a frequency of 23.5±1.3 events in 5mins. The basal SA in detrusor strips (n=13) from 4-week diabetic rats had a mean amplitude of 0.025±0.004 g/mg tissue and a frequency of 27.3±2.62 events in 5mins. The mean amplitude of basal SA in detrusor strips from 8-week diabetic rats was 0.027±0.005 g/mg tissue, whilst the frequency was 16.5±1.7 events in 5mins. Only one detrusor strip from 12-week diabetic rats demonstrated basal SA, with an amplitude of 0.007g/mg tissue and a frequency of 9 events in 5mins. No further statistical analysis was performed on 12-week diabetic tissues.

When the amplitude of basal SA of each diabetic group was compared to control (non-diabetic) tissues, detrusor strips from 1-week diabetic rats demonstrated a significantly ($p<0.01$) greater amplitude of basal spontaneous contractions compared to control tissues. There was no significant difference in the amplitude of SA between control (non-diabetic) vs. 4-week and 8-week diabetic tissues

The frequency of basal SA in detrusor strips from 1-week and 4-week diabetic rats was not significantly different compared to control (non-diabetic) tissues. However, the frequency of basal SA in 8-week diabetic tissues was significantly ($p<0.01$) lower than control (non-diabetic) tissues (Figure 31).

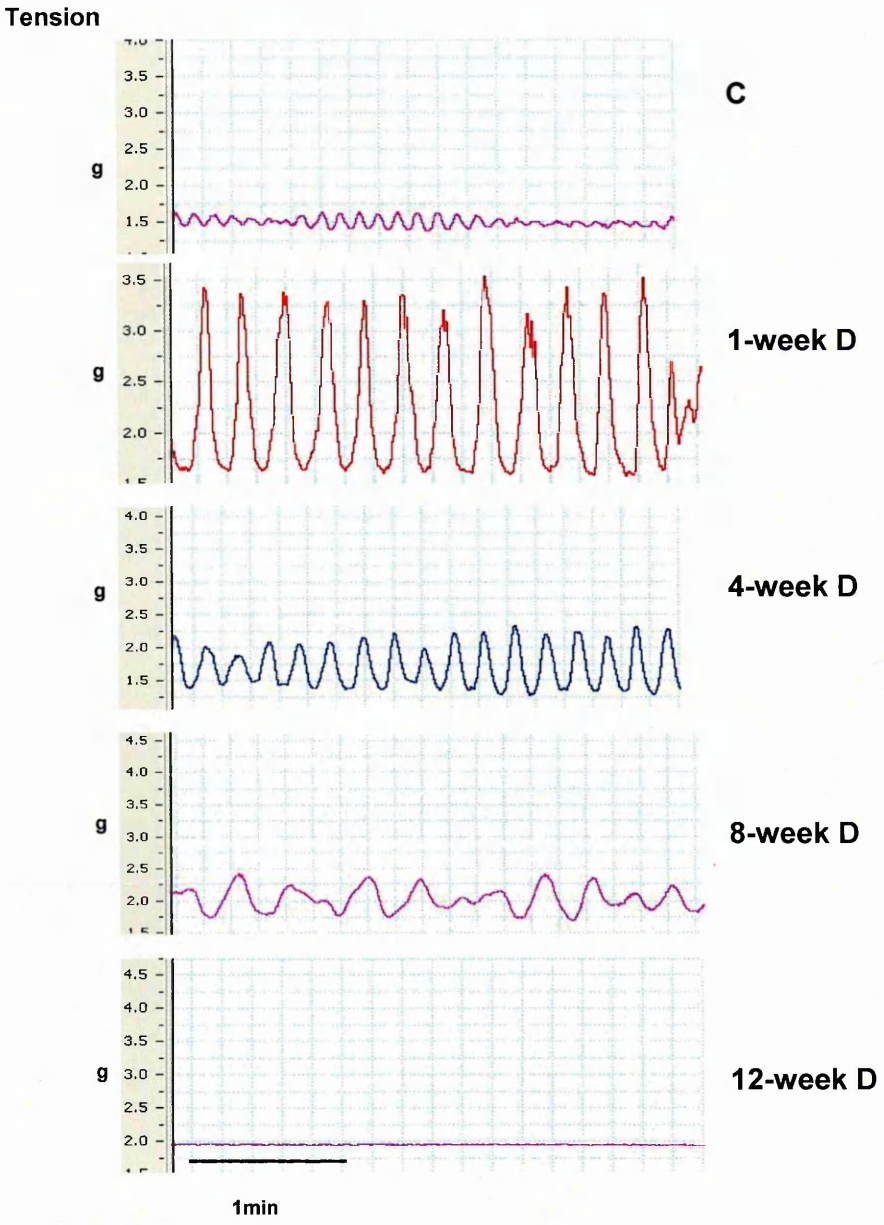


Figure 30) Typical chart recordings of basal spontaneous contractions in detrusor strips from control (non-diabetic) (C) and diabetic (D) rats.

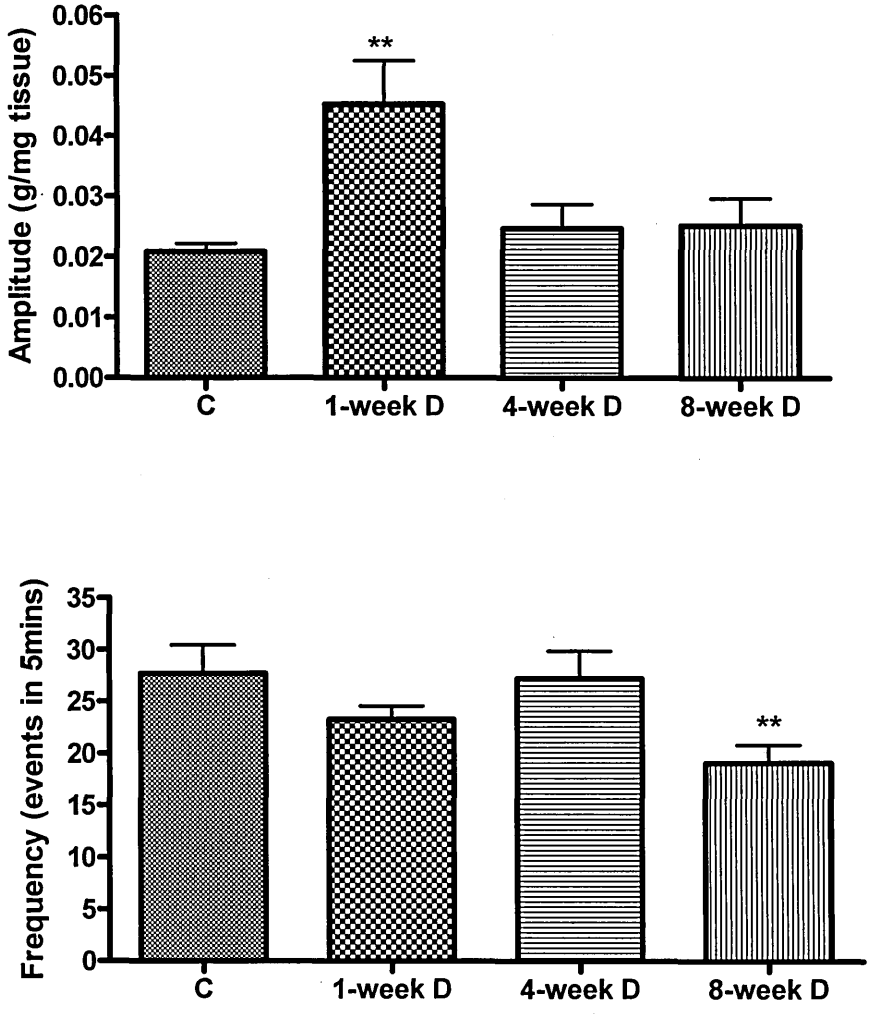


Figure 31) Comparison of the amplitude and the frequency of basal spontaneous contractions between control (non-diabetic) (C) & diabetic (D) tissues. C: n=10, 1-week D: n=16, 4-week D: n=13 & 8-week D: n=11. Data is presented as mean±SEM. **p<0.01 vs. control (non-diabetic) tissues.

4.4.2 Induction of spontaneous activity by CCH in bladder strips from control (non-diabetic) and diabetic rats.

The non-selective muscarinic agonist CCH induced spontaneous bladder contractions in a concentration-dependent manner in control (non-diabetic) and diabetic rat bladder strips (Figure 32). At low concentrations (0.05μM), CCH increased spontaneous contractions without causing a tonic contraction, whereas 0.1μM CCH caused a transient tonic contraction. 0.5μM CCH caused an even larger increase in tonic contraction, with the spontaneous contractions being superimposed on the top of this large slow tonic wave (Figure 32).

4.4.2.1 Effect of CCH on the SA in bladder detrusor from 1-week diabetic rats

CCH induced SA in a concentration-dependent manner in 1-week diabetic tissues and their age-matched controls (non-diabetic) with detrusor strips from STZ-diabetic rats showing a greater response to CCH. Figure 33 demonstrates the effect of CCH on the amplitude and frequency of SA in 1-week diabetic detrusor strips versus age-matched controls (non-diabetic). There was a significant difference in the amplitude of CCH-stimulated SA between detrusor strips from 1-week diabetic vs. their age matched control (non-diabetic) rats at both 0.1μM CCH and 0.5μM CCH (Figure 33).

The frequency of CCH-stimulated SA was significantly less in 1-week diabetic tissues compared to controls at 0.1μM and 0.5μM CCH (Figure 33).

4.4.2.2 Effect of CCH on the SA in detrusor strips from 4-week diabetics

CCH induced SA in a concentration-dependent manner in 4-week diabetic tissues and their age matched controls (non-diabetic). However, there was no significant difference in the amplitude of CCH-stimulated SA between control (non-diabetic) and diabetic tissues at all concentrations of CCH tested (Figure 34). There was also no significant difference in the frequency of CCH-stimulated

SA between both groups upon stimulation with increasing concentrations of CCH (Figure 34).

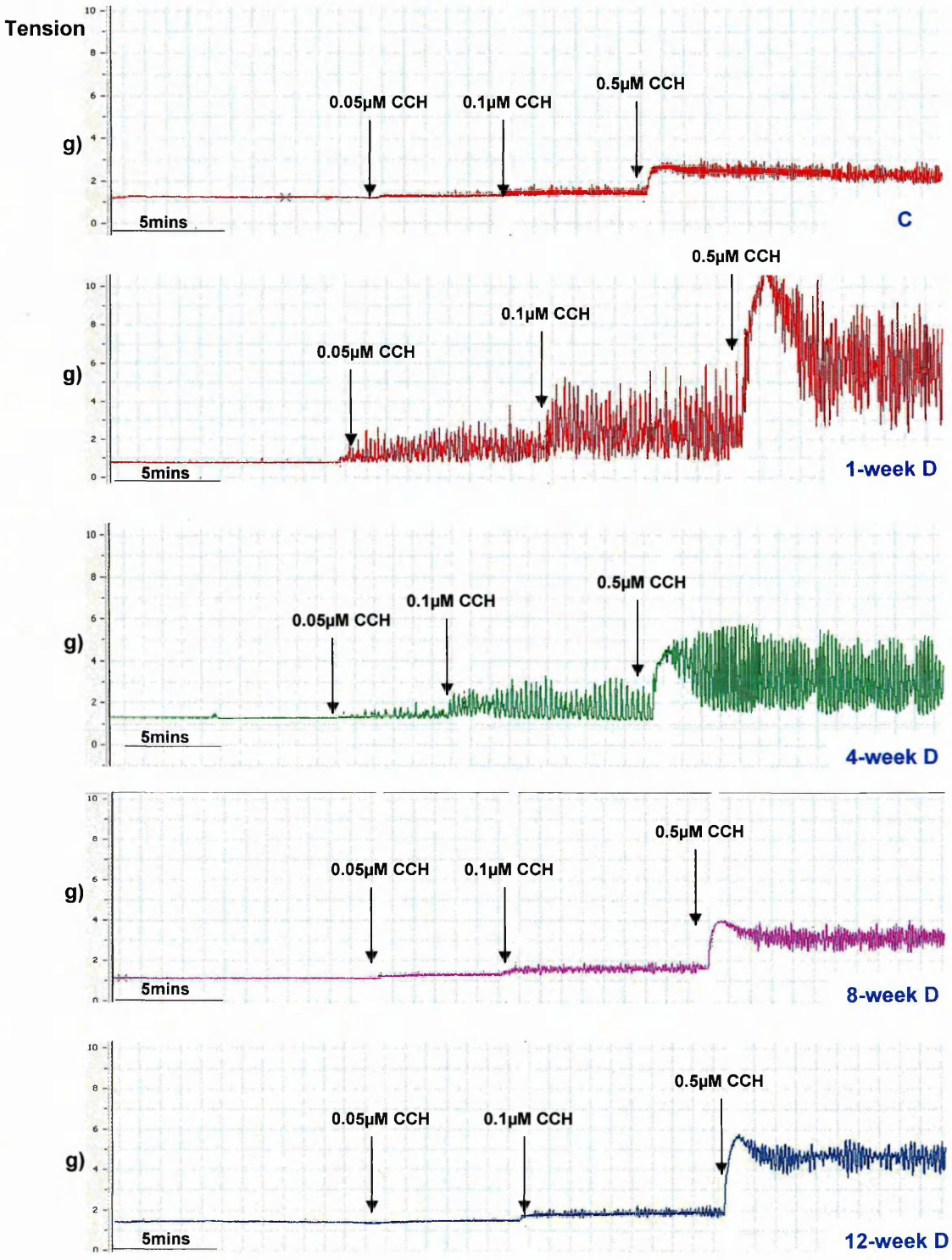


Figure 32) Typical chart traces showing the effect of increasing concentrations of CCH on spontaneous contractions in detrusor strips from a representative control (non-diabetic) (C) & diabetic (D) rats.

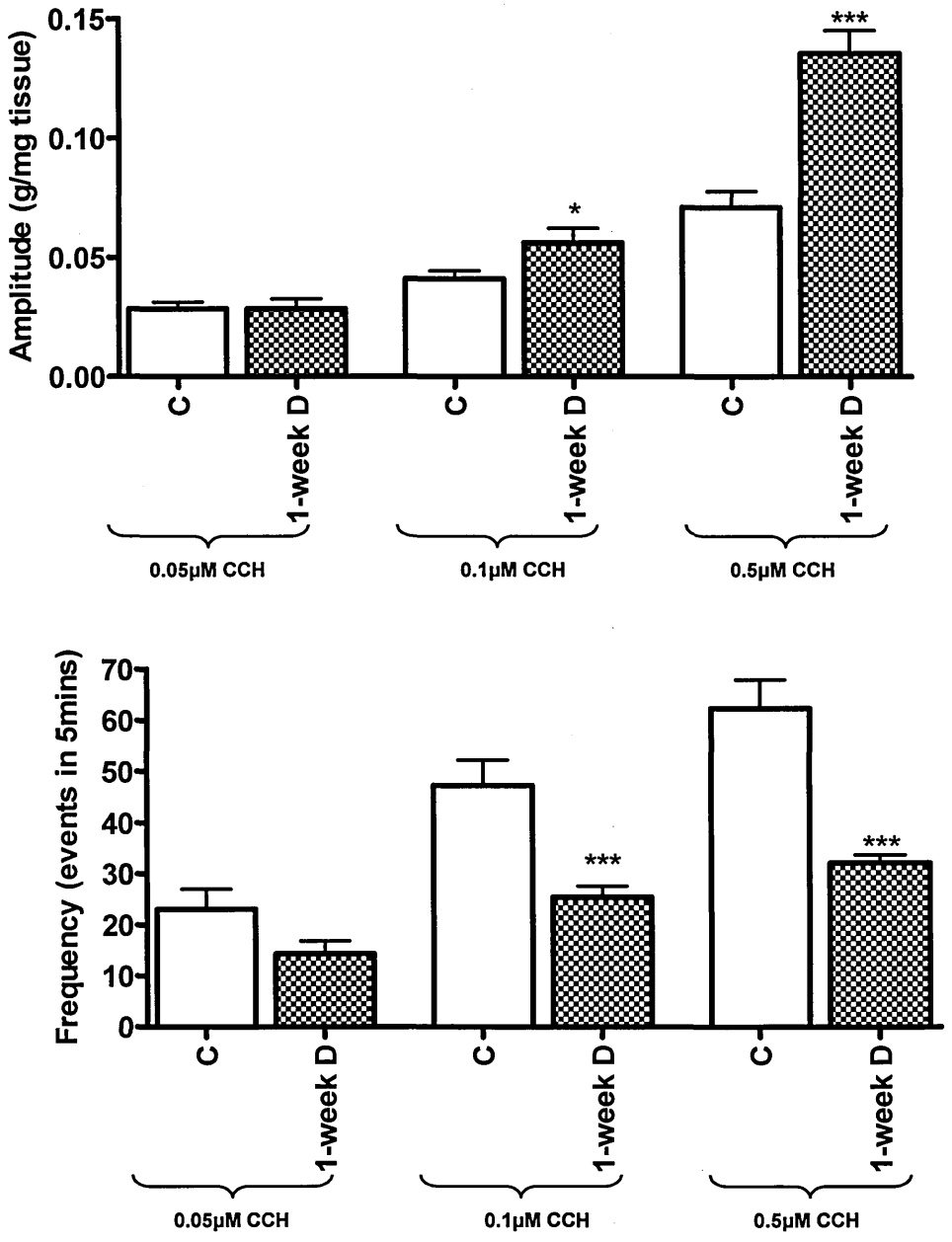


Figure 33) Comparison of the amplitude and the frequency of CCH-stimulated SA in control (C) (age-matched non-diabetic) and 1-week diabetic (D) tissues at increasing concentrations of CCH (0.05-0.5µM). C: n=24 & 1-week D: n=31. *p<0.05 & ***p<0.001 indicate a significant difference between control (non-diabetic) and diabetic tissues. Data is presented as mean±SEM

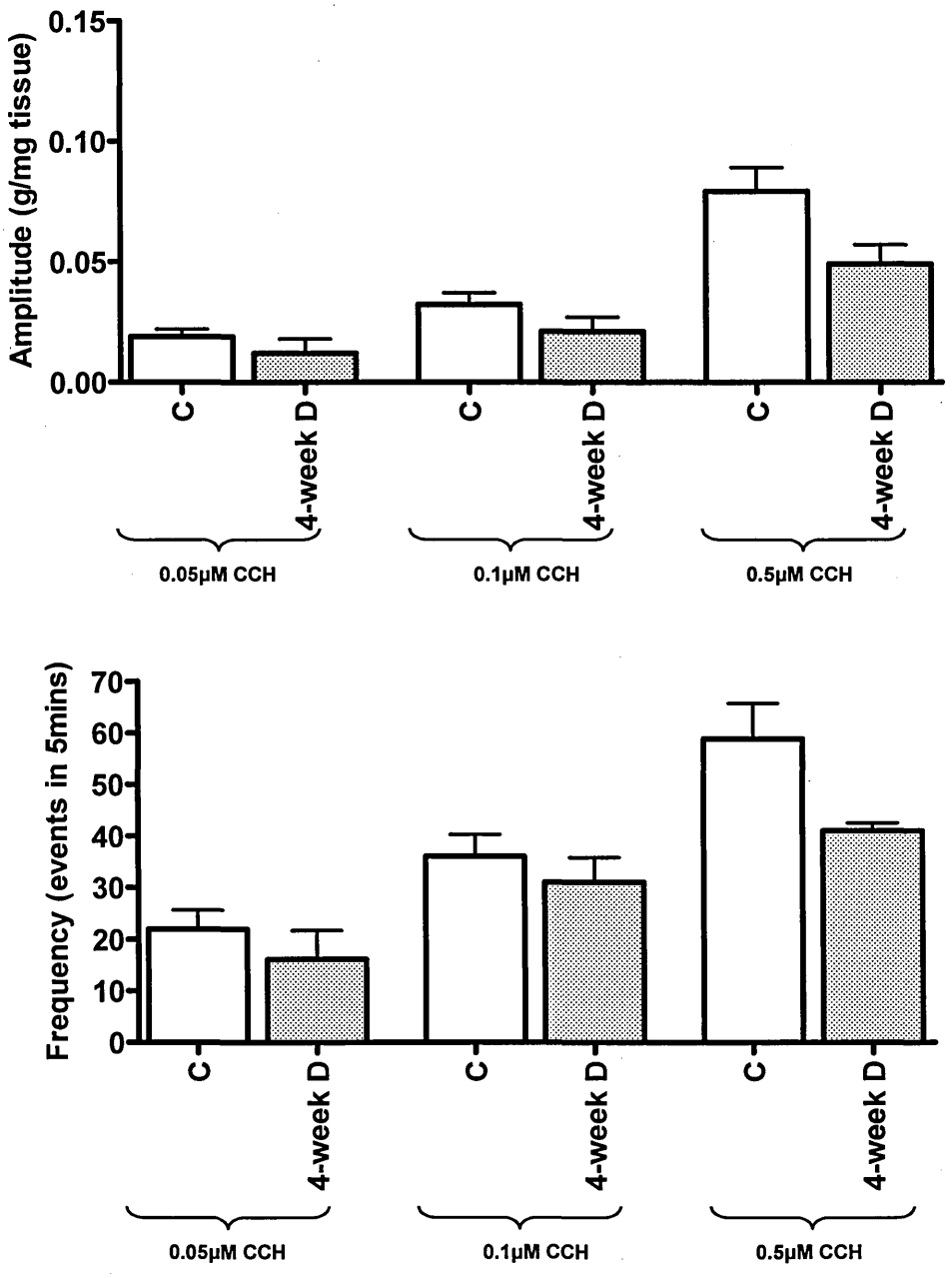


Figure 34) Comparison of the amplitude and the frequency of CCH-stimulated SA in control (C) (age-matched non-diabetic) and 4-week diabetic (D) tissues at increasing concentrations of CCH (0.05-0.5μM). C: n=19 & 4-week D: n=12. Data is presented as mean±SEM

4.4.2.3 Effect of CCH on the SA in detrusor strips from 8-week diabetic rats

CCH induced SA in a concentration-dependent manner in 8-week diabetic tissues and their age matched controls (non-diabetic). The only significant difference detected was between the amplitude of stimulated SA in 8-week diabetic tissues vs. age matched controls (non-diabetic) at 0.1μM CCH (Figure 35).

There was no significant difference in the frequency of stimulated SA between control and 8-week diabetics (Figure 35).

4.4.2.4 Effect of CCH on the SA in detrusor strips from 12-week diabetic rats

CCH also induced SA in a concentration dependent manner in 12-week diabetic tissues and their age matched controls. However, the amplitude of SA in 12-week diabetic tissues was significantly less than that of 12-week age matched control (non-diabetic) tissues upon stimulation with all concentrations of CCH (0.05-0.5μM) (Figure 36).

The frequency of SA in 12-week diabetic tissues was also significantly less than 12-week control (non-diabetic) tissues upon stimulation with 0.05 and 0.1μM CCH (Figure 36).

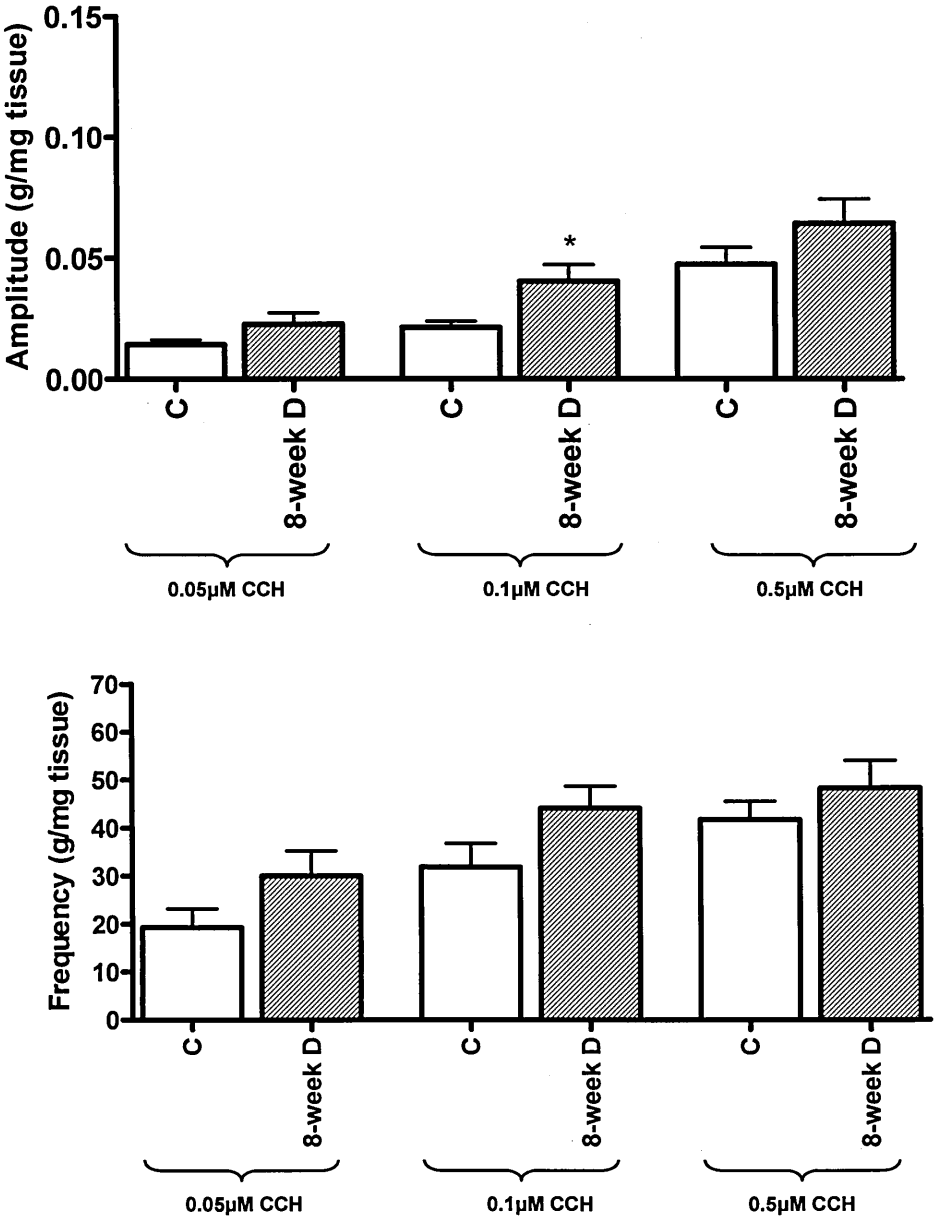


Figure 35) Comparison of the amplitude and the frequency of CCH-stimulated SA in control (C) (age-matched non-diabetic) and 8-week diabetic (D) tissues at increasing concentrations of CCH (0.05-0.5μM). C: n=12 & 8-week D: n=12. *p<0.05 indicates a significant difference between control (non-diabetic) and diabetic tissues. Data is presented as mean±SEM

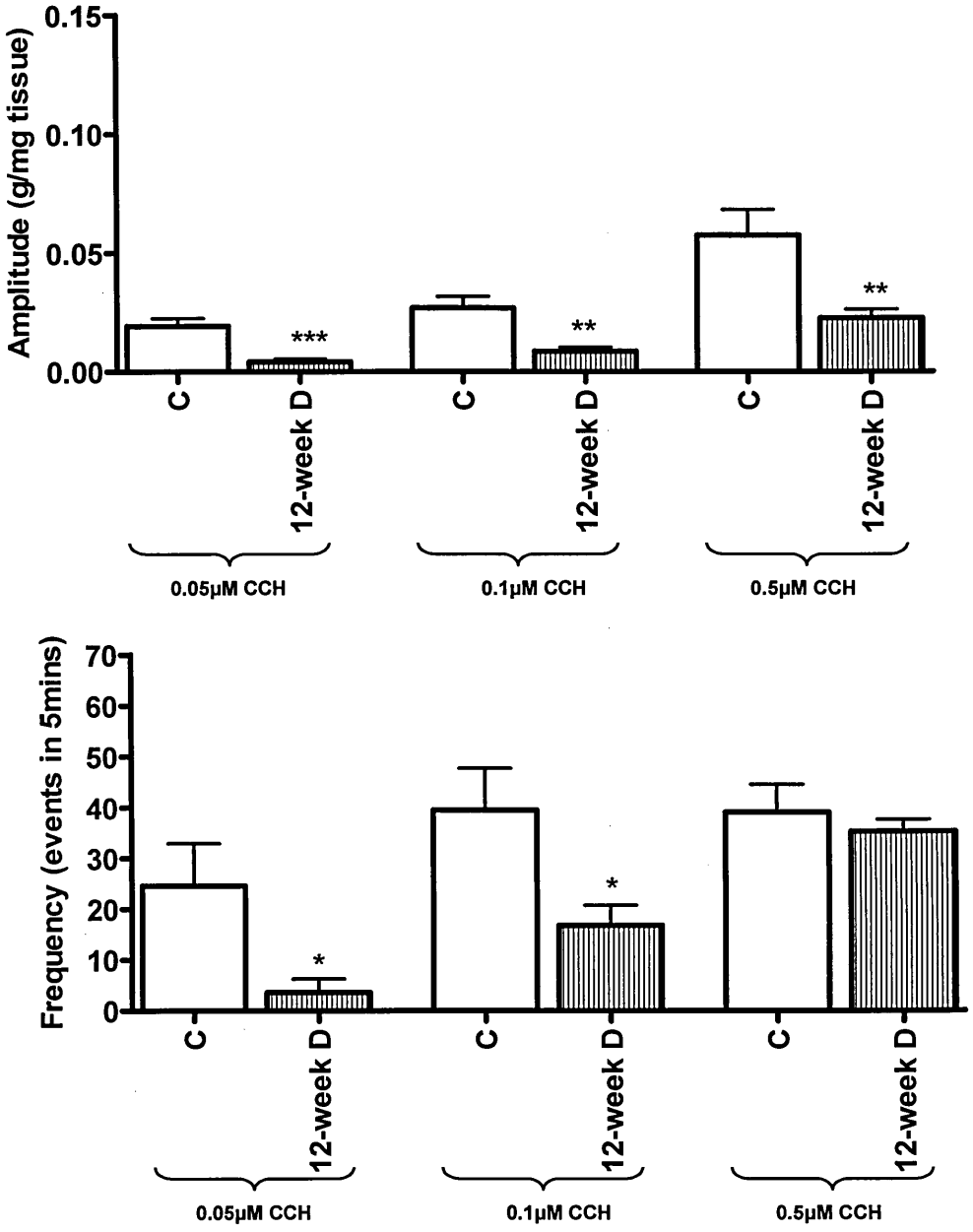


Figure 36) Comparison of the amplitude and the frequency of CCH-stimulated SA in control (C) (age-matched non-diabetic) and 12-week diabetic (D) tissues at increasing concentrations of CCH (0.05-0.5μM). C: n=13 & 12-week D: n=12. *p<0.05, **p<0.01 & ***p<0.001 indicate a significant difference between control (non-diabetic) and diabetic tissues. Data is presented as mean±SEM.

4.4.2.5 Comparison of the effect of 0.5μM CCH on SA in bladder strips from control (non-diabetic) and diabetic rats

Control (non-diabetic) rats

Since stimulation of control (non-diabetic) and diabetic tissues with 0.5μM CCH produced the largest spontaneous contractions, the changes in the amplitude and frequency of these contractions at this concentration of CCH was compared between different age-matched control groups. As demonstrated in Figure 37, the amplitude of CCH-induced SA did not change with time in control (non-diabetic) tissues and the frequency of CCH-stimulated SA was only significantly different between 1-week and 12-week control (non-diabetic) tissues (Figure 37).

Diabetic rats

In diabetic tissues, detrusor strips from 1-week diabetic rats demonstrated significantly greater amplitude of SA upon stimulation with 0.5μM CCH compared to all other diabetic groups. The frequency of CCH-stimulated SA was significantly higher in 8-week diabetic tissues compared to 1-week and 12-week diabetic tissues (Figure 37).

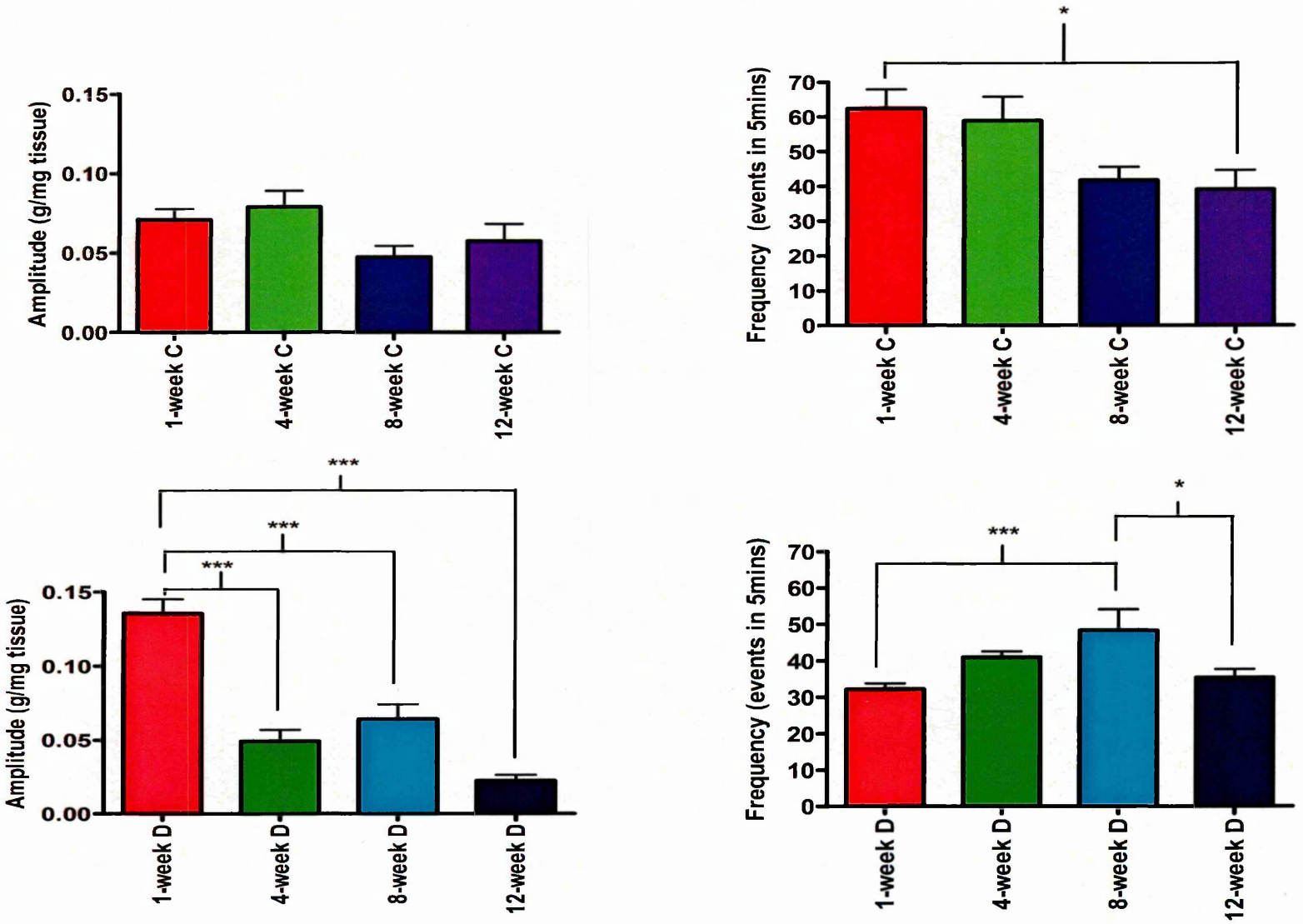


Figure 37) Duration dependent changes in the amplitude and the frequency of CCH-stimulated SA at 0.5μM CCH in detrusor strips from age-matched control (non-diabetic) and diabetic rats. n=12-31 bladder strips from control (non-diabetic) (C) and diabetic (D) animals.*p<0.05 and ***p<0.001 vs. groups indicated. Data is presented as mean±SEM.

4.4.3 Effect of removal of the mucosa on CCH-induced SA

Earlier data presented in this chapter showed that, differences in the CCH-induced SA were mainly detected in detrusor strips from 1-week and 12-week diabetic rats. Thus, to examine whether the mucosa plays a role in mediating these differences, the effect of increasing low concentrations of CCH on the SA was examined in detrusor strips denuded of mucosa from control (non-diabetic), 1-week and 12-week diabetic rats.

4.4.3.1 Effect of removal of the mucosa in control (non-diabetic) detrusor strips

The amplitude and the frequency of CCH-stimulated SA in control (non-diabetic) intact and denuded detrusor strips at each concentration of CCH were compared (Figure 38). CCH induced SA in a concentration-dependent manner in intact and denuded detrusor strips. Removal of the mucosa had no significant effect on the amplitude and the frequency of CCH-stimulated SA (Figure 38).

4.4.3.2 Effect of removal of the mucosa in 1-week diabetic detrusor strips

CCH induced SA in a concentration dependent manner in intact and denuded detrusor strips from 1-week diabetic rats. Removal of the mucosa had no effect on the amplitude and the frequency of CCH-induced SA (Figure 39).

4.4.3.3 Effect of removal of the mucosa in 12-week diabetic detrusor strips

CCH induced SA in a concentration dependent manner in intact and denuded detrusor strips from 12-week diabetic rats. The amplitude of SA in denuded detrusor strips from 12-week diabetic rats was significantly greater than intact detrusor strips from these animals at all concentrations of CCH tested (Figure 40). The frequency of SA in the denuded tissues was significantly higher than the intact tissues at 0.05 and 0.1μM concentrations of CCH (Figure 40).

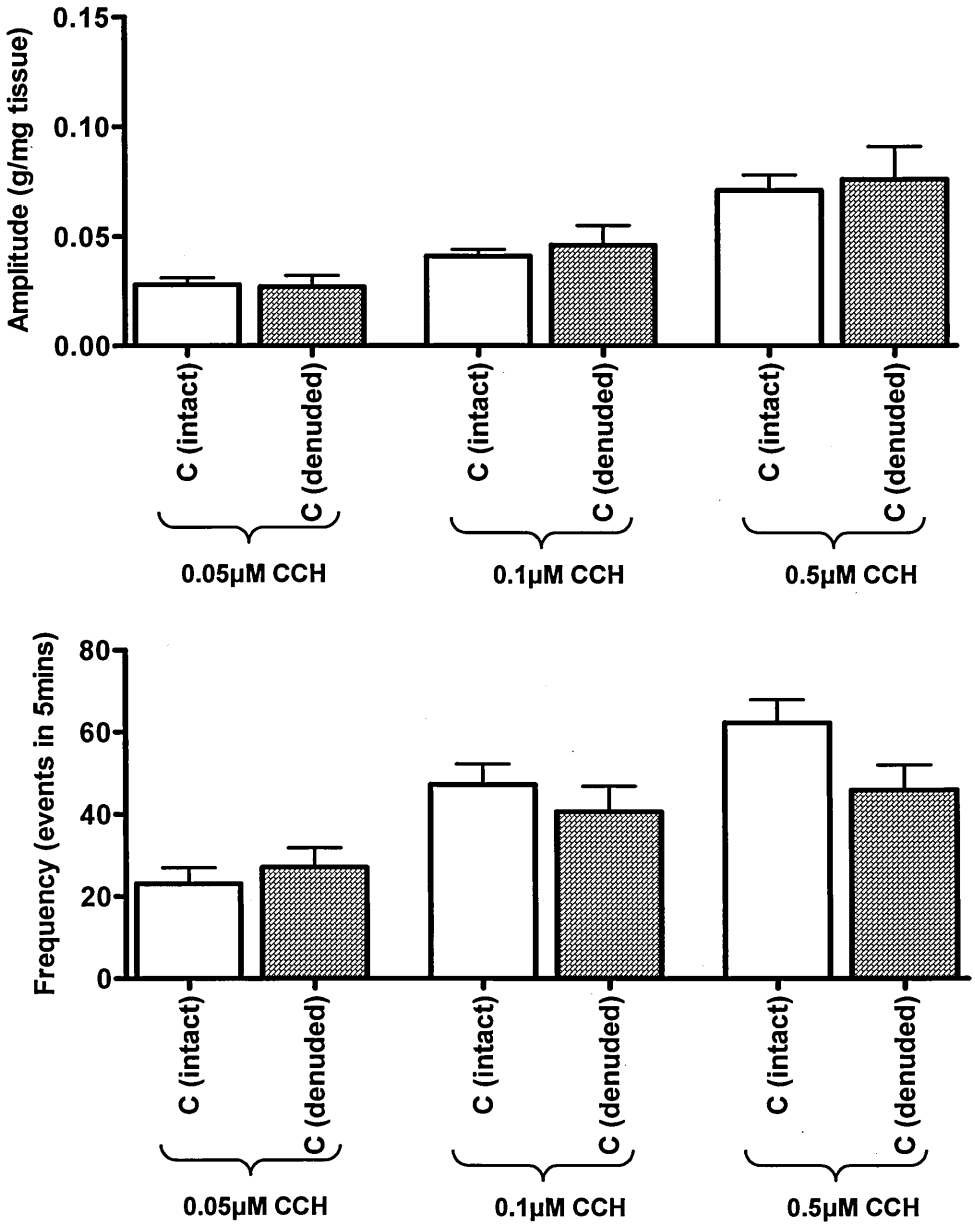


Figure 38) Comparison of the amplitude and the frequency of CCH-stimulated spontaneous contractions in control (C) (non-diabetic) intact and denuded tissues at increasing concentrations of CCH. C (intact): n=24 & C (denuded): n=14. Data is presented as mean±SEM.

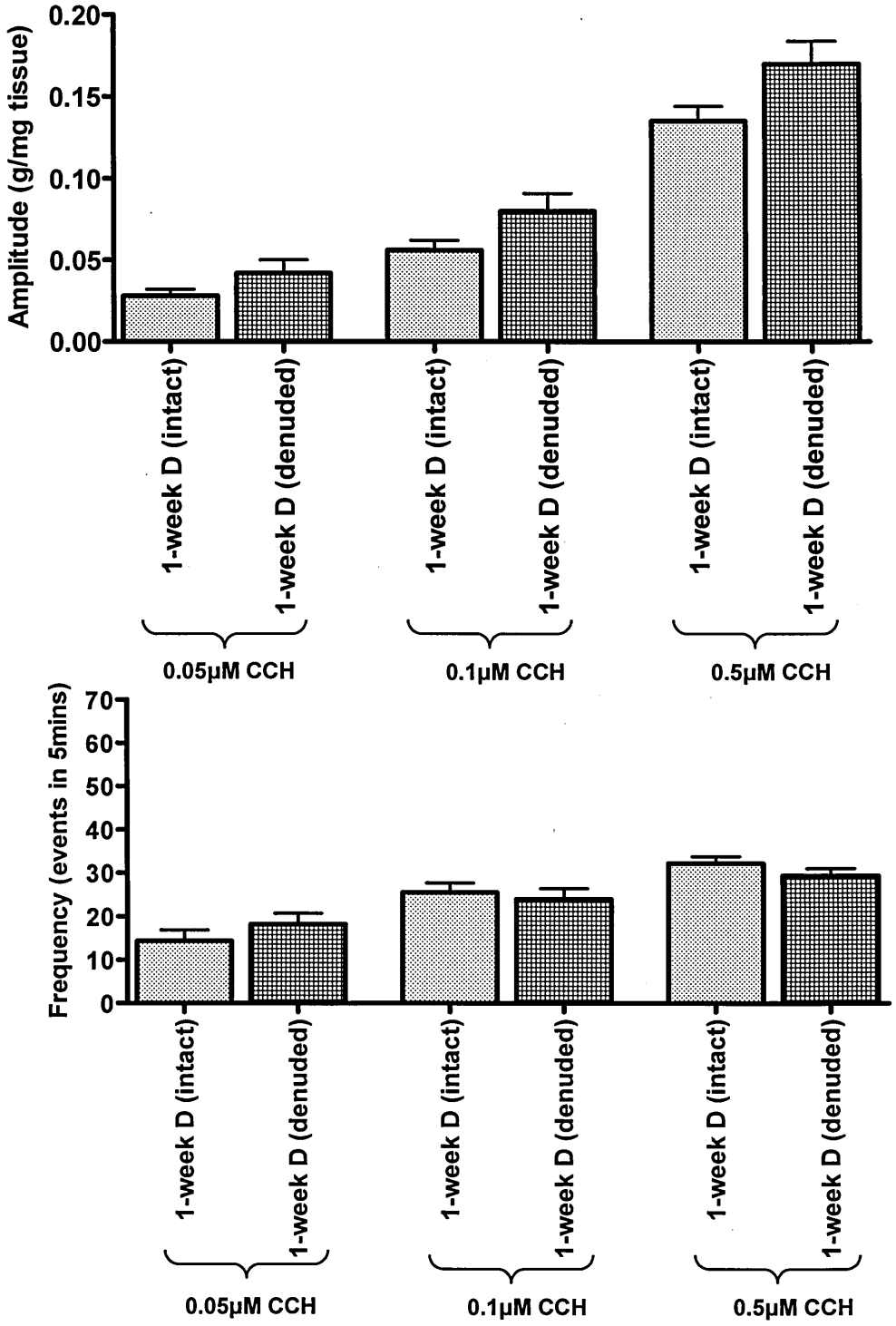


Figure 39) Comparison of the amplitude and the frequency of CCH-stimulated spontaneous contractions in 1-week diabetic (D) intact and denuded tissues at increasing concentrations of CCH. 1-week D (intact): n=31 & 1-week D (denuded): n=16. Data is presented as mean±SEM

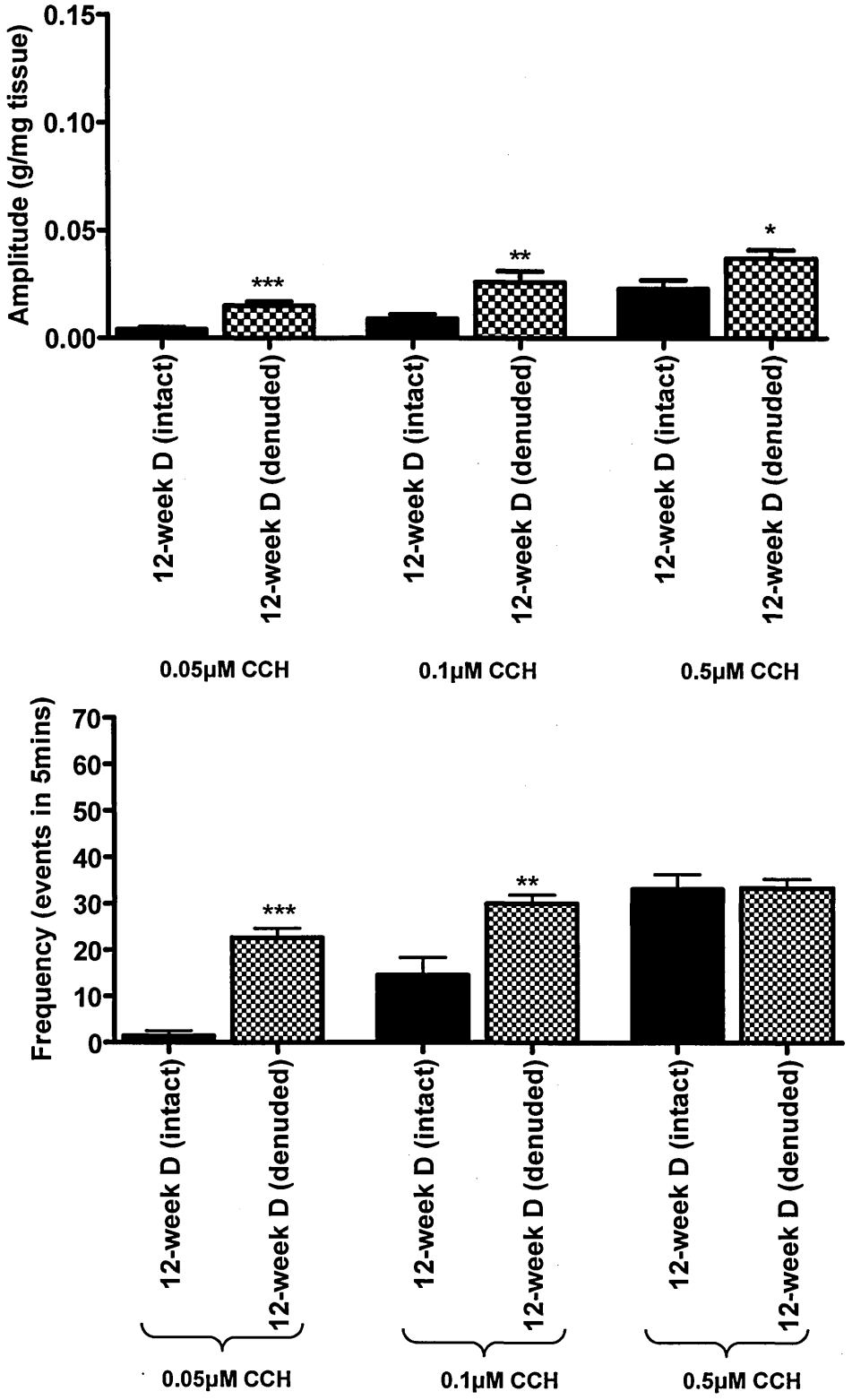


Figure 40) Comparison of the amplitude and the frequency of CCH-stimulated spontaneous contractions in 12-week diabetic (D) intact and denuded tissues at increasing concentrations of CCH. 12-week D (intact): n=12 & 12-week D (denuded): n=8. *p<0.05, **p<0.01 & ***p<0.001 indicate a significant difference between intact and denuded detrusor strips. Data is presented as mean±SEM

4.5 Discussion

4.5.1 Investigation of basal spontaneous activity in bladder detrusor strips from control (non-diabetic) and diabetic rats

Increased basal spontaneous contractions are associated with DO and OAB (Brading, 1997). In the present chapter, basal spontaneous contractions were detected in detrusor strips from control (non-diabetic), 1-week, 4-week and 8-week diabetic rats but not in 12-week diabetic rats. The detrusor strips from 1-week diabetic rats demonstrated a profound increase in amplitude of basal spontaneous contractions compared to control (non-diabetic) tissues. This significant increase in the amplitude of basal SA was not detected in the detrusor strips from 4-week and 8-week diabetic rats and 12-week diabetic tissues did not demonstrate any basal SA. The frequency of SA in detrusor strips from 1-week, 4-week and 8-week diabetic rats was not significantly different from controls.

An increased basal SA in detrusor strips from 1-week STZ-diabetic rats has also previously been reported by Stevens *et al* (2006) and can be associated with urodynamic changes (such as increased diuresis) detected by Malmgren *et al* (1989) in bladders of these animals at early stages of diabetes. The decrease in basal SA in detrusor strips from 12-week diabetic rats may also be associated with functional changes such as atonic detrusor and urinary retention detected in bladders of these animals at the later durations of diabetes as reported by Lincoln *et al* (1984) and Daneshgari *et al* (2006). Therefore, the initial increase in basal SA in 1-week diabetic tissues and the decrease in 12-week diabetic tissues may be related to time-dependent compensatory and decompensatory changes in function and structure of the STZ-diabetic urinary bladder, as previously suggested in Chapter 3. One possible compensatory mechanism could be altered expression of connexin proteins, which are important for maintenance of gap junctions in virtually all cell types. It has been reported that the expression levels of connexin proteins increases significantly within three days of diabetes, and later (after 2-weeks) returns to normal levels (Poladia *et al*, 2005). These findings may well be related to the enhanced SA detected in 1-week diabetic versus 12-week diabetic tissues, since increased number of gap-junctions could result in augmented intercellular communication

and increased propagation of action potentials within the muscle bundles and subsequent rise in SA.

The amplitude of SA may be dependent on changes in cellular Ca^{2+} levels via either VDCCs or Ca^{2+} released via the ryanodine receptors of the SR, whereas the frequency of SA reflects mechanisms that regulate UBSM action potential firing such as opening of BK_{Ca} channels (Buckner *et al*, 2002). High amplitude basal SA could therefore be associated with Ca^{2+} handling mechanisms in UBSM cells in detrusor strips from 1-week diabetic rats. Since high amplitude basal SA has been associated with DO, it could be speculated that these spontaneous contractions may also result in DO in 1-week diabetic rats, by enhancing afferent nerve firing *in vivo* and promoting efferent nerve stimulation and un-controlled urine loss as suggested by Kanai *et al*, 2007 in their study on neonatal rat bladders, although this requires confirmation by urodynamic studies.

For the initial studies on SA, bladders from male Wistar rats, supplied by Harlan were used. Due to a change in the animal supplier from Harlan to Charles River, subsequent experiments were carried out on bladder tissue from male Wistar-Hans rats. However, it became apparent throughout the course of our investigations that no basal SA developed in the bladder tissues from these animals. Ideally, further experiments would have been performed to investigate the underlying mechanisms of basal SA and the effect of the mucosa in modulation of this activity. In spite of this, it was possible to investigate cholinergic induction and modulation of SA in the bladders of these animals.

4.5.2 Induction of SA by CCH in detrusor strips from control (non-diabetic) and diabetic rats

As described previously, recent interest in cholinergic modulation of spontaneous contractions was stimulated by the hypothesis that symptoms of DO may be due to the enhancement of spontaneous contractions by leakage of small amounts of acetylcholine from intramural nerves (Andersson & Yoshida, 2003). Various studies have demonstrated increased SA in bladder strips from various species upon cholinergic stimulation (Gillespie *et al*, 2003; Drake *et al*,

2003; Ng *et al*, 2006; Gevaert *et al*, 2008). However, no investigations have looked at the cholinergic modulation of SA in STZ-diabetic rat bladder. In the present chapter, SA was induced in detrusor strips from control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic rats using low concentrations (0.05-0.5μM) of the non-selective muscarinic agonist, CCH. The amplitude and the frequency of CCH-induced SA in detrusor strips from diabetic animals and their age-matched controls (non-diabetic) were measured and compared at each concentration of CCH. The main differences observed in the amplitude of SA between control (non-diabetic) and diabetic tissues were detected in 1-week and 12-week diabetic rats. i.e. detrusor strips from 1-week diabetic tissues demonstrated a significantly higher amplitude of SA compared to their age-matched controls (non-diabetics) whilst 12-week diabetic tissues demonstrated a significantly lower amplitude of SA compared to their age-matched controls (non-diabetics). When the amplitude of SA at 0.5μM CCH was compared between all diabetic tissues, detrusor strips from 1-week and 12-week diabetic rats demonstrated the highest and the lowest amplitude of SA compared to all other diabetic tissues respectively. Since the age-matched control (non-diabetic) tissues demonstrated no change in the amplitude of SA with duration, it can be concluded that the changes seen in 1-week diabetic tissue versus all other diabetic groups are indeed true differences due to secondary responses of the UBSM to diabetes.

The reason for the increased amplitude of SA in 1-week diabetic tissues is unknown but changes in intracellular Ca^{2+} levels due to alterations in the activity of VDCCs or Ca^{2+} release mechanisms from internal stores may be important underlying factors. Also in parallel with excitation-contraction coupling, the sensitivity of contractile proteins to Ca^{2+} may have altered in the diabetic tissues, resulting in the current observations. The increased and decreased amplitude of SA in detrusor strips from 1-week and 12-week diabetic animals may also be due to a contractile transition from a compensated to a decompensated state at later durations of diabetes or an organ adaptation to diuresis and the metabolic effects of diabetes, some of which were described earlier in Chapter 3.

The frequency of stimulated SA was also measured and compared between different control and diabetic rat bladder tissues. Detrusor strips from 1-week

and 12-week diabetic rats demonstrated a significantly lower frequency of SA compared to their aged matched controls. In 1-week diabetic tissues, the decreased frequency of SA compared to the age-matched control (non-diabetic) tissues could be due to more organised pattern of Ca^{2+} and voltage activity in the detrusor resulting in greater magnitude and lower frequency of spontaneous contractions as suggested to occur in the detrusors from spinal transected rats by Ikeda & Kanai (2008). Also since the amplitude of SA is significantly greater in 1-week diabetic tissues compared to their age-matched control (non-diabetic) tissues, in the length of time taken for one contractile event to subside in detrusor strips from 1-week diabetic rats, during the same amount of time, control (non-diabetic) tissues will contract several times resulting in subsequent increase in the frequency of SA.

The decrease in frequency of stimulated SA in detrusor strips from 12-week diabetic rats compared to their age-matched control (non-diabetic) tissues could be due to the general decrease in the SA in 12-week diabetic tissues. Since the frequency of SA is dependent upon mechanisms that regulate smooth muscle action potential firing (such as opening of K^+ channels), it could be speculated that alterations in K^+ channels function in the diabetic tissues may result in the altered frequency of CCH-induced SA.

When the frequency of SA at 0.5μM CCH was compared between different diabetic tissues, a noticeable increase in the frequency of stimulated SA was detected in detrusor strips from 8-week diabetic rats compared to 1-week and 12-week diabetic tissues. In age-matched control (non-diabetic) tissues, there was a significant difference in the frequency of CCH-stimulated SA between 1-week and 12-week tissues only. Thus, it can be assumed that the differences seen in amongst the diabetic tissues are true differences as a result of diabetes and are not due to time dependent changes as a result of age in these animals. However, the reason behind the increased frequency of SA in 8-week diabetic tissues is not clear. This could also be due to contractile transition from a compensated to a decompensated state which manifests itself as more frequent SA in 8-week diabetic tissues.

It is assumed that the frequency of SA is not as important as the amplitude of these contractions in generation of DO, since reports from neonatal rat bladders

(used as a model for overactive bladder) have demonstrated that *in vitro* SA changes from high amplitude, low frequency contractions in neonatal rats to a low-amplitude high frequency phasic contractions during postnatal development. This is in line with more controlled micturition in these postnatal animals (Ng *et al*; 2006, 2007).

4.5.3 Effect of removal of the mucosa on CCH-induced SA

It has been postulated that intrinsic mechanisms in the bladder wall are responsible for modulation of SA in detrusor smooth muscle. Potential structures within the bladder wall that could regulate this SA include the urothelial layer, lamina propria and ICs (Ikeda & Kanai, 2008).

The effect of the mucosa on spontaneous contractions has been studied previously (Levin *et al*, 1995; Meng *et al*, 2008; Ikeda & Kanai, 2008). As described in chapter 1, urothelial cells have neuronal like properties and release factors such as ATP, ACH, prostaglandins and NO (Birder *et al*, 2001; Beckel *et al*, 2004; Beckel *et al* 2006; Birder, 2005; Beckel *et al*, 2006; De Groat, 2006), which could also be important in mediation of SA in the detrusor muscle. In the study by Ikeda & Kanai (2008) removal of the urothelium resulted in reduced muscarinic-stimulated spontaneous contractions in spinal cord transected rat bladder tissues. They suggested that the SA of bladder smooth muscle may be controlled by muscarinic receptors expressed on the urothelium. These results, contradict other studies suggesting an inhibitory effect of the urothelium on detrusor contractile activity (Levin *et al*, 1995; Meng *et al*, 2008). The release of a UDIF from the urothelium has been reported to inhibit bladder contractions (Hawthorn *et al*, 2000). UDIF is released under the control of two mediators, namely, ATP, through purinergic receptors and acetylcholine (ACH) via muscarinic receptors. Stimulation of tissues with CCH may enhance the release of UDIF which could influence spontaneous contractions. Therefore, removal of this barrier may enhance the spontaneous bladder contractions

In the present study, removal of the mucosa did not significantly affect the CCH-induced SA in detrusor strips from control (non-diabetic) and 1-week diabetic rats. However, removal of the mucosa had a significant effect on the CCH-

induced SA in detrusor strips from 12-week diabetic rats i.e. 12-week diabetic denuded tissues demonstrated a significantly higher amplitude and frequency of CCH-induced SA compared to 12-week diabetic intact tissues. Thus, it appears that there is an inhibitory effect of the mucosa on the spontaneous contractions of the bladder detrusor in 12-week diabetic rats, but not in control (non-diabetic) and 1-week diabetic tissues. This may suggest that stimulation of muscarinic receptors expressed on the urothelial cells does not normally play a role in mediating the spontaneous contractions in rat bladder, but they may be important in mediating the release of UDIF from the urothelial cells at later durations of diabetes.

However, it must be noted that there is a possibility that preparation of denuded muscle strips may damage the bladder tissue and the underlying structures of the urothelium such as the myofibroblasts and the ICs. This could result in the release of factors such as ACH from the various cell types present in the bladder, leading to enhanced spontaneous contractions detected in the 12-week diabetic denuded tissues.

Changes in the SA of the detrusor from STZ-diabetic rats is likely to have a number of causes that originate from defects to the cellular, multicellular and nervous mechanisms that determine normal function. In this chapter some of the possible causes leading to altered SA in detrusor strips from these animals were discussed. However, whether these altered responses result in whole bladder defects remains to be answered.

4.6 Summary

Muscarinic-receptor mediated mechanisms that modulate SA are of considerable interest, since antimuscarinics are used clinically to treat the symptoms of DO. In the present study, basal spontaneous contractions were significantly greater in detrusor strips from 1-week diabetic rats and were significantly decreased in 12-week diabetic tissues

CCH induced spontaneous contractions in control (non-diabetic) and diabetic tissues. Detrusor strips from 1-week diabetic rats demonstrated a greater increase in amplitude of spontaneous contractions, which may be important in generation of DO in these animals, whilst 12-week diabetic tissues demonstrated a significantly decreased SA, which may be associated with detrusor under-activity and atonic bladders in these animals.

The bladder urothelium did not play a role in modulation of SA in detrusor strips from control (non-diabetic) and 1-week diabetic rats, but was important in mediation of SA in 12-week diabetic tissues.

It must also be considered that bladder spontaneous contractions can be modulated by various mechanisms such as increased cell excitability and changes in the properties of other cell types that may be involved in generation of this activity. Thus, in the subsequent chapters the aim was to unravel the possible mechanisms that contribute to modulation of SA in control and diabetic rat bladders.

Chapter 5: An investigation of the role of potassium channels in the modulation of spontaneous contractions of rat bladder detrusor

5.1 Introduction

As described in Chapter 4, urinary bladder smooth muscle (UBSM) exhibits spontaneous action potentials that are associated with the spontaneous contractions in this tissue (Heppner *et al*, 1997; Brading, 2006). Ca^{2+} entry through voltage dependent Ca^{2+} channels (VDCCs) is responsible for the upstroke of the action potentials whilst the repolarisation phase is mediated by the activity of large conductance Ca^{2+} activated K^+ (BK_{Ca}) (Heppner *et al*, 1997) and perhaps voltage dependent K^+ (K_v) channels (Thorneloe & Nelson, 2003). SK_{Ca} channels are also known to be important determinants of UBSM excitability and contractility. The activation of small conductance Ca^{2+} activated (SK_{Ca}) channels generates a long-lasting hyperpolarisation termed the slow afterhyperpolarisation (sAHP) (Fujii *et al*, 1990). The resting membrane potential of UBSM is also controlled by BK_{Ca}, ATP sensitive K^+ (K_{ATP}) and probably K_V channels (Heppner *et al*, 1997; Petkov *et al*, 2001; Thorneloe & Nelson, 2003).

Since bladder detrusor spontaneous activity (SA) is dependent upon the electrical properties of smooth muscle cells, changes in K^+ channel function and expression have been suggested as possible underlying mechanisms of increased SA and subsequent detrusor overactivity (DO) (Christ *et al*, 2001; Fey *et al*, 2003; Meredith *et al*, 2004; Darblade *et al*, 2006; Werner *et al*, 2007; Brown *et al*, 2008). Opening of K^+ channels may also be an attractive way of treating overactive detrusor by eliminating undesired bladder contractions and increasing the UBSM stability (Gopalakrishnan & Shieh, 2004). Numerous studies have explored the role of BK_{Ca}, SK_{Ca} and K_{ATP} channel modulators in mediation of the SA of the bladder detrusor of various species (Herrera *et al*, 2000; Petkov *et al*, 2001; Gopalakrishnan *et al*, 2002; Herrera *et al*, 2003; Fey *et al*, 2003; Malysz *et al*, 2004; Ng *et al*, 2006), thus highlighting the fundamental role of these channels in mediating UBSM tone and contractility.

5.1.1 BK_{ca} channels in bladder smooth muscle

The role of BK_{ca} channels in stabilising membrane potential and regulating the excitability and contractility of UBSM cells was discussed briefly in chapter 1. BK_{ca} channel activity in UBSM depends primarily on depolarisation of the

membrane and Ca^{2+} influx through VDCCs, as well as so called "Ca^{2+} sparks" caused by localised Ca^{2+} release from ryanodine receptors (RyRs) (Herrera *et al*, 2001; Meredith *et al*, 2004). Moreover, it is known that BK_{Ca} channels can also be regulated by phosphorylation and G-protein-coupled receptor interactions. For example, in a study by Nakamura *et al* (2002), it was demonstrated that muscarinic receptor stimulation initially activates and then inhibits BK_{Ca} channels. It was suggested that the inhibition of BK_{Ca} channels is mediated through the M2-muscarinic receptor signal transduction pathway. However, the exact mechanism by which the M2-muscarinic receptor achieves this inhibition is unknown. An indirect modulation of BK_{Ca} channels by β-adrenoceptors has also been reported by Petkov & Nelson (2005) and Brown *et al* (2008). They demonstrated that stimulation of β-adrenoceptors leads to activation of protein kinase A (PKA), which has been shown to increase Ca^{2+} spark activity in UBSM and result in subsequent activation of BK_{Ca} channels (Petkov & Nelson, 2005; Brown *et al*, 2008).

Since the BK_{Ca} channel is one of the most physiologically relevant modulators of UBSM excitability, alterations in this channel's expression or activity should lead to dramatic functional effects and increased SA. There are several lines of evidence that confirm the important role of BK_{Ca} channels in abnormal detrusor contractions. Firstly Heppner *et al* (1997) demonstrated that inhibition of BK_{Ca} channels with the specific BK_{Ca} channel blocker iberiotoxin (IBTX) enhances the action potential frequency and detrusor contractility in guinea-pig UBSM. An analogous increase in the UBSM action potential frequency also has been reported in animal models of DO with bladder outlet obstruction (Li *et al*, 2007). Secondly Genetic deletion or inhibition of expression (via gene knock out (KO) studies) of the BK_{Ca} channel pore-forming α-subunit in mice, leads to complete absence of BK_{Ca} currents in UBSM (Meredith *et al*, 2004), and deletion of the regulatory β1-subunit leads to reduced BK_{Ca} channels activity (Petkov *et al*, 2001), resulting in DO. It was observed that the absence of functional BK_{Ca} currents significantly enhances basal and nerve-mediated UBSM contractility. Thirdly consistent with this, overexpression of the BK_{Ca} channel α-subunit, using naked DNA gene transfer techniques, eliminated DO induced by partial outlet obstruction in rat (Christ *et al*, 2001).

In the STZ-diabetic rat bladder, an elevated expression of BK_{Ca} channel regulatory α- and β-subunits was suggested to be associated with enhanced SA detected in the bladders of these animals (Nakahara *et al*, 2004). Although Nakahara *et al* (2004) failed to demonstrate this at the mRNA level, it was hypothesised that diabetes might increase the expression of BK_{Ca} channels through post-transcriptional mechanisms. Currently, there are very few studies on the role of these channels in modulation of STZ-diabetic rat bladder contractility. Since an increased SA has been reported previously in detrusor strips from STZ-diabetic rats (Stevens *et al*, 2006), and confirmed in the previous chapter, it is possible that changes in BK_{Ca} channel activity and expression may have a role in modulating this SA.

5.1.2 Pharmacology of BK_{ca} channels

Currently, there are several specific BK_{Ca} channel modulators available, which are used extensively for functional *in vitro* characterisation of these channels in pharmacological studies.

5.1.2.1 BK_{Ca} channel openers

Agents that cause BK_{Ca} channel activation are termed BK_{Ca} channel openers. Activation of BK_{Ca} channels by openers stabilises cell excitability by membrane hyperpolarisation and subsequent relaxation or inhibition of action potential generation. Thus, opening of these channels could have a profound effect on reduction of SA in DO. BK_{Ca} channels are particularly more appealing as therapeutic targets than other K^+ channel, such as K_{ATP} channels, because they are not expressed in cardiac tissue (reviewed by Ghatta *et al*, 2006), and thus fewer possible adverse effects.

There are many diverse compounds that lead to BK_{Ca} channels opening. Among these, a large series of synthetic benzimidazolone derivatives, such as NS1619 and NS004, the biarylureas like NS1609, and biaryl amines such as mefenamic and flufenamic acids, are the best characterised BK_{Ca} channel openers. These compounds can affect the channel either by modulating the Ca^{2+} binding site at the C-terminus end of the α-subunit or by strengthening the interaction of α and β subunits (reviewed by Ghatta *et al*, 2006).

The effect of several BK_{Ca} channel openers on modulation of detrusor contraction and SA, under normal and pathological conditions, has been investigated in numerous species (Imai *et al*, 2001; Malycz *et al*, 2004; Mora & Suarez-Kurtz, 2005; Darblade *et al*, 2006; Ng *et al*, 2006). Contradictory results have been obtained from these studies, with some groups reporting no change in the myogenic activity of detrusor strips after exposure to increasing concentrations of BK_{Ca} channel agents (Mora & Suarez-Kurtz, 2005), whilst other groups have demonstrated an inhibition of myogenic contractions with these drugs (Sheldon *et al*, 1997; Imai *et al*, 2001). For example in a study by Mora & Suarez-Kurtz (2005) the effect of NS1608 on the myogenic SA of detrusor strips from guinea pig urinary bladder was examined. It was demonstrated that this BK_{Ca} channel opener does not have a significant inhibitory effect on the spontaneous contractions of normal guinea-pig urinary bladder. In contrast, Imai *et al* (2001) demonstrated that NS1619 (30μM) significantly reduced the SA of detrusor strips from normal guinea-pig urinary bladder. Therefore, the effect of BK_{Ca} channel openers in mediating bladder detrusor contractions is still not fully clear.

5.1.2.2 BK_{Ca} blockers

The first potent peptide blockers of the BK_{Ca} channel were purified from *Leiurus quinquestriatus* (charybdotoxin; CHTX), *Buthus tamulus* (iberiotoxin; IBTX) and *Centruroides limbatus* (limbatotoxin; LBTX). IBTX is a potent BK_{Ca} channel blocker and acts by blocking the K^+ current flow (IC_{50}= 5-10nM) by binding to a site in the external vestibule of the pore and thus occluding the channel (reviewed by Kaczoroworski & Garcia, 1999 & Ghatta *et al*, 2006).

Other chemicals, such as quaternary ammonium compounds like tetraethylammonium (TEA) and tetrabutylammonium (TBA) (although are not specific) and alkaloids such as paxilline, penitrem A and verruculogen, are also classified as BK_{Ca} channel blockers (Reviewed by Ghatta *et al*, 2006).

A number of studies have demonstrated that BK_{Ca} channel blockers can increase SA in UBSM under normal and pathological conditions in several species (Defarias *et al*, 1996; Imai *et al*, 2001; Nakahara *et al*, 2004; Darblade *et al*, 2006; Shieh *et al*, 2007), emphasising the importance of these channels in modulating the UBSM tone and excitability.

5.1.3 SK_{Ca} channels in the bladder smooth muscle

As well as BK_{Ca} channels, SK_{Ca} channels have an important contributory role in UBSM excitability and contractility. Of the SK_{Ca} channel family (SK1-3), mRNA for the SK2 and SK3 subunits has been reported in mouse and rat UBSM (Herrera *et al*, 2003; Thorneloe *et al*, 2008; Hougaard *et al*, 2009).

SK_{Ca} channels are activated by an increase in intracellular Ca^{2+} concentrations and they underlie the sAHP phase of the action potential in UBSM (Herrera *et al*, 2003). The sAHP limits the firing frequency of repeated action potentials and subsequently limits the basal level of UBMS excitability and subsequent contractility (Herrera & Nelson, 2002).

The role of SK_{Ca} channels has been studied in the bladders of transgenic mice overexpressing SK3 channels (Herrera *et al*, 2003). It was demonstrated that suppression of SK_{Ca} channel expression by dietary doxycycline (DOX) is associated with an increase in the frequency of SA in UBSM *in vitro* and DO *in vivo* (Herrera *et al*, 2003).

Currently, there is only one study investigating the role of the SK_{Ca} channel in modulation of urinary bladder SA in STZ-diabetic rats (Nakahara *et al*, 2004). In this study, blockade of SK_{Ca} channels by the selective SK_{Ca} channel blocker, apamin, significantly increased the SA of detrusor strips from STZ-diabetic rats compared to control (non-diabetic) rats (Nakahara *et al*, 2004).

5.1.4 Pharmacology of SK_{Ca} channels

5.1.4.1 SK_{Ca} channel openers

There are as yet few studies of the effect of SK_{Ca} channel openers on inhibition of detrusor contractility. Various compounds have been classified as SK_{Ca} channel openers such as DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) and NS309 (3-oxime-6,7-dichloro-1H-indole-2,3-dione), which have been shown to inhibit VDCC activity in mouse bladder myocytes (Morimura *et al*, 2006). However, their specifity remains unclear. *In vivo* studies have shown that NS309 increases bladder capacity and micturition volume (Pandita *et al*, 2006). Recently the role of NS4591 (4,5-dichloro-1,3-diethyl-1,3-dihydro-benzoimidazol-2-one), a new modulator of SK_{Ca} channels, was

investigated in the mediation of SA in detrusor rings from normal rats. It was demonstrated that NS4591 significantly inhibits CCH-induced spontaneous contractions in bladder detrusor rings, although the specifity of this drug is under question, since it also affects IK_{Ca} channels (Hougaard *et al*, 2009).

5.1.4.2 SK_{Ca} channel blocker

Apamin is a bee venom peptide toxin that specifically blocks all types of SK_{Ca} channels (Vergara *et al*, 1998). Application of apamin increased the amplitude and decreased the frequency of spontaneous contractions in the guinea-pig urinary bladder (Imai *et al*, 2001; Herrera *et al*, 2000). Buckner *et al* (2002) also demonstrated that apamin increases both the amplitude and frequency of SA in pig urinary bladder. Electrophysiological studies show that apamin reduces UBSM action potential afterhyperpolarisation, which results in conversion of individual action potentials into bursts of action potentials in the UBSM cells. The results obtained from studies with apamin suggest that SK_{Ca} channels play an important role in the regulation of bladder function and provide rationale as a target for treatment of DO (Gopalakrishnan & Shieh, 2004).

5.1.5 K_{ATP} channels in the bladder smooth muscle

K_{ATP} channels in the bladder smooth muscle play a critical role in controlling myogenic tone and excitability. An increase in K_{ATP} channel activity can have a large effect on cell membrane potential and smooth muscle tone (Petkov *et al*, 2001). Openers of K_{ATP} channels decrease smooth muscle excitability/hyperexcitabilty by hyperpolarising the cell membrane by increasing the permeability of the cell to K^+ ions (Quayle *et al*, 1997). Small increases in K_{ATP} channel activity are likely to move the resting membrane potential away from the threshold of action potential activation and thus have significant inhibitory effects on related spontaneous contractions (Petkov *et al*, 2001). Due to their important role in regulation of membrane excitability, K_{ATP} channels have been extensively explored as possible therapeutic targets for treatment of overactive detrusor, with openers identified from diverse structural classes (Pinna *et al*, 2005; Elzayat *et al*, 2006; Shieh *et al*, 2007b; Kamiyama *et al*, 2008). However, studies on the role of these channels on modulation of detrusor contractility in diabetic bladder are scarce.

5.1.6 Pharmacology of K_{ATP} channel

5.1.6.1 K_{ATP} Channel openers

First generation K_{ATP} channel openers such as cromakalim were shown to reduce SA *in vitro*, when tested on normal and overactive detrusor smooth muscle strips from different species (Foster *et al*, 1989; Martin *et al*, 1997; Milicic *et al*, 2006). However, these drugs were unsuccessful in clinical applications due to their lack of bladder selectivity compared with vascular tissue (Nurse *et al*, 1991). This lead to development of 'uroselective' second-generation compounds such as ZD-6169 and its derivative, ZD226600. These drugs have been shown to greatly reduce DO in different animal models (Wojdan *et al*, 1999). The recently identified K_{ATP} opener, WAY-133537, has been reported to be effective *in vitro* in relaxing detrusor strips from a paraplegic rat model of neurogenic DO (Elzayat *et al*, 2006). Other K_{ATP} channel openers such as YM934 (Fey *et al*, 2003) and novel cyanoguanidine compounds (Perez-Medarno *et al*, 2007) have also been developed which have inhibitory effects on smooth muscle contractions in normal and overactive detrusor.

5.1.6.2 K_{ATP} channel blockers

The mechanism of action of K_{ATP} channel openers has been established primarily through the finding that sulphonylureas such as glibenclamide, a potent and selective K_{ATP} channel blocker, are able to reduce the functional and electrophysiological actions of the K_{ATP} channel in vascular and non-vascular smooth muscle. Apart from sulphonylureas, a number of other compounds such as imidazolines (e.g. alinidine) and chemically related compounds including phentolamine, have also been shown to inhibit the relaxant actions of K_{ATP} channel in smooth muscle cells (Mcpherson *et al*, 1999).

5.2 Aim of the chapter

The aim of this chapter was to investigate the role of K^+ channels in modulation of basal and muscarinic-receptor stimulated SA in detrusor strips from control (non-diabetic) and diabetic rats.

5.3 Materials and Methods

5.3.1 Functional investigation of the role of BK_{Ca}, K_{ATP} and SK_{Ca} channels in mediating the SA of detrusor strips from control (non-diabetic) and diabetic rats

5.3.1.1 Modulation of BK_{Ca} channels

Longitudinal strips of smooth muscle (2-4 x 6-12mm) were isolated from the bladder body of 1-week, 4-week, 8-week and 12-week diabetic rats and their age-matched controls (non-diabetic), and suspended in 15ml organ baths as described in chapter 2.

Following equilibration for 60mins, tissues were stimulated with 0.5μM concentration of carbachol (CCH) to induce spontaneous contractions.

In the continuous presence of 0.5μM CCH, increasing cumulative concentrations of NS1619 (1-30μM) (Sigma, Dorset, UK) were added to the tissues and the effects of this drug on the SA was assessed. Tissues were incubated with each concentration of NS1619 for a period of 15mins. During the last 5mins of each incubation period, the mean amplitude and the frequency of SA were calculated and analysed.

The effect of the vehicle, dimethyl sulfoxide (DMSO), (Sigma, Dorset, UK) on SA was also examined in parallel experiments to account for any time- or vehicle-dependent changes in the SA.

After assessing the effect of NS1619, detrusor strips from control (non diabetic); 1-week, 4-week, 8-week and 12-week diabetic rats and only rats were washed for 30mins with fresh Krebs bicarbonate solution. Following return to basal tension, tissues were exposed to increasing concentrations of IBTX (0.01-0.1μM) (Alomone labs, Jerusalem, Israel). Tissues were incubated with each concentration of IBTX for a period of 10-15mins. The last 5mins period with each concentration of IBTX was used to calculate the mean amplitude and the frequency of the spontaneous contractions.

5.3.1.2 Modulation of K_{ATP} channels

Longitudinal strips of smooth muscle (2-4 x 6-12mm) were isolated from the bladder body of 1-week control (non-diabetic) and diabetic rats, and suspended in 15ml organ baths as described previously.

Following equilibration for 60mins, tissues were stimulated with 0.5μM concentrations of CCH to induce SA.

In the continuous presence of 0.5μM CCH, increasing cumulative concentrations of cromakalim (0.1-10μM) (Sigma, Dorset, UK) were added to the tissues. Detrusor strips were incubated with each concentration of cromakalim for a period of 15mins. The mean amplitude and the frequency of SA in the last 5min of each incubation period were calculated and analysed.

The effect of the vehicle, DMSO, was also examined on the SA in parallel experiments to account for any time- or vehicle-dependent changes in the SA.

The effect of increasing concentrations of K_{ATP} channel blocker, glibenclamide (1-10μM), on basal SA of detrusor strips from control (non-diabetic) and diabetic rats was also investigated as described for IBTX in section 5.3.1.1.

5.3.1.3 Modulation of SK_{Ca} channels

The effect of increasing concentrations of the SK_{Ca} channel blocker, apamin (0.01-0.1μM), on basal spontaneous contractions of bladder strips from control (non-diabetic) and diabetic rats was also investigated as described for IBTX in section 5.3.1.1. Tissues were incubated with each concentration of apamin was for a period of 10-15mins. The last 5mins period with each concentration was used to calculate the mean amplitude and the frequency of the spontaneous contractions.

5.3.2 Data Analysis

To calculate the amplitude and the frequency of SA, a slightly modified method to that proposed by Imai *et al* (2001) was used to define a single spontaneous contractile event, as described previously in section 4.3.3. SA was expressed as the mean contraction amplitude/mg tissue and frequency ± SEM during the last 5mins period within a 10-15mins interval. Percentage change in the amplitude and the frequency of SA was also calculated in the presence of K^+ channel openers relative to that in the absence of the opener i.e. 0.5μM CCH only.

5.3.3 Statistical analysis

Statistical analysis was performed using repeated measures *ANOVA* followed by Dunnett's post hoc test for intra-tissue variations and unpaired Student's *t*-test for inter-tissue variations. For multiple comparisons between different control (non-diabetic) and diabetic groups, one way *ANOVA* followed by Tukey's post hoc test was performed.

5.3.4 Investigation of K^+ channel expression in the rat bladder using real-time PCR.

cDNA samples, which were prepared previously in chapter 3 from bladders of control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic rats were used in this set of experiments.

Real-time RT-PCR was carried out as described in chapter 3 to determine the expression ratios of BK_{Ca} channel subunits (Slo1 and Sloβ1) and K_{ATP} channel subunits (Kir6.1 and SUR2B) relative to the housekeeping gene HPRT in diabetic tissues vs. control (non-diabetic) tissues using previously designed and optimised primers (Table 13).

K^+ Channel	Subunits (genes)	Accession no	Primer sequence	PS
BK_{Ca}	Slo-1 (KCNMA1)	NM-031828	F: 5′GCATCAGGCAGAAGATCAGG 3′ R: 5′GGGGATGGTGGTTGTTATGG 3′	180 bp
	Sloβ-1 (KCNMAB1)	NM-019273	F: 5′GGGAAGAAGCTGGTGATGGC 3′ R: 5′TTCCTGGTCCTTGATGTTGG 3′	244 bp
K_{ATP}	Kir 6.1 (KCNJ8)	AB-043636	F: 5′TGGGTGACCTGAGGAAAAGC 3′ R: 5′TATCGTACAGGGGGCTACGC 3′	204 bp
	SUR2B (ABCC9)	AB-045281	F: 5′ACAGATGGCTGGAGGTCAGG 3′ R: 5′TCGTGAGGGCATACAGAAGG 3′	126 bp

Table 13) BK_{Ca} channel subunits (Slo-1 & Sloβ-1) and K_{ATP} channel subunits (Kir6.1 & SUR2B) gene primer sequences, gene accession numbers and product size. bp= base pairs; P.S=product size, F=forward R=reverse.

Data analysis was performed as described in chapter 2. Briefly, by calculating the relative expression ratio of the target gene (K^+ channels subunit) based on its efficiency (E) and the difference in C_t value (delta C_t) of the treated sample (diabetic) against that of a control (non-diabetic) and expressed in comparison to a reference gene (HPRT) as described by Pfaffl (2001):

$$ratio = \frac{(E_{target})^{\Delta C_t\, target\,(control-treated)}}{(E_{ref})^{\Delta C_t\, ref\,(control-treated)}}$$

E= efficiency of the target (E_{target}) or the housekeeping (E_{ref}) gene primers. ΔCt= the difference between the Ct values of the control (non-diabetic) sample and the treated (diabetic) sample.

5.4 Results

5.4.1 Effects of the BK_{Ca} channel opener, NS1619, on stimulated SA in control (non-diabetic) and diabetic intact tissues

Typical chart recordings showing the effect of the BK_{Ca} channel opener, NS1619, on CCH-induced SA detrusor strips from control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic tissues are shown in Figure 41.

5.4.1.1 The effect of NS1619 on CCH-stimulated SA in detrusor strips from 1-week diabetic rats

In 1-week diabetic tissues and their age-matched control (non-diabetic) group NS1619 had a limited effect and elicited a significant inhibition of the amplitude of CCH-induced SA only at the highest concentration (30μM) (Figure 42).

NS1619 did not affect the frequency of SA at any of the concentrations examined (Figure 42)

When the percentage change in the amplitude of the SA in the presence of 30μM NS1619 was calculated relative to spontaneous contractions in the absence of the opener (i.e. in the presence of 0.5μM CCH only), there was no significant difference between age-matched control (28.76±4.16%) and diabetic (24.35± 4.2%) tissues. The same results were observed for the frequency of SA (control: -5.45±9.77% versus diabetic: 16.83±4.16%)

The vehicle, DMSO, did not produce any significant effects on the amplitude and the frequency of SA in either control (non-diabetic) or diabetic tissues. (Figure 41).

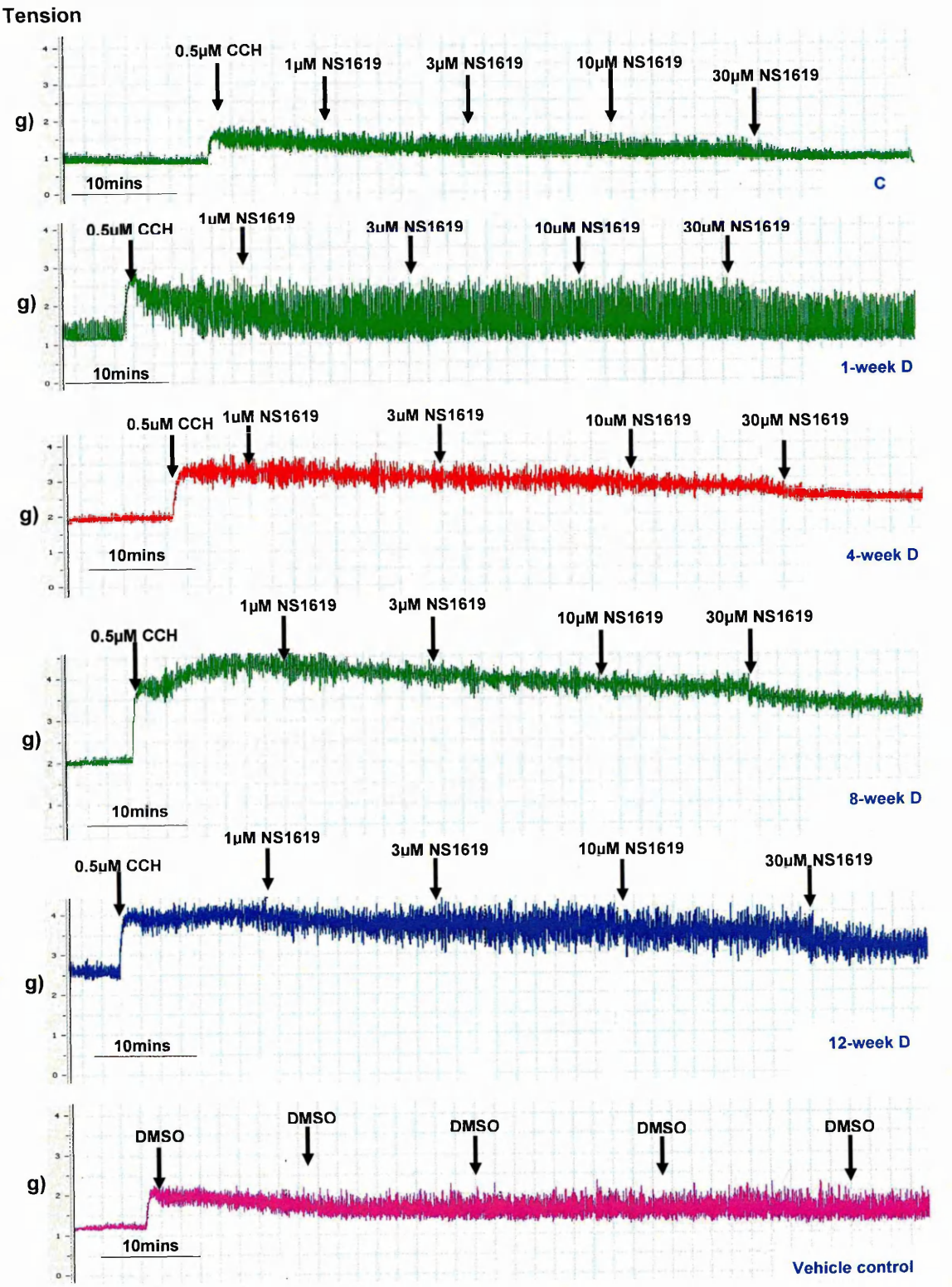


Figure 41) Typical chart recordings of the effect of increasing concentrations of NS1619 and DMSO on CCH induced SA in detrusor strips from representative control (non-diabetic) (C) & diabetic (D) rats.

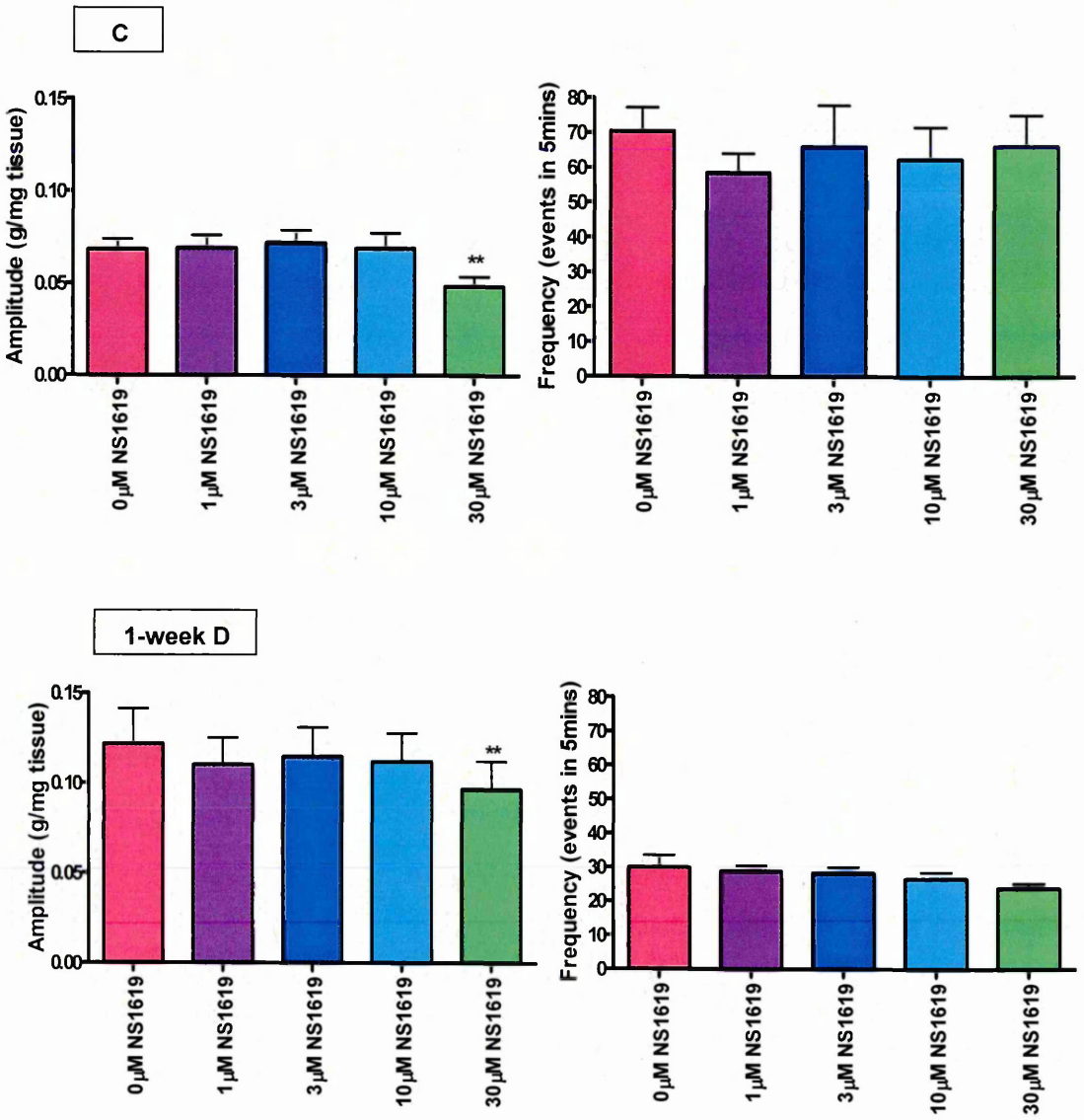


Figure 42) Effect of increasing concentrations of NS1619 on the amplitude and the frequency of SA induced by 0.5µM CCH in control (non diabetic) (C) and 1-week diabetic (D) tissues. C: n=5 (top panel) & 1-week D: n=10 (lower panel). **p<0.01 versus paired 0µM NS1619 response. Data is presented as mean±SEM.

5.4.1.2 The effect of NS1619 on CCH-stimulated SA in detrusor strips from 4-week diabetic rats

NS1619 did not affect the amplitude or the frequency of SA in 4-week age-matched control (non diabetic) tissues (Figure 43). In 4-week diabetic tissues only 30μM NS1619 significantly inhibited the amplitude of SA but had no effect on the frequency of SA (Figure 43).

When the amplitude and the frequency of SA in the presence of 30μM NS1619 were calculated as percentage change relative to spontaneous contractions in the absence of NS1619 (i.e. in presence of 0.5μM CCH only), there was no significant difference between age-matched control (non-diabetic) tissues (amplitude: 4.32±11.28% & frequency: -0.68±9.94) versus diabetic (amplitude: 29.95± 8.09% & frequency: 7.16±3.44%) tissues.

5.4.1.3 The effect of NS1619 on CCH-stimulated SA in detrusor strips from 8-week diabetic rats

NS1619 decreased the amplitude of SA in 8-week age-matched (non-diabetic) and 8-week diabetic tissues only at 30μM (Figure 44). This concentration of NS1619 also significantly decreased the frequency of SA in control (non-diabetic) tissues, but it did not affect the frequency of SA in 8-week diabetic tissues (Figure 44).

The percentage change of both the amplitude and the frequency of stimulated SA, at 30μM NS1619 relative to that of SA in the absence of NS1619 (i.e. 0.5μM CCH only) were calculated for control (non-diabetic) and diabetic tissues and compared. There was no significant difference in the percentage change of the amplitude: (28.37± 11.00% in controls (non-diabetic) vs. 16.07± 7.39% in diabetics) and the frequency (30.87± 16.24% in controls (non-diabetic) vs. 4.75± 4.60% in diabetics) of stimulated SA at 30μM NS1619 between both groups.

5.4.1.4 The effect of NS1619 on CCH-stimulated SA in detrusor strips from 12-week diabetic rats

In 12-week age-matched control (non-diabetic) tissues, the amplitude of SA was significantly decreased at 10μM and 30μM NS1619. However, the amplitude of SA in 12-week diabetic tissues was significantly decreased only at 30μM NS1619 (Figure 45). NS1619 did not affect the frequency of SA in either tissue group at the concentrations tested (Figure 45).

There was no significant difference in the %change of the amplitude of SA in the presence of 30μM NS1619 between control (non-diabetic) (31.01±6.52%) and 12-week diabetic (30.60±3.55%) tissues. The same results were observed for the frequency of SA for controls (non-diabetic) (28.97±19.66%) versus 12-week diabetic (7.98±10.05%) tissues.

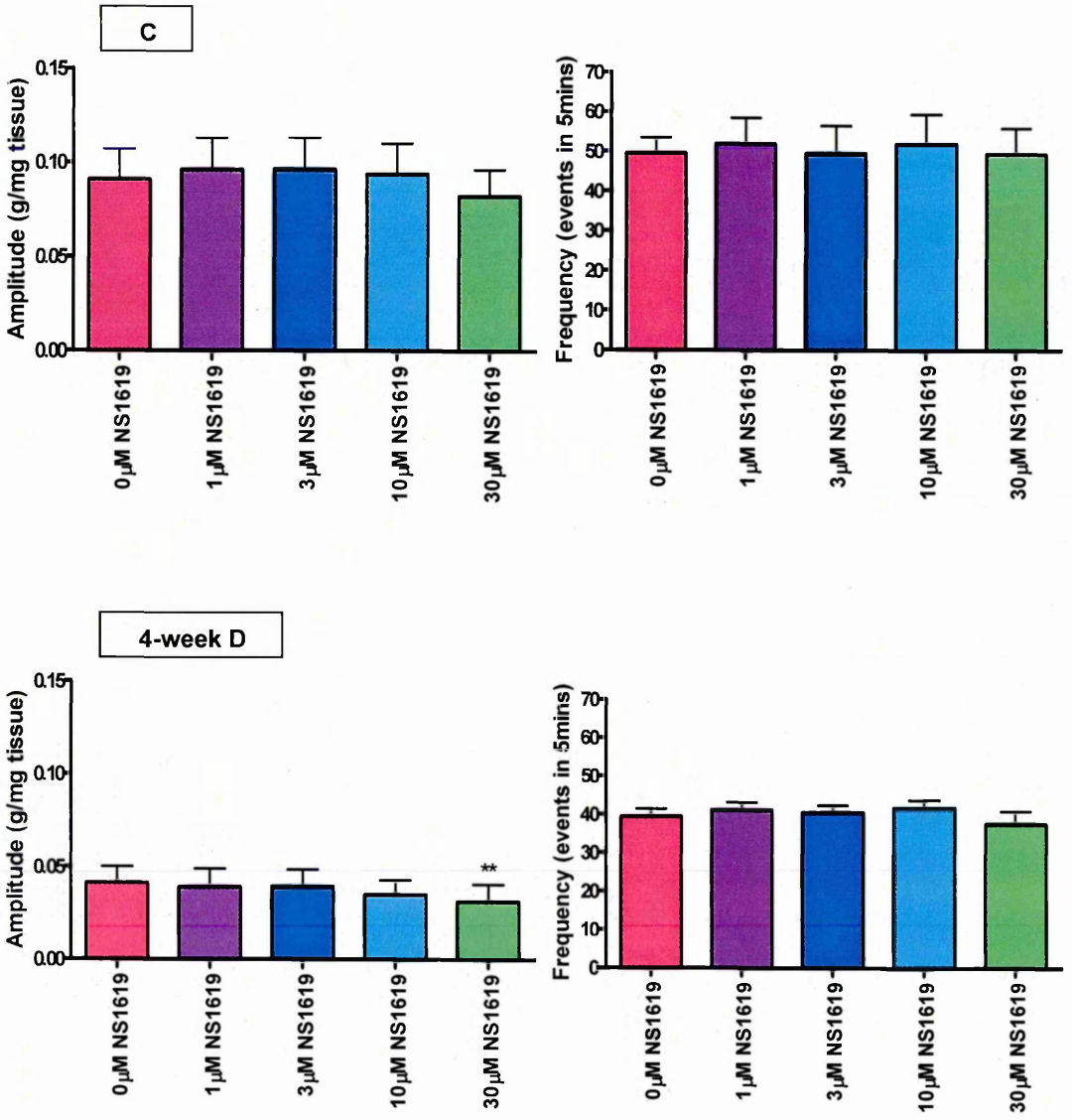


Figure 43) Effect of increasing concentrations of NS1619 on the amplitude and frequency of SA induced by 0.5μM CCH in 4-week control (non diabetic) (C) and 4-week diabetic (D) tissues. C: n=8 (top panel) & 4-week D: n=9 (lower panel). **p<0.01 versus paired 0μM NS1619 response. Data is presented as mean±SEM.

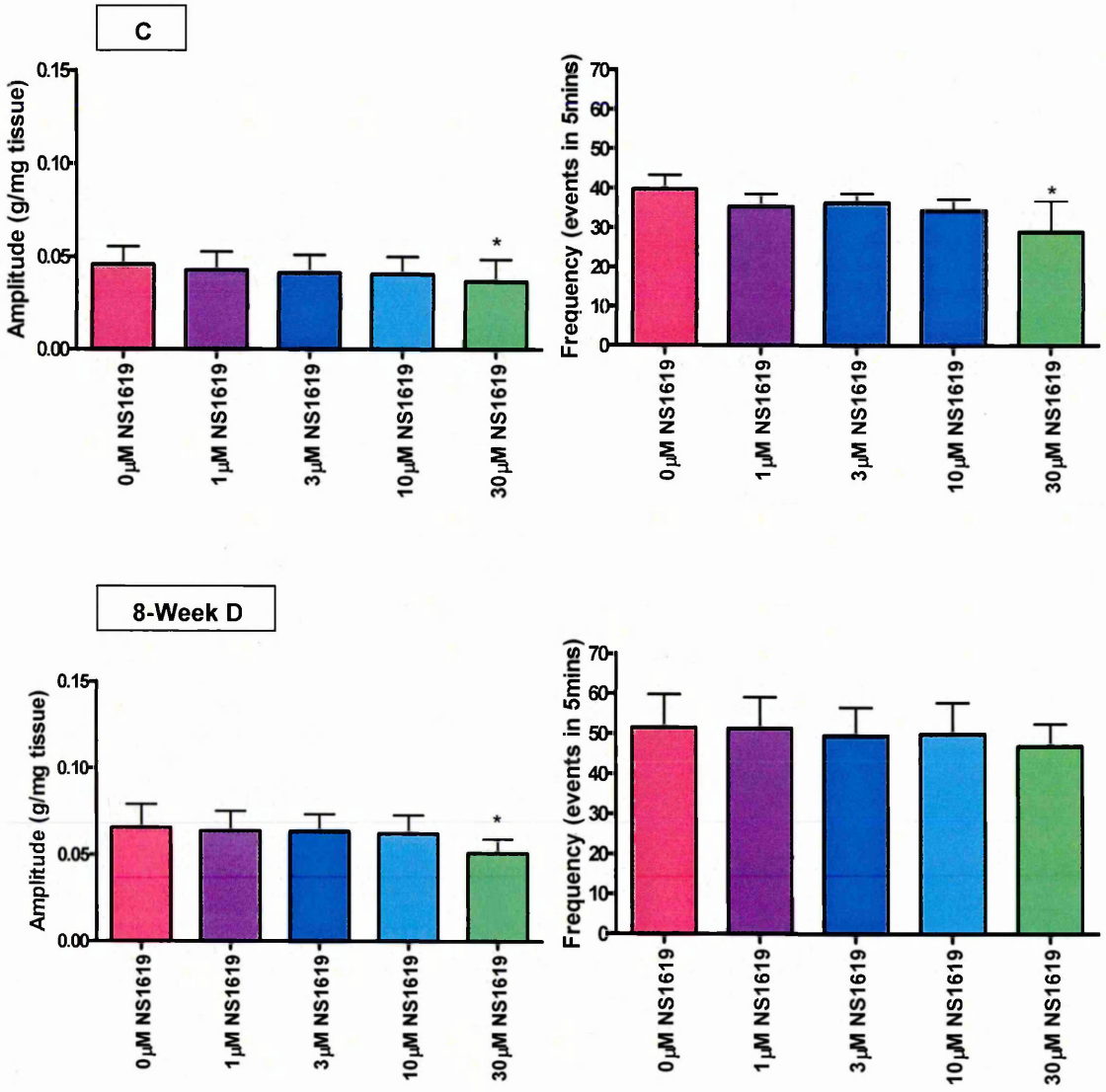


Figure 44) Effect of increasing concentrations of NS1619 on the amplitude and the frequency of SA induced by 0.5μM CCH in 8-week control (non diabetic) (C) and 8-week diabetic (D) tissues. C: n=8 (top panel) & 8-week D: n=8 (lower panel). *p<0.05 versus paired 0μM NS1619 response. Data is presented as mean±SEM.

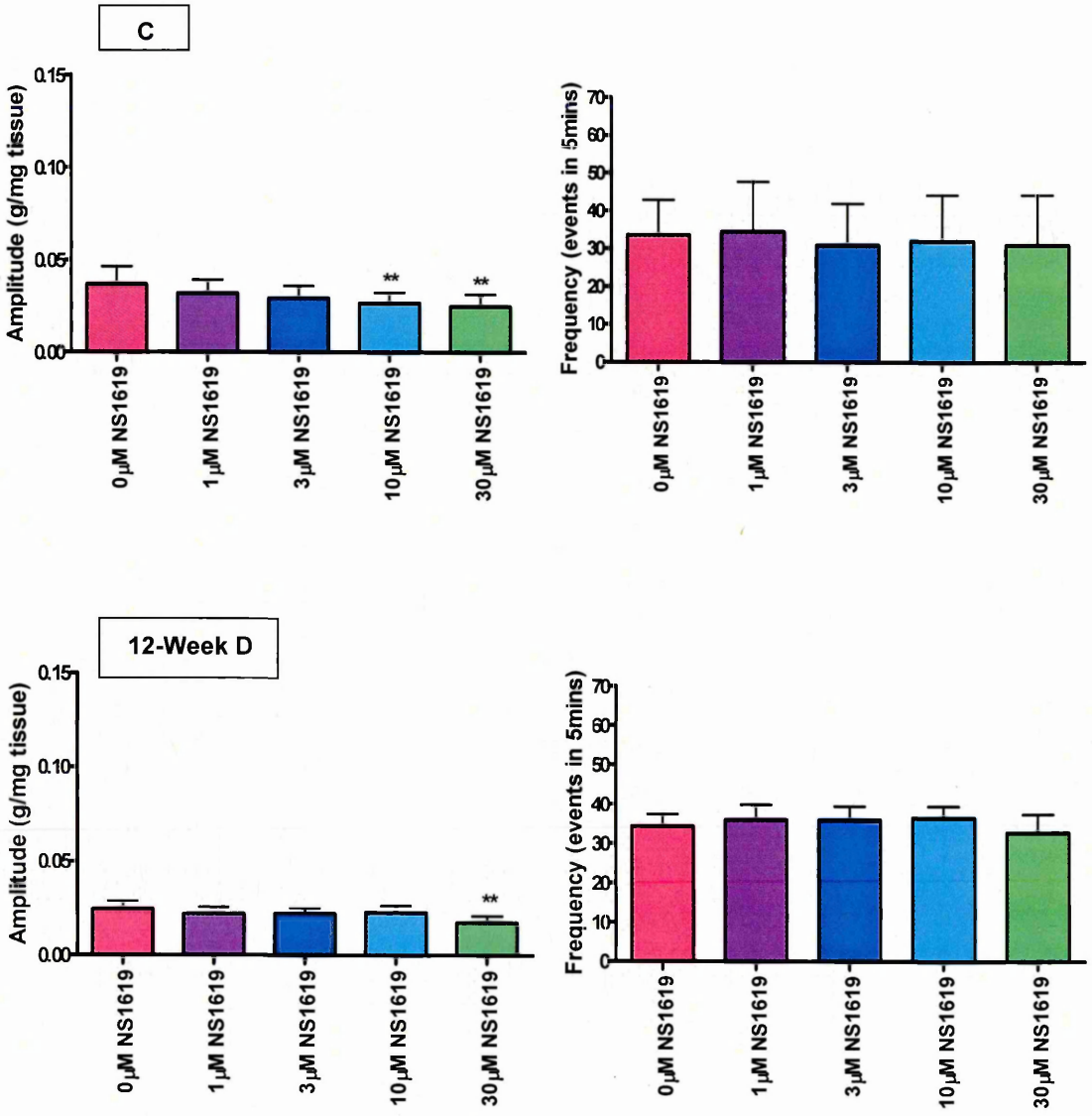


Figure 45) Effect of increasing concentrations of NS1619 on the amplitude and the frequency of SA induced by 0.5µM CCH in 12-week control (non diabetic) (C) and 12-week diabetic (D) tissues. C: n=8 (top panel) & 12-week D: n=9 (lower panel). **p<0.01 versus paired 0µM NS1619 response. Data is presented as mean±SEM

The percentage change in the amplitude and the frequency of spontaneous contractions in the presence of highest concentration of NS1619 (30 μM) relative to the absence of NS1619 i.e. in the presence of 0.5μM CCH was compared between control (non-diabetic) and diabetic tissues and plotted against the duration of diabetes (Figure 46). There was no significant difference in the percentage change of the amplitude and the frequency of CCH-stimulated SA between different age-matched control (non-diabetic) groups at 30μM NS1619. There was also no significant difference in the percentage change of the amplitude and the frequency of stimulated SA at 30μM NS1619 between different diabetic groups with duration (Figure 46).

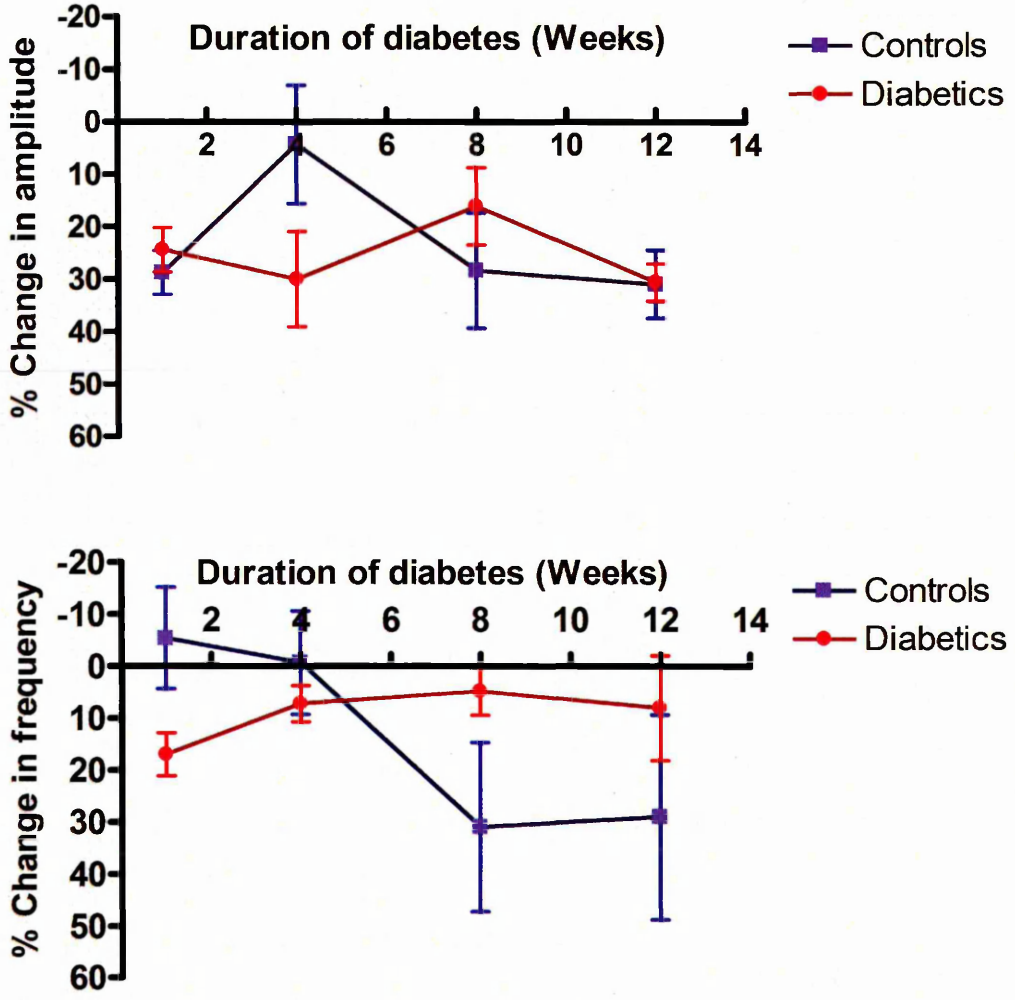


Figure 46) Percentage changes in the amplitude and the frequency of CCH-stimulated SA in the presence of 30μM NS1619. Data is plotted against the duration of diabetes (weeks) in detrusor strips from control (non-diabetic) and diabetic rats. n=5-10. Data is presented as mean±SEM.

5.4.2 Effects of the BK_{Ca} channel blocker IBTX on basal SA in control (non-diabetic) and diabetic tissues

The hypothesis that blocking BK_{Ca} channels can induce basal SA in UBSM cells was examined by incubating the detrusor strips from control (non-diabetic) (n=5), 1-week (n=8), 4-week (n=5), 8-week (n=3) and 12-week (n=4) diabetic rats with increasing concentrations (0.01, 0.03 and 0.1μM) of IBTX was investigated on basal contractile activity in tissues.

Typical chart recordings from control (non-diabetic) and diabetic tissues in the presence of increasing concentrations of IBTX are demonstrated in Figure 47. Before treatment with IBTX, little or no spontaneous contractions could be detected in detrusor strips from control (non-diabetic) and diabetic rats. However, after treatment of the detrusor strips with the highest concentration of IBTX (0.1μM), SA developed in all tissues, with the exception of detrusor strips from 12-week diabetic rats.

The amplitude and the frequency of basal SA increased upon addition of IBTX in detrusor strips from 1-week diabetic rats and their age-matched control (non-diabetic) group. However, only at 0.1μM was this significant. 0.01μM and 0.03μM IBTX did not have a significant effect on the amplitude or the frequency of SA in either group (Figure 48).

Although the effect of 0.1μM IBTX on amplitude of basal SA was greater in 1-week diabetic detrusor strips (0.041g/mg tissue) than in the control (non-diabetic) tissues (0.014g/mg tissue), this difference was not significant (p=0.07). There was also no significant difference in the effect of 0.1μM IBTX on the frequency of basal SA between 1-week diabetic and control (non-diabetic) tissues.

In 4-week diabetic tissues, 0.01 and 0.03μM IBTX did not have a significant effect on the amplitude and the frequency of SA, whilst 0.1μM IBTX significantly increased the amplitude and the frequency of basal SA (Figure 49).

A similar effect was seen in 8-week diabetic tissues i.e. only 0.1μM IBTX produced a significant increase in amplitude of SA. However, in this group the

frequency of SA in the diabetic tissues was also significantly increased upon application of both 0.03μM and 0.1μM IBTX (Figure 49).

IBTX increased the amplitude or the frequency of basal SA only slightly in 12-week diabetic tissues and this increase was not significant (Figure 49).

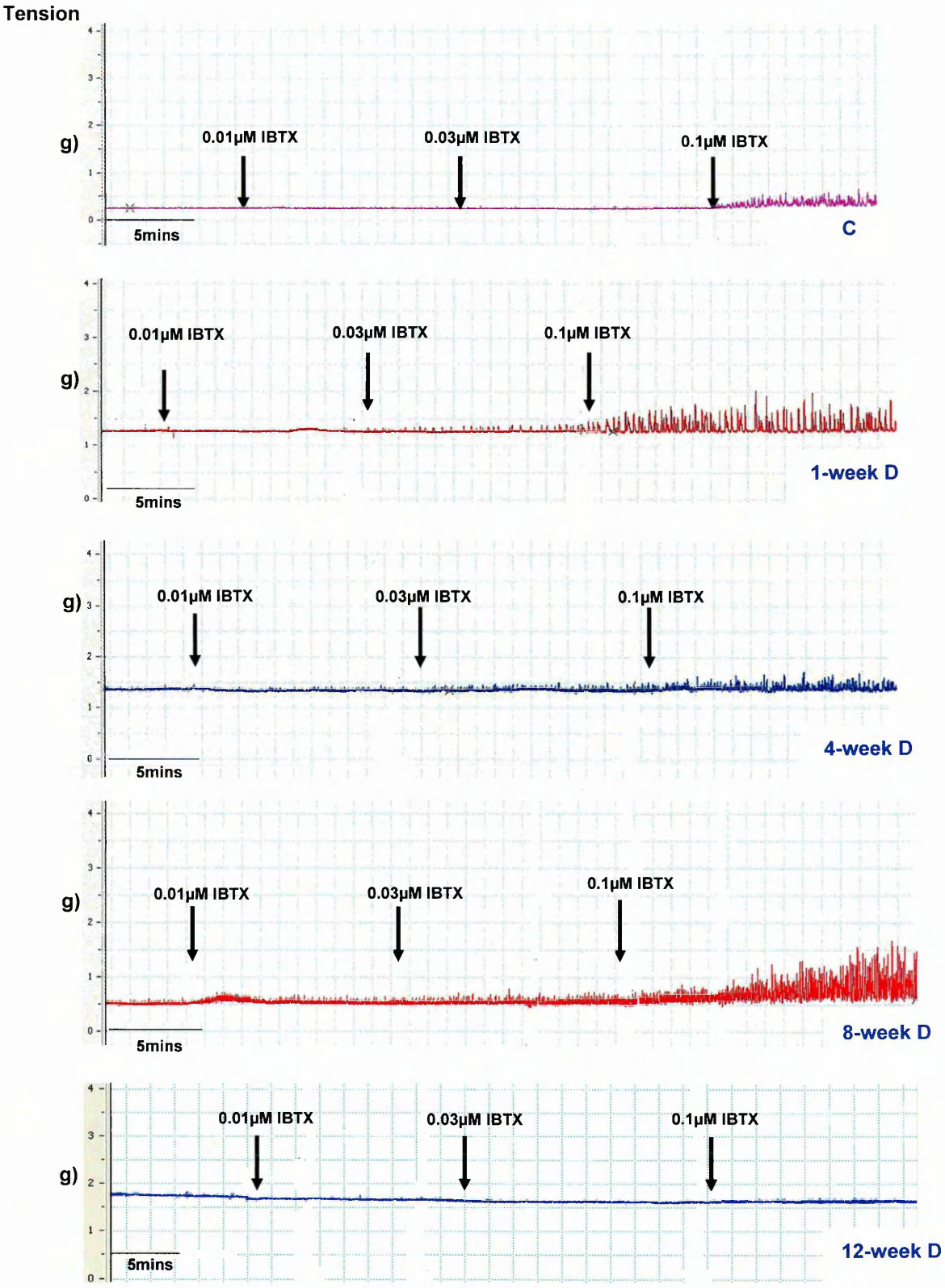


Figure 47) Typical chart recordings of basal SA in the presence of increasing concentrations of IBTX in control (non-diabetic) (C) & diabetic tissues.

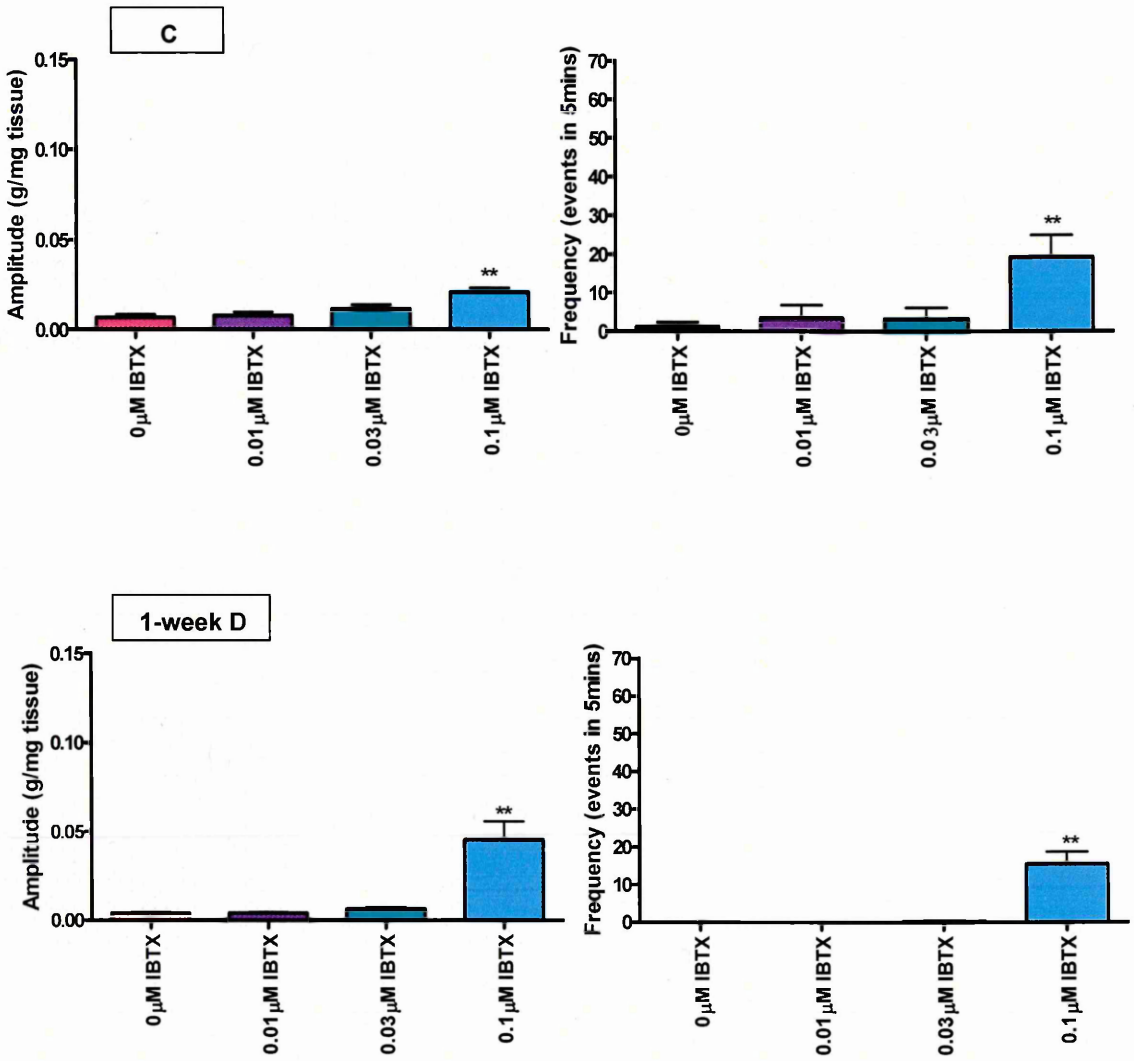


Figure 48) Effect of increasing concentrations of IBTX on the amplitude and frequency of basal SA in control (non-diabetic) (C) and diabetic (D) tissues. C: n=5 (top panel) & 1-week D: n=8 (lower panel). **p<0.01 versus paired 0µM IBTX response. Data is presented as mean±SEM.

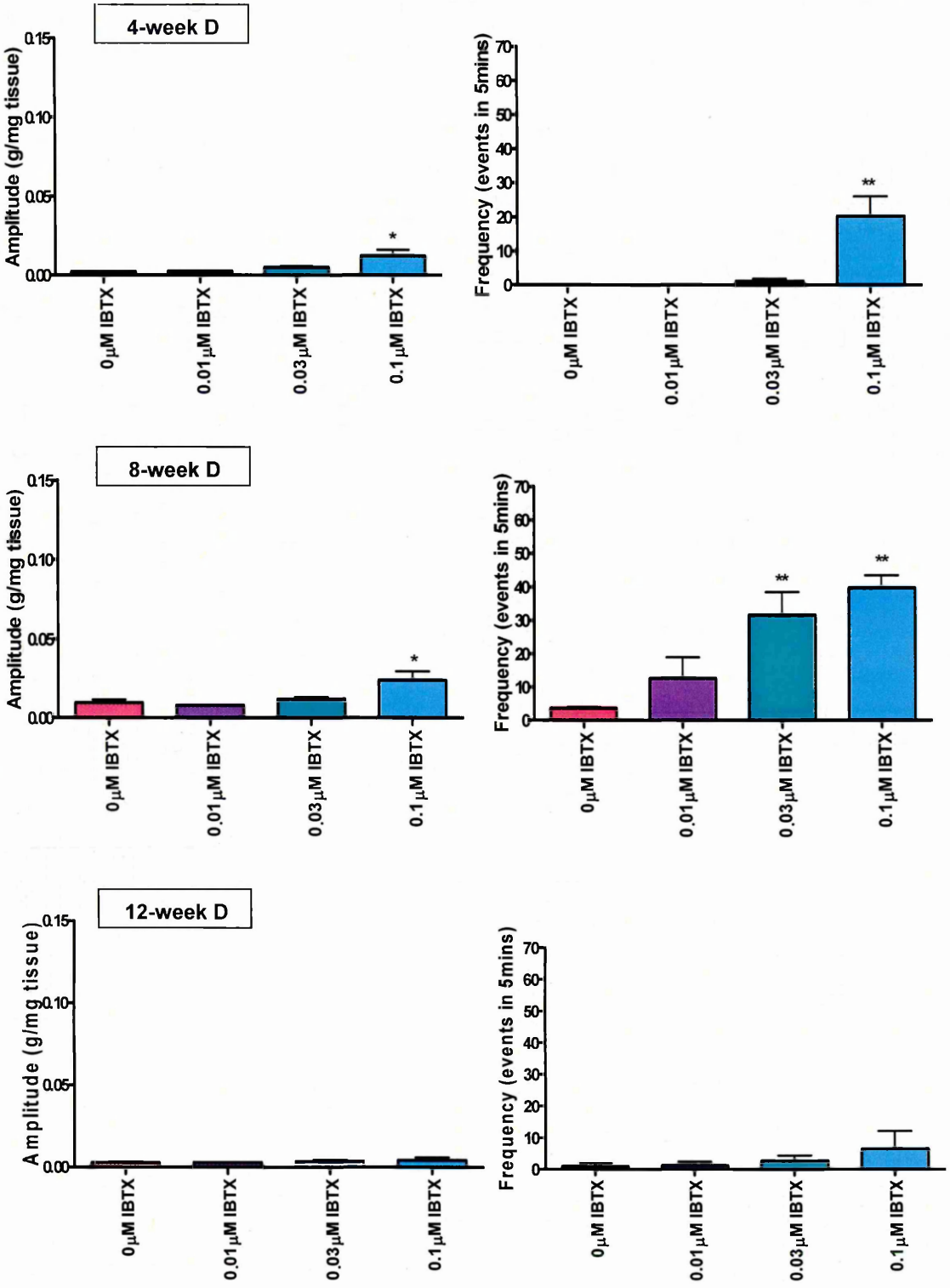


Figure 49) Effect of increasing concentrations of IBTX on the amplitude and frequency of basal SA in diabetic (D) tissues. 4-week D: n=5 (top panel), 8-week D: n=3 (middle panel) and 12-week D (n=4) (lower panel). * $p<0.05$ and ** $p<0.01$ versus paired 0μM IBTX response. Data is presented as mean±SEM.

In order to compare the effects of IBTX between different diabetic groups, the amplitude and the frequency of SA at 0.1μM IBTX were plotted against the duration of diabetes (Figure 50). Although 1-week diabetic tissues demonstrated a greater increase in the amplitude of SA upon stimulation with 0.1μM IBTX compared to all other diabetic groups, this increase was only significantly greater than the 12-week diabetic tissues. There was no significant difference in the increase of the amplitude of basal SA between all other groups (Figure 50).

The frequency of SA in the presence of 0.1μM IBTX increased with duration of diabetes up to 8-weeks post STZ-injection and then decreased in detrusor strips from 12-week diabetic rats. The frequency of SA was significantly higher in 8-week diabetic tissues at 0.1μM IBTX compared to 1-week and 12-week diabetic tissues (Figure 50).

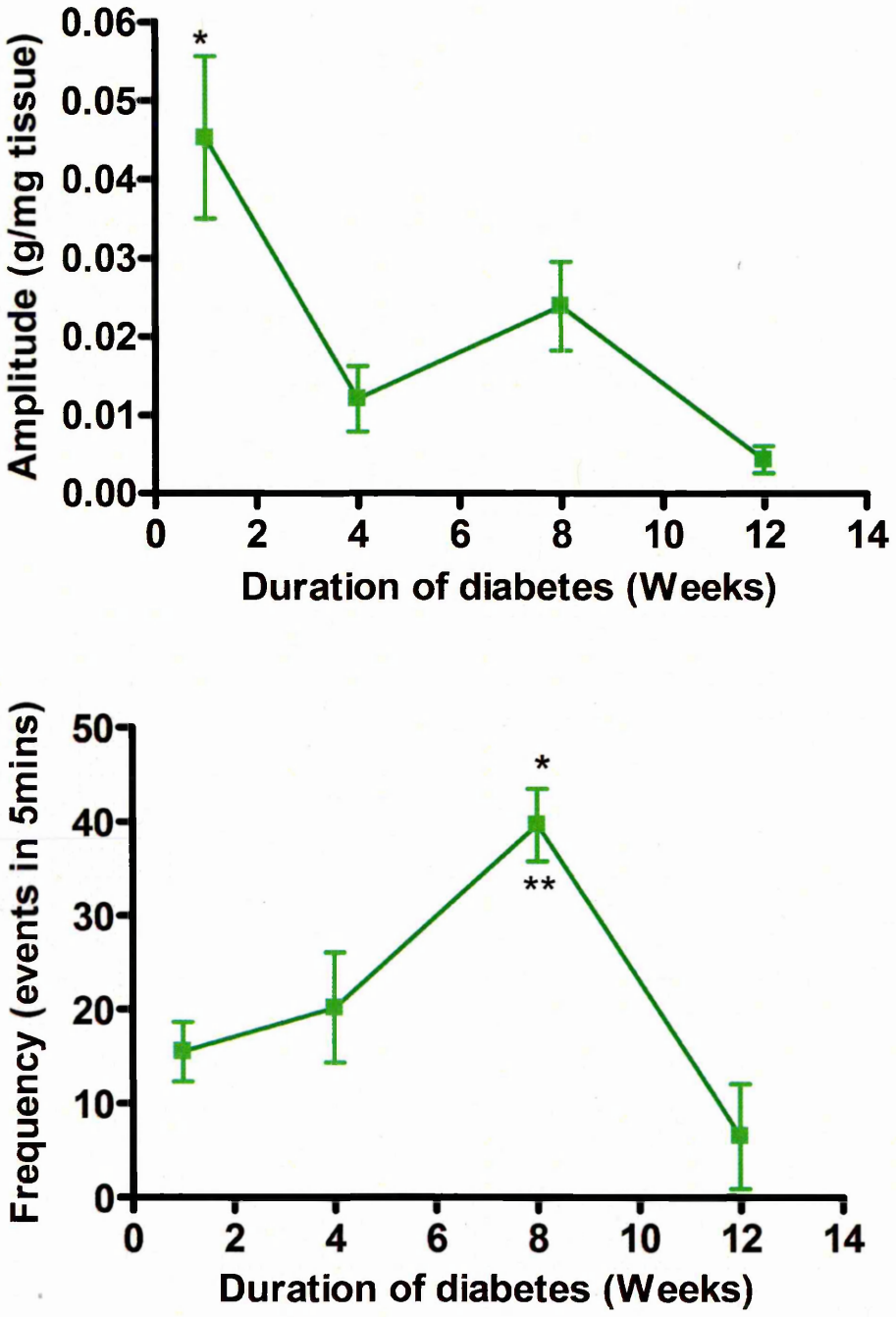


Figure 50) Duration-dependent changes in the amplitude and the frequency of SA in the presence of 0.1uM IBTX in detrusor strips from diabetic rats. Changes in the amplitude (top panel) & changes in the frequency (lower panel). n=5-10. *p<0.05 versus 12-week diabetic tissues (top panel). *p<0.05 versus 1-week diabetic tissues. **p<0.01 versus12-week diabetic tissues (lower panel). Data is presented as mean±SEM.

5.4.3 Effect of the K_{ATP} channel opener cromakalim on CCH-stimulated SA in control (non-diabetic) and diabetic tissues

Typical chart recordings of the effect of the K_{ATP} channel opener cromakalim on CCH-induced SA in detrusor strips from 1-week diabetic rats and their age-matched controls (non-diabetics) are demonstrated in Figure 51. The effect of cromakalim on inhibition of CCH-induced SA was only investigated at this duration of diabetes.

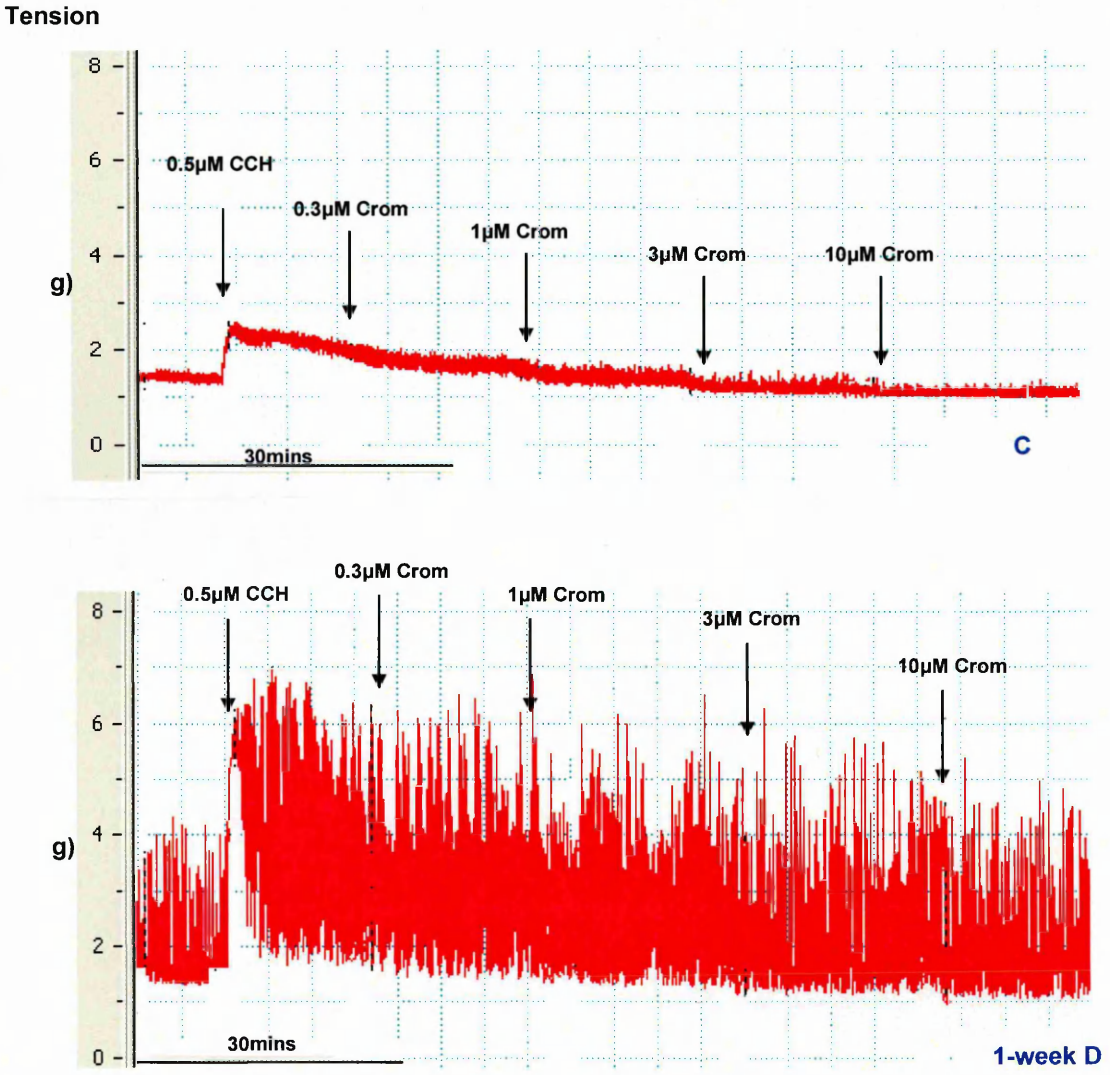


Figure 51) Typical chart recordings of the effect of increasing concentrations of cromakalim on CCH-induced SA in detrusor strips from 1-week and diabetic (D) rats and their age-matched controls (non-diabetics) (C).

Cromakalim did not have a significant effect on the amplitude of CCH-induced SA in either control (non-diabetic) (n=11) or diabetic (n=14) tissues (Figure 52). However, cromakalim did significantly decrease the frequency of SA in control (non-diabetic) tissues at 1μM, 3μM and 10μM and in the diabetic tissues at 3μM and 10μM.

The percentage change in the amplitude and the frequency of CCH-stimulated spontaneous contractions in the presence of 3μM and 10μM cromakalim relative to that in the absence of cromakalim (i.e. in presence of 0.5μM CCH only), was calculated and compared between control (non-diabetic) and diabetic tissues. There was no significant difference in the percentage inhibition of the amplitude of SA between the control (non-diabetic) (3μM: 2.46±8.94%, 10μM: 23.35±9.34%) and the diabetic (3μM:5.42±6.37%, 10μM:8.05±7.15%) tissues at both concentrations of cromakalim. However, percentage inhibition of the frequency of SA at 3μM and 10μM cromakalim was significantly greater ($p<0.05$) in control (non-diabetic) tissues (3μM: 34.70±10.41%, 10μM:41.34±10.08%) than the diabetic tissues (3μM:11.69±6.13%, 10μM: 18.60±5.70%).

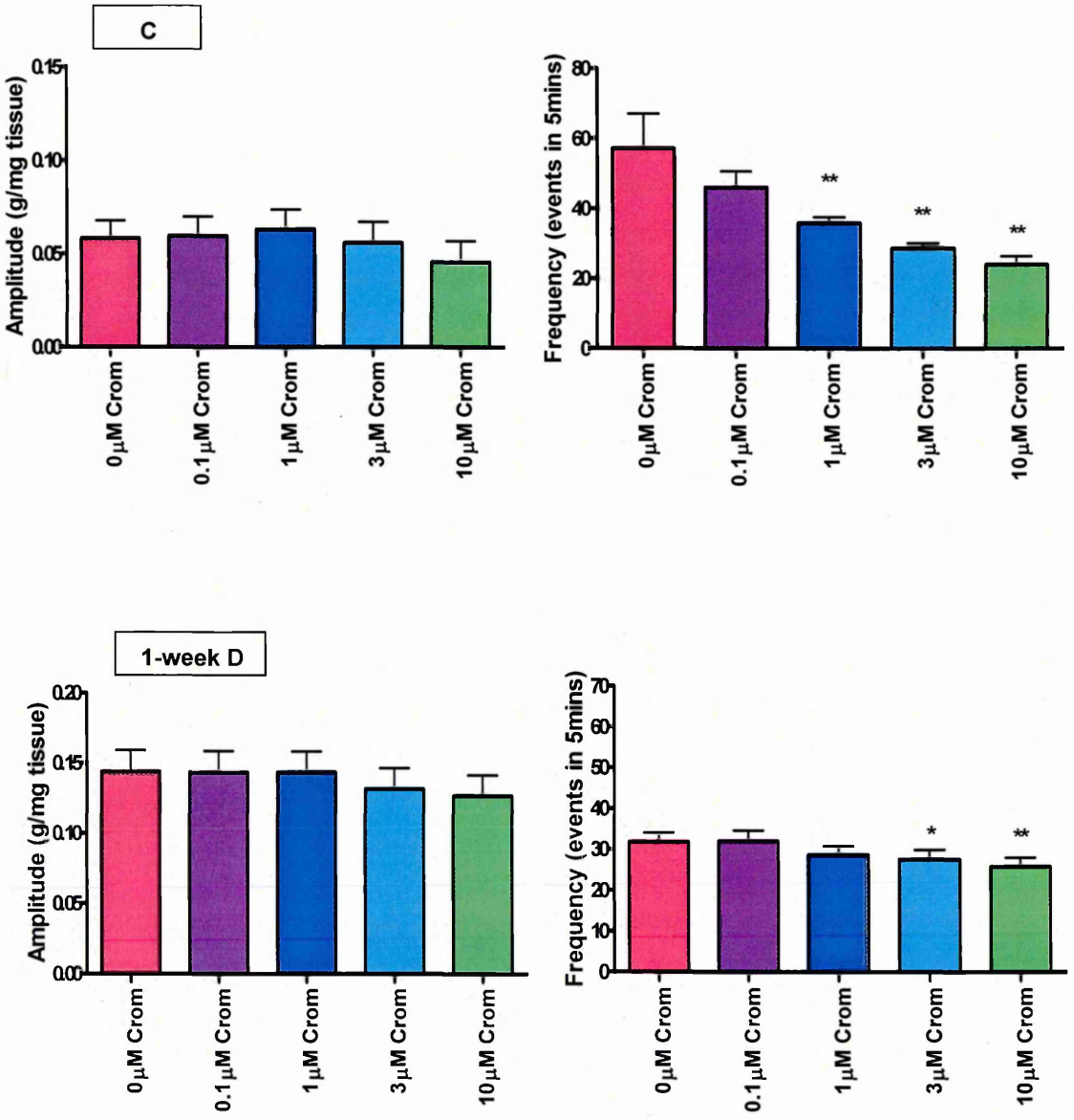


Figure 52) Effect of increasing concentrations of cromakalim on the amplitude and the frequency of SA induced by 0.5µM CCH in control (non-diabetic) (C) and diabetic (D) tissues. C: n=11 & 1-week D: n=14. *p<0.05 & **p<0.01 versus paired 0.5µM CCH response. Data is presented as mean±SEM.

5.4.4 Effects of the K_{ATP} channel blocker glibenclamide on the basal SA in control (non-diabetic) and diabetic tissues

The effect of the K_{ATP} channel blocker glibenclamide on basal SA was investigated in detrusor strips from 1-week diabetic rats and their age-matched controls. Typical chart recordings of the effect of glibenclamide on basal SA in control and diabetic rat bladder detrusor strips are demonstrated in Figure 53.

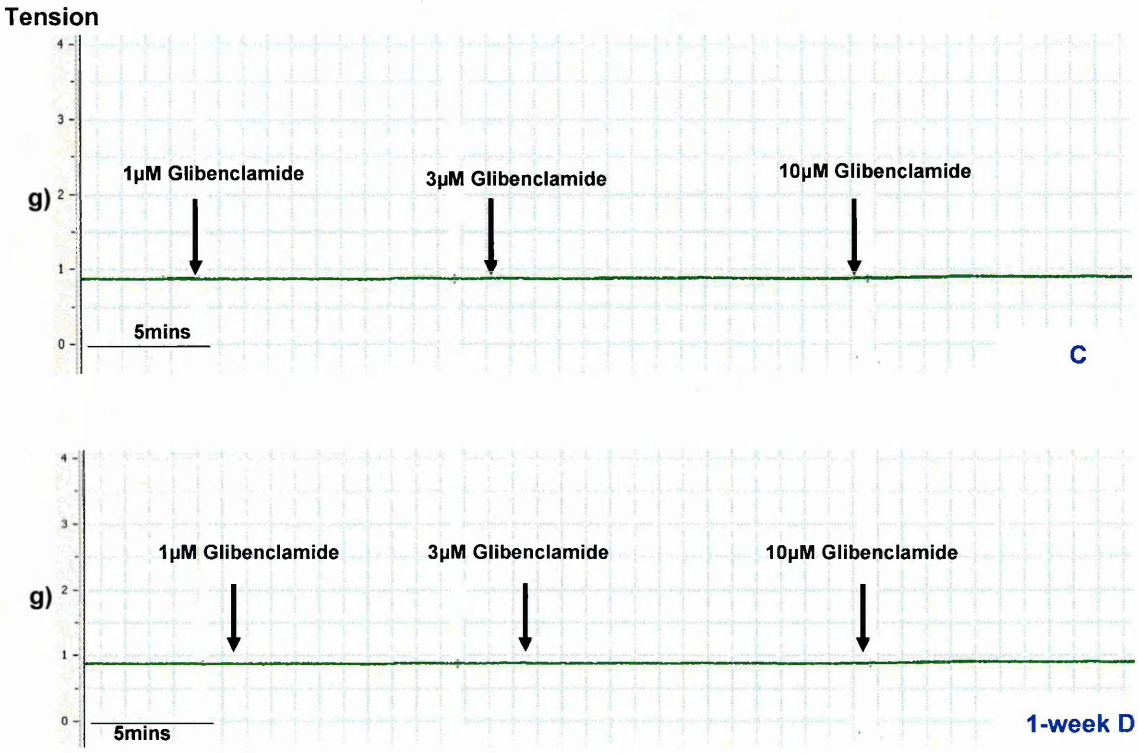


Figure 53) Typical chart recordings of the basal SA in the presence of increasing concentrations of glibenclamide in control (non-diabetic) (C) and 1-week diabetic (D) tissues.

Glibenclamide failed to induce basal SA in detrusor strips from control (non-diabetic) and diabetic rats.

5.4.5 Effects of the SK_{Ca} channel blocker apamin on basal SA in control (non-diabetic) and diabetic tissues

The effect of the SK_{Ca} channel blocker apamin on basal SA was investigated in control (non-diabetic) and 1-week diabetic rat bladder tissues. Typical chart recordings of the effect of apamin on basal SA in control (non-diabetic) and diabetic rat bladder detrusor strips are demonstrated in Figure 54.

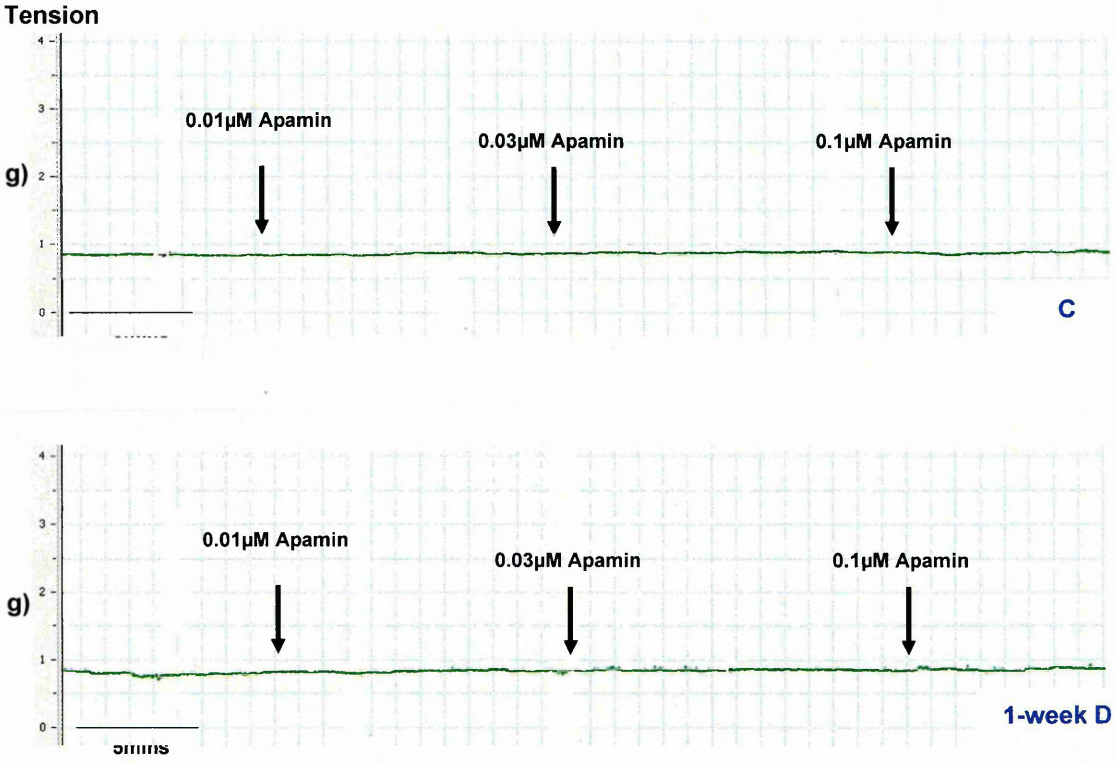


Figure 54) Typical chart recordings of the effect of increasing concentrations of apamin on basal SA in 1-week control (non-diabetic) and diabetic tissues.

Apamin failed to induce SA in detrusor strips from controls (non-diabetic) and diabetic rats.

5.4.6 Investigation of K^+ channel subunit expression in diabetic rat bladder using real-time PCR

5.4.6.1 Relative expression ratios of BK_{Ca} channel subunits in diabetic versus control (non-diabetic) rat bladders

Amplification with house-keeping (HPRT) and target gene primers (Slo1 and Sloβ1) was carried out in each control (non-diabetic) and diabetic sample. In order to calculate the relative expression ratio of BK_{Ca} channel subunits, Slo1 and Sloβ1, in diabetic versus control (non-diabetic) rat bladders, the efficiencies of each primer set (Sloβ1, Slo1 and HPRT) for each sample were determined. The amplification curves for serial dilutions of each bladder sample, control (non-diabetic) and diabetic for each set of primers (Sloβ1, Slo1 and HPRT) were analysed and the Ct values for each dilution were recorded.

Figure 55 shows an example of a real-time PCR amplification curve for serial dilutions of a control (non-diabetic) bladder sample amplified with Slo1 primers. The melt curve analysis demonstrates a clear sharp peak which is an indication of a specific amplified product. The Ct value of each dilution versus the concentration was graphed by iCycler software (Figure 55). The efficiency of the primer set was then calculated by the software according to the equation described in chapter 3 (section 3.3.4.4) (Figure 55). Figure 56 also demonstrates the amplification and melt curve analysis plus the efficiency plot for Sloβ1 primers with cDNA samples obtained from control (non-diabetic) rat bladder. The same analysis was performed for samples from diabetic rat bladder tissues.

There was no significant difference in the efficiency values between any primer pair for any sample group. Therefore, the mean efficiency values for each primer set (Slo1, Sloβ1 and HPRT) were calculated and used for determining the expression ratios using the method described by Pfaffl (2001). For each sample group, control (non-diabetic), 1-week, 4-week, 8-week and 12-week diabetic, mean Ct values for the 1:10 dilution of each sample for each primer pair were calculated and subsequently used in the equation described by Pfaffl (2001) to calculate the expression ratios.

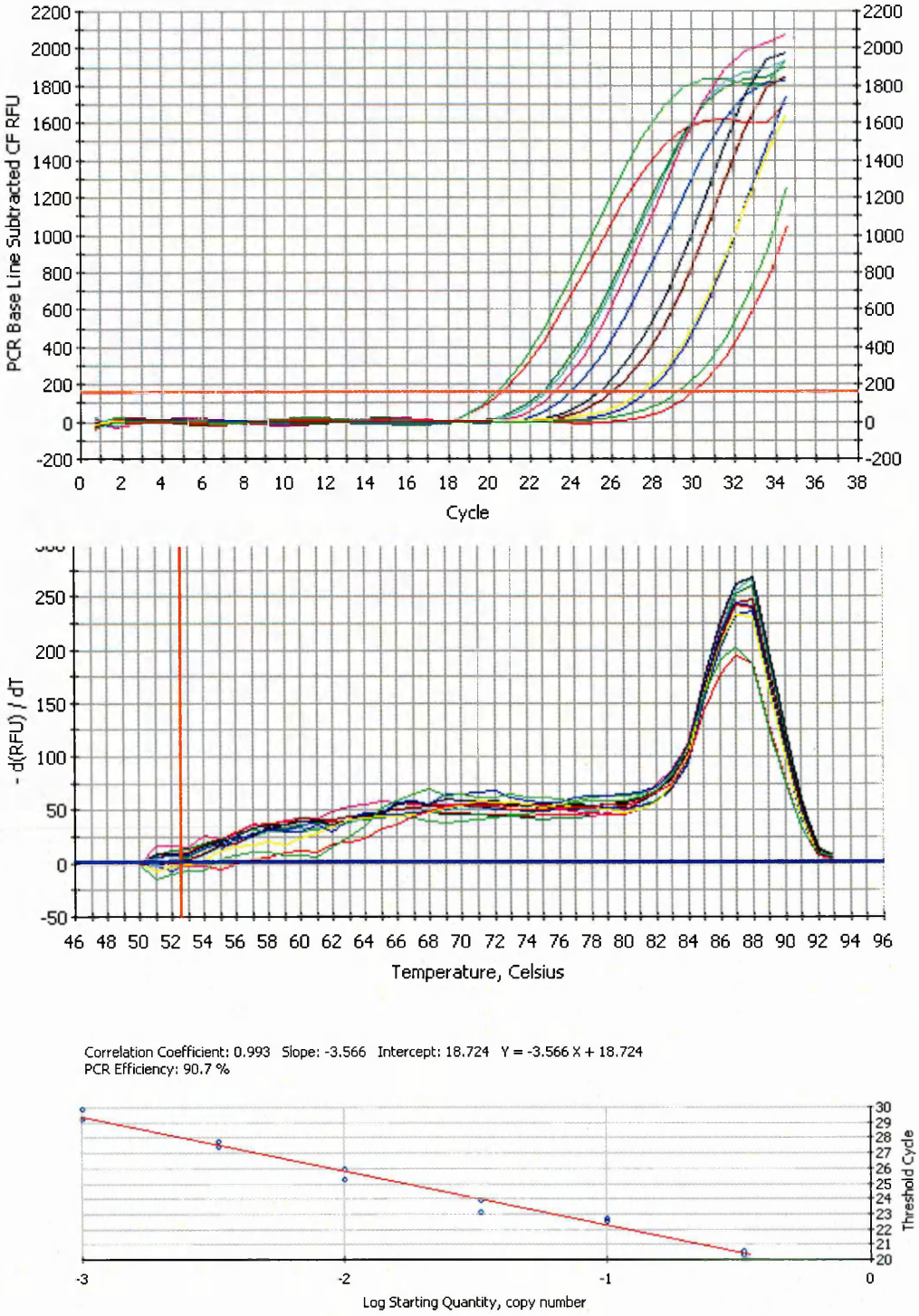


Figure 55) Real-time PCR amplification of Slo1 primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1:1000), from left to right: Red & light green:1:3, light blue and dark green: 1:10, pink and dark blue: 1:30, purple and brown:1:100, yellow and dark purple: 1:300, red and green:1:1000 dilutions respectively. ii) Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced. iii) Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

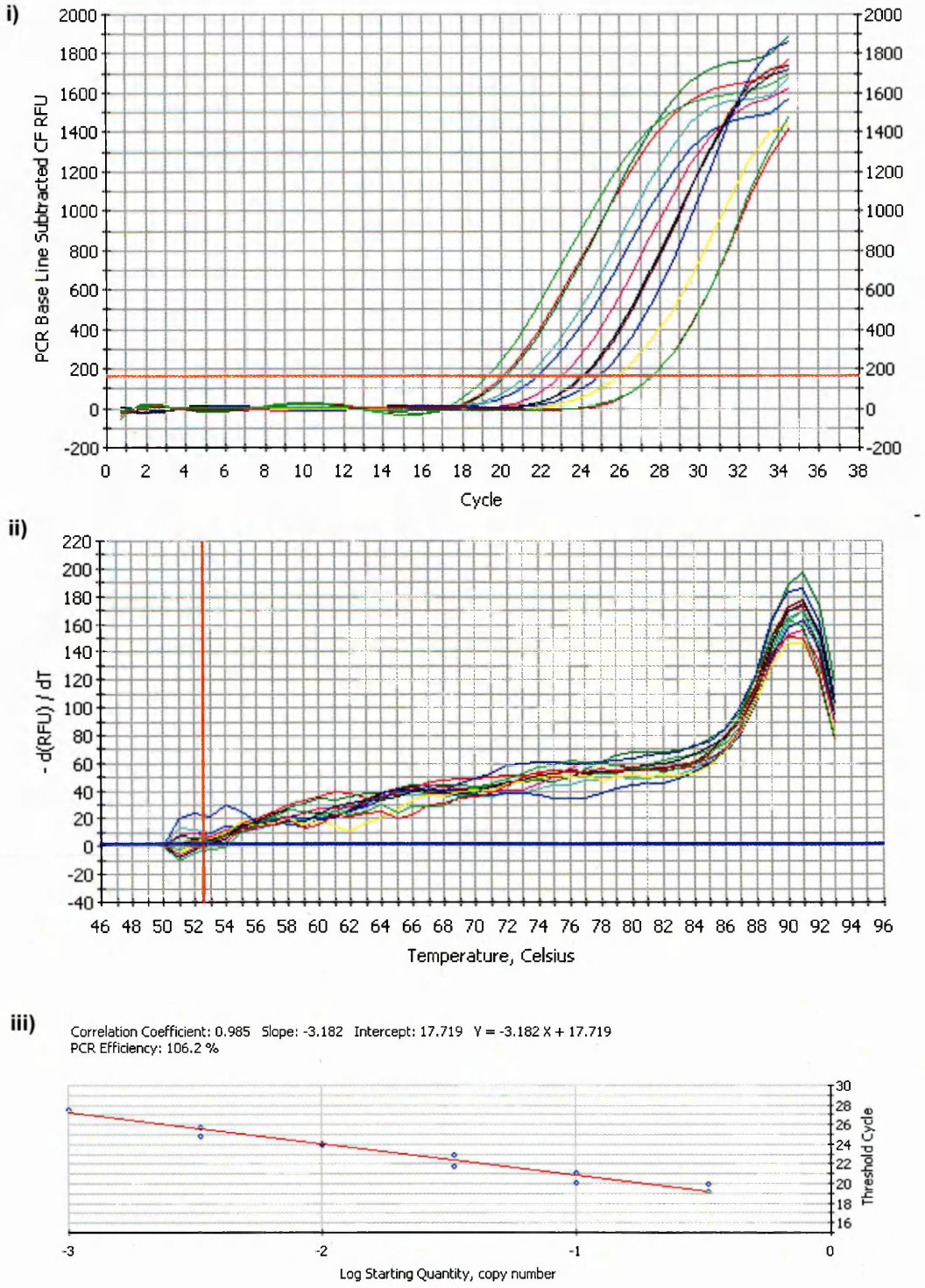


Figure 56) Real-time PCR amplification of Sloβ1 primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1:1000), from left to right Red & light green:1:3, light blue and dark green: 1:10 , pink and dark blue: 1:30, purple and brown:1:100,yellow and dark purple: 1:300, red and green:1:1000 dilutions respectively. ii) Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced. iii) Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

The relative expression ratios of BK_{Ca} channel subunits, Slo1 and Sloβ1, for 1-week (n=3), 4-week (n=4), 8-week (n=5) and 12-week (n=6) diabetic rat bladders compared to control (non-diabetic) tissues (n=5) (n=number of bladders) are shown in Table 14.

Relative expression ratios compared to the control (non-diabetic) bladders	1-week diabetics	4-week diabetics	8-week diabetics	12-week diabetics
Slo1	**0.60**	**0.25**	**0.15**	**0.17**
Sloβ1	**0.92**	**0.73**	**0.29**	**0.32**

Table 14) Relative expression ratios of Sloβ1 and Slo1 muscarinic receptors in diabetic bladders compared to control (non-diabetic) bladders. Values <1 show a decrease in expression and values >1 demonstrate an increase in expression.

The ratios demonstrated a decrease in the expression of BK_{Ca} channel subunits in all diabetic groups relative to control (non-diabetic) tissues, with bladders from 8 and 12-week animals demonstrating the greatest reduction in the expression of both subunits. The decrease in the Slo1 gene expression was more profound than the Sloβ1 gene in all groups.

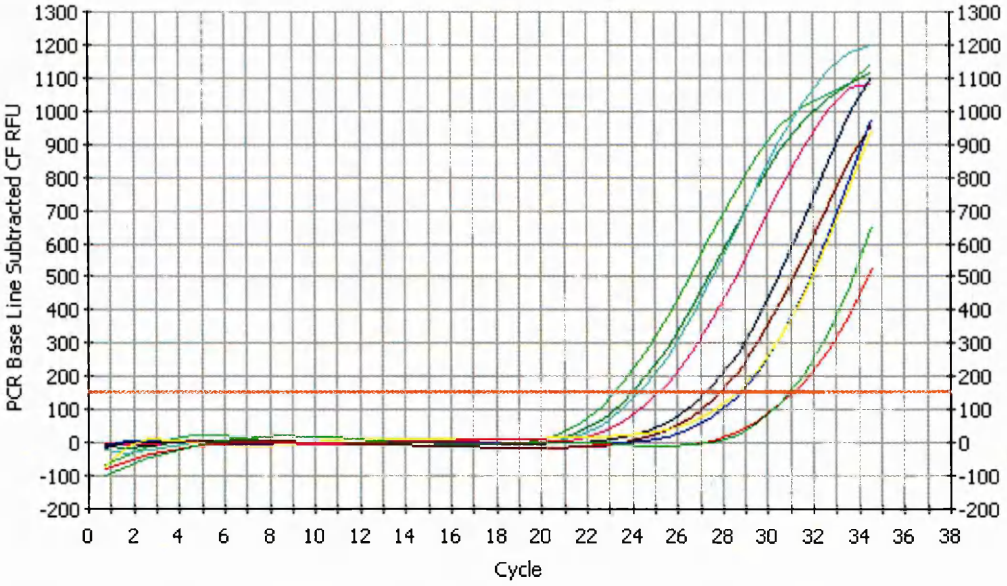
5.4.6.2 Relative expression ratios of the K_{ATP} channel subunits in diabetic rat bladders versus control (non-diabetic) rat bladders

Amplification with house keeping (HPRT) and target gene (Kir6.1 & SUR2B) primers was carried out in each control (non-diabetic) and diabetic bladder sample. In order to calculate the relative expression ratio of K_{ATP} channel subunits Kir6.1 and SUR2B in 1-week diabetic versus control (non-diabetic) rat bladders, the efficiencies of each primer set (Kir6.1, SUR2B & HPRT) for each sample were determined. The amplification curves for serial dilutions of each control (non-diabetic) and diabetic sample for each set of primers were analysed and the Ct values for each dilution were recorded.

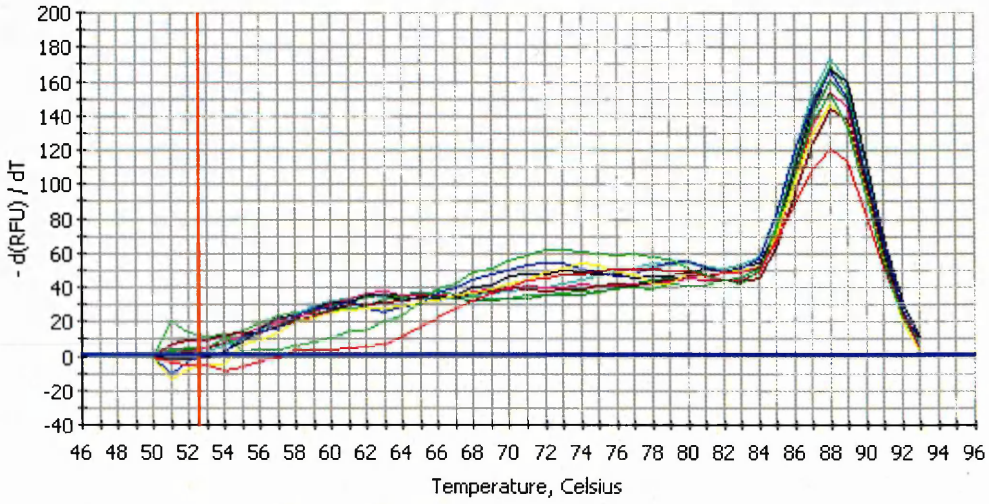
Figure 57 demonstrates an example of a real-time PCR amplification curve for serial dilutions of a control (non-diabetic) sample amplified with Kir6.1 primers. The melt curve analysis demonstrates a clear sharp peak which is an indication of a specific amplified product. The Ct value of each dilution versus the concentration was plotted and graphed by iCycler software (Figure 57). The efficiency of the primer set was then calculated by the software according to the equation described in chapter 3 (section 3.3.2.2) (Figure 57). Figure 58 also demonstrates an example of an amplification and melt curve analysis plus the efficiency plot for SUR2B primers with cDNA sample obtained from a control (non-diabetic) rat bladder. The same analysis was also performed for samples from diabetic rat bladder tissues.

There was no significant difference in the efficiency values between any primer pair for any sample group. Therefore, the mean efficiency values for each primer set (Kir6.1, SUR2B & HPRT) were calculated and used for determining the expression ratios using the method described by Pfaffl (2001). For each sample group (control (non-diabetic) and 1-week diabetic) mean Ct values for the 1:10 dilution of each sample for each primer pair were calculated and subsequently used in the equation described by Pfaffl (2001) to obtain expression ratios.

i)



ii)



iii)

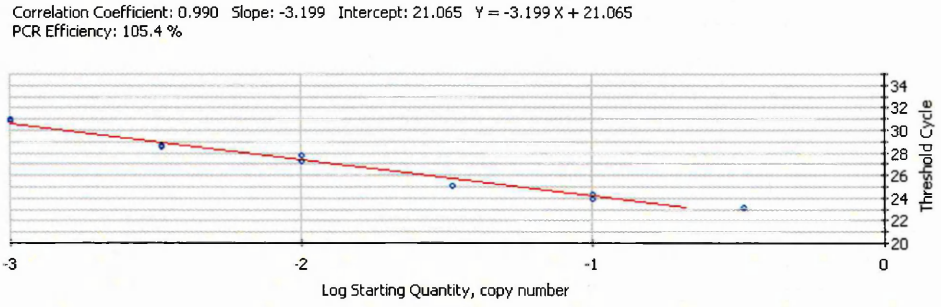


Figure 57) Real-time PCR amplification of Kir6.1 primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1:1000), from left to right: Red & light green:1:3 (duplicate), light blue and dark green: 1:10, pink and dark blue: 1:30, purple and brown:1:100,yellow and dark purple: 1:300, red and green:1:1000 dilutions respectively. ii) melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced. iii): Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

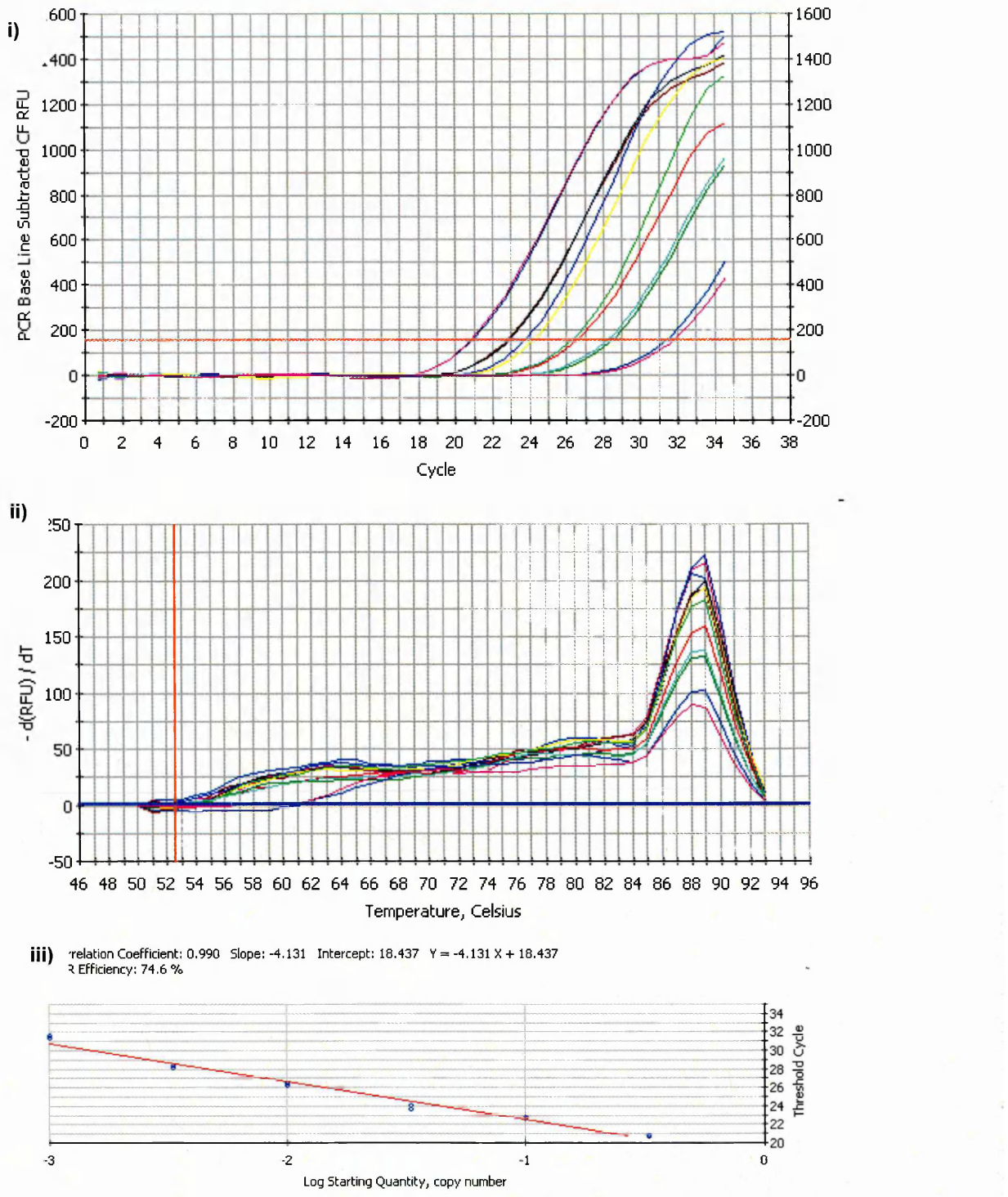


Figure 58) Real-time PCR amplification of SUR2B primers with serial dilutions of cDNA prepared from control (non diabetic) rat bladder tissue. i) Amplification curve for serial dilutions (1:3-1:1000), from left to right: light blue & dark green: 1:3, pink & blue: 1:10, dark blue & brown : 1:30 low & purple:1:100, red and light green:1:300, light blue & dark green :1:1000 dilutions. Middle panel: Melt curve analysis demonstrates a clear sharp peak for all dilutions indicating the specifity of the PCR products produced. Lower panel: Efficiency graph generated by iCycler software by plotting the Ct values of each dilution against their concentration. The PCR efficiency is demonstrated as a percentage above the graph.

The relative expression ratio of K_{ATP} channel subunits Kir6.1 and SUR2B were calculated in 1-week (n=3) diabetic compared to control (non-diabetic) tissues (n=5) (n=number of bladders) using the Pfaffl method as described in chapter 2 (Pfaffl, 2001). HPRT was used as the reference gene in the Pfaffl equation.

Table 15 demonstrates the relative expression ratios of Kir6.1 and SUR2B genes in 1-week diabetic rat bladders compared to control (non-diabetic) tissues

.

	Relative expression ratios compared to the control (non-diabetic) group
Kir6.1	**0.65**
SUR2B	**0.45**

Table 15) Relative expression ratios of Kir6.1 and SUR2B in the 1-week diabetic rat bladders compared to control (non-diabetic) tissues. Values <1 show a decrease in expression and values >1 demonstrate an increase in expression.

The ratios demonstrated a decrease in the expression of K_{ATP} channel subunits in 1-week diabetic group relative to control (non-diabetic) tissues.

5.5 Discussion

DO is associated with increased spontaneous bladder contractions, which often result in symptoms of urgency and incontinence and as described previously, the cause of DO may lie within the UBSM (Brading, 1997). Current treatments for DO have unwanted side effects, and thus a greater understanding of the underlying mechanisms involved in regulating UBSM contractility and excitability is crucial for developing new therapeutic agents. In recent years much effort has been focused on increasing our understanding of the function of K^+ channels, such as BK_{Ca}, K_{ATP} and SK_{Ca} channels, in regulating UBSM excitability and contractility. It is believed that activation of these channels may prove useful in the treatment of overactive detrusor by causing a hyperpolarisation the membrane potential in UBSM cells and a reduction in the opening of VDCCs which leads to reduced Ca^{2+} entry and a subsequent relaxation of the detrusor muscle.

There are very few studies investigating the role of K^+ channels in mediating the SA of the detrusor from the STZ-diabetic rat model. Here, a combination of molecular and pharmacological approaches were used to characterise the role of BK_{Ca}, SK_{Ca} and K_{ATP} channels in modulating UBSM SA in control (non-diabetic) and STZ-diabetic rat bladder tissues.

5.5.1 The role of BK_{Ca} channels in modulation of CCH-stimulated SA in control (non-diabetic) and diabetic tissues

Understanding the role of BK_{Ca} channels in the modulation of cholinergic stimulated bladder SA was one of the questions explored in this chapter, since it is was hypothesised that abnormal enhancement of smooth muscle excitability could be due to alterations in BK_{Ca} channel activity.

5.5.1.1 The effect of BK_{Ca} channel opener NS1619 on CCH-stimulated SA in control (non-diabetic) and diabetic rat bladder

Effect of NS1619 on the amplitude and the frequency of CCH-stimulated SA

The effect of NS1619 in modulation of bladder SA was first demonstrated by Sheldon *et al* (1997) in normal guinea-pig urinary bladder. It was shown that NS1619 can inhibit detrusor spontaneous contractions in a concentration

dependent manner. Imai *et al* (2001) also demonstrated a significant reduction in the amplitude and the frequency of basal SA in guinea-pig detrusor strips after exposure to 30μM NS1619. The effect of NS1619 on inhibition of CCH-induced SA in neonatal rat bladders was investigated by Ng *et al* (2006). They demonstrated a significant reduction in both the amplitude and the frequency of CCH-induced SA in whole urinary bladders of neonatal rats at 30μM NS1619. In the present study, the role of NS1619, in modulation of CCH-induced SA was examined in control (non-diabetic) and diabetic tissues. The results demonstrated that NS1619 had a significant effect on the amplitude of SA only at the highest concentration (30μM) tested, with no effect on the frequency of SA in all groups at any of the concentrations tested. There was no significant difference in the functional responses of control (non-diabetic) and diabetic tissues to NS1619.

The amplitude of a spontaneous contraction depends on the increase in intracellular Ca^{2+} levels as result of Ca^{2+} entry caused by membrane depolarisation, as well as CICR via RyRs in the SR (Herrera *et al*, 2000). Since NS1619 only decreased only the amplitude of SA in detrusor strips from control (non-diabetic) and diabetic rats in the current study, this may demonstrate that this drug is interfering with Ca^{2+} entry and release mechanisms in the UBSM. This hypothesis is supported by the study of Sheldon *et al* (1997), who investigated the effect of NS1619 on detrusor contractility of guinea-pig urinary bladder, and demonstrated that NS1619 acts by increasing the open probability of BK_{Ca} channels. Opening of the BK_{Ca} channels results in a decrease in the inward Ca^{2+} current and a subsequent decrease in the amplitude of UBSM SA. Thus, it was concluded that NS1619 effectively inhibits guinea-pig detrusor smooth muscle by involving both activation of BK_{Ca} channels and inhibition of Ca^{2+} channels (Sheldon *et al*, 1997).

The frequency of phasic contractions should reflect mechanisms that result in action potential firing. Since the initiation of action potentials depend on Ca^{2+} entry, accumulated inactivation of VDCC by membrane hyperpolarisation will lead to a cessation of action potentials and subsequently determine the onset and length of the interval between contractions (Herrera *et al*, 2000). The underlying reason for the lack of effect of NS1619 in inhibiting the frequency of SA in detrusor strips from control (non-diabetic) and diabetic rats in the current

study is not clear, since the opening of the BK_{Ca} channels is important in restoring the membrane potential and mediating the action potential frequency. Further experiments using electrophysiology to investigate the electrical properties of the bladder including single smooth muscle cells could enhance our understanding of the effect of NS1619 on the frequency of action potential firing. Also it must be noted that modulation of channels in *in vitro* functional experiments may not necessary translate to a measurable physiological response.

The limited inhibitory effect of NS1619 on CCH-stimulated SA

Although various studies have demonstrated that low micromolar concentrations of BK_{Ca} channel openers can cause a significant stimulation of these channels in isolated UBSM cells (Siemer *et al*, 2000; Sheldon *et al*, 1997), the present study demonstrated a relative lack of functional response in both control (non-diabetic) and diabetic tissues to NS1619. The reason for this is unknown. The lack of sensitivity could be due to diffusion barriers in detrusor strip preparations, which may affect the concentrations reaching the drug's postulated site of action. This hypothesis was proposed by Mora & Suarez-Kurtez (2005) after demonstrating that NS1608 had no effect on the myogenic contractile activity of detrusor strips from guinea-pig urinary bladder.

Another possibility may be that the membrane depolarisation and the subsequent elevation of intracellular Ca^{2+} concentration during the action potential in UBSM cells was sufficient to cause activation of BK_{Ca} currents. Thus, additional stimulation induced by NS1619 may be insufficient to affect the excitation-coupling process in functional experiments when other depolarising K^+ currents are operative.

Counter to the above possibility, another possible reason for insensitivity of diabetic and control (non-diabetic) tissues to NS1619 could be muscarinic inhibition of BK_{Ca} channels in the UBSM cells. Previous studies (Kume & Koltikoff, 1991; Nakamura *et al*, 2002) have demonstrated that Ca^{2+}-activated K^+ (K_{Ca}) currents are inhibited by muscarinic agonists in bladder smooth muscle. It is believed that muscarinic stimulation of the smooth muscle cells results in a marked rise in intracellular Ca^{2+} and a graded depolarisation, which is enhanced by K_{Ca} channel inhibition and lowering K^+ conductance (Kume &

Koltikoff, 1991). Nakamura *et al* (2002) also demonstrated a muscarinic receptor-mediated inhibition of BK_{Ca} channels in rat bladder smooth muscle following cholinergic stimulation. Nakamura *et al* (2002) believed that this inhibition was mediated via the M2 muscarinic receptor signalling pathway. In the experiments conducted in this chapter, control (non-diabetic) and diabetic tissues were stimulated with low concentrations of CCH to enhance spontaneous contractions. Thus, it could be hypothesised that CCH-induced muscarinic receptor activation may also inhibit BK_{Ca} channels in the UBSM of rat and thus render the tissue relatively insensitive to the actions of BK_{Ca} channel openers.

The relative lack of inhibition of SA in control (non-diabetic) and diabetic tissues by NS1619 does not necessarily indicate that the same underlying mechanisms are acting in the UBSM cells of both groups of animals. As seen in Table 14, the expression of the BK_{Ca} channel subunits is decreased in diabetic bladder. A similar finding was shown by Li *et al* (2008), who demonstrated a reduced BK_{Ca} channel expression in the bladders of partially outlet obstructed rats. Studies have demonstrated the importance of the presence of both of the BK_{Ca} channel subunits in the smooth muscle for regulation of contractility. In a study by Petkov *et al* (2001b), which used gene knockout mice lacking the β1-subunit of the BK_{Ca} channel, a reduced activity of these channels was observed. Petkov *et al* (2001b) also reported that detrusor strips from these mice had elevated SA, with high amplitude, low frequency contractions. In UBSM cells lacking the β-subunit, BK_{Ca} channels also had a much lower open probability. In the current chapter, a decrease in expression of the Slοβ-1 gene, which encodes the β-subunit, along with a decrease in Slo-1 gene, which encodes the pore-forming α-subunit of BK_{Ca} channels, was detected in the diabetic bladders. This decrease in the mRNA expression of BK_{Ca} channel α- and β-subunits in the diabetic bladders, if reflected at the functional protein level, could potentially explain the reduced activity and the insensitivity of these tissues to openers such as NS1619 respectively. In other words, there are simply not enough channels to have a significant effect on the UBSM's membrane potential and detrusor contractility. However, since the same pattern of insensitivity to NS1619 was also detected in detrusor strips from control (non-diabetic) rats, the decreased expression levels of BK_{Ca} channels' subunits in the diabetic bladder

can not serve as a solid argument for the lack of sensitivity of the diabetic tissues to NS1619.

5.5.1.2 The effect of BK_{Ca} channel blocker IBTX on basal SA in control (non-diabetic) and diabetic rat bladder

The effect of inhibition of BK_{Ca} channels on the spontaneous contractions of normal bladders in various species has been studied extensively. BK_{Ca} channel blockade in UBSM results is increased SA in these tissues (Imai *et al*, 2001; Buckner *et al*, 2002; Darblade *et al*, 2006). In terms of the effect of IBTX on bladders from diabetic animals, only one study by Nakahara *et al* (2004) has looked at the effect of BK_{Ca} channel modulation by IBTX in bladders from 8-10 week diabetic rats, it was demonstrated that diabetic tissues react much more strongly after exposure to IBTX than control (non-diabetic) tissues. In the present chapter, the effect of the BK_{Ca} channel blocker IBTX on basal SA of detrusor strips from control (non-diabetic) and diabetic rats was also investigated. IBTX, at the highest concentration (0.1μM) produced changes in SA similar to those induced by CCH including a rise in amplitude and frequency of basal spontaneous contractions in control (non-diabetic) and all diabetic tissues except in detrusor strips from 12-week diabetic rats, which demonstrated a relative lack of functional response to IBTX. The SA induced by 0.1μM IBTX had greater amplitude in 1-week diabetic tissues compared to their aged matched control (non-diabetic) tissues, but this was not significant. The frequency of the SA was not different between control (non-diabetic) and 1-week diabetic tissues in the presence of 0.1μM IBTX. The lack of significance between control (non-diabetic) and diabetic animals may be due to the limited number of tissues used in these experiments and further experiments may highlight a significant difference

Clear differences were detected in responses of detrusor strips from 1-week and 12-week diabetic rats to IBTX. 1-week diabetic tissues demonstrated significantly higher amplitude of SA upon stimulation with 0.1μM IBTX compared to 12-week diabetic tissues. Thus, differences between functional responses of 1-week and 12-week diabetic tissues are consistent with the results obtained from previous chapters, demonstrating an increased and decreased contractility in 1-week and 12-week diabetic tissues respectively.

These differences could be due to different underlying mechanisms controlling the membrane potential of UBSM cells or altered levels of baseline UBSM activity and excitability in these rats. Altered expression of BK_{Ca} channels may be another possible reason for the observed differences between responses of 1-week and 12-week diabetic tissues. For example, in a study by Ng *et al* (2007), who investigated the effect of IBTX on spontaneous contractions of UBSM from neonatal and postnatal rats, it was demonstrated that detrusor strips from neonatal rats (used as a model of DO) demonstrate high amplitude, low frequency spontaneous contractions upon exposure to IBTX, whilst detrusor strips from postnatal rats demonstrate low amplitude high frequency SA. This pattern of activity correlated with the expression levels of BK_{Ca} channel α-subunit in these animals. i.e. the expression levels of BK_{Ca} channels α-subunit increased during postnatal maturation of the bladders. Thus, it was concluded that changes in expression levels of BK_{Ca} channels in bladder smooth muscle might be involved in the downregulation of SA during postnatal maturation (Ng *et al*, 2007). The authors suggested that the lack of feedback pathway of BK_{Ca} channels in UBSM cells in neonatal rat bladders, as seen in studies using BK_{Ca} knock out mice (Meredith *et al*, 2004; Thorneloe *et al*, 2005), excitability of UBSM remains unchecked, leading to detrusor muscle instability which is a primary cause of DO. In the current chapter, a decreased expression of BK_{Ca} channel subunits mRNA was also detected in bladders form diabetic rats. However, in contrast to the study by Ng *et al* (2007), the expression levels of BK_{Ca} channel subunits in the diabetic bladders decreased with duration of diabetes, which does not correlate with the functional data obtained for these tissues i.e. if a decrease in the expression of BK_{Ca} channel subunits is associated with an increase in the SA, then 12-week diabetic tissues should have demonstrated the highest amplitude and frequency of IBTX-induced SA compared to all other groups, whilst they were insensitive to modulation with IBTX. One possible explanation for these contradictory results could be that in 12-week diabetic tissues, the expression levels of BK_{Ca} channels have declined to such low levels that blockade of these channels does not have any further effect on the membrane potential and other compensatory mechanisms or channels may be playing a role in the modulation of UBSM membrane potential. In addition, it has been shown that residues on the extracellular loop of the β1 subunit of BK_{Ca} channels are very important in creating high affinity IBTX

binding sites (reviewed by Ghatta *et al*, 2006). Thus, the observed reduction in β1 subunit expression may affect IBTX binding to BK_{Ca} channels and result in insensitivity of 12-week diabetic tissues to IBTX In 1-week, 4-week and 8-week diabetic tissues, the reduction in BK_{Ca} β1 subunit expression, may not have such a dramatic effect on IBTX binding affinity. Also modulating the channel's activity in *in vitro* tissue bath experiments may not necessarily have a measurable functional response. In order to make definitive conclusions, further investigations on the expression of these channels at the protein level, as well as studying the channel's activity at single cell level are needed.

The role of other cell types in mediating detrusor contractions upon modulation of BK_{Ca} channels must also be considered, since ICs found in the bladder are believed to demonstrate calcium activated and voltage dependent currents (McCloskey, 2005). Therefore, further investigations on the role of these cells in mediating spontaneous contractions and their density in bladder tissues at the different stages of diabetes, may provide a better understanding of the underlying mechanisms that lead to the current results.

5.5.2 The role of K_{ATP} channel in modulation of CCH-stimulated SA in control (non-diabetic) and diabetic rat bladder

The effect of K_{ATP} channel modulation by cromakalim on the SA of detrusor muscle has been investigated previously in the guinea-pig bladder (Foster *et al*, 1989; Imai *et al*, 2001). In these studies, cromakalim had a significant inhibitory effect on both the amplitude and the frequency of SA. The results of other experiments demonstrated that activation of <1% of K_{ATP} channels was enough to significantly inhibit the action potentials and the related SA (Petkov *et al*, 2001) Therefore, it was hypothesised that UBSM may be very sensitive to K_{ATP} channel openers, since small changes in K_{ATP}-channel activity are likely to move the resting membrane potential away from the threshold of action potential activation (Petkov *et al*, 2001). In the present chapter, the role of K_{ATP} channel opener cromakalim was investigated in detrusor strips from control (non-diabetic) and diabetic rats. In comparison to the effect of BK_{Ca} channel opening, which only had an effect on the amplitude of SA, activation of K_{ATP} channels had a significant effect only on the frequency of SA. This suggests

that opening of these channels may have a profound effect on action potential activation and firing in UBSM of the rat.

K_{ATP} channel openers such as cromakalim, YM934, ZD6169, A-278637 and WAY-133537 have been shown to reduce the amplitude and the frequency of spontaneous contractions evoked by acetylcholine (Martin *et al*, 1997; Wojdan *et al*, 1999; Gopalakrishnan *et al*, 2002). However, it has also been shown that muscarinic receptor activation could suppress 40-70% of K_{ATP} currents in UBSM (Bonev and Nelson, 1993b), oesophageal muscularis mucosae (Hatakeyama *et al*, 1995) and trachea (Nuttle and Farley, 1997) through protein kinase C-mediated pathways. In the present study, the reason for the lack of effect of cromakalim on the amplitude of muscarinic stimulated SA in control (non-diabetic) and diabetic rat bladder tissues is not clear. One possible explanation again could be the untranslated electrical activity of channels to functional responses in rat urinary bladder.

SA in diabetic tissues was less responsive to the activation of K_{ATP} channels by cromakalim compared to controls (non-diabetic). The reason for this is not clear. In a similar study by Jarajapu *et al* (2006) it was demonstrated that the responses of bladder tissues from 2-months diabetic rats to pinacidil were also significantly less than in control (non-diabetic) tissues. In the present study, Kir6.1 and SUR2B expression levels were decreased in diabetic rat bladders, which could explain the reduced functional effect to cromakalim. It maybe that there are not enough K_{ATP} channels expressed in the diabetic tissues to demonstrate a functional response to cromakalim. Also, the general belief, from reconstitution studies on K_{ATP} channels, is that their K^+ selectivity, inward rectification and unitary conductance are determined by Kir6.x subunits, whilst their pharmacology and sensitivity to drugs and nucleotides depends on their SUR subunits (Babenko *et al*, 1998). Thus, the reduction in expression of the SUR2B subunit could explain the reduced sensitivity of the diabetic tissues to cromakalim. However, changes in the expression levels of K_{ATP} channels subunits at the mRNA level do not necessarily correlate with the number of functional channels at the protein level, thus any conclusion based on the expression levels of these channels at the molecular level must be interpreted with care.

The K_{ATP} channel blocker glibenclamide failed to have a significant effect in inducing basal SA in both control (non-diabetic) and diabetic rat bladder tissues in this study. The same has been observed in human (Darblade *et al*, 2006), pig (Buckner *et al*, 2002) and guinea-pig (Imai *et al*, 2001) UBSM. The lack of functional responses to glibenclamide in control (non-diabetic) and diabetic bladder tissues may indicate that in rat UBSM cells that K_{ATP} channels are closed and thus do not participate in the regulation of phasic contractions under basal conditions.

5.5.3 The role of SK_{Ca} channel in modulation of SA in control (non-diabetic) and diabetic rat bladder

The role of SK_{Ca} channels in modulating the SA has been investigated by various studies in mouse and rat urinary bladder (Herrera et al, 2003; Hougard et al, 2009). These studies have demonstrated that the modulation of SK_{Ca} is important in mediating SA of the bladder under pathological conditions leading to DO in these species.

In the current study, detrusor strips from control (non-diabetic) and diabetic rats were insensitive to modulation by apamin. This suggests that SK_{Ca} channels may not be important in mediating UBSM membrane potential or spontaneous bladder contractions in this species under the current experimental setup.

5.6 Summary

The effect of various K^+ channel modulators on basal and CCH-stimulated spontaneous contractions in control (non-diabetic) and diabetic rat bladders was investigated. Overall, it was demonstrated that only at 30μM could NS1619 reduce the amplitude of SA in both control (non-diabetic) and diabetic tissues with no effect on the frequency of the SA. However, closure of BK_{Ca} channels had a significant effect on both the amplitude and the frequency of SA in control (non-diabetic) and diabetic tissues (with the exception of 12-week diabetic tissues). IBTX had a greater effect in increasing the amplitude of the SA in detrusor strips from 1-week diabetic tissues compared to all other groups. A decrease in expression of BK_{Ca} channels subunits was detected in all diabetic tissues.

Opening of K_{ATP} channels by cromakalim had a significant inhibitory effect on the frequency of CCH-induced SA in both control (non-diabetic) and diabetic rat bladder tissues. Diabetic tissues seemed to be less sensitive to this modulation compared to the control (non-diabetic) tissues, suggesting a possible down regulation of K_{ATP} channel activity in the diabetic tissues which was confirmed at the mRNA level for the SUR2B and Kir6.1 subunits by real-time PCR.

SK_{Ca} channels do not appear to play a role in the modulation of SA in these tissues.

A better understanding of the K^+ channel defects in the urinary bladder smooth muscle could be achieved by electrophysiological techniques that allow the study of ion channels function at a single cell level.

Chapter 6: Identification and characterisation of c-kit positive cells in rat urinary bladder

6.1 Introduction

It is believed that the urothelial and the sub-urothelial cells of the bladder play a significant role in the generation and modulation of bladder sensation (Gillespie *et al*, 2005). Recently a specialised type of cell, similar to Interstitial cells of Cajal (ICCs) in the gut, have been found in various parts of the urinary tract of human, guinea pig, pig and mouse, where they are believed to play a role in processing sensory information, pacemaking and modulating contractile function (Klemm *et al*, 1999; Lang *et al*, 2001; Pezzone *et al*, 2003; Drake *et al*, 2006; Metzger *et al*, 2008). Various terms have been used to describe these cells, including myofibroblasts, interstitial cells (ICs), interstitial cells of Cajal-like cells and pacemaking cells. In practice, the precise distinction between these cells is unclear due to their phenotypic plasticity. However a consistent terminology is needed for these cells to clarify their physiological role in the bladder. In this chapter they will be referred to as interstitial cells (ICs).

6.1.1 Characterisation of interstitial cells (ICs) and c-kit signalling pathways

ICs were first discovered in the gut by Ramon Cajal (Cajal, 1983). He described a network of ICs, which he believed were a type of premature neuron, and suggested that these cells were influenced by various neurotransmitters and exerted a direct regulatory effect on smooth muscle cells (reviewed by Brading & McCloskey, 2005). It has been established that there are different subsets of ICs present throughout the alimentary tract, each with different regulatory roles (Faussone-Pellegrini & Thuneberg, 1999). A pacemaker function has been demonstrated in cells lying close to the myenteric plexus (IC-MY) and the colonic ICs associated with the submuscular plexus (IC-SMP). Other subsets of these cells are found intramuscularly, in circular muscle layers in the small intestine, in the deep muscular plexus and the submucous plexus and are believed to be involved in mediation and modulation of inhibitory and excitatory innervation and mechanoreception of the muscle (reviewed by Faussone-Pellegrini & Thuneberg, 1999 & Brading & McCloskey, 2005).

ICs were first detected by standard histological techniques that had been developed for identification of neurons, such as silver staining and methylene

blue. With the advent of electron microscopy it was possible to observe the morphological features of these cells in the gut, which allowed a clear distinction from smooth muscle cells and fibroblasts (reviewed by Faussone-Pellegrini & Thuneberg, 1999; Sanders & Ward, 2006). However, it is worth mentioning that there remains a group of cells which have ultrastructural characteristics of both smooth muscle cells and fibroblasts and therefore it is convenient to have a set of characteristics that defines what could be called an IC "prototype". IC prototypes in the gut have an elongated cell body and several slender processes, branching to give numerous secondary and tertiary processes. They can be distinguished from fibroblasts by their narrow rounded or slightly flattened processes which in fibroblasts are very thin and broad (reviewed by Faussone-Pellegrini & Thuneberg, 1999).

ICs have a close relation with smooth muscle cells and nerves. Contact with smooth muscle cells occurs through areas of close opposition, interdigitation, intermediate junctions or gap junctions, depending on the location and the species studied. A heavy innervation is a common feature of ICs, with close opposition with transmitter-containing varicosities. This characteristic of ICs suggests a role in neurotransmission and pacemaker activity (reviewed by Faussone-Pellegrini & Thuneberg, 1999; Sanders & Ward, 2006)

Although electron microscopy allows vigorous examination of cell to cell interactions, this method is limited in obtaining a widespread image of cellular distribution and function and is also a very labour intensive technique (Sanders & Ward, 2006). Therefore, various markers for identification of ICs in histochemical and immunochemical studies have been used such as different enzymes such as diaphorases (Xue *et al*, 1993), nitric oxide synthase (Xue *et al*, 1994), maganese superoxide dismutase (Fang & Christensen, 1995), heme oxygenase 2 (Miller *et al*, 1998), intermediate filaments such as vimentin (Torihashi *et al*, 1994), receptor proteins such as c-kit (Maeda *et al*, 1992), tachykinin receptors (Grady *et al*, 1996), as well as other antigenic proteins like cyclic GMP (Ward *et al*, 1998) and CD34 (Hirota *et al*, 1998).

6.1.2 c-kit structure and function

Labelling of the c-kit receptor as an immuno-marker for ICs is one of the most widely used methods for identification of these cells, since genuine or identifiable fibroblasts and smooth muscle cells are c-kit negative.

c-kit is a receptor tyrosine kinase which is encoded by the proto-oncogene c-kit and signalling via this receptor is essential for development of IC phenotype and electrical rhythmicity (Maeda *et al*, 1992; Torihashi *et al*, 1995). The proto-oncogene c-kit is the normal cellular homolog of the oncogene v-kit of the HZ4-feline sarcoma virus (McCulloch & Minden, 1993). c-kit maps to the long arm of human chromosome 4 and to mouse chromosome 5 and is allelic to the murine White spotting locus (W) (Lev *et al*, 1994; Viagoftis *et al*, 1997).

c-kit is a type III receptor kinase and is structurally similar to platelet-derived growth factor receptors (PDGFRs) (Viagoftis *et al*, 1997). These receptors have unique features, such as an extracellular (EC) domain made up of five immunoglobulin-like repeats and a tyrosine kinase (TK) domain that is split into two domains (TK1 and TK2) by an insert sequence of variable length (Kitamura & Hirota, 2004; Webseter *et al*, 2006). A schematic diagram of the kit receptor is demonstrated in Figure 59.

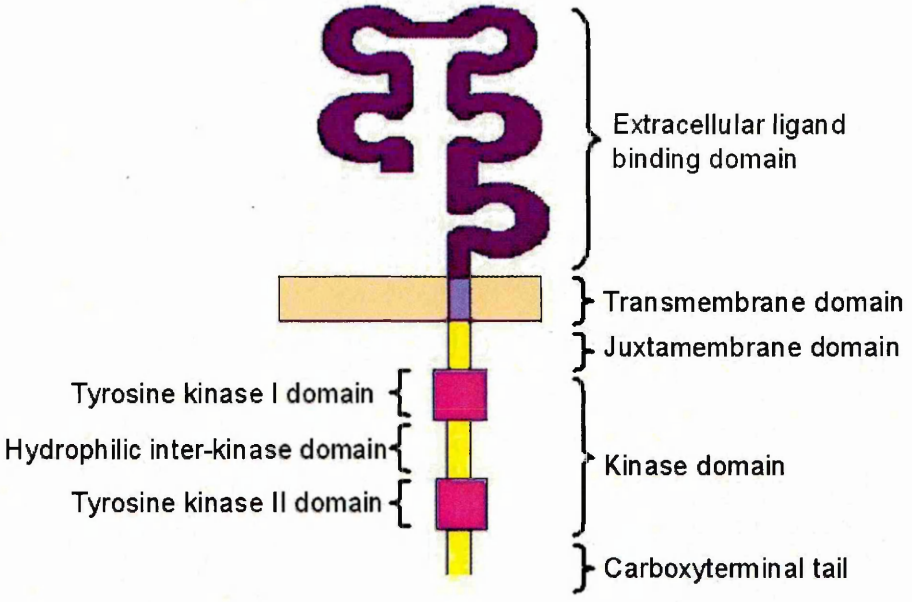


Figure 59) Schematic diagram of the receptor tyrosine kinase, c-kit. Adapted from Kitamura & Hirota, 2004; Webster *et al*, 2006.

Immunohistochemistry using c-kit labelling has improved our understanding of the structure and distribution of IC networks and has enhanced our perception of the anatomic relationship between ICs and enteric neurons, smooth muscle cells and other cells such as macrophages (reviewed by Ward & Sanders, 2001). Also identification of c-kit, lead a team of Japanese scientists to develop a neutralising antibody, ACK2, to block c-kit signalling in order to determine the consequences of reduced c-kit function (Nishikawa et al, 1991; Sanders & Ward, 2006). Administration of c-kit-neutralising antibodies and blocking the c-kit pathway greatly reduced the population of ICs in the GI tract by inducing apoptosis in these cells. This had a negative impact on neural responses and generation of electrical slow waves, which are the basic electrical rhythm of the GI tract and are essential for the normal motility patterns of the gut. This supported the idea that signalling via c-kit is essential for the development and maintenance of the IC phenotype (Torihashi et al, 1995). Animal models with mutations in the gene encoding c-kit, surgical treatment, infections and animals treated with chemicals have also been used to gain a better understanding of the function of IC cells (Ward et al, 1994; Huizinga et al, 1995; Sanders, 2006). IC networks do not develop in animals in which the function of the c-kit protein is genetically impaired i.e. by inducing loss-of-function mutations in the c-kit gene or the gene for the ligand for c-kit, stem cell factor gene (Sanders, 2006).

The signalling pathways associated with c-kit activation are numerous. Binding of the c-kit ligand, stem cell factor, to the receptor results in dimerisation and autophosphorylation on key tyrosine residues within the receptor. These phosphorylated tyrosine residues associate with substrates of various kinds (Kitamura & Hirota, 2004; Ronnstrand, 2004). The phosphatidylinositol-3-kinase (PI3K)/Akt system is one of the major pathways which is dependent on interaction with c-kit. Activation of PI3K via c-kit produces phosphatidylinositol-3,4-biphosphate, which, in turns activates Akt, a serine-theorine kinase. The activated Akt is involved in the inhibition of apoptosis of cells, whose survival depends on c-kit signalling (Kitamura & Hirota, 2004).

6.1.3 c-kit inhibition by Glivec (imatinib mesylate)

Recently, selective tyrosine kinase inhibitors have been developed which have been used for functional analysis of ICs in different tissues. Glivec (imatinib mesylate) is one of these tyrosine kinase inhibitors, and has been used in the treatment of Philadelphia chromosome positive chronic myeloid leukaemia and c-kit positive gastrointestinal stromal tumours (Kubota *et al*, 2004). The active substance of this drug is the mesylate salt of Glivec which is known to specifically inhibit the tyrosine kinase activity of c-kit and platelet-derived growth factor receptor (PDGFR) (Heinrich *et al*, 2000; Kraus & Van Etten 2005). Glivec can block autophosphorylation of c-kit, as well as Akt, which can result in induction of apotosis in c-kit signalling-dependent cells. This drug has been used extensively for studying the role of ICs in generation of spontaneous electrical activity, which is present in various smooth muscle organs such as the uterus, intestine, myometrium and bladder (Biers *et al*, 2006; Hutchings *et al*, 2006; Kubota *et al*, 2006; Popescue *et al*, 2006). In a recent study by Hashitani *et al* (2008) it was demonstrated that Glivec can significantly downregulate the spontaneous contractions of guinea-pig stomach by inhibiting the increase in intracellular Ca^{2+} in smooth muscle cells rather than by specifically inhibiting the activity of c-kit signalling. However, the effects of Glivec on isolated ICs needs to be investigated directly.

6.1.4 Electrical properties of ICs

Microelectrode recordings from ICs dissociated from the myentric plexus of the gut show regular large depolarisations (slow waves), which then depolarise neighbouring smooth muscle cells (Ward *et al*, 1994). This can lead to opening of VDCCs in smooth muscle cells, resulting in action potential generation (Ward *et al*, 1994). Ca^{2+} entering through these channels, elicits Ca^{2+} induced Ca^{2+} release (CICR) from smooth muscle sarcoplasmic reticulum (SR) via ryanodine receptors (RYRs) and subsequent activation of the contractile machinery. These contractions, which occur coincidently with the slow waves of depolarization in ICs, are ideal for gut motility and peristalic movements (Brading & McCloskey, 2005). It is believed that ICs are responsible for generation of spontaneous electrical activity, as they show spontaneous

changes in membrane potential, which may influence the SA detected in different smooth muscle organs (Koh *et al*, 1998; Sergeant *et al*, 2000).

6.1.5 ICs in the bladder

The SA of the bladder (discussed in chapter 4) is similar to peristaltic movement of the GI tract. Since the co-ordinated contractions in the GI tract are modulated by ICs, it was postulated that a similar type of cell may be present in the bladder. Many investigations have been carried out in different species in order to identify and characterise these cells using various bio-markers and methodologies.

6.1.5.1 Immunological markers for identification of ICs in the urinary bladder

ICs were first described in the guinea-pig and human bladder as target cells for nitric oxide (NO), and responded to this stimulus with an increase in immuno-reactivity for cGMP (Smet *et al*, 1996). ICs in human and guinea-pig bladders also showed immunoreactivity for vimentin (Van der AA, 2004; Davidson & McCloskey, 2005).

ICs in human bladder also express the vanilloid receptor, VR1/TRPV1, and the vanilloid receptor-like protein, VRL1/TRPV2, indicating a possible role in noxious stimuli transduction (Ost *et al*, 2002; Van der AA *et al*, 2004).

Sui *et al* (2002) also reported a network of ICs in human urinary bladders, which demonstrated immunoreactivity to connexin 43. They believed that this network is well-placed beneath the urothelium to act as an integrating network for signals to and from afferent and motor nerves respectively. However, it was found that this suburothelial cell layer stained poorly for the c-kit receptor. Sui *et al* (2002) also demonstrated that c-kit and a coincidental staining for desmin were apparent in the basal layer and even in the intermediate regions of the urothelial layer rather than in the connexion 43/vimentin positive cell layer in the suburothelial space and thus concluded that c-kit staining cannot be used as a marker to distinguish suburothelial interstitial cells (Sui *et al*, 2002).

ICs have also been identified in the guinea-pig bladder by McCloskey and Gurney (2002), using antibodies to the c-kit receptor. ICs with immunoreactivity

to c-kit, and similar morphology to that described by McCloskey and Gurney (2002), were also reported by Hashitani *et al* (2004) in guinea pig urinary bladder.

While a wide range of c-kit antibodies are available commercially, many fail to detect ICs in positive control tissue. c-kit antibodies have failed to detect ICs in murine bladders, thus immunoreactivity for cGMP after exposure to a NO donor, has been used as a marker for identification of these cells in the bladder of this species (Smet *et al* 1996; Lagou *et al*, 2006b).

6.1.5.2 Location of ICs in the urinary bladders

There have been numerous reports on the location of ICs in the bladder. Smet el al (1996) reported that ICs were more numerous in the guinea-pig bladder body than in the urethra. ICs were present in large numbers throughout the muscle bundles and within the interstitium of the bladder, but were concentrated more in the outer fibromuscular coat where the processes formed a dense interconnected network (Smet *et al*, 1996). Multiple labelling studies in guinea-pig urinary bladders using confocal microscopy revealed that ICs were located on the boundary of smooth muscle bundles, running axial with smooth muscle cells and were positioned in close proximity to intramural nerves, particularly to nNOS-containing neurons (McCloskey & Gurney, 2002). Hashitani *et al* (2004) reported three subpopulations of these cells in the guinea-pig urinary bladder. In their study, ICs were found adjacent to the boundary of muscle bundles, some ICs were found scattered among smooth muscle cells within muscle bundles and in addition to these, ICs were also found in connective tissues between smooth muscle bundles (Hashitani *et al*, 2004). Gillespie *et al* (2004, 2005) also reported cGMP positive cells in guinea-pig urinary bladder, which were present immediately beneath the urothelium and on the outer muscle layers.

In comparison with the study by Smet *et al* (1996), Van Der Aa *et al* (2004) found that in humans, ICs seem to be smaller in number in the bladder body than in the bladder neck. Co-localisation of c-kit immunostaining and connexin 43 immunostaining demonstrated that a network of ICs localised beneath the urothelium and between smooth muscle cells of the bladder (Van der AA, 2004). Sui *et al* (2002) reported a population of these cells beneath the urothelium in human bladder that formed a functional syncytium (Sui *et al*,

2002), whilst Shafik *et al* (2004) also found ICs throughout the dome of the human bladder and suggested a vesical pacemaker activity of these cells (Shafik *et al*, 2004).

In mouse urinary bladder, ICs were identified in the outer part of the detrusor muscle layer and between the muscle bundles (Lagou *et al*, 2006). No cGMP staining was seen in the suburothelium or the urothelium of the mouse bladder (Lagou *et al*, 2006). No studies have so far reported the location of these cells in rat urinary bladder.

6.1.5.3 Functional role of ICs in the urinary bladder of various species

The observation that ICs are immunopositive for connexin 43 and VRL1/TPRV2 with the previously reported immunopositivity for cGMP, and their close proximity to nitregic and peptidergic nerve fibres, suggests a functional role for these cells (Smet *et al*, 1996; Van der Aa *et al*, 2004; Lagou *et al*, 2006b). It has been suggested that ICs may function as a sensing network, receiving/sending signals from/to the urothelium, modulating afferent bladder innervation and/or activating a spinal or intramural reflex arc (Van der Aa *et al*, 2004). It has also been shown that ICs in the bladder are capable of generating Ca^{2+} transients (Hashitani *et al*, 2004), but do not have an obvious role in pacemaking. Instead these cells might modulate communication between subgroups of smooth-muscle cells in the bladder and thus might help to explain the micromotions of the bladder wall (Hashitani *et al*, 2004).

ICs in the outer muscle layer also seem to be involved in muscarinic-induced SA of mouse urinary bladder, demonstrating a further functional role of ICs in the bladder (Lagou *et al*, 2006).

6.1.6 Implications of ICs in overactive bladder

As described in chapter 1, both a myogenic and a neurogenic basis of DO have been proposed, although both factors may be implicated and are often comorbid. Recent studies have added ICs, as a new component, into the complexity of the pathophysiology of DO (Kubota *et al*, 2008).

In overactive detrusors increased electrical coupling between smooth muscle cells may explain the enhanced excitability. Therefore, spontaneous action

potentials that result in spontaneous contractions may spread further and cause synchronous contractions of multiple smooth muscle bundles which results in elevation of intravesical pressure. It has been reported that micromotions of the bladder wall are enhanced in a rat model of DO (Drake *et al*, 2003b). This abnormality of increased SA in overactive detrusor may result from changes in the properties of ICs, as well as increased coupling between smooth muscle cells. In a bladder outlet obstructed (BOO) guinea-pig model, it was demonstrated that ICs were more widely distributed in the suburothelial layers than normal animals (Kubota *et al*, 2008). Since it has been shown that these cells form a dense electrical network in the space between afferent nerves and the urothelium, it was suggested that these cells may cause altered signal transmission from the urothelium to afferent nerves which results in DO. It was also shown that suburothelial connective tissue ICs increase in BOO bladders and that initial changes in suburothelial ICs may contribute to 'neurogenic' DO. BOO progresses 'myogenic' DO may result from an increased population of muscular ICs subsequent to changes in suburothelial ICs (Kubota, 2008).

An increased number of c-kit positive cells in patients with idiopathic and neurogenic DO has also been reported (Biers *et al*, 2006). However, ICs can also decrease in number or be completely abolished in conditions such as megacystis microcolon intestinal hypoperistalis syndrome in infants, which results in distended non-functional, nonobstructed bladders (Piotrowska *et al*, 2004). These studies, again, suggest a link between ICs and contractile activity and thus, inhibitors of these cells such as Glivec could be used to inhibit undesired SA and subsequent DO.

Although there are various reports on the role of ICs in mediating detrusor SA in normal and pathological conditions, no studies have reported the presence of these cells in rat urinary bladder, and therefore there are no reports on the functional implications of ICs in mediating SA of the STZ-diabetic rat bladder.

6.2 Aim of the chapter

The aim of this chapter was to identify and characterise ICs in control (non-diabetic) and STZ-diabetic rat bladder using molecular, immunological and functional methods.

6.3 Material and Methods

6.3.1 Investigation of c-kit expression in rat bladder using RT-PCR

Total cellular RNA was extracted from control (non-diabetic) (n=5) and 1-week diabetic (n=6) rat bladders according to the protocol described in chapter 3.

RNA integrity and quantity was analysed using the Experion automated electrophoresis system as described in chapter 3.

cDNA synthesis was carried out as described in chapter 3.

6.3.1.1 Primer design

The sequence of rat -c-kit tyrosine kinase mRNA (*Rattus norvegicus* mRNA for c-kit receptor tyrosine kinase. Accession no: D12524.1) was retrieved from "NCBI" website: "http://www.ncbi.nlm.nih.gov" (Figure 60) and primers specific to this sequence were designed using primer 3 (web) software. The parameters used for primer design were:

- 15-20bp primer length
- Primer GC content of 40-60%
- Primer Tm of 57-63°C
- The primer Tm for both primers were within 2-3 °C of each other
- The amplified region between the two primers was set at 400-500 base pairs.

The programme returned a range of sequences meeting the parameters, from which the primer pair (sequence demonstrated in Table 16) was selected and subsequently synthesised (Invitrogen, UK). The synthesised primers were then reconstituted with nuclease free water at concentration of 200µM and kept at -20°C until use. The amplification region between the set of primers is demonstrated in Figure 60.

Target gene	Accession no	Primer sequence	Start base	Tm	P.S
c-kit tyrosine kinase receptor	D12524.1	F: 5′ GGCCTAGCCAGAGACATCAG 3′ R: 5′ GAGAGGCTGTGTGGAAGAGG 3′	2481 2960	59.97 59.99	480 bp

Table 16) Primer sequences for the rat c-kit gene. Tm=melting temperature. bp= base pairs; P.S=product size, F=forward R=reverse.

2481GGCCTAGCCAGAGACATCAGGAATGATTCGAATTACGTGGTAAAAGGAAATGCACG
GCTGCCCGTGAAGTGGATGGCACCGGAGAGCATTTTCAACTGCGTGTACACATTTGAAA
GTGACGTCTGGTCCTATGGGATTTTCCTCTGGGAGCTATTCTCTCTAGGAAGCAGCCCC
TACCCAGGGATGCCGGTCGATTCCAAGTTTTACAAGATGATCAAGGAAGGTTTCCGAAT
GCTCAGCCCTGAGCACGCGCCTGCCGCAATGTATGAAGTTATGAAGACTTGCTGGGATG
CTGATCCCCTGAAAAGGCCAACATTCAAGCAGGTTGTTCAGCTCATTGAGAAGCAGATC
TCAGACAGCAGCAAACATATTTACTCCAACTTAGCAAACTGTAACCCCAACCCAGAGAA
CCCCGTGGTGGTGGACCATTCTGTGAGGGTCAATTCCGTCGGCAGCAGCACCTCTTCCA
CACAGCCTCTC2960

Figure 60) Partial sequence of Rattus norvegicus mRNA for c-kit receptor tyrosine kinase. Accession no: D12524.1. The primer sequence are indicated in red. The sequence of the amplified region is highlighted in grey.

6.3.1.2 Polymerase chain reaction (PCR)

A 25µl PCR reaction consisting of the components described in Table 17 was prepared to the indicated end concentrations.

Reagent	Stock concentration	Final concentration in the reaction	Volume added
PCR mastermix (Abgene)	1.1x	1x	22.5µl
Forward primer	5µM	0.2µM	1µl
Reverse primer	5µM	0.2µM	1µl
cDNA	0.5µg/ul	0.01µg/ul	0.5µl
		Total Volume=	25 µl

Table 17) The PCR reaction components used for amplification of cDNA samples obtained from control (non-diabetic) and diabetic rat bladders with c-kit and GAPDH primers.

Each control (non-diabetic) and diabetic sample was first amplified using GAPDH primers (Table 4) to confirm the presence of amplifiable cDNA after reverse transcription (positive control). In the negative control, sterile water was added instead of the cDNA template. After confirmation of the presence of amplifiable cDNA, samples were then amplified using the designed c-kit primers (Table 16).

The PCR reaction was carried out in a GeneE thermocycler (Techno Cambridge Ltd) with the following cycle parameters:

- Initial denaturation at 95°C for 5 mins
- 15 seconds denaturation at 95°C
- 1 min annealing step at 59°C
- 1min elongation step at 72°C

} x35 cycles

- Final elongation step at 72°C for 10 min

PCR products were then analysed on a 1% (w/v) agarose gel containing 0.5ng/ml ethidium bromide. Electrophoresis was carried out at 100 V for 45min. Gel images were obtained using a UVP Bioimaging system (Upland, USA).

PCR products were identified by comparison with a DNA ladder covering the range of 100-1000bp. Specific PCR bands were excised from the gel and were then purified with QIAquick Gel extraction kit (Qiagen) according to manufacturer's protocol. The absorbance of DNA solution was determined by spectrophotometer at 260nM wavelength and the concentration was determined using the following formula:

Concentration (µg/ml) = (A_{260} reading) x 50 x dilution factor

1 absorbance unit (A_{260}) = 50µg/ml of DNA

The PCR products purified from the agarose gel were sent to GATC-Biotech (Germany) for direct sequencing to confirm the identity of amplified products. The sequenced data was then downloaded from www.GATC-biotech.com.

The sequenced data was identified by comparison with genome database using the BLAST tool from NCBI website (http://blast.ncbi.nlm.nih.gov/blast.cgi) which is a basic logical alignment tool and finds regions of similarity between biological sequences.

6.3.2 Immunohistochemistry for detection of c-kit protein expression in rat urinary bladder

6.3.2.1 Tissue Preparation

Immunohistochemistry to identify c-kit expression in the rat urinary bladder was carried out according to that described by McCloskey & Gurney (2002), with modifications.

Whole mount preparations of the bladder from control (non-diabetic) rats were prepared for immunohistochemistry. Briefly, the bladder was cut into two longitudinal sections. The top and the bottom of each longitudinal strip were removed and the mucosa was removed by dissection with curved scissors. Bladder preparations were fixed in ice cold acetone for 10mins followed by 3x5min washes in PBS (phosphate buffered saline) (Sigma, Dorset, UK). Tissues were blocked for 1-hour in PBS containing 1%(v/v) donkey serum (Sigma, Dorset, UK) before being incubated with 1:200 v/v of anti c-kit goat

polyclonal IgG primary antibody (C-19)(Santa-Cruz, Heidelberg, Germany) diluted in PBS containing 1% donkey serum for 1 hour at room temperature. For negative controls, tissues were incubated in PBS containing 1% donkey serum only. Following incubation with the primary antibody, tissues were washed 3x10mins with PBS, and were then incubated with the secondary donkey anti goat IgG fluorescent antibody (Alexa 488) (Invitrogen, Paisely, UK) diluted (1µg/ml) in PBS containing 5% donkey serum and 0.05% (v/v) Tween, for 1.5 hour at room temperature. After 3x5min washes in PBS, tissues were mounted on L-polylysine-coated slides using aqueous mounting media (70% Glycerol / 30% PBS).

6.3.2.2 Confocal image analysis

Images were captured using a Zeiss 510 confocal scanning laser microscope equipped with krypton/ argon laser as the source of the ion beam. Alexa 488 labeled antibodies were visualised by excitation at 488nm with a 506-538 band-pass emission filter. Acquiring 10-20 optical sections in the Z-plane and running the series-scanning mode from the deepest focus point to the highest focus point allowed a projected image and data set to be generated from which composite projected images were saved. Images were analysed using Zeiss LSM5 software.

6.3.3 Functional investigation of the role of ICs in mediating SA of detrusor strips from control (non-diabetic) and diabetic rats

Longitudinal mucosa-intact and denuded strips of smooth muscle (2-4 x 6-12mm) were isolated from the body of rat bladders from, 1-week, 4-week, and 12-week diabetic animals and their age-matched controls (non-diabetics), and suspended in 15ml organ baths as described in chapter 2. Following equilibration (60mins in Krebs), tissues were stimulated with 0.1μM CCH to induce SA as previously described by Ng *et al* (2006). In the continuous presence of CCH, increasing cumulative concentrations of Glivec (1-50μM) were added to the tissues and the effects of this drug on the amplitude and the frequency of SA was assessed. The tissues were incubated with each concentration of Glivec for a period of 10-15mins.

6.3.3.1 Data analysis

To calculate the amplitude and the frequency of SA, a slightly modified method to that proposed by Imai *et al* (2001) was used to define a single spontaneous contraction event, as described in section 4.3.3. SA was expressed as the mean contraction amplitude/mg tissue and frequency ± SEM during a 5mins period within a 10-15mins interval. The percentage change in the amplitude and the frequency of SA at each concentration of Glivec relative to that of SA in the absence of Glivec i.e. in the presence of 0.1μM CCH only, was calculated for both control (non-diabetic) and diabetic tissues.

6.3.3.2 Statistical analysis

Statistical analysis was performed using repeated measures *ANOVA* followed by Dunnett's post hoc test for intra-tissue variations and unpaired Student's *t*-test for inter-tissue variations.

6.4 Results

6.4.1 Investigation of c-kit expression in rat bladder using PCR

6.4.1.1 Identification of c-kit gene expression in control (non-diabetic) and diabetic rat bladders.

RT-PCR revealed the expression of c-kit in cDNA samples from both control (non-diabetic) and 1-week diabetic rat bladders, at the expected product size of 480bp (Figure 61). The expression of GAPDH was also confirmed in the cDNA samples by observing a band at ≈170bp.

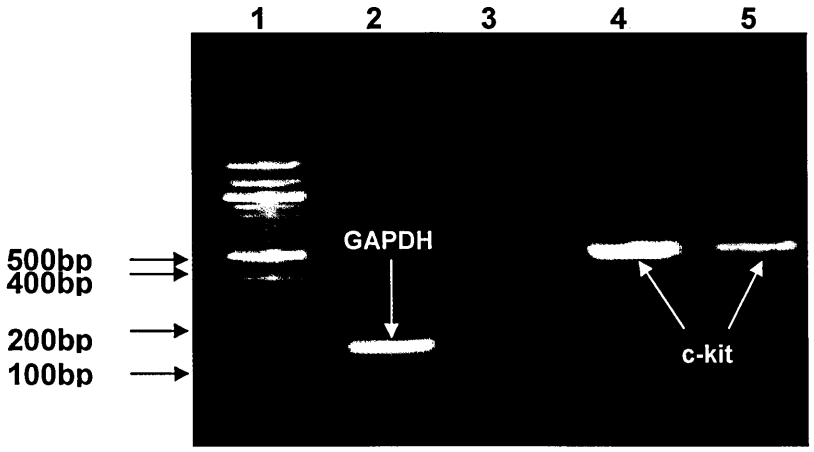


Figure 61) c-DNA samples from control (non-diabetic) and 1-week diabetic rat bladders amplified with c-kit primers. The gel shows amplified products of c-kit and GAPDH. Lane 1: 100bp ladder. Lane 2: Positive control amplified with GAPDH primers (≈171bp). Lane 3: negative control (water). Lane 4: 1-week diabetic rat bladder sample amplified with c-kit primers (≈480bp). Lane 5: Control (non-diabetic) rat bladder sample amplified with c-kit primers.

6.4.1.2 *Sequencing of the PCR products for confirmation of c-kit expression in rat urinary bladder*

PCR products from both control (non-diabetic) and 1-week diabetic rat bladder samples amplified with c-kit primers were isolated from the gel and dispatched to GATC-biotech for direct sequencing. Partial sequences (272bp for 1-week

diabetic and 113bp for control (non-diabetic) samples) obtained from the direct sequencing showed homology with *R.rattus* mRNA for c-kit receptor tyrosine kinase isoform (accession no: D12524.1) confirming the expression of c-kit tyrosine receptor kinase in rat urinary bladder (Figure 62).

C

2481 CAGGAATATCCTCCTCACTCACGGGCGGATCACAAAGATTTGCGATTTCGGCCTAGCCAGAGACATC
AGGAATGATTCGAATTACGTGGTAAAAGGAAATGCACGGCTGCCCGTGAAGTGGATGGCACCGGAGAGCA
TTTTCAACTGCGTGTACACATTTGAAAGTGACGTCTGGTCCTATGGGATTTTCCTCTGGGAGCTATTCTC
TCTAGGAAGCAGCCCCTACCCAGGGATGCCGGTCGATTCCAAGTTTTACAAGATGATCAAGGAAGGTTTC
CGAATGCTCAGCCCTGAGCACGCGCCTGCCGCAATGTATGAAGTTATGAAGACTTGCTGGGATGCTGATC
CCCTGAAAAGGCCAACATTCAAGCAGGTTGTTCAGCTCATTGAGAAGCAGATCTCAGACAGCAGCAAACA
TATTTACTCCAACTTAGCAAACTGTAACCCCAACCCAGAGAACCCCGTGGTGGTGGACCATTCTGTGAGG
GTCAATTCCGTCGGCAGCAGCACCTCTTCCACACAGCCTCTCCTCGT 2960

1-week D

2481 CAGGAATATCCTCCTCACTCACGGGCGGATCACAAAGATTTGCGATTTCGGCCTAGCCAGAGACATC
AGGAATGATTCGAATTACGTGGTAAAAGGAAATGCACGGCTGCCCGTGAAGTGGATGGCACCGGAGAGCA
TTTTCAACTGCGTGTACACATTTGAAAGTGACGTCTGGTCCTATGGGATTTTCCTCTGGGAGCTATTCTC
TCTAGGAAGCAGCCCCTACCCAGGGATGCCGGTCGATTCCAAGTTTTACAAGATGATCAAGGAAGGTTTC
CGAATGCTCAGCCCTGAGCACGCGCCTGCCGCAATGTATGAAGTTATGAAGACTTGCTGGGATGCTGATC
CCCTGAAAAGGCCAACATTCAAGCAGGTTGTTCAGCTCATTGAGAAGCAGATCTCAGACAGCAGCAAACA
TATTTACTCCAACTTAGCAAACTGTAACCCCAACCCAGAGAACCCCGTGGTGGTGGACCATTCTGTGAGG
GTCAATTCCGTCGGCAGCAGCACCTCTTCCACACAGCCTCTCCTCGT 2960

Figure 62) Partial sequence of Rattus norvegicus mRNA for c-kit gene amplified by designed primers in control (non-diabetic) (C) and 1-week diabetic rat bladder samples. Amplified mRNA sequence with c-kit primers is highlighted in Grey. Bases of the c-kit primers are in red. The sequenced product sent back by GATC-biotech is indicated in blue.

6.4.2 Immunohistochemistry for detection of c-kit protein expression in rat urinary bladder

Using immunohistochemistry, a network of c-kit-positive cells could be identified in the rat urinary bladder. Figures 63 and 64 demonstrate immunostaining for c-kit on a network of connected cells on the edge, as well as between smooth muscle bundles in the rat urinary bladder. No immunostaining for c-kit could be seen in the negative control (minus the primary antibody) (Figure 63).

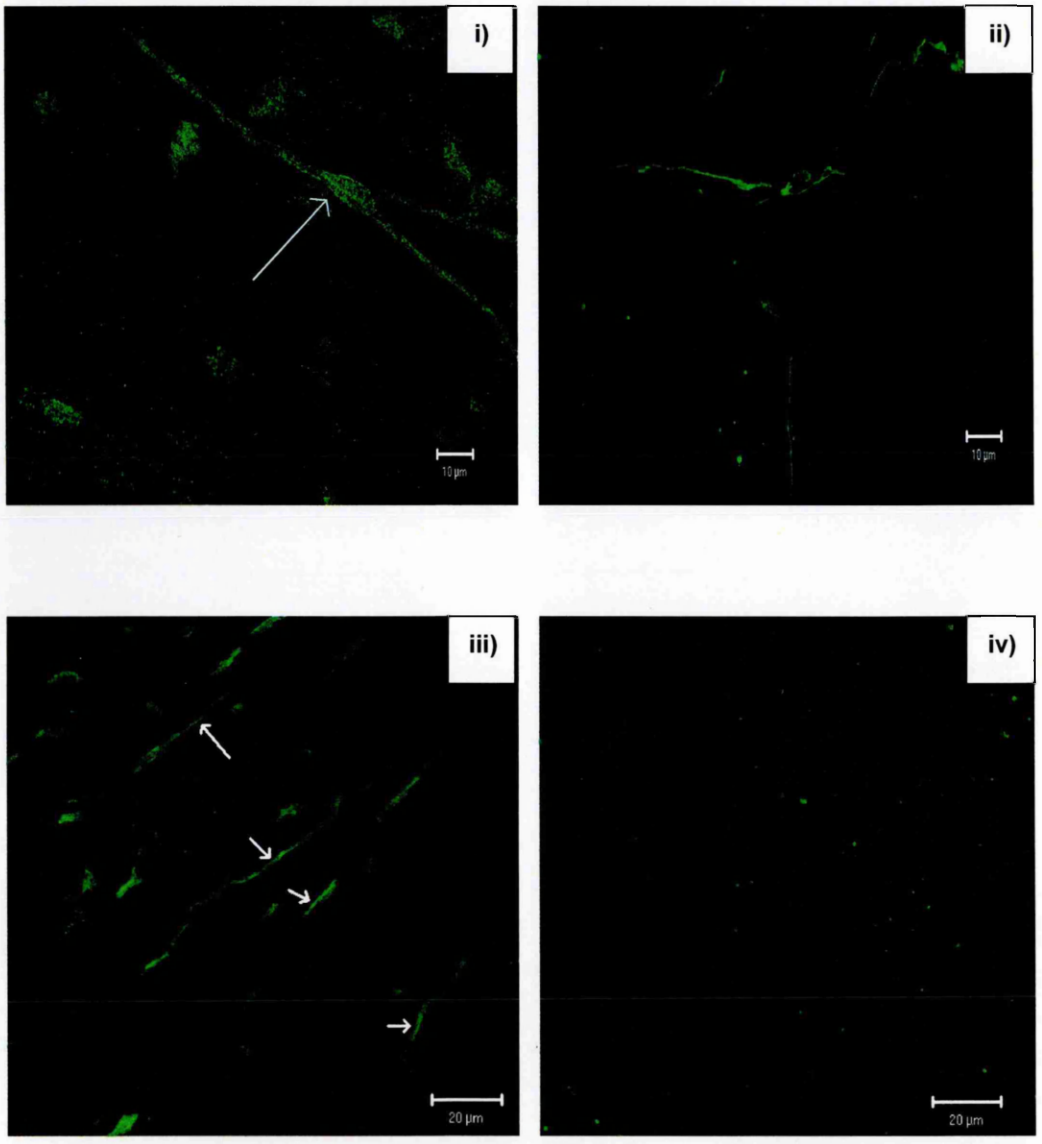


Figure 63) c-kit positive cells in the rat urinary bladder. Whole mount denuded preparations of control (non-diabetic) rat bladder blocked with donkey 1% serum and incubated overnight with 1:50 dilution of c-Kit antibody (C-19). i) c-kit immunoreactivity can be seen as a network of cells within the rat detrusor. ii) A c-kit positive cell with elongated cell body and several slender processes. iii) c-kit positive cells found between smooth muscle bundles iv) Negative control.

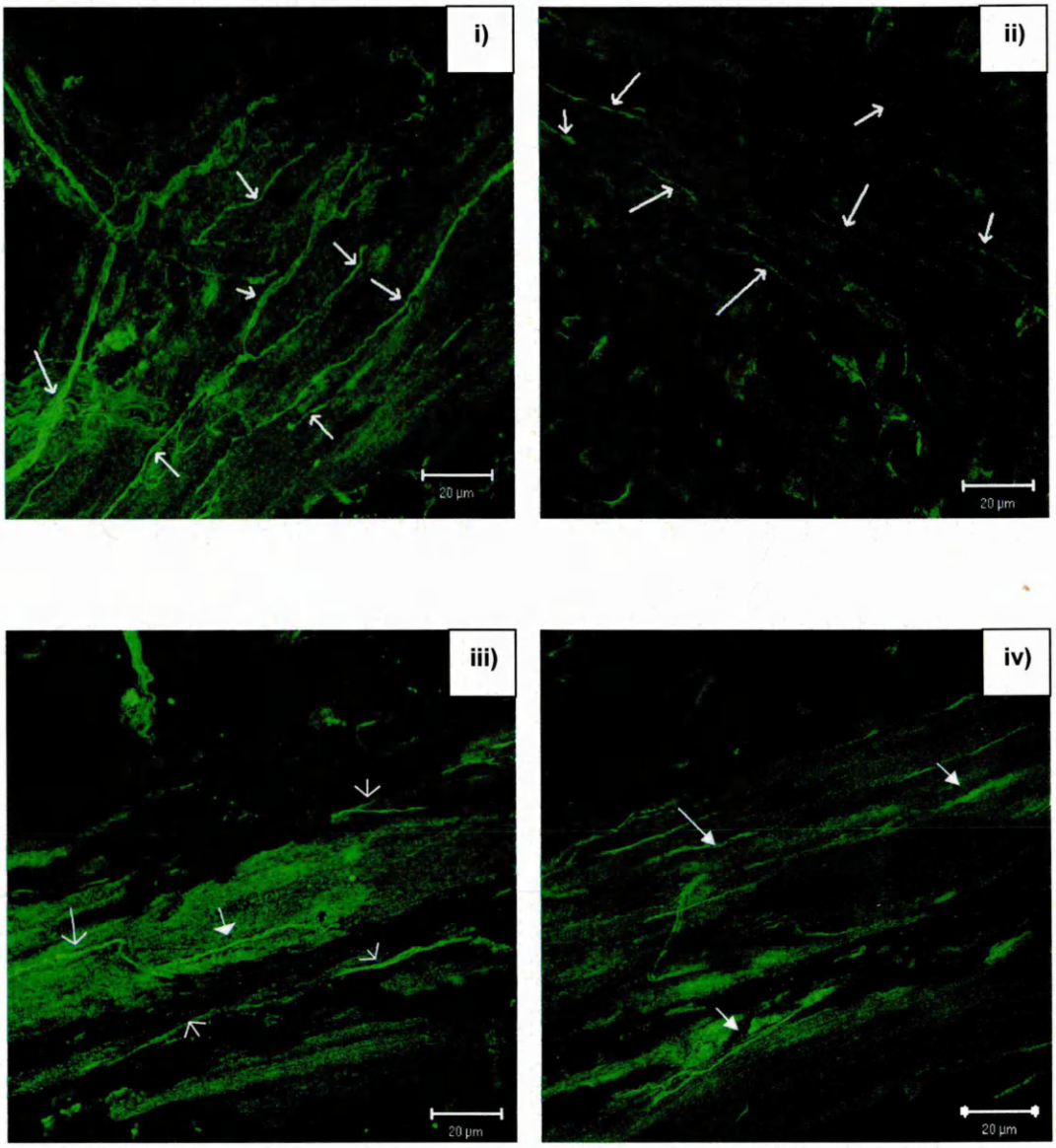


Figure 64) C-kit positive cells in rat urinary bladder. Whole mount denuded preparations of control (non-diabetic) rat bladder blocked in donkey 1% serum and incubated with 1:50 dilution of c-kit antibody (C-19) overnight. White arrows indicate the c-kit positive cells between (i, ii & iv) smooth muscle bundles and (iii) outside of smooth muscle bundles.

6.4.3 Induction of spontaneous activity by CCH in bladder strips from control (non-diabetic) and diabetic rats.

The non-selective muscarinic agonist CCH (0.1μM) induced spontaneous contractions in bladder strips from control (non-diabetic) and diabetic rats (Figure 65).

As demonstrated in Figure 66, the amplitude/mg tissue of SA in 1-week diabetic tissues was significantly greater than in the age-matched controls (non-diabetic) group. There was no significant difference in the amplitude of CCH-stimulated SA of the detrusor strips from 4-week and 12-week diabetic rats vs. their aged matched control (non-diabetic) tissues.

In contrast to amplitude, the frequency of SA in 1-week diabetic tissues was significantly less than in the age-matched control (non-diabetic) tissues. However, no significant difference was seen between 4-week diabetics and 12-week diabetic tissues vs. their aged matched control (non-diabetic) tissues.

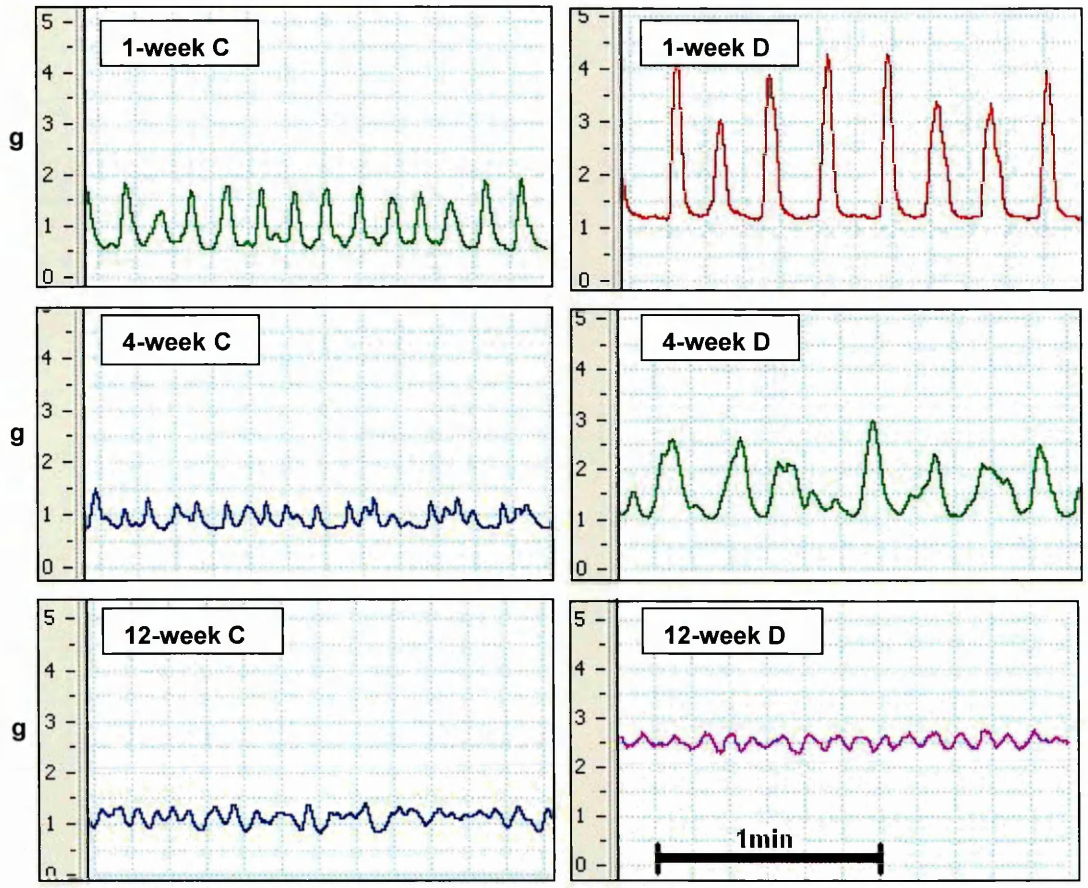


Figure 65) Typical recordings of spontaneous activity induced by 0.1μM CCH in control (non-diabetic) (C) and diabetic (D) bladder strips.

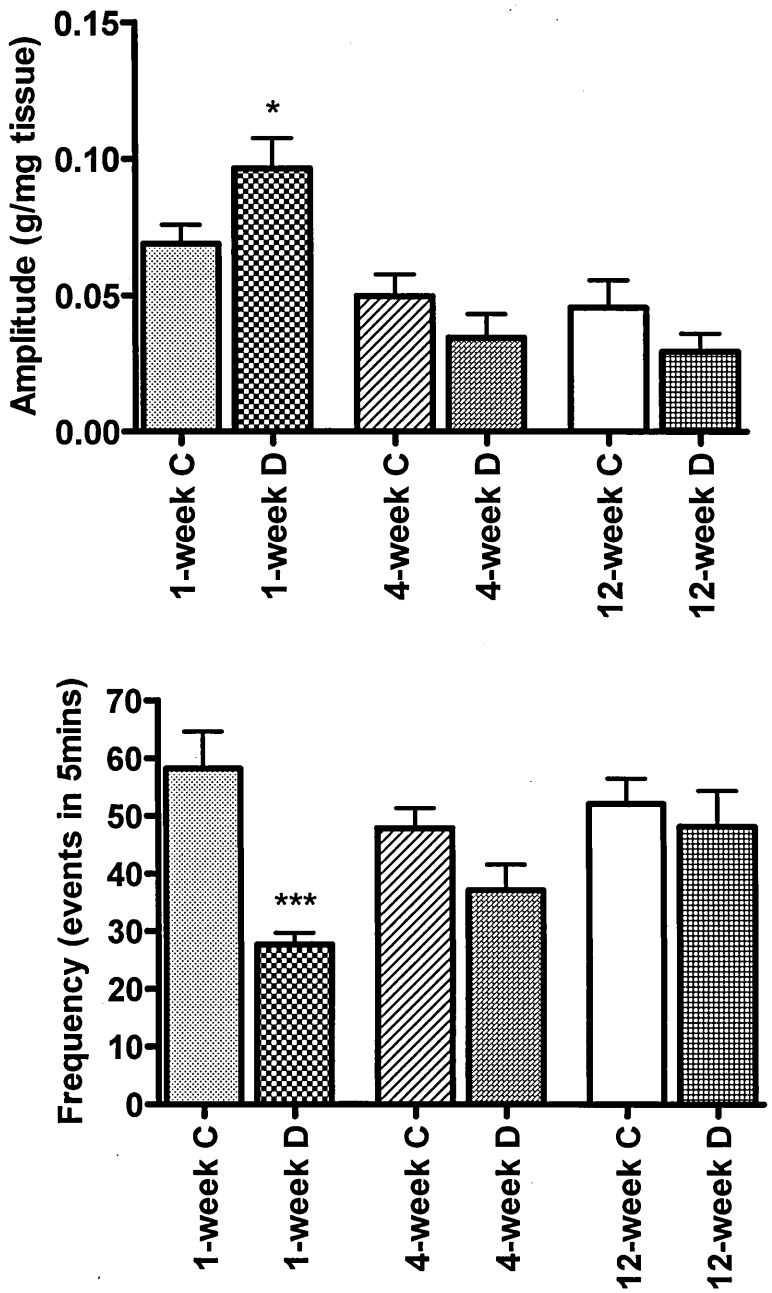


Figure 66) The amplitude and frequency of SA in detrusor strips from diabetic (D) and age-matched control (non-diabetic) rats (C) upon stimulation with 0.1μM CCH. * $p<0.05$, *** $p<0.001$ vs. the relevant age-matched control (non-diabetic) groups (unpaired t-test). Data is presented as mean±SEM. .

6.4.4 Functional investigation of the role of ICs in mediating the SA of detrusor strips from control (non-diabetic) and diabetic rats

Glivec inhibited the amplitude and frequency of CCH-induced SA in a concentration-dependent manner. Typical chart recordings of the effect of Glivec on CCH-induced SA in control (non-diabetic) and 1-week, 4-week and 12-week diabetic bladder strips are demonstrated in Figure 67.

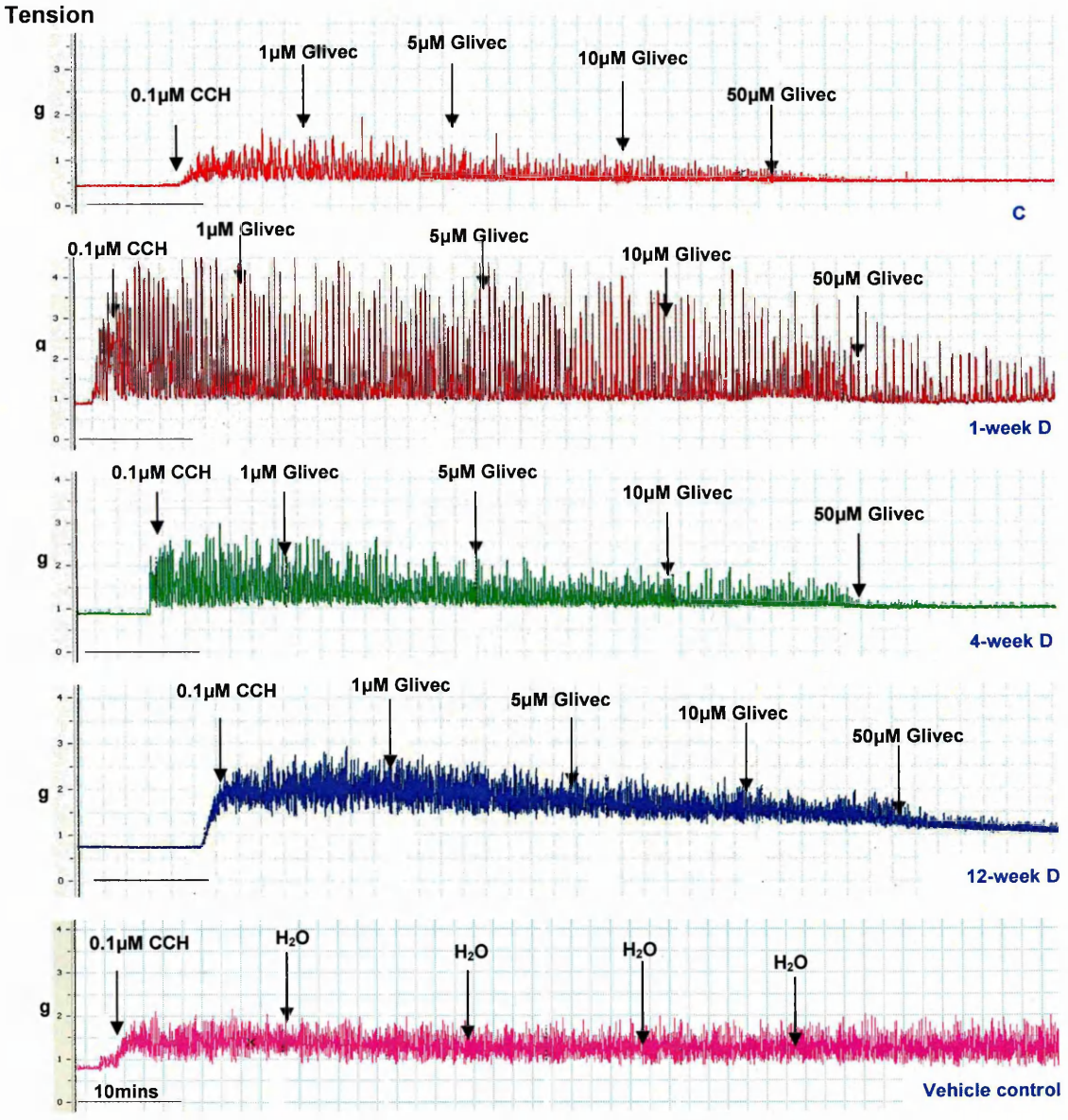


Figure 67) Typical chart recordings of the effect of increasing concentrations of Glivec and vehicle (H_2O) on CCH-induced SA in detrusor strips from representative control (non-diabetic) (C) & diabetic (D) rats.

The effect of vehicle (time control) was non significant in all groups (Figure 67).

6.4.4.1 Effects of Glivec on CCH-stimulated SA in detrusor strips from 1-week diabetic rats

Glivec significantly inhibited the amplitude of SA in detrusor strips from 1-week diabetic and aged-matched control (non-diabetic) rats at all concentrations (Figure 68).

Glivec also significantly inhibited the frequency of SA in detrusor strips from control (non-diabetic) animals at all concentrations (Figure 68). However, in 1-week diabetic tissues, only 10μM and 50μM Glivec significantly inhibited the frequency of SA (Figure 68).

When the amplitude of SA at each concentration of Glivec was calculated as a percentage inhibition relative to SA in the absence of Glivec (i.e. 0.1μM CCH only), there was no significant difference between control (non-diabetic) and diabetic tissues (Figure 69).

When the reduction in the frequency was calculated as a percentage inhibition relative to SA in the absence of Glivec (i.e. 0.1μM CCH only), there was a significant difference between control and diabetic tissues only at 10μM Glivec (Figure 69).

6.4.4.2 Effects of Glivec on CCH-stimulated SA in detrusor strips from 4-week diabetic rats

Glivec significantly decreased the amplitude of SA in age-matched control (non-diabetic) tissues at all concentrations apart from 1μM (Figure 70). In 4-week diabetic tissues Glivec significantly decreased the amplitude of SA only at 10μM and 50μM (Figure 70).

A significant decrease in the frequency of SA in 4-week control (non-diabetic) tissues was detected at all concentrations of Glivec apart from 1μM (Figure 70). However, Glivec only had a significant effect on the frequency of SA in 4-week diabetic tissues at the highest concentration tested (i.e. 50μM) (Figure 70).

When the amplitude of SA at each concentration of Glivec was calculated as a percentage inhibition relative to SA in the absence of Glivec (i.e. 0.1μM CCH

only), there was no significant difference between control (non-diabetic) and diabetic tissues (Figure 71).

When the reduction in the frequency was calculated as a percentage inhibition relative to the SA in the absence of Glivec (i.e. 0.1μM CCH only), there was a significant difference between control (non-diabetic) and diabetic tissues only at 5μM Glivec (Figure 71).

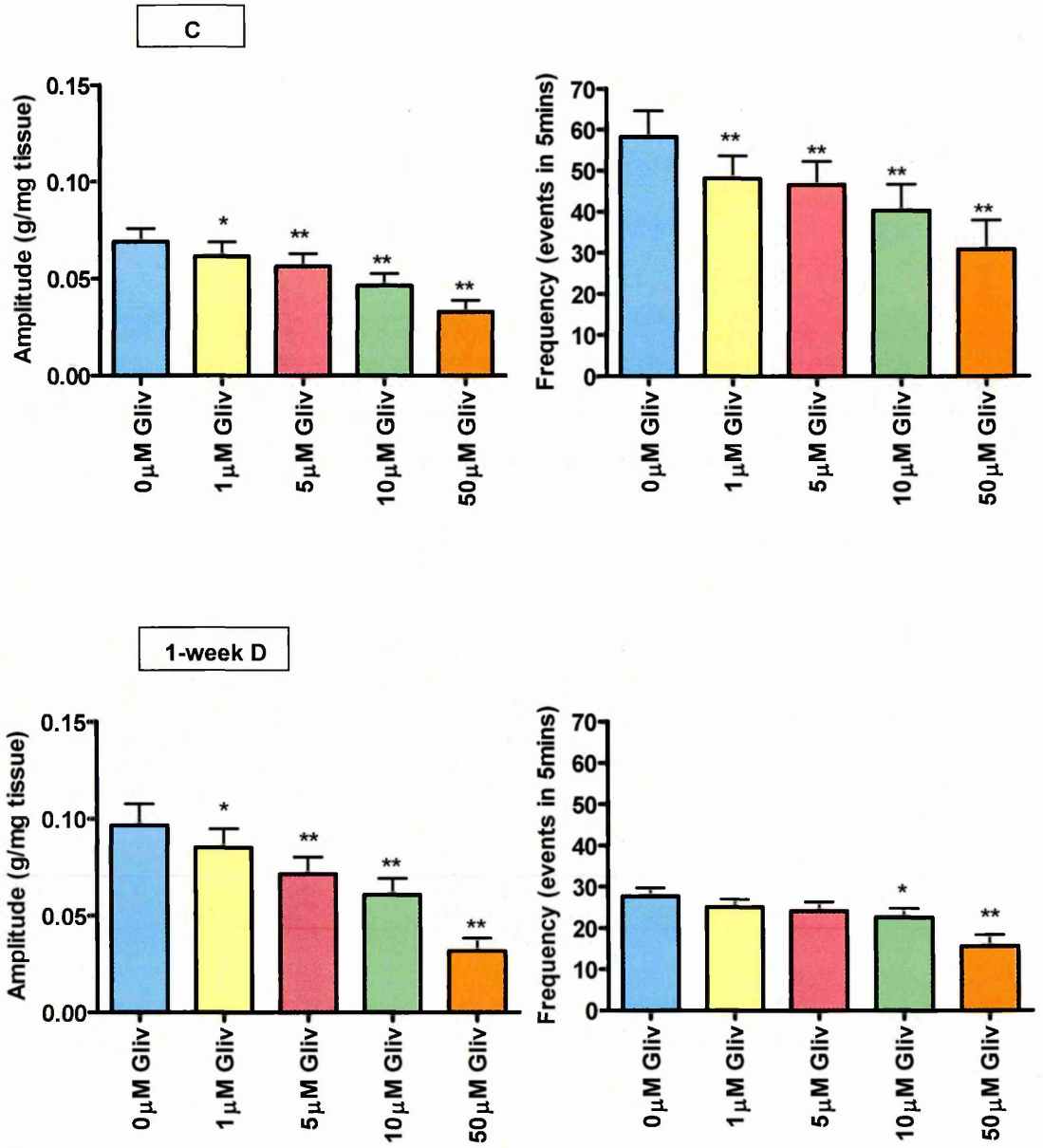
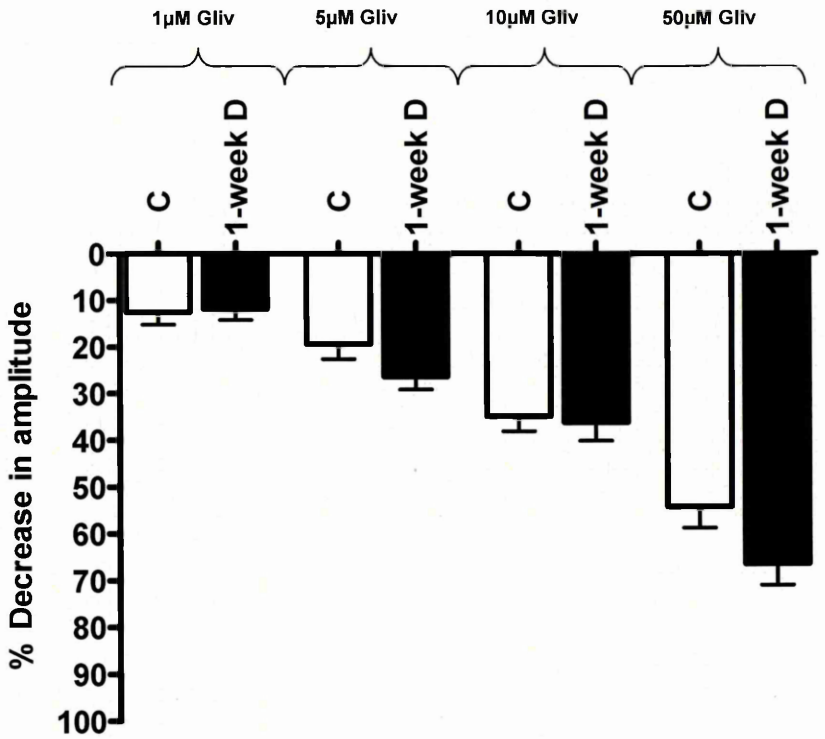


Figure 68) Effect of increasing concentrations of Glivec on the amplitude and the frequency of SA induced by 0.1µM CCH in control (non-diabetic) (C) & 1-week diabetic (D) tissues. C: n=17 (top-panel) & 1-week D: n=15 (lower panel). *p<0.05 & **p<0.01 vs. 0.1µM CCH response in absence of Glivec. Data is presented as mean±SEM.

i)



ii)

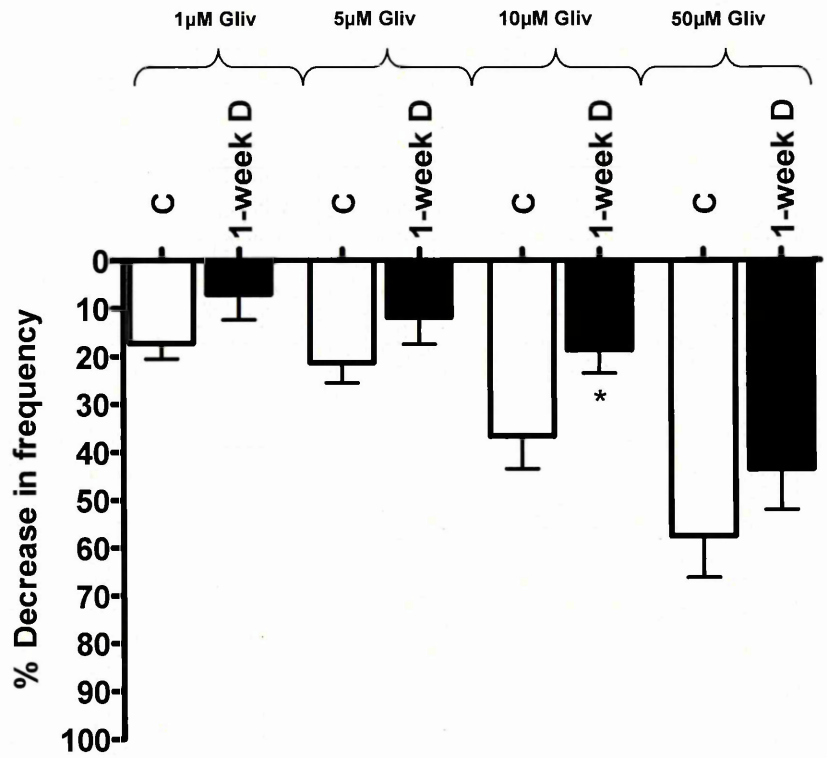


Figure 69) Percentage decrease in the amplitude and the frequency of SA in detrusor strips from control (non-diabetic) versus 1-week diabetic (non-diabetic) rats in presence of Glivec. i) Percentage decrease in amplitude & ii) percentage decrease in frequency. *p<0.05 vs. the control (non-diabetic) tissues. Data is presented as mean±SEM.

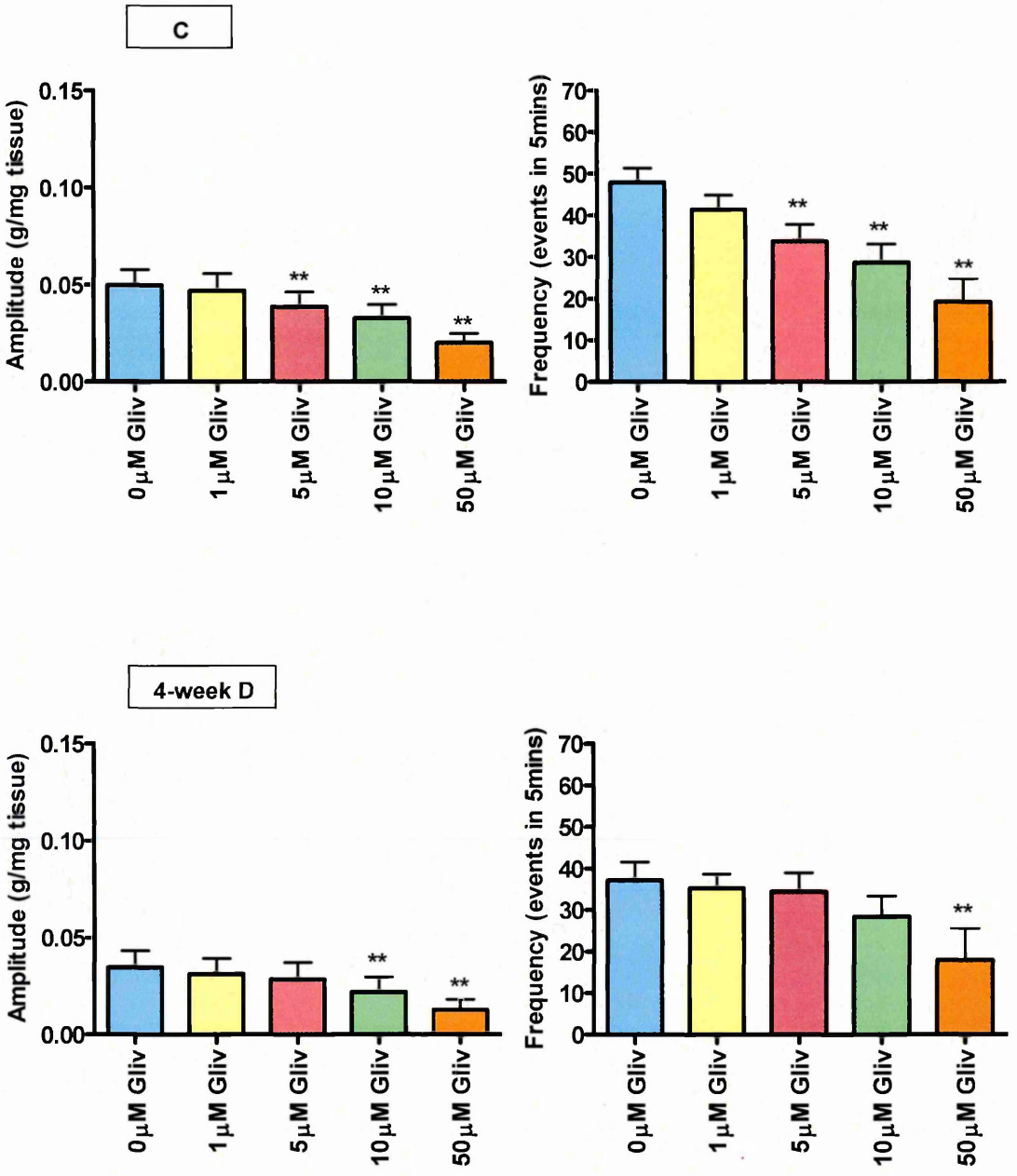


Figure 70) Effect of increasing concentrations of Glivec on the amplitude and the frequency of SA induced by 0.1µM CCH in control (non-diabetic) (C) & 4-week diabetic (D) tissues. C: n=13 (top-panel) & 4-week D: n=8 (lower panel) **p<0.01 vs. 0.1µM CCH response in absence of Glivec. Data is presented as mean±SEM

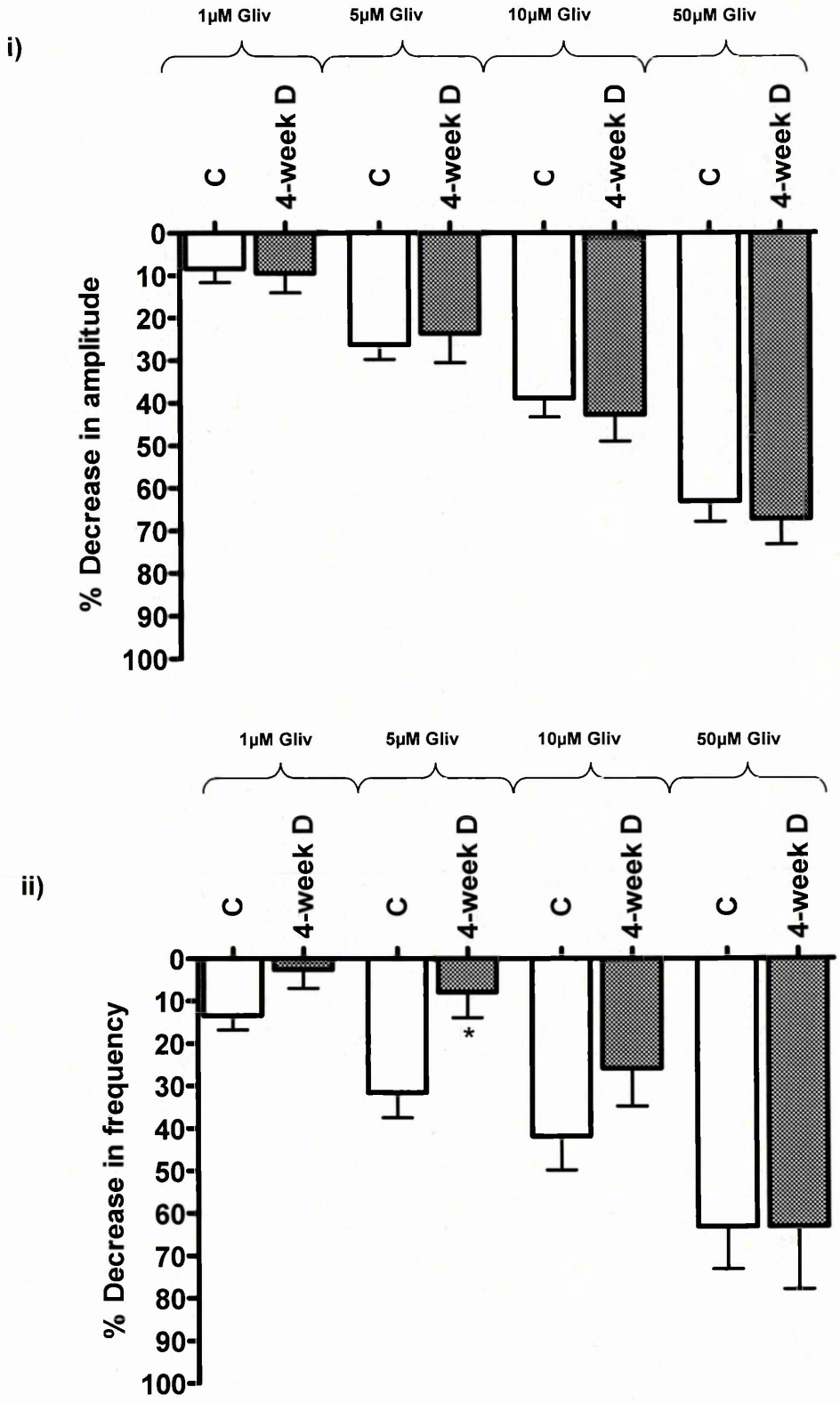


Figure 71) Percentage decrease in the amplitude and the frequency of SA in detrusor strips from control (non-diabetic) versus 4-week diabetic (non-diabetic) rats in presence of Glivec. i) Percentage decrease in amplitude & ii) percentage decrease in frequency *p<0.05 vs. the control (non-diabetic) tissues. Data is presented as mean±SEM.

6.4.4.3 Effects of Glivec on CCH-stimulated SA in detrusor strips from 12-week diabetic rats

Glivec significantly decreased the amplitude of SA in age-matched control (non-diabetic) tissues at all concentrations apart from 1μM (Figure 72). However, Glivec significantly decreased the amplitude of SA in 12-week diabetic tissues only at 50μM.

10μM and 50μM Glivec decreased the frequency of SA in 12-week control (non-diabetic) tissues (Figure 72). However, in 12-week diabetic tissues a significant effect on the frequency of SA was seen only at the highest concentration tested (i.e. 50μM).

When the percentage decrease in the amplitude of SA in the presence of all concentrations of Glivec relative to SA in the absence of Glivec (i.e. 0.1μM CCH only) was compared, there was no significant difference between control and diabetic tissues (Figure 73).

When the reduction in the frequency was calculated as a percentage inhibition relative to the SA in the absence of Glivec (i.e. 0.1μM CCH only), there was no significant difference between control and diabetic tissues (Figure 73).

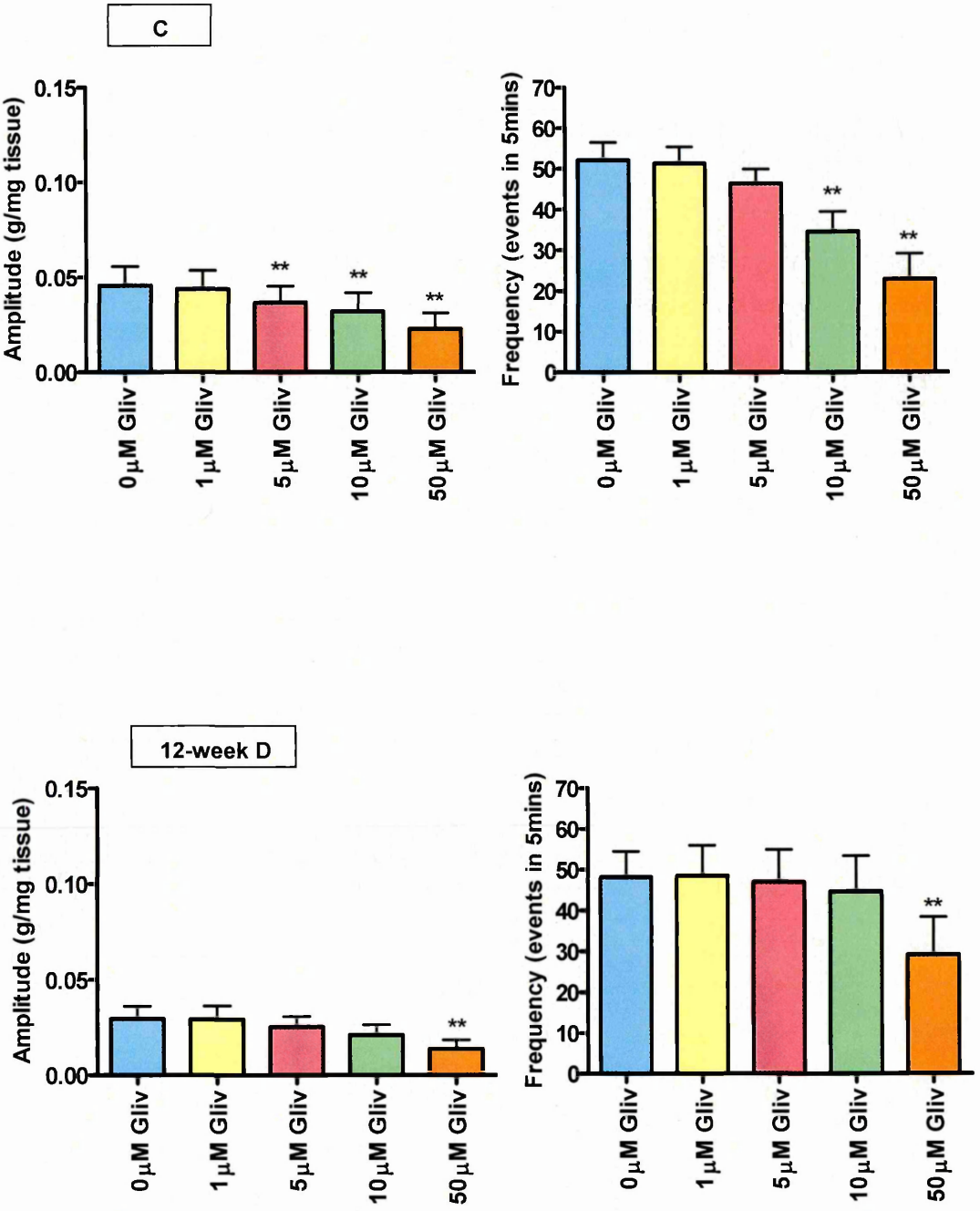


Figure 72) Effect of increasing concentrations of Glivec on the amplitude and the frequency of SA induced by 0.1μM CCH in control (non-diabetic) (C) & 12-week diabetic (D) tissues. C: n=13 (top-panel) & 12-week D: n=8 (lower panel). **p<0.01 vs. 0.1μM CCH response in absence of Glivec. Data is presented as mean±SEM.

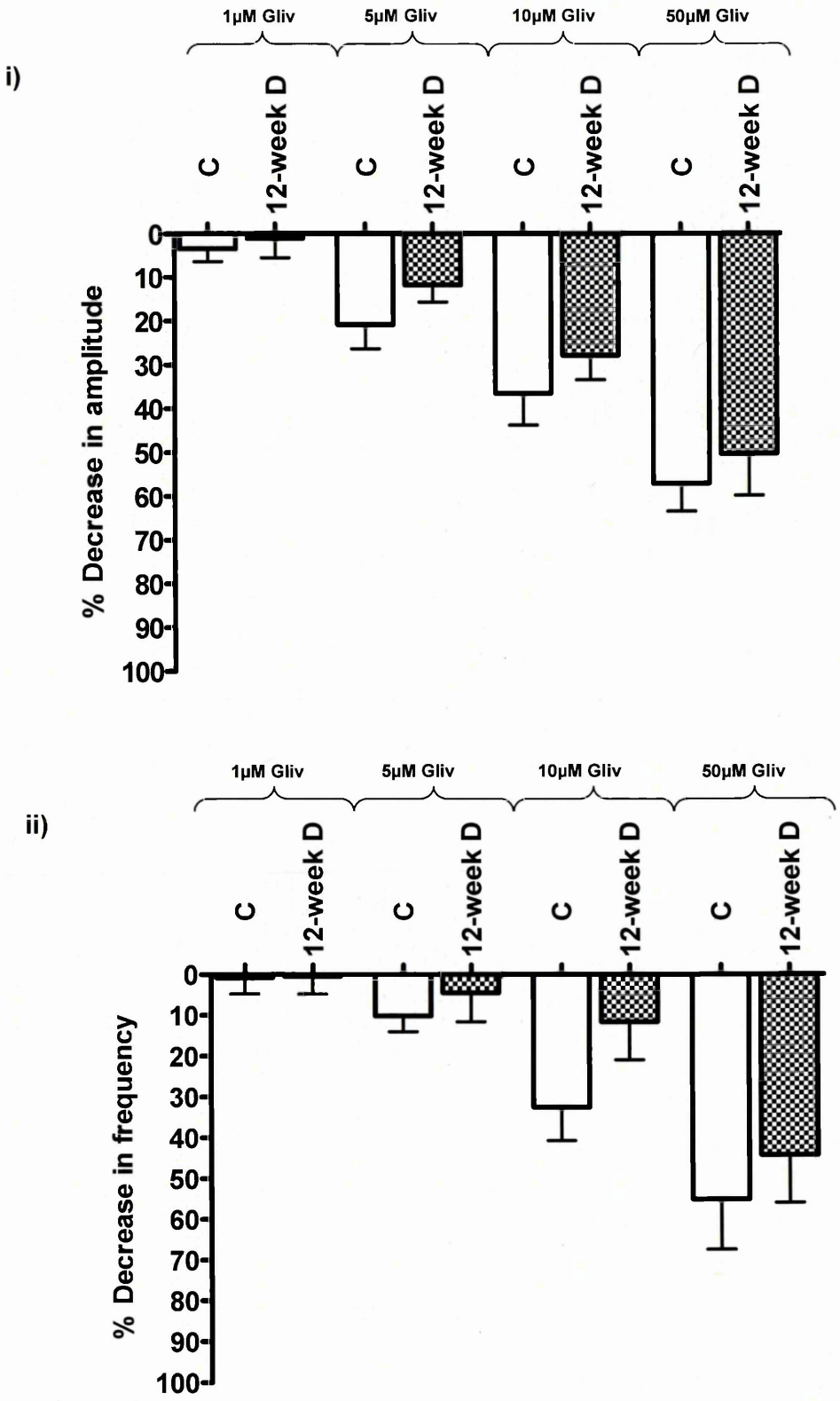


Figure 73) Percentage decrease in the amplitude and the frequency of SA in detrusor strips from control (non-diabetic) versus 12-week diabetic (non-diabetic) rats in presence of Glivec. i) Percentage decrease in amplitude & ii) percentage decrease in frequency. Data is presented as mean±SEM.

6.4.5 The role of the mucosa on the inhibitory action of Glivec on CCH-stimulated SA in detrusor strips from the rat.

To examine whether the urothelium plays a role in mediating the inhibitory responses to Glivec, the effect of increasing concentrations of this drug on CCH-stimulated SA was compared in mucosa-intact and denuded detrusor strips from control (non-diabetic) and 1-week diabetic rats.

The changes in the amplitude and the frequency of CCH-stimulated spontaneous contractions in the presence of Glivec were compared between mucosa-intact and denuded detrusor strips from control (non-diabetic) animals. The mucosa had no significant effect on the changes in amplitude and frequency of SA in the presence of increasing concentrations of Glivec (Figure 74).

In 1-week diabetic rats, the mucosa also had no significant effect on the changes in amplitude and the frequency of SA in the presence of increasing concentrations of Glivec (Figure 75).

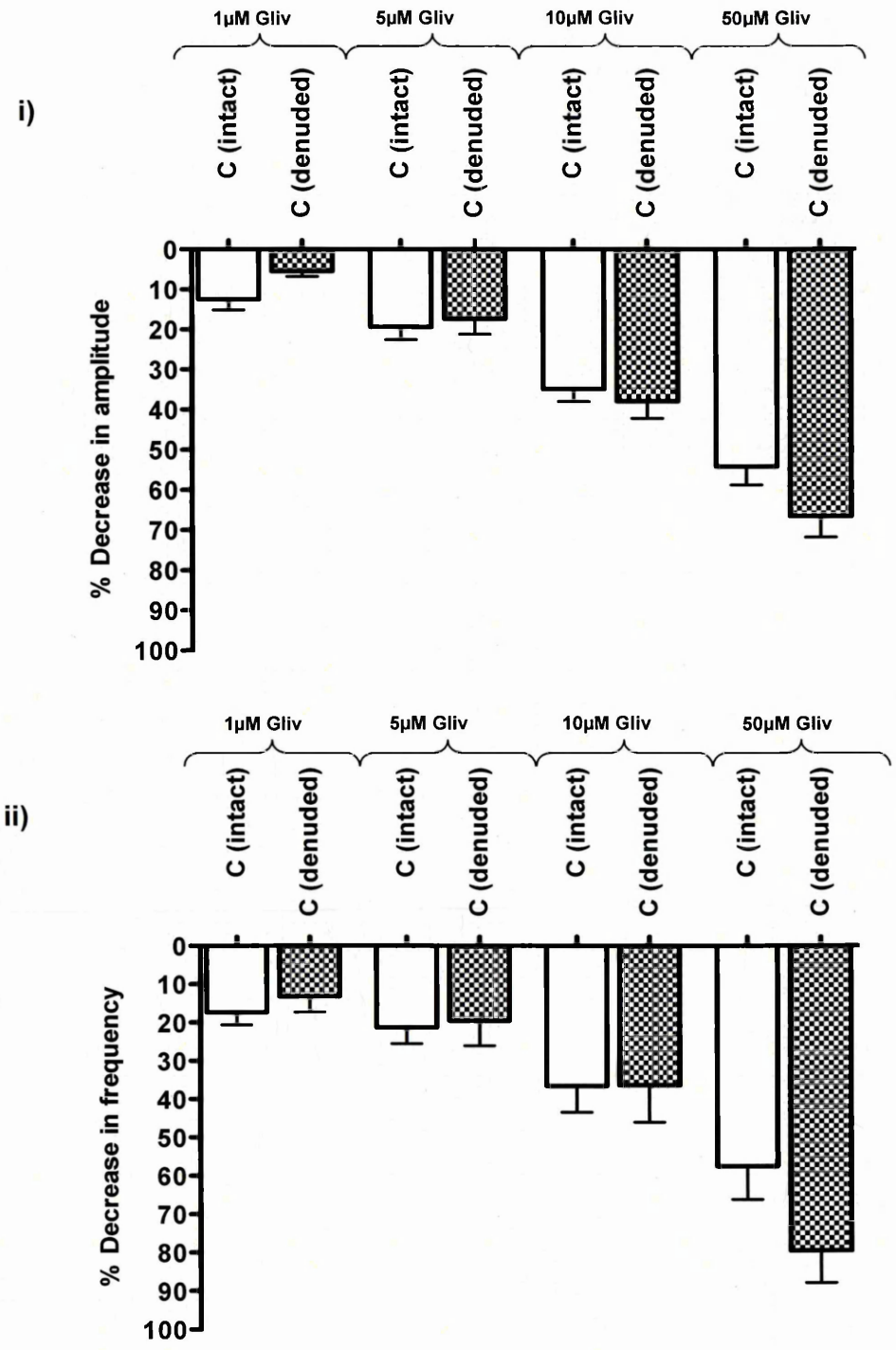


Figure 74) Percentage decrease in the amplitude and frequency of CCH-stimulated SA in intact and denuded detrusor strips from control (non-diabetic) (C) rats in the presence of increasing concentrations of Glivec. i) Percentage decrease in amplitude. ii) Percentage decrease in frequency. Data is presented as mean±SEM

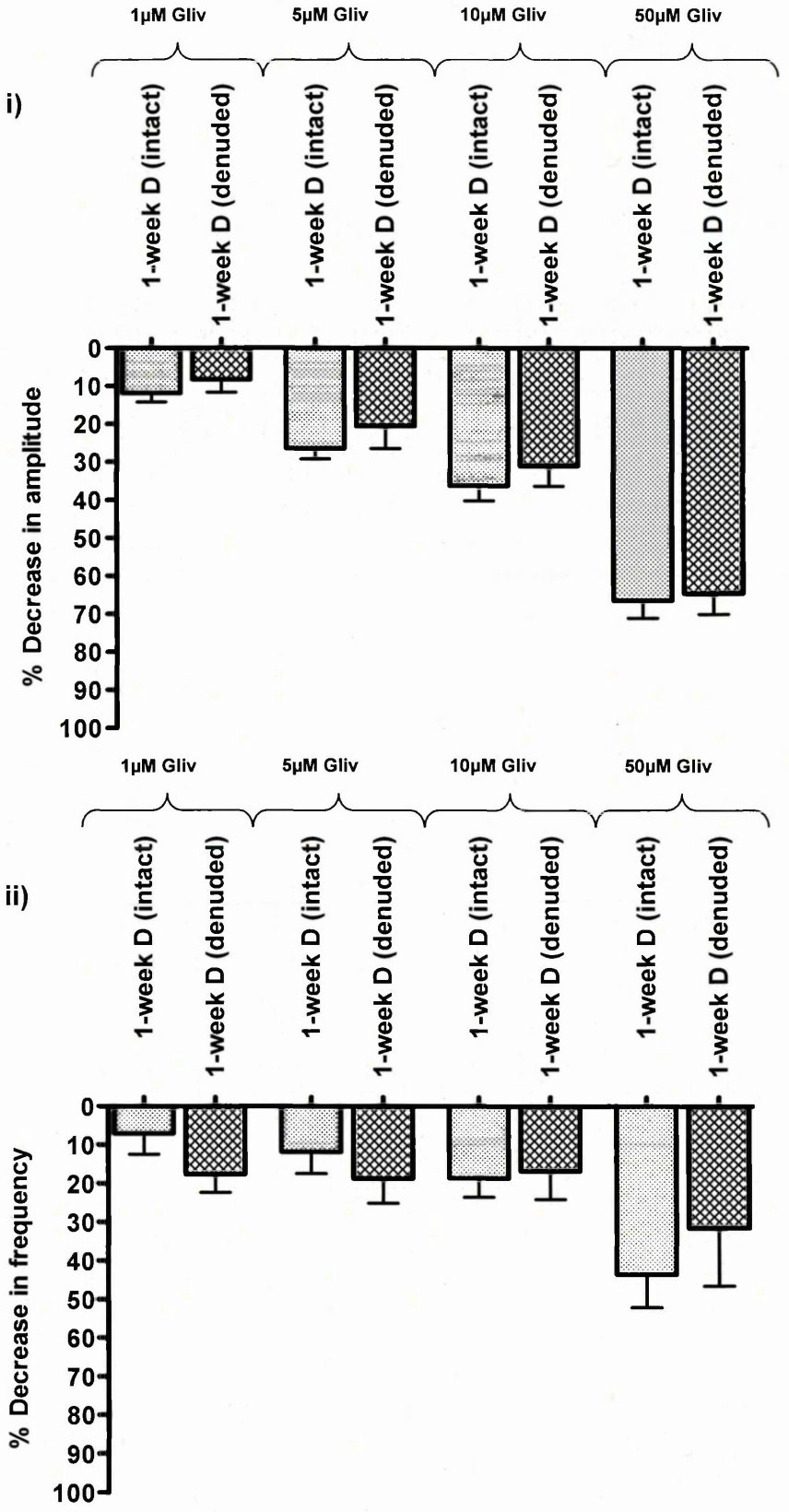


Figure 75) Percentage decrease in the amplitude and frequency of CCH-stimulated SA in intact and denuded detrusor strips from 1-week diabetic (D) rats in the presence of increasing concentrations of Glivec. i) Percentage decrease in amplitude. ii) Percentage decrease in frequency. Data is presented as mean±SEM.

6.5 Discussion

It is speculated that in addition to structural and functional changes to nerve and muscle in DO, alterations may also occur in the network of ICs in the bladder, leading to increased bladder sensation and afferent output from the detrusor and subsequent symptoms of urgency and frequency (De Jongh *et al*, 2007). Since in the detrusor of the STZ-diabetic rat, increased basal and CCH-induced SA was seen, it was postulated that ICs may be present in the rat urinary bladder and their function might be changed in the diabetic state, accounting for these changes in the SA. Since no studies have so far reported the existence of ICs in the rat urinary bladder, the first aim of this chapter was to identify the expression of the c-kit gene, the proto-oncogene that encodes the tyrosine receptor kinase c-kit, which is a specific marker for these cells.

6.5.1 Identification of c-kit positive cells in the rat urinary bladder

Amplified products obtained from RT-PCR experiments showed homology to the *Rattus norvegicus* c-kit receptor tyrosine kinase gene, confirming c-kit mRNA expression in the rat urinary bladder. c-kit positive cells were also identified in the rat urinary bladder by use of immunohistochemistry. c-kit immunoreactivity was detected on a network of cells between smooth muscle bundles and at the edge of the bundles. Thus, using the current data, it can be confirmed that c-kit positive cells are present between smooth muscle bundles (interbundle interstitial cells (IC-IB)) as well as on the boundary of the bundles (intramuscular IC (IC-IM)) in the rat urinary bladder. Morphological studies on guinea-pig and human bladders have similarly identified ICs on the boundary of smooth muscle bundles (IC-IMs) and in the spaces between the bundles (IC-IBs) (McCloskey & Gurney, 2002; Van der AA *et al*, 2004; Shafik *et al*, 2004). In addition, these studies have confirmed the presence of ICs in the suburothelium/lamina propria region. We were unable to confirm this in the present chapter due to removal of the mucosal layer. Although care was taken during the course of the experiments to maintain the orientation of the tissues, it was difficult to be absolutely certain about the presence of ICs in the sub-mucosa and the wall of the urinary bladder, and thus caution was taken on concluding the location of ICs in rat urinary bladder, other than their immediate

detected sites within the bladder wall. However, the presence of ICs in the sub-mucosa and on the outer wall of rat bladder can not be ruled out.

6.5.2 Functional investigation of the role of ICs in mediating the SA of detrusor strips from control (non-diabetic) and diabetic rats

To investigate the role of ICs in mediating the spontaneous contractions of rat bladder detrusor, *in vitro* functional pharmacological studies using Glivec (a c-kit tyrosine kinase inhibitor) were performed. As both development and maintenance of ICs require c-kit, Glivec can be used as a specific blocker for the function of ICs and allows characterisation of the role of these cells in mediating detrusor spontaneous contractility.

SA was induced in detrusor strips from control (non-diabetic) and diabetic bladders by a low concentration of CCH (0.1μM). In response to the low concentration of CCH, there was a significant increase in the amplitude and a decrease in the frequency of spontaneous contractions respectively in 1-week diabetic tissues compared to their aged-matched controls (non-diabetics), further confirming the results observed in chapter 4.

Following induction of SA, increasing concentrations of Glivec were added to the tissues to assess the effect of this drug in mediating the spontaneous detrusor contractions. Glivec inhibited the amplitude and frequency of SA in a concentration-dependent manner in both control (non-diabetic) and diabetic tissues, confirming a role for c-kit positive cells in mediating SA in rat bladder detrusor.

Glivec has been shown to suppress contractile activity in various phasic smooth muscles, including guinea-pig bladder (Kubota *et al*, 2004, 2006), stomach (Hashitani *et al*, 2008) and gallbladder (Lavoie *et al*, 2007); mouse small intestine (Shimojima *et al*, 2005); human uterus and small intestine (Popescue *et al* , 2006); and human bladder (Biers *et al*, 2006). However, there are reports questioning the specificity of Glivec, particularly at the higher concentrations of this drug. Some studies have suggested that acute application of Glivec *in vitro* is unlikely to suppress IC activity through its inhibition of c-kit signalling, and instead Glivec may suppress SA of the guinea-pig stomach by inhibiting

intracellular Ca^{2+} handling mechanisms (Hashitani *et al*, 2008). However, others studies have claimed that Glivec does not have non-specific inhibitory actions on smooth muscle and that c-kit signalling does play an essential role in the spontaneous contractions of circular muscles of the mouse small intestine (Shimojima *et al*, 2005). In bladder smooth muscle, relatively high concentrations of Glivec (50-100μM) have been shown to suppress VDCCs and K^+-selective outward currents, indicating a non-specific action (Kubota *et al*, 2004). In the current chapter, the effect of Glivec on inhibiting SA via modulation of intracellular Ca^{2+} concentrations was not investigated and thus an action of this drug, especially at higher concentrations (50μM), on intracellular Ca^{2+} handling mechanisms can not be ruled out. However, since lower concentrations of Glivec (1μM and 5μM) also inhibited the SA, especially in 1-week diabetics and their age-matched control tissues, it may be postulated that Glivec is indeed acting via inhibition of the c-kit tyrosine kinase receptor.

However, it is possible that Glivec may act on other receptors and cell types present within the tissue and that this may result in the differences in the sensitivities of various tissues to the drug. It is known that Glivec can inhibit not only the c-kit tyrosine kinase receptor but also PDGFR and mast cells (which have been shown to express c-kit) (Popescue *et al*, 2006). Signalling through the PDGFR is a potent activator of DNA synthesis in bladder smooth muscle (Adam, 2006), whereas mast cells are important in interstitial cystitis, in which their number increases (Boucher *et al*, 1995). In order to rule out the role of PDGFR in mediating the contractile activity of smooth muscle tissues, Popescue *et al* (2006), have used neutralising antibodies against PDGFR to reduce the activity of this receptor. It was found that these antibodies do not have an effect on uterine smooth muscle SA. They also ruled out the role of mast cells in generation of SA in human myometrium by use of Ketotifen (an inhibitor of degranulation of these cells). It was then suggested that the inhibitory effect of Glivec on SA is mediated via other means (Biers *et al*, 2006; Propescu *et al*, 2006). In order to rule out whether some of the effect of Glivec on UBSM SA in the present study is mediated through PDGFRs and mast cells, further experiments, using specific inhibitors of these would be needed.

6.5.3 Comparison of the effect of Glivec in inhibition of SA in detrusor strips from control (non-diabetic) and diabetic animals.

Glivec reduced the amplitude and the frequency of SA in control (non-diabetic) and diabetic tissues in a concentration-dependent manner. However, although non-significant, detrusor strips from all diabetic groups showed a trend towards being less sensitive to Glivec than the control (non-diabetic) tissues. In contrast to this, studies investigating the spontaneous contractions of human overactive detrusors have shown that Glivec has a more profound effect in reducing the SA in overactive detrusor than normal tissues (Biers *et al*, 2006). In a study by Sui *et al* (2008), Glivec also reduced the SA in bladder sheets from spinal cord-transected (SCT) rats, but not in normal rats. The reason for these discrepancies is not clear, although it may be due to species and model differences, as well as diversity in the experimental procedures and tissue preparation techniques. For example in the study by Sui *et al* (2008), the effect of a single dose of Glivec (30μM) on reduction of basal SA in sheets of rat whole bladder was assessed, whilst in the present study the effect of increasing concentrations of Glivec on CCH-stimulated SA was measured. Also changes in the diffusibility of the drug in the diabetic rat detrusor as a result of increased bladder mass might render these tissues relatively insensitive to Glivec, although there is no data in the literature to suggest this. In addition, all tissues were exposed to each concentration of Glivec for only 10mins before the next concentration of drug was added. This may not be a sufficient length of time for Glivec to have an effect on the diabetic bladders. Differences in the sensitivity of normal and overactive detrusor from various species to Glivec may also be explained by the proposed non-specific actions of Glivec, such as inhibition of VDCCs and K^+-selective outward currents and/or by an inhibitory effect on other participating cells (e.g. mast cells) and receptors (PDGFR) within the detrusor which may have been altered as a result of bladder dysfunction.

The trend towards a reduced sensitivity of diabetic tissues to Glivec could also be due to a reduction in the population of ICs, or changes in the location of these cells in the rat bladder, resulting in fewer, or altered, available targets for Glivec. In previous studies (Kanai *et al*, 2007; Sui *et al*, 2008; Kubota *et al*, 2008) it has been proposed that ICs provide the focus for generation of SA and that an increase in their number would enhance this coordination. A decrease in

their number may also lead to decreased SA and sensitivity to Glivec. However, the population of ICs in control (non-diabetic) and diabetic rat bladders were not investigated in this study and thus further conclusions of the role of altered population of these cells in the diabetic rat bladder can not be made.

Removal of the urothelium/mucosa from control (non-diabetic) and 1-week diabetic rat bladders did not have a significant effect on the responsiveness and sensitivity of detrusor strips to Glivec. This may suggest that removal of the mucosa does not effect the population of c-kit positive ICs and that in the rat urinary bladder the targets of Glivec are situated elsewhere. This hypothesis is supported by the studies that suggest that ICs in the bladder belong to multipotent group of functional regulatory cells (reviewed by Drake *et al*, 2006). It has been demonstrated that the suburothelial ICs/myofibroblasts have immunoreactivity for vimentin and connexin 43 (reviewed by Drake *et al*, 2006; Roosen *et al*, 2008). Some of vimentin positive cells beneath the urothelium are c-kit positive but are too sparsely distributed. Other c-kit positive ICs are found between smooth muscle bundles where they are believed to precipitate calcium waves and generation of pacemaking activity (reviewed by Drake *et al*, 2008). In this study, we did not manage to characterise the ICs in the suburothelium of the rat bladder but the data from the effect of Glivec on denuded strips as well as immunohistochemistry data suggests that c-kit positive ICs, which are the targets for the inhibitory effect of Glivec, may be situated between smooth muscle bundles as well as between smooth muscle cells rather than in the suburothelial region. Further characterisation of these cells using PCR to investigate the expression of c-kit in the mucosa as well as immunohistochemistry would aid understanding of the role and location of ICs in the rat bladder.

6.6 Summary

In this chapter, the presence of ICs was confirmed in the rat urinary bladder by demonstrating the expression of c-kit mRNA and its protein product, c-kit, (a specific marker of ICs) by means RT-PCR and immunohistochemistry.

The functional role of these cells in mediating rat detrusor SA was confirmed by the use of the tyrosine kinase inhibitor Glivec, using *in vitro* tissue bath experiments. Glivec inhibited both the amplitude and the frequency of SA in control (non-diabetic) and diabetic tissues. Diabetic tissues were slightly, although not significantly, less sensitive to Glivec.

The removal of the mucosa had no effect on the sensitivity of control (non-diabetic) and diabetic tissues to Glivec.

Chapter 7: General discussion

In recent decades, one of the major forces driving lower urinary tract research has been the aim of discovering the origins of detrusor overactivity (DO) and overactive bladder syndrome (OABS), as well as effective ways to treat these conditions (Gillespie, 2005). The consequence of these studies has been the emergence of new insights into the physiological factors controlling the human bladder. It is now becoming clear that the general view of the control of bladder function is far more complicated than once thought and involves complex interactions between smooth muscle cells, urothelium, suburothelial myofibroblasts, interstitial cells (ICs) and sensory nerves. These complex interactions must be explored and taken into account, especially when considering new therapeutic targets for treatment of bladder dysfunction. One example of this is the development of antimuscarinic drugs to treat DO and urgency. The initial clinical rationale for using these agents was that they exerted their effects by acting solely on muscarinic receptors of the urinary bladder smooth muscle (UBSM), and it was thought that alterations in these receptors may occur in bladder dysfunction associated with DO. This concept has now become controversial and recently it has been hypothesised that antimuscarinic drugs may act via other mechanisms related to the afferent mechanosensory pathways rather than the efferent system.

Other examples of the oversimplified view of bladder function include the lack of clear physiological roles for purinergic, adrenergic, nitrergic nerves, the intramural and pelvic ganglia, the urothelium, ion channels and specialised cell types such as ICs. For a full picture of the physiology of the bladder and its control mechanisms, all of these component systems should be considered. In this respect, experiments using animal model to investigate the integrated control of bladder function can be extremely useful. A common animal model to investigate the physiology and the pathophysiology of urinary bladder dysfunction and DO is the streptozotocin (STZ)-diabetic rat. This animal model was used in the present study, at different durations post STZ injection in an attempt to understand the underlying mechanisms mediating normal and abnormal detrusor contractility.

7.1 Cholinergic mediation of contractile function in rat urinary bladder

Anticholinergic drugs are the first line of pharmacotherapy for treatment of DO and changes in expression and function of muscarinic receptors, which mediate the main contractile pathway of the detrusor, have long been thought to be associated with the pathology of DO. Therefore, in an attempt to elucidate the underlying mechanisms of bladder dysfunction in the STZ-diabetic rat, in the initial stages of the present study (Chapter 3) the role of muscarinic receptor subtypes in mediating the detrusor contraction was investigated. In the current study, an enhanced contractile response to cholinergic stimulation was only detected in 1-week diabetic rats, with the detrusor strips from other diabetic groups demonstrating no change in their sensitivity or functional responses to cholinergic stimulation. This enhanced functional response in detrusor strips from 1-week diabetic rats was mediated via the M3-muscarinic receptor subtype only, similar to control (non-diabetic) and other diabetic tissues (4-week, 8-week and 12-week diabetics), with the role of M2-muscarinic receptor remaining unclear. Results of this thesis are in line with a recent study investigating the role of muscarinic receptor subtypes in patients with idiopathic DO (Stevens et al, 2007). In this recent study it was demonstrated that although detrusor from patients with idiopathic DO demonstrates an enhanced contractile response to cholinergic stimulation, the main receptor mediating the detrusor contraction is the M3-muscarinic receptor subtype (Stevens et al, 2007).

An increased expression of both M2- and M3-muscarinic receptors was also detected at the mRNA level in bladders from 1-week diabetic rats which could potentially explain the increased responses to cholinergic stimulation in these tissues. If this increase in expression of both M2- and M3-muscarinic receptors' mRNA is indeed translated at the protein level, it could shift the contractile-relaxant neurological balance towards increased contractility in these tissues. However, confirmation of the expression of these receptors at the protein level is required.

As described earlier, it was initially postulated that antimuscarinic drugs blocked muscarinic receptors on the detrusor, which are normally stimulated via

acetylcholine (ACH) released from parasympathetic nerves; thereby they decrease the ability of the bladder to contract. However, it is now speculated that antimuscarinics might have a putative sensory mode of action and it is possible that at least part of the therapeutic effect of these agents could be via modulation of sensory pathways (Finney et al, 2006); especially since new evidence has led to the suggestion that antimuscarinics may act by binding to muscarinic receptors within the mucosa (Mansfield et al, 2008). It has been postulated that muscarinic receptors on the mucosa and the UBSM cells are the target of endogenous ACH released from non-neuronal sources such as the urothelium (Yoshimura et al, 2003; Ng et al, 2006). Activation of the muscarinic receptors within the bladder via this endogenous ACH release is believed to be partly responsible for generation of spontaneous contractions detected during the filling phase of the micturition cycle. Spontaneous contractions of the urinary bladder are believed to be important in maintaining bladder tone under normal conditions, but are enhanced in pathological states leading to DO. The origin of such SA and the mechanisms that facilitate it are unknown and may be coordinated by complex interactions between smooth muscle, urothelial, suburothelial cells and other non-neuronal structures within the bladder wall such as ICs. In this study, during the equilibration of *in vitro* isolated detrusor strips, both control (non-diabetic) and diabetic tissues demonstrated basal SA. The amplitude of this basal SA was significantly higher in 1-week diabetic tissues compared to detrusor strips from control (non-diabetic) and other diabetic (4-week, 8-week and 12-week) rats which may demonstrate hyper-contractility of the bladder in these rats. However, at 12-weeks post STZ-injection, basal SA could not be detected in the diabetic tissues which may reflect hypo-contractility of the bladder in these animals (Chapter 4).

It has previously been reported that atropine (a non-selective antimuscarinic agent) can inhibit the SA of detrusor strips from STZ-diabetic rats (Stevens et al, 2006). Therefore, it was hypothesised that the spontaneous contractions detected in detrusor strips from diabetic rats in the current study may be modulated by endogenous ACH release from non-neuronal sources within the bladder. To further explore the cholinergic modulation of SA in control (non-diabetic) and diabetic rats, detrusor strips from these animals were stimulated with low concentrations of the muscarinic agonist CCH. CCH induced

spontaneous contractions in detrusor strips of both control (non-diabetic) and diabetic rats, confirming the importance of cholinergic modulation of SA in normal and diseased bladders. In detrusor strips from 1-week diabetic rats this CCH-induced SA was significantly enhanced relevant to controls (non-diabetic). In addition, a clear difference in basal and CCH-induced SA was demonstrated in the detrusor strips from 1-week and 12-week diabetic rats. 1-week diabetic rats demonstrated significantly higher amplitude of spontaneous contractions compared to 12-week diabetic tissues. The altered expression of M2- and M3-muscarinic receptors observed in the present study could be an important factor in mediating these differences between 1-week and 12-week diabetic tissues. However, additional possible underlying reasons for the differences in the SA of detrusor strips from 1-week and 12-week diabetic tissues were discussed in chapter 4.

High amplitude SA has been associated with DO (Ng et al, 2006), since it may result in synchronisation of the bladder's autonomous activity, increased afferent output and the subsequent increase in intravesical pressure and symptoms of urgency and frequency associated with DO. Therefore, it appears that bladders from 1-week diabetic rats may provide a good model for the study of DO in these animals.

Since stimulation of control (non-diabetic) and diabetic tissues with CCH resulted in an increased SA, it was important to elucidate the muscarinic receptor targets for this agent in the urinary bladder of the rat. Since muscarinic receptors are known to be expressed in the mucosa (reviewed by de Groat, 2004), their role in mediating the SA of detrusor strips from control (non-diabetic) and diabetic rats was investigated. CCH-stimulated SA was measured in intact and mucosa-denuded detrusor strips from control (non-diabetic) and diabetic rats. However, removal of the mucosa/urothelium did not alter the CCH-induced SA of detrusor strips from either control (non-diabetic) or diabetic rats, except for 12-week diabetic tissues. Therefore, it was concluded that muscarinic receptors on the mucosa are not important in mediating the phasic detrusor contractions in control (non-diabetic) or younger diabetic rats (1-week, 4-week and 8-week). In 12-week diabetic tissues, removal of the mucosa resulted in increased spontaneous contractions compared to intact tissues.

UDIF is known to be released upon muscarinic stimulation of the mucosa and has been reported to inhibit bladder contractions (Hawthorn *et al*, 2000). Therefore, the release of UDIF may be important in inhibiting the SA of the detrusor at later durations of diabetes i.e. 12-weeks.

Immunocytochemical evidence from rodents has demonstrated the presence of muscarinic receptors on specialised types of cells such as ICs in the suburothelial region of the bladder. It is possible that these cells could be an additional target for muscarinic receptor agonists such as CCH within the bladder, and could contribute to the increased SA of this tissue. In order to characterise the role of ICs in mediating the cholinergic induced SA of rat urinary bladder, the effect of inhibition of these cells, using the c-kit tyrosine kinase inhibitor Glivec, was investigated in detrusor strips from control (non-diabetic) and diabetic rats (chapter 6). Glivec inhibited CCH-induced SA of detrusor strips from both groups suggesting an involvement of ICs in regulation of SA in the rat bladder. This hypothesis is supported by studies demonstrating the role of ICs in mediating the SA in human bladders (Biers et al, 2005). Since ICs belong to a multipotent group of functional regulatory cells and it is hypothesised that some of these cells may be situated in the suburothelial region and some within the muscle bundles and around smooth muscle cells. Thus, it is important to determine which population of these cells is more important in mediating the cholinergic mediated SA in rat bladder. In the current study, the role of suburothelial ICs in mediating the SA in rat bladder can be ruled out to some extent, since removal of the mucosa did not affect the detrusor responses to Glivec in either control (non-diabetic) or diabetic rats. This suggests that ICs within the smooth muscle bundles and between smooth muscle cells may be more important in mediating this activity in rat urinary bladder and that the suburothelial ICs (if they exist in rat bladder) may be more important in sensory transduction and communication between afferent nerves, urothelial cells and smooth muscle cells.

Since changes in muscarinic-mediated contractions of the detrusor have been suggested to be important in pathological conditions, it was also important to determine which muscarinic receptor subtype was important in mediating the SA. Studies have demonstrated an increased expression of both M2- and M3-muscarinic receptors in the detrusor and the urothelium of the STZ-diabetic rat

(Tong et al, 1999, 2002 & 2006; Cheng et al, 2007). We were unable to comment on the expression levels of muscarinic receptors on the urothelium of STZ-diabetic rats, but we did demonstrate that the expression of M2-muscarinic receptor mRNA was increased in the detrusors of all diabetic rats except 12-week diabetic tissues, which demonstrated no change in the expression level of this receptor subtype. The expression levels of M3-muscarinic receptors were decreased in all diabetic tissues except 1-week diabetic tissues, which demonstrated an increased expression of this receptor subtype. It must be noted that levels of mRNA will not always reflect the level of the encoded protein. For example it has been demonstrated in humans that levels of M3- and M4-muscarinic receptors mRNA do not correlate with their protein densities, although M2-muscarinic receptor expression did show a positive correlation between amount of mRNA expressed and its transcription (Arrighi *et al*, 2008). These results are in line with those reported by Braverman & Ruggieri (2006), who have shown that M2-muscarinic receptor protein density correlates directly with the respective transcripts, whilst no correlation exists between the protein and transcript levels of the M3-muscarinic receptor subtype in rat urinary bladder. Indeed if this is true, then the decreased expression of M3-muscarinic receptors found in 4-week to 12-week diabetic rat bladders in the present study may not be reflected at the protein level. This may also mean that the increased SA detected in diabetic rat bladders (except 12-week diabetics) may be mediated via a combination of both receptors. The latter hypothesis is supported by a recent study demonstrating that activation of M2- as well as M3-muscarinic receptors by endogenous ACH can enhance spontaneous contractions in whole bladder preparations from neonatal and postnatal rats (Ng et al, 2006). Mukerji et al (2006) also showed increased immunostaining for M2- and M3-muscarinic receptors in suburothelial myofibroblast-like cells in painful bladder syndromes and idiopathic DO, which correlated with clinical scores of urgency, suggesting a role in pathophysiology. Further studies looking at the expression levels of these receptors at the protein level in the detrusor, mucosa and ICs and using more specific muscarinic receptor agonists and antagonists would enhance our understanding of their role in mediating detrusor contractions in this animal model.

Endogenous ACH released in the bladder may also induce the release of other neurotransmitters, such as ATP, by stimulation of muscarinic receptors in the urothelium. This could induce DO by stimulating purinergic receptors expressed on the afferent nerves and ICs, further adding to the complicated interactions between different components of the bladder wall mediating detrusor contractility (Wu et al, 2004; reviewed by Fowler et al, 2008). The role of ATP and purinergic receptors was not investigated in the rat bladder in this study, but may be worth future investigation.

7.2 Smooth muscle cell excitability mediating contractile function in rat urinary bladder

As well as changes in cholinergic modulation of bladder contractility, changes in UBSM and IC excitability may also be important in bladder dysfunction, especially since over recent years it has become clear that SA of the urinary bladder is dependent upon electrical properties of smooth muscle cells. Cholinergic stimulation of UBSM contraction and M3-muscarinic receptor pathway, as well as the electrical properties of smooth muscle cells and ICs, are dependent upon ion conductance across the cell membrane. It has been demonstrated that M3-muscarinic receptor activation results in urinary bladder smooth muscle (UBSM) membrane depolarisation as a result of Ca^{2+} entry through voltage dependent Ca^{2+} channels (VDCCs) (Ekman et al, 2006; Ekman et al, 2009). Ca^{2+} entry through VDCCs leads to an increase in global intracellular Ca^{2+}, which is important in initiation of action potentials that underlie the spontaneous contractions (Ekman et al, 2009). The repolarisation phase of the UBSM action potential, regulation of Ca^{2+} entry and subsequent relaxation of UBSM is mediated by the activity of various K^+ channels, especially BK_{Ca} channels. Since opening of K^+ channels leads to relaxation of the detrusor, agents that mediate the activation of these channels are being considered as an alternative therapeutic treatment for DO and OABS (reviewed by Sellers & McKay, 2007).

The role of K^+ channels is not only important in mediating the repolarisation of UBSM action potentials and relaxation, but it has also been shown that their inhibition via the M2-muscarinic receptor pathway contributes to UBSM

contraction (Nakamura et al, 2002), highlighting the complex interactions of these channels with various components of signalling pathways mediating USBM contractile activity. IC cells within the bladder have also been shown to possess Ca^{2+}-activated and voltage-dependent K^+ currents (McCloskey, 2005). These studies highlight the importance of K^+ channels in mediating detrusor contractility. Thus, the role of K^+ channels in mediating CCH-induced SA was investigated in this study.

In an attempt to elucidate the role of large conductance Ca^{2+} activated K^+ BK_{Ca} channels in modulating basal and CCH-induced SA in detrusor strips from control (non-diabetic) and diabetic rats, tissues were incubated with a blocker (iberiotoxin (IBTX)) or an opener (NS1619) of this channel respectively (chapter 5). It was demonstrated that opening of BK_{Ca} channels reduces only the amplitude of SA in detrusor strips from both control (non-diabetic) and diabetic rats and that this effect was seen only at the highest concentration of NS1619. The lack of functional responses of detrusor strips from control (non-diabetic) and diabetic rats to NS1619 in this study are in line with a recent report demonstrating that modulation of BK_{Ca} channels is not important in mediating CCH-induced SA of detrusors from newborn and adult mice (Ekman et al, 2009). The possible reasons for this lack of functional response of detrusor strips from the rat to NS1619 in the current study could be that modulation of channels under the current experimental set up may not necessarily translate to a functional physiological response. In this study the measured variable is the contractile response of detrusor strips, which is modulated by various complicated mechanisms and signalling pathways. For example as well as smooth muscle cells, ICs within the bladder in guinea-pigs and humans also demonstrate BK currents (Sui et al, 2004; McCloskey, 2005) and it could be speculated that ICs may also be involved in the functional responses of rat bladders to BK_{Ca} channel modulators. These complex interactions may act together to produce the final result: contraction of the bladder. Therefore, in order to understand the role of NS1619 in modulating the SA of the bladder, each of these contributing factors must be dissected and studied individually.

Although opening of BK_{Ca} channels had limited effect in mediating the SA, inhibition of these channels by the specific BK_{Ca} channel blocker IBTX did significantly affect the basal SA of detrusor strips from control (non-diabetic)

and diabetic rats, demonstrating that these channels may be important in modulating the basal membrane excitability in UBSM of the rat. These results are further supported by various studies demonstrating that BK_{Ca} channel blockade in UBSM cells results in increased basal SA in detrusors from various species (Imai et al, 2001; Buckner et al, 2002; Darblade et al, 2006; Ng et al, 2007). 1-week diabetic tissues appeared to be more sensitive to modulation by IBTX, whilst 12-week diabetic tissues were relatively insensitive to this blocker, again highlighting the clear differences in bladder function between 1-week and 12-week diabetic rats. The underlying reasons for the differences in the sensitivity of 1-week and 12-week diabetic tissues to IBTX are discussed in Chapter 5, but may be due to different underlying mechanisms controlling the membrane potential of UBSM cells or altered levels of UBSM activity and excitability in these rats. The role of ICs in modulating the basal SA in rat bladder must also be considered. Whatever the underlying cause, the increased spontaneous contractions as a result of BK_{Ca} channel inhibition may result in increased afferent output from the bladder and cause DO.

An important finding from the current study was that the mRNA expression of BK_{Ca} channel subunits decreased with duration in diabetic tissues. This observation did not correlate with the pattern of SA detected in 1-week-12-week diabetic tissues, but could explain the altered levels of UBSM activity and excitability. Measuring the BK_{Ca} currents at single cell level in UBSM cells and ICs using electrophysiology in control (non-diabetic) and diabetic tissues could further clarify the role of these channels in mediating the SA of the detrusor.

In terms of the role of other Ca^{2+} activated K^+ channels, only the effect of blocking small conductance Ca^{2+} activated K^+ (SK_{Ca})channels on basal SA was investigated in control (non-diabetic) and diabetic tissues. SK_{Ca} channels did not play a role in mediating the UBSM membrane potential and SA in these rats, which is in contrast to the results of other studies demonstrating the importance of SK_{Ca} channels in modulating the SA in detrusor strips of various species (Herrera *et al,* 2003; Hougard *et al*, 2009). However, to confirm this, further analysis with various SK_{Ca} channel openers and studying the expression levels of these channels in UBSM would aid our understanding of the role of these channels in rat urinary bladder.

The role of ATP sensitive K^+ (K_{ATP}) channels in modulating CCH-induced and basal SA was also investigated in detrusor strips from controls (non-diabetic) and diabetic rats. In contrast to the effect of the BK_{Ca} channel opener NS1619, opening of K_{ATP} channels by cromakalim inhibited the frequency of SA only, demonstrating that opening of these channels in UBSM may be affecting action potential activation and firing. Detrusor strips from diabetic rats demonstrated a trend towards being less sensitive to inhibition by cromakalim than detrusor strips from control (non-diabetic) tissues. This may be due to downregulation of K_{ATP} channel activity in the diabetic tissues as a result of decreased expression of K_{ATP} channel subunits, which was detected at the mRNA level in the current study. Regulation of K_{ATP} channel function and expression by hyperglycaemia in various cell types has been reported previously (Acosta-Martinez & Levine, 2007; Kinoshita et al, 2008). For example in the study by Acosta-Martinez & Levin (2007), it was demonstrated that hyperglycaemia reduces the expression of K_{ATP} channels in brain cells and neurons, and this reduction may result in altered excitability of these cells. The same mechanism could be postulated in UBSM cells of diabetic rat bladder. However, measuring the glucose levels in UBSM cells and its' role in mediating various regulatory pathways leading to detrusor contractility and excitability were beyond the scope of this thesis and could be investigated in future research. Further experiments elucidating the expression of K_{ATP} channels at the protein level could also be useful. Also since the SA was induced by stimulation of the tissues with CCH, it could be postulated that muscarinic receptor activation may inhibit K_{ATP} channel activity, especially where an increased expression of M2- and M3-muscarinic receptors mRNA has been detected (1-week diabetic rats). In other words cholinergic stimulation of muscarinic receptors may initiate a cascade of events in UBSM leading to decreased activity of K_{ATP} channels, and since there is an increased expression of muscarinic receptors in 1-week diabetic tissues, this inhibitory effect on the K_{ATP} channels may be enhanced, resulting in a decreased functional response to K_{ATP} channel opener, cromakalim.

In contrast to the effect of cromakalim, the K_{ATP} channel blocker glibenclamide, failed to have a significant effect on the basal SA in both control (non-diabetic) and diabetic tissues, indicating that the blockade of K_{ATP} channels at single channel level may not be adequate to result in an altered measurable functional

response in tissue bath experiments. Another possibility may be that blocking K_{ATP} channels is not important in regulating the SA under basal conditions.

Since opening of BK_{Ca} channels resulted in inhibition of the amplitude of SA and opening of the K_{ATP} channels inhibited the frequency of SA, it could be postulated that BK_{Ca} and K_{ATP} channels on smooth muscle act together via complex mechanisms, which may also involve interactions with ICs and other signalling pathways associated with muscarinic receptor stimulation in UBSM cells, to modulate the SA in rat urinary bladder.

IK_{Ca} and K_v channels may also be important in mediating the UBSM membrane potential and the SA of the detrusor from control (non-diabetic) and diabetic rats. Thus, future investigations on the role of these channels may also be useful.

7.3 Summary and conclusions

In conclusion the results of the present study demonstrate that in order to understand the mechanisms underlying normal and abnormal detrusor contractions, it is important to consider not only the pathways that mediate direct UBSM cell contraction, but also the complex interaction of various mechanosensory components of the urinary bladder wall such as the mucosa, afferent nerves and ICs, that have recently become a focus of interest in this area.

In this thesis, the physiology and pathophysiology of detrusor in normal and diabetic rat bladders were explored. It was demonstrated some changes within the cholinergic pathways mediating detrusor contraction in the animal model, especially those mediating the spontaneous contractions of the bladder in these animals. Marked differences were detected in SA of the bladders from 1-week diabetic and 12-week diabetic animals.

This study also identified a network of specialised cells (ICs) in the rat urinary bladder and it was demonstrated that these ICs have a role in mediating the spontaneous contractions of the detrusor under normal and diabetic states.

The role of various K^+ channels modulators on modulating the cholinergic induced SA was investigated. Some changes were detected in the diabetic tissues in their response to K^+ channel modulation.

Since the detrusor strips from 1-week diabetic animals demonstrated the most striking characteristics, some of which have also been demonstrated in overactive bladders, then it can be concluded that diabetic animals at this duration of diabetes could be used as a model of DO.

The data presented in this thesis indicates that complex mechanisms serving basic physiological processes are present in the urinary bladder. However, it is clear that not enough is known about the detailed integrated physiology of the bladder wall and the structures involved. In the case of spontaneous contractions of the bladder, data are highly suggestive that this system plays an important role in generating and modulating sensations originating in the

bladder wall and involves complex interactions between various components present in the urinary bladder. It would only be possible to gain a better perspective of this sensory system from detailed analysis of the integrated whole isolated organ. Such complex events can not be predicted from data on small pieces of tissue obtained from human patients, emphasising the importance of animal models in revealing basic principles of bladder function and control.

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