

**The Pharmacology of the Autonomic  
Control of the Female Rat Urethra:  
Relevance to Micturition**

**Thesis submitted for the  
Degree of Doctor of Philosophy**

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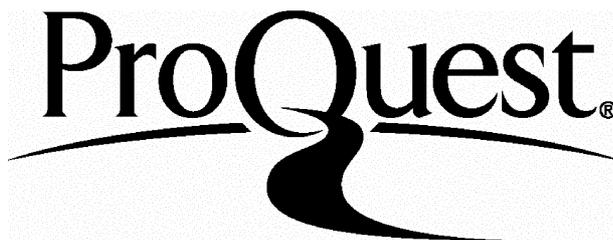
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*To my family*

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## LIST OF ABBREVIATIONS

ATP	Adenosine 5' Triphosphate
DMPP	1,1-Dimethylphenylpiperazinium
EUS	External Urethral Sphincter
cGMP	Guanosine 3':5'-cyclic monophosphate
HR	Heart Rate
IML	Intermediolateral Cell Column
L-NA	N $\omega$ -nitro-L-arginine
L-NAME	N $\omega$ -nitro-L-arginine methyl ester
LUT	Lower Urinary Tract
MAP	Mean Arterial Pressure
MPG	Major Pelvic Ganglion
MUI	Mixed Urinary Incontinence
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
ON	Onuf's Nucleus
OUI	Overflow Urinary Incontinence
PAG	Periaqueductal Grey
PDE	Phosphodiesterase
PMC	Pontine Micturition Centre
PSS	Pontine Storage Centre
SNP	Sodium Nitroprusside

SPN	Sacral Parasympathetic Nucleus
TTX	Tetrodotoxin
SUI	Stress Urinary Incontinence
UI	Urinary Incontinence
UUI	Urge Urinary Incontinence

## ABSTRACT

The storage and periodic elimination of urine (micturition) is dependent upon a complex co-ordination of activities in the bladder and urethra. Nitric oxide (NO) has been suggested to play a role in the urethral control. The effects of NO are mediated by increases in guanosine 3':5'-cyclic monophosphate (cGMP) formation, which is broken down by a family of phosphodiesterases (PDEs), including PDE 5. However, the exact roles of this transduction system in the urethra are unknown. Therefore, the present experiments examined the effects of this pathway on changes in urethral and bladder pressures caused by bladder distension (micturition reflex) in urethane-anaesthetised female rats. As changes in urethral tone are due to both smooth and striated muscle (external urethral sphincter; EUS) EMG recordings were made to assess changes in EUS tone (EUS-EMG). The roles of NO/cGMP signalling *in vitro* were examined in the isolated female rat urethra.

L-NAME, a NO synthase (NOS) inhibitor significantly attenuated reflex-evoked urethral relaxations and increased EUS-EMG activity and baseline urethral tone. Following neuromuscular blockade with  $\alpha$ -Bungarotoxin ( $\alpha$ -BT), L-NAME still attenuated urethral relaxations, but did not cause an increase in EUS-EMG or baseline tone. This supports the view that NO mediates urethral smooth muscle relaxations during voiding, and also indicates that there is a tonic release of NO inhibiting EUS-EMG activity. As would be expected, zaprinast, a PDE types 1, 5, 6 and 9 inhibitor, significantly potentiated reflex-evoked urethral relaxations. However, zaprinast also increased background and reflex-evoked EUS-EMG activity. Surprisingly, this ability to potentiate reflex-evoked relaxations was blocked by  $\alpha$ -BT indicating that it is due to the ability of zaprinast to increase EUS-EMG activity. It is possible that this ability to increase EUS-EMG is indirect by causing changes in smooth muscle tone. This was investigated by examining the effects of urethral smooth muscle relaxation with intraurethral perfusion of isoprenaline and sodium nitroprusside on EUS-EMG activity. These relaxations were associated with an increase in EUS activity, and were inhibited by the ganglion blocker, chlorisondamine, supporting this view. Further experiments in the isolated female rat urethra demonstrated that L-NAME increased urethral tone and attenuated electrically-evoked (TTX sensitive) urethral relaxations. Nicotinic agonists evoked urethral relaxations, which were attenuated by L-NAME and inhibited by hexamethonium and chlorisondamine, but were TTX resistant. Zaprinast decreased urethral tone, although re-tensioning tissues to pre-drug levels unmasked a potentiation of the duration of electrically-evoked urethral relaxations at low frequencies of stimulation (1-4 Hz).

These results indicate that reflex-, electrical- and nicotinic agonist-evoked urethral relaxations are mediated by NO. *In vitro*, these effects appear to be mediated by increases in cGMP. However, the ability of zaprinast to potentiate urethral relaxations *in vivo* are due to an ability to increase EUS activity, and conclusive evidence for the involvement of cGMP in the urethra *in vivo* could not be demonstrated. Further, the data indicates that there is an interaction between urethral smooth and striated muscles, mediated by ganglionic nicotinic receptors. In addition to the complex effects of the NO/cGMP pathway on this musculature, the above interactions complicate investigations regarding the effects of zaprinast in the urethra *in vivo*.

# **Chapter One**

## **Introduction**

## **Introduction**

The storage and periodic elimination (micturition) of urine is dependent on a complex co-ordination of activities in two important structures in the body, the bladder and urethra, which constitute the main proportion of the lower urinary tract (LUT). The ability to store and void urine has evolved into important aspects of both physiological and behavioural functioning in a number of species. In all species, in eliminating urine from the body, the bladder and urethra act in the excretion of waste products, foreign compounds and excess water and ions, thereby contributing to homeostasis (see Frings, Purves & Macknight, 1990). In some species, the elimination of urine also plays a large role in behavioural activities, such as territory marking (see Van Asselt, Groen & Van Mastrigt, 1995). In the human, the bladder and urethra confer an ability to temporarily store urine until a socially convenient time to eliminate the contents of the bladder becomes available. In this respect, the bladder and urethra are unique from other visceral structures in that they are under voluntary control (see de Groat, 1993). The combined activities of the bladder and urethra are effected by a complex integration of parasympathetic, sympathetic, somatic and sensory systems, which form an intricate series of reflexes (see de Groat, Booth & Yoshimura, 1993). Furthermore, these co-ordinated reflexes are completely dependent on complex neural control systems located in the central nervous system. This distinguishes the control of the LUT from many other physiological systems, such as the cardiovascular system, that maintain a certain level of function even after elimination of neural input. Disturbances in any aspect of this complex relationship between the nervous system and the bladder and urethra

results in disorders of normal LUT function. The neural control of the bladder and urethra is further complicated by the presence a large number of neurotransmitters. However, this does provide many selective targets for the treatment of LUT dysfunction (see Andersson, 1993).

### 1.1. Structure of the bladder and urethra

The bladder, in the rat and other species, lies in the pelvis, ventral to the rectum (and also to the uterus, cervix and vagina in the female), and dorsal to the symphysis pubis. The bladder receives urine from the kidneys via the ureters, which enter the dorsal wall of the lower region of the bladder. Just below the level of these vesico-ureteric junctions, the walls of the bladder narrow to form the bladder neck, from which the urethra projects, which is a tubular structure through which urine is expelled to the outside (Blandy & Fowler, 1996).

The bladder is divided into two distinct regions on the basis of embryonic origin in all mammalian species, including the rat and human. The *detrusor*, derived from endoderm, forms the smooth muscle of the fundus of the bladder, and is highly distensible. In contrast, the *trigone*, derived from mesoderm, is a triangular area located dorsally at the bladder neck that undergoes little change in size during bladder filling. The principal function of the trigone is thought to be in control of the ureteric orifices, to prevent reflux of urine into the ureters when the pressure in the bladder rises during voiding (Shafik, 1998). Histologically, the innervation of the trigonal elements of the bladder base is similar to those of

the detrusor elements of the bladder base (Raezer *et al.*, 1976), and therefore the division of the bladder into these segments is considered to be inappropriate pharmacologically. However, the bladder can be divided into distinct regions based on physiological and pharmacological criteria at the level of the ureteric orifices into rostral and caudal segments, termed *body* and *base*, respectively (see de Groat & Booth, 1980a).

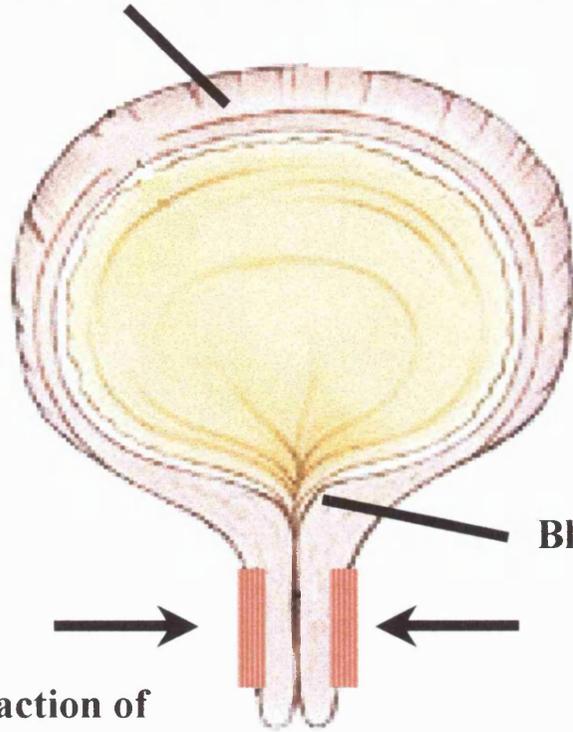
In all mammalian species, the smooth muscle of the bladder consists of three layers, inner and outer longitudinal and middle circular, which interlace to form a complex meshwork, such that discrete layers are not readily discernible (Gosling & Dixon, 1975). This has functional relevance since a contraction of this interconnected muscle bundle will cause a reduction in all dimensions of the bladder lumen during micturition (Gosling, 1979). In the bladder neck, circular smooth muscle is present, which extends distally to surround the proximal urethra completely. This muscle is more prominent in the male than the female urethra, and extends further along the length of the proximal urethra (Gosling & Dixon, 1975). The urethra also possesses an inner longitudinal muscle layer that is continuous with the inner longitudinal coat of the bladder, and forms the greater portion of the urethral wall. In all species, a well-developed sleeve of striated muscle surrounds the urethra, with its thickest part at the middle two-thirds (Jonas & Tanagho, 1976; Thind, 1995). These striated muscle fibres have a circular orientation and are collectively termed the *rhabdosphincter* or external urethral sphincter (EUS; see Lincoln & Burnstock, 1993).

## **1.2. Functional changes in the bladder and urethra during urine storage and voiding**

The storage and elimination of urine are dependent on a complex co-ordination of activities in the bladder and urethra. In mechanical terms, the bladder essentially acts as a reservoir during storage and a pump during voiding, with the urethra providing not only the necessary resistance to maintain continence, but also to allow complete evacuation of urine from the bladder (see de Groat, Booth & Yoshimura, 1993). During urine storage, the bladder neck and proximal urethra are closed. Sphincteric mechanisms within the urethra mediate this closure mechanism (Figure 1.1; see also section 1.3 of this chapter). The smooth muscle of the detrusor is inhibited from contracting during this period, thus allowing the bladder to accommodate increasing volumes of urine. Involuntary voiding occurs in adult mammalian species and the neonatal human when the bladder reaches capacity, however it can also be initiated voluntarily in the adult human. The initial event of micturition is a reduction in urethral pressure, followed by a large detrusor contraction, which is maintained until the bladder empties. The detrusor contraction is accompanied by an opening of the bladder neck and urethra. In addition, the closure mechanisms of the bladder neck and urethra that were essential during urine storage are now inhibited, further contributing to an overall reduction in outlet resistance. In rats and dogs, but not in humans, the striated muscle of the urethra also exhibits high frequency bursting during this period that is thought to be necessary for efficient voiding (Kakizaki, Fraser & de Groat, 1997; see section 1.6 of this chapter).

## URINE STORAGE

**Detrusor inhibited from contracting**

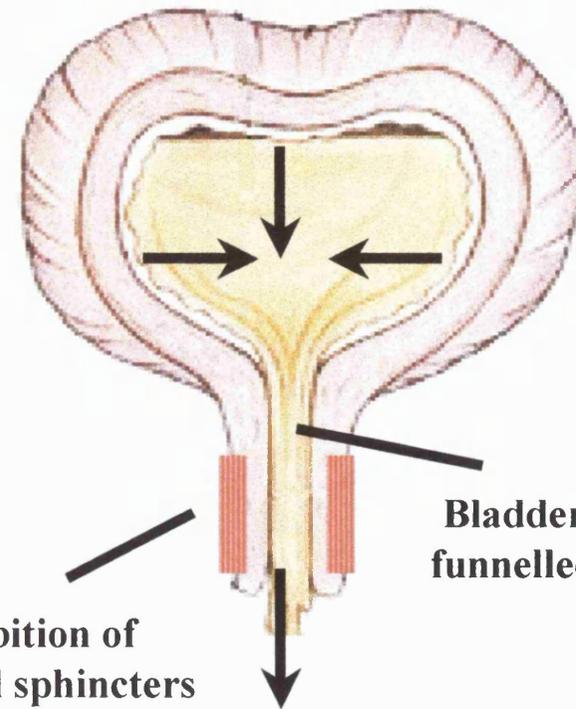


**Bladder neck closed**

**Contraction of urethral sphincters (EUS and smooth muscle)**

## VOIDING

**Active detrusor contraction**



**Bladder neck funnelled open**

**Inhibition of urethral sphincters**

**Figure 1.1.** Functional changes in the bladder and urethra during urine storage and voiding (EUS, external urethral sphincter).

### **1.3. Urethral sphincters and mechanisms of urinary continence**

The precise mechanisms of urinary continence during urine storage have been the subject of wide controversy for more than 100 years (Kleeman, 1970). Essentially, two sphincteric mechanisms in the urethra have now been described. Although not a distinct anatomical structure, the internal sphincter is a proximal physiological mechanism that consists of the circular smooth musculature of the bladder neck and proximal urethra. The concept of this internal sphincter mechanism was first suggested by Krane & Olsen (1973), and is thought to be mediated not only by the urethral smooth muscle, but also by an abundance of elastic tissue in the submucosa and the presence of a rich vascular plexus (de Groat & Booth, 1980; Gosling, 1979). More distally, the EUS comprises the striated muscle fibres of the urethra that become intermingled with smooth muscle as they extend proximally and distally along the urethra (see Lincoln & Burnstock, 1993). The striated muscle of the urethra is more prominent in males than in females in all species, and as a result, the EUS can maintain continence in males even when the internal sphincter has been damaged (Turner-Warwick, 1975). Similarly, blockade of the nerves that supply the striated muscle of the urethra, or the use of striated muscle relaxants, do not cause urinary incontinence provided the internal sphincter is competent, in both sexes (Kleeman, 1970).

The overall contribution of the smooth and striated muscle urethral components in maintaining urinary continence remains to be fully elucidated. Studies in human

females have demonstrated that urethral smooth muscles contribute only a minor degree to resting urethral pressure (Thind, 1995). In contrast, the striated muscle is believed to play a paramount role in not only the maintenance of urethral tone at rest, but also during dynamic events such as physical activity and coughing. Indeed, blockade of the nerves that supply the striated muscle in the human female produces a notable reduction in the pressure and power generation of the urethra during coughing and pelvic floor squeezing (Thind, 1995).

#### **1.4. Bladder compliance and mechanisms of urine storage**

The bladder has a remarkable ability to maintain low intravesical pressures during bladder filling (Coolsaet, 1985). In this way, bladder pressure remains lower than intraurethral pressure, and continence is maintained until the volume of fluid in the bladder reaches threshold and micturition is initiated. The exact mechanisms mediating these effects are the subject of much dispute, with conflicting theories emerging. The myogenic theory holds that bladder tonus and accommodation result from the intrinsic properties of the bladder wall and are therefore independent of neural activity. The neurogenic theory argues an involvement of neural input (see Finkbeiner, 1999). To date, a large body of evidence provides support for the former theory in that if visceral smooth muscle is stretched, it first exerts increased tension, which gradually decreases to pre-stretched levels, a phenomenon termed 'stress-relaxation' (see Finkbeiner, 1999; Benson *et al.*, 1975). Furthermore, the response of the bladder to filling is unchanged following sympathectomy, parasympathectomy

or complete denervation (Klevmark, 1974, 1977; Tang & Ruch, 1955), suggesting that the properties of the bladder wall play a paramount role in bladder compliance.

## **1.5. Autonomic innervation of the bladder and urethra: history and current knowledge**

### **1.5.1. Parasympathetic nervous system: innervation and function**

Investigations regarding the role of the parasympathetic nervous system in the control of the LUT began in 1863 when Gianuzzi demonstrated that fibres causing a strong contraction of the bladder in the dog arose from sacral spinal segments. Langley & Anderson (1895) also showed that stimulation of motor fibres in the sacro-coccygeal region of the dog and rabbit produced micturition. During the course of his extensive work on the reflex control of the LUT between 1915 and 1941, Barrington demonstrated that bladder distension caused a bladder contraction, and that the efferent pathways concerned with this response ran in the pelvic nerves (1915, 1921 & 1925). Studies since this time have provided further evidence that parasympathetic innervation provides the main excitatory input to the bladder to evoke a contraction during micturition (Burnstock *et al.*, 1970; Elmer, 1975; Theobald, 1986).

The pelvic nerves originate from the sacral parasympathetic nucleus, located in the intermediolateral grey matter of the spinal cord. Parasympathetic preganglionic

neurones leave the spinal cord and enter the pelvic plexus, from which postganglionics emerge to innervate the LUT (see section 1.5.2 of this chapter). Species variation exists with regard to the lumbosacral segments from which preganglionic parasympathetic neurones arise (Table 1.1). In the rat, the sacral parasympathetic nucleus is localised to spinal cord segments L6-S1 (Fletcher & Bradley, 1978; Hancock & Peveto, 1979), although Pascual, Insausti & Gonzalo (1989) have suggested that differences exist amongst strains of rat with respect to the lumbosacral levels from which parasympathetic preganglionic neurones arise, originating in the S1 and S2 spinal segments of the Wistar rat, and the L6 and S1 segments in Sprague Dawley strains. However, further studies have demonstrated that bladder pathways project to the L6 and S1 spinal segments in both strains of this species (Birder & de Groat, 1992; Jansco & Maggi, 1987), and electrical stimulation of these spinal roots evokes bladder contractions in both Sprague-Dawley and Wistar rats, with no responses observed in either of these strains following stimulation of S2 spinal cord segments (Martinez-Pineiro *et al.*, 1992).

The pelvic nerves also convey parasympathetic input to the urethra, where the parasympathetic nervous system has important roles in urethral function during micturition. One of the earliest reports of a parasympathetic function in the urethra was by Barrington in his series of reflexes (1931, 1941), who demonstrated that bladder distension caused a relaxation of the urethra in cats, and that this response involved a pelvic nerve efferent pathway. These findings in the anaesthetised cat have been corroborated by other investigators (Slack & Downie, 1983; McGuire &

Herlihy, 1978), and stimulation of the parasympathetic spinal roots has also been shown to evoke urethral relaxations in the anaesthetised rat (Fraser, Flood & de Groat, 1995) and in man (Torrens, 1978; Van Kerrebroeck *et al.*, 1991). There have also been suggestions of a parasympathetically-mediated contractile response of the urethra during micturition, although the exact functional importance of these effects remain to be fully elucidated (see section 1.11.2. of this chapter)

Species	Afferent Pathway			Efferent Pathway		
	Pelvic	Hypogastric	Pudendal	Pelvic	Hypogastric	Pudendal
Rat	L6-S1 <sup>a</sup>	T13-L3 <sup>d</sup>	L5-L6 <sup>g</sup>	L1-L6 <sup>j</sup>	L1-L2 <sup>m</sup>	L5-L6 <sup>p</sup>
Cat	S1-S3 <sup>b</sup>	L2-L5 <sup>e</sup>	S1-S2 <sup>h</sup>	S1-S3 <sup>k</sup>	L3-L5 <sup>n</sup>	S1-S2 <sup>q</sup>
Human	S2-S4 <sup>c</sup>	L3-S1 <sup>f</sup>	S2-S3 <sup>i</sup>	S2-S4 <sup>l</sup>	L1-L4 <sup>o</sup>	- *

**Table 1.1.** The spinal cord segment origin of afferent and efferent neurones innervating the bladder and urethra in the rat, cat and human.

<sup>a</sup> Fletcher & Bradley, 1978, <sup>b</sup> Morrison, 1987, <sup>c</sup> Thomas, 1982, <sup>d</sup> Nadelhaft & McKenna, 1987, <sup>e</sup> Morgan, de Groat & Nadelhaft, 1986, <sup>f</sup> Steers, 1994, <sup>g</sup> McKenna & Nadelhaft, 1986, <sup>h</sup> Thor *et al.*, 1989, <sup>i</sup> Schroder, 1981, <sup>j</sup> Birder *et al.*, 1991, <sup>k</sup> Morgan, Nadelhaft & de Groat, 1981, <sup>l</sup> White, 1943, <sup>m</sup> Nadelhaft & Vera, 1991, <sup>n</sup> Appelbaum, Vance & Coggeshall, 1980, <sup>o</sup> Janig & Morrison, 1986, <sup>p</sup> Hulsebosch & Coggeshall, 1982, <sup>q</sup> Thor *et al.*, 1989

\* To the author's knowledge, there are no studies regarding the spinal cord origin of pudendal afferents in man. For references in full see chapter seven.

### **1.5.2. The pelvic plexus**

The pelvic plexus is a bilateral association of neurones and ganglia that lie at the base of the bladder, and receive inputs from the pelvic and hypogastric nerves. The pelvic plexus varies considerably between species and genders, in both morphology and location. In many mammals, including cat and man, the ganglia of the pelvic plexus are numerous and distributed broadly among the pelvic organs (Kuntz & Moseley, 1936; Wozniak & Skowronska, 1967). In addition, many of these ganglion cells are located close to the wall of the pelvic organs or within their outer layers (Fletcher & Bradley, 1969; Gosling & Thompson, 1977). Examples include ganglia in the human (Fletcher & Bradley, 1969), cat (Dixon & Gosling, 1974) and guinea pig (Crowe, Haven & Burnstock, 1986) bladder wall, often referred to as 'vesical ganglia', and in the human urethral smooth and striated muscles (Crowe, Burnstock & Light, 1988). In the rat, a simpler arrangement of pelvic autonomic neurones exists, in which the pelvic neurones are aggregated into bilateral macroscopic collections of ganglion cells, which are collectively known as the major pelvic ganglion (MPG) in the male rat (Langworthy, 1965), and paracervical ganglion or, in older studies, Frankenhausers ganglion, in the female rat (Keast, 1999). Furthermore, in the male rat, smaller satellite or 'accessory ganglia' occur around the ureter and prostatic end of the vas deferens (Keast, Booth & de Groat, 1989). However, ganglia in the bladder wall and urethra have not been identified in the rat (Gabella & Uvelius, 1990), and therefore this species provides a simple model to study the effects of denervation on detrusor morphology and function. The

removal of both pelvic ganglia in the rat results in complete denervation (Carpenter & Rubin, 1967). There is a marked sexual dimorphism in the pelvic plexus, with many more pelvic neurones in males than in females. Greenwood, Coggeshall & Hulsebosch (1985) have demonstrated that there are approximately 15,000 and 6,000 pelvic neurones in male and female rats, respectively. These authors suggested that this difference is largely explained in terms of the relative density of autonomic nerve terminals within the reproductive organs, with an extensive distribution to the male reproductive organs, but a sparse supply to the female organs. The pelvic plexus exhibits viscerotopic organisation in that neurones occupy dorsal or ventral regions based upon their target organs. For example, neurones which innervate the penis and colon are largely found in the dorsal portion of the MPG, whereas bladder neurones are evenly distributed throughout the MPG in the male rat (Dail, 1996). Interestingly, the pelvic plexus is the only site in the autonomic nervous system where sympathetic and parasympathetic neurones occur in the same ganglia (Dail, 1996). These two groups of nerves are easily identifiable and discrete, entering the complex of the pelvic ganglia and subsequently sending off numerous branches to supply individual groups of neurones (Keast, 1999). As a result, the pelvic plexus is not just considered to function merely as a relay station, but is thought to participate in the complex regulation of neural activities responsible for effecting co-ordinated activities in the bladder and urethra during urine storage and voiding.

### **1.5.3. Sympathetic nervous system: innervation and function**

In all species, the sympathetic supply to the bladder and urethra arises from the intermediolateral nuclei of the low thoracic and upper lumbar segments of the spinal cord (Table 1.1). Preganglionic sympathetic fibres travel in the splanchnic nerves either to the inferior mesenteric ganglia, or pass caudally in the paravertebral chains to the level of the lumbosacral sympathetic chain ganglia. The hypogastric nerves arise from the inferior mesenteric ganglia, and carry a mixture of both preganglionic and postganglionic sympathetic nerve fibres to the bladder and urethra. The ratio of pre- to postganglionic axons in the hypogastric nerve varies considerably between species, being largely postganglionic in the cat, and preganglionic in the rat (Janig & McLachlan, 1987). Interestingly, sympathetic fibres originating from the sympathetic chain account for approximately 80-90% of the sympathetic innervation of the bladder in the rat (Vera & Nadelhaft, 1992), compared to only 40% for the cat (Downie, Champion & Nance, 1984). From the sympathetic chain, sympathetic fibres join preganglionic parasympathetic axons in the pelvic nerve (de Groat & Booth, 1980a). Therefore, the pelvic nerve cannot be described as exclusively parasympathetic. Indeed, it has been estimated that there are at least twice as many sympathetic postganglionic fibres as parasympathetic preganglionic fibres in the pelvic nerve of the cat (Kuo, Hisamitsu & de Groat, 1984). In addition, sympathetic axons represent approximately 25% of the efferent fibres in the pelvic nerve of the rat (Hulsebosch & Coggeshall, 1982). Sympathetic nerves therefore reach the pelvic plexus via both the hypogastric and pelvic nerves. Studies of sympathetic neurones

in the pelvic ganglia have shown they are resistant to reserpine and 6-hydroxydopamine treatment (see Keast, 1999). Furthermore, due to their close proximity to their target organs, these neurones are distinct from 'classic' sympathetic noradrenergic neurones, and are therefore often termed 'short adrenergic neurones' (see Keast, 1999).

The role of the sympathetic nervous system in the control of the bladder is the subject of much debate. The observation that an intact sympathetic supply is not a requirement for micturition to take place (de Groat & Booth, 1984), has questioned whether the sympathetic nervous system possesses any essential functions in the LUT, especially with regard to the human. Sympathectomy and operations that affect sympathetic ganglia rarely cause a lasting effect on the micturition of an individual (Nordling, Christensen & Gosling, 1980). However, conversely, side effects such as urinary retention caused by sympathomimetics (e.g. ephedrine for asthma) or urinary incontinence following administration of sympatholytics (e.g. prazosin for hypertension) are frequently observed (Bradley & Sundin, 1982). The debate has been complicated by a variety of conflicting experimental evidence from a number of animal species. Early studies by Gianuzzi (1863) demonstrated that pricking the spinal cord at the level of the lower part of the third lumbar vertebra in the dog produced a strong bladder contraction. This contractile response was abolished after section of the nerves running from this spinal segment, which have since been identified as the hypogastric nerves. Contractile responses of the bladder to hypogastric nerve stimulation have also been demonstrated in the rat (Elmer,

1975), guinea pig (Mantegazza & Naimzada, 1967) and cat (de Sy, Lacroix & Leusen, 1974). Von Zeissl first questioned the functional relevance of this sympathetic contractile response in the bladder in 1893. In accordance with the theory of 'crossed innervation' (that visceral organs receive both excitatory and inhibitory inputs from different sections of the nervous system), that was becoming increasingly popular around this time, this author suggested that the bladder must also receive 'inhibitor' nerves from the sympathetic roots. Indeed, Von Zeissl went on to demonstrate the existence of these fibres. In this respect, de Groat & Saum (1972) have observed a transient contraction of the bladder following hypogastric nerve stimulation, followed by a fall in bladder pressure below control levels in the anaesthetised cat. These inhibitory responses were shown to be due to a direct inhibition of bladder smooth muscle cells, as they were present in both decentralised and normally innervated bladder preparations. A direct inhibitory action on human bladder smooth muscle cells by isoprenaline, which activates  $\beta$ -adrenoceptors (see section 1.11.4 of this chapter), has also been reported (Hindmarch *et al.*, 1977).

Evidence has been provided for the presence of a sympathetic regulatory mechanism in the pelvic excitatory pathways to the bladder. Transection of the sympathetic nerves to the bladder in the cat results in an enhancement of both spontaneous and reflex-evoked bladder contractions (Edvardsen, 1968; Gjone, 1965b). Similarly, stimulation of the hypogastric nerve decreases both spontaneous and pelvic nerve-evoked bladder contractions in the anaesthetised cat (de Groat & Saum, 1972) and

dog (Ohtsuka *et al.*, 1980; Creed, 1979). An important site of action of this inhibition is thought to exist at the level of the pelvic ganglia. De Groat & Saum (1971) showed that stimulation of the hypogastric nerve possesses inhibitory effects on transmission in parasympathetic ganglia of the bladder in the cat. Moreover, a facilitatory effect of hypogastric nerve stimulation on transmission in these ganglia has since been described, and it has been shown that these contrasting inhibitory and facilitatory responses are mediated by specific receptor types (Keast, Kawatani & de Groat, 1990; see section 1.11.4 of this chapter). These authors have suggested that these two actions of the sympathetic nervous system on parasympathetic transmission may be important during different stages of urine storage or voiding. These interactions have also been demonstrated in the anaesthetised dog (Bosch *et al.*, 1990) and guinea pig (Dave & Dhattiwala, 1976), and histochemical studies have identified the presence of noradrenaline-containing nerve terminals alongside cholinergic ganglion cells in the human pelvic ganglion (Gosling & Thompson, 1977). However, there is no evidence for modulation of pelvic ganglionic synaptic transmission in the rat (Elmer, 1975; Mallory, Steers & de Groat, 1989; Tabatabai, Booth & de Groat, 1986). Indeed, there are few dendritic processes on pelvic ganglion cells of the rat, compared with numerous dendritic processes capable of integrating input from several neurones in the cat (Tabatabai, Booth & de Groat, 1986).

The contractile sympathetic input to the bladder has now been attributed to stimulation of different populations of sympathetic receptors according to different

stimulus characteristics and species used (Levin & Wein, 1995; see section 1.11.4 of this chapter), and it is now generally accepted that the physiological roles of sympathetic nervous system in the control of the bladder are inhibitory. These inhibitory effects are evoked following activation of bladder afferents in the pelvic nerve in response to bladder distension (de Groat & Lalley, 1972; de Groat & Theobald, 1976), and are thought to represent an intersegmental spinal 'storage' reflex whereby elevations in intravesical pressure trigger sympathoinhibitory input to the bladder smooth muscle and pelvic ganglia, to allow greater urine accommodation (Khadra, Satchell & Vaughan, 1995; see Steers, 1995). This spinal storage mechanism is augmented by further urine storage control sites that are located supraspinally (see section 1.8 of this chapter). Indeed, sympathectomy has been reported to decrease bladder capacity in the cat (Edvardsen, 1968; Gjone, 1965b) and dog (Nishizawa *et al.*, 1985). The importance of the sympathetic nervous system in the control of the bladder in man remains to be established owing to a lack of conclusive experimental evidence. However, the functions of the sympathetic nervous system in healthy humans may differ from those in conditions of bladder dysfunction. After long-term parasympathetic decentralisation, an increase in sympathetic innervation and a change in sympathetic receptor function has been observed in both the cat (Sundin & Dahlstrom, 1973; Norlen, 1977) and human (Sundin *et al.*, 1977) bladder, suggesting that the physiology of the sympathetic nervous system may be altered in various disease states.

The sympathetic nervous system also plays a role in the control of the bladder outlet. Sympathetic stimulation produces contractile responses of the bladder neck and urethra in the rat (Maggi *et al.*, 1989; Martinez-Pineiro *et al.*, 1992), cat (Elliot, 1907; McGuire & Herlihy, 1979; Kakizaki, Koyanagi & Kato, 1991) and dog (Creed, 1979; Poirier *et al.*, 1988). In these species, these responses are thought to be concerned with the maintenance of continence during urine storage, whereby contraction of the smooth muscle of the bladder neck and urethra acts to close the bladder outlet. These effects are closely co-ordinated with the functions of the somatic nervous system which act to increase the activity of the EUS to augment this urethral closure mechanism (see section 1.6 of this chapter). However, the exact roles of the sympathetic nervous system in the control of the urethra in the human are controversial. Sympathomimetic agents have been found to produce significant increases in urethral pressure (Ek *et al.*, 1977a; Nordling, 1983), a finding that is exploited therapeutically (see section 1.11.4 of this chapter). However, acute transection or chemical blockade of sympathetic and pudendal nerves in humans does not produce urinary incontinence (Bors & Comarr, 1971), and likewise, patients with dopamine  $\beta$ -hydroxylase deficiency who lack noradrenergic nerves are continent and have no voiding complaints (Gary & Robertson, 1994). These findings may reflect the complex combination of urethral smooth and striated muscles that are involved in the activities of the urethral sphincters, and thereby urinary continence.

Inhibitory urethral responses to stimulation of the hypogastric nerves in both the cat (Slack & Downie, 1983) and rat (Kontani & Shiraoya, 2000) have also been reported. Furthermore, urethral constrictions in response to hypogastric nerve stimulation in the cat are attenuated by ganglionic blockade, whereas hypogastric nerve-evoked urethral relaxations are resistant to ganglionic blockade, suggesting that these responses are organised differently and may be modulated independently (Slack & Downie, 1983). In similarity to the integration of sympathetic and parasympathetic inputs at the level of the pelvic plexus in the control of the bladder, it has been proposed that the activities of these neurones could also be regulated in the control of urethral function. Indeed, afferent and efferent discharge in the pelvic nerve seems to exert a modulatory effect on the urethral response to sympathetic stimulation in the anaesthetised cat (McGuire & Herlihy, 1979)

#### **1.6. Somatic nervous system: innervation and function**

In all species, the pudendal nerves supply the somatic innervation to the striated muscle of the EUS. Species variation exists with regard to the spinal cord segments from which somatic motoneurones arise (Table 1.1). Anatomically, somatic motoneurones are contained in dorsolateral and dorsomedial nuclei in spinal cord segments L5-L6 in the rat (McKenna & Nadelhaft, 1986), whereas in the human, monkey and cat, these neurones are contained within a single well defined nucleus, Onuf's nucleus (Onufrowicz, 1899).

A storage role has been ascribed to the pudendal nerves, based on findings that pudendal motoneurone firing occurs upon bladder filling to increase urethral striated muscle activity, and therefore increase urethral resistance (Diokno, Koff & Bender, 1974; Blaivas *et al.*, 1977). In addition to a sympatho-excitatory input to the urethra via hypogastric nerve inputs, this phenomenon constitutes the ‘guarding’ or ‘continence’ reflex, that is active during bladder filling (Park, Bloom & McGuire, 1997). During voiding, pudendal motoneurons in Onuf’s nucleus are inhibited, thereby inhibiting the EUS to promote an opening of the bladder outlet. This is achieved by the activation of supraspinal control centres (see section 1.8 of this chapter). In rats and dogs, but not humans, the striated muscle of the urethra also exhibits high frequency bursting during voiding that is mediated by the somatic nervous system (Kakizaki, Fraser & de Groat, 1997). This phasic activity may function like a pump to enhance urine flow along the urethra, and is therefore necessary for efficient voiding (Kakizaki, Fraser & de Groat, 1997). It has also been suggested that this phasic activity is involved in territory marking (Van Asselt, Groen & Van Mastrigt, 1995) or more recently, that it is purely a side effect of relaxations of the urethra that occur during micturition (Le Feber & Van Asselt, 1999).

### **1.6.1. Innervation of the EUS: The ‘triple innervation’ theory**

In addition to an established somatic innervation to the EUS, a number of histochemical and ultrastructural studies have suggested that the EUS also receives

parasympathetic and sympathetic innervation (Elbadawi & Schenk, 1974; Elbadawi & Atta, 1985). Hypogastric nerve stimulation has been shown to elicit electrical potentials of the EUS in anaesthetised cats with transected pudendal nerves (Kakizaki *et al.*, 1994). Moreover, normal electromyographic recordings of the urethral striated muscle have been observed in anaesthetised dogs following bilateral transection of the pudendal nerves, but these are inhibited after subsequent bilateral pelvic neurotomy (Morita *et al.*, 1984). Horseradish peroxidase studies have also suggested that the pelvic nerve may contain somatic fibres innervating the EUS (Morita *et al.*, 1984). However, other workers have disputed the triple innervation theory. Evidence for a lack of adrenergic innervation to the EUS has been provided from functional studies (Gajewski, Downie & Awad, 1984; Wein, Benson & Jacobowitz, 1979), and the fact that there is a considerable overlap of smooth and striated muscle fibres in the urethra raises the possibility that any autonomic nerves observed within the latter may simply be en route to the urethral smooth muscle (see Lincoln & Burnstock, 1993).

### **1.7. Afferent pathways from the bladder and urethra**

Bladder afferents carry information from tension receptors in the detrusor muscle to sacral spinal cord neurones, and provide a sensory input that controls the activities of the bladder, urethra and EUS (see Morrison, Wen & Kibble, 1999). These receptors are 'in series' tension receptors, so called because they increase their rate of discharge in response to bladder distension with bladder filling and also during

bladder contraction (Iggo, 1955; Habler, Janig & Koltzenburg, 1990, 1993). The former responses are physiologically important during the filling phase in that when a threshold level of afferent discharge is reached, micturition will be initiated, and the latter are thought to be important in the maintenance of a bladder contraction during voiding.

Afferent fibres from the lower urinary tract are carried in the pelvic, hypogastric and pudendal nerves. Species variation exists with regard to the level at which these afferents enter the spinal cord (Table 1.1). Nevertheless, in all species, there is a close correlation between the level of entry of pelvic nerve afferents and the level of the sacral parasympathetic nucleus (see Morrison, 1987), such that afferent information from the LUT is conveyed to this nucleus, which then feeds this information to higher control centres located supraspinally (see section 1.8 of this chapter). The most important afferents concerned with the initiation of the micturition reflex are carried in the pelvic nerve and comprise small myelinated A $\delta$  fibres (Mallory, Steers & de Groat, 1989; Vera & Nadelhaft, 1990b). Recordings from afferent fibres in the pelvic nerve in rats have found that 48% of these fibres are A $\delta$  fibres, with the rest comprising unmyelinated C-fibre afferents (Sengupta & Gebhart, 1994). In addition, approximately 95% and 75% of the afferent fibres in the hypogastric (Nadelhaft & Vera, 1991) and pudendal (Hulsebosch & Coggeshall, 1982) nerves, respectively, in the rat, have been classified as C-fibres. The exact function of these C-fibre afferents remains to be determined. In the rat, these fibres

have been found to be active during bladder distension (Sengupta & Gebhart, 1994). Furthermore, these afferents respond to changes in volume, not bladder contractions, and are therefore not 'in series' tension receptors (see Morrison, Wen & Kibble, 1999). However, activity in these afferents is triggered by chemical irritation of the bladder mucosa (Habler, Janig & Koltzenburg, 1990) or cold (Fall, Lindstrom & Mazieres, 1990), suggesting functions in the signalling of inflammatory or noxious events in the LUT. In chronic spinal cats, there is an emergence of a spinal C-fibre evoked bladder reflex (Shaker *et al.*, 1998), and this has been implicated in the pathogenesis of hyperreflexia in patients with spinal cord injury (Yoshimura, 1999).

Autonomic nerves arising from the urethra also contain afferents responsible for reflexes that influence bladder filling and emptying. Barrington (1931) demonstrated that urine flow or mechanical stimulation of the urethra evoked bladder contractions to maintain increased intravesical pressure during micturition in the anaesthetised cat. He demonstrated that the urethral afferent fibres mediating these responses course in the pelvic and pudendal nerves and that the pelvic nerves mediate the efferent arc of this reflex. Further investigations have corroborated the existence of these reflexes (Langworthy, Kolb & Lewis, 1940; Todd, 1964). Recently, Jung *et al.* (1999) have demonstrated that saline flow through the urethra also increases the frequency of reflex-evoked bladder contractions in the anaesthetised female rat. Moreover, these authors have suggested that this particular mechanism has a clinical relevance in patients with urinary incontinence, as leakage of urine into the proximal urethra may stimulate urethral afferents and facilitate

voiding reflexes, thus inducing or increasing bladder instability (Hindmarsh, Gosling & Deane, 1983; see section 1.10 of this chapter). To date, little evidence has been provided for the existence of this reflex in the normal human subject. Pudendal nerve anaesthesia reduces the rate of urine flow by approximately half, and this has been suggested to be due to loss of the aforementioned vesicourethral reflexes (Brindley, Rushton & Craggs, 1974). However, other studies have been unable to corroborate these findings (Nathan, 1952; Sutherst & Brown, 1978).

### **1.8. Central influences on the bladder and urethra: spinal and supraspinal sites**

The normal cycle of micturition involves a spinal-bulbo-spinal reflex loop that passes through the pons. The central control of micturition is considered to comprise a simple “on-off” switching circuit, as this pathway is “switched on” when mechanoreceptors in the bladder respond to a threshold tension (Iggo, 1955; see section 1.7 of this chapter). The area in the pons concerned with micturition was first described by Barrington (1921, 1925, and 1928) in the anaesthetised cat, and is therefore known as Barrington’s nucleus, or, more recently as the pontine micturition centre (PMC). In the cat, the PMC refers to an area in the dorsolateral pons that includes the locus coeruleus and subcoeruleus. The location of this pontine control site varies between species, and has been shown to comprise neurones in the lateral dorsolateral tegmental nucleus and the locus coeruleus in the rat (Satoh *et al.*, 1978). Evidence for a comparable region in the dorsolateral pons in humans has been provided through brain imaging studies during micturition in

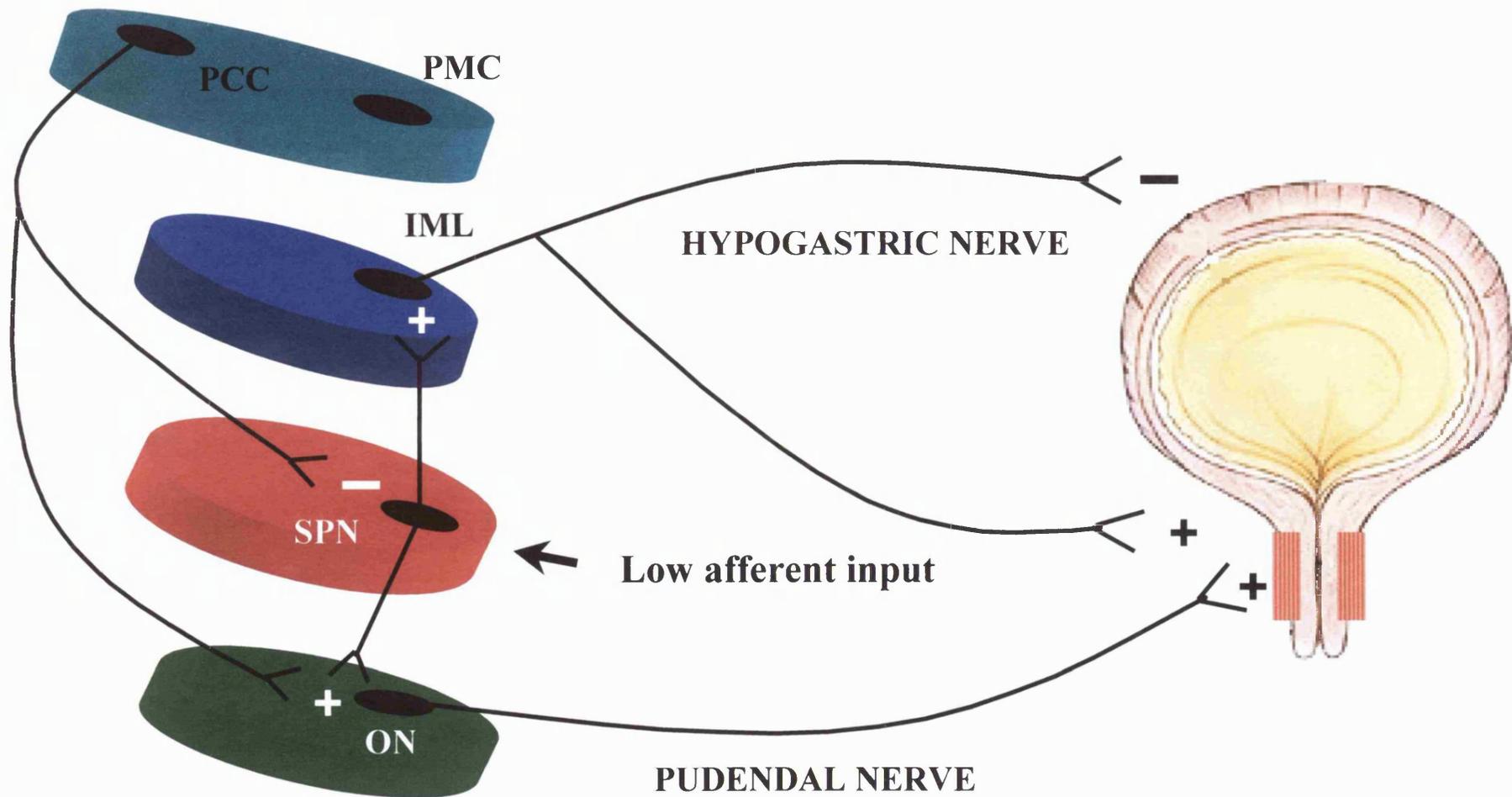
normal subjects (Blok, Willemsen & Holstege, 1997) and from patients with brainstem stroke (Sakakibara *et al.*, 1996). The PMC receives pelvic visceral information via projections from the lumbosacral spinal cord. Interestingly, retrograde tracing studies have demonstrated the presence of projections from the spinal cord to the periaqueductal grey (PAG) in both the rat (Ding *et al.*, 1997) and cat (Blok, de Weerd & Holstege, 1995). In addition, micturition is attenuated following injection of cobalt chloride into the PAG in anaesthetised rats (Matsuura, Allen & Downie, 1998). The PAG, in turn, sends projections to the PMC, suggesting that this area serves as a 'relay centre' for the transmission of afferent input to the pons. However, direct projections from the lumbosacral spinal cord to the PMC have also been identified, suggesting that both these pathways are operational (Ding *et al.*, 1997). Upon activation, the PMC sends excitatory projections to parasympathetic preganglionic motoneurons in the sacral spinal cord, which in turn, promote detrusor contractions and urethral relaxations (Blok & Holstege, 1997; Ding *et al.*, 1997). Interestingly, neurones that project supraspinally or are preganglionic are organised as distinct subpopulations in the rat sacral parasympathetic nucleus (Hamilton *et al.* 1995). Projections from the PMC to neurones in the sacral dorsal grey commissure are also activated during micturition, which inhibit urethral sphincter motoneurons and produce a relaxation of the EUS during micturition (Nadelhaft & Vera, 1996; Blok, Van Maarseveen & Holstege, 1998).

Evidence has also been provided for the existence of a pontine storage centre (PSC) or L-region in the central control of the LUT. This area has been localised to the subcoeruleus nucleus of the dorsolateral pontine tegmentum in the rat (Sugaya *et al.*, 1998), dog and cat (Nishizawa & Sugaya, 1994), and sends excitatory projections to Onuf's nucleus in the sacral spinal cord (Holstege, 1996). Electrical stimulation of this region has been shown to increase the activity of the EUS in cats and rats (Koyama, Ozaki & Kuru, 1966; Kruse *et al.*, 1988). Furthermore, bilateral destruction of this area induces bladder hyperactivity and urinary incontinence (Kruse *et al.*, 1988; Griffiths *et al.*, 1990). Interestingly, recent studies have shown that the PMC and PSC are not interconnected directly, suggesting that these regions represent separate functional systems that act independently (Blok & Holstege, 1999).

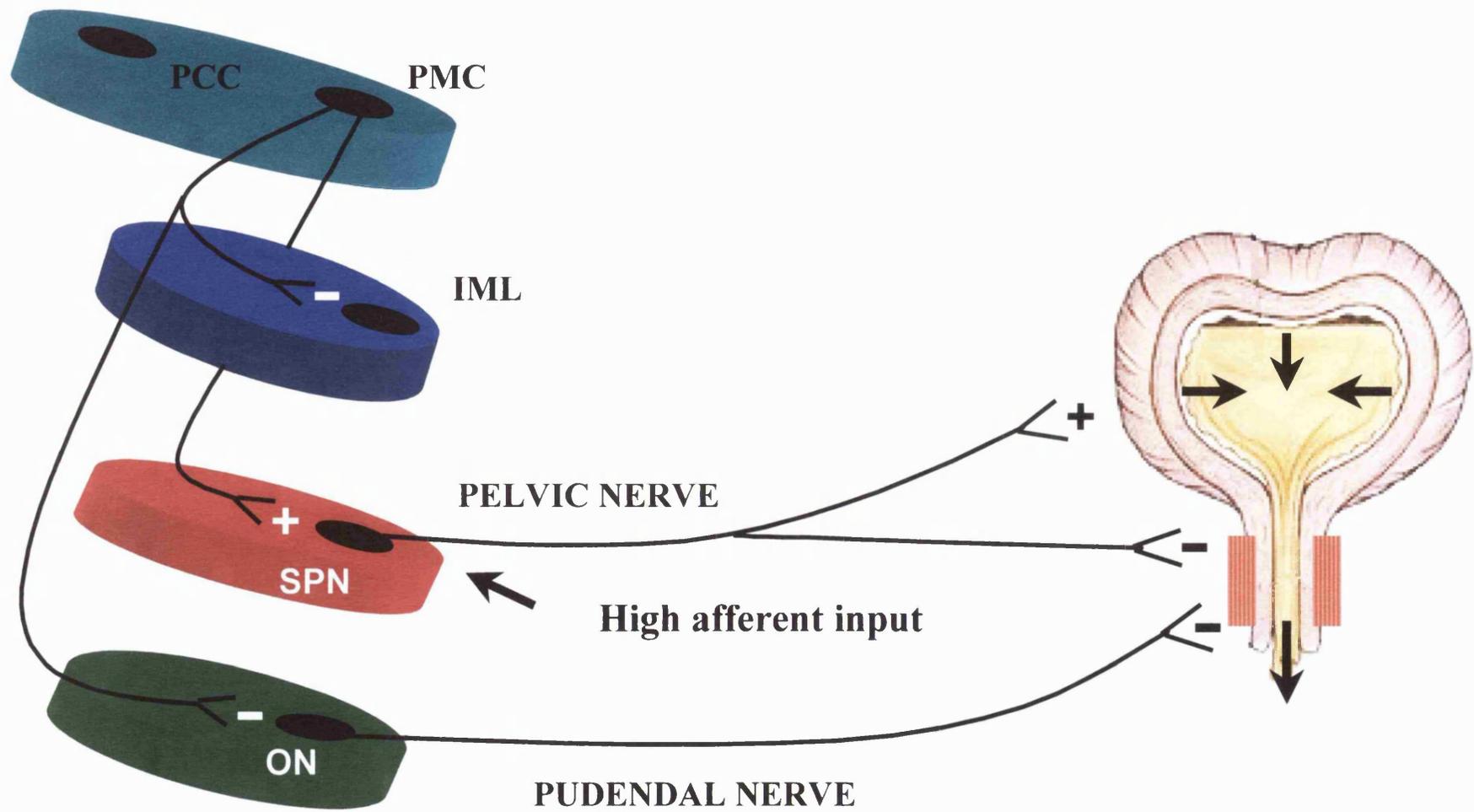
A number of suprapontine sites also influence the activities of the bladder and urethra, including the cerebral cortex and diencephalon (Vizzard *et al.*, 1995; Grill *et al.*, 1999). In this respect, the complex circuitry involved in the voluntary control of micturition is believed to be highly dependent on these suprapontine sites (see de Groat, 1998).

### **1.9. Reflex mechanisms in the bladder and urethra: An integration of systems**

Each of the components of the reflex control of the bladder and urethra are integrated to effect the functions of urine storage and voiding. The main reflexes controlling the activities of the bladder and urethra, and are essentially dependent on the level of afferent activity from the bladder in response to bladder distension (Figures 1.2 and 1.3; Table 1.2). Reflexes promoting the storage of urine are active at low levels of afferent activity and spino-bulbo-spinal micturition pathways are “switched off”. When a threshold level of afferent activity is attained, these central micturition pathways are “switched on”. These pathways not only excite efferent pathways to initiate micturition, but also inhibit the activity of the pathways that were previously active during urine storage.



**Fig 1.2.** Reflexes concerned with the storage of urine. Sympathoinhibitory effects on transmission in pelvic ganglia also prevent contraction of the bladder during this period (not shown for the sake of clarity). (PCC, pontine continence centre; PMC, pontine micturition centre; IML, intermediolateral cell column; SPN, sacral parasympathetic nucleus; ON, Onuf's nucleus).



**Figure 1.3.** Reflexes concerned with micturition. Sympathoinhibitory effects on transmission in pelvic ganglia are also inhibited during voiding (not shown for the sake of clarity). (PCC, pontine continence centre; PMC, pontine micturition centre; IML, intermediolateral cell column; SPN, sacral parasympathetic nucleus; ON, Onuf's nucleus).

	<b>Response</b>	<b>Efferent Pathway</b>	<b>Central Pathway</b>
<b>Urine Storage</b>	<b>Inhibition of detrusor contractility at bladder smooth muscle and pelvic ganglia</b>	Hypogastric	Spinal
	<b>Contraction of urethral and bladder neck smooth muscle</b>	Hypogastric	Spinal
	<b>Increased urethral striated muscle activity</b>	Pudendal	Spinal and Pontine (PSC)
<b>Micturition</b>	<b>Bladder contraction</b>	Pelvic	Pontine (PMC)
	<b>Relaxation of urethral and bladder neck smooth muscle</b>	Pelvic/inhibition of hypogastric outflow	Pontine (PMC)
	<b>Inhibition of urethral striated muscle activity</b>	Neurones in the sacral dorsal grey commissure	Pontine (PMC)
	<b>High frequency bursting of urethral striated muscle (rats and cats)</b>	Pudendal	Pontine (PMC)

**Table 1.2.** Reflex pathways controlling the activities of the bladder and urethra in the storage and elimination of urine. PSC, pontine storage centre; PMC, pontine micturition centre

### 1.10. Disorders of bladder and urethral function

Disturbances in any aspect of the complex relationship between the activities of the bladder and urethra, and in the essential neural mechanisms that ultimately control these events, results in disorders of normal LUT function. These disorders generally manifest as an inability to store urine, termed urinary incontinence (UI), or conversely as an inability to empty the bladder, described as urinary retention, which is often associated with a type of UI termed overflow incontinence (see Andersson *et al.*, 1999).

#### *Urinary incontinence (UI)*

UI is defined by the International Continence Society (ICS) as an involuntary loss of urine that is objectively demonstrable and is a social or hygiene problem (Hampel *et al.*, 1999). Accurate prevalence estimates of UI are difficult to determine because of varying definitions of the condition, and the fact that many cases are not reported due to patient embarrassment (Thom, 1998). Indeed, it has been estimated that up to 50% of all cases of UI may not be reported (Umlauf, Goode & Burgio, 1996). Despite these problems, it has been estimated that UI affects about 5% of women and less than 1% of men in adults younger than 30 years (Hampel *et al.*, 1997). UI is a condition closely associated with ageing, especially in the female population (Herzog & Fultz, 1990), and for men aged 30-60 years, the prevalence remains at

less than 1%, but approximately 29% of women in this age group are estimated to suffer from UI (Lagro-Janssen, Smits & Van Weel, 1992).

*Stress urinary incontinence*

The most commonest form of UI is stress UI (SUI), which has been estimated to account for 35% of all UI, 40% of all female UI and 5% of all male UI (Bos, Vu & Massiah, 1994). Patients describe losses of small volumes of urine during activities involving raised intra-abdominal pressure, for example during coughing, sneezing and running, caused by a failure of the EUS to contract the urethra during these events. The causes of this type of UI are wide-ranging. In women, parturition may damage the pelvic support so that the bladder neck and urethra are not supported and thus slip from their normal position above the pelvic diaphragm (Couture & Valiquette, 2000). This leads to changes in the position and functioning of the bladder neck and urethral sphincters. Furthermore, these changes may then be exacerbated during childbirth, which further causes pelvic relaxation. Other causes include postmenopausal loss of oestrogens, peripheral neuropathy, use of  $\alpha$ -adrenoceptor antagonists for hypertension (see section 1.11.4 of this chapter) and in men, damage to the EUS during urological surgery (Couture & Valiquette, 2000).

### *Urge Urinary Incontinence*

Urge UI (UUI) is defined as an involuntary urine loss associated with a strong desire to void, even when the bladder contains only a small amount of urine (Couture & Valiquette, 2000). UUI is estimated to account for 25% of all cases of UI, 20% of all female UI, and 35% of all male UI (Bos, Vu & Massiah, 1994). The primary cause of UUI is an oversensitive detrusor muscle that contracts unexpectedly causing the bladder neck to open. This is termed detrusor instability, detrusor hyperactivity, overactive bladder or uninhibited bladder (Hampel *et al.*, 1997). Non-neurological causes of detrusor instability include local genitourinary infections such as bladder irritation or interstitial cystitis, obstruction, and drug therapy for other complaints, including diuretics (Couture & Valiquette, 2000). When associated with a neurogenic aetiology, this condition is termed detrusor hyperreflexia. Indeed, neurological causes of detrusor instability are the most common and include dementia, Parkinson's Disease, multiple sclerosis and suprasacral spinal cord injury (see Blaivas, 1997).

### *Urinary retention and overflow incontinence*

Overflow UI (OUI) is characterised by a reduced urinary stream, incomplete micturition, and the sensation of incomplete voiding (Chutka *et al.*, 1996; Couture & Valiquette, 2000). Prevalence estimates suggest that this condition constitutes 10% of all cases of UI, 5% of all female UI and 30% of all male UI (Bos, Vu & Massiah,

1994). There are two main causes of OUI, bladder outlet obstruction and bladder contractile dysfunction. Obstruction commonly results from an enlarged prostate in men (benign prostatic hyperplasia, BPH) and urogenital cancers (Couture & Valiquette, 2000). A lack of contractile tone in the bladder can be caused by diabetes, sacral spinal cord lesions, or the use of drug therapy for other conditions, especially those with anticholinergic properties such as narcotics and some tricyclic antidepressants (Busby-Whitehead & Johnson, 1998).

#### *Mixed Urinary Incontinence*

Mixed UI (MUI) typically involves a combination of both SUI and UUI, and is thought to account for 25% of all UI, 30% of all female UI, and 25% of all male UI (Bo, Vu & Massiah, 1994). However, in men with an obstruction, symptoms of both UUI and OUI can also be detected (Chutka *et al.*, 1996).

## **1.11. Pharmacology of the bladder and urethra and treatment of lower urinary tract dysfunction**

### **1.11.1. Nitric oxide and cGMP signalling**

Nitric oxide (NO) plays a role in many physiological functions (see Moncada, Palmer & Higgs, 1991). In the peripheral nervous system, NO is released by a widespread network of nerves that have been categorised as non-adrenergic non-cholinergic (NANC). These nerves are known to regulate a number of gastrointestinal, respiratory and genitourinary functions (see Rand, 1992). NO is formed from the amino acid L-arginine by a family of enzymes, the NO synthases (NOS). This family comprises a number of isoforms, including two which are constitutive and one which is inducible by immunological stimuli (see Knowles & Moncada, 1994; Nathan & Xie, 1994). The constitutive NOS that was first discovered in the vascular endothelium has been designated eNOS, whereas that present in the brain, spinal cord and peripheral nervous system is termed nNOS. In addition, the form of NOS induced by immunological or inflammatory stimuli is known as iNOS (see Moncada, Higgs & Furchgott, 1997). In the LUT, both these constitutive types of NOS have been identified. Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase has commonly been used as a specific marker for nNOS in neurones, and NADPH-diaphorase immunoreactive nerve fibres have been demonstrated extensively in the urethra of the rat (Vizzard *et al.*, 1994), rabbit (Dokita *et al.*, 1994) and pig (Persson *et al.*, 1993). Conversely, neurones

innervating the bladder exhibit little NADPH-diaphorase immunoreactivity in these studies. These histochemical studies are in strong agreement with the results of both *in vivo* and *in vitro* functional studies, suggesting an important role for NO in the urethra, with few roles in the bladder (see below). Constitutive eNOS has been localised to urothelial cells of the hamster proximal urethra (Pinna *et al.*, 1999), human bladder (Smet *et al.*, 1996) and mouse bladder and urethra (Burnett *et al.*, 1997), suggesting a role for urothelial NO in the control of LUT function. Indeed, Birder *et al.* (1998) have found that NO released from rat urinary bladder is largely of non-neuronal origin and arises by about 85% from the urothelial layer. Moreover, removal of the urothelium reduces NO-mediated urethral relaxations in the hamster urethra (Pinna *et al.*, 1999).

NO has been shown to mediate electrically-evoked relaxations of the smooth muscle of the isolated bladder neck and urethra in the rat (Persson *et al.*, 1992), rabbit (Andersson *et al.*, 1992), sheep (Garcia-Pascual & Triguero, 1994), guinea-pig (Werkstrom *et al.*, 1998), dog (Hashimoto, Kigoshi & Muramatsu, 1992) and human (Klarskov *et al.*, 1983), suggesting that NO mediates relaxation of the bladder outlet during voiding. These findings are supported by the results of *in vivo* studies showing that reflex-evoked urethral relaxations are mediated by NO in anaesthetised female rats (Bennett *et al.*, 1995), and results from nNOS knockout mice which possess dysfunctional urinary outlets (Burnett *et al.*, 1997). Electrical stimulation of parasympathetic pathways in the pelvic nerve evoke urethral relaxations in a number of species (see section 1.5.1 of this chapter). In addition, immunohistochemical and

functional studies have suggested that NO-releasing nerves in the female rat LUT do not belong to nerve populations sensitive to the sympathetic neurotoxin, 6-hydroxydopamine, or the sensory neurotoxin, capsaicin (Persson *et al.*, 1997). Therefore, in functional terms, the parasympathetic nervous system essentially acts to promote micturition by releasing NO to produce urethral relaxations and other transmitters to evoke bladder contractions (see sections 1.11.2 and 1.11.3 of this chapter).

The effects of NO in various physiological systems are believed to be mediated by activation of guanylate cyclase, resulting in increased formation of cGMP. The biological effects of cGMP are dependent not only on its rate of formation, but also on its rate of breakdown, which is fulfilled by a family of PDE isoenzymes, primarily through the actions of PDE 5 (see Beavo, 1995). Increases in cGMP levels have been associated with NO-mediated inhibitory responses of the isolated rabbit urethra (Persson & Andersson, 1994; Dokita *et al.*, 1994), and cGMP related drugs (e.g. 8-bromo-cyclic GMP) evoke relaxant responses in this tissue (Persson & Andersson, 1994). However, the exact roles of this transduction system in the urethra remain to be determined.

The exact roles of NO in the bladder are the subject of debate, and to date, a substantial role has not been identified. Studies using the photo-induced adequate nitric oxide (PIANO) system demonstrate that NO generation relaxes isolated rat detrusor (Chung, Choi & Chang, 1996). Moreover, James, Birmingham & Hill

(1991) observed electrically-evoked NO-mediated relaxations in pre-contracted preparations of human detrusor. These studies have led to the suggestion that NO has an inhibitory effect on the bladder during bladder filling (Persson & Andersson, 1992). Indeed, studies in the conscious rat have found that inhibition of NOS causes bladder hyperactivity and decreased bladder capacity (Persson *et al.*, 1991). However, these effects are more likely initiated from the bladder outlet region and conclusive evidence for a functional role of nitrenergic transmission in the bladder has not been obtained.

NADPH-diaphorase immunoreactivity has been detected in afferent neurones within the lumbosacral DRGs and dorsal horn of the spinal cord (Vizzard, Erdman & de Groat, 1993a, 1993b). Furthermore, NO and NO donors have inhibitory effects on rat afferent neurones (Ozawa *et al.*, 1999; Yoshimura & de Groat, 1997) and NO release from afferent nerves has been detected in the rat bladder (Birder *et al.*, 1998). It is still unclear whether these effects of NO play a physiological role in the transmission of afferent input from the LUT. However, increased expression of nNOS has been observed in bladder afferent pathways in the rat following chronic bladder irritation (Vizzard, Erdman & de Groat, 1996) and spinal cord injury (Vizzard, 1997). Moreover, induction of an acute cystitis in rats also increases NOS immunoreactivity in sensory neurones (Callsen-Cencic & Mense, 1997) and nNOS activity and cGMP levels are decreased in cystitis in humans (Korting *et al.*, 1999). Thus, the use of intravesical L-arginine and NO donors to treat cystitis, mediated, in

part, by an inhibitory effect on bladder afferent pathways, remains an ongoing area of research (Korting *et al.*, 1999; Ozawa *et al.*, 1999).

nNOS has been identified in sympathetic and parasympathetic preganglionic neurones in the spinal cord of the rat (see Anderson *et al.*, 1993; Burnett *et al.*, 1995). However, it appears that spinal NO does not play a role in the central reflex pathway during normal micturition and may be more relevant in conditions of chronic irritation. Indeed, Kakizaki & de Groat (1996) have found that intrathecal injection of a NOS inhibitor reverses the irritant effects of intravesical administration of acetic acid in the anaesthetised female rat.

### **1.11.2. Acetylcholine: Histochemical studies, receptor types and distribution, and relevance to functions of the parasympathetic nervous system**

The parasympathetic nervous system provides the main excitatory input to the bladder during micturition (see section 1.5.1 of this chapter). One of the main neurotransmitters released from these nerves innervating the bladder is acetylcholine, and in the bladder, this transmitter is co-released with adenosine 5'-triphosphate (ATP; see section 1.11.3 of this chapter). Abundant cholinergic innervation of the bladder body has been demonstrated in the rat (Alm & Elmer, 1975), rabbit (Khanna *et al.*, 1981) dog (Raezer *et al.*, 1973), cat (Elbadawi, 1982) and man (Kluck, 1980). Cholinergic supply to the bladder neck and urethra has also been demonstrated in the rabbit (Mattiasson *et al.*, 1990), rat (Ekstrom & Malmberg,

1984), guinea pig, cat (Gosling & Dixon, 1975) and human (Gosling, Dixon & Lendon, 1977), although this is less extensive than that supplying the bladder body (Ek *et al.*, 1977).

Studies in the rat (Maggi *et al.*, 1989), rabbit (Mutoh *et al.*, 1997) and cat (Hassouna *et al.*, 1983) have demonstrated a strong contractile urethral response to cholinergic stimulation, whereas low contractile responses to acetylcholine have been reported in the guinea-pig (Persson & Andersson, 1976) and human (Ek *et al.*, 1977a) urethra. During micturition, the parasympathetic nervous system evokes NO-mediated urethral relaxations (see sections 1.5.1 and 1.11.1 of this chapter), and therefore the functional relevance of a contractile cholinergic response in the urethra remains unclear. Interestingly, there have been reports of differential effects of cholinergic stimulation on the different layers of the urethra, with strong cholinergic contractile responses present in urethral longitudinal smooth muscles and weaker contractions in circular urethral muscles of the rabbit (Mattiason *et al.*, 1990) and cat (Hassouna *et al.*, 1983). It has been suggested that a cholinergically-mediated contraction of the longitudinal muscle of the urethra would contribute to an opening of the urethra during voiding (Mattiason *et al.*, 1990). However, blockade of cholinergic receptors has no effect on the relaxatory response of the urethra to sacral root stimulation in the cat (Craggs & Stephenson, 1982), and the exact functional roles of the cholinergic supply to the urethra remain to be fully elucidated.

There are currently five identified subtypes of muscarinic receptors, namely  $M_{1,5}$  (see Eglén, Hedge & Watson, 1996). The  $M_{1,4}$  receptors are now relatively well pharmacologically characterised, although investigations regarding the pharmacology and function of the  $M_5$  receptor have been hampered due to an inability to distinguish it from the  $M_3$  receptor (see Eglén & Nahorski, 2000). Radioligand binding studies have demonstrated a high density of muscarinic receptors in rat (Monferini, Giraldo & Ladinsky, 1988), rabbit (Lepor & Kuhar, 1984), guinea-pig (Nilvebrant & Sparf, 1983) and human (Lepor *et al.*, 1989) bladder. Furthermore, parasympathetically-evoked bladder contractions that can be blocked by atropine have been demonstrated in the rat (Elmer, 1974), cat (Nergårdh, 1975), dog (Raezer *et al.*, 1973), rabbit (Levin, Shofer & Wein, 1980) and human (Levin, Staskin & Wein, 1983). In the rat, pig (Maeda *et al.*, 1988) and human (Yamaguchi *et al.*, 1996) bladder, mRNA encoding the  $M_2$  and  $M_3$  subtypes, but not the  $M_1$  or  $M_4$  subtypes, has been identified. Furthermore, a predominance of  $M_2$  over  $M_3$  receptors has been reported in the rat, rabbit and human bladder (Wang, Luthin & Ruggieri, 1995). Interestingly, despite this predominance of  $M_2$  receptors,  $M_3$  receptors have been suggested to represent the functional muscarinic receptor subtype in the bladder in the rat (Longhurst, Leggett & Briscoe, 1995; Tong *et al.*, 1997), rabbit (Tobin & Sjogren, 1995), mouse (Durant *et al.*, 1991), guinea-pig (Noronha-Blob *et al.*, 1989) and human (Newgreen & Naylor, 1996). Nevertheless, studies utilising  $M_2$  and  $M_4$  receptor knockout mice have shown that muscarinic agonist-evoked contractions of the bladder are attenuated in  $M_2$  receptor knockout mice (Stengel *et al.*, 2000). It has been suggested that  $M_2$  receptor activation

opposes  $\beta$ -adrenoceptor mediated bladder relaxation (that is activated during urine storage; see section 1.11.4 of this chapter) during micturition, thereby facilitating the contractile effects of  $M_3$  receptor activation (Hedge *et al.*, 1997). Indeed, recently,  $M_2$  muscarinic receptor-mediated inhibition of adenylate cyclase has been demonstrated in cultured human detrusor cells (Daniels, Meloy & Loury, 1999).

Muscarinic receptors have been identified on cholinergic nerve terminals innervating the bladder, which upon stimulation affect acetylcholine release (see Eglén, Hedge & Watson, 1996). These prejunctional muscarinic receptors comprise both facilitatory and inhibitory types that are differentially activated depending on the frequency of parasympathetic nerve stimulation (see Somogyi & De Groat, 1999). Prejunctional facilitatory muscarinic receptors comprise the  $M_1$  subtype in the rat (Braverman *et al.*, 1998), rabbit (Tobin & Sjogren, 1998) and human (see Somogyi & De Groat, 1999) bladder. It has been proposed that these receptors serve as an amplification mechanism during voiding to ensure complete bladder emptying (Hedge & Eglén, 1999). However, to date, no *in vivo* evidence for this mechanism has been obtained. For example, pirenzepine, a selective  $M_1$  receptor antagonist, has no effect on volume-evoked bladder contractions in the anaesthetised rat (Hedge *et al.*, 1997). Inhibitory prejunctional muscarinic receptors have been identified as the  $M_2$  subtype in the rabbit (Tobin & Sjogren, 1998),  $M_4$  in the guinea-pig (Alberts, 1995) and the  $M_2$  (Braverman *et al.*, 1998) and  $M_4$  (D'Agostino *et al.*, 1997) subtypes in the rat bladder. It has been suggested that these receptors may act in an

autoinhibitory fashion to stop the release of acetylcholine from parasympathetic nerve endings and therefore end reflex-evoked bladder contractions (Braverman *et al.*, 1998).

Prejunctional muscarinic receptors have also been identified on adrenergic nerve terminals, where they influence the release of noradrenaline in the rat (Somogyi & De Groat, 1999), rabbit, cat and human (Mattiason *et al.*, 1987) bladder. Interestingly, in the rat bladder, these M<sub>4</sub> receptors show the same stimulation-dependent functions as those at cholinergic terminals (Somogyi & De Groat, 1990; Somogyi *et al.*, 1996), and it has been suggested that the cholinergic facilitation of adrenergic transmission might have a modulatory influence on blood flow to the bladder during micturition (Somogyi *et al.*, 1996).

The muscarinic receptor subtypes in the urethra have not been extensively studied. In the rabbit urethra, contractile responses to cholinergic agonists have been suggested to comprise the M<sub>1</sub> and/or M<sub>3</sub>, and possibly the M<sub>2</sub> subtypes (Mutoh *et al.*, 1997). Furthermore, the presence of presynaptic muscarinic receptors on cholinergic and adrenergic nerve terminals in the urethra have not been investigated, although stimulation of muscarinic receptors has been shown to inhibit noradrenaline release in human erectile tissue (Hedlund, Andersson & Mattiason, 1984), suggesting that prejunctional muscarinic receptor types may also be present in the urethra.

It has been suggested that the cholinergic supply to the bladder may be altered by disease. Bladder strips from patients with neuropathic bladder dysfunction are supersensitive to muscarinic receptor agonists (German *et al.*, 1995). Moreover, morphometric studies have revealed a lower density of cholinergic nerves in neuropathic detrusor compared with normal tissue, and it has been suggested that an increased postjunctional muscarinic receptor supersensitivity secondary to a partial parasympathetic denervation of the bladder smooth muscle may be an important factor in the aetiology of detrusor hyperreflexia (German *et al.*, 1995). This type of 'denervation supersensitivity' has also been demonstrated in detrusor obtained from animal models of outflow obstruction (Speakman *et al.*, 1987) and in patients with detrusor instability resulting from prostatic obstruction (Harrison *et al.*, 1987). Despite these possible changes in cholinergic innervation during disease, it is well established that acetylcholine-induced stimulation of muscarinic receptors on detrusor smooth muscle is involved in both normal and involuntary bladder contractions (see Eglen & Hedge, 1997). This finding underlies the rationale for the use of muscarinic receptor antagonists in the treatment of overactive bladder (see Chapple, 2000). However, side effects such as dry mouth, constipation and blurred vision have restricted the therapeutic usefulness of previously available agents, such as oxybutynin, and recent research has been directed towards the development of more tolerable agents for the long term management of the symptoms of bladder overactivity. In this respect, antagonists such as darifenacin and tolterodine have shown selectivity for the bladder over other tissues in animal models (Nilvebrant *et al.*, 1997; Wallis, Burges & Cross, 1995). Further, the recent development of

extended-release oxybutynin is expected to offer improved tolerability in antimuscarinic therapy (Comer & Goa, 2000). Surprisingly, despite the dependence of the control of the micturition reflex on spinal and supraspinal mechanisms, the role of central muscarinic receptors in LUT function has seldom been studied. Sillen, Rubenson & Hjalmas (1982) have shown that that activation of central muscarinic receptors induces a hyperactive bladder response. Furthermore, these authors proposed that this response involves pontine-mesencephalic structures, suggesting that muscarinic receptors modulate bladder function at the central level. Indeed, it is possible that the therapeutic efficacy of antimuscarinics in the treatment of bladder dysfunction involves blockade of these receptors.

### **1.11.3. Adenosine-5'-triphosphate, the concept of 'atropine resistance' and relevance to the functions of the parasympathetic nervous system**

The phenomenon of 'atropine-resistance' in the bladder has long been demonstrated since the work of Langley & Anderson in 1895, who found that atropine, reduced, but did not abolish, contractile responses of the bladder to stimulation of sacral nerves in the rabbit, cat and dog. A number of theories have been proposed to explain this anomaly. The oldest is the 'proximity theory', which suggested that parasympathetic nerves in the bladder impinge so closely to the muscarinic receptor complex that atropine cannot penetrate sufficiently to produce a complete inhibition (Dale and Gaddum, 1930). Other explanations have included that some of the cholinceptors in the bladder are nicotinic, rather than muscarinic (Gyermek, 1961),

or that there are differences in the degree of blockade of pre- and postjunctional muscarinic receptors (Hammarstrom & Sjostrand, 1984). These theories were deemed unsatisfactory after the vast amount of work of Burnstock, beginning in the early 1970s. Burnstock proposed that a major portion of parasympathetic excitatory transmission to the bladder is noncholinergic (and nonadrenergic), being mediated by ATP (Burnstock, Dumsday & Smythe, 1972). Indeed, ATP evokes contractile responses of the bladder in a wide variety of species, including rat (Bolego *et al.*, 1995), rabbit (Levin, Shofer & Wein, 1980), dog (Matsumura, Taira & Hashimoto, 1968), guinea-pig (Cusack *et al.*, 1987) and human (Hoyle, Chapple & Burnstock, 1989). In addition, pelvic nerve-evoked bladder contractions are attenuated by ATP antagonists in the rat, guinea pig (Burnstock *et al.*, 1970) and cat (Theobald, 1986). The receptors mediating the biological effects of ATP and also adenosine, the breakdown product of ATP, are termed P1 and P2 receptors, respectively (Burnstock, 1978; Fredholm *et al.*, 1997). There are currently four identified P1 receptors for adenosine, namely A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors. These receptors have a wide tissue distribution and have varied physiological roles (see Nyce, 1999). In the bladder, adenosine evokes relaxations in isolated bladder strips of the rat (King, Huddart & Staff, 1997) and guinea-pig (Burnstock *et al.*, 1978). In the rat, the A<sub>2B</sub> receptor subtype mediates these responses (Nicholls, Hourani & Kitchen, 1992). Adenosine and adenosine re-uptake inhibitors reduce contractile responses of the bladder to electrical stimulation in the rat (Parija, Raviprakash & Mishra, 1991), mouse (Acevedo *et al.*, 1992) and human (Husted, Sjorgen & Andersson, 1983),

suggesting an additional role for P1 receptors in the prejunctional modulation of excitatory neurotransmission to the bladder.

P2 receptors have been subdivided into P2X and P2Y subtypes on the basis of pharmacological properties (Burnstock & Kennedy, 1985). To date, seven mammalian P2X receptors (P2X<sub>1-7</sub>) and five mammalian P2Y receptors (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub>) have been cloned, characterised and accepted as members of the P2 receptor family (see Ralevic & Burnstock, 1998). The main class of P2 receptors mediating contraction of bladder to ATP have been suggested to comprise the P2X subtype. These receptors have been traditionally distinguished from other purine receptors owing to their susceptibility to desensitisation with  $\alpha,\beta$ -methylene ATP (Kasakov & Burnstock, 1983), and the use of this agent, in combination with atropine, abolishes contractile responses to electrical stimulation in the rat (Brading & Williams, 1990; Hegde *et al.*, 1998), guinea-pig (Kasakov & Burnstock, 1983), rabbit (Creed, Ito & Katsuyama, 1991), marmoset (Moss & Burnstock, 1985) and human (Hoyle, Chapple & Burnstock, 1989) bladder. Residual contractile responses following atropine and  $\alpha,\beta$ -methylene ATP treatment have been reported in the rabbit (Chen and Brading, 1991) and rat (Luheshi & Zar, 1990) bladder, leading to suggestions that receptors other than the P2X subtype mediate the effects of ATP in the bladder, or that ATP is not the sole non-cholinergic motor transmitter in this tissue (Palea *et al.*, 1994). However, some P2X receptor-mediated responses are remarkably prone to tachyphylaxis following exposure to  $\alpha,\beta$ -methylene ATP, while

others are not (Evans & Kennedy, 1994; Khakh, Surprenant & Humphrey, 1995). Further, P2X receptor subunits are known to assemble as hetero-oligomeric complexes in which different subunits associate to generate channels with unique functional properties (Torres, Egan & Voigt, 1999). Therefore, the use of this agent to pharmacologically characterise the exact P2X receptor subtypes mediating the responses of ATP in the bladder is limited, and the development of selective agonists and antagonists should prove useful in this respect.

P2Y receptor-mediated relaxatory responses have been demonstrated in the rat (Bolego *et al.*, 1995), mouse (Boland *et al.*, 1993) and marmoset (McMurray, Dass & Brading, 1998) bladder. The exact P2Y receptor involved in these responses remains to be identified, although recently P2Y<sub>1</sub> receptor transcripts have been localised in the rat bladder (Obara, Lepor & Walden, 1998). Interestingly, P2Y-mediated relaxant responses of the rat bladder are increased by removal of the urothelium, suggesting that this response is modulated by the release of excitatory substances from bladder epithelial cells (Bolego *et al.*, 1995).

The proportions of cholinergic and purinergic excitatory transmission to the bladder during micturition varies between species. In rats and mice, cholinergic transmission accounts for approximately 50% of excitatory responses of the bladder, whereas in cats, excitatory transmission is almost all non-cholinergic. Furthermore, in primates, such as baboons and rhesus monkeys, and man, largely all of excitatory neuromuscular transmission in the bladder is mediated by cholinergic components

(see Cowan & Daniel, 1983; Hoyle & Burnstock, 1995). It has been proposed that each of these components differs in the nature of their effects on the bladder during micturition, with ATP being largely responsible for the initiation of voiding, while cholinergic transmission affects the maintenance of voiding (Theobald, 1995; Maggi *et al.*, 1985; Luheshi & Zar, 1990; Igawa, Mattiason & Andersson, 1993). However, the reason why the relative proportions of purinergic and cholinergic excitatory responses in the bladder should differ between species remains unknown. It has been suggested that the large ATP-evoked contraction in animals such as rats and cats is used for territory marking, rather than urine emptying, and is therefore not present in man (Craggs, Rushton & Stephenson, 1986).

ATP has recently been suggested to be involved in the activation of pelvic nerve afferents in both the bladder (Namasivayam, Eardley & Morrison, 1999) and gastrointestinal tract (Kirkup *et al.*, 1999). An extensive plexus of suburothelial sensory nerve fibres has been described in the bladder of several mammalian species (Alm *et al.*, 1978), and there is evidence for the release of ATP from urothelial cells close to this plexus in the rabbit (Ferguson, Kennedy & Burton, 1997; see Ferguson, 1999). The receptors involved in these responses have yet to be identified, although previous studies have demonstrated the presence of P2X<sub>2</sub> and P2X<sub>3</sub> heteromultimers on sensory nerve terminals (Lewis *et al.*, 1995). Furthermore, recently, P2X<sub>3</sub> receptors have been localised on nerves in the bladder (Lee, Bardini & Burnstock, 2000), and P2X<sub>3</sub> knockout mice display reduced bladder distension-evoked afferent nerve activity (Vlaskovska *et al.*, 2000).

The role of purinergic transmission to the urethra has not been extensively studied. No significant  $\alpha,\beta$ -methylene ATP binding is detected in the smooth muscle of the rat, guinea pig and rabbit urethra (Bo & Burnstock, 1992), suggesting a lack of a role of P2X receptors in this tissue. However, a relaxation of the urethra in response to ATP has been reported in the hamster (Pinna, Puglisi & Burnstock, 1998), and P2Y receptor antagonists attenuate electrically-evoked circular urethral smooth muscle in the rabbit (Ohnishi *et al.*, 1997), suggesting that ATP may have a role in this tissue.

Interestingly, responses to ATP in the human bladder have been shown to be increased in interstitial cystitis (Palea *et al.*, 1993) and in the neurogenic bladder (Saito *et al.*, 1993), suggesting that although the purinergic innervation to the bladder may not be important physiologically, it may have significant roles in pathological conditions of the bladder. Therefore, the receptors mediating the effects of ATP represent a possible drug target in the development of novel therapies for bladder dysfunction.

#### **1.11.4. Noradrenaline: Histochemical studies, receptor types and distribution, and relevance to the functions of the sympathetic nervous system**

Noradrenaline (NA)-immunoreactive nerve fibres are sparse in the body of the bladder, whereas an abundance of these fibres have been demonstrated in the bladder neck and proximal urethra of the rat (Alm & Elmer, 1975), guinea pig (Gosling &

Dixon, 1975), cat (Owman, Owman & Sjöberg, 1971; Wakabayashi *et al.*, 1994), dog (Benson *et al.*, 1976; Dokita, Nishimoto & Morita, 1987) and human (Benson, McConnel & Wood, 1979; Ek *et al.*, 1977a, 1977b; Gosling, Dixon & Jen, 1999). These studies suggest a lack of a role of the sympathetic nervous system in the bladder, but supplies a strong sympathetic influence on the bladder neck and urethra, consistent with the results of functional studies (see section 1.5.3 of this chapter). Moreover, studies on the types and distributions of the receptors mediating the effects of noradrenaline in the urethra have provided experimental support for a role of the sympathetic nervous system in this tissue. However, the presence of these receptors in the bladder suggests that the sympathetic nervous system may indeed possess a physiological role in the control of bladder function during urine storage (see below).

Adrenoceptors mediate the responses to the endogenous catecholamines NA and adrenaline, and have been subdivided into two subtypes, the  $\alpha$ - and  $\beta$ -adrenoceptors (Ahlquist, 1948). The  $\alpha$ -adrenoceptors have since been subdivided into  $\alpha_1$  and  $\alpha_2$  subtypes, and four  $\beta$ -adrenoceptors have been identified (see Docherty, 1998). Three subtypes of  $\alpha_1$ -adrenoceptors have been cloned and pharmacologically characterised, the  $\alpha_{1A}$ ,  $\alpha_{1B}$  and  $\alpha_{1D}$ -adrenoceptors (see Hieble, 2000). The  $\alpha_2$ -adrenoceptor family comprises the  $\alpha_{2A}$ ,  $\alpha_{2B}$ , and  $\alpha_{2C}$ -adrenoceptor subtypes (Docherty, 1998). A species variation of the  $\alpha_{2A}$ -AR, the  $\alpha_{2D}$ -adrenoceptor has been identified, but this variant is not considered a separate receptor subtype

(Simonneaux, Ebadi & Bylund, 1991; Trendelenburg, Limberger & Starke, 1993). Furthermore, a fourth  $\alpha_1$ -adrenoceptor with a low affinity for prazosin, the so-called  $\alpha_{1L}$ -adrenoceptor, has been postulated (Ford *et al.*, 1997). However, this receptor has not been cloned, and it has been suggested that it may represent an energetically favourable conformational state of the  $\alpha_{1A}$ -adrenoceptor (Daniels *et al.*, 1999).

#### *Peripheral $\alpha_1$ -adrenoceptors in the bladder and urethra*

Contractile responses to  $\alpha_1$ -adrenoceptor agonists have been demonstrated in the rat (Szell *et al.*, 2000), rabbit (Salimi, Setekleiv & Skobba, 1969), guinea pig (Dave & Dhattiwala, 1976) and human (Awad *et al.*, 1974) bladder. The predominant  $\alpha_1$ -adrenoceptor subtype in the rat, monkey and human bladder has been identified as the  $\alpha_{1A}$ -adrenoceptor (Walden *et al.*, 1997). However, recently, Mallory *et al.* (1998) have demonstrated a predominance of  $\alpha_{1D}$ - over  $\alpha_{1A}$ -adrenoceptors, with no  $\alpha_{1B}$ -adrenoceptors, in the human bladder, and an equal number of  $\alpha_{1A}$ -,  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenoceptors in the rat bladder. Furthermore, Szell *et al.* (2000) have recently shown that postjunctional  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenoceptors mediate  $\alpha_1$ -adrenoceptor-mediated contractile responses in the isolated female rat bladder. Therefore, selective  $\alpha_{1D}$ , or combined  $\alpha_{1A}/\alpha_{1D}$ -adrenoceptor, antagonists may be useful in the treatment of the irritative bladder symptoms associated with detrusor overactivity (Mallory *et al.*, 1998). In this respect, the density of  $\alpha$ -adrenoceptors increases significantly in the unstable bladder compared to normal bladder (Restorick &

Mundy, 1989), and non-subtype selective  $\alpha_1$ -adrenoceptor antagonists relieve the irritative symptoms of this disorder in both men and women (Serels & Stein, 1998).

$\alpha_1$ -adrenoceptors have been identified in the bladder base in the dog (Rohner *et al.*, 1971), cat and man (Nergardh & Boreus, 1972). Stimulation of these receptors evokes contractile responses of the rat (Chess-Williams, Aston & Couldwell, 1994), rabbit (Modiri *et al.*, 2000), and human (Nishimatsu *et al.*, 1999) urethra, and the rabbit (Shannon Kava *et al.*, 1998) and human (Taki *et al.*, 1999) bladder neck. The  $\alpha_1$ -adrenoceptor subtype mediating these responses have been suggested<sup>to</sup> comprise the  $\alpha_{1A}$ -adrenoceptor in the rat (Chess-Williams, Aston & Couldwell, 1994), rabbit (Auguet, Delaflotte & Chabrier, 1995) and dog (Taniguchi *et al.*, 1996). However, a large number of studies have provided convincing evidence for a functional role of the  $\alpha_{1L}$ -adrenoceptor in rabbit, dog (Leonardi *et al.*, 1997) and human (Ford *et al.*, 1996; Nishimatsu *et al.*, 1999) urethral tissues. The exact roles of the  $\alpha_{1L}$ -adrenoceptor, and its relevance to the  $\alpha_{1A}$ -adrenoceptor subtype in these tissues requires further investigation.  $\alpha_1$ -adrenoceptor-evoked contractile urethral responses are considered to play an important role in the maintenance of urethral tone and mediate sympathetic nerve-evoked urethral contractions to promote urinary continence during bladder filling (Taki *et al.*, 1999).  $\alpha$ -adrenoceptor antagonists decrease urethral pressure in normal women (Donker, Ivanovici & Noach, 1972) and in patients with lower motor neurone lesions (Andersson *et al.*, 1981). Furthermore,  $\alpha_1$ -adrenoceptor agonists such as midodrine have proved effective in the treatment of

female stress incontinence, by increasing urethral tone (Jonas, 1977). However, the use of these agents is limited by their cardiovascular side effects, and recent research is focussed on the development of selective  $\alpha_{1A}$ - and  $\alpha_{1L}$ -adrenoceptor agonists for the treatment of this disorder (Modiri *et al.*, 1999).

*Peripheral  $\alpha_2$ -adrenoceptors in the bladder and urethra.*

$\alpha_2$ -adrenoceptors have been identified in the bladder and urethra of rabbits (Latifpour *et al.*, 1990), although  $\alpha_2$ -adrenoceptor stimulation does not evoke contractile bladder responses in this species (Ueda, Satake & Shibata, 1984). However, electrically-evoked contractions of the isolated rat, guinea-pig and pig bladder have been shown to be inhibited by  $\alpha_2$ -adrenoceptor agonists (Ali *et al.*, 1998). These presynaptic receptors have been identified as  $\alpha_{2D}$ -adrenoceptors in the rat and guinea-pig, and  $\alpha_{2A}$ -adrenoceptors in the pig (Ali *et al.*, 1998). In the rabbit bladder, high stimulation frequencies are needed to activate these inhibitory receptors, and it has been suggested that these receptor subtypes operate as rate-limiting components in extremes of sympathetic activity (Tobin & Sjogren, 1998).

Radioligand binding studies have suggested that the rabbit urethra contains almost equal (Latifpour *et al.*, 1990; Shapiro & Lepor, 1986) or more (Morita *et al.*, 1987)  $\alpha_2$ -adrenoceptors compared to  $\alpha_1$ -adrenoceptors. Indeed,  $\alpha_2$ -adrenoceptor stimulation produces a contraction of the rat (Willette, Sauermelch & Hieble, 1989)

and rabbit (Morita *et al.*, 1987) urethra. However, there is no evidence for a role for postsynaptic  $\alpha_2$ -adrenoceptors in the human urethra (Kunisawa *et al.*, 1985).  $\alpha_2$ -adrenoceptor antagonists have been shown to enhance urethral responses to hypogastric nerve stimulation in the rat (Willette, Sauermelch & Hieble, 1990). These presynaptic receptors have been identified as the  $\alpha_{2A}$  subtype (Alberts, 1995), although, other studies have also suggested an involvement of the  $\alpha_{2D}$ -adrenoceptor (Trendelenburg *et al.*, 1997). Interestingly, Seshita *et al.* (2000) have identified the presence of inhibitory and facilitatory  $\alpha_1$ - and  $\alpha_2$ -adrenoceptors, respectively, on nitrergic nerve terminals in the rabbit urethra, that modulate the release of NO. Further,  $\alpha_2$ -agonists reduce electrically-evoked urethral relaxations in the pig urethra (Werstrom, Persson & Andersson, 1997). The exact physiological relevance of these receptors remains to be determined.

$\alpha_1$ - and  $\alpha_2$ -adrenoceptors also exert facilitatory and inhibitory effects, respectively, on transmission in vesical ganglia in the anaesthetised cat (Keast, Kawatani & De Groat, 1990). These opposing effects on ganglionic transmission suggests that the differential activation of each these receptor types may be important during different stages of urine storage and voiding.

*Central roles of  $\alpha$ -adrenoceptors.*

Intrathecal (i.t.) administration of  $\alpha_1$ -adrenoceptor antagonists attenuate locus-coeruleus-evoked bladder contractions in the rat (Ishizuka *et al.*, 1996b, 1997a) and cat (Yoshimura *et al.*, 1988, 1990), and modulate bladder distension-evoked micturition reflexes in both conscious (Ishizuka *et al.*, 1997b) and anaesthetised (Jeong & Lee, 2000) rats. These studies suggest the presence of a descending noradrenergic excitatory pathway from the locus coeruleus to preganglionic neurones in the sacral spinal cord. This input may also be mediated by interneurones in the spinal cord via the action of noradrenaline acting on  $\alpha_1$ -adrenoceptors (Ishizuka *et al.*, 1997b).  $\alpha_1$ -adrenoceptor antagonists also depress somatic (Gajewski, Downie & Awad, 1984) and sympathetic (Ramage & Wyllie, 1995) outflows to the bladder in the anaesthetised cat, suggesting that central facilitatory  $\alpha_1$ -adrenoceptors are tonically active in the neural control of the LUT. In this respect, the use of prazosin in the treatment of hypertension has been reported to cause stress incontinence in a number of patients (Dwyer & Teele, 1992).

$\alpha_2$ -adrenoceptor agonists (i.t.) exert inhibitory effects on micturition in the rat (Ishizuka, Mattiason & Andersson, 1996a) and cat (Galeano *et al.*, 1989). Conversely,  $\alpha_2$ -adrenoceptor antagonists facilitate micturition reflexes in conscious (Ishizuka, Mattiason & Andersson, 1996) and anaesthetised (Jeong & Lee, 2000) rats. Krier, Thor & De Groat (1979) have reported that  $\alpha_2$ -adrenoceptor agonists

depress lumbar sympathetic outflows to the bladder in the anaesthetised cat. In man,  $\alpha_2$ -adrenoceptor agonists decrease urethral pressure, presumably through a centrally mediated decrease in sympathetic activity (Nordling, Meyhoff & Christensen, 1979).

*$\beta$ -adrenoceptors: classification and roles.*

$\beta$ -adrenoceptors have been identified in rat (Longhurst & Levendusky, 1999), rabbit (Oshita, Hiraoka & Watanabe, 1997), dog (Yamazaki *et al.*, 1998), cat (Nergardh, Boreus & Naglo, 1977) and human (Awad *et al.*, 1974) bladder, where they mediate relaxatory responses to adrenergic stimulation. Radioligand binding studies have shown a predominance of  $\beta$ -adrenoceptors over  $\alpha$ -adrenoceptor subtypes in detrusor muscle of these species, suggesting that these receptors possess important functional roles (see Caine, 1984). It has been suggested that activation of these receptors during bladder filling promotes relaxation of the bladder and contributes to the storage of urine (Vaughn & Satchell, 1995).

$\beta$ -adrenoceptor subtypes were initially classified into  $\beta_1$ - and  $\beta_2$ -adrenoceptors on the basis of the relative potencies of NA and adrenaline (Lands, Luduena & Buzzo, 1967). Suggestions for the presence of additional  $\beta$ -adrenoceptor subtypes arose from the observations that  $\beta$ -adrenoceptors mediating the metabolic functions of endogenous catecholamines such as lipolysis and thermogenesis (Arch *et al.*, 1984) were insensitive to typical  $\beta$ -adrenoceptor antagonists. This led to the

characterisation and cloning of a  $\beta_3$ -adrenoceptor subtype (Emorine *et al.*, 1989; Nahmias *et al.*, 1991). More recently, pharmacological evidence has also suggested the presence of another atypical  $\beta$ -adrenoceptor subtype, the  $\beta_4$ -adrenoceptor (Kaumann, 1997).

The  $\beta$ -adrenoceptor subtypes mediating the effects of adrenergic stimulants in the bladder differ between species.  $\beta_2$ - and  $\beta_3$ -adrenoceptors have been shown to mediate relaxatory responses in the rat detrusor (Yamazaki *et al.*, 1998). Recently,  $\beta_1$  mRNA has been identified in this species (Fujimura *et al.*, 1999), and functional responses to  $\beta_1$ -adrenoceptor stimulation have also been demonstrated (Longhurst and Levendusky, 1999).  $\beta_1$ -adrenoceptors mediate relaxatory responses in the cat (Nergardh, Boreus & Naglo, 1977) and guinea-pig (Yamamoto, Mori & Koike, 1998),  $\beta_2$ -adrenoceptors are functional in the rabbit, and  $\beta_3$ -adrenoceptor responses predominate in the dog (Yamazaki *et al.*, 1998). A predominance of  $\beta_2$ -adrenoceptors have been identified in human detrusor (Levin, Ruggieri and Wein, 1988), although, recently, a functional role for the  $\beta_3$ -adrenoceptor in this tissue has been proposed (Fujimura *et al.*, 1999; Takeda *et al.*, 1999; Igawa *et al.*, 1998). Thus, it has been suggested that  $\beta_3$  adrenoceptor-agonists may be effective in the treatment of urinary frequency (Fujimura *et al.*, 1999). However, the use of  $\beta_2$ -adrenoceptor agonists in the treatment of LUT disorders has not been dismissed and research still continues in this area. Recently,  $\beta_2$ -selective adrenoceptor agonists have been shown to inhibit purinergic, not cholinergic, responses to electrical stimulation in the rat

detrusor, suggesting that activation of  $\beta_2$ -adrenoceptors modulates the response to ATP released from purinergic nerves (Hudman, Elliot & Norman, 2000a, 2000b). Furthermore, these effects are mediated by the opening of ATP-sensitive potassium channels ( $K_{ATP}$ ). A number of disturbances of bladder function have been linked with the appearance of a purinergic excitatory component, and the potential for drug therapies involving  $\beta_2$ -adrenoceptor agonists is therefore an important area for further investigation.

The presence and functional roles of  $\beta$ -adrenoceptors in the urethra have been not been studied extensively. Activation of  $\beta_2$ -adrenoceptor subtypes evokes urethral relaxations both *in vivo* and *in vitro* in the dog (Garcia-Sacristan *et al.*, 1986). In humans,  $\beta$ -adrenoceptor antagonists inhibit urethral relaxations evoked by sacral root stimulation (McGuire, 1978; McGuire & Herlihey, 1978). Furthermore, in a small group of patients, these antagonists have been shown to be of value in the treatment of stress incontinence (Kaisary, 1984). However, the increased levels of continence exhibited by these patients are thought to be related to an unopposed effect of  $\alpha$ -adrenoceptors following  $\beta$ -adrenoceptor blockade.

**1.11.5. Other peripheral and central neurotransmitters relevant to the functions of the bladder and urethra.**

*Glutamate*

Glutamate is the principal excitatory neurotransmitter in the brain and spinal cord (see Meldrum, 2000). Ionotropic glutamate receptors are categorised on the basis of their agonist properties into N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (see Vandenberg, 1998). Glutamate receptor antagonists depress micturition reflexes in the rat (Matsumoto, Kawatani & Takeshige, 1991; Yoshiyama, Roppolo & de Groat, 1995a, 1995b; Nishizawa *et al.*, 1999). Furthermore, experiments using selective glutamate receptor antagonists have shown that NMDA receptors are involved in descending pathways from the PMC to the spinal cord (Matsumoto, Hisamitsu & de Groat, 1995). AMPA and NMDA glutamergic synaptic mechanisms are thought to play a role in the spinal processing of afferent input from the bladder and in the ascending limb of the spino-bulbo-spinal micturition reflex pathway (Kakizaki *et al.*, 1998). Furthermore, AMPA and kainate receptors have been shown to be essential for the activation of EUS activity during micturition in the anaesthetised rat (Yoshiyama, Roppolo & de Groat, 1995b). Activation of NMDA receptors in the spinal cord has been associated with the processing of nociceptive input from the LUT (Birder & de Groat, 1992), and AMPA antagonists have been shown to reduce the LUT symptoms

associated with spinal cord injury (Yoshiyama *et al.*, 1999), suggesting that glutamergic transmission may be relevant in the pathophysiology of LUT dysfunction. Glutamate receptors may therefore represent an attractive therapeutic target in the treatment of LUT disorders.

### GABA

Gamma-amino-butyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (see Chebib & Johnston, 1999). Three major GABA receptors, termed GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptors have been identified (see Chebib & Johnston, 1999). In the control of the LUT, both peripheral and central roles of GABA have been suggested. Peripherally, GABA receptor agonists have been shown to inhibit electrically evoked bladder contractions in the rabbit (Santicioli, Maggi & Meli, 1984; Chen, Doyle & Ferguson, 1992), guinea pig (Maggi, Santicioli & Meli, 1985a) and human (Chen, Doyle & Ferguson, 1994) bladder. Moreover, these studies have suggested that these inhibitory effects on excitatory neurotransmission in the bladder are mediated by the GABA<sub>B</sub> subtype, and have suggested the use of GABA<sub>B</sub> analogues in the treatment of detrusor instability. Furthermore, GABA<sub>A</sub> and GABA<sub>B</sub> receptors are located in parasympathetic ganglia (Kataoka *et al.*, 1994), and mediate the depressant effects of GABA on excitatory transmission in these ganglia in the rat (Maggi, Santicioli & Meli, 1985b) and cat (Mayer *et al.*, 1983). GABAminergic neurons have been identified in the sacral spinal cord and locus coeruleus (Iijima, Oktomo & Iijima,

1988). Stimulation of central GABA receptors inhibits micturition and increases bladder capacity in rats (Igawa, Mattiason & Andersson, 1993) and cats (Roppolo *et al.*, 1987). Furthermore, GABA agonists also inhibit afferent nociceptive input from the bladder (Araki & de Groat, 1996) and sacral preganglionic neurons (Todd & Sullivan, 1990). These studies have identified a role for GABA<sub>A</sub> and GABA<sub>B</sub> receptors in these effects. The results of these, and other, studies have suggested that GABAminergic neurones essentially facilitate urine storage by inhibition of afferents and interneurons in the sacral spinal cord and supraspinal pathways (see Steers, 1995). Furthermore, GABA also plays a role in the inhibition of reflexes controlling urine storage during micturition. GABAminergic neurones in the sacral spinal cord are activated during micturition, which inhibit urethral sphincter motoneurons and therefore relax the EUS (Blok, Van Maarseveen & Holstege, 1998). Thus, pharmacological manipulation of the GABAminergic pathways controlling the activities of the LUT remains a potential therapeutic target in the treatment of LUT dysfunction. Indeed, Baclofen, a GABA<sub>B</sub> agonist, has been shown to have beneficial effects on bladder dysfunction in patients with myelopathy (Frost *et al.*, 1989).

### 5-HT

5-hydroxytryptamine (5-HT or serotonin) receptors comprise seven subfamilies (5-HT<sub>1</sub>-5HT<sub>7</sub>) based on agonist and antagonist affinities (see Gerhardt & Van Heerikhuizen, 1997). The 5-HT<sub>1</sub>, 5-HT<sub>2</sub> and 5-HT<sub>5</sub> receptor classes are also

subdivided into various subtypes. 5-HT exerts effects on the LUT at both peripheral and central sites. Peripherally, 5-HT evokes contractile responses of the bladder in a number of mammalian species including the rat (Vanov, 1965), dog (Gyermek, 1962), cat (Saum & de Groat, 1973) and human (Tiara, 1972). These excitatory effects on bladder smooth muscle are blocked by 5-HT<sub>2</sub> receptor antagonists in the cat (Saum & de Groat, 1973), dog (Matsumura, Taira & Hashimoto, 1968) and mouse (Holt, Cooper & Wyllie, 1986) bladder, and 5-HT<sub>3</sub> receptor antagonists in the rabbit bladder (Chen, 1990). 5-HT potentiates electrically-evoked bladder contractions in the guinea pig (Messori *et al.*, 1995), mouse (Holt, Cooper & Wyllie, 1986), rabbit (Barras, Van Der Graaf & Angel, 1996) and human (Corsi *et al.*, 1991; Tonini *et al.*, 1994) bladder. Interestingly, species variation exists with regard to the characteristics of the presynaptic 5-HT receptor involved in this effect. These receptors comprise 5-HT<sub>1B</sub> and 5-HT<sub>2</sub> receptors in the mouse (Holt, Cooper & Wyllie, 1986), 5-HT<sub>2A</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> subtypes in the guinea pig (Messori *et al.*, 1995), and the 5-HT<sub>4</sub> receptor in man (Tonini *et al.*, 1994). Moreover, evidence has been provided for the existence of an inhibitory 5-HT<sub>4</sub> receptor attenuating excitatory neurotransmission to the bladder in the monkey (Waikar, Ford & Clarke, 1994). These findings have raised the possibility of the use of 5-HT<sub>4</sub> receptor agonists in the treatment of bladder disorders, for example in the management of voiding disorders associated with reduced cholinergic drive, such as in the ageing bladder (Tonini & Candura, 1996). Further peripheral roles of 5-HT in the control of the LUT involve effects at the level of the pelvic ganglia, where 5-HT primarily inhibits, and occasionally facilitates, cholinergic ganglionic transmission (Saum &

De Groat, 1973). In the urethra, 5-HT has been localised to neuroendocrine cells in the dog (Hanyu *et al.*, 1987), and 5-HT<sub>2</sub> and 5-HT<sub>3</sub> agonists produce contractile responses of this tissue in a number of species (see Steers, 1995). 5-HT receptors are widely distributed in the central nervous system including a number of areas concerned with the control of the LUT. The importance of central 5-HT in this control is particularly illustrated by the observation that urge incontinence occurs more frequently in depressed individuals, who are known to have lower levels of 5-HT or decreased activity in serotonergic pathways (Hafner, Stanton & Guy, 1977). Lumbosacral sympathetic and parasympathetic autonomic nuclei receive serotonergic projections from the raphe nuclei in the brain stem (Loewy & Neil, 1981), and electrical stimulation of these 5-HT-containing neurones inhibits bladder activity in the cat (McMahon & Spillane, 1982). Moreover, stimulation of this central area inhibits the pelvic nerve afferent-evoked firing of spinal dorsal horn neurones (Lumb, 1986), suggesting the presence of an inhibitory descending raphe-spinal pathway that is activated by afferent input from the bladder, and inhibits the micturition reflex (Testa *et al.*, 1999). A role for inhibitory 5-HT<sub>1A</sub> autoreceptors in the control of raphe firing has been proposed (Aghajanian & Vander-Maelen, 1986), raising the potential for the use of 5-HT<sub>1A</sub> receptor antagonists to increase raphe firing and facilitate this descending inhibitory input on the micturition reflex in the treatment of bladder overactivity (Lecci *et al.*, 1992; Testa *et al.*, 1999).

## Dopamine

Dopamine is a neurotransmitter in the peripheral and central nervous systems that is involved in the control of a wide variety of autonomic systems. There are two families of dopamine receptors, termed D<sub>1</sub> and D<sub>2</sub>. The D<sub>1</sub> family consists of D<sub>1</sub> and D<sub>5</sub> receptor subtypes, and the D<sub>2</sub> family comprises the D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptor subtypes (for review see Emilien *et al.*, 1999). It is well known that urinary symptoms are a common finding amongst patients with Parkinson's disease, suggesting that dopamine has important roles in the control of the LUT (Singer, 1998). However, despite these observations, the role of dopamine in the control of the bladder and urethra has not been extensively studied. D<sub>1</sub> and D<sub>2</sub> receptors have been localised in the human bladder (Escaf *et al.*, 1994), and dopamine has been found to depress purinergic transmission in the rat bladder (Lot, 1993; El-Mas *et al.*, 1999). Interestingly,  $\alpha_2$ -adrenoceptor antagonists attenuate these inhibitory effects of dopamine in the rat bladder, suggesting that presynaptic  $\alpha_2$ -adrenoceptors mediate these effects (El-Mas *et al.*, 1999). Pharmacological experiments in a number of animal species have also implicated a central role of dopamine in the control of the LUT. Stimulation of central dopamine receptors with the dopamine precursor L-DOPA and apomorphine in rats produces produces a er response (Sillen, Rubenson & Hjalmas, 1981; Kontani, Inoue & Sakai, 1990b; Ishizuka *et al.*, 1997a, 1997b). Furthermore, these responses are mediated by activation of central D<sub>2</sub> receptors, and potentiated by the simultaneous stimulation of D<sub>1</sub> receptors (Kontani,

Inoue & Sakai, 1990b). The site for dopaminergic facilitation of bladder activity has been suggested to reside in the PMC in the rat (Sillen, Rubenson & Hjalmas, 1982) and cat (Roppolo *et al.*, 1987). These limited studies on the dopaminergic control of the bladder have suggested that activation of central D<sub>1</sub> and D<sub>2</sub> receptors may have therapeutic value in the treatment of LUT dysfunction, especially in those patients with Parkinson's disease. It is thought that the mechanism underlying the emergence of detrusor hyperreflexia in Parkinson's disease involves a degeneration of dopaminergic neurons in the substantia nigra and a subsequent lack of activation of D<sub>1</sub> receptors in the PMC (Yoshimura *et al.*, 1993). Indeed, D<sub>1</sub> (Yoshimura *et al.*, 1993) and combined D<sub>1</sub> and D<sub>2</sub> (Yoshimura *et al.*, 1998) receptor agonists have been shown to suppress the symptoms of detrusor hyperreflexia in parkinsonian monkeys.

### *Others*

The neurotransmitters described in this introduction are by no means a complete list of those involved in the control of the LUT. Indeed, there are a number of other transmitters, peptides and regulatory substances that play a role in the complex activities of the bladder and urethra. These include potassium-channels, vanilloid receptors, prostaglandins, endothelins, neurokinins, opiates and a large number of neuropeptides (see Steers, 1995; Andersson, 1993, 1999). The large number of substances involved in the control of the storage and elimination of urine offers a number of potential therapeutic targets, and current research is particularly focused on the development of novel treatments for LUT dysfunction.

### 1.12. Aims of this study

From this introductory chapter, it is clear that although the pharmacological control of the bladder has been well studied and characterised, the pathways and neurotransmitters concerned with the activity of the urethra are less known. Of the previous investigations that have focussed on urethral control, a large proportion of these have investigated the physiological role of NO in relaxation of the smooth muscle of the bladder neck and urethra during voiding (see section 1.11.1. of this chapter). Ho *et al.*, (1998, 1999) have also recently identified the presence of nitrergic nerve fibres in the human striated urethral sphincter, suggesting that NO may possess an additional role in the control of the urethral striated muscle. However, the exact roles of NO in this musculature remain to be determined. The inhibitory responses of NO in the urethral outlet during voiding have been suggested to be mediated by cGMP on the basis of both immunohistological (Smet *et al.*, 1996; Waldeck *et al.*, 1998) and functional (Persson & Andersson, 1994; Dokita *et al.*, 1994) studies (see section 1.11.1 of this chapter). However, evidence for a role for this transduction system in the control of the urethra has not been provided from *in vivo* investigations. Furthermore, the presence of NO/cGMP signalling has, to the author's knowledge, never been demonstrated in the rat urethra. Therefore, a proportion of the studies in this thesis were concerned with determining the presence and roles of NO/cGMP signalling in the smooth and striated muscles of the female rat urethra *in vivo*.

The rat offers an important animal model to investigate bladder and urethral function in that it is inexpensive, requires low maintenance and is readily accessible (Steers, 1994). Furthermore, in this species, a detailed knowledge of the anatomical organisation and connectivity of the central and peripheral pathways has been obtained (Steers, 1994). Although certain aspects of LUT function in the rat differ significantly from humans, experiments in this thesis were performed on female rats, as the urethral response during micturition in the female rat (Fraser, Flood & De Groat, 1995) and human (Torrens, 1978; Van Kerrebroeck *et al.*, 1991) is a relaxation, whereas in the male rat, a urethral contraction is more commonly observed (Kakizaki *et al.*, 1997). Therefore, the results of studies employing the female rat may be more closely correlated to the physiological and pharmacological control of the human urethra. Fraser, Flood & De Groat (1995) have developed a novel experimental system in the anaesthetised rat, whereby bladder and urethral pressures can be recorded simultaneously without surgically disconnecting the bladder and urethra or tying ligatures between them, a procedure which could potentially disrupt the fine control of the urethral outlet during voiding. The *in vivo* experiments performed in this thesis used a modified version of this experimental system, and examined the effects of pharmacological manipulation of the NO/cGMP signalling pathway on changes in bladder and urethral pressures evoked by the micturition reflex with saline infusion into the bladder, and by stimulation of postganglionic neurones with 1,1-dimethylphenylpiperazinium (DMPP), a neuronal nicotinic receptor agonist, in anaesthetised female rats. In addition, changes in the activity of the urethral striated muscle during the micturition reflex were measured

by electromyographic (EMG) recordings, and experiments were also carried out in the presence of  $\alpha$ -Bungarotoxin, a neuromuscular blocker, to examine the exact roles of nitregeric transmission in both the smooth and striated muscles of the urethra. These initial *in vivo* investigations revealed the presence of possible reflex interactions between the urethral smooth and striated muscles, with implications for the control of overall urethral tone, and thereby continence. These interactions were therefore further investigated locally, at the level of the urethra, by examining the effects of pharmacological manipulation of smooth muscle tone with intraurethral perfusion of agents, on the activity of the urethral striated muscle, *in vivo*.

In the interpretation of the results of *in vivo* studies in this thesis investigating the pharmacological control of the female rat urethra, an involvement of both the smooth and striated muscles of the urethra must be considered in the effects of the various drugs used. Thus, to be able to further investigate the mechanisms of action of these drugs, and to determine the relevance of these findings to the pharmacological control of the female rat urethra *in vitro*, it is important to determine that both the smooth and striated muscles of the urethra are present in this isolated tissue preparation. Therefore, initial *in vitro* studies were concerned with determining the presence of striated muscle in the isolated female rat urethral preparation using a combination of histological and functional techniques. Experiments were then performed to further investigate both the roles of NO/cGMP signalling and the mechanism(s) of action of reflexes controlling tone in the urethra,

in the female rat urethra *in vitro*. These *in vitro* findings were then correlated with the results of *in vivo* investigations in an attempt to obtain a clearer understanding of the complex physiological and pharmacological control mechanisms that are operational in

## **Chapter Two**

**Nitric Oxide and cyclic GMP in the**

**Female Rat Urethra *in vivo*:**

**Interactions between Urethral**

**Smooth and Striated Muscles**

## INTRODUCTION

The activities of the urinary bladder and urethra are under a complex neural control system to act synergistically in the storage and periodic elimination (micturition) of urine. During urine storage the bladder accommodates increasing volumes of urine with little change in internal pressure, whereas there is an increase in the activity of the bladder neck and urethra to close the urethral outlet and thereby maintain continence. When the volume of urine in the bladder reaches the threshold, the micturition reflex is evoked which comprises a contraction of the bladder and relaxation of the urethral outlet (see de Groat, Booth & Yoshimura, 1993). The urethra consists of a combination of circular and longitudinal smooth muscle layers which are surrounded by striated muscle fibres along the middle third in a number of species (see Elbadawi, 1987). This area of striated muscle constitutes the external urethral sphincter (EUS). Relaxation of both these types of muscle is essential for effective voiding, and similarly both muscles are thought to be important in maintaining urinary continence (see Brading, 1999). In addition, in the rat, rapid contractions and relaxations of the striated musculature occur during voiding, which are thought to be involved in efficient bladder emptying (Conte, 1991).

A large number of *in vitro* studies have implicated nitric oxide (NO) as the main neurotransmitter involved in mediating relaxation of the urethral smooth muscle during voiding (see Andersson & Persson, 1994). Furthermore, Ho *et al.*, (1998, 1999) have identified the presence of nitrergic nerve fibres in the human striated urethral sphincter, suggesting that NO may also play a role in the control of the

urethral striated muscle. The effector system for the actions of NO in various physiological systems is thought to involve activation of guanylate cyclase, resulting in increased formation of guanosine 3':5'-cyclic monophosphate (cGMP) and subsequent smooth muscle relaxation (Zhang & Synder, 1995). The biological effects of cGMP are dependent not only on its rate of formation, but also on its rate of breakdown, which is fulfilled by a family of cyclic nucleotide phosphodiesterase (PDE) isoenzymes (Beavo, 1995). In particular, PDE 5 has been shown to fulfil this role in a number of biological systems (see Beavo, 1995; Yanaka *et al.*, 1998). Inhibition of cGMP-selective PDEs has been shown to potentiate NO-mediated relaxations in gastrointestinal (Williams & Parsons, 1995), genital (Cellek & Moncada, 1998) and vascular (Liu *et al.*, 1992) smooth muscle. In addition, increases in cGMP levels have been associated with NO-mediated inhibitory responses of the isolated rabbit urethra (see Persson & Andersson, 1994; Dokita *et al.*, 1994), suggesting that this transduction system is also involved in the control of urethral smooth muscle. However, the presence of NO/cGMP signalling in urethral smooth muscle relaxation has never been demonstrated *in vivo*. Furthermore, the potential roles of this transduction system in the control of the striated muscle of the urethra have not been investigated. Therefore, the aim of the present study was to investigate the role of nitrergic transmission in the lower urinary tract using two complementary experimental approaches: (a) inhibition of NO synthesis with N $\omega$ -nitro-L-arginine methyl ester (L-NAME; Moore & Handy, 1997), and (b) inhibition of cGMP-selective PDEs, with zaprinast (a PDE types 1, 5, 6 and 9 inhibitor; Ballard *et al.*, 1998; Fisher *et al.*, 1998). The effects of these agents were examined on bladder and urethral responses evoked by the micturition reflex

with saline infusion into the bladder and to stimulation of postganglionic autonomic nerves with 1,1-dimethylphenylpiperazinium (DMPP), a neuronal nicotinic receptor agonist (see Broadley, 1996). To determine the roles of NO/cGMP signaling in the urethral striated musculature, the effects of these agents were examined on electromyographic recordings of the external urethral sphincter (EUS-EMG). Furthermore, experiments were carried out in the absence and presence of neuromuscular blockade with  $\alpha$ -Bungarotoxin, a non-competitive inhibitor of neuromuscular nicotinic receptors (see Chiappinelli, 1985), to investigate the contribution of urethral smooth and striated muscle in the effects of these antagonists.

These preliminary experiments suggested the presence of interactions between the urethral smooth and striated muscles. Therefore, these interactions were further investigated by examining the effects of urethral smooth muscle relaxation with intraurethral (IU) perfusion of either sodium nitroprusside (SNP), a NO donor, or isoprenaline, a  $\beta$ -adrenoceptor agonist, on EUS-EMG activity. The effects of IU administration of these agents were also examined following pre-treatment with chlorisondamine, a neuronal nicotinic receptor antagonist.

## METHODS

The experiments were carried out under the Animals (Scientific Procedures) Act, 1986. After completion of experiments, animals were killed by an overdose of intravenous (i.v.) pentobarbitone sodium.

### General preparation

Experiments were performed on ninety six female Sprague-Dawley rats (200-345 g) anaesthetised with isoflurane (4 % in 100 % oxygen), and maintained with urethane (1.2 g kg<sup>-1</sup>, i.v.). Depth of anaesthesia was assessed by the stability of blood pressure and heart rate, and by an absence of hindlimb withdrawal in response to paw pinch. In preparations where neuromuscular blockade was produced by  $\alpha$ -Bungarotoxin (0.4 mg kg<sup>-1</sup>, i.v.), depth of anaesthesia was assessed by stability of cardiovascular variables and by an absence of cardiovascular response to paw pinch. Supplementary doses of urethane (0.1 g kg<sup>-1</sup>, i.v.) were given where necessary. A midline incision was made in the neck and the trachea intubated to maintain a patent airway. The left jugular vein was cannulated for anaesthetic and drug administration. The left common carotid artery was cannulated with a heparinised cannula (20 units ml<sup>-1</sup> heparin in 0.9% w/v saline) for the measurement of arterial blood pressure and for sampling arterial blood for blood gas analysis. Blood pressure was measured using a pressure transducer (Gould Statham P23Db), and the heart rate (HR) derived electronically on-line from the blood pressure signal using *AcqKnowledge version 3.5.3* software (Biopac Systems Inc, USA). The left femoral artery was

cannulated with a heparinised cannula for close intra-arterial (i.a.) administration of drugs to the urinary bladder and urethra. Body temperature was monitored with a rectal temperature probe and maintained between 36-38 °C using a homeothermic blanket system (Harvard). Animals were either spontaneously breathing oxygen enriched air, or artificially ventilated (rate 80 min<sup>-1</sup>, stroke volume 8 ml kg<sup>-1</sup>) with oxygen enriched room air by use of a positive pressure pump (Harvard Rodent Ventilator 683) in preparations where neuromuscular blockade was produced by  $\alpha$ -Bungarotoxin. Blood samples were taken from the carotid arterial cannula and blood gases and pH were monitored with a Corning pH/blood gas analyser (Model 238). Blood gases were maintained between 90-130 mmHg Po<sub>2</sub>, 40-50 mmHg Pco<sub>2</sub> and pH 7.3-7.4. Adjustments of the supplemented oxygen levels (spontaneously breathing animals) and respiratory pump rate and volume (artificially ventilated animals) were made as necessary to maintain blood gas and pH balance. The animals were infused (6 ml kg<sup>-1</sup> h<sup>-1</sup>, i.v.) with a solution comprising 10 ml plasma substitute (Gelofusine), 10 ml distilled water, 0.04 g glucose and 0.168 g sodium bicarbonate, to prevent the development of non-respiratory acidosis and to maintain blood volume. The rats were placed in a stereotaxic frame and the head tilted approximately 10-15° to allow the animal to lie in the supine position. This was to prevent the weight of the animal interfering with bladder and urethral pressure recordings.

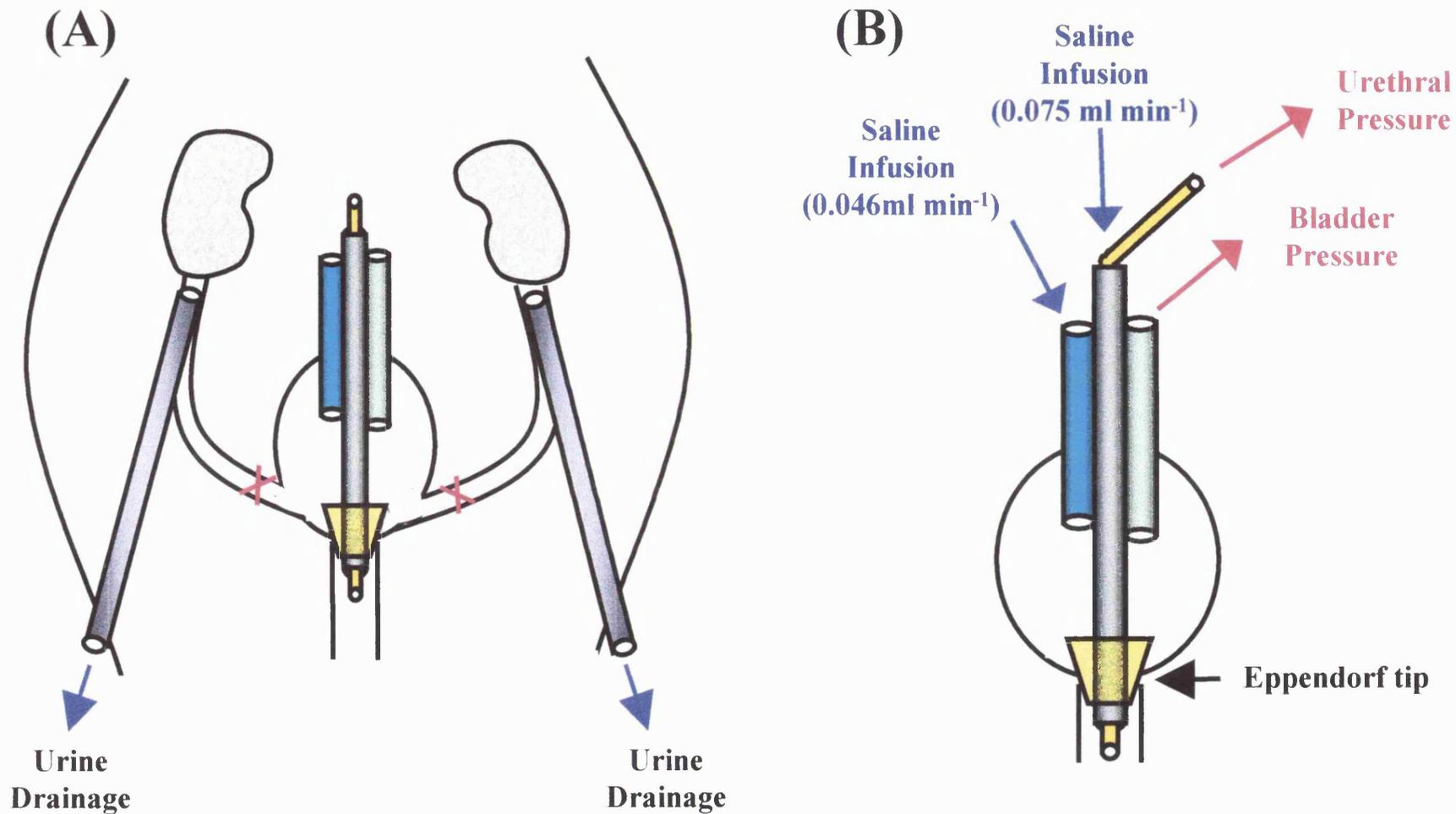
*Measurement of bladder and urethral pressures*

The urinary bladder was exposed by a midline abdominal incision and the ureters emerging from the base of the bladder were tied distally and cut. The proximal ends of each ureter leading from the kidneys were exposed by retroperitoneal incisions and cannulated (0.28 mm internal diameter, 0.61 mm outer diameter) to prevent the bladder filling with urine during experiments. A cut was made in the bladder dome and a cannula (0.5 mm internal diameter, 1.2 mm outer diameter) was inserted into the bladder and connected to a pressure transducer (Gould Statham P23Db) to record intravesical bladder pressure. A second cannula (0.58 mm internal diameter, 0.96 mm outer diameter) was inserted into the bladder and connected to a syringe pump (Semat, St Albans, UK) for the infusion of saline (0.9 % w/v) to evoke the micturition reflex. Backflow through this cannula allowed the bladder to be emptied of saline. Urethral pressure was measured based on a method developed by M.Fraser (Fraser, Flood & de Groat, 1995; Kakizaki *et al.*, 1997; Figure 2.1). A double lumen cannula with its tip embedded in a Gilson eppendorf pipette tip (1000  $\mu$ l) was inserted through the cut in the bladder dome, and wedged into the bladder neck, permitting separation of the bladder and urethra, without surgical interruption of the vesicourethral junction. The outer cannula (0.86 mm internal diameter, 1.52 mm outer diameter) was connected to a syringe pump (Semat, St Albans, U.K.) for the continuous perfusion of saline through the urethra ( $0.075 \text{ ml min}^{-1}$ ), that was allowed to drain freely through the urethral opening. The inner cannula (0.40 mm internal diameter, 0.80 mm outer diameter) was connected to a pressure transducer (Gould Statham P23Db). Urethral pressure was therefore measured as

a resistance to flow of saline through the urethra. All 3 cannulae inserted into the bladder were secured with a purse-string suture.

#### *EUS-EMG recordings*

In preparations where the effects of test drugs were investigated on the striated muscle of the urethra, two fine copper wire electrodes (0.2 mm diameter) were inserted into the external urethral sphincter. The tip of an electrode was positioned in the bevel of a needle (25G) and the needle was inserted percutaneously approximately 0.5 cm lateral and 0.5 cm caudal to the urethral opening. The needle and wire were advanced approximately 1 mm through the skin and the needle slowly withdrawn leaving the wire inserted in the external urethral sphincter. A second electrode was inserted in the same position on the contralateral side of the urethral opening. The electrodes were connected to a Neurolog head stage (NL100; Digitimer, Welwyn Garden City, U.K.) and the signal was amplified (20 kHz; NL104) and filtered (5 kHz; NL125, and displayed on an oscilloscope (Tektronix, 5103N). At the end of experiments, animals were neuromuscular blocked with decamethonium ( $3 \text{ mg kg}^{-1}$ , i.v.) to confirm the position of the electrodes in the striated muscle of the urethra.



**Figure 2.1.** Schematic representation of the experimental methods used. (A) The ureters were tied and cut at the level of the bladder and cannulated at the level of the kidneys. (B) A double lumen cannula embedded in an eppendorf tip that was wedged in the bladder neck allowed simultaneous measurement of bladder and urethral pressures. Two cannula inserted into the bladder allowed measurement of intravesical bladder pressure and saline infusion to evoke the micturition reflex.

## Experimental Protocols

### *Effects of test substances on reflex- and DMPP-evoked responses*

Following surgery, the animals were allowed to stabilise for 30 min. Warmed saline was then infused into the bladder ( $0.046 \text{ ml min}^{-1}$ ) to evoke the micturition reflex (the “initial” reflex). This rate of infusion of saline into the bladder was chosen to simulate the maximal hourly diuresis rate within the physiological range (see Klevmark, 1974). After the appearance of three consecutive reflex-evoked bladder contractions of similar amplitude, saline infusion was discontinued. These bladder contractions were associated with urethral relaxations and, usually, the appearance of high frequency oscillations in urethral pressure (Figure 2.2). The infused volume of saline was left in the bladder for a 5 min period, during which time the reflex was ongoing. The bladder was then emptied and filled with half the volume of saline that was required to evoke this initial reflex so baseline bladder and urethral pressures could be attained without reflex activity. The preparation was then allowed to stabilise for 10 min, after which DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.) was administered (the “initial” DMPP response). This dose was chosen as it was found to produce reproducible bladder and urethral responses without any effect on skeletal muscle. After a further 10 min the bladder was emptied, and 5 min later a test drug or vehicle was administered. After a 10 min period, to allow stabilisation of any changes in baseline variables caused by these substances, a second micturition reflex was evoked, followed by DMPP administration, as described above. In some of these experiments L-arginine or vehicle was further administered, after which additional reflexes and

DMPP injections were carried out. In a separate group of animals, following the initial reflex and administration of DMPP, zaprinast or vehicle was infused i.v. for 30 min and reflex- and DMPP-evoked responses were repeated as above. In experiments involving neuromuscular blockade, the initial reflex and DMPP responses were carried out as above after which the animal was artificially ventilated and  $\alpha$ -Bungarotoxin was administered. A 30 min period then followed to allow stabilisation of blood gases and baseline variables, after which additional reflexes and DMPP injections were carried out following further administration of test substances. In a fourth group of experiments, where the effects of manipulation of the NO/cGMP signalling pathway were assessed on the activity of the external urethral sphincter, a reflex was carried out after the 30 min post-surgical stabilisation period to ensure that an adequate EMG signal was being recorded. A 20 min period then followed during which any alterations in the position of the electrodes could be made. The initial micturition reflex was then evoked and further reflexes were obtained following the administration of test substances as outlined above. The effects of DMPP on EUS-EMG activity were not investigated.

#### *Effects of intraurethral administration of test substances*

Following a 30 min post-surgical stabilisation period, baseline urethral perfusion pressures and EUS-EMG activities were recorded with saline perfusion through the urethra ( $0.075 \text{ ml min}^{-1}$ ) for 20 min. Baseline bladder pressures were not recorded as the cannula used to fill the bladder with saline was left unconnected to the syringe pump to ensure that activity from the bladder did not affect

urethral pressure and EUS-EMG recordings. The perfusate was then changed to drug or vehicle (0.9% w/v saline) by changing the syringe on the syringe pump. This resulted in a loss of urethral perfusion pressure recordings for a period of no more than 30 s, after which urethral pressure returned to previous levels. Drug or vehicle was then perfused ( $0.075 \text{ ml min}^{-1}$ ) for 20 min. In experiments investigating the effects of ganglionic blockade, after the 30 min post-surgical stabilisation period, chlorisondamine was administered i.v., and effects on baseline variables were allowed to stabilise (10-15 min). The protocol outlined above was then followed.

### **Data capture and analysis**

Arterial blood pressures, bladder and urethral pressures were continuously displayed on a chart recorder (Grass Instruments) and captured (1500 samples per second) by a MP 100 WSW interface (Biopac Systems Inc, USA) to allow data to be acquired and analysed off-line using *AcqKnowledge version 3.5.3* software (Biopac Systems Inc, USA). Heart rates (HR) were derived electronically on-line from the blood pressure signal using this software (Biopac Systems Inc, USA). The amplified EMG signal was captured (1500 samples per second) and the input integrated off-line using *AcqKnowledge version 3.5.3* software (Biopac Systems Inc, USA).

*Reflex- and DMPP-evoked bladder and urethral responses*

Saline infusion into the bladder produced bladder contractions of increasing amplitude that were followed by rhythmic contractions with comparable amplitudes as the volume of saline in the bladder increased. These large amplitude rhythmic contractions have been assumed to represent a micturition reflex (Maggi, Santicioli & Meli, 1986). After the appearance of three consecutive reflex-evoked bladder contractions of similar amplitude, saline infusion was discontinued, after which the reflex was ongoing. The mean amplitude (mmHg) and duration (s) of the first three bladder contractions that occurred after saline infusion was discontinued were measured. These three bladder contractions were analysed as they represented isovolumetric reflex bladder contractions, i.e. they occurred when the amount of saline in the bladder, and therefore resting bladder pressure, was constant. The mean amplitude (mmHg) and duration (s) of the three urethral relaxations that accompanied these bladder contractions were also measured. High frequency oscillations in urethral pressure occurred with each reflex bladder contraction, that were associated with high frequency EUS-EMG bursting (Figures 2.2 and 2.3). The mean amplitude (mmHg) and duration (s) of these high frequency oscillations in urethral pressure and the mean area of these integrated EMG bursts that occurred with the three isovolumetric bladder contractions were measured. For DMPP effects, the amplitude (mmHg) and duration (s) of bladder contractions, and amplitude (mmHg) and duration (s) of urethral relaxations were measured. The micturition reflex pressure thresholds were taken as the bladder pressure (mmHg) at which

the first reflex bladder contraction with concomitant reflex urethral activity (i.e. relaxation and high frequency oscillations) occurred. The volume threshold (ml) was calculated by expressing the time to evoke this first bladder contraction in volumes of saline. The frequency of the reflex was determined by measuring the number of reflex bladder contractions that occurred from the first reflex bladder contraction to the point where the bladder was emptied of saline. This was then divided by the total time over this period to express frequency as bladder contractions per min. Baseline values for bladder and urethral pressures, mean arterial pressure (MAP) and heart rate (HR) were measured 2 min before the reflex. The effects of bolus administration of test substances on baseline bladder and urethral pressures, MAP and HR were measured 5 min after their administration. This time was chosen as it was adjudged that effects evoked on these variables had stabilised. The effects of zaprinast or vehicle on these baseline variables were measured 30 min after start of infusion i.v. The total area of integrated EUS-EMG activity was measured over a 3 min period, 2 min before and 30 min after administration of various test substances as above. The area of integrated noise (measured after administration of decamethonium at the end of experiments) was subtracted from these integrated EUS-EMG values. Changes in baseline and reflex- and DMPP-evoked effects on the variables measured were expressed as percentage changes before and after the administration of test substances and compared with vehicle controls by unpaired Student's t-test. Changes in baseline values caused by the infusion of zaprinast for 30 min were expressed as percentage changes before and after administration, and compared with vehicle controls and experiments involving pre-treatment with  $\alpha$ -Bungarotoxin by two-way analysis of variance followed by the least significance

difference test (Sokal & Rohlf, 1969). P values of less than 0.05 were considered indicative of a statistically significant difference. All values are expressed as mean  $\pm$  s.e.mean.

*Urethral and EUS-EMG responses to intraurethral perfusion of test substances*

Baseline urethral pressures, MAP and HR were measured 5 min before the start of intraurethral administration of vehicle or test substances. The effects of test substances on these parameters were measured every 5 min until 20 min after the start of intraurethral perfusion. The total area of integrated EUS-EMG activities were measured over a 3 min period 5 min before the start of intraurethral administration of test substances, and every 5 min after the start of perfusion, as described above. The area of integrated noise (measured after administration of decamethonium at the end of experiments) was subtracted from these integrated EUS-EMG values. The effects of bolus administration of chlorisondamine on baseline bladder and urethral pressures, MAP and HR were measured 5 min after administration. This time was chosen as it was adjudged that effects evoked on these variables had stabilised. The effects of intraurethral perfusion of substances were then examined according to the protocol outlined above. Changes in baseline values were expressed as percentage changes before and after the administration of test substances and compared with vehicle controls and experiments involving pre-treatment with chlorisondamine by two-way analysis of variance followed by the least significance difference test (Sokal & Rohlf, 1969). P values of less than 0.05 were considered indicative as a statistically significant difference. All values are expressed as mean  $\pm$  s.e.mean.

*Drugs and solutions*

Drugs and chemicals were obtained from the following sources: urethane, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), N $\omega$ -nitro-L-arginine methyl ester (L-NAME; hydrochloride), L-arginine (hydrochloride),  $\alpha$ -Bungarotoxin, zaprinast, decamethonium bromide, sodium nitroprusside, isoprenaline (hydrochloride) and chlorisondamine (iodide) from Sigma Aldrich Chemicals., Poole, Dorset, U.K.; pentobarbitone sodium from Rhône Mérieux Ltd, Harlow, Essex; isoflurane from Abbott Labs, Queenborough, Kent; gelofusine from Braun Medical Ltd, Aylesbury, Bucks; sodium chloride, glucose, sodium bicarbonate and triethanolamine from Merck/BDH, Poole, Dorset; heparin from CP Pharmaceuticals Ltd, Wrexham. DMPP, L-NAME, L-arginine,  $\alpha$ -Bungarotoxin, decamethonium bromide, sodium nitroprusside and isoprenaline were all dissolved in 0.9% w/v saline. Zaprinast was dissolved in 5% triethanolamine in 0.9% w/v saline. All i.v. agents except zaprinast were administered in a 0.03 ml dose volume followed by a flush of 0.1 ml saline. Zaprinast or vehicle was infused i.v. at a rate of 6 ml kg<sup>-1</sup> min<sup>-1</sup>. DMPP i.a. was administered in a 0.05 ml dose volume followed by a flush of 0.1 ml heparinised saline.

## RESULTS

### REFLEX- AND DMPP-EVOKED RESPONSES

#### Initial reflex- and DMPP-evoked responses

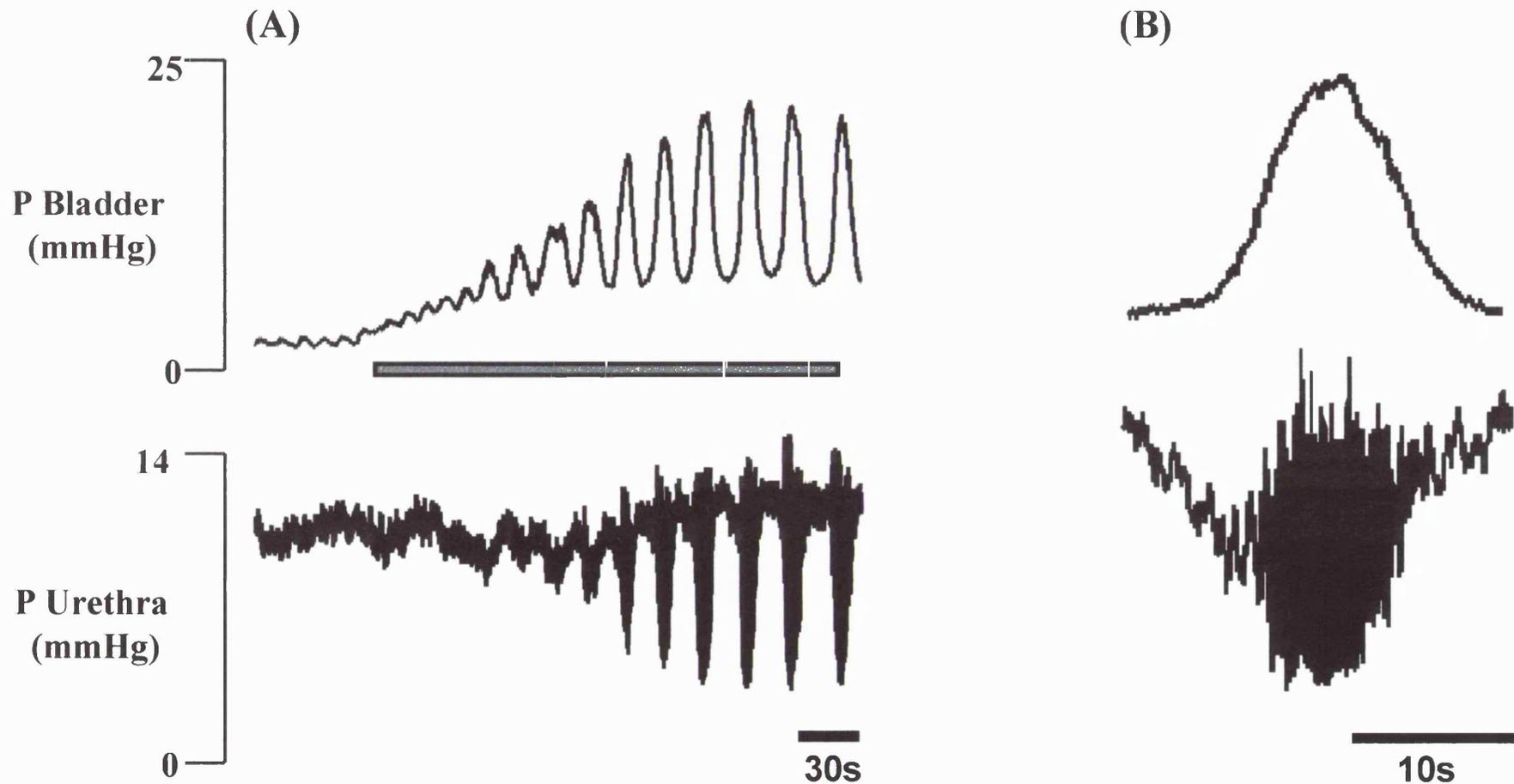
##### *Initial reflex-evoked responses*

Infusion of saline into the bladder in seventy two female rats caused distension of the bladder, which in turn evoked the micturition reflex, which was characterised by the appearance of rhythmic bladder contractions of  $24.7 \pm 1.0$  mmHg (Figure 2.2). These contractions had a mean duration of  $23.3 \pm 1.1$  s and occurred at a frequency of  $1.6 \pm 0.1$  contractions  $\text{min}^{-1}$ . The mean bladder pressure threshold to evoke the micturition reflex was  $8.5 \pm 0.5$  mmHg, which was reached when  $0.13 \pm 0.02$  ml of saline had been infused into the bladder. Each rhythmic bladder contraction was accompanied by a fall in urethral pressure of  $9.3 \pm 0.5$  mmHg that continued for  $23.7 \pm 1.3$  s before returning to baseline, in some cases above baseline. During these bladder contractions baseline urethral pressure rose by  $3.1 \pm 0.4$  mmHg. In fifty three out of these seventy six animals, high frequency oscillations in urethral pressure occurred at the peak of each bladder contraction that had a mean amplitude of  $12.0 \pm 1.0$  mmHg and continued for  $11.3 \pm 1.0$  s (Figure 2.2). In the remaining eighteen animals, high frequency oscillations in urethral pressure were not observed. This was probably due to variations in the position of the urethral cannula. In animals where urethral striated muscle activity was recorded ( $n = 22$ ), these high frequency oscillations

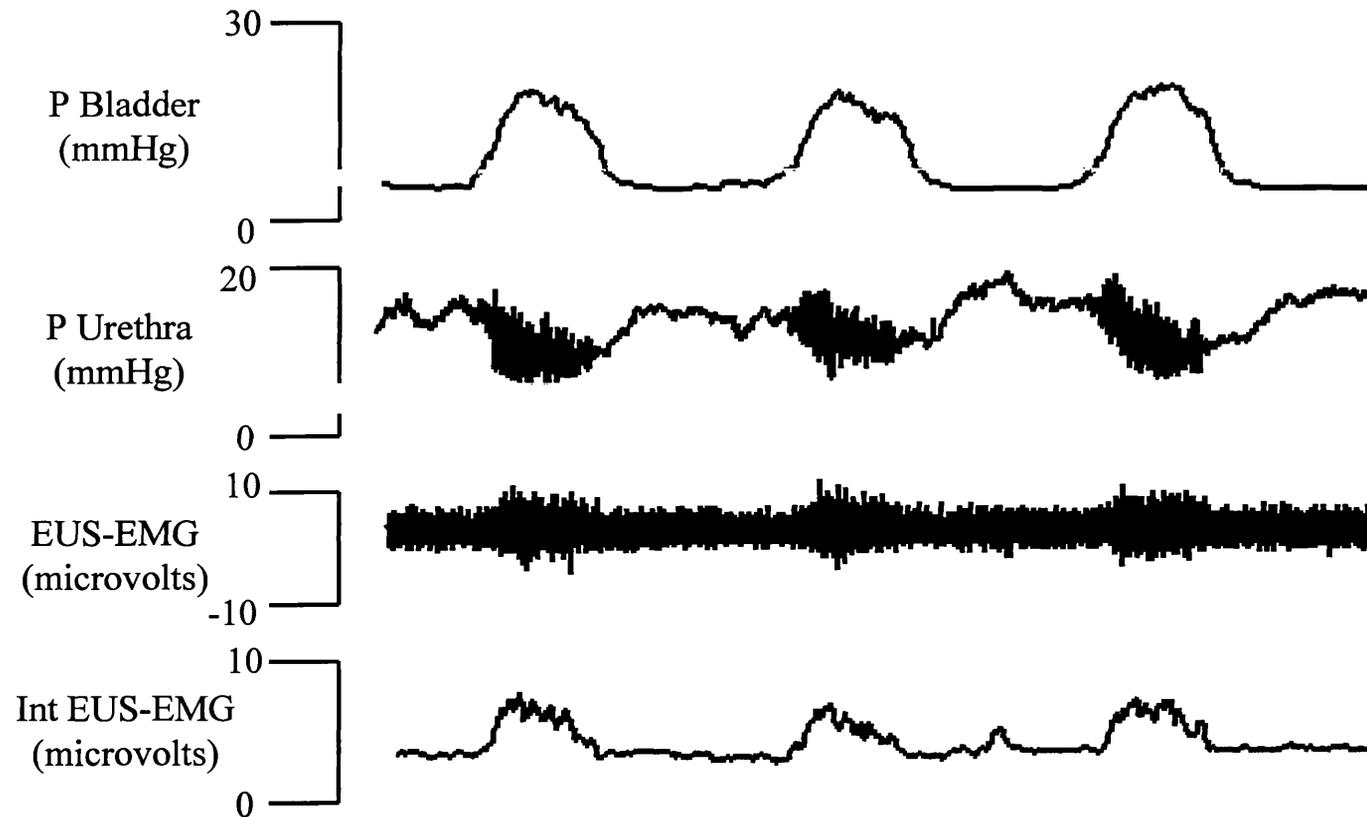
were accompanied by bursting of the EUS-EMG (Figure 2.3). The mean data for the “initial” reflex for each experimental group is shown in Table 2.1. Each reflex bladder contraction was accompanied by an increase in MAP and HR, which were not fully analysed, but ranged between 5 - 23% and 2 - 7%, respectively. The combined mean baseline bladder and urethral pressures, MAP and heart rate were  $3.2 \pm 0.3$ ,  $14.5 \pm 0.6$ ,  $120.6 \pm 1.7$  mmHg and  $406.0 \pm 4.5$  beats  $\text{min}^{-1}$ , respectively. In experiments where EUS-EMG activity was recorded, these animals had a combined mean integrated EUS-EMG activity of  $5.2 \pm 1.2$  microvolts ( $n = 14$ ). The mean baseline data for individual experimental groups is shown in Table 2.2.

#### *Initial DMPP-evoked responses*

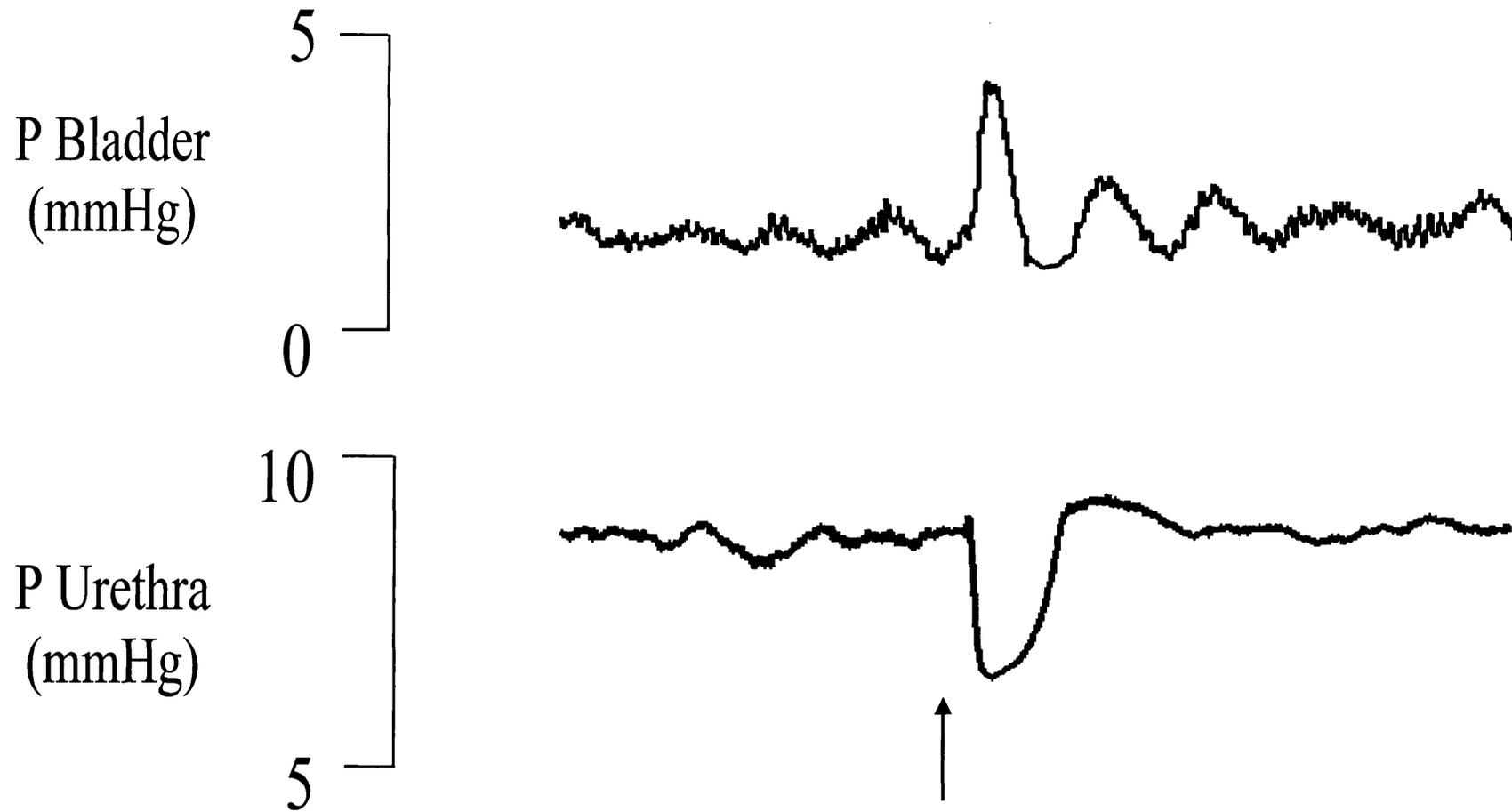
DMPP ( $n = 45$ ;  $0.5 \text{ mg kg}^{-1}$ , i.a.) evoked an increase in bladder pressure of  $3.4 \pm 0.3$  mmHg that lasted for  $11.1 \pm 1.7$  s (Figure 2.4). This bladder contraction was accompanied by fall in urethral pressure that lasted for  $20.7 \pm 2.2$  s reaching a maximum of  $6.3 \pm 0.6$  mmHg. DMPP failed to cause any oscillations in urethral pressure. The mean data for the “initial” DMPP response for each experimental group is shown in Table 2.3. DMPP also evoked a transient increase in MAP and HR that were not fully analysed but ranged between 15 - 28% and 6 - 18%, respectively. The combined mean baseline primed bladder and urethral pressures, MAP and heart rate, i.e. the baseline values before administering DMPP, were  $4 \pm 0.4$ ,  $11 \pm 1.0$ ,  $118 \pm 3.0$  mmHg and  $417 \pm 7$  beats  $\text{min}^{-1}$ , respectively. The mean baseline primed data for individual experimental groups is shown in Table 2.2.



**Figure 2.2.** (A) Trace showing changes in bladder and urethral pressures in response to bladder distension with saline infusion (the micturition reflex) in the urethane-anaesthetised female rat. (B) An individual reflex-evoked bladder contraction showing accompanying urethral relaxation and high frequency oscillations in urethral pressure. Shaded bar represents period of infusion of saline into the bladder.



**Figure 2.3.** Trace showing changes in bladder and urethral pressures, raw and integrated EUS-EMG activities in response to bladder distension with saline infusion (the micturition reflex) in the urethane-anaesthetised female rat.



**Figure 2.4.** Trace showing DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.)-evoked changes in bladder and urethral pressures in the urethane-anesthetised female rat. Arrow denotes administration of agonist.

Experimental Group	"Initial" reflex baselines						"Initial" DMPP response baselines					
	n	P Urethra (mmHg)	P Bladder (mmHg)	EUS-EMG ( $\mu$ V)	MAP (mmHg)	HR (beats min <sup>-1</sup> )	n	P Urethra (mmHg)	P Bladder (mmHg)	MAP (beats min <sup>-1</sup> )	HR (beats min <sup>-1</sup> )	
<b>Non-neuromuscular blocked</b>												
L-NAME (20 mg kg <sup>-1</sup> )	8	12.1 ± 1.7	4.5 ± 0.9	-	134 ± 8	397 ± 7	7	9.7 ± 0.4	6.6 ± 1.8	113 ± 8	386 ± 17	
L-NAME vehicle (saline)	9	10.8 ± 2.9	2.1 ± 0.2	-	116 ± 2	422 ± 17	6	8.9 ± 2.3	5.1 ± 0.9	109 ± 10	458 ± 36	
zaprinast (0.6 mg kg <sup>-1</sup> min <sup>-1</sup> )	6	13.7 ± 1.9	4.8 ± 0.7	-	121 ± 6	397 ± 21	7	12.4 ± 0.9	5.7 ± 0.5	122 ± 4	408 ± 13	
zaprinast vehicle (6 ml kg <sup>-1</sup> hr <sup>-1</sup> ) (5% triethanolamine in saline)	6	12.0 ± 1.7	2.6 ± 0.9	-	111 ± 10	412 ± 15	6	15.3 ± 1.7	3.5 ± 0.5	113 ± 15	396 ± 14	
<b>Neuromuscular blocked</b>												
Before $\alpha$ -Bungarotoxin (0.4 mg kg <sup>-1</sup> )	20	12.0 ± 1.4	2.5 ± 0.6	-	120 ± 5	412 ± 12	20	12.9 ± 2.3	4.3 ± 1.1	126 ± 4	420 ± 9	
After $\alpha$ -Bungarotoxin L-NAME	5	14.0 ± 0.6	3.2 ± 0.6	-	116 ± 10	405 ± 9	5	13.5 ± 0.8	5.6 ± 0.5	118 ± 10	410 ± 12	
L-NAME vehicle	5	13.3 ± 1.1	2.9 ± 0.7	-	120 ± 7	401 ± 10	5	13.6 ± 0.5	3.5 ± 0.4	120 ± 6	400 ± 14	
zaprinast	5	10.7 ± 2.2	3.6 ± 1.2	-	127 ± 2	442 ± 7	5	11.8 ± 3.1	4.8 ± 1.4	124 ± 6	428 ± 8	
zaprinast vehicle	5	13.6 ± 0.6	1.7 ± 0.5	-	115 ± 11	389 ± 15	5	15.1 ± 1.7	3.4 ± 1.1	131 ± 3	403 ± 5	
<b>EUS-EMG recordings</b>												
L-NAME	5	13.6 ± 2.7	2.9 ± 1.3	5.1 ± 1.6	121 ± 10	406 ± 15	-	-	-	-	-	
L-NAME vehicle	5	15.6 ± 2.3	3.6 ± 0.6	6.2 ± 1.3	117 ± 6	401 ± 11	-	-	-	-	-	
zaprinast	6	17.1 ± 2.1	4.6 ± 0.9	6.1 ± 1.2	123 ± 4	390 ± 20	-	-	-	-	-	
zaprinast vehicle	6	18.4 ± 2.2	3.7 ± 1.0	5.9 ± 1.1	125 ± 3	407 ± 8	-	-	-	-	-	

**Table 2.1.** Baseline values of urethral and bladder pressure, external urethral sphincter (EUS-EMG) activity, mean arterial blood pressure (MAP) and heart rate (HR) for all experimental groups in urethane-anaesthetised female rats. All drugs and solutions were given i.v.

Experimental Group	n	Thresholds		Rhythmic bladder contractions			Urethral relaxations		Urethral high frequency oscillations		EUS-EMG activity	
		Bladder Pressure (mmHg)	Saline volume (ml)	Amplitude (mmHg)	Duration (s)	Frequency (contraction min <sup>-1</sup> )	Amplitude (mmHg)	Duration (s)	Amplitude (mmHg)	Duration (s)	Total amount (area/duration)	Mean burst size ( $\mu$ v)
<b>Non-neuromuscular blocked</b>												
L-NAME (20 mg kg <sup>-1</sup> )	8	7.4 ± 0.8	0.2 ± 0.03	24.7 ± 1.8	23.4 ± 2.6	1.7 ± 0.2	9.2 ± 1.0	20.9 ± 2.6	15.9 ± 2.4 (5)	10.7 ± 2.2	-	-
L-NAME vehicle (saline)	9	7.8 ± 1.0	0.1 ± 0.03	29.4 ± 4.3	26.7 ± 2.5	1.4 ± 0.3	9.2 ± 1.3	23.7 ± 4.3	14.3 ± 1.91 (6)	10.9 ± 2.8	-	-
zaprinast (0.6 mg kg <sup>-1</sup> min <sup>-1</sup> )	6	6.2 ± 1.1	0.1 ± 0.01	25.4 ± 1.6	29.1 ± 5.1	2.0 ± 0.5	11.8 ± 1.3	36.9 ± 5.1	6.2 ± 3.5 (4)	9.5 ± 6.1	-	-
zaprinast vehicle (6 ml kg <sup>-1</sup> hr <sup>-1</sup> ) (5% triethanolamine in saline)	6	5.6 ± 0.4	0.1 ± 0.02	32.8 ± 3.0	21.5 ± 2.1	1.6 ± 0.1	9.3 ± 2.0	29.3 ± 2.3	8.8 ± 3.7 (5)	14.0 ± 1.6	-	-
<b>Neuromuscular blocked</b>												
Before $\alpha$ -Bungarotoxin (0.4 mg kg <sup>-1</sup> )	20	7.8 ± 1.2	0.2 ± 0.05	24.1 ± 1.3	21.0 ± 2.0	1.5 ± 0.1	7.6 ± 1.2	22.3 ± 2.5	8.7 ± 2.0 (15)	11.6 ± 1.8	-	-
After $\alpha$ -Bungarotoxin L-NAME	5	8.6 ± 1.2	0.2 ± 0.04	28.3 ± 2.1	23.6 ± 2.4	1.7 ± 0.3	9.3 ± 1.5	24.6 ± 3.4	-	-	-	-
L-NAME vehicle	5	7.4 ± 2.4	0.2 ± 0.06	30.6 ± 1.5	22.6 ± 1.4	2.0 ± 0.4	8.5 ± 1.0	28.4 ± 4.6	-	-	-	-
zaprinast	5	7.2 ± 2.3	0.2 ± 0.03	27.2 ± 1.9	22.2 ± 1.1	1.3 ± 0.1	8.0 ± 1.6	38.4 ± 3.2	-	-	-	-
zaprinast vehicle	5	6.3 ± 1.0	0.2 ± 0.07	20.4 ± 2.1	20.2 ± 1.3	1.1 ± 0.2	6.2 ± 1.6	25.7 ± 5.0	-	-	-	-
<b>EUS-EMG recordings</b>												
L-NAME	5	8.9 ± 3.2	0.2 ± 0.03	24.3 ± 6.2	21.8 ± 5.6	1.5 ± 0.6	8.6 ± 1.0	21.1 ± 8.2	6.2 ± 3.6 (4)	7.9 ± 2.4	22.9 ± 3.6	10.5 ± 1.1
L-NAME vehicle	5	9.6 ± 2.4	0.2 ± 0.08	25.3 ± 3.1	25.7 ± 1.4	1.7 ± 0.2	7.4 ± 1.5	23.8 ± 4.6	7.3 ± 1.2 (4)	8.1 ± 1.6	32.2 ± 6.5	10.7 ± 2.2
zaprinast	6	7.8 ± 1.5	0.2 ± 0.09	26.9 ± 2.8	23.6 ± 2.6	1.4 ± 0.2	10.0 ± 3.3	30.1 ± 4.8	7.6 ± 2.6 (5)	11.4 ± 2.6	31.5 ± 7.2	10.6 ± 1.3
zaprinast vehicle	6	8.9 ± 1.0	0.2 ± 0.06	23.6 ± 1.9	20.7 ± 5.4	1.7 ± 0.4	10.5 ± 1.9	20.9 ± 6.5	12.1 ± 3.1 (5)	10.9 ± 4.6	24.4 ± 5.4	10.0 ± 2.3

**Table 2.2.** Baseline values of control "initial" reflex-evoked changes in bladder and urethral pressures and external urethral sphincter activities caused by saline infusion into the bladder for each experimental group in urethane-anaesthetised female rats. All drugs and solutions were given i.v.

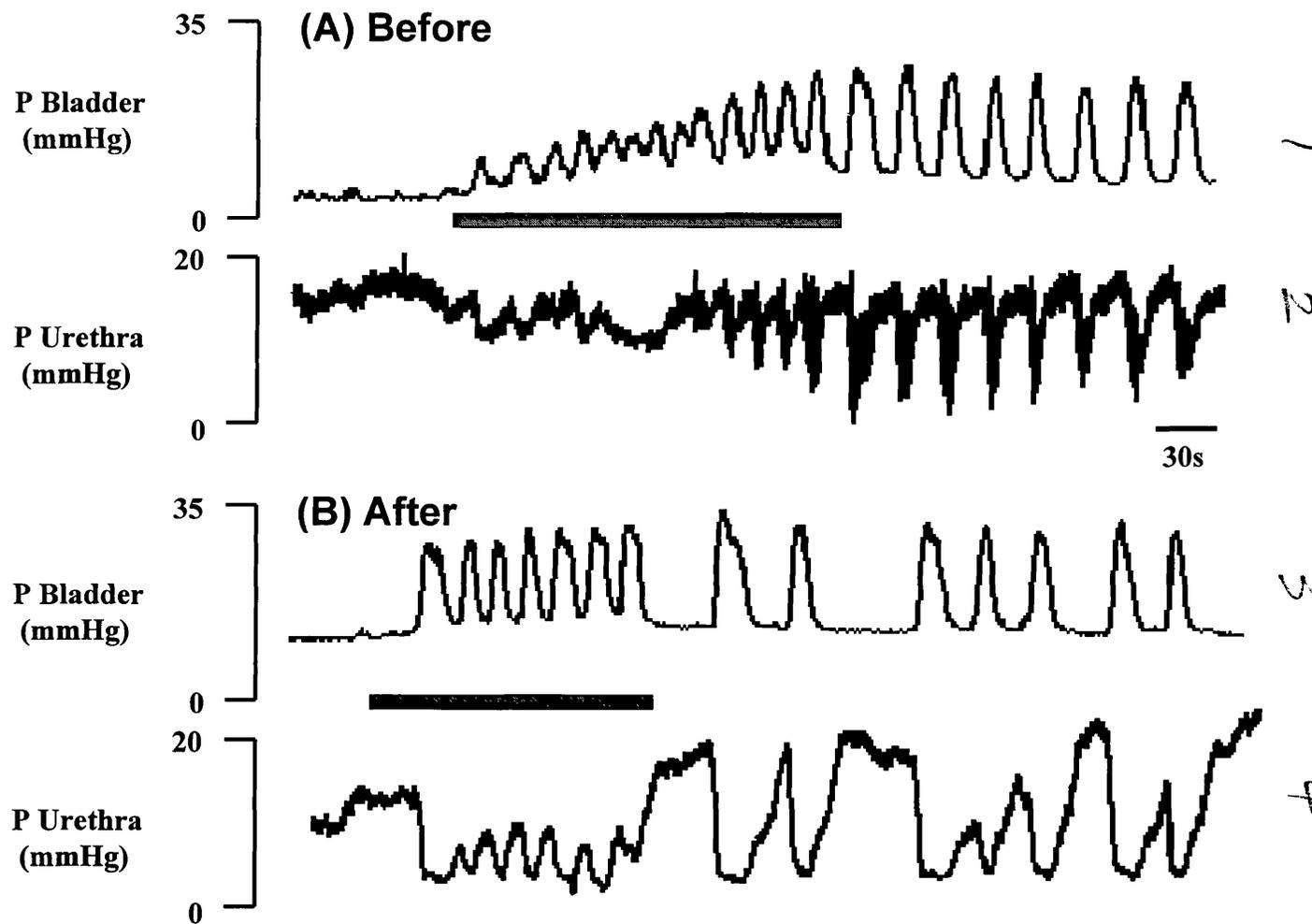
Experimental Group	n	Bladder contraction		Urethral relaxation	
		Amplitude (mmHg)	Duration (s)	Amplitude (mmHg)	Duration (s)
<b>Non-neuromuscular blocked</b>					
L-NAME (20 mg kg <sup>-1</sup> )	7	3.97 ± 1.19	11.34 ± 1.46	8.03 ± 3.29	22.80 ± 9.60
L-NAME vehicle (saline)	6	2.66 ± 0.37	13.17 ± 5.73	6.45 ± 2.22	25.78 ± 4.02
zaprinast (0.6 mg kg <sup>-1</sup> min <sup>-1</sup> )	6	3.29 ± 0.83	9.50 ± 1.44	6.38 ± 1.16	24.89 ± 3.05
zaprinast vehicle (6ml kg <sup>-1</sup> hr <sup>-1</sup> ) (5% triethanolamine in saline)	6	3.32 ± 0.69	11.75 ± 1.28	7.77 ± 0.97	25.32 ± 2.19
<b>Neuromuscular blocked</b>					
Before α-Bungarotoxin (0.4 mg kg <sup>-1</sup> , i.v.)	20	3.79 ± 0.44	9.16 ± 3.08	7.46 ± 0.60	20.37 ± 0.69
After α-Bungarotoxin L-NAME	5	3.51 ± 0.85	10.78 ± 1.52	8.23 ± 0.77	21.61 ± 1.52
L-NAME vehicle	5	4.26 ± 1.06	9.73 ± 2.51	7.05 ± 1.48	24.62 ± 3.21
zaprinast	5	4.38 ± 0.77	8.60 ± 2.0	7.77 ± 2.19	22.83 ± 4.01
zaprinast vehicle	5	2.40 ± 1.30	8.82 ± 0.62	8.39 ± 0.49	21.40 ± 0.31

**Table 2.3.** Control "initial" DMPP (0.5 mg kg<sup>-1</sup>, i.a.)-evoked changes in bladder and urethral pressures, external urethral sphincter activities (EUS-EMG) for each experimental group in urethane-anaesthetised female rats. All drugs and solutions were given i.v.

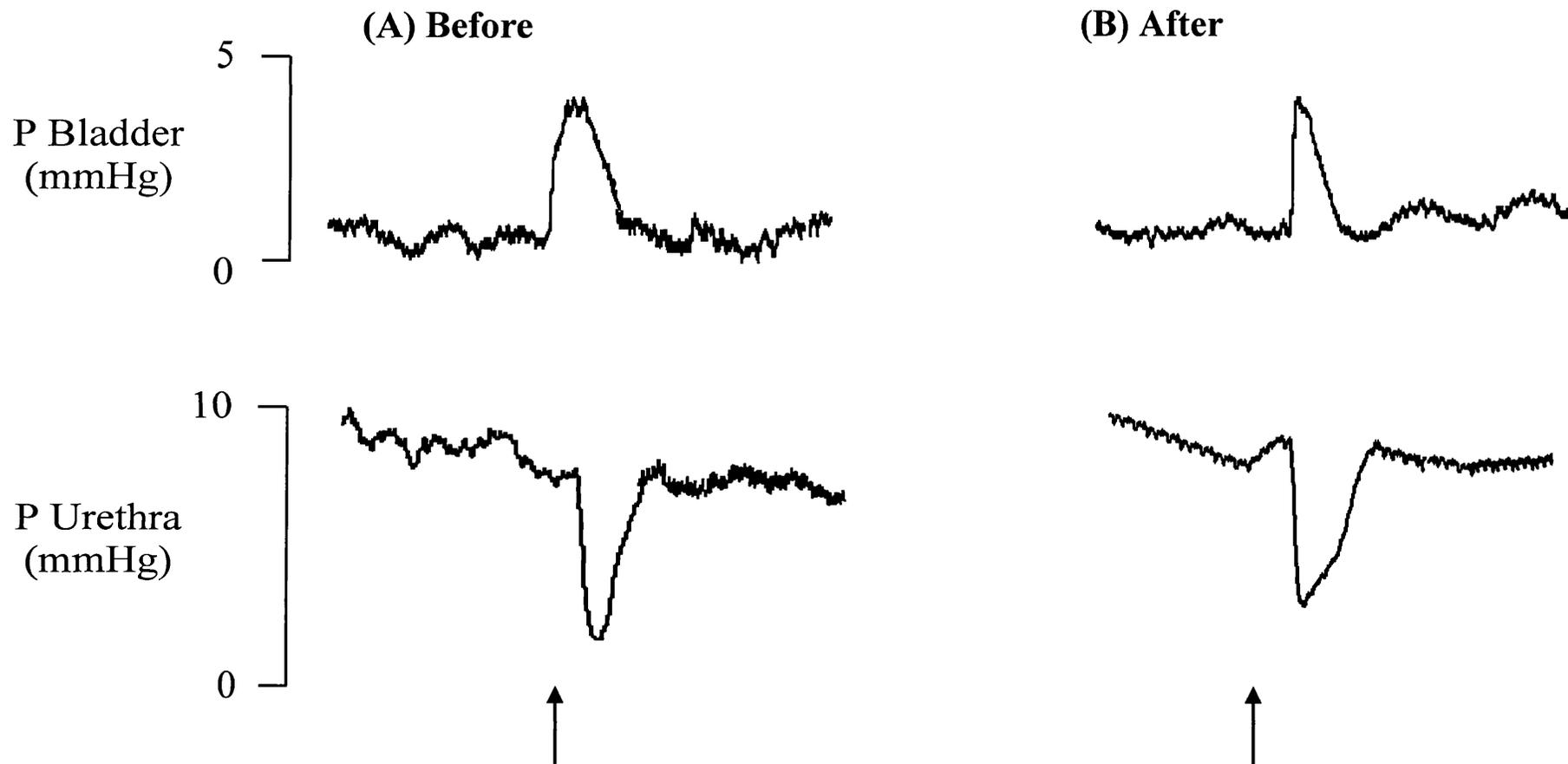
**Effects of vehicles and  $\alpha$ -Bungarotoxin on reflex- and DMPP-evoked responses and baseline values**

Administration of vehicles for L-NAME, L-arginine (0.9% w/v saline) and zaprinast (5% triethanolamine in 0.9% w/v saline) had no effect on reflex- and DMPP-evoked changes in bladder and urethral pressures and EUS-EMG activities in all experimental groups. In addition, none of these vehicles had any effect on baseline bladder and urethral pressures, MAP and HR.

$\alpha$ -Bungarotoxin (n = 15; 0.4 mg kg<sup>-1</sup>, i.v.) had no effect on reflex- and DMPP-evoked changes in bladder and urethral pressures (Figures 2.5 and 2.6). However, the reflex-evoked high frequency oscillations in urethral pressure that accompanied reflex bladder contractions and urethral relaxations were abolished (Figure 2.5).  $\alpha$ -Bungarotoxin also had no effect on baseline bladder and urethral pressures, MAP and HR after 30 min (allowing stabilisation of blood gases and pH comparable to the spontaneously breathing animal).



**Figure 2.5.** Trace showing reflex-evoked changes in bladder and urethral pressures before (A) and after (B) the administration of  $\alpha$ -Bungarotoxin ( $0.4 \text{ mg kg}^{-1}$ , i.v.) in the urethane-anaesthetised female rat. Shaded bars represent period of infusion of saline into the bladder ( $0.046 \text{ ml min}^{-1}$ ).



**Figure 2.6.** Traces showing DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.)-evoked changes in bladder and urethral pressures before (A) and after (B) the administration of  $\alpha$ -Bungarotoxin ( $0.4 \text{ mg kg}^{-1}$ , i.v.) in the urethane-anaesthetised female rat. Arrows denote administration of agonist.

**Effects of L-NAME, L-arginine and zaprinast on reflex -evoked changes in non- and  $\alpha$ -Bungarotoxin pre-treated rats**

*Urethral Pressure*

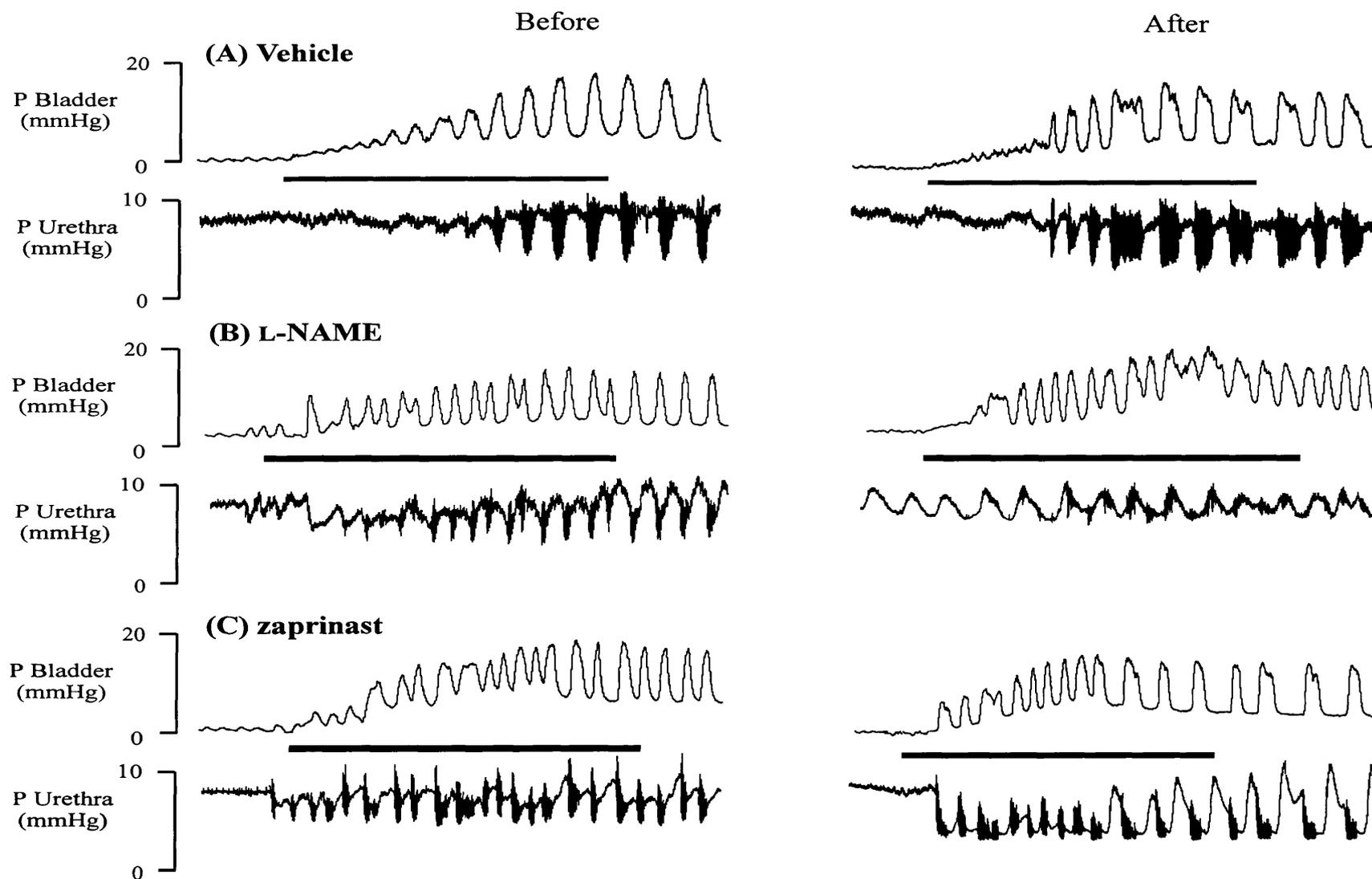
L-NAME (n = 13; 20 mg kg<sup>-1</sup>, i.v.) significantly attenuated the amplitude and duration of reflex-evoked urethral relaxations by 65 ± 10% and 51 ± 13%, respectively (Figure 2.7). In addition, the amplitude of reflex-evoked high frequency oscillations in urethral pressure were significantly reduced by 56 ± 12% (n = 9), but L-NAME had no effect on the duration of this high frequency bursting. In 4 animals, further treatment of L-arginine (150 mg kg<sup>-1</sup>, i.v.) reversed these effects of L-NAME. In  $\alpha$ -Bungarotoxin pre-treated animals administration of L-NAME (n = 5) also significantly attenuated the amplitude and duration of reflex-evoked urethral relaxations by 69 ± 9% and 46 ± 15%, respectively (Figure 2.8). These effects of L-NAME were not significantly different to those obtained in non- $\alpha$ -Bungarotoxin pre-treated animals. These effects of L-NAME were also reversed after further administration of L-arginine (n = 5).

Zaprinast (n = 12; 0.6 mg kg<sup>-1</sup> min<sup>-1</sup>, i.v., for 30 minutes) significantly potentiated the amplitude and duration of reflex-evoked urethral relaxations by 68 ± 24 % and 97 ± 31 %, respectively (Figure 2.7). Furthermore, zaprinast significantly attenuated the amplitude of reflex-evoked high frequency oscillations in urethral pressure by 30 ± 10 % (n = 9), but had no effect on the duration of this high frequency bursting. (However, these inhibitory effects of zaprinast and L-NAME on this oscillatory urethral activity are likely to be due to

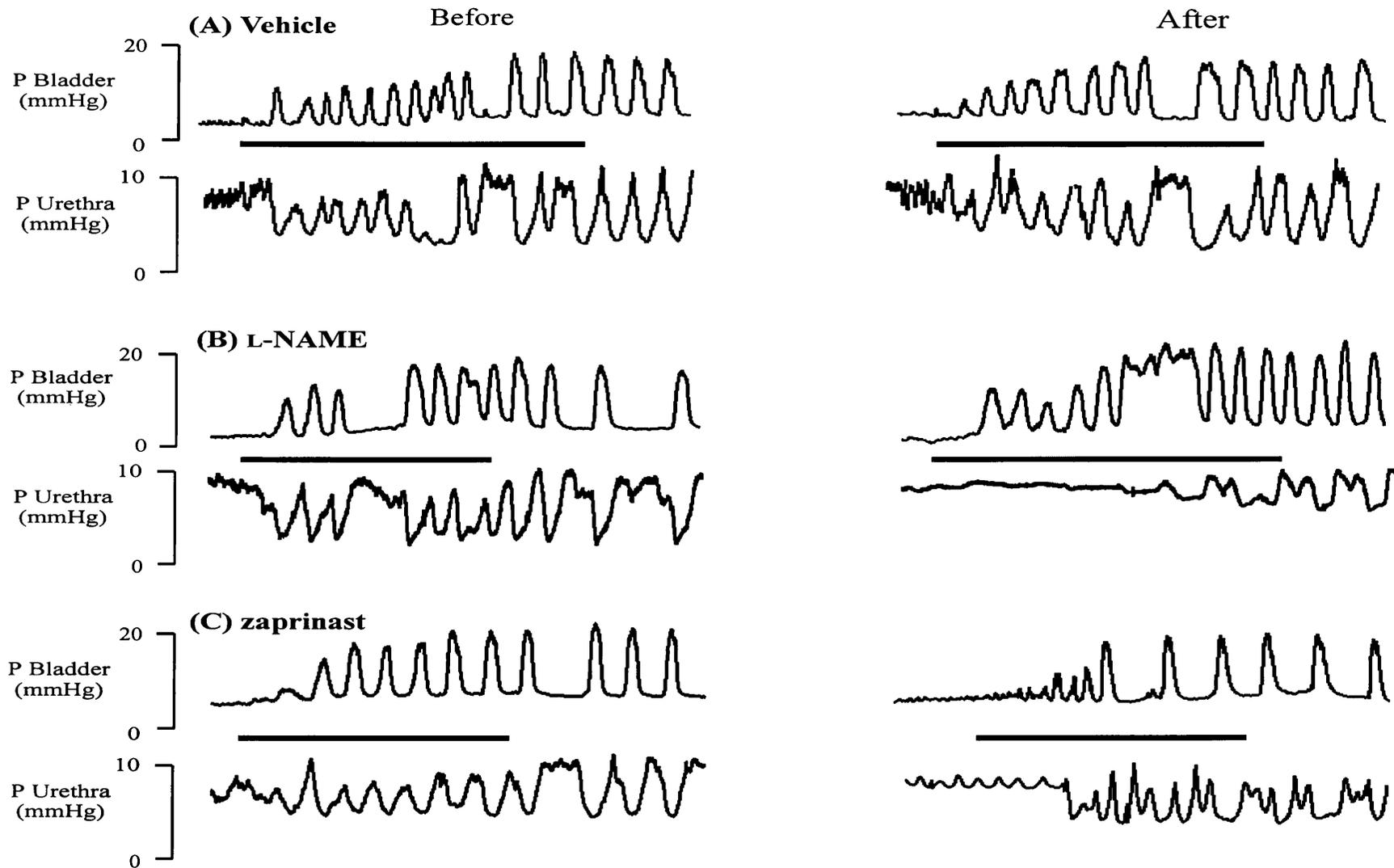
changes in recording sensitivities as both these antagonists increased EUS-EMG bursting, see further results). In  $\alpha$ -Bungarotoxin pre-treated animals (n = 6), zaprinast had a non-significant effect on the amplitude of reflex-evoked urethral relaxations causing an overall inhibition of  $20 \pm 8 \%$ , but inhibited the rise in baseline urethral pressure between the rhythmic bladder contractions observed during the reflex by  $95 \pm 7\%$  (Figure 2.8). In addition, zaprinast did not affect the duration of these relaxations. No high frequency oscillations were observed in  $\alpha$ -Bungarotoxin pre-treated animals.

#### *Bladder Pressure*

In non- and  $\alpha$ -Bungarotoxin pre-treated animals L-NAME and zaprinast had no significant effects on the amplitude, duration or frequency of reflex-evoked bladder contractions (Figures 2.7 and 2.8). In addition, the volume and pressure thresholds to evoke the micturition reflex were unaffected following administration of these test substances. Further treatment of L-arginine had no effect on any reflex-evoked bladder variables that were measured.



**Figure 2.7.** Traces showing reflex-evoked changes in bladder and urethral pressures before and after the administration of vehicle (A), L-NAME ( $20 \text{ mg kg}^{-1}$ , i.v.; B) and zaprinast ( $0.6 \text{ mg kg}^{-1} \text{ min}^{-1}$ , i.v.; C) in urethane-anesthetized female rats. Shaded bars represent infusion of saline into the bladder.

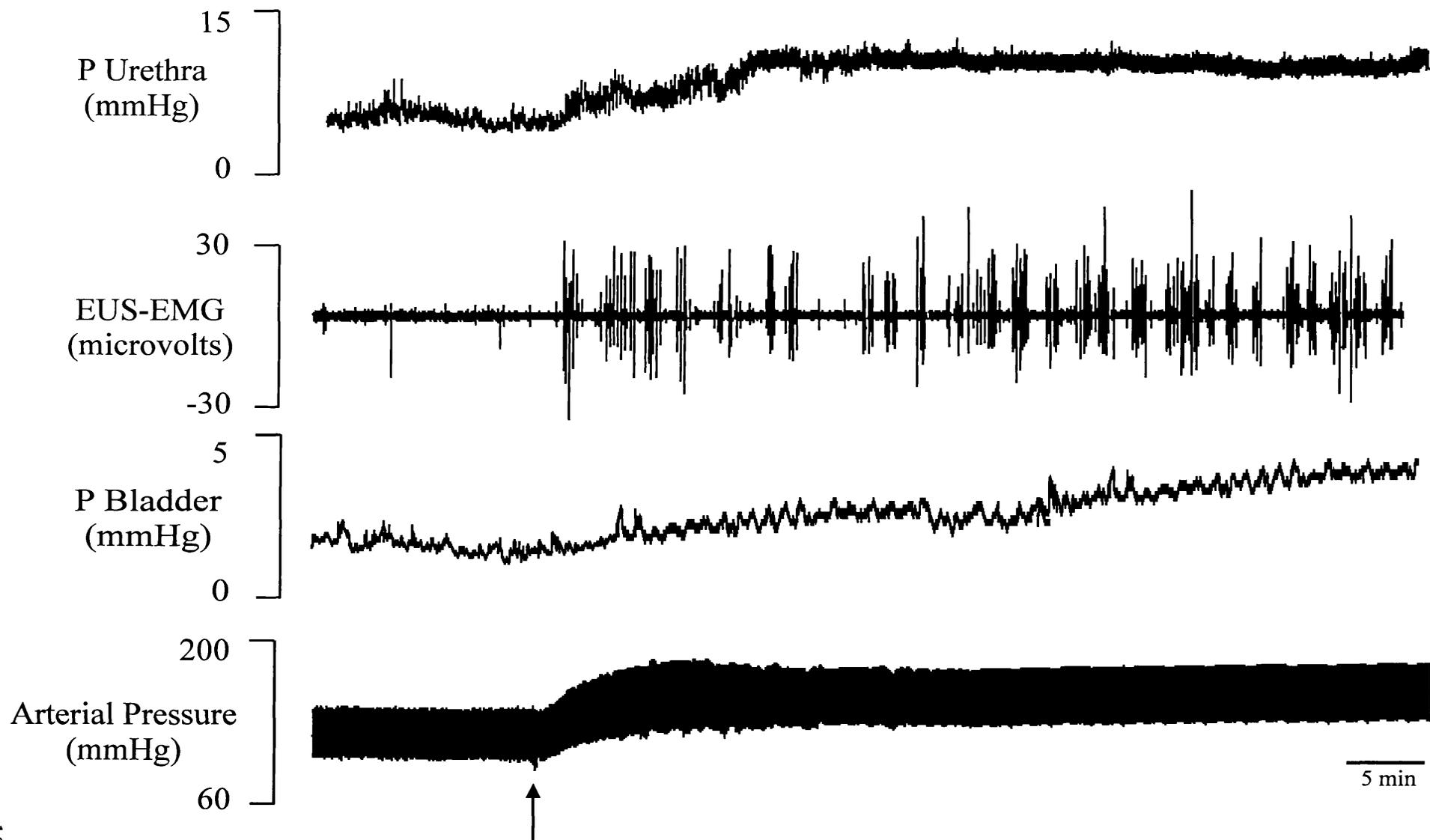


**Figure 2.8.** Traces showing reflex-evoked changes in bladder and urethral pressures before and after the administration of vehicle (A), L-NAME ( $20 \text{ mg kg}^{-1}$ , i.v.; B) and zaprinast ( $0.6 \text{ mg kg}^{-1} \text{ min}^{-1}$ , i.v.; C) in urethane-anaesthetised female rats pre-treated with  $\alpha$ -Bungarotoxin ( $0.4 \text{ mg kg}^{-1}$ , i.v.). Shaded bars represent period of infusion of saline into the bladder ( $0.046 \text{ ml min}^{-1}$ ).

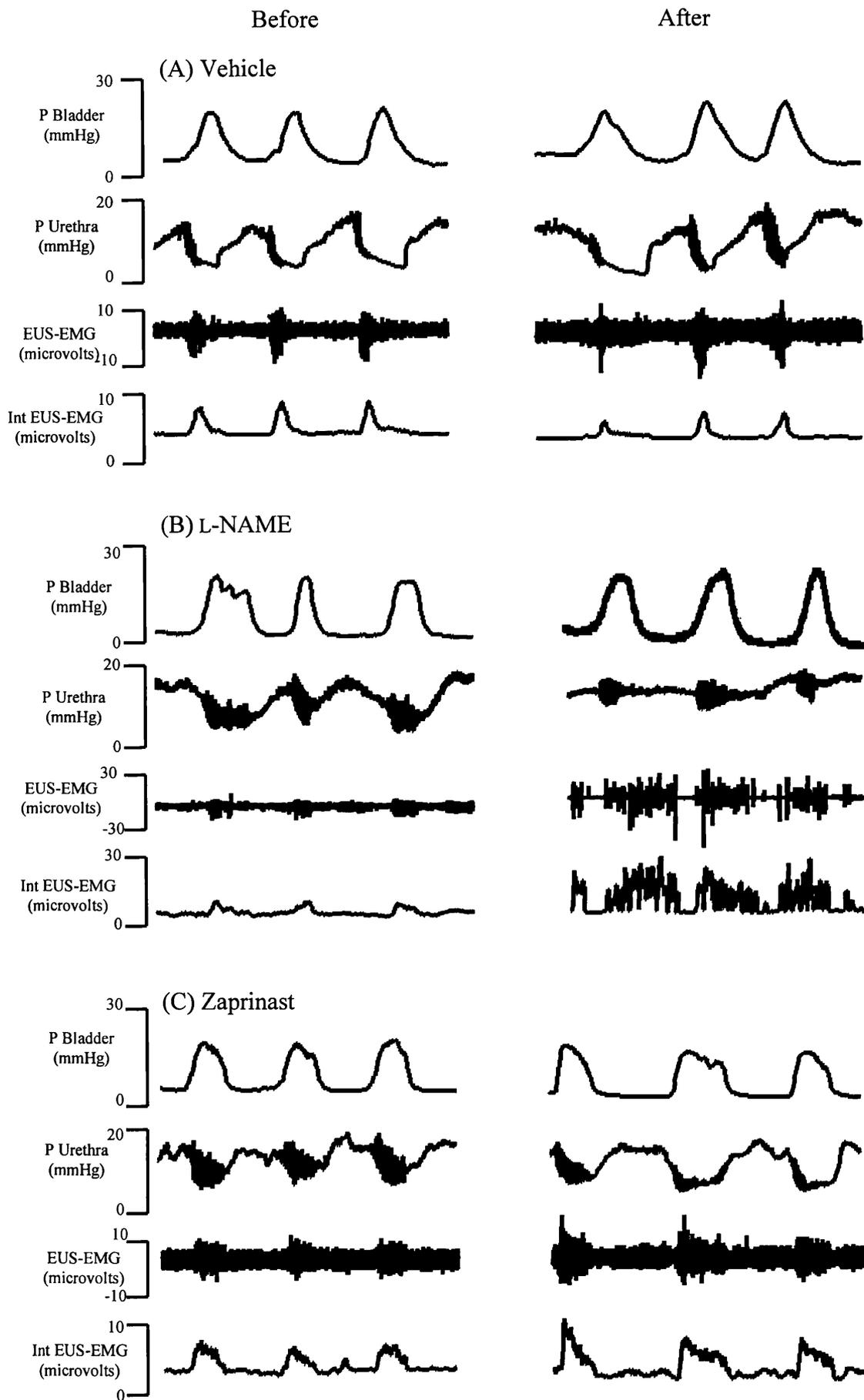
**Effects of L-NAME and zaprinast on EUS-EMG activity in non  $\alpha$ -Bungarotoxin pretreated rats**

L-NAME significantly increased baseline EUS-EMG activity by  $908 \pm 201\%$  (Figure 2.9). L-NAME also caused reflex-evoked EUS-EMG bursting to become unsynchronised with the reflex-evoked high frequency oscillations in urethral pressure, such that there was now a pattern of continuous bursting rather than increases in EUS-EMG in synergy with these oscillations (n = 4; Figure 2.10).

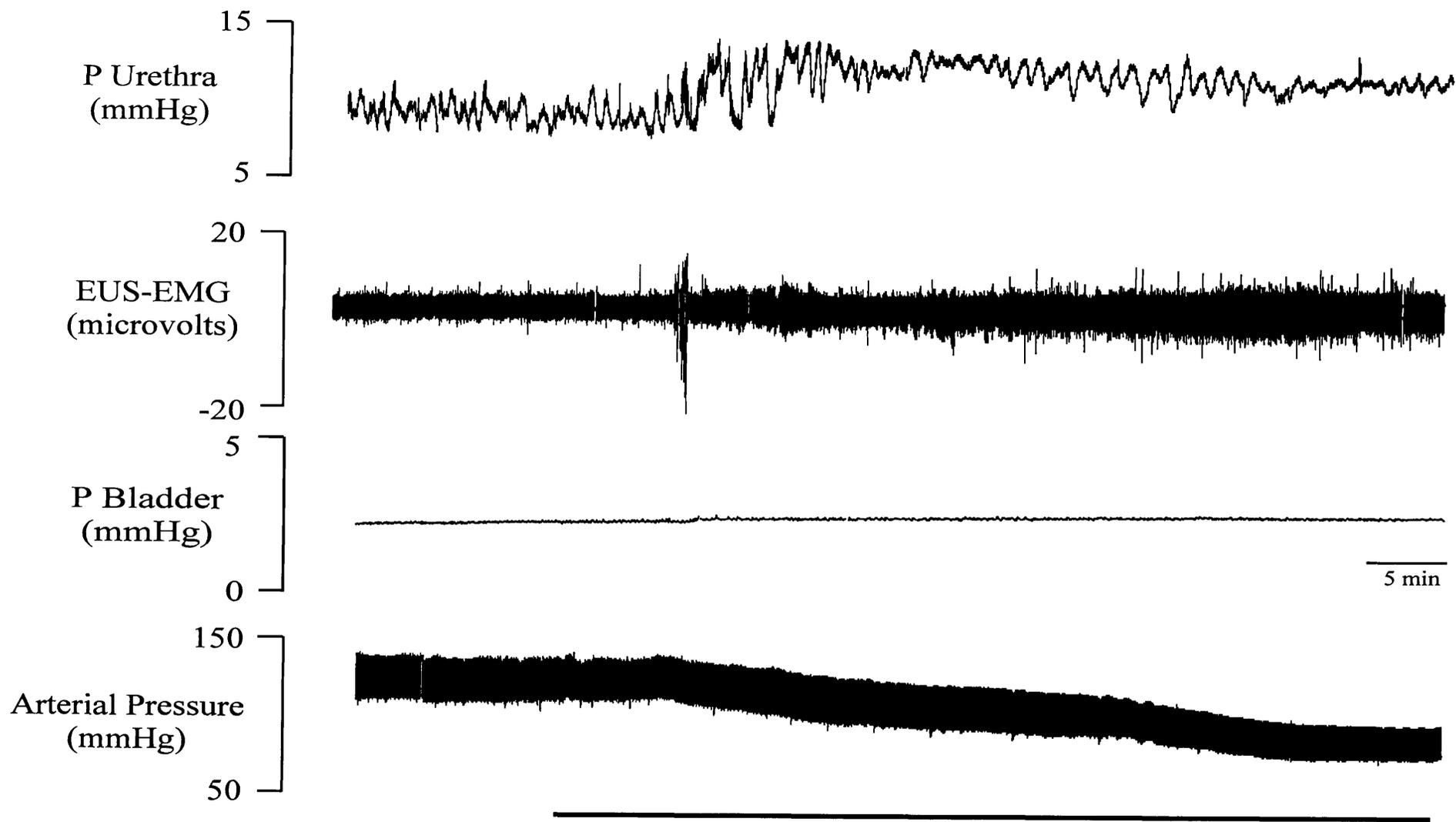
Zaprinast significantly increased baseline EUS-EMG activity by  $120 \pm 44 \%$  (n = 6; Figure 2.11). However, the pattern of continuous bursting in EUS-EMG recordings caused by L-NAME, as described above, was not observed following zaprinast treatment. Zaprinast also significantly increased the burst size of reflex-evoked increases in EUS-EMG activities that accompanied the high frequency oscillations in urethral pressure by  $178 \pm 85\%$  (n = 6; Figure 2.10).



**Figure 2.9.** Trace showing the effects of L-NAME ( $20 \text{ mg kg}^{-1}$ , i.v.) on baseline urethral pressure, EUS-EMG activity, bladder pressure and arterial pressure in the urethane-anaesthetised female rat. Arrow denotes administration of antagonist.



**Figure 2.10.** Traces showing reflex-evoked changes in bladder and urethral pressures and raw and integrated EUS-EMG activities before and after the administration of vehicle (A), L-NAME (20 mg kg<sup>-1</sup>, i.v.; B) and zaprinast (0.6 mg kg<sup>-1</sup> min<sup>-1</sup>, i.v.; C) in urethane-anaesthetised female rats.



**Figure 2.11.** Trace showing the effects of zaprinast ( $0.6 \text{ mg kg}^{-1} \text{ min}^{-1}$ , i.v.) on baseline urethral pressure, EUS-EMG activity, bladder pressure and arterial pressure in the urethane-anaesthetised female rat. Shaded bar represents period of infusion of zaprinast.

**Effects of L-NAME, L-arginine and zaprinast on DMPP-evoked changes in non- and  $\alpha$ -Bungarotoxin pre-treated rats**

*Urethral Pressure*

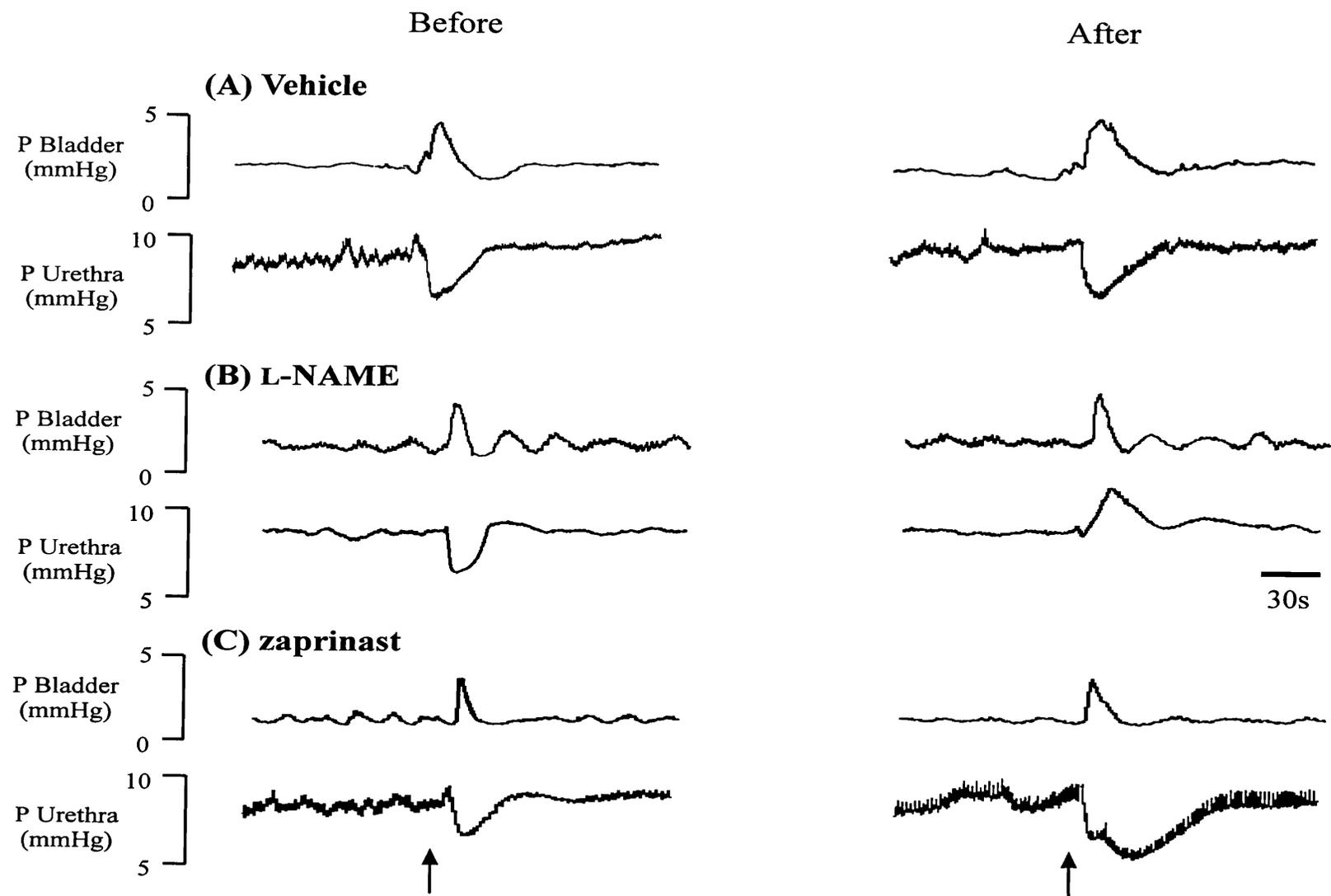
In non  $\alpha$ -Bungarotoxin pre-treated animals, L-NAME significantly attenuated the amplitude and duration of DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.)-evoked urethral relaxations by  $83 \pm 11\%$  and  $93 \pm 6\%$ , respectively ( $n = 7$ ; Figure 2.12). In 5 out of 7 animals, DMPP-evoked urethral relaxations were completely abolished, and in 4 out of these 5 animals, DMPP-evoked contractions of the urethra were now observed (Figure 2.12). These contractions had a mean amplitude of  $8.3 \pm 2.8$  mmHg and a mean duration of  $19.1 \pm 7.1$  s. These effects of L-NAME were reversed after further administration of L-arginine ( $n = 4$ ). In  $\alpha$ -Bungarotoxin pre-treated animals, L-NAME also significantly attenuated the amplitude and duration of DMPP-evoked urethral relaxations by  $79 \pm 9\%$  and  $91 \pm 6\%$ , respectively ( $n = 5$ ; Figure 2.13). In 3 out of these 5 animals, DMPP-evoked urethral contractions were now observed, that had a mean amplitude and duration of  $6.2 \pm 4.4$  mmHg and  $18.3 \pm 8.3$  s, respectively (Figure 2.13). These effects of L-NAME were not significantly different from those in non- $\alpha$ -Bungarotoxin pre-treated animals. These effects of L-NAME were also reversed after further administration of L-arginine ( $n = 4$ ).

Zaprinast significantly potentiated the amplitude and duration of DMPP-evoked urethral relaxations by  $62 \pm 25\%$  and  $109 \pm 46\%$ , respectively, in non  $\alpha$ -Bungarotoxin pre-treated animals ( $n = 7$ ; Figure 2.12). However, in the presence

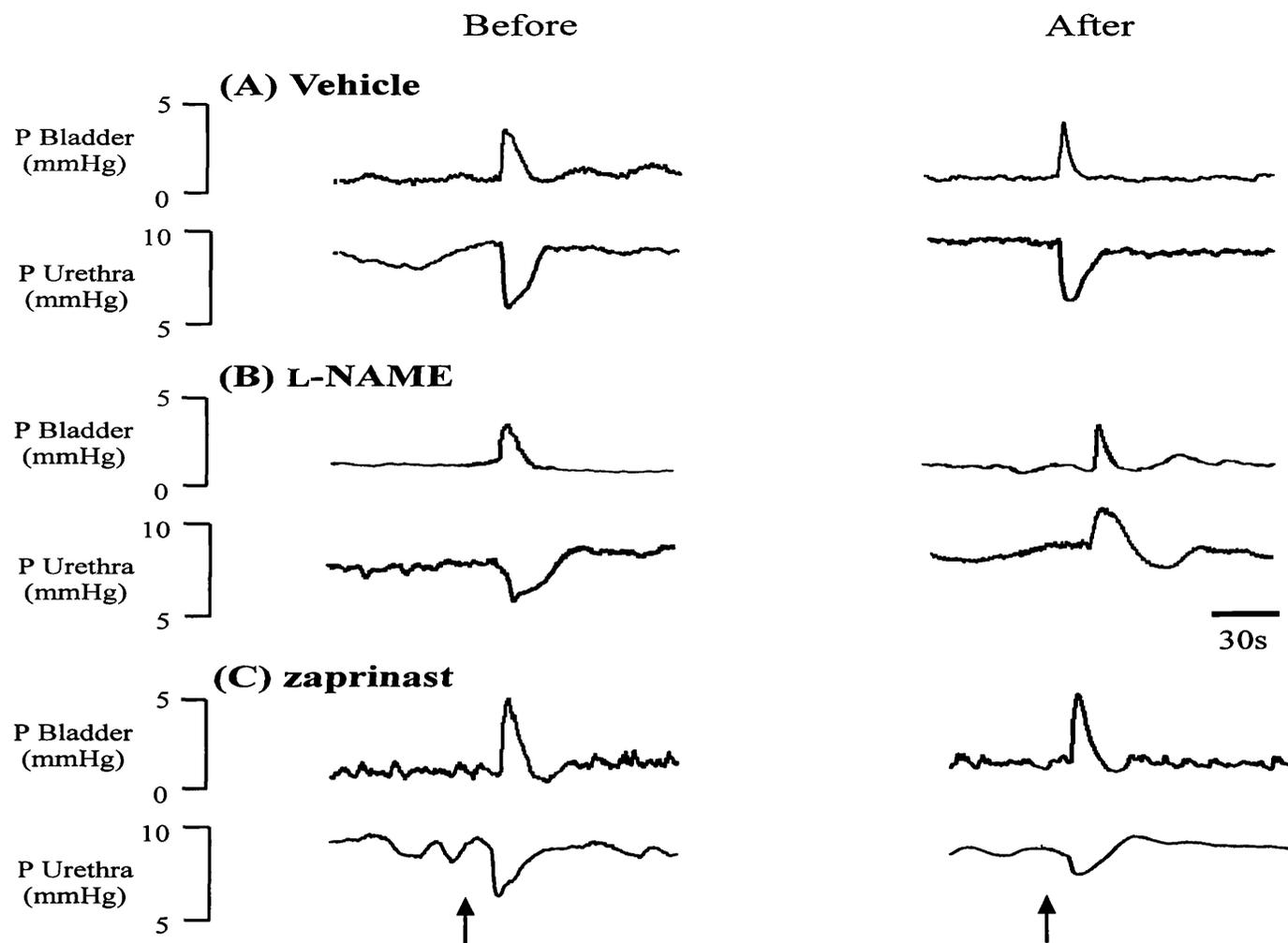
of  $\alpha$ -Bungarotoxin, zaprinast significantly attenuated the amplitude or duration of DMPP-evoked urethral relaxations by  $46 \pm 26\%$  and  $61 \pm 14\%$  ( $n = 5$ ; Figure 2.13).

#### *Bladder Pressure*

L-NAME, L-arginine and zaprinast had no effect on the amplitude or duration of DMPP-evoked bladder contractions in all experimental groups ( $n = 23$ ; Figures 2.12 and 2.13).



**Figure 2.12.** Traces showing DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.)-evoked changes in bladder and urethral pressures before and after the administration of vehicle (A), L-NAME ( $20 \text{ mg kg}^{-1}$ , i.v.; B) and zaprinast ( $0.6 \text{ mg kg}^{-1} \text{ min}^{-1}$ , i.v.; C) in urethane-anaesthetised female rats. Arrows denote administration of agonist.



**Figure 2.13.** Traces showing DMPP ( $0.5 \text{ mg kg}^{-1}$ , i.a.)-evoked changes in bladder and urethral pressures before and after the administration of vehicle (A), L-NAME ( $20 \text{ mg kg}^{-1}$ , i.v.; B) and zaprinast ( $0.6 \text{ mg kg}^{-1}$ , i.v.; C) in urethane-anaesthetised female rats pre-treated with  $\alpha$ -Bungarotoxin ( $0.4 \text{ mg kg}^{-1}$ , i.v.). Arrows denote administration of agonist.

**Effect of L-NAME, L-arginine and zaprinast on baseline values in non- and  $\alpha$ -Bungarotoxin pre-treated rats**

L-NAME (n = 13) significantly increased baseline urethral pressure by  $12 \pm 10\%$  after 5 min. (Figure 2.9). In addition, L-NAME caused a significant increase in baseline bladder pressure of  $82 \pm 21\%$  (Figure 2.9). However, in  $\alpha$ -Bungarotoxin pre-treated rats (n = 5), a significant increase in baseline bladder pressure of  $43 \pm 10\%$ , with no change in baseline urethral pressure, was observed. Furthermore, in non- and  $\alpha$ -Bungarotoxin pre-treated animals, L-NAME produced a significant increase in MAP of  $35 \pm 3\%$  and  $32 \pm 6\%$  respectively (Figure 2.9). These increases were associated with a significant decrease of  $11 \pm 2\%$  and  $10 \pm 3\%$ , respectively, in baseline HR. Administration of L-arginine following L-NAME pre-treatment produced a significant decrease of  $9 \pm 2\%$  from L-NAME-induced rise in MAP but had no effect on the raised HR, urethral and bladder pressures (n = 4). Infusion of zaprinast caused a significant increase of  $20 \pm 8\%$  in baseline urethral pressure after 10 min (Figure 2.11). This change in urethral pressure remained significantly increased for a further 10 min after which urethral pressure decreased to baseline levels. After 30 min baseline urethral pressure was non-significantly increased by  $14 \pm 12\%$ . Baseline bladder pressure was unaffected by zaprinast. Infusion of zaprinast caused a significant decrease in baseline MAP of  $21 \pm 4\%$  after 30 min, and a significant increase initial HR of  $7 \pm 3\%$  after 10 min (n = 12; Figure 2.11). None of the test substances had any effect on DMPP baseline bladder and urethral pressures in both non- and  $\alpha$ -Bungarotoxin pre-treated rats.

## **EFFECTS OF INTRAURETHRAL (I.U.) PERFUSION OF TEST SUBSTANCES ON BASELINE URETHRAL EUS-EMG**

### **Effects of vehicles and chlorisondamine on baseline values**

Administration of vehicle for i.u. infusion of sodium nitroprusside and isoprenaline (0.9% w/v saline; 0.075 ml min<sup>-1</sup>) had no effect on baseline urethral pressures, EUS-EMG activities, MAP and HR in all experimental groups (n = 8; Figure 2.14). The combined mean baseline urethral pressures, integrated EUS-EMG activities, MAP and heart rate were 13.2 ± 0.3 mmHg, 5.3 ± 1.1 microvolts, 121.5 ± 2.3 mmHg and 410.5 ± 6.7 beats min<sup>-1</sup>, respectively. The mean baseline data for individual experimental groups is shown in Table 2.4. Baseline bladder pressures were not recorded in these experiments as the bladder was left to drain to prevent EUS-EMG activities in response to bladder activity occurring.

Administration of chlorisondamine (10 mg kg<sup>-1</sup>, i.v.) had no effect on baseline urethral pressures and EUS-EMG activities. However, this drug caused a decrease in MAP of 14.1 ± 0.2% and a decrease in HR of 20.3 ± 4.7%, that remained at this level for the course of the experiments. The mean baseline data for these experimental groups is shown in Table 2.4.

Experimental Group	n	P Urethra (mmHg)	P Bladder (mmHg)	EUS-EMG ( $\mu\text{V}$ )	MAP (mmHg)	HR (beats $\text{min}^{-1}$ )
<b>Non-chlorisondamine pre-treated</b>						
sodium nitroprusside	4	12.2 $\pm$ 0.4	2.6 $\pm$ 0.6	6.1 $\pm$ 0.9	120 $\pm$ 11	401 $\pm$ 12
isoprenaline	4	13.5 $\pm$ 0.7	3.5 $\pm$ 0.4	5.9 $\pm$ 1.3	116 $\pm$ 6	397 $\pm$ 9
vehicle (saline)	4	10.9 $\pm$ 5.3	4.2 $\pm$ 0.9	5.7 $\pm$ 1.2	119 $\pm$ 10	400 $\pm$ 21
<b>Chlorisondamine pre-treated</b>						
Before chlorisondamine (10 mg $\text{kg}^{-1}$ i.v.)	12	13.5 $\pm$ 0.9	3.5 $\pm$ 0.9	6.2 $\pm$ 1.5	121 $\pm$ 12	399 $\pm$ 22
After chlorisondamine sodium nitroprusside	4	11.7 $\pm$ 0.6	3.7 $\pm$ 1.2	5.1 $\pm$ 1.2	78 $\pm$ 12	350 $\pm$ 12
isoprenaline	4	13.8 $\pm$ 1.4	2.9 $\pm$ 0.8	5.4 $\pm$ 1.1	69 $\pm$ 8	335 $\pm$ 15
vehicle (saline)	4	12.9 $\pm$ 0.7	3.2 $\pm$ 1.4	5.6 $\pm$ 1.2	75 $\pm$ 9	336 $\pm$ 21

**Table 2.4.** Baseline values of bladder and urethral pressure, external urethral sphincter (EUS-EMG) activity, mean arterial pressure (MAP) and heart rate (HR) for all experimental groups in urethane-anaesthetised female rats. All drugs and solutions were given intraurethrally (i.u.) unless otherwise indicated.

**Effects of sodium nitroprusside and isoprenaline on baseline urethral pressure and EUS-EMG activity in non-chlorisondamine pre-treated rats**

SNP (n = 4; 0.075 ml min<sup>-1</sup>, i.u.) and isoprenaline (n = 4; 0.075 ml min<sup>-1</sup>, i.u.) caused a significant fall in baseline urethral pressure after 10.4 ± 2.3 min and 12.3 ± 3.5 min, respectively, reaching a maximum of 25.4 ± 5.1 % and 28.9 ± 7.2 %, respectively, after 20 min (Figures 2.14 and 2.15). i.u. infusion of SNP and isoprenaline both evoked bursting in baseline EUS-EMG recordings, after 11.2 ± 4.5 min and 14.6 ± 4.2 min, respectively, reaching a maximum of 68.7 ± 12.4 % and 75.6 ± 14.3 %, respectively, after 20 min (Figures 2.14 and 2.15).

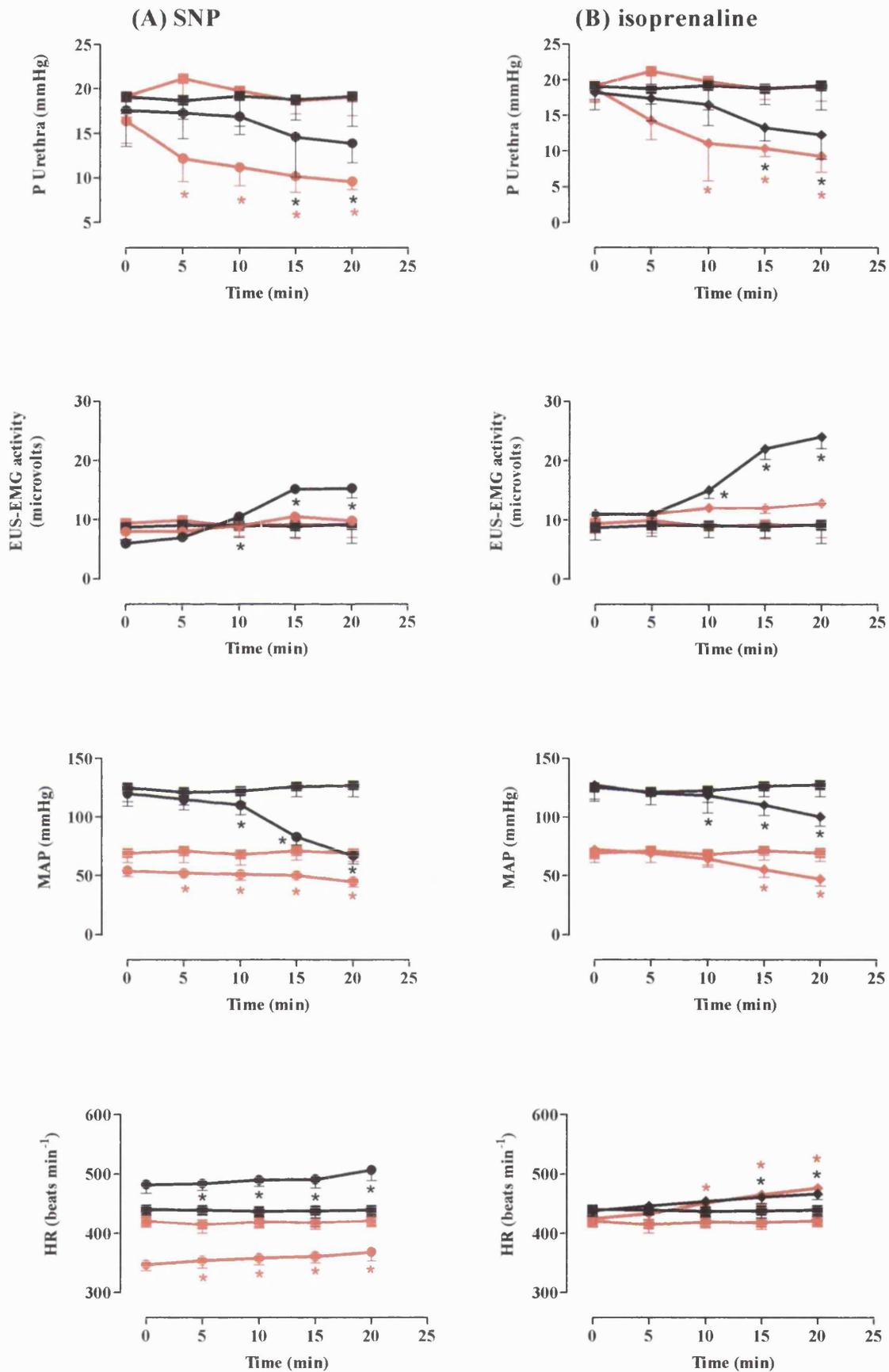
**Effects of sodium nitroprusside and isoprenaline on baseline urethral pressure and EUS-EMG activity in chlorisondamine pre-treated rats**

SNP (n = 4; 0.075 ml min<sup>-1</sup>, i.u.) and isoprenaline (n = 4; 0.075 ml min<sup>-1</sup>, i.u.) caused a significant fall in baseline urethral pressure after 5.7 ± 1.6 min and 4.3 ± 2.7 min, respectively, reaching a maximum of 35.9 ± 4.1 % and 40.8 ± 9.5 %, respectively, after 20 min in chlorisondamine pre-treated animals (Figures 2.14 and 2.15). The maximum fall in urethral pressure evoked by these agents was significantly greater than that produced in non-chlorisondamine pre-treated animals. Furthermore, the time point when urethral pressure began to fall following i.u. administration of these agents was significantly earlier than the point at which this occurred in non-chlorisondamine pre-treated rats. In addition,

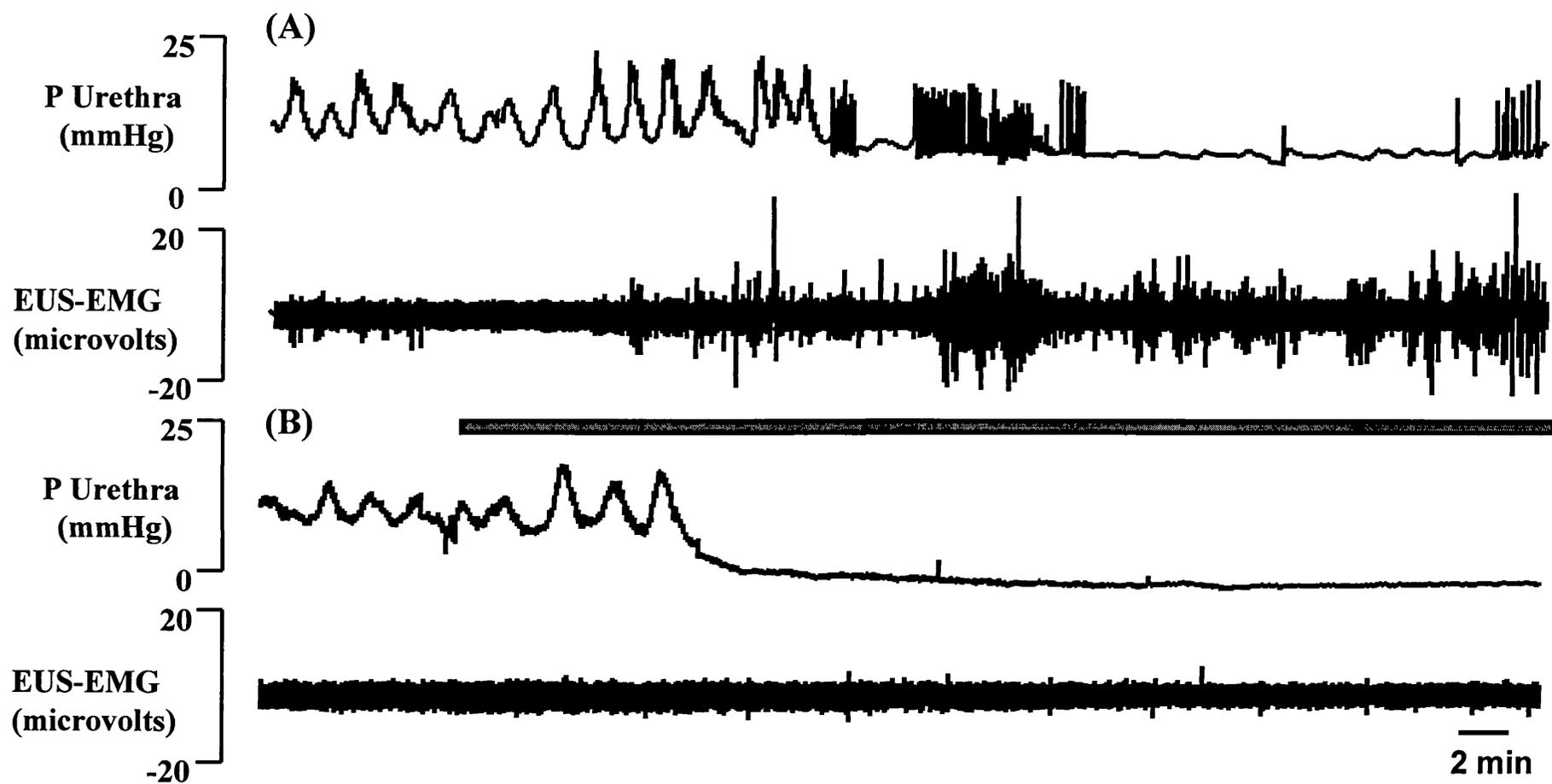
i.u. administration of SNP and isoprenaline now had no effect on EUS-EMG activities in chlorisondamine pre-treated animals (Figures 2.14 and 2.15).

**Effects of sodium nitroprusside and isoprenaline on baseline MAP and HR in non- and chlorisondamine pre-treated rats**

i.u. infusion of SNP evoked a significant fall of  $34.1 \pm 5.4\%$  and  $26.4 \pm 4.3\%$  in MAP in both non- and chlorisondamine-pretreated rats, respectively, after 20 min ( $n = 8$ ; Figure 2.14). Furthermore, this agent caused a significant increase in HR of  $6.4 \pm 1.3\%$  and  $8.2 \pm 3.4\%$ , respectively, after 20 min (Figure 2.14). Similarly, I.U. isoprenaline evoked a significant fall of  $20.3 \pm 6.5$  and  $22.3 \pm 7.8\%$  in MAP in both non- and chlorisondamine-pretreated animals, respectively, after 20 min ( $n = 8$ ; Figure 2.14). This agent also produced a significant increase in HR of  $5.2 \pm 2.2\%$  and  $6.3 \pm 1.4\%$ , respectively, after 20 min (Figure 2.14).



**Figure 2.14.** Urethral pressure, external urethral sphincter (EUS-EMG) activity, mean arterial pressure (MAP) and heart rate (HR) during intraurethral perfusion of vehicle ( $0.075 \text{ ml min}^{-1}$ ; ■), SNP ( $0.075 \text{ ml min}^{-1}$ ,  $1 \text{ mM}$ ; ●) and isoprenaline ( $0.075 \text{ ml min}^{-1}$ ,  $1 \text{ mM}$ ; ◆) in non- (black symbols) and chlorisondamine pre-treated (red symbols) urethane-anaesthetised female rats. \*  $P < 0.05$  based on percentage changes in baseline values compared with vehicle controls in non (\*) and chlorisondamine pre-treated (\*) groups.



**Figure 2.15.** Traces showing the effects of intraurethral infusion of SNP (1 mM; 0.075 ml min<sup>-1</sup>) on baseline urethral pressures and EUS-EMG activities in non- (A) and chlorisondamine (10 mg kg<sup>-1</sup>, i.v.)-pretreated (B) urethane-anaesthetised female rats. Intraurethral perfusion of isoprenaline (1 mM) had a similar effect. Shaded bar represents period of intraurethral perfusion of SNP.

## DISCUSSION

### *Effects of L-NAME on reflex- and DMPP-evoked urethral relaxations*

The present data demonstrates that reflex- and DMPP-evoked urethral relaxations are mediated by NO in the anaesthetised female rat, as these responses were attenuated by L-NAME, a NOS inhibitor (Moore & Handy, 1997) and further reversed after administration of L-arginine, a NOS substrate. The dose of L-NAME used was chosen as this concentration caused changes in urodynamic parameters in the unanaesthetised female rat in a previous study (Persson *et al.*, 1992). The dose of L-arginine was chosen on its ability to reverse the effects of L-NAME treatment in the same study (Persson *et al.*, 1992). Reflex- and DMPP-evoked urethral relaxations were mediated by relaxation of the smooth muscle layers of the urethra, as they were unchanged following neuromuscular blockade with  $\alpha$ -Bungarotoxin. In the presence of  $\alpha$ -Bungarotoxin, L-NAME was found to attenuate these relaxations by approximately the same amount as in the absence of neuromuscular blockade, suggesting that a nitrenergic pathway to smooth muscle mediates reflex- and DMPP-evoked relaxations of the urethra. These conclusions are in agreement with a number of *in vitro* studies showing that inhibitory non-adrenergic non-cholinergic (NANC) responses of isolated rat (Persson *et al.*, 1992), rabbit (Andersson *et al.*, 1992), sheep (Garcia-Pascual *et al.*, 1991) and human (Klarskov *et al.*, 1983) urethral smooth muscle are mediated by NO (see also chapter four). Furthermore, Bennett *et al.*, (1995) has also shown that reflex-evoked urethral smooth muscle relaxations are mediated by NO in anaesthetised

female rats using an experimental system whereby the bladder and urethra were functionally separated with a ligature around the bladder neck. Close arterial administration of DMPP was employed in the present study to stimulate local autonomic ganglia and promote neurotransmitter release from postganglionic neurones (Bissada, Welch & Finkbeiner, 1978). The ability of DMPP in the present study to mimic reflex-evoked NO-mediated urethral smooth muscle relaxations, in addition to evoking bladder contractions and changes in cardiovascular parameters, suggests that these responses are mediated via the stimulation of ganglionic nicotinic acetylcholine receptors, with the release of NO from postganglionic neurones. Indeed, the ability of DMPP to mimic urethral relaxations in response to electrical stimulation of postganglionic nerves *in vitro* (see chapter four), provides further evidence for this mechanism of action of DMPP. Furthermore, the observation that removal of the pelvic ganglion abolishes urethral relaxations to electrical stimulation in female rats *in vitro* (Persson *et al.*, 1998), suggests that these nicotinic acetylcholine receptors are located at the level of the pelvic ganglia. However, the release of NO from stimulation of neuronal nicotinic acetylcholine receptors within the urethra itself cannot be ruled out (see chapter five).

Immunohistochemical and functional *in vitro* studies have suggested that NO-releasing nerves in the female rat lower urinary tract do not belong to nerve populations sensitive to the sympathetic neurotoxin, 6-hydroxydopamine, or the sensory neurotoxin, capsaicin (Persson *et al.*, 1997). Furthermore, *in vivo* studies have suggested that in the female rat urethra, NO is released from parasympathetic postganglionic neurons (Fraser, Flood and De Groat, 1995).

Therefore, these combined data indicate that a parasympathetic nitrergic pathway to smooth muscle mediates reflex- and DMPP-evoked relaxations of the urethra. Interestingly, following the administration of L-NAME, close arterial DMPP evoked a contraction of the urethra. Previous studies have shown that intravenous administration of DMPP evokes a contraction of the female rat urethra *in vivo* (Wibberley, Naylor & Ramage, 1999). These studies also found that this contraction was attenuated by atropine, and abolished following further treatment with prazosin, suggesting an involvement of acetylcholine and noradrenaline acting on muscarinic and  $\alpha$ -adrenoceptors, respectively, in mediating this contractile urethral response to intravenous DMPP. This raises the possibility that the urethral contraction observed in the present studies following L-NAME administration, occurs by the same mechanisms. Further studies are needed to elucidate the exact mechanism(s) by which DMPP produces these contractile responses. Interestingly, electrical stimulation of the isolated female rat urethra also evoked a contractile response following L-NAME treatment (see chapter four). Thus the ability of DMPP in the present study to mimic urethral responses to electrical stimulation of postganglionic nerves provides further evidence that DMPP effects urethral responses *in vivo* via a stimulation of ganglionic nicotinic receptors, presumably at the level of the pelvic ganglia (see chapter one).

*Effects of L-NAME on urethral striated muscle activity*

In addition to producing an attenuation of reflex- and DMPP-evoked urethral smooth muscle relaxations, inhibition of NOS also affected urethral striated muscle activity, producing non-reflex tonic EUS-EMG bursting and disorganised reflex-evoked EUS-EMG activity. In addition, L-NAME also increased baseline urethral pressure in non  $\alpha$ -Bungarotoxin pre-treated animals, but did not affect urethral pressure following neuromuscular blockade, suggesting that the ability of L-NAME to increase baseline urethral tone reflect changes in urethral striated, and not smooth, muscle activity. These effects of NOS inhibition on urethral striated muscle activity have been previously observed in anaesthetised female rats where the junction between the bladder and urethra (termed the vesico-urethral junction) is open (Bennett *et al.*, 1995). This in contrast to the present studies where this junction was sealed with the eppendorf tip in the bladder neck to record urethral pressure. These authors suggested that urethral smooth muscle relaxation is necessary for co-ordinated urethral striated muscle activity, and inhibition of this relaxation subsequently results in disorganised reflex-evoked EUS-EMG bursting (Bennett *et al.*, 1995). However, this does not explain the appearance of non-reflex tonic EUS-EMG bursting, and a direct effect of NOS inhibition on the urethral striated muscle can not be ruled out. Indeed, the observation that L-NAME also evokes an increase in baseline urethral tension *in vitro* in the female rat suggests a direct effect of this drug on this musculature (see chapter four). NOS immunoreactivity has recently been identified in human urethral striated muscle (Ho *et al.*, 1998, 1999). Further, human skeletal muscle is known to express high levels of nNOS messenger RNA, protein, and NOS

activity (Nakane *et al.*, 1993), and NOS immunoreactivity has been identified in a number of rat skeletal muscles, including soleus (Kobzik *et al.*, 1994) and oesophageal (Worl *et al.*, 1994) muscles. In addition, a close association of  $\alpha$ -Bungarotoxin binding sites and NOS immunoreactivity has been demonstrated in rat oesophageal striated muscle (Neuhuber *et al.*, 1994). A number of studies have suggested a variety of roles for NO in skeletal muscles including modulation of cellular respiration and contractile force (Kobzik *et al.*, 1994). Indeed, recently, NO has been shown to inhibit the release of acetylcholine at the rat diaphragmatic neuromuscular junction (Mukhtarov *et al.*, 2000). Furthermore, NO is released from rat extensor digitorum longus muscle preparations *in vitro* and *in vivo* (Balon & Nadler, 1994), where it has been suggested to act as a 'breaking' mechanism to inhibit force production (Kobzik *et al.*, 1994). Therefore, this would suggest the presence of a tonic release of NO in the female rat EUS that inhibits EUS-EMG activity. Removal of this 'break' with NOS inhibitors subsequently results in an increase in urethral striated muscle activity. Interestingly, a physiological role for NO in the prevention of excessive increases in urethral tone during periods of increased tension has previously been suggested on the basis of *in vitro* investigations (Persson *et al.*, 1992), and these effects of NO on the urethral striated muscle may contribute to this role (see chapter four). Thus, the ability of L-NAME to attenuate reflex-evoked urethral relaxations in the absence of neuromuscular blockade cannot be entirely attributed to the effects of this drug on the urethral smooth muscle, in that an increase in urethral striated muscle tone must also be considered.

*Effects of zaprinast on reflex- and DMPP-evoked urethral relaxations*

The biological effects of NO are thought to be mediated by the activation of guanylate cyclase, producing an increase in cGMP formation and subsequent smooth muscle relaxation (Zhang & Synder, 1995). The cGMP signal is terminated by the activities of cyclic nucleotide PDE isoenzymes which catalyse the breakdown of cGMP as well as other transduction molecules, including adenosine 3' : 5'-cyclic monophosphate (cAMP; Beavo, 1995). A role for cGMP in NO-mediated urethral smooth muscle relaxations has been suggested following the observation that cGMP-immunoreactivity is present in the smooth muscle of the guinea pig and human urethra (Smet *et al.*, 1996). Spindle-shaped cGMP-immunoreactive cells have also been shown to form a network around and between the smooth muscle bundles of the rabbit urethra (Waldeck *et al.*, 1998). Furthermore, Persson & Andersson (1994) have shown that nerve-induced relaxation of the rabbit urethra increases the smooth muscle content of cGMP, and that this increase is more pronounced following treatment with zaprinast, a cGMP-selective PDE inhibitor. In the present study, zaprinast potentiated both reflex- and DMPP-evoked urethral relaxations in the absence of neuromuscular blockade. The dose of zaprinast used was chosen as it produced a similar decrease in MAP as reported by Dundore *et al.* (1993) who positively correlated this effect with significant increases in plasma and aortic cGMP levels. Thus, these experiments with zaprinast suggest that reflex- and DMPP-evoked urethral relaxations are mediated by NO via increases in cGMP formation. To date, eleven families of PDEs have been identified in a number of mammalian tissues (Conti & Jin, 1999; Fujishige *et al.*, 1999; Fawcett *et al.*, 2000). Of these

PDE families, only PDE 5 and PDE 6 have been shown to be specific for the hydrolysis of cGMP, although recent reports suggest that PDE 9 is also selective for cGMP as a substrate (Fisher *et al.*, 1998). Zaprinas has significant inhibitory effects on the PDE types 1, 5, 6 (Ballard *et al.*, 1998) and 9 (Fisher *et al.*, 1998) isoenzymes, and also inhibits PDEs 10 (Fujishige *et al.*, 1999) and 11 (Fawcett *et al.*, 2000), although to a lesser extent (Table 2.5). High levels of PDE 1 isoenzymes are found in the heart and brain (Yu *et al.*, 1997), PDE 6 isoenzymes are expressed almost exclusively in photoreceptors, where they are involved in visual signalling pathways (Beavo, 1995), whereas the PDE 9 family has a broad tissue distribution (Fisher *et al.*, 1998). PDE 10 transcripts are abundant in a number of brain areas (Fujishige *et al.*, 1999) and PDE 11 occurs at highest levels in skeletal muscle, prostate, kidney, liver and testes (Fawcett *et al.*, 2000). High levels of PDE 5 are found in most smooth muscle preparations, including genital tissues (Beavo, 1995; Loughney *et al.*, 1998; Yanaka *et al.*, 1998). Moreover, zaprinast has been shown to potentiate NO-mediated relaxations in gastrointestinal (Williams & Parsons, 1995), genital (Cellek & Moncada, 1998) and vascular (Liu *et al.*, 1992) smooth muscles. Thus, it is likely that the potentiating effects of zaprinast on urethral relaxations are mediated via an inhibition of PDE 5 in urethral smooth muscle. Therefore, the effects of zaprinast were examined on reflex-evoked urethral smooth muscle relaxations after blockade of the striated muscle with  $\alpha$ -Bungarotoxin. However, interestingly, in these experiments, zaprinast did not potentiate reflex- or DMPP-evoked urethral relaxations. In fact, these relaxations were now actually attenuated.

Compound	IC <sub>50</sub> (nM)										
	PDE 1	PDE 2	PDE 3	PDE 4	PDE 5	PDE 6	PDE 7	PDE 8	PDE 9	PDE 10	PDE 11
zaprinast	6650 <sup>1</sup>	>100,000 <sup>1</sup>	>100,000 <sup>1</sup>	77400 <sup>1</sup>	856 <sup>1</sup>	385 <sup>1</sup>	-	-	3500 <sup>2</sup>	14000 <sup>3</sup>	12000 <sup>4</sup>

**Table 2.5.** IC<sub>50</sub> values for inhibition of human PDE isoenzymes and bovine photoreceptor PDE 6 by zaprinast.

PDE 2,3 and 5 were isolated from human corpus cavernosum, PDE 1 from human cardiac ventricle, PDE 4 and PDE 11 from human skeletal muscle, PDE 9 from human brain and PDE 10 from human foetal lung.

All values are mean (nM); standard errors could not be shown as these were not calculated in all studies.

<sup>1</sup> Ballard *et al.*, 1998, <sup>2</sup> Fisher *et al.*, 1998, <sup>3</sup> Fujishige *et al.*, 1999, <sup>4</sup> Fawcett *et al.*, 2000

For references in full see chapter seven.

*Effects of zaprinast on urethral striated muscle activity*

The ability of zaprinast to potentiate reflex-evoked urethral relaxations in the absence of  $\alpha$ -Bungarotoxin is presumably related to the surprising observation that zaprinast increased urethral striated muscle activity. The urethral striated muscle exhibits a distinct type of activity during micturition in the rat. In this species, reflex-evoked urethral relaxations are associated with high frequency oscillations in urethral pressure that are eliminated after application of  $\alpha$ -Bungarotoxin. Furthermore, these oscillations are associated with EUS-EMG high frequency bursting in the EUS and have previously been shown to be abolished after sectioning of the pudendal nerves, which provide the somatic innervation to the LUT (Conte *et al.*, 1991b), strongly suggesting that they are the result of striated muscle activity. In addition, these oscillations are still present following dissection of the extra-urethral striated muscles of the pelvic floor showing that they are the direct result of urethral striated muscle activity (Conte *et al.*, 1991b). In the present study, zaprinast significantly increased both baseline and reflex-evoked EUS-EMG activities. In addition, these zaprinast-evoked increases in baseline EUS-EMG activity were associated with increases in baseline urethral pressure that were not observed following neuromuscular blockade, suggesting, surprisingly, that these changes in urethral tone reflect effects on urethral striated, and not smooth, muscle activity. Therefore, it appears that the potentiating effects of zaprinast on DMPP- and reflex-evoked urethral relaxations are the result <sup>of</sup> the ability of this agent to increase urethral striated muscle activity, thereby increasing urethral tone. Indeed, urethral relaxant responses to electrical stimulation have been found to be more

pronounced at high than low tension levels (Andersson, Mattiason & Sjogren, 1983; Garcia-Pascual *et al.*, 1991).

*NO and cGMP signalling in the urethral striated muscle?*

If the inhibitory effects of NO on the urethral striated muscle (suggested by the ability of L-NAME to increase EUS-EMG activity) are mediated by increases in cGMP levels, then cGMP-selective PDE inhibitors would be expected to potentiate these effects of NO and thus further decrease urethral striated muscle activity. Evidence suggesting that the effects of NO on striated muscle are indeed mediated by cGMP is provided from findings that cGMP dependent protein kinase type I, a primary effector for NO, is concentrated at the neuromuscular junction in rat and mouse skeletal muscles (Chao *et al.*, 1997). Moreover, the cGMP signalling pathway is thought to be active in resting skeletal muscle, where it plays a role in the inhibition of force production (Kobzik *et al.*, 1994). However, the effects of zaprinast on the urethral striated musculature would not seem to support a role for NO/cGMP signalling in the EUS, although other actions of this agent on this musculature should be considered (see later). It is possible that the inhibitory effects of NO on the urethral striated muscle are not mediated by the cGMP transduction pathway, and thus inhibitors of PDE will not potentiate these effects. Indeed, NO inhibits skeletal muscle contraction in the rabbit by inhibiting a Ca<sup>2+</sup>-ATPase, thereby involving a mechanism independent of the NO/cGMP signalling pathway (Ishii *et al.*, 1998). Alternatively, PDEs other than PDE 5 may be involved in the transduction mechanisms mediating these effects of NO in the urethral striated

muscle, and the PDE inhibitors used in the present study may have varied effects on these isoenzymes. In this respect, it is possible that the inhibitory effects of NO in striated muscle not only involve cGMP, but also other signalling molecules, such as cAMP. Indeed, it is well known that both cGMP-activated and cGMP-inhibited cAMP PDEs exist, termed PDE 2 and PDE 3, respectively, and a role for these isoenzymes in the NO-mediated control of urethral striated muscle cannot be excluded (Beavo, 1995). For example, NO has been shown to lower renal vascular resistance via cGMP formation in the isolated rat kidney. However, cGMP, in this case, does not activate 'classical' transduction mechanisms involved in smooth muscle relaxation (for example, activation of cGMP-dependent protein kinases), but inhibits PDE 3, increasing intracellular levels of cAMP, which mediates the vasodilatory action of cGMP induced by NO (Sandner *et al.*, 1999). Therefore, it is conceivable that NO-evoked inhibition of urethral striated muscle activity involves PDEs, in addition to or other than PDE 5, and as such, inhibition of this enzyme will not produce a potentiation of the inhibitory effects of NO on the urethral striated muscle. Alternatively, cAMP levels may be increased by a direct modulation of the adenylyl cyclase-dependent protein kinase pathway by NO in urethral striated muscle, to mediate the inhibitory responses of NO on this musculature (Vila-Petroff *et al.*, 1999). Moreover, the effects of NO may be entirely direct on the striated musculature, and as such, the effects of zaprinast on the EUS are due to mechanisms entirely independent of these effects of NO. In addition, the effects of zaprinast on the urethral striated muscle may be due to other unknown mechanisms not related to PDE inhibition (see later).

*Effects of zaprinast on urethral striated muscle activity: interactions between urethral smooth and striated muscles*

There are a number of possible explanations for the ability of zaprinast to increase urethral striated muscle activity. In animals pre-treated with  $\alpha$ -Bungarotoxin, administration of zaprinast attenuated reflex-evoked urethral smooth muscle relaxations. During the period of the micturition reflex, an increase in baseline urethral pressure, that was measured between the rhythmic reflex-evoked bladder contractions, was observed (Figure 2.2). These reflex-evoked increases in baseline urethral pressure are mediated by urethral smooth muscle, as they are unaffected by neuromuscular blockade. Zaprinast attenuated these increases in baseline urethral pressure. In addition, other studies in this thesis have shown that zaprinast evokes a reduction in baseline urethral tension and attenuates electrically-evoked urethral relaxations in the female rat urethra *in vitro* (see chapter four). These results suggest that zaprinast causes relaxations by reducing urethral smooth muscle tone. Indeed, cGMP analogues have been shown to relax isolated rabbit urethral smooth muscle (Waldeck *et al.*, 1998), and Smet *et al.* (1996) have demonstrated the presence of cGMP-immunoreactivity in the smooth muscle cells of the human urethra. Furthermore, a reduction in tone after zaprinast has also been reported in isolated genital (Cellek & Moncada, 1998) and gastrointestinal (Williams & Parsons, 1995) smooth muscles. Therefore, the possibility exists that zaprinast increases EUS indirectly by causing changes in urethral smooth muscle tone. This was investigated by examining the effects of urethral smooth muscle relaxation with intraurethral perfusion of SNP and isoprenaline on baseline EUS activity. Both these agents

produced a decrease in urethral tone, which were associated with increases in urethral striated muscle activity. These effects were inhibited following pre-treatment with chlorisondamine, a neuronal nicotinic receptor antagonist. Further, chlorisondamine had no effect on baseline EUS-EMG activity, showing that this agent did not affect neuromuscular nicotinic receptors, providing further evidence that these drug-evoked increases in urethral striated muscle activity involve the activation of neuronal nicotinic receptors.

The drugs used for this set of experiments were assumed to decrease urethral smooth muscle tone based on the observations that they relax this musculature in isolated tissue studies (Garcia-Sacristan *et al.*, 1986; Bridgewater, MacNeil & Brading, 1993; Garcia-Pascual & Triguero, 1994; Persson & Andersson, 1994; Garcia-Pascual *et al.*, 1999). The mechanisms of action of these drugs are likely to involve increases in cGMP (zaprinast and SNP) and cAMP (isoprenaline) levels in the urethra to produce smooth muscle relaxation. However, an effect of zaprinast on urethral smooth muscle tone by a mechanism of action other than PDE 5, or other PDE, inhibition cannot be excluded. Indeed, caffeine, a non-selective PDE inhibitor, has been reported to lower urethral pressure in the anaesthetised female dog (Palermo & Zimskind, 1977). These authors suggested that one of the possible mechanisms of action of this agent may involve the mobilisation of  $\text{Ca}^{2+}$  ions from the urethral smooth muscle membrane, which increases  $\text{Na}^{2+}$  permeability, leading to an inactivation of the contractile mechanism of this musculature. Furthermore, recently, caffeine has been shown to inhibit ATP-sensitive  $\text{K}^+$  channels in pig urethral smooth muscle cells, by a mechanism independent of PDE inhibition or  $\text{Ca}^{2+}$  release (Teramoto *et al.*,

2000). These authors suggested that these effects were most likely effected by a direct action of caffeine on these channels, raising the possibility that zaprinast may also lower smooth muscle tone by these mechanisms. These possibilities require further investigation.

The increase in urethral striated muscle activity in response to zaprinast, SNP and isoprenaline may also involve responses to relaxation of the lamina propria, which constitutes the innermost layers of the urethra, between the urethral lumen and urethral smooth muscle, and comprises connective tissue and extensive vasculature (see Brading, 1999; see chapter three). The blood supply to the lamina propria has been suggested to contribute to pressure generation within the urethra, and to the maintenance of urethral tone (Rud *et al.*, 1980), and therefore the possible vasodilatory effects of zaprinast, SNP and isoprenaline on this urethral layer may, in turn, evoke an increase in urethral striated muscle activity. Indeed, these agents lowered mean arterial pressure in the present study suggesting that decreases in urethral tone and resultant increases in urethral striated muscle activity could reflect changes in overall blood pressure and urethral blood flow. However, Brading *et al.* (1996) have shown that intravenous infusion of SNP produces a fall in mean blood pressure and urethral pressure, but evokes increases in lamina propria blood flow in the female pig. These authors therefore concluded that the ability of this agent to decrease urethral tone is mediated by the urethral smooth muscle, independently of changes in overall blood pressure or urethral blood flow.

It is also possible that these increases in urethral striated muscle activity following zaprinast, SNP or isoprenaline treatment, occur in response to a decrease in urethral tone caused by a relaxation of the urethral striated muscle directly. Indeed, it has been reported that SNP produces a pronounced relaxation of the urethral striated muscle in the guinea pig (Von Heyden *et al.*, 1995). However, these authors also found that this region of urethral 'striated' musculature also responded most strongly to calcium depletion. Despite these authors previously stating (in the same paper) that the 'smooth muscle component is more dependent on extracellular calcium than is striated', they still reached the surprising conclusion that SNP relaxes urethral striated muscle. However, it is more likely that this relaxation is effected by the smooth muscle fibres of this urethral region which are closely intermingled with striated muscle fibres (see Lincoln & Burnstock, 1993).

Alternatively, these agents may not directly relax the urethral striated muscle, but cause an inhibition of the activity of this musculature, which may, in turn, evoke a decrease in urethral striated muscle tone and a subsequent reflex increase in the activity of the EUS. Previous experiments in these studies have demonstrated an inhibitory effect of NO on the urethral striated muscle, raising the possibility that SNP may also exert a similar inhibitory effect on this musculature. In addition, isoprenaline exerts a depressant effect on skeletal muscle in the cat (see Bowman & Nott, 1969; Merican & Nott, 1981) and guinea pig (Waldeck, 1977), via the formation of cAMP. However, none of these agents had any overt effects on baseline EUS-EMG activities, preceding the activation of the aforementioned reflex. Furthermore, the extent to which urethral striated muscle activity was

increased in these experiments, especially in response to SNP and isoprenaline, suggests a lack of inhibitory effects of these agents on this musculature, as this increase would have been opposed to some degree. However, the previously published evidence suggesting that if any of these agents have a direct effect on urethral striated muscle activity, then it will be a depressant effect, rules out a direct effect of these agents on the striated muscle fibres to increase their activity.

Alternatively, the stimulus to activate this reflex may involve a direct effect of these agents on urethral afferent nerves, to increase their activity and evoke a reflex increase in urethral striated muscle activity, independently of changes in urethral tone. However, it is unlikely that SNP directly activates urethral afferents to evoke these changes in urethral striated muscle activity, as NO is known to directly inhibit urethral afferent nerves, via a suppression of Ca<sup>2+</sup> ion channels (Quignard *et al.*, 1997). In addition, although cAMP has been postulated to play a role in the sensitisation of afferents to noxious stimuli in the skin (Kress, Rodl & Reeh, 1996) and abdominal viscera (Guo & Longhurst, 2000), a physiological role for this substance in afferent activation has not been identified, and it is unlikely that the similar effects of SNP and isoprenaline on EUS activity would be mediated by two separate chlorisondamine-sensitive mechanisms.

It can therefore be hypothesised that zaprinast, SNP and isoprenaline evoke increases in urethral striated muscle activity indirectly, in response to evoked decreases in urethral tone, most likely mediated by a decrease in urethral smooth

muscle tone, and constitute a reflex involving the activation of neuronal nicotinic acetylcholine receptors.

*Interactions between urethral smooth and striated muscles: mechanisms of action*

A number of reflexes influencing the fine control of the smooth and striated muscles of the urethra have previously been described. Urethral distension with saline at the level of the urethral striated muscle has been shown to elicit a series of slow phasic urethral contractions in male rats (Conte *et al.*, 1991a). Furthermore, this distension-induced rhythmic urethral activity was abolished by hexamethonium, a neuronal nicotinic receptor antagonist, but was unaffected by bilateral section of the pudendal nerves, bilateral removal of the major pelvic ganglia or spinalisation (T12-S1), suggesting a local site of action. Moreover, intraurethral administration of capsaicin has been shown to produce activation of the urethral striated muscle in urethane-anaesthetised male rats (Conte *et al.*, 1993). These responses were absent after application of tetrodotoxin on the pudendal nerves and in spinalised (T13-L1) animals, but were unaffected by hexamethonium. These authors suggested the presence of a physiological interaction between capsaicin-sensitive afferents innervating the urethra and the somatic efferent innervation to the EUS, constituting a chemonociceptive urethra-urethral neural loop, which, via pudendal nerves, leads to a supraspinally-mediated activation of the EUS (Conte *et al.*, 1993). The present results have suggested the presence of interactions between the various components of the urethra, whereby a drug-evoked decrease in urethral tone

produces an increase in the activity of the urethral striated muscle, via the activation of neuronal nicotinic acetylcholine receptors.

The exact sites and mechanisms of action involved in this reflex remain to be elucidated. Chlorisondamine is a centrally acting neuronal nicotinic receptor antagonist (Clarke *et al.*, 1994), and it is therefore possible that these reflexes involve activation of these receptors at spinal and supraspinal sites. Indeed, immunohistochemical studies have shown that neurones in the locus coeruleus, an important central site in the control of bladder and urethral function, synthesise nicotinic receptor protein (Caffe, 1994). Moreover, nicotinic acetylcholine receptors have been shown to depolarise interneurons in the rat lateral geniculate nucleus (Zhu & Uhlrich, 1997), and play a role in the modulation of neuronal circuits in the human cerebral cortex (Alkondon *et al.*, 2000). Neuronal nicotinic receptors mediate ganglionic transmission in the pelvic plexus, a bilateral association of neurones and ganglia which lie at the base of the bladder (see Keast, 1999), raising the possibility that these reflexes also involve activation of the receptors at this site. In addition, ganglionic cell bodies have been identified in the human urethral smooth and striated muscles (Crowe, Burnstock & Light, 1988), and therefore an involvement of local ganglionic nicotinic acetylcholine receptors in the urethra cannot be excluded. However, experiments investigating the effects of nicotine and the selective neuronal nicotinic receptor agonists, DMPP and epibatidine, on the isolated female rat urethra have shown that these agents evoke urethral relaxations by TTX resistant mechanisms, providing evidence against the presence of autonomic ganglia at the level of the urethra (see chapter five). Furthermore,

histologically, ganglionic cell bodies have not been identified in the rat urethra (Gabella & Uvelius, 1990). Thus, the results of these studies provide evidence against the involvement of ganglionic neuronal nicotinic receptors at the level of the urethra in this reflex, although an involvement of non-ganglionic neuronal nicotinic receptors at the level of the urethra, or other peripheral (for example, at the level of the pelvic plexus) and central sites cannot be excluded.

The autonomic afferent and efferent pathways involved in these reflexes remain to be determined. Both functional (Maggi *et al.*, 1993) and immunohistochemical (Su *et al.*, 1986) studies have identified the presence of capsaicin sensitive primary afferents in the rat proximal urethra. Further, stimulation of these fibres with intraurethral administration of capsaicin leads to an activation of the EUS (Conte *et al.*, 1993), suggesting that these afferents may be involved in the reflex described in the present studies. Furthermore, it has been suggested that urethral pudendal afferents may have direct access to a spinal circuitry that can co-ordinate bladder and EUS activity in decerebrate cats (Shefchyk & Buss, 1998), raising the possibility that afferents in the pudendal nerve, in particular, may play a role in this reflex. The autonomic efferent arc of these reflexes is also unknown. The somatic efferent supply to the EUS is carried in the pudendal nerves (Elbadawi & Schenk, 1974). However, a number of histochemical and ultrastructural studies have suggested that the EUS also receives parasympathetic and sympathetic innervation, via the pelvic and hypogastric nerves, respectively (Elbadawi & Schenk, 1974; Elbadawi & Atta, 1985). Indeed, if this reflex involves activation of ganglionic nicotinic receptors at the level of the pelvic plexus, it is likely that the efferent pathway involves

parasympathetic and sympathetic transmission to the EUS, as somatic fibres do not synapse at this site. Further studies examining the effects of nerve transection, bilateral removal of the pelvic ganglia and spinal cord transection will prove useful in delineating the exact mechanisms involved in the reflex described in these studies.

*Interactions between urethral smooth and striated muscles: physiological relevance*

The exact physiological relevance of this reflex remains to be elucidated. In the presence of chlorisondamine, intraurethral administration of SNP and isoprenaline produced larger decreases in baseline urethral pressure than in the absence of this antagonist, suggesting that this mechanism operates to minimise decreases in overall urethral tone. In this respect, it can be speculated that these changes in urethral striated muscle activity may act to maintain continence when the urethral smooth muscle is compromised. Indeed, studies have shown that the EUS can maintain continence in males even when the smooth muscle of the internal urethral sphincter has been damaged, for example during transurethral resection of the prostate (Turner-Warwick, 1975). Furthermore, in the rat, if these changes occur in response to urethral smooth muscle relaxation, then it can be hypothesised that this reflex plays a role in the initiation of the high frequency oscillations in urethral pressure associated with urethral smooth muscle relaxations during voiding (Van Asselt, Groen & Van Mastrigt, 1995). The observation that L-NAME simultaneously attenuates reflex-evoked urethral

smooth muscle relaxations and produces disorganised reflex-evoked high frequency oscillations in urethral pressure supports this possibility.

*Effects of zaprinast on urethral striated muscle activity: alternative mechanisms of action*

In addition to the possible involvement of the aforementioned reflex interactions within the urethra in the effects of zaprinast on the urethral striated muscle, there a number of alternative explanations for this surprising ability of zaprinast to increase urethral striated muscle activity. An inhibition of PDE 5 in the urethral striated musculature remains a possibility. Indeed, recently, two splice variants of human PDE 5, PDE 5A1 and PDE 5A2, have been identified (Loughney *et al.*, 1998), with the potential for varied distributions and roles of these subtypes. However, to the author's knowledge, there is no published evidence for the presence of PDE 5 in striated muscle. An alternative explanation is that inhibition of PDEs other than PDE 5 following zaprinast treatment causes an increase in urethral striated muscle activity. In this respect, in addition to inhibition of the PDE 5 isoenzyme, zaprinast also has significant inhibitory effects on the PDE 1 isoenzyme, being only approximately 8 fold more selective for PDE 5 than PDE 1 (Table 2.5). In this respect, pharmacokinetic studies would prove useful to establish whether the plasma concentrations of these drugs would reach the pharmacological levels required to inhibit these various PDE isoenzymes. Furthermore, these data on the affinities of zaprinast for PDE isoenzymes are obtained from human PDE isoenzymes, and it remains to be determined whether this agent would show the same selectivity profiles in the

rat. One indication that zaprinast is inhibiting additional PDEs other than PDE 5 in the present study, arises from the ability of this agent to evoke a pronounced fall in blood pressure. Zaprinast has been shown to lower mean arterial pressure by decreasing peripheral vascular resistance and by antagonising the activities of neurohumoral vasoconstrictor systems, such as those mediated by phenylephrine (Trapani *et al.*, 1991). Indeed, PDE 1 and 5 isoenzymes have been identified in vascular smooth muscles (Trapani *et al.*, 1991; Yanaka *et al.*, 1998), and it is therefore possible that the pronounced ability of this agent to lower blood pressure may reflect an ability to inhibit both these enzymes. Therefore, the increase in urethral striated muscle activity following zaprinast treatment may reflect inhibition of PDE 1. Indeed, PDE 1 has been identified in human skeletal muscle (Yu *et al.*, 1997). Furthermore, three genes encoding three related PDE 1 isoenzymes have been identified, namely PDE 1A, PDE 1B and PDE 1C (Beavo *et al.*, 1995). PDE 1B isoenzymes have been detected in high levels in human skeletal muscle, and are inhibited by zaprinast (Yu *et al.*, 1997). A large density of PDE 1 isoenzymes have also been identified in the human detrusor, where a role in the regulation of detrusor smooth muscle tone has been suggested (Truss *et al.*, 1996). However, a functional role for this PDE in the bladder has not been unequivocally demonstrated, and the lack of effect of zaprinast on reflex- and DMPP-evoked changes in bladder pressures (see below) does not necessarily imply a lack of inhibition of PDE 1.

A direct effect of zaprinast on urethral striated muscle remains a distinct possibility. Increases in cGMP levels in isolated rat soleus muscle preparations following incubation with zaprinast have been reported (Young, Radda &

Leighton, 1996). Furthermore, zaprinast has been shown to increase glycogen synthesis and glucose oxidation in this muscle (Young & Leighton, 1998), suggesting that the effects of zaprinast on urethral striated muscle activity could be related to modulation of glucose utilisation. Zaprinast may also directly inhibit the actions of other neurotransmitters or regulatory substances that are involved in the control of urethral striated muscle activity. These effects may occur independently of PDE inhibition. Indeed, caffeine has previously been shown to evoke skeletal muscle contraction in the frog (Weber & Herz, 1968; McCallister & Hadek, 1973). These authors have suggested that these effects are due to the modulation of  $Ca^{2+}$  release from this musculature. Furthermore, caffeine and theophylline, a further non-selective PDE inhibitor, have been reported to modulate noradrenaline release in the guinea pig and rat brain (Berkowitz, Tarver & Spector, 1970). Therefore, the effects of zaprinast on the urethral striated muscle reported in the present study may reflect an inhibitory or modulatory effect of this agent on one, or more, of these pathways.

*Does NO/cGMP signalling play a physiological role in the female rat urethra?*

Previous studies have shown that NO-mediated relaxations of the isolated rabbit (Dokita *et al.*, 1994; Persson & Andersson, 1994) and sheep (Garcia-Pascual *et al.*, 1999) urethra are associated with increases in cGMP levels. Furthermore, these relaxations are attenuated by guanylate cyclase inhibitors, providing further evidence that in the urethra, NO activates guanylate cyclase, which in turn, increases cGMP levels to produce urethral smooth muscle relaxation. However, conclusive evidence for the involvement of cGMP in NO-mediated effects in the

urethra could not be obtained from these *in vivo* investigations. The ability of zaprinast to potentiate reflex-evoked urethral relaxations *in vivo* are due to an ability to increase urethral striated muscle activity, and potentiations of these responses with zaprinast treatment following neuromuscular blockade are presumably not observed because of a reduction in urethral tone. In this respect, the amplitude of these relaxations may not be potentiated because urethral smooth muscle tone has reached a maximal level of relaxation. As such, the baseline urethral pressure that is recorded following zaprinast treatment in the presence of  $\alpha$ -Bungarotoxin may be mediated by the elastic tissue in the submucosa and/or the rich vascular plexus that are known to contribute to the maintenance of urethral tone (De Groat & Booth, 1980; Gosling, 1979). The possibility that any potential potentiating effects of zaprinast on NO-mediated urethral relaxations are masked by the reduction in urethral tone that this agent caused, is particularly illustrated from the results of *in vitro* studies (see chapter four). These experiments demonstrated a potentiation of the duration of electrically-evoked urethral relaxations in the isolated female rat urethra with zaprinast only in experiments where the tissues were re-tensioned to pre-drug levels following incubation with this agent, to compensate for the evoked decrease in urethral tone. Indeed, the effects of zaprinast to increase urethral striated muscle activity *in vivo* may also 'compensate' for the decrease in urethral tone caused by this agent, and as such potentiations of reflex-evoked urethral relaxations are observed. Alternative methods for determining the presence and roles of cGMP in the urethra *in vivo* including measurement of urethral cGMP levels during micturition and the effects of zaprinast on these responses may be more appropriate. Furthermore, the use of inhibitors of guanylate cyclase, rather

than PDE inhibitors, may provide conclusive evidence for a role for NO/cGMP signalling in the female rat urethra.

*Effects of manipulation of the NO/cGMP signalling pathway on reflex- and DMPP-evoked changes in bladder pressure*

Pharmacological manipulation of the NO/cGMP signalling pathway had no effects on reflex- and DMPP-evoked changes in bladder pressure. Persson *et al.* (1991, 1992) showed that inhibition of NOS induced bladder hyperactivity and decreased bladder capacity in the unanaesthetised female rat, although these changes may reflect an effect on the urethral outlet rather than a direct effect on the bladder. Furthermore, experiments involving isolated strips of detrusor muscle from various species have provided evidence for NO-mediated inhibitory neurotransmission (James, Birmingham & Hill, 1993; Chung, Choi & Chang, 1996), but Persson & Andersson (1992) failed to confirm these findings. In addition, immunohistochemical studies have shown that only a small percentage of neurons innervating the bladder exhibit NOS immunoreactivity (Vizzard *et al.*, 1994). The findings in the present study provide further evidence for a lack of a role for NO in reflex- and DMPP-evoked bladder responses. However, administration of L-NAME caused an increase in baseline bladder pressure, suggesting that there is a small tonic release of NO to the bladder. In this respect, NO has been suggested to play a role in maintaining low bladder pressures during the storage phase of the micturition cycle (Andersson & Persson, 1994), although in the present experiments L-NAME had no effect on the volume and pressure thresholds to evoke the micturition reflex. In addition,

these effects of L-NAME on baseline bladder pressure were observed in both non- and  $\alpha$ -Bungarotoxin pre-treated animals, indicating that there are effected by the urethral smooth muscle. Furthermore, the fact that these changes were observed in both non- and  $\alpha$ -Bungarotoxin pre-treated animals, suggests that they are not caused indirectly as the result of the effects of L-NAME on the urethral striated muscle, in that an increased urethral tone will evoke a concomitant increase in bladder tone. These effects of L-NAME on baseline bladder tone may be due to the effects of L-NAME on the vasculature, as this antagonist evoked an increase in MAP in both non- and  $\alpha$ -Bungarotoxin pre-treated animals. Zaprinast has been shown to have no effect on the contractile responses of isolated guinea pig (Longhurst *et al.*, 1997) and porcine (Truss *et al.*, 1995) bladder strips. Furthermore, Morita, Tsujii & Dokita (1992) found that in rabbits, cGMP is mainly related to urethral relaxation and cAMP to bladder relaxation. Therefore, the present findings are in agreement with these and other *in vitro* studies, suggesting a lack of a role for NO/cGMP signalling in the bladder.

### *Conclusions*

In conclusion, the present study has demonstrated that reflex- and DMPP-evoked smooth muscle relaxations are mediated by NO. Furthermore, NO has a tonic inhibitory effect on the urethral striated muscle, which may operate as a breaking mechanism in the maintenance of urethral tone. The ability of zaprinast to potentiate reflex-evoked urethral relaxations and presumably this nitrenergic pathway are due to an ability to increase urethral striated muscle activity. This

increase in urethral striated muscle tone may involve neuronal nicotinic receptor-mediated reflex changes in response to decreases in urethral tone, which can also be demonstrated with intraurethral administration of SNP and isoprenaline. Further, these decreases in urethral tone are most likely mediated by an effect on urethral smooth muscle. In addition to the complex effects of manipulation of the NO/cGMP pathway on urethral tone, the aforementioned reflexes complicate investigations regarding the effects of zaprinast in the urethra *in vivo*, and the present study could not unequivocally demonstrate the presence of NO/cGMP signalling in urethral responses to the micturition reflex in anaesthetised female rats. Experiments involving manipulation of the NO/cGMP pathway other than PDE inhibition, may prove more useful in future investigations regarding the exact roles of this transduction pathway in this complex physiological system.

## **Chapter Three**

# **The Isolated Female Rat Urethra: Histological and Functional Studies**

## INTRODUCTION

The urethra, in the rat and other species, consists of a combination of longitudinal and circular smooth muscles, and a region of striated musculature, which has a circular orientation and is termed the *rhabdosphincter* or external urethral sphincter (EUS; see Lincoln & Burnstock, 1993). The studies described in this thesis have investigated the complex interactions between the smooth and striated muscles of the urethra in the anaesthetised female rat (see chapter two). These findings have been subsequently further investigated using *in vitro* functional tissue studies with the whole isolated female rat urethra (see chapters four and five). To be able to correlate the results of these *in vitro* studies with *in vivo* investigations, it is important to determine whether both these muscle types are present in the isolated female rat urethral preparation. Indeed, it is possible that owing to the close anatomical relationship between the urethra and vagina in the female rat, and the dissection procedures required to separate the two, the striated muscle may have been lost in this preparation. Furthermore, it would be useful to know that if the striated muscle is indeed intact in this preparation, whether it is functional. In this respect, Brading (1999) has previously noted that the properties of the striated muscle are difficult to study *in vitro*, as damage to the fibres and subsequent depolarisation of their membranes inactivates the action potential mechanism. Therefore, histological studies were carried out to determine the presence of these muscular components in the female rat urethral preparation. In addition, the effects of electrical field stimulation and succinylcholine, a striated muscle depolarising agent, were examined on the

isolated female rat urethra. The effects of these stimulants were also examined on the isolated female rat diaphragm, which is composed entirely of striated muscle, to provide a comparative preparation for the functional effects of striated muscle stimulation in these experimental conditions.

## **METHODS**

### **Histological studies**

The preparation and staining of urethral tissues for histological examination was carried out by Julie Owen, Histology Department, Pfizer Global Research and Development (Kent, U.K.). Briefly, transverse sections of urethral tissue were cut and mounted onto glass microscope slides. These sections were then treated with a phosphotungstic acid haematoxylin (PTAH) stain, which is particularly good for the demonstration of striated musculature. The PTAH solution used comprised 0.5 g haematoxylin, 10 g phosphotungstic acid, 25 ml aqueous potassium permanganate solution and 500 ml distilled water. Before applying this stain, the tissues were treated with acid permanganate solution (0.5% potassium permanganate and 3% sulphuric acid), bleached in 1% oxalic acid, rinsed, treated with 5% iron alum and rinsed again. Urethral sections were then immersed in the PTAH solution overnight.

### **Functional studies**

Twelve female Sprague-Dawley rats (200-300g; Charles River, Kent, U.K.) were stunned by a blow to the head and killed by cervical dislocation. Through a midline incision the bladder and proximal urethra emerging from the pubic bone were exposed. The pubic bone was cut and the bladder and urethra were removed. The urethra was cleared of connecting vascular and fatty tissues and

the bladder was disconnected from the urethra by a cut just below the bladder neck. Through a midline thoracic incision, the diaphragm was exposed, and dissected from the connecting bone. Diaphragmatic longitudinal strips of roughly the same dimensions as the isolated whole rat urethral preparations (approximately 1.0 cm by 0.2 cm), along the 'grain' of the muscular striations, were prepared. The whole urethras were mounted longitudinally under 1g resting tension in 5ml tissue baths. Diaphragmatic preparations were mounted under 0.5g resting tension as preliminary experiments showed that tensions of 1g could not be maintained in this tissue. The tissues were bathed and perfused (3.43 ml min<sup>-1</sup>, MCP peristaltic pump, Ismatec, Weston-super-Mare, U.K.) with Krebs solution of the following composition (in mM): NaCl 118; KCl 4.7; NaHCO<sub>3</sub> 25; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 0.58; CaCl<sub>2</sub> 2.5 and glucose 11. The temperature of the baths were maintained at 37°C using a heater circulator system (C-85A, Techne, Cambridge, U.K.) and were aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Tissues were stimulated using an electrical stimulator (in house, Pfizer Global Research and Development, Kent, U.K.).

### *Experimental Protocols*

The tissues were equilibrated under their respective resting tensions for a period of 60 min. Frequency-response curves to electrical field stimulation were carried out using the following parameters: 20 volts, 0.8 ms pulse width for 5 s, at 5 min intervals, at frequencies of 1 - 64 Hz. It was observed from preliminary experiments that stopping wash-out of the tissues caused an increase in resting

urethral tension, although this was not observed in diaphragmatic preparations. This could be related to changes in the concentration of oxygen in the bath, which has previously been shown to affect resting urethral tensions in the pig (Greenland & Brading, 1997), or due to mechanical factors. Alternatively, an excitatory substance may be released from this tissue, which upon stopping perfusion of tissues, evokes a contractile response. These possibilities require further investigation. Therefore, before any test substance was applied, a period of 10 min after stopping perfusion of the tissues was allowed to re-equilibrate resting urethral tension. After this period, tissues were incubated with vehicle or tetrodotoxin (TTX) for 30 minutes. Second frequency response curves were then constructed.

In experiments investigating the effects of succinylcholine, frequency-response curves were performed in all urethral and diaphragmatic tissues to test the viability of these preparations. Perfusion of tissues was then stopped and a 10 min equilibration period allowed, as described above. Preliminary experiments investigating the effects of succinylcholine showed that this agent evoked relaxations of isolated rat urethral preparations. These relaxations are mediated by activation of neuronal nicotinic receptors as they are abolished by hexamethonium, a selective neuronal nicotinic receptor antagonist (data not shown). Furthermore, the presence of these receptors in the female rat urethra has been provided from further studies in this thesis (see chapter five). Therefore, in subsequent experiments, tissues were pre-treated with hexamethonium (100  $\mu$ M), an antagonist of neuronal nicotinic receptors. This

antagonist was added to the baths following the cessation of perfusion of tissues, and allowed to incubate for 30 min. All tissue preparations were then exposed to succinylcholine.

### **Data capture and analysis**

Changes in urethral and diaphragmatic tensions were measured by means of 10 g isometric force transducers (Maywood Instruments Ltd, Basingstoke, U.K.) and were acquired (4 samples per second) using a DART system (in house, Pfizer Global Research and Development, Kent, U.K.), and analysed off-line using ADA software (in house, Pfizer Global Research and Development, Kent, U.K.). The amplitudes (g) and durations (s) of the relaxatory and contractile tissue responses to electrical field stimulation were measured. Responses to succinylcholine could not be analysed accurately owing to the classical contractile fasciculations that this agent produced (Anttila & Ertama, 1978; Fletcher & Rosenberg, 1986). Therefore, either the presence or absence of succinylcholine-evoked responses were reported. Changes in electrically-evoked urethral relaxations were expressed as percentage changes before and after the administration of antagonists, and compared with vehicle controls by unpaired student's t-test. Contractile urethral responses to field stimulation observed following the administration of test substances could not be expressed as percentage controls owing to the fact that these responses were not observed at all frequencies of electrical stimulation in control curves. Therefore, where

agents had an overt effect on these responses they were described numerically.

All values are mean  $\pm$  s.e.mean.

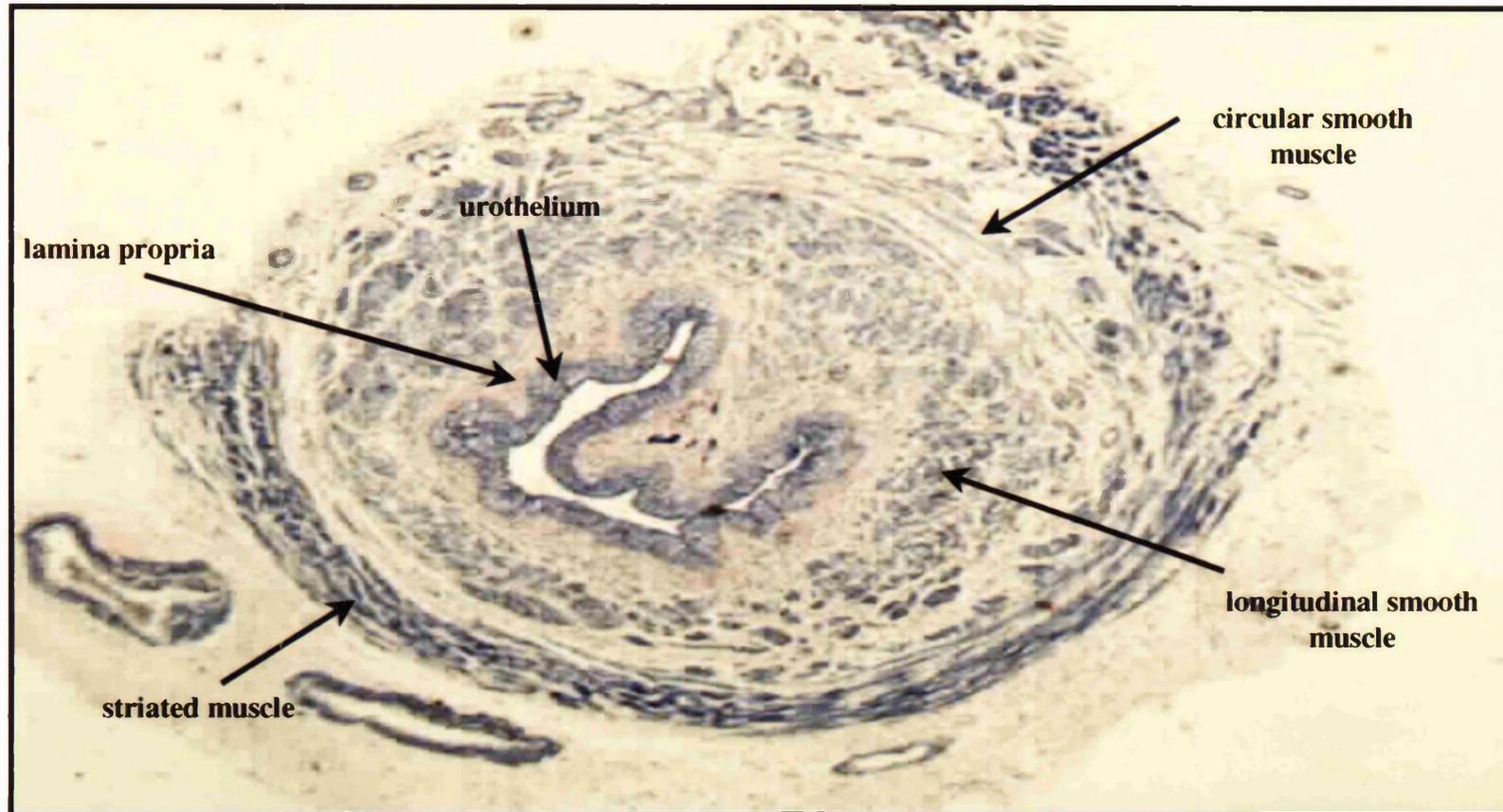
### **Drugs and solutions**

Hexamethonium (chloride), tetrodotoxin and succinylcholine (chloride) were obtained from Sigma Aldrich Chemicals, Poole, Dorset, U.K. All drugs were dissolved in Krebs solution. Test substances were added to baths in a maximum of 50  $\mu$ l volume and the concentrations reported are final bath concentrations.

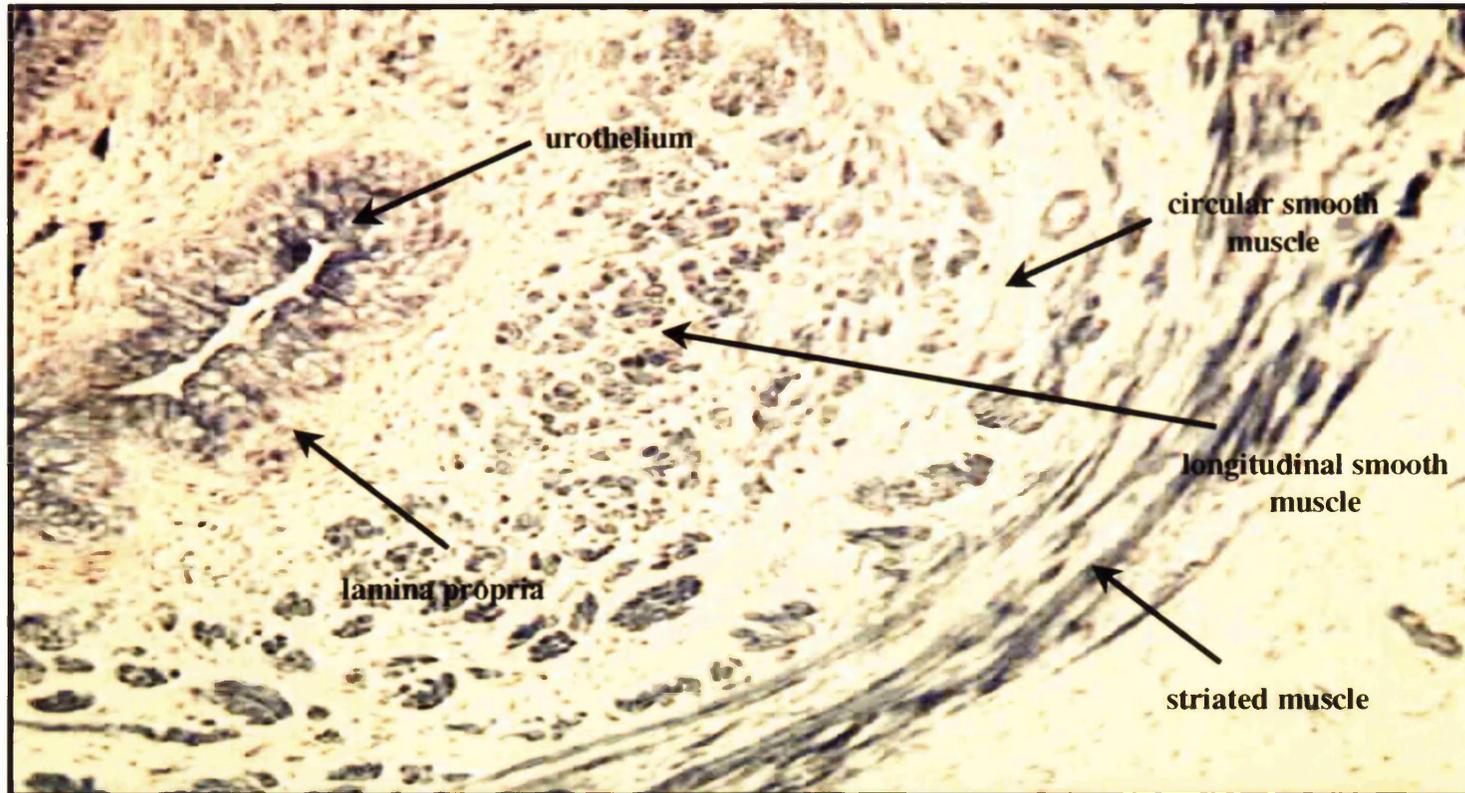
## **RESULTS**

### **Histological studies**

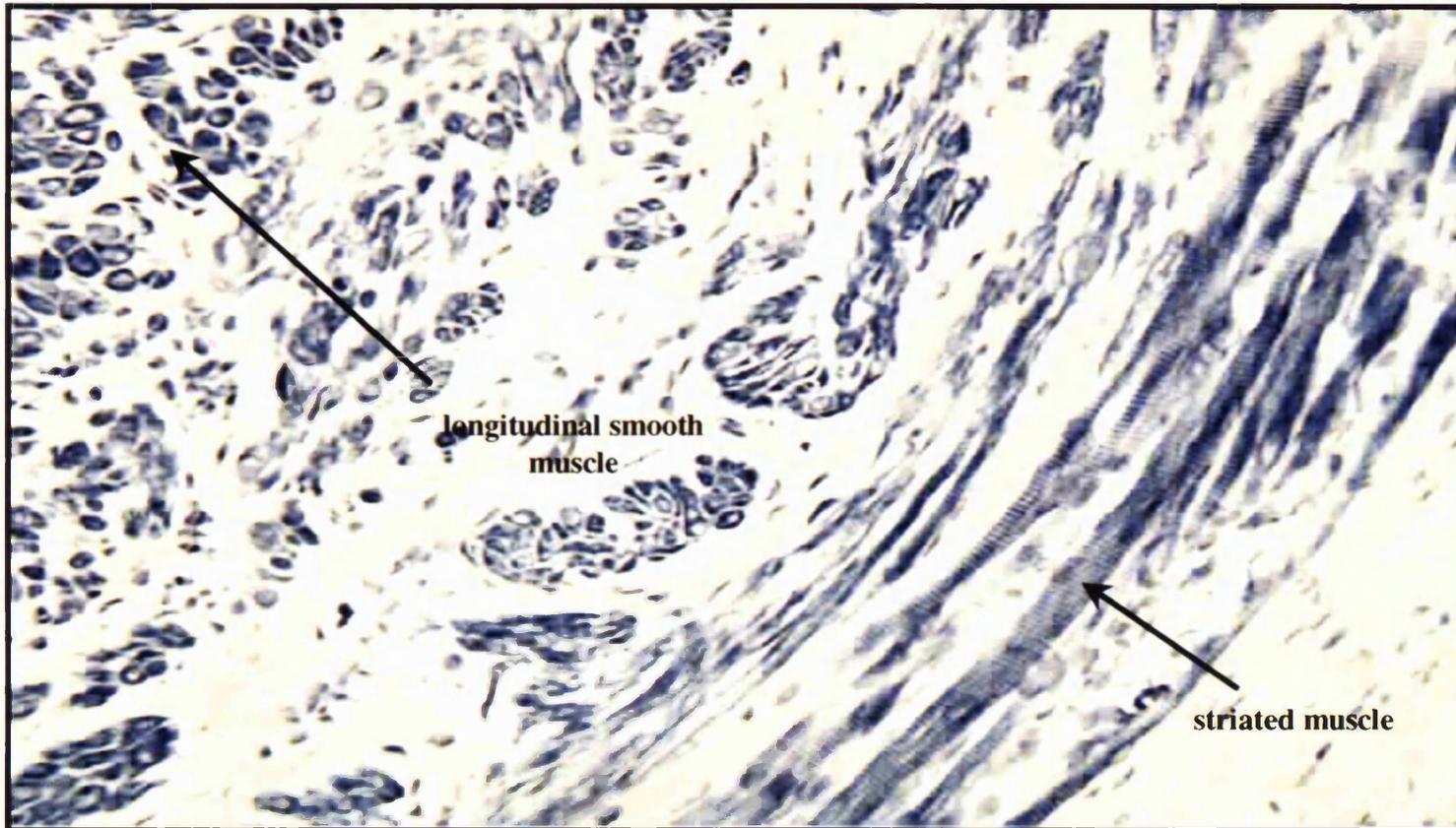
Distinct regions of smooth and striated musculature are clearly visible in urethral sections stained with PTAH (Figures 3.1, 3.2 and 3.3). Sections of the mid part of the female rat urethra viewed at low magnification (x 4) show the presence of a distinct inner lamina propria underlying the urothelium, surrounded by layers of circular and longitudinal smooth muscle (Figure 3.1). A well developed sleeve of C-shaped striated musculature constitutes the outer region of the female rat urethra, that is deficient posteriorly. Higher magnifications (x 10 and x 40) of the urethral sections provide more detailed views of the urothelium and underlying lamina propria and smooth and striated muscles (Figures 3.2 and 3.3).



**Figure 3.1.** Transverse section of the mid part of the female rat urethra. (PTAH stained, viewed at x4 magnification)



**Figure 3.2.** Transverse section of the mid part of the female rat urethra.  
(PTAH stained, viewed at x10 magnification)



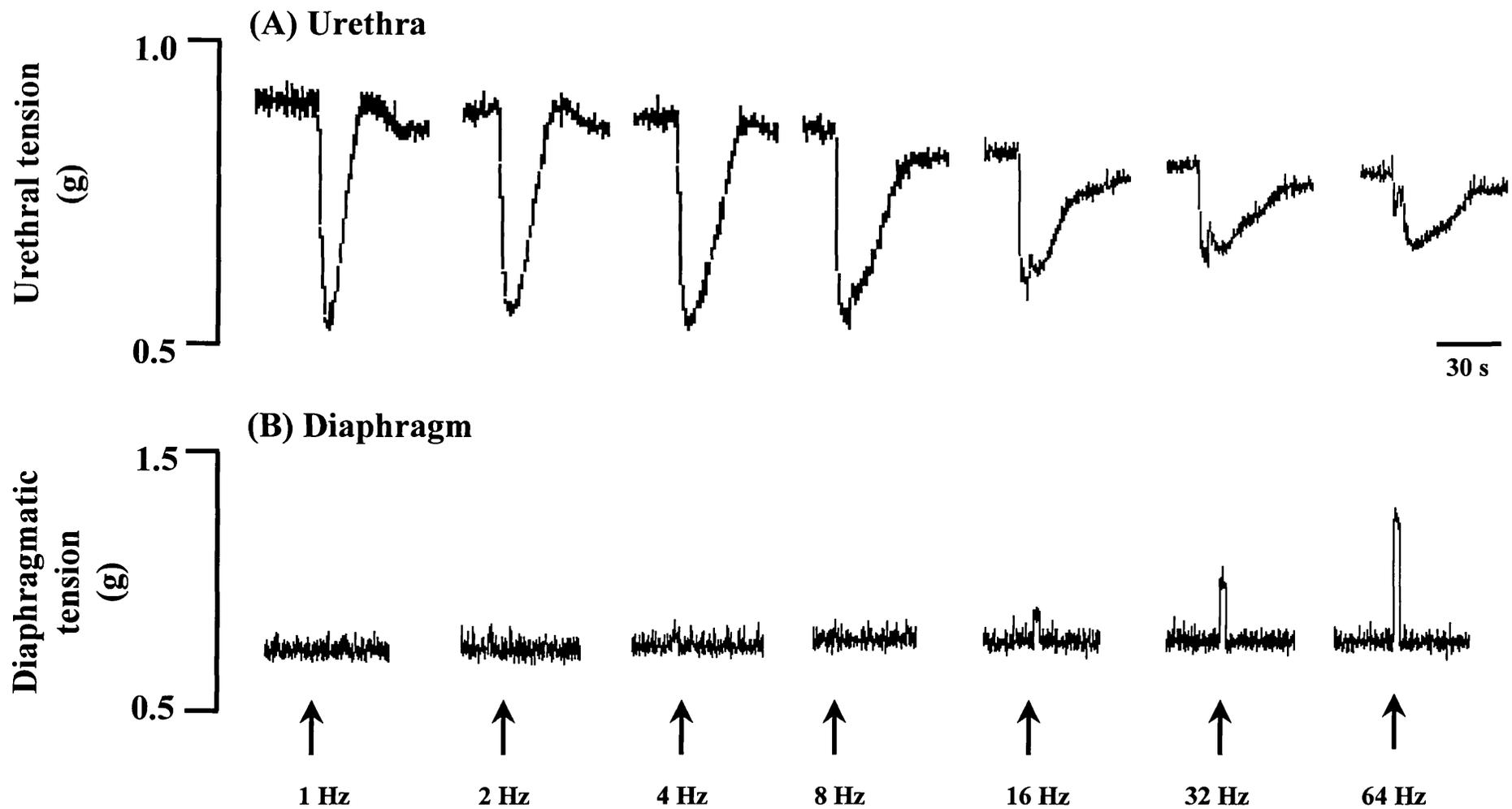
**Figure 3.3.** Transverse section of the mid part of the female rat urethra. (PTAH stained, viewed at x40 magnification)

## Functional studies

### Effects of electrical field stimulation on rat urethral and diaphragmatic preparations

Electrical stimulation evoked relaxations of the isolated female rat urethra at 1, 2, 4 and 8 Hz (Figure 3.4). These relaxations had a mean amplitude of  $0.66 \pm 0.03$  g,  $0.67 \pm 0.03$  g,  $0.67 \pm 0.03$  g and  $0.66 \pm 0.02$  g ( $n = 8$ ), respectively, and a mean duration of  $20.8 \pm 0.6$  s,  $22.3 \pm 1.3$  s,  $25.0 \pm 1.1$  s and  $27.0 \pm 1.9$  s, respectively. At frequencies of 16 Hz and above, frequency-dependent contractions of the urethra were also observed in response to electrical stimulation in some tissues. These contractions occurred 'mid-way' through the urethral relaxations, and had a mean amplitude of  $0.08 \pm 0.05$  g ( $n = 4$ ),  $0.21 \pm 0.04$  g ( $n = 6$ ) and  $0.17 \pm 0.06$  g ( $n = 8$ ) at 16, 32 and 64 Hz, respectively (Figure 3.4). Furthermore, these contractions had a mean duration of  $3.8 \pm 0.73$  s,  $8.5 \pm 1.7$  s and  $9.5 \pm 0.58$  s, respectively. The urethral relaxations in which these contractions occurred had a mean amplitude of  $0.64 \pm 0.12$  g,  $0.55 \pm 0.11$  g and  $0.48 \pm 0.07$  g at 16, 32 and 64 Hz, respectively (measured from baseline to the minimal point reached), hence the amplitude of these relaxations decreased as the contractile urethral responses increased with higher frequencies of stimulation. These inhibitory responses of the urethra had a mean duration of  $30.5 \pm 0.75$  s,  $29.8 \pm 0.99$  s and  $30.0 \pm 0.47$  s, respectively (which were taken from the total duration of the relaxations before and after the contractile responses).

Electrical stimulation of the isolated rat diaphragm had no effect at frequencies of 1, 2, 4 and 8 Hz ( $n = 8$ ; Figure 3.4). Contractile responses to field stimulation were observed at frequencies of 16 Hz and above. Interestingly, this frequency of stimulation also produced contractile responses of the urethra, that were not observed at lower stimulation parameters (Figure 3.4). Electrically-evoked contractions of the rat diaphragm had a mean amplitude of  $0.49 \pm 0.13$  g,  $0.74 \pm 0.12$  g and  $1.08 \pm 0.32$  g, and a mean duration of  $8.5 \pm 0.33$  s,  $8.8 \pm 0.56$  s and  $7.76 \pm 0.87$  s ( $n = 4$ ), at 16, 32 and 64 Hz, respectively.



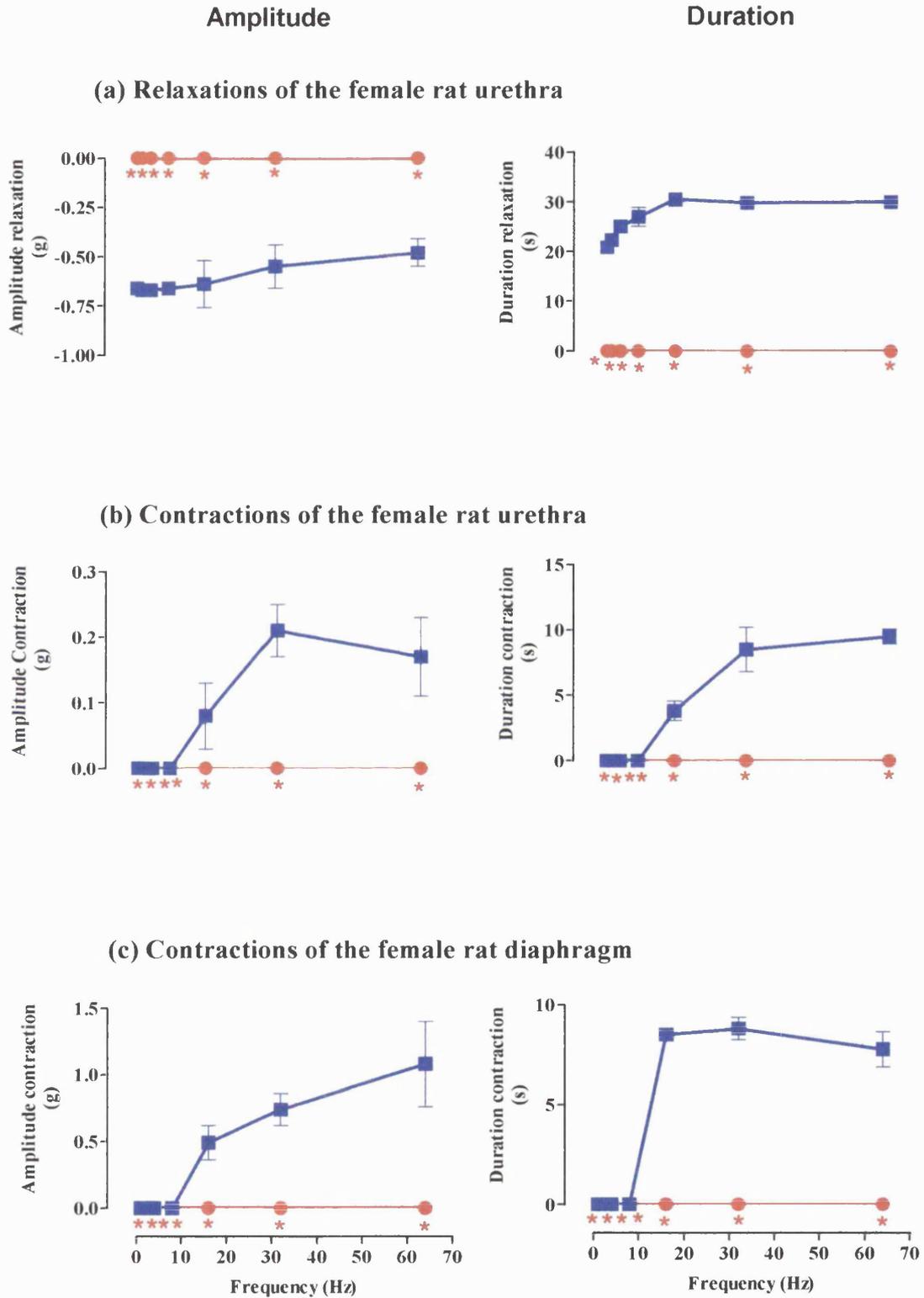
**Figure 3.4.** Traces showing the effects of electrical field stimulation (20 volts, 0.8 ms, for 5s, at 5 min intervals) on the isolated female rat urethra (A) and diaphragm (B).

### **Effects of tetrodotoxin on electrical field stimulation-evoked rat urethral and diaphragmatic responses**

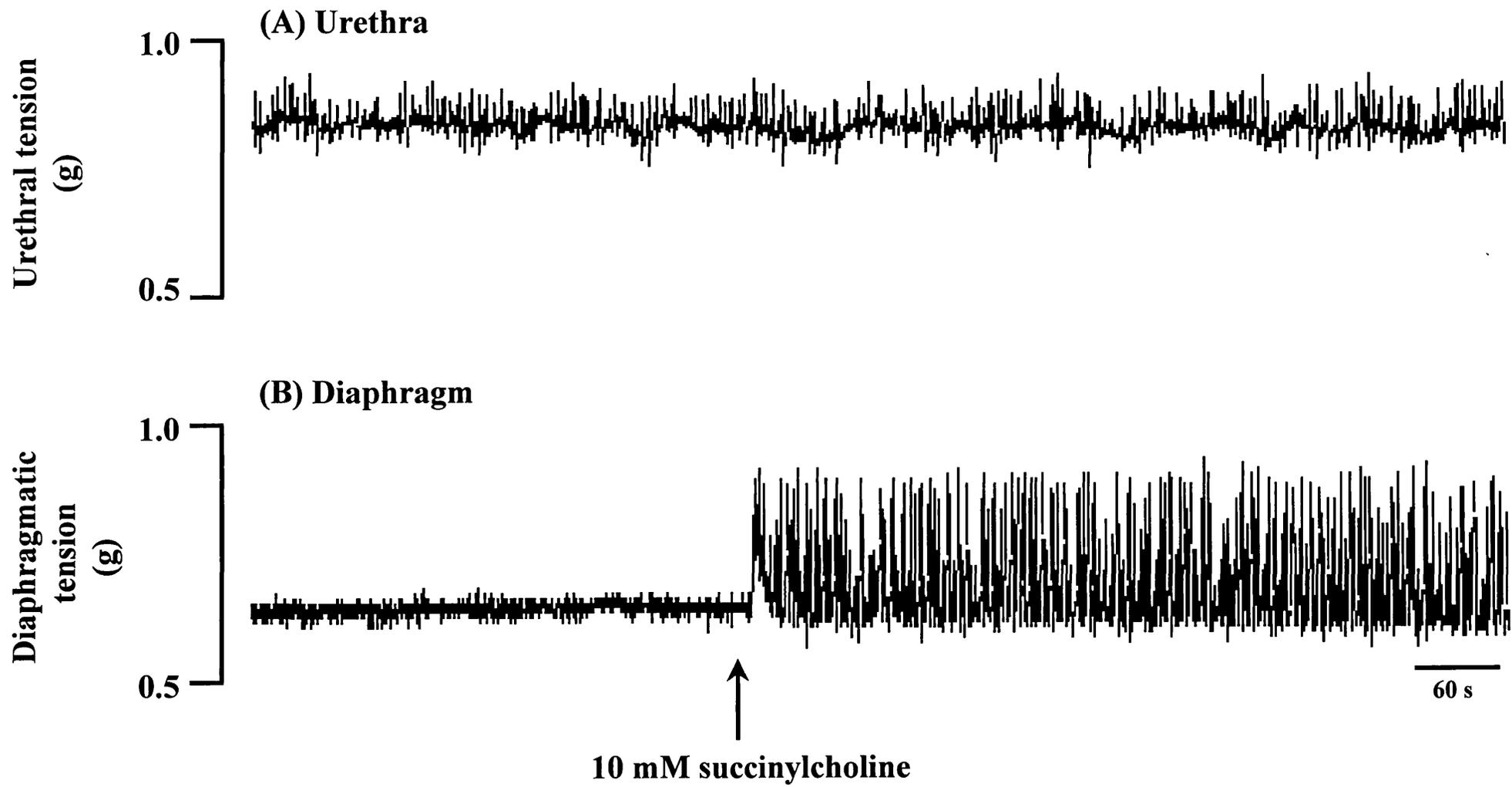
Administration of TTX (1  $\mu$ M) inhibited both the relaxatory and contractile urethral responses to electrical stimulation at all frequencies tested (n = 4; Figure 3.5). Furthermore, diaphragmatic contractions evoked by electrical stimulation were inhibited by TTX at all frequencies of stimulation that evoked these responses (n = 4; Figure 3.5).

### **Effects of succinylcholine on rat urethral and diaphragmatic preparations**

Succinylcholine (10 mM) had no effect on the isolated female rat urethra in any of the preparations tested (n = 4; Figure 3.6). Conversely, this depolarising agent caused rapid contractile fasciculations of the isolated female rat diaphragm in all preparations (n = 4; Figure 3.6). In separate experiments, a higher concentration (300 mM) of succinylcholine evoked relaxations of urethral tissues by the activation of neuronal nicotinic receptors (data not shown; see chapter five). However, rapid contractions of the urethra similar to those occurring in diaphragmatic tissues were still not observed.



**Figure 3.5.** Amplitude and duration of electrically-evoked relaxations and contractions of the isolated female rat urethra (a and b, respectively) and contractions of the isolated female rat diaphragm (c) following incubation of tissues with vehicle (■) or TTX (1 μM; ●). Each point represents mean ± s.e. mean of 4 experiments. \* P<0.05.



**Figure 3.6.** Traces showing the effects of succinylcholine on the isolated female rat urethra (A) and diaphragm (B).

## **DISCUSSION**

### *Histological studies*

The present histological studies have confirmed the presence of both smooth and striated muscles in the whole isolated female rat urethral preparation. Sections of urethral tissue from the mid-urethra showed distinct layers of urothelium, lamina propria, longitudinal and circular smooth muscles, and an outer region of striated musculature that was deficient posteriorly. These findings are in agreement with those of Andersson, Malgren & Uvelius (1990) who also observed the presence of both these muscle types in the female rat urethra. These authors also examined the muscle types in each region of the urethra from proximal (bladder) to distal (urethral orifice) ends, and found that circular smooth muscle was predominant in the proximal part, and striated muscle was predominant in the middle part. Furthermore, similar histological observations have also been demonstrated for the female human (Hutch & Rambo, 1967) and dog (Cullen, Fletcher & Bradley, 1981) urethra.

### *Effects of electrical field stimulation on the isolated female rat urethra and diaphragm*

Electrical stimulation of the isolated female rat urethra evoked urethral responses that were inhibited by TTX, indicating that they are the result of action potential generation. The response of the isolated female rat urethra to low frequencies of

stimulation was urethral relaxation, whereas at higher frequencies (16 Hz and above) a combination of field stimulation-evoked urethral contractions and relaxations were observed. Electrical stimulation of the isolated rat diaphragm at high frequencies of stimulation (16 Hz and above) evoked contractions that were also inhibited by TTX. Electrically-evoked urethral relaxations have been previously reported in isolated longitudinal strips from the female rat urethra (Andersson, Malgren & Uvelius, 1990). Furthermore, relaxatory responses to electrical stimulation are not observed in circular female rat urethral preparations, suggesting that the urethral relaxations observed in the present study reflect responses of the longitudinal urethral smooth muscle. Conversely, rapid contractile responses of the circular female rat urethra have been shown to constitute the primary response to electrical stimulation in this region of the urethra (Andersson, Malgren & Uvelius, 1990). Analysis of this contractile urethral response revealed a fast twitch response during electrical stimulation with single impulses, and summation and tetanus with increasing frequencies. In addition, these responses were resistant to autonomic blockade and the depletion of extracellular  $Ca^{2+}$  (thought to be selective for smooth muscle), suggesting they are the result of urethral striated muscle activity that is orientated circularly. Interestingly, in the present study, electrically-evoked urethral contractions were observed at the same frequencies of stimulation that contractions of the isolated rat diaphragm were first observed (16 Hz). Furthermore, the histological observations that the whole isolated female rat urethral preparation comprises both smooth and striated muscles, raises the possibility that these contractions are mediated, at least in part, by contractions of the urethral striated muscle.

However, an involvement of electrically-evoked contractions of the urethral longitudinal and circular smooth muscles, mediated by acetylcholine (Mattiason *et al.*, 1990) and/or noradrenaline (Chess-Williams, Aston & Couldwell, 1994) release in these urethral responses cannot be excluded. Examination of the effects of Ca<sup>2+</sup> depletion and neuromuscular nicotinic and muscarinic receptor and adrenoceptor antagonists would prove useful in delineating the exact contributions of smooth and striated muscles in these contractile urethral responses.

*Effects of succinylcholine on the isolated female rat urethra and diaphragm*

To further investigate the functional presence of striated muscle in the urethra, the effects of succinylcholine were investigated on this preparation. The effects of this agent were also examined on the isolated rat diaphragm as a comparative control for the effects of succinylcholine on striated muscle. Succinylcholine is a depolarising neuromuscular relaxant, that interacts with nicotinic receptors to produce depolarisation, which is commonly observed as fasciculations and twitching in striated musculature (see Broadley, 1996). Indeed, in the isolated rat diaphragm, administration of succinylcholine evoked rapid fasciculations of this tissue. These responses of rat diaphragmatic preparations have been extensively described previously, and are commonly used as an assay in the development of neuromuscular blocking agents (Anttila & Ertama, 1978; Fletcher & Rosenberg, 1986). The dose of succinylcholine used in the present study was chosen on the basis of these previous investigations. In contrast to the effects on the rat

diaphragm, succinylcholine had no effect on the isolated female rat urethra. These results are in contrast to previous studies that have demonstrated contractile responses to succinylcholine in circular female rat urethral preparations (Andersson, Malgren & Uvelius, 1990). Furthermore, further studies in this thesis (see chapter five) have shown that nicotinic agonists do not evoke any contractile responses in this tissue. However, as the urethral tissue used in the present studies comprised the whole female rat urethra, in contrast to individual circular urethral sections, it is possible that any effects of succinylcholine on the striated muscle are masked by the increased amount of urethral smooth muscle that is present in this preparation. Conversely, the isolated rat diaphragm comprises only striated muscle, and therefore succinylcholine-evoked responses may be more easily observed. Indeed, these previous studies (Andersson, Malgren & Uvelius, 1990) were carried out in exactly the same experimental conditions as the present study, suggesting that contractile responses mediated by activation of urethral striated muscle may be masked by the combined presence of longitudinal and circular smooth musculature in the whole female rat urethra. Another possible explanation for these surprising observations is related to the orientation of the striated muscle fibres, in that this region of musculature in the female rat urethra is circular, C-shaped and deficient posteriorly (see chapter three), and therefore the mounting of this preparation longitudinally may not record an increase in tension in this plane. Examination of the effects of succinylcholine on urethral preparations comprising only the mid-part of the whole female rat urethra, where striated muscle dominates, and mounted in the 'plane' of the striated muscle fibres may

prove useful in delineating some of these issues. Alternatively, the striated muscle fibres may be damaged in this preparation as a result of the dissection techniques or the experimental conditions, or the neuromuscular nicotinic receptors in this musculature may be distinct from those of the rat diaphragm and therefore not respond to succinylcholine. These possibilities require further investigation.

### *Conclusions*

In conclusion, the results of the present histological studies have identified the presence of both smooth and striated muscles in the whole female rat urethral preparation. Furthermore, the presence of both relaxatory and contractile responses of this tissue to electrical stimulation suggests a functional difference in the responses of longitudinal and circular smooth and striated muscles in this preparation. Surprisingly, succinylcholine had no effect on the isolated female rat urethra, although the reasons for this remain unclear. However, the histological presence of striated muscle and the contractile responses to field stimulation that were observed in this tissue, suggests that a role for the urethral striated muscle in the effects of various test substances examined in further studies in this thesis on the female rat urethra cannot be excluded.

## **Chapter Four**

**Nitric Oxide and cyclic GMP in the**

**Female Rat Urethra *in vitro***

## INTRODUCTION

Previous studies in this thesis have demonstrated that L-NAME, a NOS inhibitor, inhibits reflex- and DMPP (a neuronal nicotinic receptor agonist)-evoked urethral relaxations in the anaesthetised female rat, showing that these effects are mediated by NO (see chapter two). In addition, L-NAME also increased urethral striated muscle activity in these experiments, suggesting that NO acts as a 'break' to inhibit the activity of this musculature. The effects of zaprinast (a selective PDE 1, 5, 6 and 9 inhibitor) were also examined in these *in vivo* experiments to determine if the effects of NO are mediated by cGMP. Zaprinast potentiated reflex- and DMPP-evoked urethral relaxations in non  $\alpha$ -Bungarotoxin pre-treated animals, and interestingly, also exhibited an ability to increase urethral striated muscle tone. Further experiments showed that zaprinast did not potentiate reflex- and DMPP-evoked urethral relaxations following pre-treatment with  $\alpha$ -Bungarotoxin, a neuromuscular blocker, suggesting that the potentiating effects of this agent are due to an ability to increase urethral striated muscle activity, which may involve reflex interactions between the urethral smooth and striated muscles (see chapter two). Therefore, conclusive evidence for a role of NO-mediated cGMP signalling in relaxations of the urethra during voiding could not be obtained from these *in vivo* studies. Moreover, the drugs used in these studies also had effects on baseline urethral pressures, suggesting a possible role for NO/cGMP signalling in the control of urethral tone, which may have a functional relevance in the complex interactions between the activities of the smooth and striated muscles *in vivo*, as described previously (see chapter two). It has

previously been suggested that L-NAME also inhibits muscarinic acetylcholine receptors from functional organ bath and radioligand binding studies (Buxton *et al.*, 1993). Although other investigations have failed to identify any significant antimuscarinic properties of this agent *in vivo* (Hellmich & Gyermek, 1997; Koss, 1997), the possibility exists that any effects of L-NAME on baseline urethral tensions in the present experiments reflect an involvement of these receptors. Thus, the aim of the present study was to investigate the effects of L-NAME, N $\omega$ -nitro-L-arginine, another NOS inhibitor (L-NA, Moncada, Higgs & Furchgott, 1997) and zaprinast on the female rat urethra *in vitro*. The effects of these agents were examined on electrically-evoked relaxations and resting urethral tensions in this tissue.

## METHODS

### General preparation

Thirty-six female Sprague-Dawley rats (200-300g; Charles River, Kent, U.K.) were stunned by a blow to the head and killed by cervical dislocation. Through a midline incision the bladder and proximal urethra emerging from the pubic bone were exposed. The pubic bone was cut and the bladder and urethra were removed. The urethra was cleared of connecting vascular and fatty tissues and the bladder was disconnected from the urethra by a cut just below the bladder neck. The whole urethras were then mounted longitudinally under 1g resting tension in 5ml tissue baths. The tissues were bathed and perfused (3.43 ml min<sup>-1</sup>, MCP peristaltic pump, Ismatec, Weston-super-Mare, U.K.) with Krebs solution of the following composition (in mM): NaCl 118; KCl 4.7; NaHCO<sub>3</sub> 25; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 0.58; CaCl<sub>2</sub> 2.5 and glucose 11. The temperature of the baths were maintained at 37°C using a heater circulator system (C-85A, Techne, Cambridge, U.K.) and were aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Tissues were stimulated using an electrical stimulator (in house, Pfizer Global Research and Development, Kent, U.K.).

### *Experimental Protocols*

The tissues were equilibrated under 1 g resting tension for a period of 60 min. Control frequency-response curves to electrical field stimulation were carried out using the following parameters: 20 volts, 0.8 ms pulse width for 5 s, at 5 min

intervals, at frequencies of 1 - 64 Hz. It was observed from preliminary experiments that stopping wash-out of the tissues caused an increase in resting urethral tension. The possible explanations for these observations are outlined in previous sections of this thesis (chapter three). Therefore, before any test substance was applied, a period of 10 min after stopping the wash out of the tissues was allowed to re-equilibrate resting urethral tension. After this period, tissues were incubated with antagonist or vehicle for a period of 30 min. Second frequency-response curves were then constructed.

### **Data capture and analysis**

Changes in urethral tensions were measured by means of 10 g isometric force transducers (Maywood Instruments Ltd, Basingstoke, U.K.), acquired (4 samples per second) using a DART system (in house, Pfizer Global Research and Development, Kent, U.K.), and analysed off-line using ADA software (in house, Pfizer Global Research and Development, Kent, U.K.). The amplitudes (g) and durations (s) of the relaxatory and contractile urethral responses to electrical field stimulation were measured. Baseline urethral tensions were measured 1 min before administration of test substances, and changes in resting urethral tensions from baseline caused by test substances were measured 10 min after their administration. This time was chosen as it was adjudged that any effects of agents on baseline urethral tensions had stabilised. Changes in baseline urethral tensions and urethral relaxatory responses to field stimulation were expressed as percentage changes before and after the administration of antagonists, and

compared with vehicle controls by unpaired student's t-test. P values of less than 0.05 were considered indicative of a statistically significant difference. Contractile urethral responses to field stimulation following the administration of test substances could not be consistently expressed as percentage controls as these responses were not observed at all frequencies of electrical stimulation in control curves. Therefore, these responses were expressed as percentage of controls where possible, and where agents had an overt effect on these responses they were described numerically. All values are mean  $\pm$  s.e.mean.

### **Drugs and solutions**

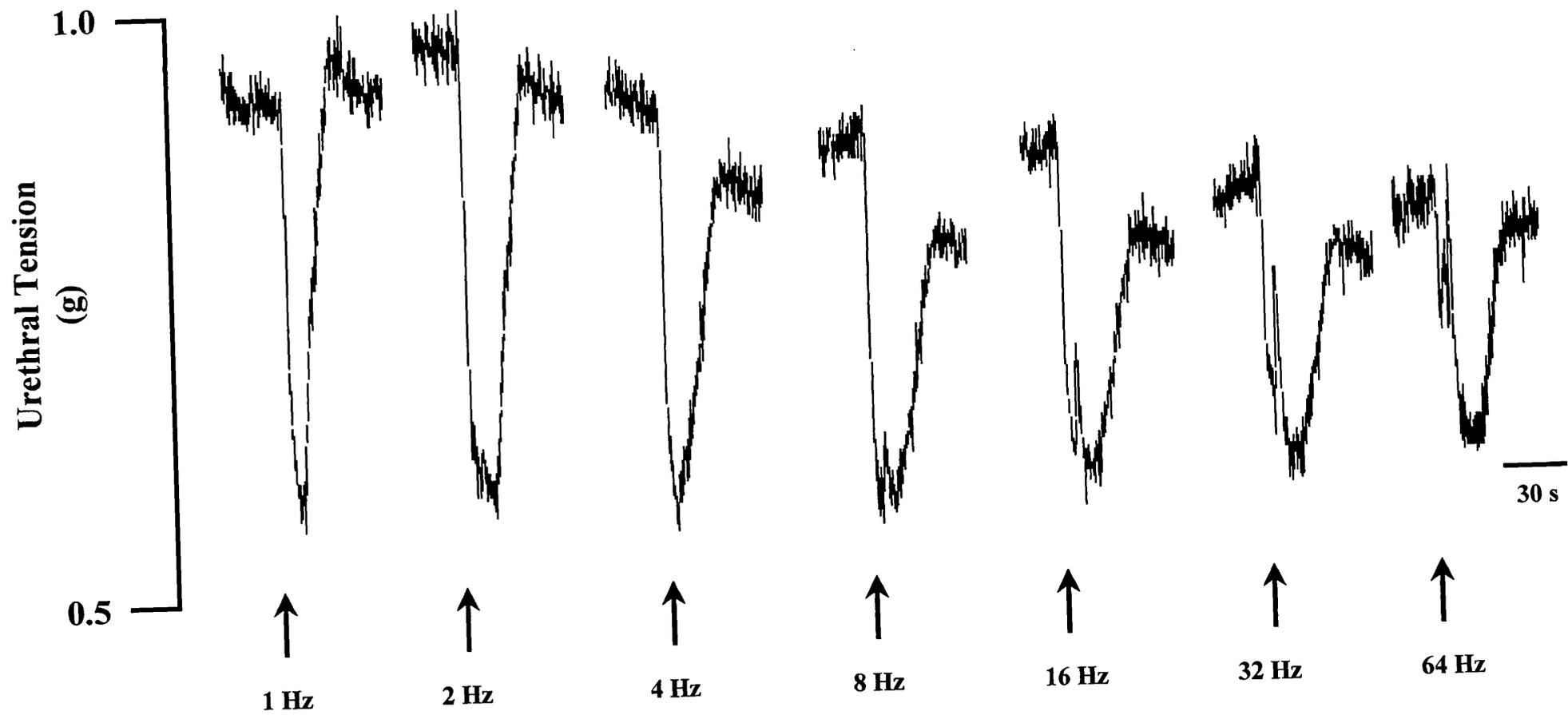
Drugs and chemicals were obtained from the following sources: N $\omega$ -nitro-L-arginine methyl ester (L-NAME; hydrochloride), N $\omega$ -nitro-L-arginine (L-NA), zaprinast and Krebs ringer solution were purchased from Sigma Aldrich Chemicals, Poole, Dorset, U.K.; lactic acid from Merck/BDH, Poole, Dorset, U.K. All drugs were dissolved in Krebs solution, except zaprinast that was dissolved in 5% triethanolamine in Krebs solution. Test substances were added to baths in a maximum of 50  $\mu$ l volume and the concentrations reported are final bath concentrations.

## RESULTS

### Effects of electrical field stimulation on the female rat urethra

Electrical stimulation evoked relaxations of the isolated female rat urethra at 1, 2 and 4 Hz (Figure 4.1). The mean amplitude of these control electrically-evoked urethral relaxations for all experimental groups were  $0.40 \pm 0.04$  g,  $0.42 \pm 0.03$  g and  $0.42 \pm 0.03$  g (n = 28) at 1, 2 and 4 Hz, respectively. Furthermore, these relaxations had a mean duration of  $32.0 \pm 1.0$  s,  $35.1 \pm 0.86$  s and  $40.1 \pm 1.1$  s. At frequencies of 8 Hz and above, frequency-dependent contractions of the urethra were also observed in response to electrical stimulation in some of the 28 tissues used in these experiments. These contractions occurred during electrically-evoked urethral relaxations, and had a mean amplitude of  $0.01 \pm 0.01$  g (n = 3),  $0.05 \pm 0.02$  g (n = 7),  $0.13 \pm 0.03$  g (n = 15) and  $0.22 \pm 0.04$  g (n = 21) at 8, 16, 32 and 64 Hz, respectively (Figure 4.1; the n numbers in brackets denote the number of tissues out of a total of 28 in which EFS-evoked urethral contractions were observed). These contractions had a mean duration of  $0.42 \pm 0.40$  s,  $2.46 \pm 0.82$  s,  $5.92 \pm 1.13$  s and  $8.08 \pm 1.08$  s, respectively. The urethral relaxations in which these contractions occurred had a mean amplitude of  $0.40 \pm 0.03$  g,  $0.36 \pm 0.02$  g,  $0.32 \pm 0.02$  g and  $0.28 \pm 0.02$  g at 8, 16, 32 and 64 Hz, respectively, (which were measured from baseline to minimum urethral tension reached, despite the occurrence of urethral contractile responses). Thus, the amplitude of these relaxations decreased as the contractile urethral responses increased with higher frequencies of stimulation. These inhibitory responses of the urethra had a mean duration of  $45.0 \pm 1.5$  s,  $45.1 \pm 2.9$  s,  $48.0 \pm 3.5$  s and

40.1 ± 3.3 s, respectively (which were taken from the total duration of the relaxations before and after the contractile responses).



**Figure 4.1.** Trace showing the effects of electrical field stimulation (20 volts, 0.8 ms, for 5 s, at 5 min intervals) on the isolated female rat urethra.

**Effects of vehicles on electrical field stimulation-evoked urethral responses and baseline urethral tensions**

Administration of vehicles for L-NAME, L-NA (Krebs solution) and zaprinast (5% triethanolamine in Krebs solution) had no significant effects on the amplitude or duration of field stimulation-evoked urethral relaxations (Tables 4.1 and 4.2) and baseline urethral tensions (Figure 4.4).

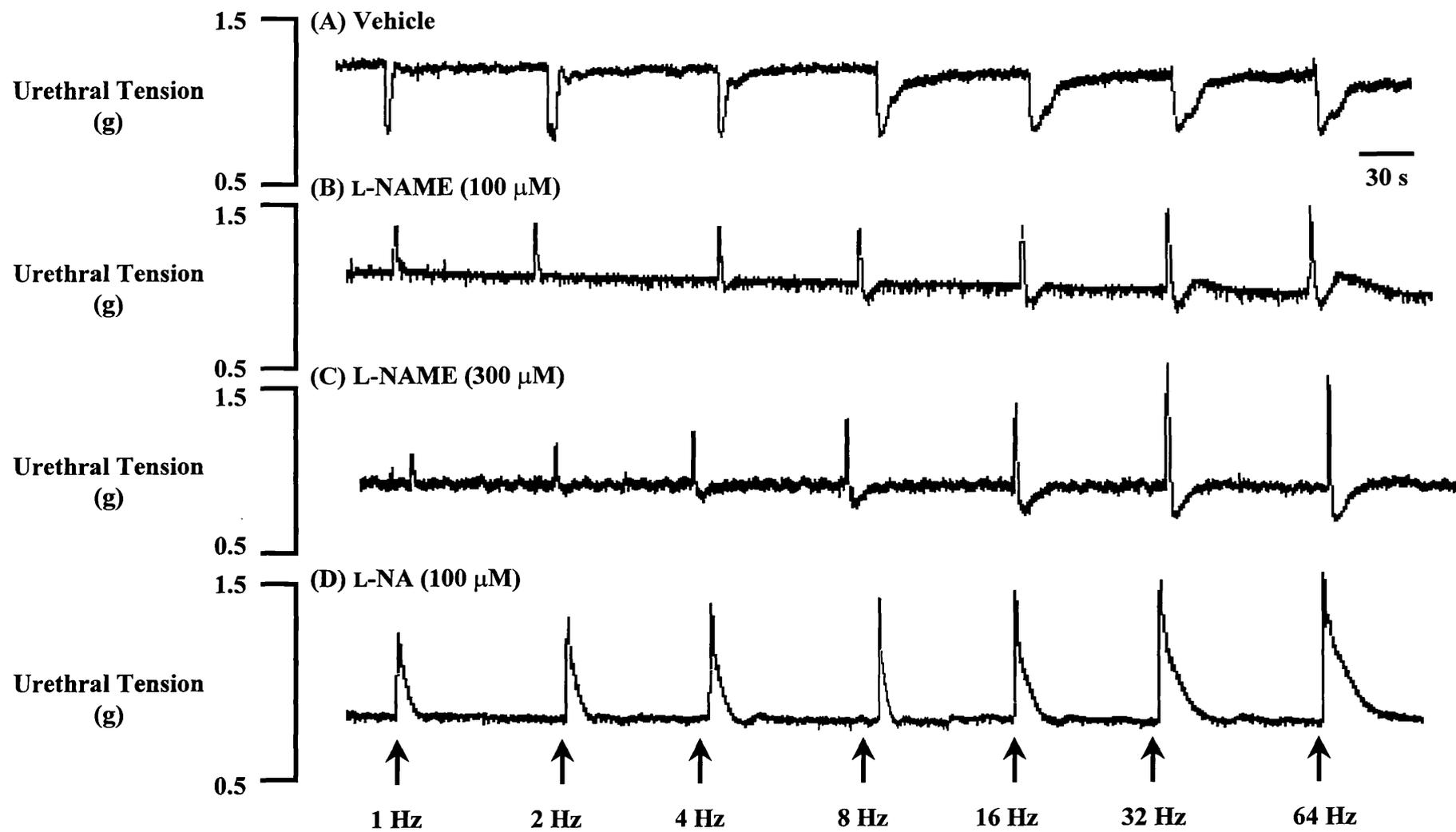
**Effects of L-NAME on electrical field stimulation-evoked urethral responses and baseline urethral tensions**

L-NAME (100  $\mu$ M) abolished the urethral relaxations evoked by field stimulation at 1 Hz, and significantly attenuated the amplitude and duration of electrically-evoked urethral relaxations at frequencies of 2 Hz and above ( $n = 4$ ; Figures 4.2 and 4.3; Tables 4.1 and 4.2). Furthermore, contractile responses to field stimulation were now observed at all frequencies of electrical stimulation, and those at higher frequencies of stimulation (32 and 64 Hz), that were present in control frequency-response curves, were increased by  $100.1 \pm 31.6 \%$  and  $148.3 \pm 41.9 \%$ , respectively ( $n = 4$ ; Figure 4.2). A higher concentration of L-NAME (300  $\mu$ M) abolished the urethral relaxations evoked by field stimulation at 1 and 2 Hz, and significantly attenuated the amplitude and duration of electrically-evoked urethral relaxations at frequencies of 4 Hz and above ( $n = 4$ ; Figures 4.2 and 4.3; Tables 4.1 and 4.2). The degree of inhibition produced by this concentration of L-NAME was not significantly different to that observed

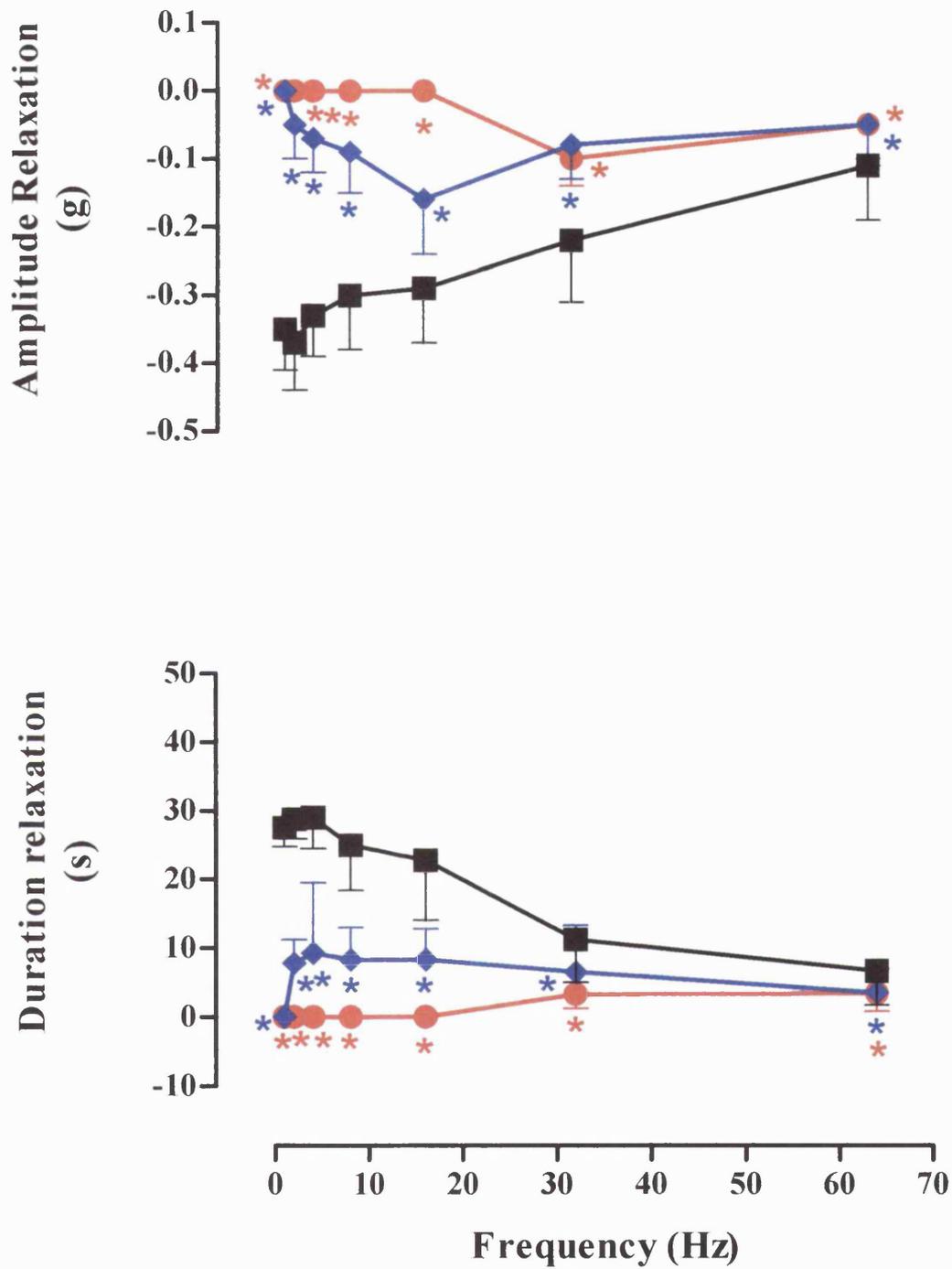
following 100  $\mu$ M L-NAME treatment. Furthermore, contractile responses to field stimulation were now observed at all frequencies of stimulation, and those at higher frequencies (32 and 64 Hz), that were observed in control frequency-response curves, were increased by  $370.1 \pm 43.2 \%$  and  $548.7 \pm 64.2\%$ , respectively ( $n = 4$ ; Figure 4.2). Both concentrations of L-NAME (100 and 300  $\mu$ M) caused a significant increase in baseline urethral tension of  $17.4 \pm 11.5 \%$  ( $0.14 \pm 0.06$  g) and  $11.6 \pm 3.5 \%$  ( $0.11 \pm 0.02$  g), respectively, after 10 min ( $n = 8$ ; Figure 4.4). The degree to which baseline urethral tension was increased by L-NAME treatment was not significantly different between the two concentrations of this drug tested.

#### **Effects of L-NA on electrical field stimulation-evoked urethral responses and baseline urethral tensions**

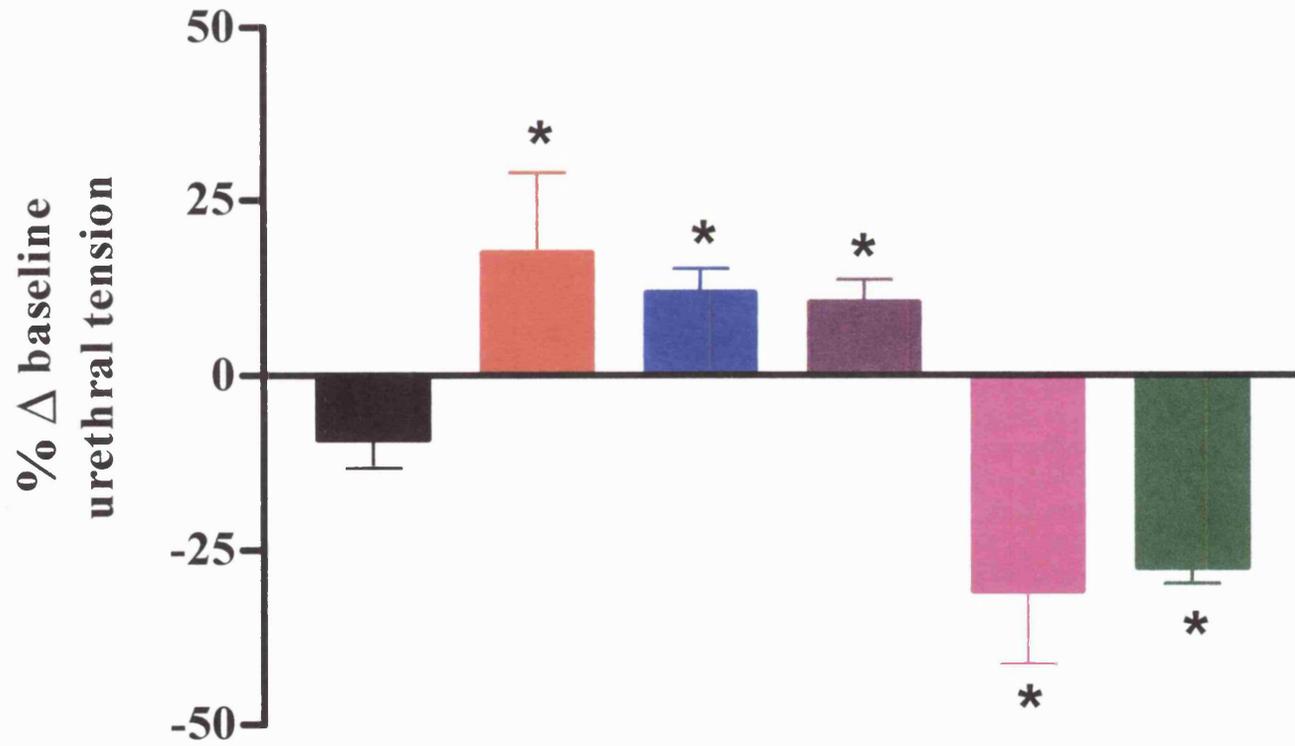
L-NA (100  $\mu$ M) abolished electrically-evoked urethral relaxations at frequencies of 1 - 16 Hz, and significantly attenuated the amplitude and duration of relaxations evoked at 32 and 64 Hz ( $n = 4$ ; Figures 4.2 and 4.3; Tables 4.1 and 4.2). Furthermore, urethral contractions were now evoked at all frequencies of electrical stimulation, and those evoked at higher frequencies (64 Hz) that were observed in control curves, were increased by  $527.5 \pm 37.2 \%$  ( $n = 4$ ; Figure 4.2). L-NA caused a significant increase in baseline urethral tension of  $10.3 \pm 3.2 \%$  ( $0.12 \pm 0.05$  g) after 10 min ( $n = 4$ ; Figure 4.4).



**Figure 4.2.** Traces showing frequency-response curves to electrical field stimulation in the isolated female rat urethra following incubation of tissues with vehicle (A), L-NAME (100 μM, B and 300 μM, C), or L-NA (100 μM; D).



**Figure 4.3.** Amplitude and duration of electrically-evoked relaxations of the isolated female rat urethra following incubation of tissues with vehicle (■), L-NAME (100 μM; ◆) or L-NA (100 μM; ●). All points are mean ± s.e.mean of 4-8 experiments. \* P<0.05, based on percentage changes in urethral relaxations following L-NAME (\*) or L-NA (\*), compared with vehicle controls.



**Figure 4.4.** Percentage changes in baseline urethral tension following administration of vehicle (■), L-NAME (100 μM; ■ and 300 μM; ■), L-NA (100 μM; ■) and zaprinast (100 μM; ■ and 300 μM; ■) in the isolated female rat urethra. \* P<0.05 as compared with vehicle controls.

Treatment	n	Frequency (Hz)						
		1	2	4	8	16	32	64
Vehicle	8	-22.4 ± 8.8	-20.6 ± 11.4	-32.9 ± 8.6	-38.2 ± 14.1	-36.1 ± 14.5	20.3 ± 10.2	-15.2 ± 9.8
L-NAME (100 µM)	4	-100 ± 0.0 *	-89.5 ± 12.1 *	-77.2 ± 16.2 *	-70.9 ± 19.8 *	-45.9 ± 27.7 *	-74.6 ± 17.2 *	-79.3 ± 24.0 *
L-NAME (300 µM)	4	-100 ± 0.0 *	-100 ± 0.0 *	-81.1 ± 26.8 *	-55.3 ± 4.0 *	-55.6 ± 0.1 *	-41.8 ± 17.2 *	-73.6 ± 37.3 *
L-NA (100 µM)	4	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-79.5 ± 25.1 *	-91.0 ± 11.1 *
Zaprinast (100 µM)	4	-49.9 ± 26.2	-56.1 ± 22.1	-56.9 ± 20.7	-33.7 ± 14.3	-46.4 ± 10.5	-54.1 ± 23.4	-42.0 ± 5.6
Zaprinast (300 µM)	4	-72.0 ± 19.0 *	-83.6 ± 19.0 *	-87.4 ± 14.5 *	-86.8 ± 15.2 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *

**Table 4.1.** Percentage changes in the amplitude (g) of electrically-evoked urethral relaxations following administration of test substances. Vehicles for NOS inhibitors and zaprinast were grouped together as they had no effect on these responses.  
\* P<0.05 compared with vehicle controls by unpaired Student's t-test. All values are mean ± s.e.mean.

Treatment	n	Frequency (Hz)						
		1	2	4	8	16	32	64
Vehicle	8	4.8 ± 18.9	-8.9 ± 15.3	-24.0 ± 10.2	-25.4 ± 14.9	-26.7 ± 19.1	-22.8 ± 14.3	-22.4 ± 12.9
L-NAME (100 μM)	4	-100 ± 0.0 *	-80.1 ± 22.9 *	-56.1 ± 9.5 *	-65.1 ± 30.1 *	-55.7 ± 24.9 *	-76.6 ± 16.0 *	-79.8 ± 23.3 *
L-NAME (300 μM)	4	-100 ± 0.0 *	-100 ± 0.0 *	-59.4 ± 24.6 *	-40.3 ± 2.0 *	-64.3 ± 7.4 *	-65.4 ± 11.3 *	-76.2 ± 18.4 *
L-NA (100 μM)	4	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *	-95.8 ± 5.1 *	-86.3 ± 16.8 *
Zaprinast (100 μM)	4	-23.2 ± 15.6	-28.1 ± 10.2	-25.5 ± 6.5	-14.7 ± 6.4	-15.1 ± 8.5	-30.1 ± 12.5	-28.4 ± 9.8
Zaprinast (300 μM)	4	-58.3 ± 28.2 *	-80.1 ± 22.9 *	-84.2 ± 18.2 *	-82.9 ± 19.7 *	-100 ± 0.0 *	-100 ± 0.0 *	-100 ± 0.0 *

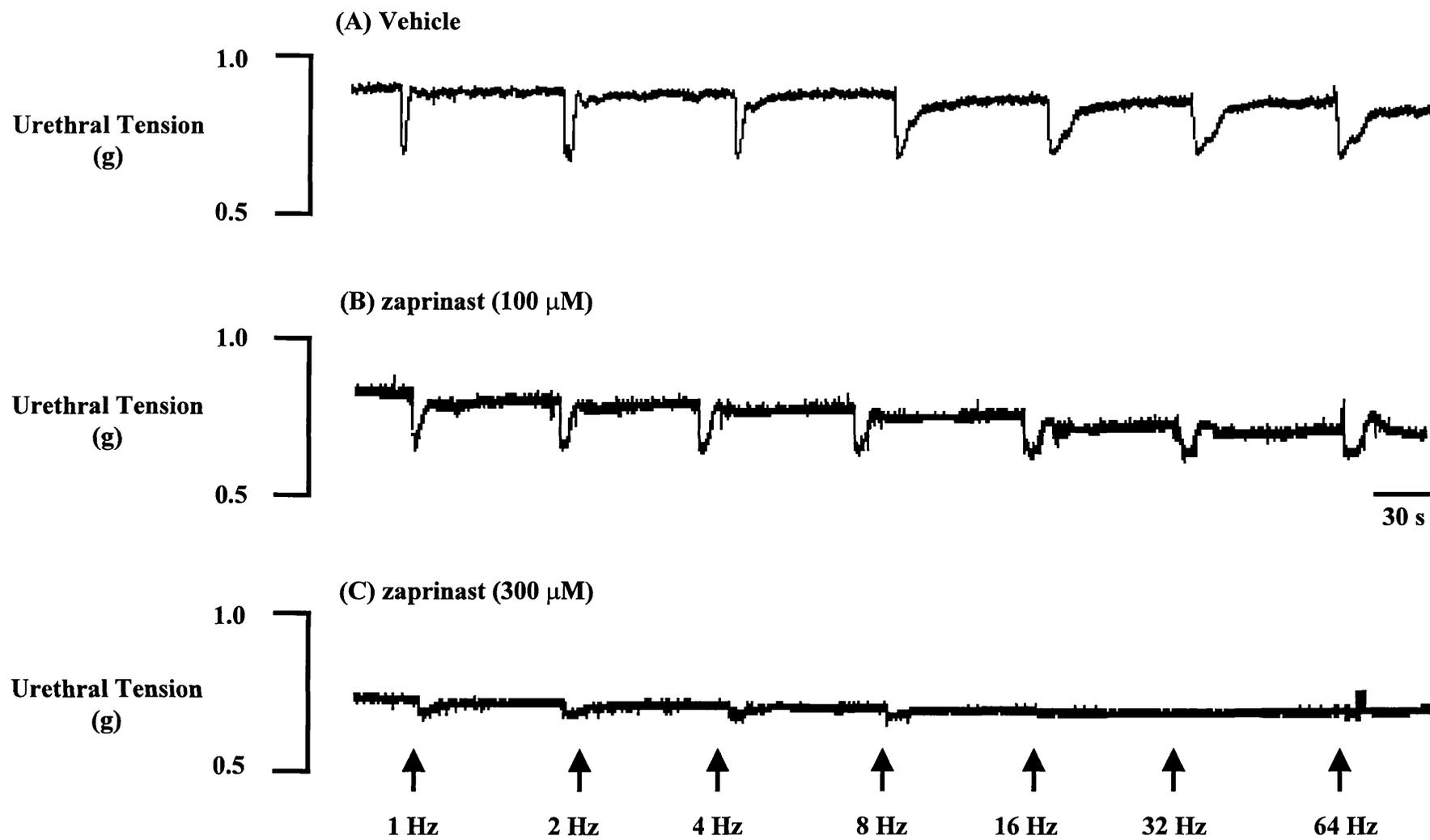
**Table 4.2.** Percentage changes in the duration (s) of electrically-evoked urethral relaxations following administration of test substances.

Vehicles for NOS inhibitors and zaprinast were grouped together as they had no effect on these responses.

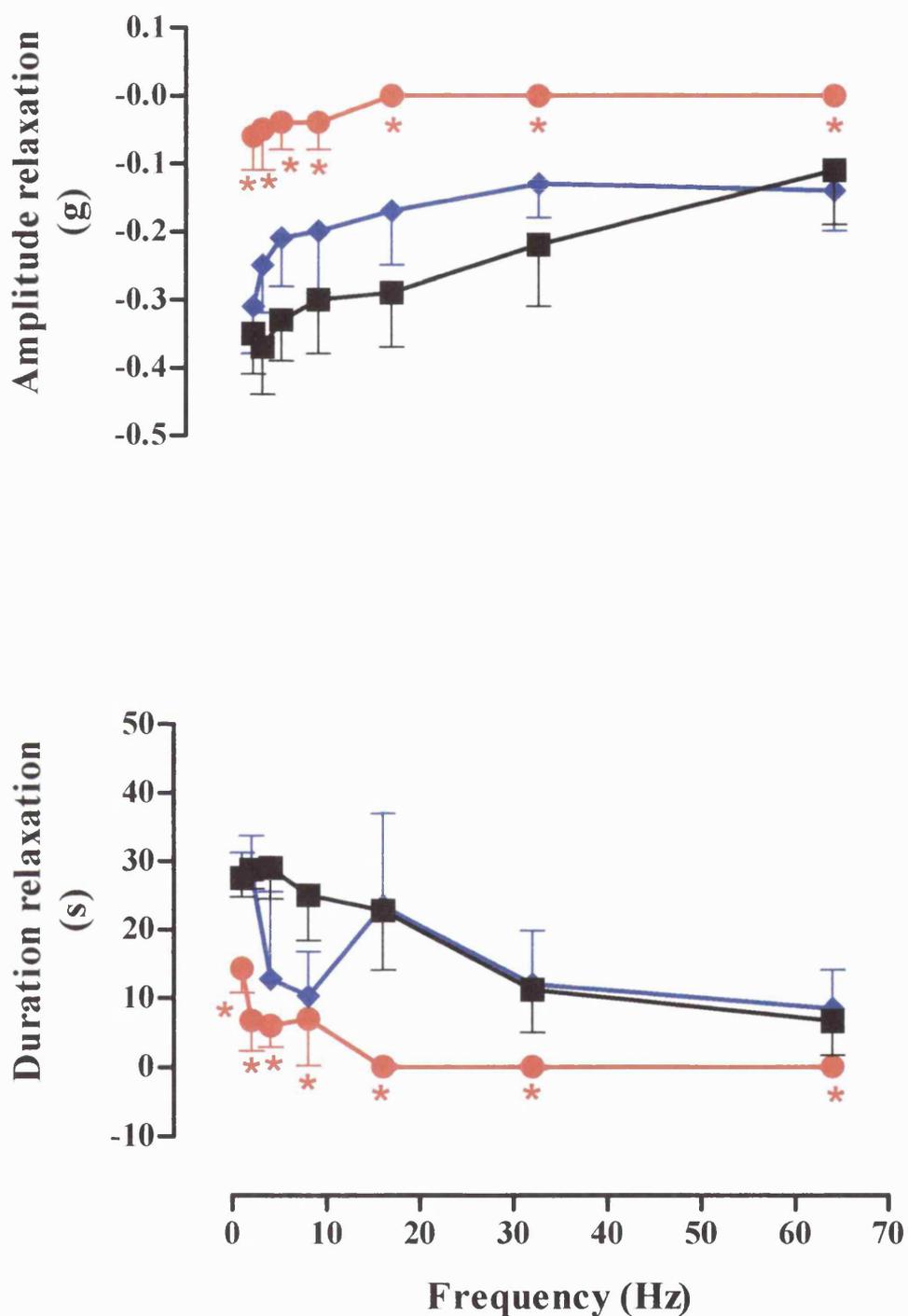
\* P<0.05 compared with vehicle controls by unpaired Student's t-test. All values are mean ± s.e.mean.

### **Effects of zaprinast on EFS-evoked urethral responses and baseline urethral tensions**

Zaprinast (100  $\mu$ M) had no effect on the amplitude and duration of electrically-evoked urethral relaxations at all frequencies of stimulation, although these responses were reduced, albeit not significantly ( $n = 4$ ; Figures 4.5 and 4.6; Tables 4.1 and 4.2). Furthermore, zaprinast had no effect on the contractile urethral responses to high stimulatory frequencies (32 and 64 Hz) that were observed in control frequency-response curves. Zaprinast (300  $\mu$ M) significantly attenuated the amplitude and duration of electrically-evoked relaxations of the female rat urethra at frequencies of 1-8 Hz, and abolished these responses at frequencies of 16 Hz and above ( $n = 4$ ; Figures 4.5 and 4.6; Tables 4.1 and 4.2). Contractile responses evoked by field stimulation in control frequency-response curves (32 and 64 Hz) were unaffected by zaprinast ( $n = 3$ ). Both concentrations of zaprinast (100 and 300  $\mu$ M) caused a significant decrease in baseline urethral tension of  $30.8 \pm 10.6 \%$  ( $0.31 \pm 0.15$  g) and  $27.5 \pm 2.41 \%$  ( $0.18 \pm 0.02$  g), respectively, after 10 min ( $n = 8$ ; Figure 4.4).



**Figure 4.5.** Traces showing frequency-response curves to electrical field stimulation in the isolated female rat urethra following incubation of tissues with vehicle (A) or zaprinast (100  $\mu$ M, B and 300  $\mu$ M, C).

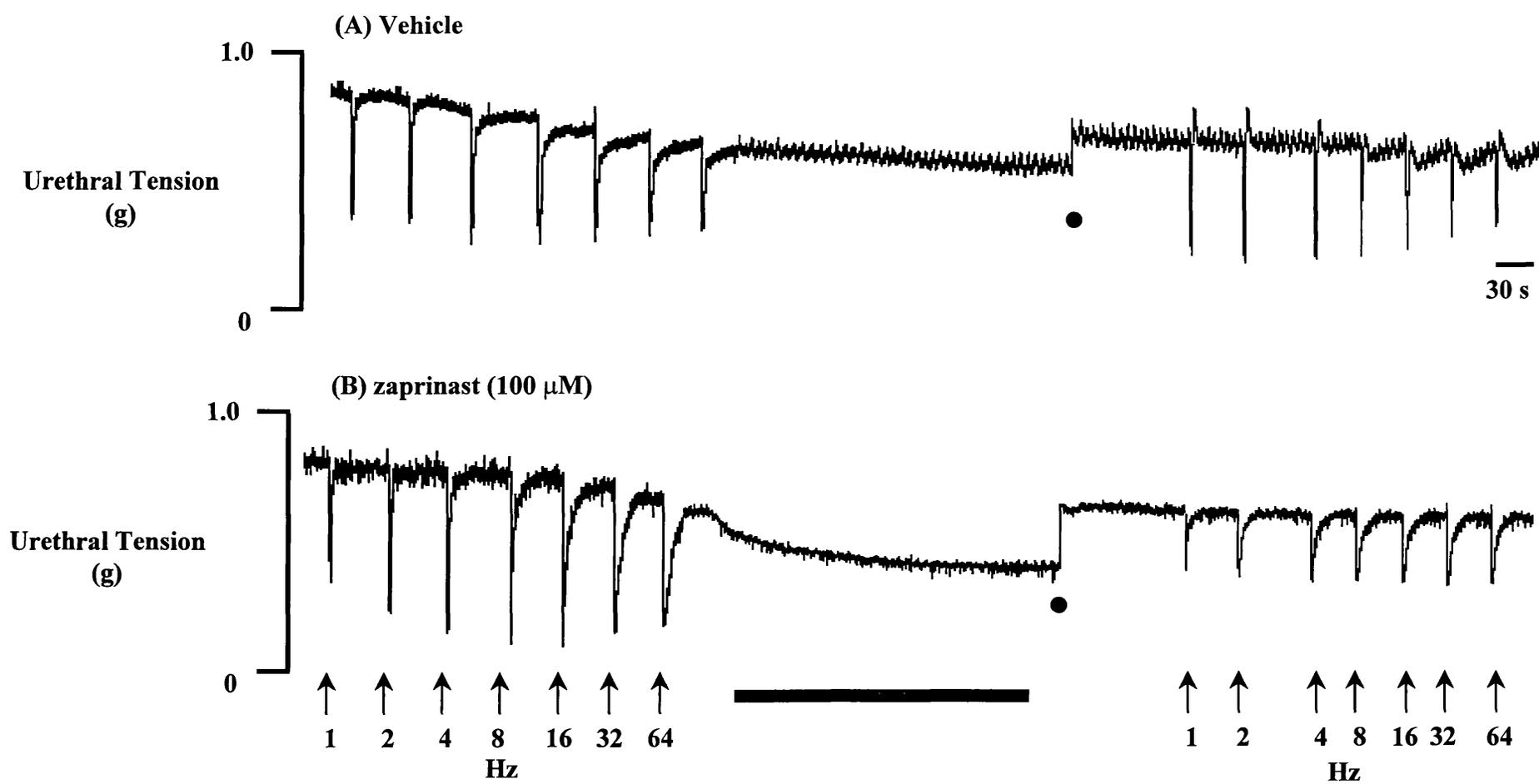


**Figure 4.6.** Amplitude and duration of electrically-evoked relaxations of the isolated female rat urethra following incubation of tissues with vehicle (■) or zaprinast (100  $\mu$ M; ◆ and 300  $\mu$ M; ●). All points are mean  $\pm$  s.e.mean of 4-8 experiments. \*  $P < 0.05$ , based on percentage changes in urethral relaxations following zaprinast (300  $\mu$ M) compared with vehicle controls.

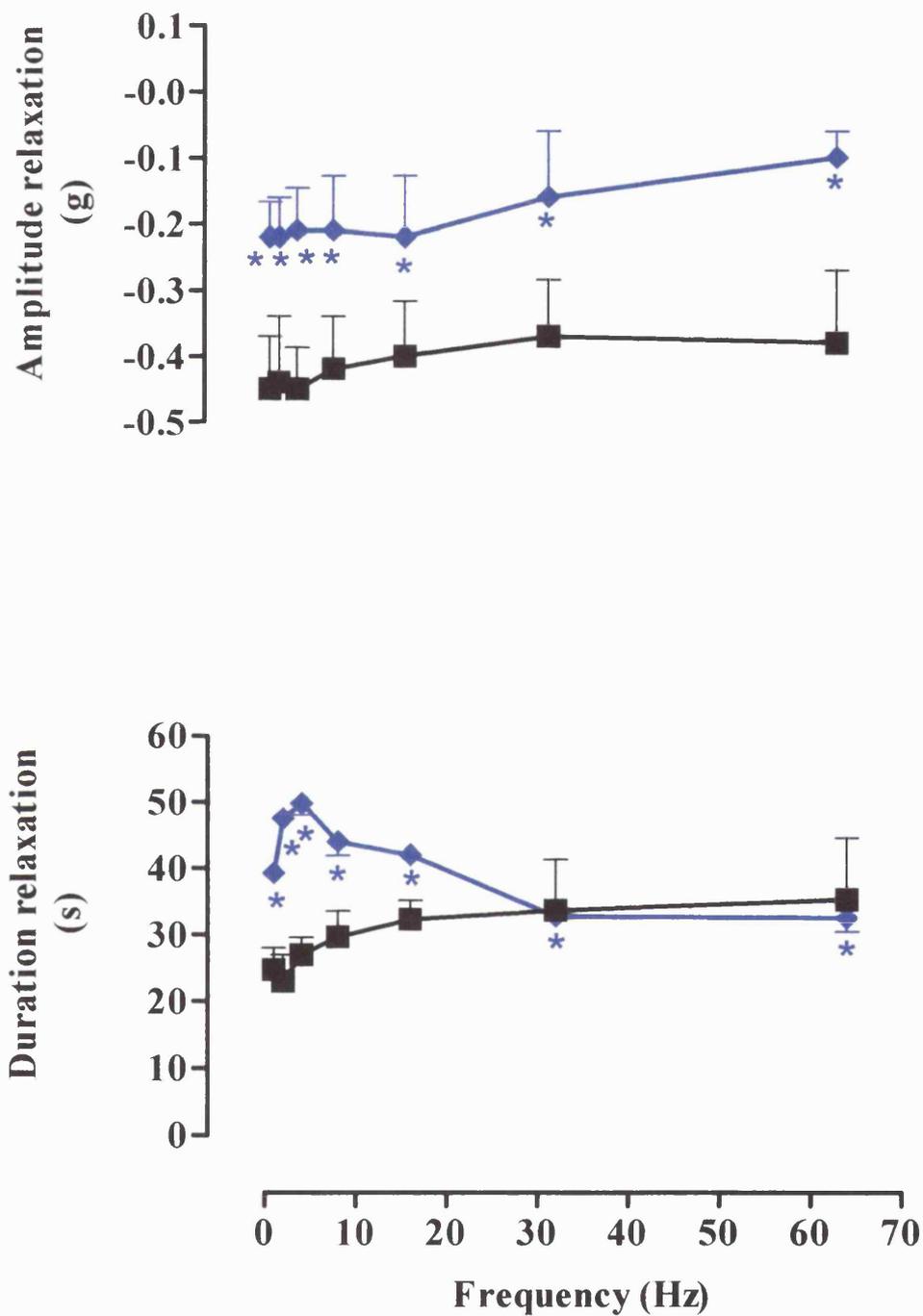
**Effects of zaprinast on electrical field stimulation-evoked responses following 'compensation' of changes in baseline urethral tension**

Following the observation that zaprinast decreased baseline urethral tensions and attenuated electrically-evoked urethral relaxations, experiments were performed to investigate if potentiations of electrically-evoked relaxations occurred if these effects on resting tensions were 'compensated' for. In this respect, Williams & Parsons (1995) also reported a decrease in tone following cGMP-selective PDE inhibition in isolated gastrointestinal tissues, but found they could compensate for these effects by re-contracting the tissues with carbachol, a muscarinic agonist, following incubation with antagonists. These authors were then able to demonstrate a potentiation of EFS-evoked NO-mediated relaxations following treatment with zaprinast and the selective PDE 5 inhibitor, SKF 96231, in these tissues. Therefore, this approach was attempted in the present experiments. However, it was found that carbachol evoked a relaxation of the isolated female rat urethra, possibly via the activation of neuronal nicotinic receptors, that have previously been identified in this tissue (data not shown, see chapter five). Furthermore, attempts to contract the urethral tissues with phenylephrine, a selective  $\alpha_1$ -adrenoceptor agonist also proved unsuccessful, as the maximal contraction produced by this agent was not sufficient to overcome the decrease in urethral tension following zaprinast treatment (data not shown). However, experiments were performed whereby tissues were 'manually' re-tensioned to 1g pre-drug levels following incubation with zaprinast or vehicle, before second frequency-response curves were constructed. Tissues were allowed to equilibrate

at re-tensioned levels for a period of 10 min. In these experiments, zaprinast (n = 4; 100  $\mu$ M) significantly attenuated the amplitude of electrically-evoked urethral relaxations at all frequencies of stimulation (Figures 4.7 and 4.8; Table 4.3). Furthermore, these effects of zaprinast were not significantly different from experiments in which tissues were not re-tensioned following incubation with this agent. However, zaprinast significantly potentiated the duration of field stimulation-evoked urethral relaxations at 2, 4, 8 and 16 Hz (Figures 4.7 and 4.8; Table 4.4). These effects of zaprinast were not observed in experiments in which tissues were not re-tensioned. Zaprinast potentiated electrically-evoked urethral relaxations at 1 Hz, although these effects were not significant. At higher frequencies of stimulation (32 and 64 Hz), zaprinast had no effect on field stimulation-evoked urethral relaxations (Figures 4.7 and 4.8; Tables 4.3 and 4.4).



**Figure 4.7.** Traces showing frequency-response curves to electrical stimulation in the isolated female rat urethra before and after the incubation of tissues with vehicle (A) and zaprinast (100 μM; B) in tissues that were re-tensioned to pre-drug urethral tensions (indicated by ●) after incubation with test substances. Shaded bar represents period of incubation of tissues with vehicle or zaprinast.



**Figure 4.8.** Amplitude and duration of electrically-evoked relaxations of the isolated female rat urethra following incubation of tissues with vehicle (■) or zaprinast (100  $\mu$ M; ◆) in tissues that were re-tensioned to pre-drug levels. Each point represents mean  $\pm$  s.e.mean of 4 experiments. \*  $P < 0.05$ , based on percentage changes in urethral relaxations following zaprinast compared with vehicle controls.

Treatment	n	Frequency (Hz)						
		1	2	4	8	16	32	64
Vehicle	4	1.2 ± 2.6	-15.0 ± 7.6	-17.2 ± 6.3	-22.5 ± 5.4	-27.1 ± 3.5	-24.9 ± 8.9	-19.1 ± 6.7
Zaprinast (100 µM)	4	-54.6 ± 15.6 *	-57.8 ± 13.1 *	-58.7 ± 12.8 *	-59.8 ± 15.1 *	-51.9 ± 21.1 *	-64.1 ± 21.9 *	-75.9 ± 14.6 *

**Table 4.3.** Percentage changes in the amplitude (g) of electrically-evoked urethral relaxations following administration of zaprinast in tissues that were re-tensioned following incubation with antagonist.

\* P<0.05 compared with vehicle controls by unpaired Students t-test. All values are mean ± s.e.mean.

Treatment	n	Frequency (Hz)						
		1	2	4	8	16	32	64
Vehicle	4	-5.1 ± 3.2	-17.1 ± 4.6	-12.0 ± 2.4	-10.3 ± 4.5	-19.9 ± 13.5	-23.0 ± 12.5	-26.4 ± 6.7
Zaprinast (100 µM)	4	14.7 ± 4.5	31.0 ± 7.8 *	21.5 ± 4.7 *	17.1 ± 3.2 *	9.7 ± 2.1 *	-30.5 ± 2.5	-36.9 ± 24.3

**Table 4.4.** Percentage changes in the duration (s) of electrically-evoked urethral relaxations following administration of zaprinast in tissues that were re-tensioned following incubation with antagonist.

\* P<0.05 compared with vehicle controls by unpaired Students t-test. All values are mean ± s.e.mean.

## DISCUSSION

### *Effects of NOS inhibition on electrical field stimulation-evoked responses*

The present study has demonstrated that electrically-evoked relaxations of the isolated female rat urethra are mediated by NO as these responses were significantly attenuated by the NOS inhibitors L-NAME and L-NA. Interestingly, L-NA attenuated electrically-evoked urethral relaxations to a greater extent than L-NAME, especially at lower frequencies of stimulation. This may reflect differences in the diffusion of these agents within the various layers of the urethra (see below). These findings are in agreement with those of Persson *et al.* (1992), who demonstrated that L-NAME significantly attenuated field stimulation-evoked urethral relaxations of isolated rat longitudinal urethral preparations. Previous studies have also found that only a small proportion of female rat circular urethral preparations respond with relaxations when stimulated electrically. It has therefore been suggested that circularly orientated urethral preparations comprise mostly circular smooth and striated muscle components, and longitudinal smooth muscle is the major component of longitudinal urethral preparations (Andersson, Malmgren & Uvelius, 1990). The whole isolated female rat urethra used in the present studies comprises all these muscle types (see chapter three). This suggests that in the present study, field stimulation-evoked relaxations of the whole female rat urethra reflect NO mediated longitudinal urethral smooth muscle relaxation. Furthermore, these findings also correlate with *in vivo* investigations from both the present studies

(see chapter two) and other authors (Bennett *et al.*, 1995), showing that reflex-evoked urethral smooth muscle relaxations are mediated by NO in anaesthetised female rats.

Following the attenuation of electrically-evoked urethral relaxations with NOS inhibition, contractile urethral responses to low frequencies of electrical stimulation were now observed that were not present in control frequency-response curves. Furthermore, contractile urethral responses to high frequencies of stimulation were observed in a large proportion of control frequency-response curves. Interestingly, previous studies in this thesis have demonstrated a contractile urethral response to close arterial administration of DMPP, a ganglionic stimulant, following L-NAME treatment in the anaesthetised female rat (see chapter two). Furthermore, a contractile urethral response is also observed following intravenous administration of DMPP that is abolished following concomitant atropine and prazosin treatment, suggesting an involvement of acetylcholine and noradrenaline release in mediating these contractile DMPP-evoked responses (Wibberley, Naylor & Ramage, 1999). Indeed, the presence of  $\alpha_1$ -adrenoceptor- and muscarinic receptor-mediated contractile responses have been shown in the urethrae of several species (see Brading, 1999, and chapter one), suggesting that the electrically-evoked contractile urethral responses in the present studies observed following L-NAME treatment are mediated by similar mechanisms. In addition, contractile responses of the urethral striated musculature may also be involved in the effects of field stimulation on the whole isolated female rat urethra. This possibility is discussed

further in previous studies in this thesis (see chapter three). Further studies are therefore needed to investigate the exact mechanisms involved in these contractile responses.

*Effects of NOS inhibition on baseline urethral tensions: mechanisms of action*

In addition to effects on electrically-evoked urethral responses, both L-NAME and L-NA increased baseline urethral tension in the isolated female rat urethra. Since both these agents produced this effect, it is unlikely that previously reported effects of L-NAME on muscarinic receptors are involved (Buxton *et al.*, 1993). Previous studies have demonstrated that L-NAME increases baseline urethral tone and the activity of the urethral striated muscle in anaesthetised female rats (see chapter two). Furthermore, in neuromuscular-blocked animals, these increases in urethral tone were not observed, suggesting that they are the result of increased striated muscle activity. These results therefore suggest that NO acts as a 'breaking' mechanism to inhibit the activity of the urethral striated muscle. These mechanisms have been discussed further in chapter two. As these effects of NOS inhibition were observed in both *in vivo* and *in vitro* studies, this suggests that the effects of NO on urethral tone *in vivo* are mediated by a direct action of this drug on the urethra, and are not caused by changes in other factors affecting urethral tone, for example changes in blood pressure (Bump, Friedman & Copeland, 1988). Furthermore, the possibility exists that the ability of L-NAME and L-NA to increase urethral tone *in vitro* may also reflect effects on the urethral striated, and not smooth, musculature. Persson *et al.* (1992) have also

demonstrated an increase in baseline urethral tension in noradrenaline-contracted isolated female rat longitudinal urethral strips following L-NAME treatment. As these urethral preparations have been suggested to comprise mostly of longitudinal smooth muscle (Andersson, Malmgren & Uvelius, 1990), and the whole isolated female rat urethra used in the present studies comprises both these muscle types (see chapter three), this would support the view that the effects of L-NAME on the whole isolated female rat urethral preparation are mediated by longitudinal urethral smooth muscle. The possible roles of NO in the control of urethral smooth muscle tone are discussed further in later sections of this discussion. However, it is possible that isolated longitudinal urethral strips also contain striated muscle fibres, especially in light of the fact that these two muscle types are intermingled at the level of the urethral striated muscle (see Lincoln & Burnstock, 1993). Experiments investigating the effects of NOS inhibition on both female rat longitudinal and circular urethral preparations would prove useful in determining the exact site of action of the NOS-evoked increase in baseline urethral tension. Indeed, an effect of L-NAME on one, or more, other urethral components in addition to the urethral smooth and striated muscles, such as the lamina propria and urothelium, to evoke an increase in urethral tone cannot be excluded. These possibilities will be discussed with regard to the effects of zaprinast in later sections of this discussion.

*Effects of NOS inhibition on baseline urethral tension: physiological roles*

Interestingly, Persson *et al.* (1992) have noted that L-NAME has no effect on baseline urethral tension when applied to non-contracted preparations. This suggests that NO synthesis contributes to tension in the urethra. In this respect, it may be speculated that the functional role of NO is to prevent excessive increases in urethral tone during periods of increased tension. The proposed functional role of NO in the regulation of urethral tone may have a similar mechanism of action to the well established NO-mediated vasodilator tone in the cardiovascular system (see Moncada, Palmer & Higgs, 1991). The physiological stimuli for the generation of NO in this system include pulsatile flow (Pohl *et al.*, 1986) and shear stress (Rubanyi, Romero & Vanhoutte, 1986), both of which increase tensions in cardiovascular tissues. Furthermore, Griffith & Edwards (1990) have also shown that the activity of endogenous NO is greatest in large arterioles in which hydraulic resistance and shear stress are highest. Similarly, the actions of NO in the control of urethral tone *in vivo* may also involve a 'breaking' action on the urethral striated muscle that have previously been demonstrated (see chapter two).

*Effects of zaprinast on electrical field stimulation-evoked responses*

The effects of NO in various physiological systems are believed to be mediated by activation of guanylate cyclase, resulting in increased formation of cGMP. The biological effects of cGMP are dependent not only on its rate of formation,

but also on its rate of breakdown, which is fulfilled by a family of PDE isoenzymes (see Beavo, 1995). In particular, PDE 5 has been shown to fulfil this role in a number of biological systems (see Beavo, 1995; Yanaka *et al.*, 1998). A role for cGMP in NO-mediated urethral smooth muscle relaxations has been suggested following the observation that cGMP-immunoreactivity is present in the smooth muscle of the guinea pig and human urethra (Smet *et al.*, 1996). Spindle-shaped cGMP-immunoreactive cells have also been shown to form a network around and between the smooth muscle bundles of the rabbit urethra (Waldeck *et al.*, 1998). In addition, zaprinast has been shown to potentiate electrically-evoked urethral relaxations (Dokita *et al.*, 1991) and increases in cGMP levels (Persson & Andersson, 1994) in the isolated rabbit urethra. Therefore, in the present experiments, inhibitors of PDE 5 would be expected to increase the availability of cGMP and so potentiate NO-mediated urethral relaxant responses evoked by electrical field stimulation. Surprisingly, zaprinast attenuated, albeit not significantly, electrically-evoked urethral relaxations at low concentrations, although zaprinast at this concentration, decreased urethral tone, showing that it was having an effect on this preparation. Experiments using a higher concentration of zaprinast, demonstrated that electrically-evoked urethral relaxations were significantly attenuated both in amplitude and duration. These surprising effects of supposed PDE inhibition by zaprinast on the isolated female rat urethra could be related to the effects of zaprinast on baseline urethral tensions. Indeed, it is possible that any potentiating effects of zaprinast on NO-mediated urethral relaxations are masked by the reduction in urethral tone that this agent produced. In this manner, electrical field stimulation may not evoke

relaxant responses because the tissues have reached a maximal level of relaxation. A reduction in tone following inhibition of cGMP-selective PDEs has also been reported in isolated genital (Cellek & Moncada, 1998) and gastrointestinal (Williams & Parsons, 1995) smooth muscles. In gastrointestinal preparations, these authors employed a different experimental design to overcome these problems. This involved conducting initial frequency-response curves in carbachol-contracted tissues, washing out the carbachol, incubating with PDE inhibitors, recontracting the tissues and then constructing further frequency-response curves. In this way, these authors were able to demonstrate a potentiation of NO-mediated EFS-evoked relaxations in gastrointestinal tissues following treatment with zaprinast and the selective PDE 5 inhibitor SKF 96321. However, preliminary experiments using this experimental approach in the present studies found that carbachol evoked a relaxation of female rat urethral preparations, most likely through the activation of neuronal nicotinic receptors that have been identified in this tissue (see chapter five). Furthermore, the use of phenylephrine, a selective  $\alpha_1$ -adrenoceptor agonist, also proved unsuccessful, as the degree to which this agonist contracted urethral preparations was insufficient to compensate for the zaprinast-evoked decrease in urethral tone. However, in an attempt to demonstrate a clear potentiating effect of zaprinast on NO-mediated relaxations in the female rat urethra, experiments were performed whereby following the administration of zaprinast, tissues were 'manually' re-tensioned to pre-drug levels in an attempt to 'compensate' for the relaxant effects of this agents. Using this approach, a potentiation of the duration of electrically-evoked urethral relaxations could now be demonstrated following zaprinast treatment.

Therefore, it appears that the decrease in urethral tone caused by zaprinast could be masking any potentiating effects of NO-mediated responses. Additionally, *in vivo* studies (see chapter two) have also demonstrated an attenuation of reflex-evoked urethral relaxations following zaprinast treatment in the presence of  $\alpha$ -Bungarotoxin. A zaprinast-evoked decrease in baseline urethral tone was also thought to be present in these experiments, suggesting that these changes could also mask possible potentiations of reflex-evoked urethral smooth muscle relaxations *in vivo* (see below). Surprisingly, a potentiation of the amplitude of electrically-evoked urethral relaxations could not be demonstrated following treatment with zaprinast, despite this revised experimental approach. However, it is possible that the relaxations induced by electrical field stimulation are maximal and therefore potentiations of these responses may not be observed. In this respect, relaxations of the urethra evoked at 1, 2 and 4 Hz (where no contractions of the urethra were observed) were not frequency-dependent and were roughly of the same magnitude. Conversely, the duration of electrically-evoked relaxations were frequency-dependent at these stimulation parameters, suggesting that these responses are not maximal, and can therefore be potentiated following inhibition of PDE isoenzymes. Measurement of cGMP levels in the urethra may provide more definitive evidence for a role for this transduction system in NO-mediated relaxations of the female rat urethra. Indeed, Barnette *et al.* (1993) have demonstrated an increase in cGMP levels but no potentiation of the relaxant response in the canine colon following PDE inhibition.

*Effects of zaprinast on baseline urethral tension: mechanisms of action and physiological roles*

The reduction in urethral tone following zaprinast treatment may reflect effects on a number of urethral components. The ability of cGMP related drugs (e.g. 8-bromo-cyclic GMP) to relax isolated rabbit smooth muscle urethral preparations (Persson & Andersson, 1994), and the abundance of PDE 5 isoenzymes in a variety of smooth muscles (Yanaka *et al.*, 1998), including the corpus cavernosum (Kuthe *et al.*, 2000), suggests that zaprinast reduces urethral tone *in vitro* via an effect on the urethral smooth musculature. Indeed, an effect of zaprinast to decrease tone via an action on the urethral smooth muscle has also been also suggested from the results of *in vivo* studies (see chapter two). Furthermore, this effect of zaprinast on smooth muscle tone has significant implications for the contributions of both the smooth and striated urethral muscles in the regulation of overall urethral tone *in vivo*. Indeed, the ability of zaprinast, in addition to SNP and isoprenaline, to relax urethral smooth muscle in the anaesthetised female rat, may activate reflex mechanisms to produce an increase in urethral striated muscle activity and thereby maintain urethral tone. These reflex interactions and their implications for the complex regulation of urethral tone, and thereby continence, are discussed in further detail in chapter two. Aside from the implications of these findings to the complex reflex interactions between the urethral smooth and striated muscles *in vivo*, these *in vitro* results also raise the possibility that PDEs play a role in the maintenance of baseline urethral smooth muscle tone. Indeed, the fact that PDE action can be

modified by changes in protein concentration or by the modification of enzymatic activity by phosphorylation and dephosphorylation mechanisms, suggests that PDE regulation may be involved in the physiological control of the biological actions of cyclic nucleotides, including smooth muscle tone (Carvajal *et al.*, 2000). If cGMP does play a physiological role in the control of urethral smooth muscle tone, then it is likely that the 'activator' of cGMP levels in this tissue is NO. Indeed, since NO evokes a relaxation of the urethral smooth muscle during voiding, and NO donors such as SNP have been shown to relax isolated rabbit (Persson & Andersson, 1994) and sheep (Garcia-Pascual *et al.*, 1999) urethral smooth muscle, this raises the possibility that NO/cGMP in the urethral smooth muscle may contribute to the inhibitory role of NO during periods of increased urethral tension, as discussed earlier. Thus, in these tensioned isolated female rat urethral preparations, NO is tonically released to the urethral smooth muscle to prevent excessive increases in tension. Similarly, inhibitors of NOS will prevent the synthesis of NO and increase urethral smooth muscle tone, and PDE inhibitors will augment this mechanism, and conversely decrease urethral smooth muscle tone. However, L-NAME has no effect on baseline urethral smooth muscle tone *in vivo*, and it remains to be determined if NO-mediated cGMP signalling possesses a physiological role in the control of smooth muscle tone. Indeed, the presence of additional mechanisms controlling urethral tone *in vivo*, for example the presence of a large number of neurotransmitters (see Steers, 1995) and an extensive vascular plexus (Bump, Friedman & Copeland, 1988), may mask the effects of NO on smooth muscle

tone. Further, the inhibitory effects of NO on urethral striated muscle activity may be more important in the control of urethral tone *in vivo*.

An effect of zaprinast on urethral smooth muscle tone by a mechanism of action other than PDE inhibition cannot be excluded. Indeed, caffeine, which is also a PDE inhibitor, has been reported to lower urethral pressure in the anaesthetised female dog (Palermo & Zimskind, 1977). These authors suggested that the mechanism of action of this agent may involve the mobilisation of  $\text{Ca}^{2+}$  ions from the urethral smooth muscle membrane, which increases  $\text{Na}^{2+}$  permeability, leading to an inactivation of the contractile mechanism of this musculature. Furthermore, recently, caffeine has been shown to inhibit ATP-sensitive  $\text{K}^{+}$  channels in pig urethral smooth muscle cells, by a mechanism independent of PDE inhibition or  $\text{Ca}^{2+}$  release (Teramoto *et al.*, 2000). These authors suggested that these effects were most likely effected by a direct action of caffeine on these channels, raising the possibility that zaprinast may also lower smooth muscle tone by these mechanisms. These possibilities require further investigation.

In addition to effects on urethral smooth and striated muscles, it is also possible that some, or all, of the effects of manipulation of the NO/cGMP pathway on baseline urethral tensions are mediated by changes in the tone of the urethral lamina propria. The lamina propria constitutes the innermost layers of the urethra between the urethral lumen and urethral smooth muscle, and comprises connective tissue and extensive vasculature (see Brading, 1999). The blood supply to the lamina propria has been suggested to contribute to pressure

generation within the urethra (Rud *et al.*, 1980), and to the maintenance of urethral tone, and thereby continence, by contributing to the 'inner urethral softness' (Zinner, Sterling & Ritter, 1980). Zygmunt *et al.* (1993) have found that isolated lamina propria preparations respond to electrical field stimulation with NO-mediated frequency-dependent relaxations in the female rabbit. Although these authors did not examine the effects of L-NAME on pre-contracted lamina propria preparations, an increase in baseline tension following incubation with methylene blue (MB), an inhibitor of soluble guanylate cyclase (Martin, 1985), was observed in this tissue. Pharmacologically, both L-NAME and MB act to inhibit the effects of NO and it is therefore possible that the ability of L-NAME to increase baseline urethral tension involves effects on this urethral component, in addition to effects on urethral striated and /or smooth muscle. However, the results studies employing methylene blue should be interpreted with caution, as in addition to an inhibition of guanylate cyclase, this agent has also been shown to inhibit muscarinic receptors (Abi-Gerges *et al.*, 1997a) and Ca<sup>2+</sup> channels (Abi-Gerges *et al.*, 1997b) in frog cardiac myocytes.

A contribution of the urothelium in the effects of L-NAME, L-NA and zaprinast in the present study must also be considered. Removal of the urothelium from hamster urethral preparations has been shown to reduce relaxations in response to electrical stimulation (Pinna *et al.*, 1996). Furthermore, these authors have since demonstrated the presence of NOS immunoreactivity in the urothelial layer of this tissue (Pinna *et al.*, 1999). However, the contribution of the urothelium in the effects of L-NAME, L-NA and zaprinast on baseline urethral tension has not

been investigated, and it therefore remains a distinct possibility that the effects observed in the present study reflect effects on this urethral layer. In this respect, it is possible that the greater degree of inhibition of electrically-evoked urethral relaxations by L-NA compared with L-NAME observed in the present experiments may reflect differences in the diffusion of these agents to the lamina propria and/or smooth muscle layers. Indeed, recently, Jung *et al.* (1999) have demonstrated that intraurethral perfusion of saline and NO donors affect reflex-evoked bladder activity via the activation of urethral afferents in the anaesthetised female rat. However, these authors found that intraurethral administration of L-NAME had no effect on reflex-evoked bladder activity, and suggested that this was due to insufficient penetration of this agent into the urethral layers that contain the nerve endings of these afferents, for example in the urothelium.

### *Conclusions*

In conclusion, the present study has demonstrated that electrically-evoked urethral relaxations are mediated by NO. In addition, it appears that these responses are mediated by increases in cGMP levels as potentiations of the duration of these responses were observed following zaprinast treatment. However, these effects were only observed in tissues where the relaxatory effects of this agent on baseline urethral tone were 'compensated' for. The effects of zaprinast on urethral tone therefore presents as a complicating factor in the interpretation of results on the effects of this agent on electrically-evoked urethral

responses, and has implications for the effects of zaprinast on reflex-evoked urethral responses *in vivo*. Further, these effects have significant implications for the reflex interactions and contributions of both the smooth and striated urethral muscles in the maintenance of urethral tone, and thereby continence, *in vivo*.

## **Chapter Five**

### **Effects of Nicotinic Agonists on the**

### **Female Rat Urethra *in vitro***

## INTRODUCTION

Nicotinic receptors are a family of ligand-gated ion channels whose opening is controlled by acetylcholine and nicotinic receptor agonists (see Clementi, Fornasari & Gotti, 2000 and Lloyd & Williams, 2000). The nicotinic receptor family have been subdivided on the basis of their subunit composition. Neuronal nicotinic receptors consist of both heteromeric and homomeric types, the former comprising pair wise combinations of  $\alpha 2$ -,  $\alpha 3$ -,  $\alpha 4$  and  $\alpha 6$ -subunits with  $\beta 2$ - or  $\beta 4$ -subunits, and the latter consisting of  $\alpha 7$ -,  $\alpha 8$ - or  $\alpha 9$ -subunits (Linstrom, 1997). Furthermore, neuromuscular nicotinic receptors consist of pairs of  $\alpha 1$ -subunits and one each of  $\beta 1$ -,  $\gamma$ - and  $\delta$ -subunits. Neuronal nicotinic receptors have a broad tissue distribution, especially in the peripheral and central nervous systems. Most importantly, these receptors are present in autonomic ganglia, where they mediate synaptic transmission. Neuromuscular nicotinic receptors are extensively distributed in skeletal musculature where they mediate contraction in response to somatic stimulation.

Previous studies in this thesis (see chapter two) have demonstrated an increase in urethral striated muscle activity following intraurethral administration of SNP and isoprenaline in the anaesthetised female rat. These complex interactions between the urethral smooth and striated muscles were not observed following pre-treatment with chlorisondamine, an antagonist of neuronal nicotinic receptors (Plummer *et al.*, 1955), suggesting an involvement of reflex mechanisms via the activation of these receptors. Chlorisondamine is a centrally acting and

ganglionic neuronal nicotinic receptor antagonist (Clarke *et al.*, 1994), and it is therefore possible that these reflexes involve activation of these receptors at spinal and supraspinal sites. Moreover, these receptors mediate ganglionic transmission in the pelvic plexus, a bilateral association of neurones and ganglia which lie at the base of the bladder (see Keast, 1999), raising the possibility that these reflexes are effected at this site. However, an involvement of neuronal nicotinic receptors at the level of the urethra cannot be ruled out. Furthermore, these receptors may comprise neuronal nicotinic receptors of the ganglionic subtype. In this respect, ganglia have been identified in the pig (Crowe & Burnstock, 1989) and human (Crowe, Burnstock & Light, 1988) urethra. The presence of these receptors in the urethra can be investigated using functional tissue studies *in vitro*. If neuronal nicotinic receptors are present in urethral tissue, agonists of these receptors would be expected to produce responses in the isolated female rat urethra. In addition, if these responses are mediated by activation of ganglionic nicotinic receptors, then they would be expected to be sensitive to tetrodotoxin (TTX), which inhibits the propagation of action potentials, and therefore prevents activation of postganglionic neurones following ganglionic nicotinic receptor stimulation (Narahashi, Moore & Scott, 1964). Therefore, the present study investigated the effects of nicotine, and the selective neuronal nicotinic receptor agonists 1,1-dimethylphenylpiperazinium (DMPP; see Broadley, 1996) and epibatidine (Gerzanich *et al.*, 1995), on the isolated female rat urethra. Furthermore, the pharmacology of these nicotinic agonist-evoked urethral responses was investigated by examining the effects of the neuronal nicotinic receptor antagonists, chlorisondamine and

hexamethonium, TTX and various autonomic receptor antagonists on these responses.

## **METHODS**

### **General preparation**

One hundred and eight female Sprague-Dawley rats (200-300g; Charles River, Kent, U.K.) were stunned by a blow to the head and killed by cervical dislocation. Through a midline incision the bladder and proximal urethra emerging from the pubic bone were exposed. The pubic bone was cut and the bladder and urethra were removed. The urethra was cleared of connecting vascular and fatty tissues and the bladder was disconnected from the urethra by a cut just below the bladder neck. The whole urethras were then mounted longitudinally under 1g resting tension in 5ml tissue baths. The tissues were bathed and perfused (3.43 ml min<sup>-1</sup>, MCP peristaltic pump, Ismatec, Weston-super-Mare, U.K.) with Krebs solution of the following composition (in mM): NaCl 118; KCl 4.7; NaHCO<sub>3</sub> 25; KH<sub>2</sub>PO<sub>4</sub> 1.2; MgSO<sub>4</sub> 0.58; CaCl<sub>2</sub> 2.5 and glucose 11. The temperature of the baths were maintained at 37°C using a heater circulator system (C-85A, Techne, Cambridge, U.K.) and were aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

### **Experimental Protocols**

The tissues were allowed to equilibrate for a period of 60 min. During this time the tissues were continuously perfused with Krebs solution and maintained a steady resting tension of approximately 1g. However, it was observed from early experiments that stopping the perfusion of Krebs to allow incubation of the

tissues with a test substance resulted in an increase in the resting tension of the tissues. The possible explanations for these observations are outlined in previous chapters of this thesis (see chapter three). Therefore, before any test substance was applied a period of 10 min after stopping perfusion of the tissues was allowed to re-equilibrate resting urethral tension. After this 10 min period, tissues were exposed to a single concentration of nAChR agonist. The agonist was left in the bath for a period of 10 min to allow responses to develop and was then washed out thoroughly over a period of 40 min. Wash out of the tissues was then stopped, and after 10 min tissues were incubated with antagonist or vehicle for a 30 min period. A second exposure to the agonist was then carried out. Owing to desensitisation of the responses of the tissues to nicotinic agonists, cumulative concentration-response curves in the same tissues could not be performed. Therefore, a single concentration of each agonist was applied to individual tissues and concentration-response curves were constructed from separate experiments. In experiments investigating the contractile responses of DMPP, tissues were pre-treated for 30 min with L-NAME, a NOS inhibitor, to inhibit the occurrence of DMPP-evoked relaxations of the urethra. Control responses to DMPP were then performed, and the protocol continued as above.

### **Data capture and analysis**

Changes in urethral tensions were measured by means of 10 g isometric force transducers (Maywood Instruments Ltd, Basingstoke, U.K.) and were acquired (4 samples per second) using a DART system (in house, Pfizer Global Research and

Development, Kent, U.K.), and analysed off-line using ADA software (in house, Pfizer Global Research and Development, Kent, U.K.).

The amplitude (g) and duration (s) of relaxations evoked by application of nicotinic agonists were measured. When a contraction of the urethra was observed following application of a nicotinic agonist, this contraction was maintained and therefore only the amplitude (g) was measured from the baseline urethral tension to the maximum tension recorded when the contraction had reached a plateau. Baseline urethral tensions were measured 1 min before administration of antagonist or vehicle, and changes in resting urethral tensions from baseline caused by test substances were measured 10 min after their administration. This time was chosen as it was adjudged that any effects evoked on baseline urethral tensions had stabilised. Baseline and drug-evoked changes in urethral tension were expressed as percentage changes before and after the administration of antagonists and compared with time-matched vehicle controls by Student's unpaired t-test.  $P < 0.05$  was considered indicative of a statistically significant difference. All values are mean  $\pm$  s.e.mean.

## **Drugs and solutions**

(-)-nicotine (hydrogen tartrate), 1,1-dimethylphenylpiperazinum, (+)-epibatidine (hydrochloride), hexamethonium (chloride), chlorisondamine (iodide), tetrodotoxin, N $\omega$ -nitro-L-arginine methyl ester (L-NAME; hydrochloride), atropine (sulphate), d-tubocurarine (chloride), prazosin (hydrochloride) and Krebs ringer solution were purchased from Sigma-Aldrich Ltd, Poole, Dorset, U.K. All drugs were dissolved in Krebs solution, except prazosin which was dissolved in distilled water. Test substances were added to baths in a maximum of 50  $\mu$ l volume and the concentrations reported are final bath concentrations.

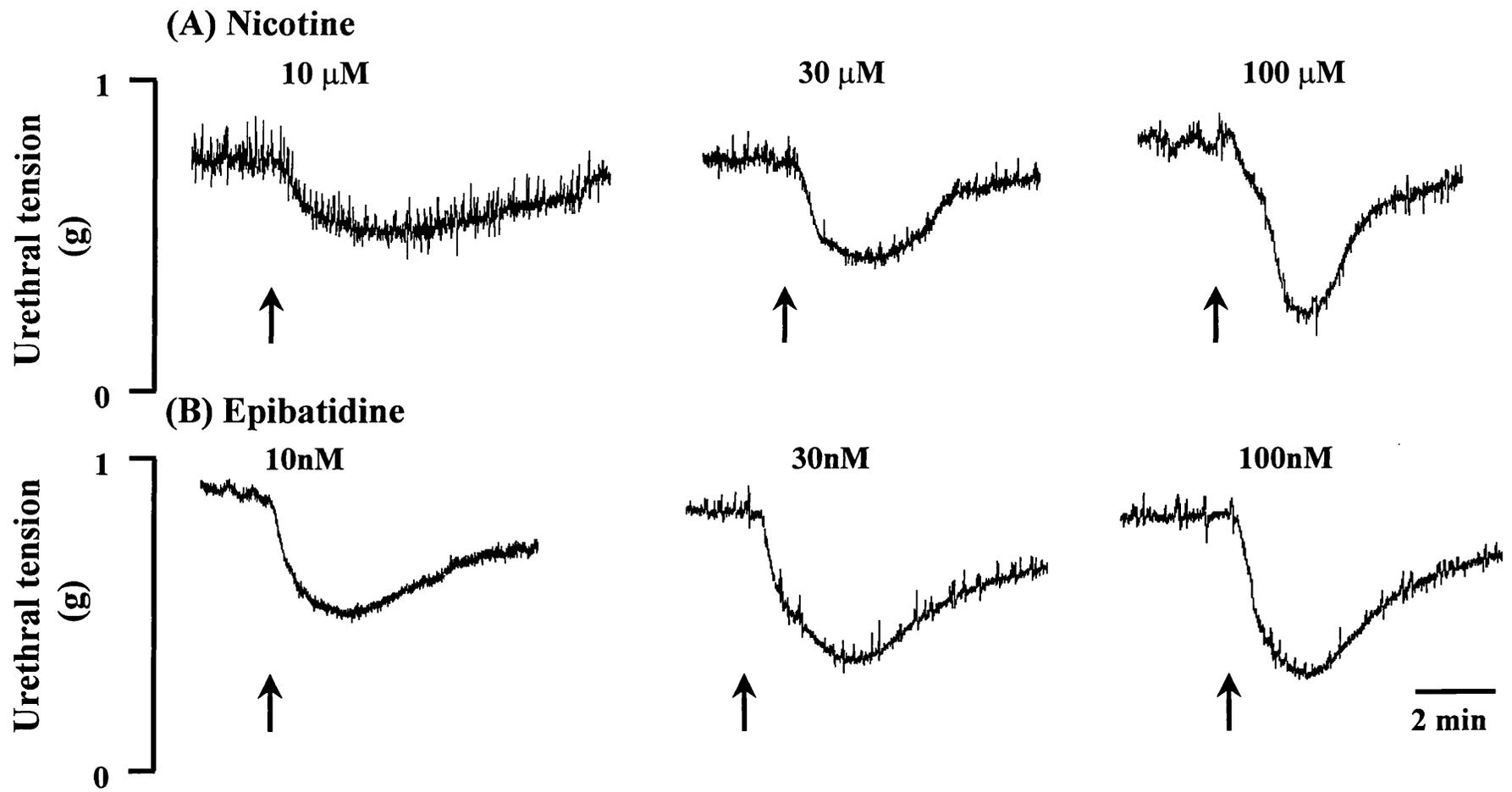
## RESULTS

### Effects of nicotine

Nicotine (10  $\mu$ M, 30  $\mu$ M and 100  $\mu$ M) evoked relaxations of the isolated female rat urethra (n = 48; Figure 5.1). No responses were observed at 1  $\mu$ M and 3  $\mu$ M nicotine. The mean amplitude of the control nicotine-evoked urethral relaxations for all experimental groups were  $0.41 \pm 0.05$  g (10  $\mu$ M; n=16),  $0.60 \pm 0.08$  g (30  $\mu$ M; n=15) and  $0.70 \pm 0.03$  g (100  $\mu$ M; n = 17). These relaxations had a mean duration of  $292.5 \pm 14.3$  s,  $283.6 \pm 25.2$  s and  $159.4 \pm 8.9$  s, respectively, after which urethral tensions returned to pre-drug levels.

### Effects of epibatidine

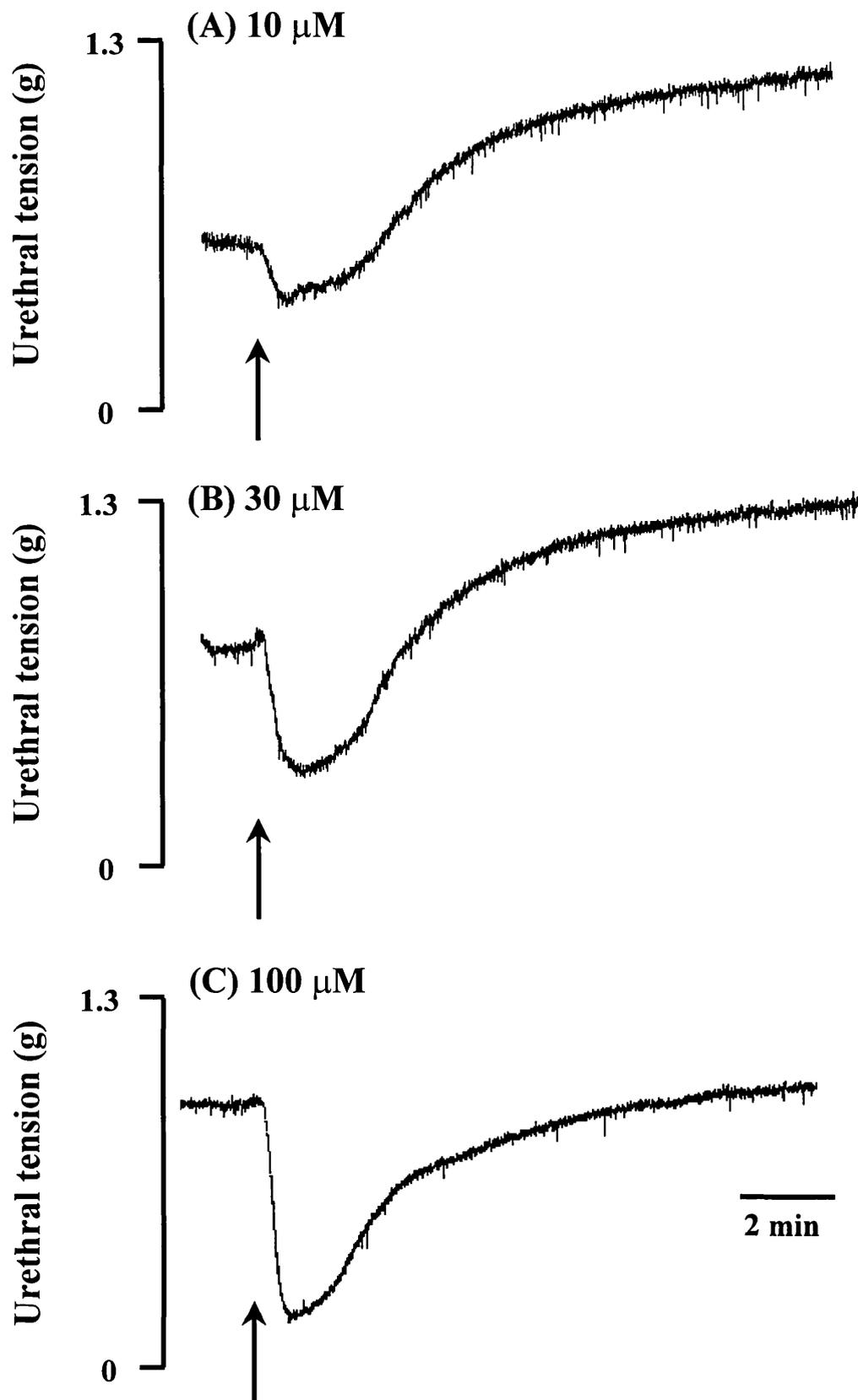
Epibatidine (30 nM, 100 nM and 300 nM) evoked relaxations of the isolated female rat urethra (n = 48; Figure 5.1). The mean amplitude of the control epibatidine-evoked urethral relaxations for all experimental groups were  $0.47 \pm 0.03$  g (30 nM; n = 16),  $0.54 \pm 0.03$  g (100 nM; n = 16) and  $0.56 \pm 0.05$  g (300 nM; n = 16). The mean duration of these urethral relaxations were  $289.1 \pm 10.2$  s,  $272.6 \pm 8.0$  s and  $210.0 \pm 11.2$  s, respectively, following which the urethral tensions returned to pre-drug levels.



**Figure 5.1.** Traces showing the effects of nicotine (A) and epibatidine (B) on the isolated female rat urethra. Arrows denote administration of agonist.

## **Effects of DMPP**

DMPP (10  $\mu\text{M}$ ) evoked delayed maintained contractile responses of the isolated female rat urethra that had a mean amplitude of  $0.30 \pm 0.03$  g ( $n = 12$ ). At higher concentrations (30  $\mu\text{M}$ , 100  $\mu\text{M}$  and 300  $\mu\text{M}$ ), DMPP evoked urethral relaxations were followed by maintained urethral contractions at 30  $\mu\text{M}$  and 100  $\mu\text{M}$  ( $n = 59$ ; Figure 5.2). At a concentration of 300  $\mu\text{M}$ , DMPP-evoked maintained contractions were not consistently observed. The mean amplitudes of the control DMPP-evoked urethral relaxations for all experimental groups were  $0.19 \pm 0.02$  g (30  $\mu\text{M}$ ;  $n = 19$ ),  $0.35 \pm 0.03$  g (100  $\mu\text{M}$ ;  $n = 24$ ) and  $0.47 \pm 0.02$  g (300  $\mu\text{M}$ ;  $n = 16$ ). These relaxations had a mean duration of  $115.3 \pm 15.0$  s,  $138.7 \pm 28.9$  s and  $122.1 \pm 5.8$  s, respectively, after which urethral tensions returned to pre-drug levels, and maintained contractions of the urethra were then evoked (at the lower two of these concentrations as discussed above). These contractions had a mean amplitude of  $0.33 \pm 0.02$  g (30  $\mu\text{M}$ ) and  $0.38 \pm 0.03$  g (100  $\mu\text{M}$ ).



**Figure 5.2.** Traces showing the effects of DMPP (10  $\mu\text{M}$ , A; 30  $\mu\text{M}$ , B and 100  $\mu\text{M}$ , C) on the isolated female rat urethra. Arrows denote administration of agonist.

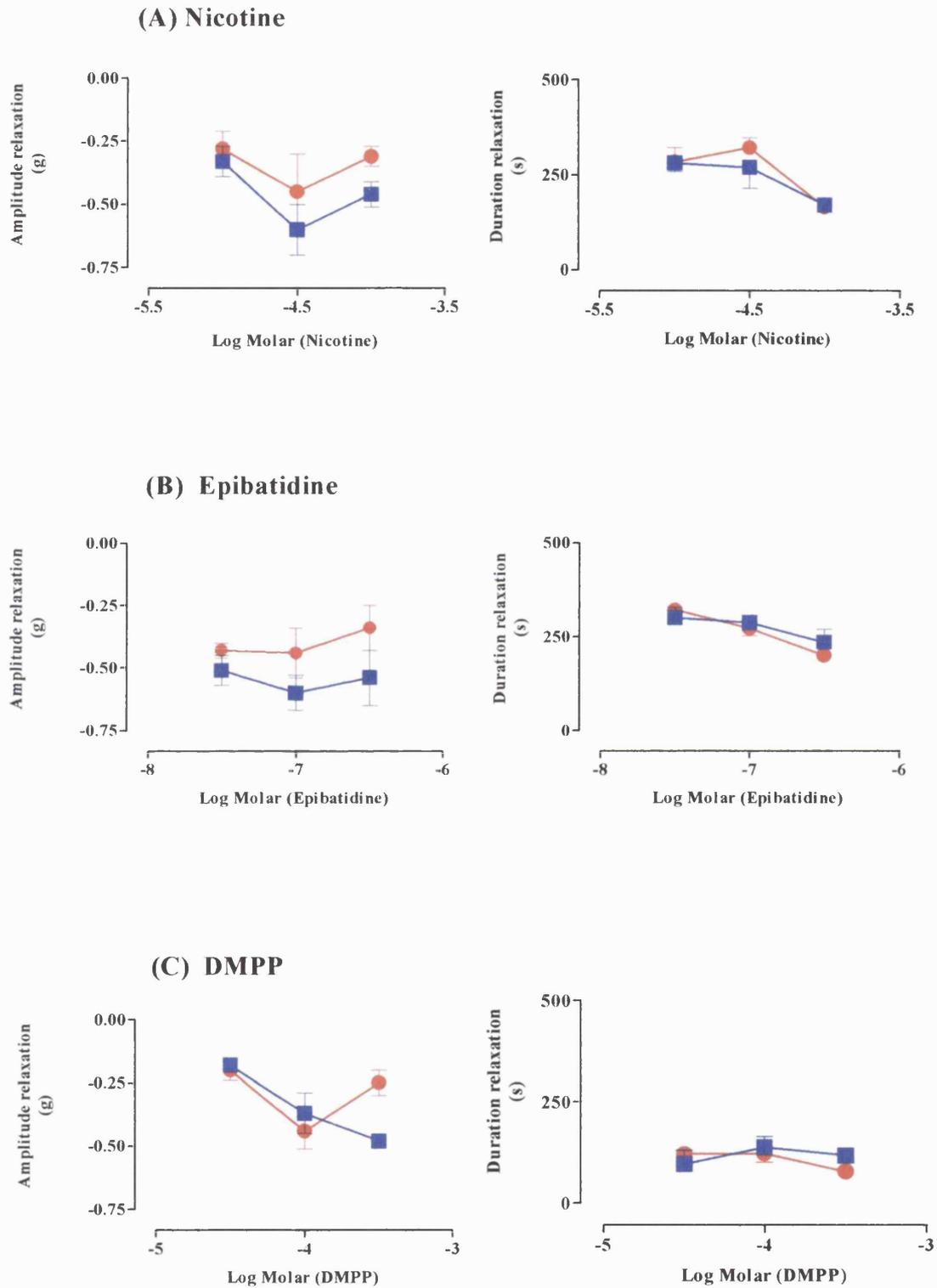
### **Effects of vehicles on nicotine-, epibatidine- and DMPP-evoked urethral responses**

From administration of vehicles (distilled water for prazosin and Krebs solution for all other agents), it was observed that the second responses to nicotine were reduced in amplitude from the control responses ( $n = 12$ ; Figure 5.3). This desensitisation was pronounced at  $100 \mu\text{M}$ , and as such the effects of concentrations of nicotine above  $100 \mu\text{M}$  on the isolated female rat urethra could not be investigated. However, the duration of nicotine-evoked urethral relaxations did not desensitise between the first and second responses (Figure 5.3).

In similarity to the effects of nicotine, the second responses to epibatidine were reduced in amplitude from the control responses at all concentrations following administration of vehicle, although these were not statistically significant ( $n = 13$ ; Figure 5.3). However, the effects of epibatidine could not be investigated at concentrations above  $300 \text{ nM}$  owing to desensitisation of the responses to this nicotinic agonist (data not shown). The duration of epibatidine-evoked urethral relaxations were unchanged between first and second responses (Figure 5.3).

DMPP-evoked urethral relaxations did not undergo a significant reduction in either amplitude or duration between the control and second responses at the lower two concentrations of agonist that evoked relaxatory responses ( $30 \mu\text{M}$ ,

n = 6, and 100  $\mu\text{M}$ , n = 6; Figure 5.3). However, urethral relaxations evoked by 300  $\mu\text{M}$  DMPP were desensitised between the control and second responses, both in amplitude and duration (n = 4; Figure 5.3), which prevented the use of higher concentrations than 300  $\mu\text{M}$  of this nicotinic agonist in these experiments. Urethral contractions evoked by DMPP (10  $\mu\text{M}$ , 30  $\mu\text{M}$  and 100  $\mu\text{M}$ ) did not desensitise between control and second responses (n = 16; Figure 5.8).

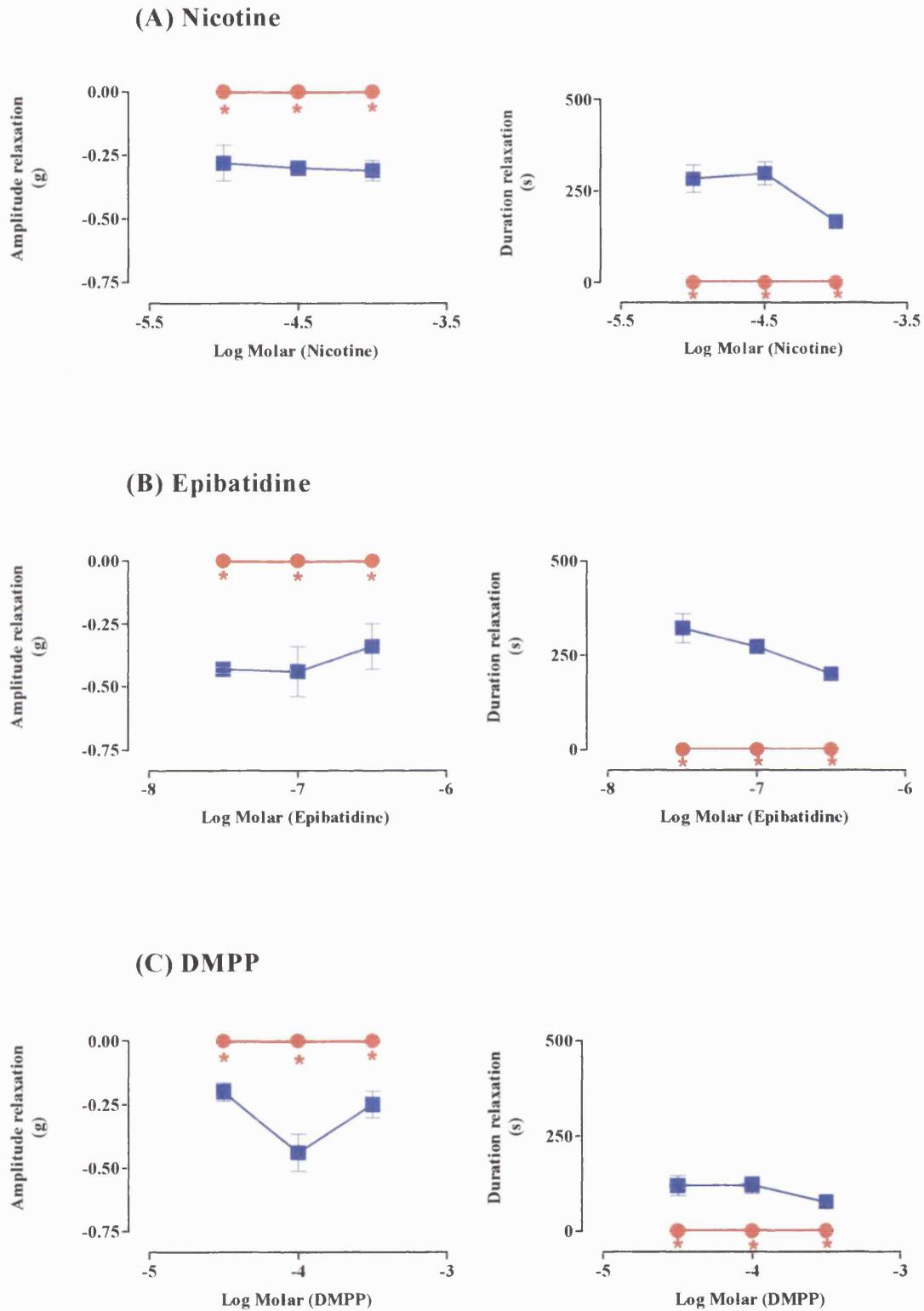


**Figure 5.3.** Amplitude and duration of urethral relaxations evoked by nicotine (A), epibatidine (B) and DMPP (C) before (■) and after (●) administration of vehicle. All points are mean  $\pm$  s.e. mean of 4-6 experiments.

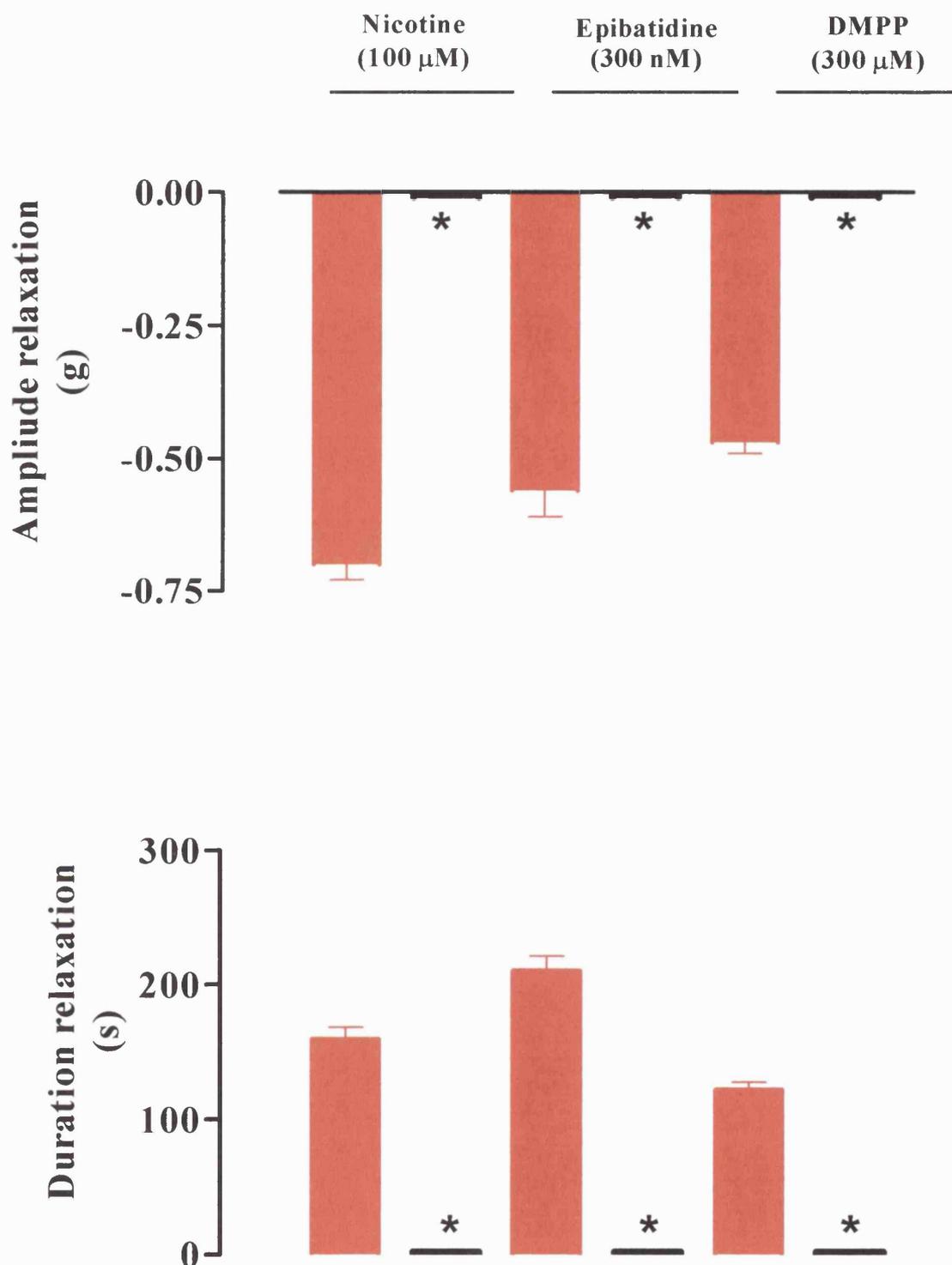
### **Effects of hexamethonium and chlorisondamine on nicotine-, epibatidine- and DMPP-evoked urethral responses**

Hexamethonium (100  $\mu\text{M}$ ) fully inhibited the urethral relaxations evoked by nicotine, epibatidine and DMPP ( $n = 36$ ; Figure 5.4). However, urethral contractions evoked by all concentrations of DMPP were unaffected following hexamethonium treatment ( $n = 12$ ; Figure 5.8).

The effects of chlorisondamine (100  $\mu\text{M}$ ), a neuronal nAChR antagonist, were examined on urethral relaxations evoked by the highest concentrations of nicotine, epibatidine and DMPP. The effects of hexamethonium were examined on the responses to all concentrations of nicotinic agonists as chlorisondamine has been reported to attenuate glutamate-mediated responses *in vitro* in the hippocampal slice preparation (Clarke *et al.*, 1994). These experiments with chlorisondamine were therefore performed to provide further evidence that the nicotinic agonist-evoked responses described in the present study are mediated via the activation of neuronal nAChRs. Chlorisondamine fully inhibited the urethral relaxations evoked by nicotine (100  $\mu\text{M}$ ;  $n = 3$ ), epibatidine (300 nM;  $n = 3$ ) and DMPP (300  $\mu\text{M}$ ;  $n = 3$ ; Figure 5.5). Furthermore, urethral contractions evoked by this concentration of DMPP were unaffected by chlorisondamine treatment (Figure 5.8).



**Figure 5.4.** Amplitude and duration of urethral relaxations evoked by nicotine (A), epibatidine (B) and DMPP (C), before (■) and after (●) administration of hexamethonium (100  $\mu$ M). All points are mean  $\pm$  s.e.mean of 4-6 experiments.  
 \*  $P < 0.05$ , based on percentage changes in urethral relaxations compared with vehicle controls.



**Figure 5.5.** Amplitude and duration of urethral relaxations evoked by nicotinic agonists before (■) and after (■) the administration of chlorisondamine (100 μM). Bars represent mean ± s.e. mean of 3 experiments.  
\* P<0.05 based on percentage changes in urethral relaxations compared with vehicle controls.

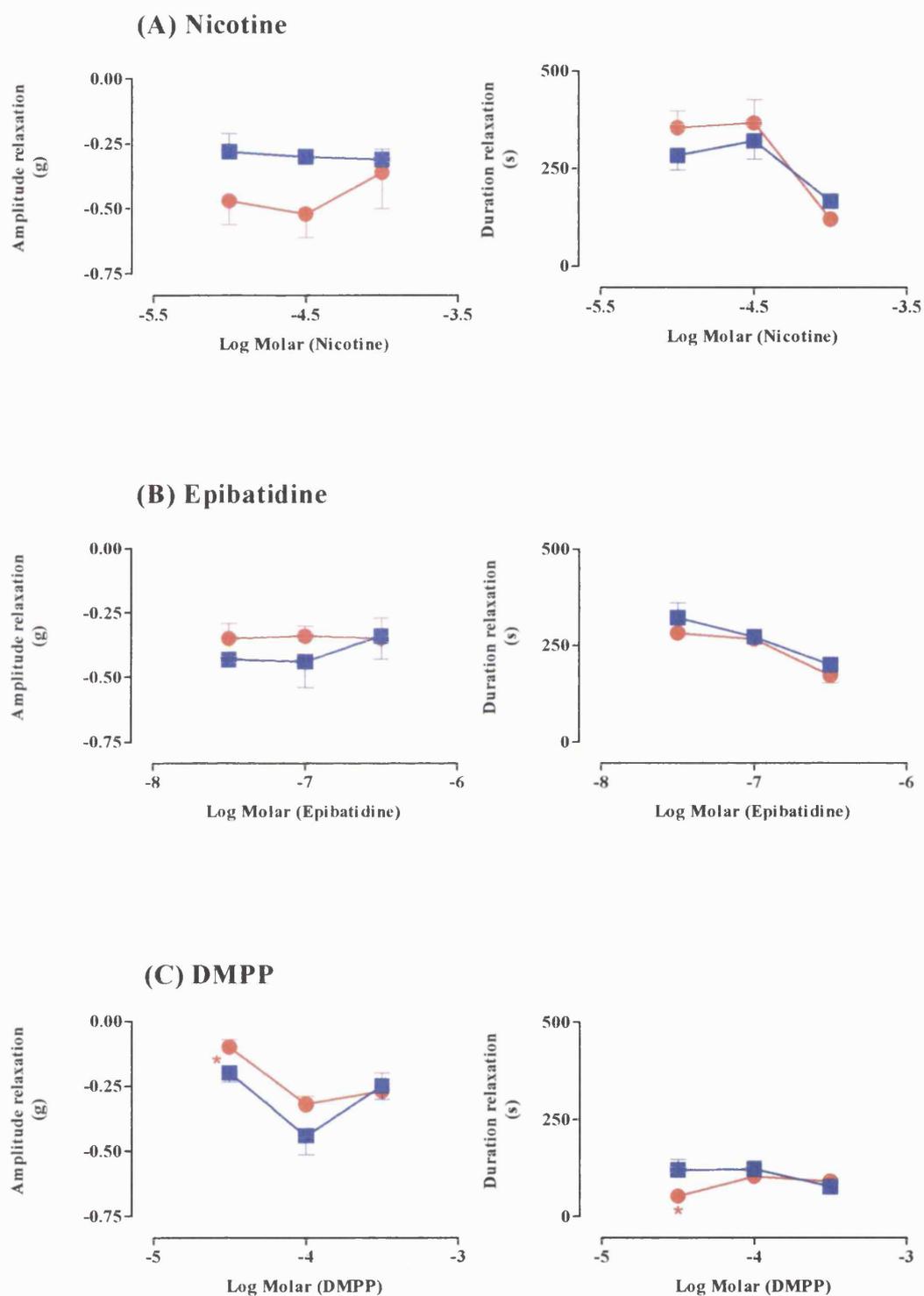
### **Effects of TTX on nicotine-, epibatidine- and DMPP-evoked urethral responses**

TTX (1  $\mu$ M) had no effect on the amplitude and duration of nicotine- and epibatidine-evoked urethral relaxations at all concentrations tested (n = 36; Figure 5.6). However, DMPP-evoked urethral relaxations were significantly attenuated by  $36.7 \pm 9.3\%$  (n = 6) at 30  $\mu$ M, following incubation with TTX (Figure 5.6). At higher concentrations of DMPP (100  $\mu$ M and 300  $\mu$ M), the urethral relaxations evoked by this agonist were TTX resistant (n = 13; Figure 5.6). Furthermore, DMPP-evoked urethral contractions were unaffected by TTX, at all concentrations of agonist that evoked contractile responses (n = 18; Figure 5.8).

### **Effects of L-NAME on nicotine-, DMPP- and epibatidine-evoked urethral responses**

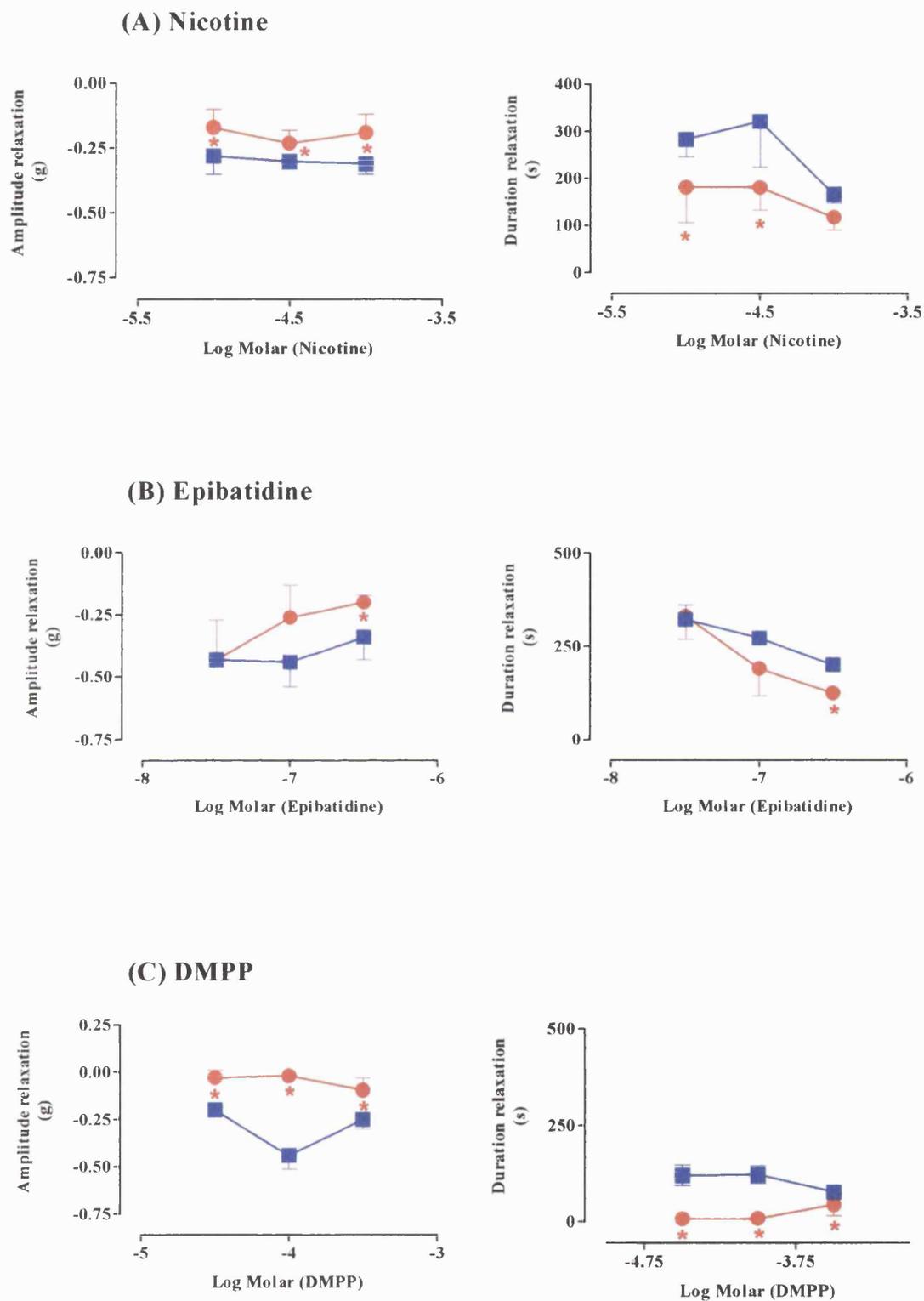
L-NAME (100  $\mu$ M) significantly attenuated the amplitude of nicotine-evoked urethral relaxations by  $63.3 \pm 17.7\%$  (10  $\mu$ M; n = 4),  $60.5 \pm 8.4\%$  (30  $\mu$ M; n = 4) and  $50.7 \pm 12.3\%$  (100  $\mu$ M; n = 4; Figure 5.7). Furthermore, the duration of nicotine-evoked urethral relaxations were significantly attenuated at the two lower of these three concentrations by  $41.1 \pm 23.2\%$  and  $31.6 \pm 18.9\%$ , respectively, although at 100  $\mu$ M nicotine, L-NAME had no effect on the duration of these responses (Figure 5.7). Epibatidine (30 nM and 100 nM)-evoked urethral relaxations were unchanged following L-NAME treatment in

both their amplitude and duration (n = 8; Figure 5.7). However, the amplitude and duration of epibatidine-evoked relaxations at 300 nM were significantly attenuated by  $39.6 \pm 6.0\%$  and  $63.3 \pm 5.6\%$ (n=4), respectively (Figure 5.7). DMPP (30  $\mu$ M, 100 $\mu$ M and 300 $\mu$ M)-evoked urethral relaxations were significantly attenuated by  $87.9 \pm 13.6\%$  (n = 5),  $95.2 \pm 5.2\%$  (n = 6) and  $80.4 \pm 14.7\%$  (n = 4) in amplitude, and by  $87.9 \pm 13.6\%$ ,  $95.2 \pm 5.2\%$  and  $61.2 \pm 26.1\%$  in duration, respectively (Figure 5.7). L-NAME had no effect on the amplitude of DMPP-evoked urethral contractions at all concentrations of agonist evoking this response (n = 12; Figure 5.8).



**Figure 5.6.** Amplitude and duration of urethral relaxations evoked by nicotine (A), epibatidine (B) and DMPP (C), before (■) and after (●) administration of tetrodotoxin (1  $\mu$ M). All points are mean  $\pm$  s.e. mean of 4-6 experiments.

\*  $P < 0.05$ , based on percentage changes in urethral relaxations compared with vehicle controls.



**Figure 5.7.** Amplitude and duration of urethral relaxations evoked by nicotine (A), epibatidine (B) and DMPP (C), before (■) and after (●) administration of L-NAME (100  $\mu$ M). All points are mean  $\pm$  s.e.mean of 4-6 experiments.

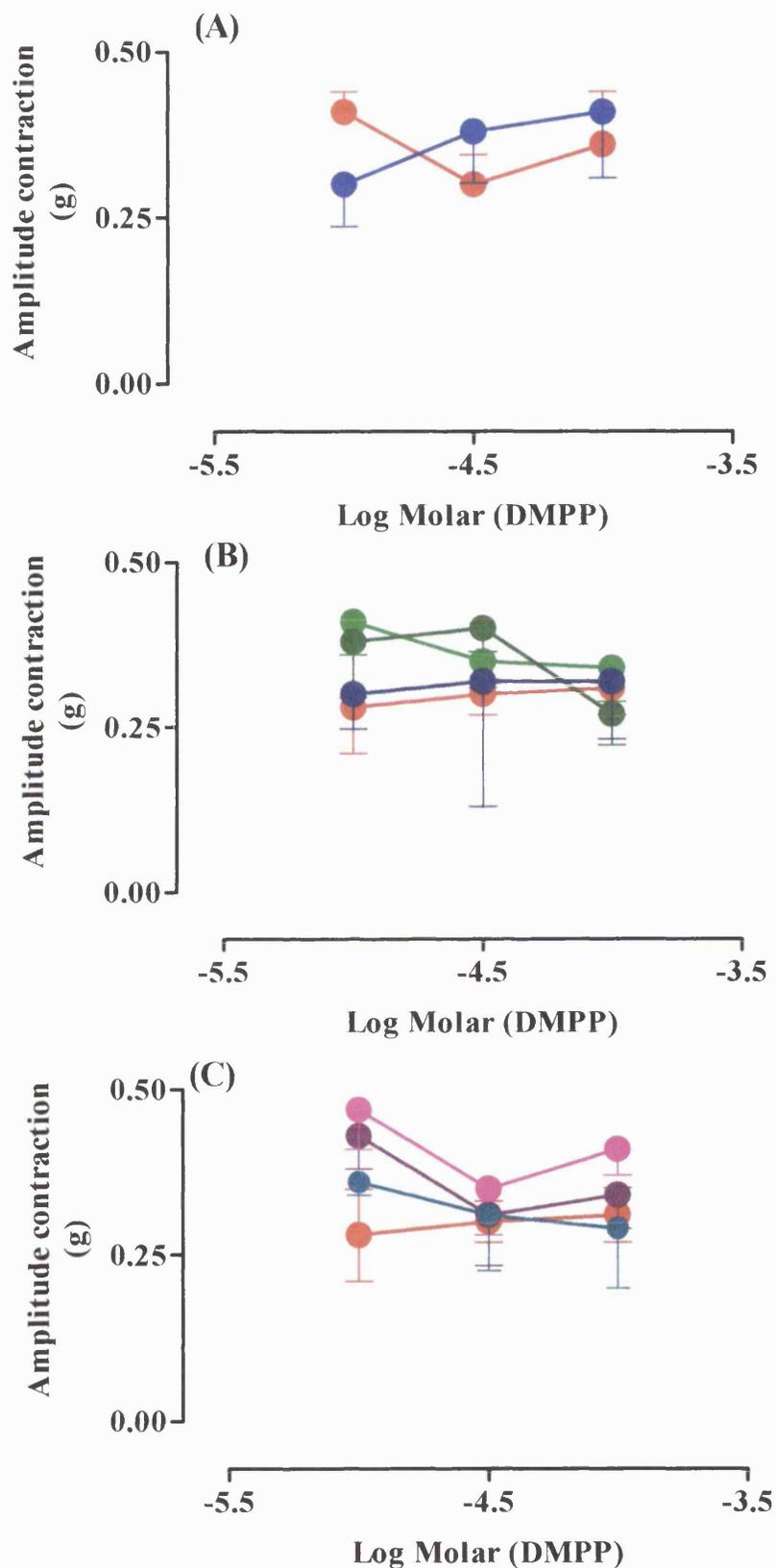
\*  $P < 0.05$  based on percentage changes in urethral relaxations compared with vehicle controls.

### **Effects of atropine, prazosin and d-tubocurarine on DMPP-evoked urethral contractions**

Atropine, a muscarinic receptor antagonist (100  $\mu\text{M}$ ;  $n = 4$ ), prazosin, an adrenergic receptor antagonist (24  $\mu\text{M}$ ;  $n = 4$ ) and d-tubocurarine, a neuronal and neuromuscular nicotinic receptor antagonist (1  $\text{mM}$ ;  $n = 4$ ) had no effect on DMPP (100  $\mu\text{M}$ )-evoked urethral contractions, in tissues pre-treated with L-NAME (100  $\mu\text{M}$ ; Figure 5.8).

### **Effects of test substances on resting urethral tensions**

All test substances, including vehicles, had no effect on baseline urethral tensions in all experiments, except L-NAME, which caused an increase of  $19.1 \pm 2.5 \%$  ( $0.16 \pm 0.03 \text{ g}$ ) after 10 min ( $n = 39$ ). This increase in baseline urethral tension following L-NAME treatment has been reported and discussed in chapters two and four of this thesis, and therefore will not be discussed in this chapter.



**Figure 5.8.** Effects of test substances on the amplitude of urethral contractions evoked by DMPP. (A) Before (●) and after (●) administration of vehicle. (B) Effects of vehicle (●), hexamethonium (100  $\mu$ M; ●), TTX (1  $\mu$ M; ●) and L-NAME (100  $\mu$ M; ●). (C) Effects of vehicle (●), atropine (100  $\mu$ M; ●), prazosin (24  $\mu$ M; ●) and d-tubocurarine (1 mM; ●). All points are mean  $\pm$  s.e. mean of 4-6 experiments.

## DISCUSSION

### *Nicotinic agonist-evoked urethral relaxations: role of nicotinic receptors*

The present study has demonstrated that nicotine-, epibatidine- and DMPP-evoked relaxations of the isolated female rat urethra are mediated via activation of nicotinic receptors. The observation that the urethral responses to nicotinic agonists were desensitised between the first and second challenges provides evidence for an involvement of nicotinic receptors in the mediation of these responses, as the agonist-evoked desensitisation of tissue responses is a classical characteristic of these receptor types (see Broadley, 1996). Indeed, the desensitisation of these responses affected the experimental protocols, in that dose-response curves could not be constructed from individual tissues, and the dose range that could be examined comprised only three log units. A role for neuronal nicotinic receptors in these responses is supported by the fact that urethral relaxations evoked by these agonists were abolished by hexamethonium and chlorisondamine, which are considered to be selective neuronal nicotinic receptor antagonists, with little or no antagonist activity at neuromuscular nicotinic receptor subtypes (see Broadley, 1996). Interestingly, despite previous studies in this thesis confirming the presence of striated muscle in the isolated female rat urethral preparation (see chapter three), a neuromuscular nicotinic agonist-evoked contraction of this tissue was not observed following inhibition of the neuronal nicotinic receptor-mediated relaxation with hexamethonium or chlorisondamine. (Evidence against a role for neuromuscular nicotinic receptors

in the DMPP-evoked urethral contractions evoked in these studies was provided from the lack of effect of d-tubocurarine, a neuromuscular nicotinic receptor antagonist; see later). Indeed, previous studies in this thesis have demonstrated that succinylcholine, a striated muscle depolarising agent, also has no effect on this preparation, but conversely, evoked rapid contractile fasciculations of the isolated female rat diaphragm (see chapter three). However, nicotine and epibatidine are selective for neuronal nicotinic receptors over neuromuscular nicotinic receptors (Table 5.1; note that to the author's knowledge, there is no published data regarding the affinity of DMPP at neuromuscular nicotinic receptors). Furthermore, the concentrations of nicotine and epibatidine used in the present study would be insufficient to interact with neuromuscular nicotinic receptors, based on the previously published data (Table 5.1). However, the reasons why succinylcholine was also without effect on this tissue (see chapter three) remain unclear. It is possible that changes in striated muscle tension caused by succinylcholine, and possibly nicotinic receptor agonists, were not recorded in these experiments. This may be due to the orientation of the striated muscle fibres, in that this region of musculature in the female rat urethra is circular, C-shaped and deficient posteriorly (see chapter three), and therefore the mounting of this preparation longitudinally may not record an increase in tension in this plane. Alternatively, as the isolated female rat urethral preparation comprises both striated and longitudinal and circular smooth muscle types, the effects of nicotinic agonists on the urethral smooth muscles may mask any contractile effects of striated muscle stimulation. Experiments involving urethral preparations comprising only the mid-part of the whole female rat urethra, where

striated muscle dominates, and mounted in the 'plane' of the striated muscle fibres would prove useful in delineating some of these issues.

Receptor	EC <sub>50</sub> (μM)					
	Nicotine	Species	(+) Epibatidine	Species	DMPP	Species
α3 β4	410	Chicken (oocyte) <sup>1</sup>	0.021	Chicken (oocyte) <sup>1</sup>	18.67	Human (oocyte) <sup>2</sup>
	110	Human (oocyte) <sup>1</sup>	0.073	Human (oocyte) <sup>1</sup>	2.5	Human (oocyte) <sup>3</sup>
	80.3	Human (oocyte) <sup>2</sup>	0.017	Human (oocyte) <sup>3</sup>		
α4 β2	0.35	Chicken (oocyte) <sup>1</sup>	0.0045	Chicken (oocyte) <sup>1</sup>	17.99	Human (oocyte) <sup>2</sup>
	5.47	Human (oocyte)				
α3 β2	200	Chicken (oocyte) <sup>1</sup>	0.017	Chicken (oocyte) <sup>1</sup>	55.87	Human (oocyte) <sup>2</sup>
	6.7	Human (oocyte) <sup>1</sup>	0.022	Human (oocyte) <sup>1</sup>		
	132.44	Human (oocyte) <sup>2</sup>	-			
α2 β2	19.23	Human (oocyte) <sup>1</sup>	-	-	11.18	Human (oocyte) <sup>2</sup>
α2 β4	20.71	Human (oocyte) <sup>2</sup>	-	-	22.77	Human (oocyte) <sup>2</sup>
α4 β4	5.02	Human (oocyte) <sup>2</sup>	-	-	18.71	Human (oocyte) <sup>2</sup>
α1 β1 δγ (Neuro-muscular)	3762	Mouse (HEK 293) <sup>3</sup>	1.6	Torpedo (oocyte) <sup>1</sup>	-	-
	250	Torpedo (oocyte) <sup>1</sup>	7.3	Human (oocyte) <sup>1</sup>		

**Table 5.1.** EC<sub>50</sub> values for the affinities of nicotinic agonists at nicotinic receptor subtypes. All values are mean (μM); standard errors could not be shown as these were not calculated in all studies.

<sup>1</sup> Gerzanich et al., 1995, <sup>2</sup> Chavez-Noriega et al., 1995, <sup>3</sup> Akk & Auerbach, 1999.

For references in full see chapter seven.

*Nicotinic agonist-evoked urethral relaxations: effects of TTX*

The present studies were performed to determine if the subtype of neuronal nicotinic receptors that are located in ganglia and play a role in the mediation of cholinergic transmission, are present in the isolated female rat urethra. These findings can then be correlated with the results obtained from *in vivo* studies investigating the mechanisms of action of previously reported reflex interactions between the urethral smooth and striated muscles (see chapter two). If the nicotinic receptors that are present in the urethra mediate transmission in ganglia located in this tissue, then stimulation of these receptors with nicotinic agonists would be expected to cause the release of neurotransmitters from post-ganglionic neurones to evoke urethral relaxations. Therefore these nicotinic agonist-evoked urethral relaxations would be inhibited by TTX, which is known to preferentially block the increase in sodium conductance associated with the action potential and thus abolish nerve conduction (Narahashi, Moore & Scott, 1964). However, the results of the present studies demonstrate that nicotine- and epibatidine-evoked urethral relaxations are TTX resistant, providing evidence against these neuronal nicotinic receptors in the female rat urethra comprising neuronal nicotinic receptors that are located and function in ganglia. Conversely, DMPP-evoked urethral relaxations were significantly attenuated by TTX at the lowest concentration of agonist examined, but were surprisingly TTX-resistant at higher concentrations. However, only approximately 35% of the DMPP-evoked urethral relaxation was sensitive to TTX, and based on the affinities of these nicotinic agonists at nicotinic receptor subtypes, it is unlikely that DMPP would

stimulate neuronal nAChRs of the ganglionic subtype more selectively than epibatidine (see below). Furthermore, the observation that DMPP-evoked urethral relaxations were TTX resistant at higher concentrations of agonist suggests that this result is an anomaly. Therefore, the results of the present study provide evidence against a role for neuronal nAChRs of the ganglionic subtype at the level of the urethra in the chlorisondamine-sensitive interactions between urethral smooth and striated muscles previously described in this thesis (see chapter two).

*Nicotinic agonist-evoked urethral relaxations: role of NO*

NO is well known to have a pronounced relaxatory effect on the bladder neck and urethra in the rat and other species, which is physiologically relevant in producing inhibitory responses of the bladder outlet during voiding (see Burnett, 1995; see also chapters three and four). Therefore, the primary candidate for the substance mediating nicotine-, epibatidine- and DMPP-evoked urethral relaxations in the present study is NO. To investigate this, the effects of L-NAME, a NOS inhibitor, were examined on these urethral responses to nicotinic agonists. L-NAME significantly attenuated the amplitude and duration of nicotine-evoked urethral relaxations at the lower two concentrations of agonist tested, but only attenuated the amplitude of these responses at the higher concentration of nicotine. Furthermore, epibatidine-evoked urethral relaxations were unaffected by L-NAME at the lower two concentrations of agonist tested, but were significantly attenuated both in amplitude and duration at the highest

concentration of agonist used. Conversely, the amplitude and duration of DMPP-evoked urethral relaxations were significantly attenuated at all concentrations of agonist used. In addition, DMPP-evoked urethral relaxations were inhibited to a greater degree by L-NAME than nicotine- and epibatidine-evoked urethral relaxations. This is most likely to be due to the fact that DMPP also evoked urethral contractions and therefore a 'physiological antagonism' of DMPP-evoked urethral relaxations is present (see below). This is particularly illustrated by the observation that urethral relaxations evoked by 300  $\mu\text{M}$  of DMPP, which did not consistently evoke urethral contractions, were less sensitive to L-NAME than lower concentrations of DMPP (30 and 100  $\mu\text{M}$ ), which frequently evoked these contractile responses.

There are a number of possible explanations for the L-NAME-resistant components of urethral relaxations evoked by nicotinic-agonists. This concentration of L-NAME was chosen from its ability to attenuate electrically-evoked urethral relaxations in the isolated female rat urethra (see chapter four). However, these previous studies have also shown that another NOS inhibitor, N $\omega$ -nitro-L-arginine (L-NA) inhibits electrically-evoked urethral relaxations to a greater extent than L-NAME in this tissue (see chapter four). Therefore, these L-NAME-resistant components of nicotinic agonist-evoked urethral relaxations may be due to an incomplete block of NOS in the urethra. Examination of the effects of L-NA on nicotine-, epibatidine- and DMPP-evoked urethral relaxations would prove useful in delineating some of these issues. Furthermore, there are a variety of possible mechanisms of action of nicotinic agonist-evoked urethral

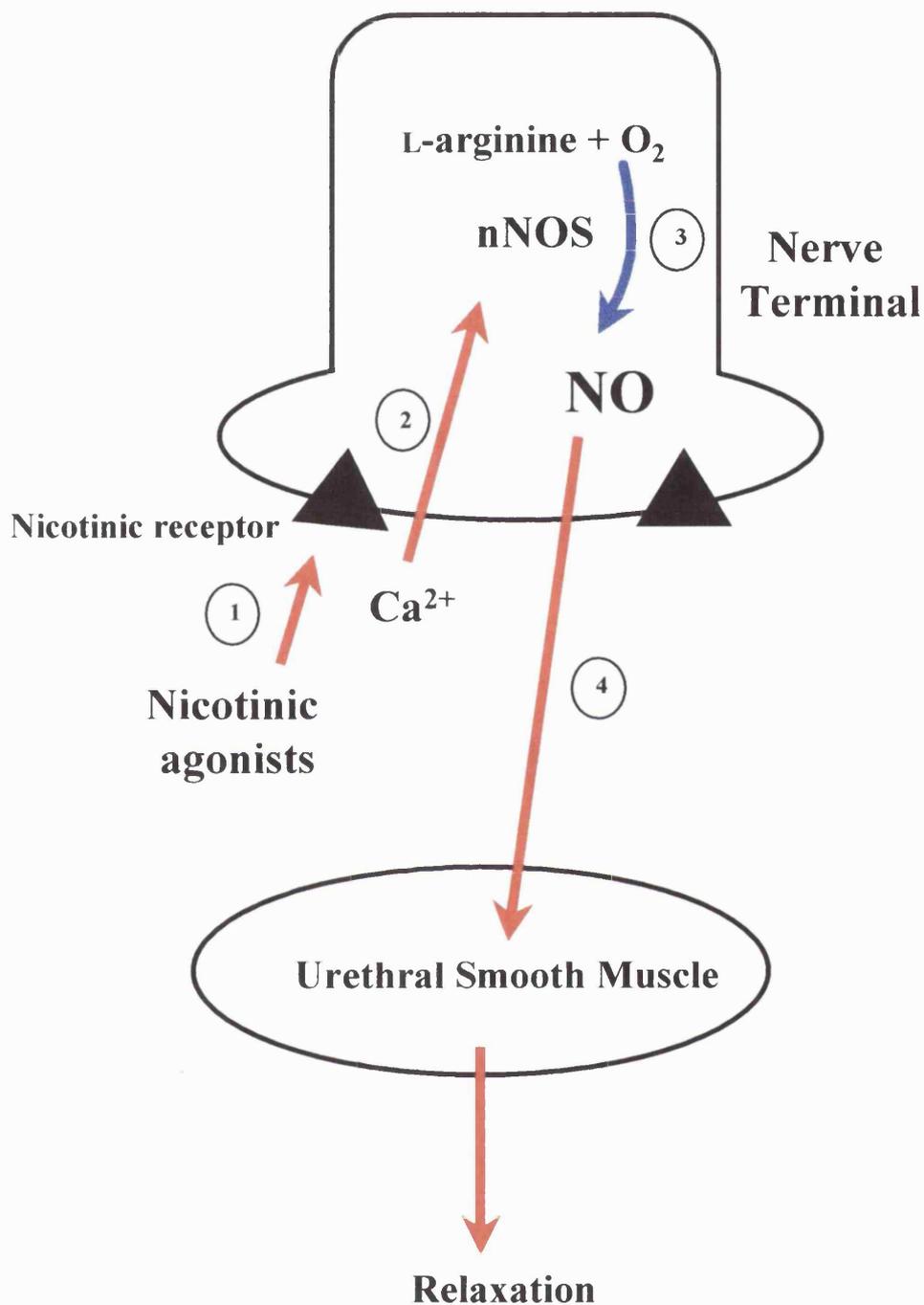
relaxations, involving a number of components of the urethra (see below), and the L-NAME resistant components of nicotinic agonist-evoked urethral relaxations may reflect an inability of L-NAME to penetrate these layers. Furthermore, the varied degree of blockade of urethral relaxations at different doses of nicotine and epibatidine following L-NAME treatment may also reflect activation of different neuronal nicotinic receptors at different sites in the urethra (see below). Alternatively, nicotinic agonist-evoked urethral relaxations may also be mediated by other inhibitory substances. The identity of these alternative relaxatory neurotransmitters or regulatory substances may be dependent on the exact mechanisms of actions of these nicotinic agonists in the urethra, and will be discussed in more detail in later sections of this discussion.

*TTX-resistant responses in the female rat urethra: mechanisms of action*

TTX-resistant responses have been demonstrated in a wide variety of tissues. Contractile responses of guinea pig bronchial strips to nicotine and DMPP have been shown to be inhibited by hexamethonium, but unaffected by TTX (Takayanagi & Kizawa, 1983). Furthermore, these authors have demonstrated that nicotine-evoked contractions of rabbit bronchial smooth muscle are sensitive to hexamethonium, but are TTX resistant (Takayanagi, Kizawa & Hiruta, 1984). TTX-resistant relaxatory responses to nicotinic agonists have also been reported in pre-contracted pig tracheal smooth muscle strips (Kannan & Johnson, 1992). In genitourinary tissues, nicotine has been shown to relax the bovine retractor penis muscle via activation of neuronal nicotinic receptors (Klinge, Alaranta &

Sjostrand, 1988). In addition, these responses were only partially sensitive to TTX. In the isolated guinea-pig urinary bladder, application of nicotine evokes a contractile response that is antagonised by hexamethonium, but insensitive to TTX (Hisayama *et al.*, 1998b). There are a number of possible explanations for the TTX resistance of urethral relaxations evoked by nicotinic agonists reported in the present study. Nicotine has been shown to induce the release of several neurotransmitters in the central nervous system by the activation of nicotinic receptors on nerve terminals (see Wonnacott, 1997). This phenomenon has been extensively investigated in the nigrostriatal dopaminergic system, where nicotine stimulates the release of dopamine both *in vitro* (Rowell & Wonnacott, 1990; Grady *et al.*, 1992) and *in vivo* (Imperato, Mulas & Di Chiara, 1986). Therefore, the possibility exists that nicotine, epibatidine and DMPP are stimulating neuronal nicotinic receptors that are located on urethral nerve terminals, thereby evoking the release of NO, and possibly other neurotransmitters, from these neurones. Unlike a number of metabotropic receptors that only influence stimulated release of neurotransmitters, presynaptic nAChRs can elicit Ca<sup>2+</sup> dependent transmitter release under resting conditions (see Wonnacott, 1997). As neuronal nicotinic receptors have a high Ca<sup>2+</sup> permeability (Mulle, Lena & Changeux, 1992), it is likely that activation of these receptors leads to sufficient Ca<sup>2+</sup> influx via the nicotinic channel itself to promote the release of neurotransmitters (Figure 5.9; see Wonnacott, 1997). Therefore, it can be hypothesised that the nicotinic agonist-evoked activation of presynaptic neuronal nicotinic receptors in the female rat urethra, involves the release of NO, and possibly other substances, from nerve terminals by a sodium action potential-

independent mechanism, and is therefore resistant to TTX. Indeed, nicotine-evoked relaxations of cerebral arteries and gastrointestinal tissues have been shown to be mediated by the release of NO via a TTX resistant mechanism (Ayajiki *et al.*, 1998).



**Figure 5.9.** Schematic representation of the possible involvement of presynaptic nicotinic receptors in nicotinic agonist-evoked relaxations of the isolated female rat urethra. (1) Nicotinic agonists stimulate nicotinic receptors located on urethral nerve terminals. (2)  $\text{Ca}^{2+}$  enters the nerve terminal directly via the nicotinic channel. (3)  $\text{Ca}^{2+}$ -dependent nNOS is activated promoting the formation of NO. (4) NO diffuses from the nerve terminal to the urethral smooth muscle to evoke a relaxation.

A role for presynaptic neuronal nicotinic receptors in the TTX resistant contractile response of the guinea pig bladder to nicotine has also been suggested (Hisayama *et al.*, 1988b). These nicotine-evoked contractile bladder responses were abolished following atropine (a muscarinic receptor antagonist) and  $\alpha,\beta$ -methylene ATP (a desensitising P2X receptor agonist; see Ralevic & Burnstock, 1998) treatment, suggesting that if presynaptic nicotinic receptors are involved, they are located on parasympathetic and non-adrenergic, non-cholinergic (NANC) nerve terminals. Immunohistochemical and functional *in vitro* studies have suggested that NO-releasing nerves in the female rat lower urinary tract do not belong to nerve populations sensitive to the sympathetic neurotoxin, 6-hydroxydopamine, or the sensory neurotoxin, capsaicin (Persson *et al.*, 1997). Furthermore, *in vivo* studies have suggested that in the female rat urethra NO is released from parasympathetic postganglionic neurons (Fraser, Flood & De Groat, 1995). This suggests that if the nicotinic agonist-evoked urethral relaxations reported in the present study are mediated by activation of presynaptic nicotinic receptors, then it is likely that these receptors are located on parasympathetic nerve terminals in the urethra. In this way, activation of presynaptic neuronal nicotinic receptors on parasympathetic nerve terminals would lead to an influx of  $\text{Ca}^{2+}$  into the nerve terminal, stimulation of  $\text{Ca}^{2+}$ -dependent neuronal NOS, and the production of NO which diffuses from the nerve terminal to the urethral smooth muscle cells to evoke urethral relaxation. As such, the nicotinic agonist-evoked release of substances other than NO from parasympathetic nerve terminals could account for the L-NAME resistant components of the urethral relaxations evoked by these agonists. For example,

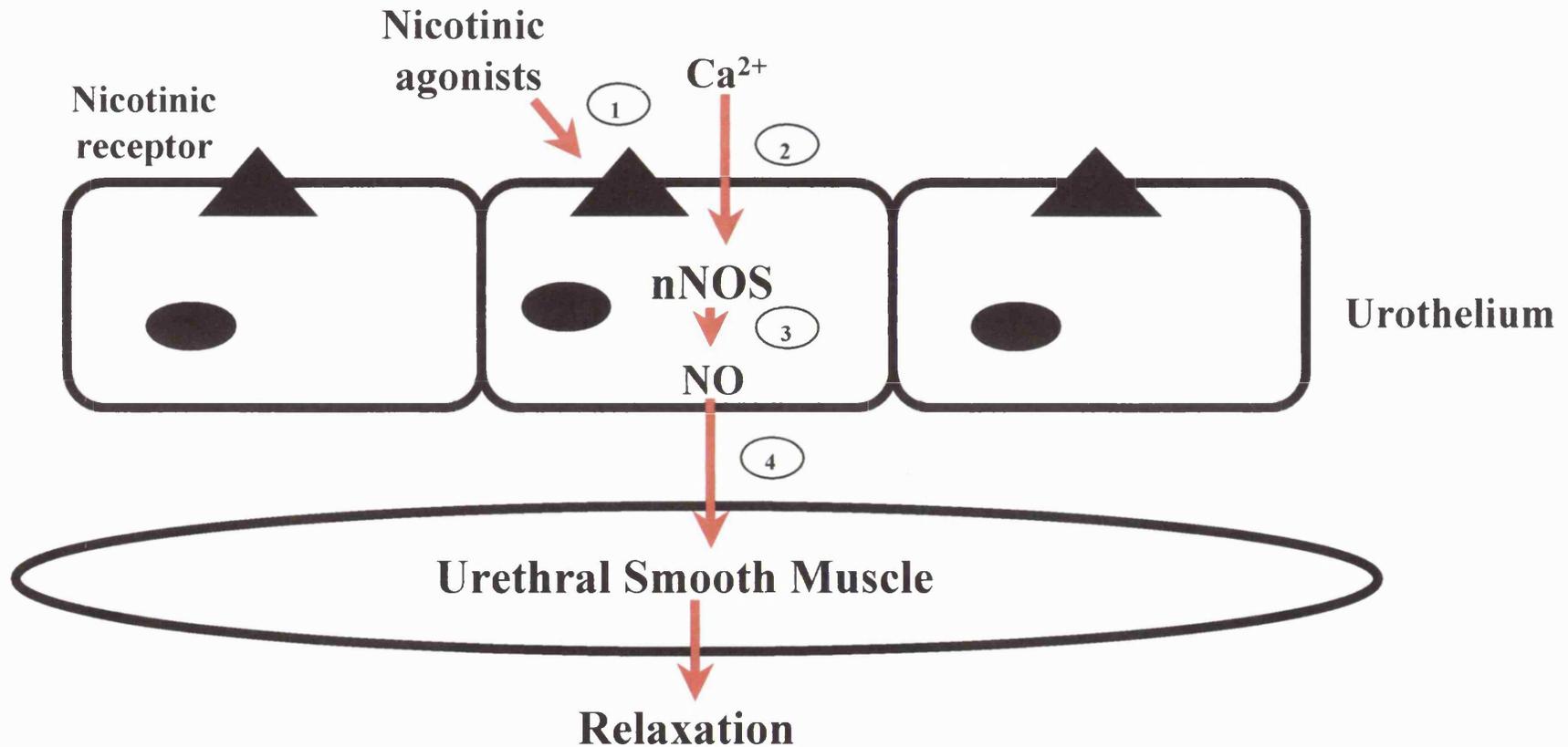
the release of vasointestinal polypeptide, neuropeptide Y and prostanoids from these nerves to evoke changes in urethral tone remains a possibility. Alternatively, the release of other transmitters and regulatory substances from sympathetic nerve terminals, such as noradrenaline to evoke urethral relaxations via the activation of  $\beta$ -adrenoceptors, cannot be excluded (see Steers, 1996). Despite previous studies suggesting that NO-releasing nerves do not belong to nerve populations sensitive to capsaicin (Persson *et al.*, 1997), a role for sensory nerves in the urethral responses to nicotinic agonists cannot be excluded. Indeed, NOS immunoreactivity has been detected in afferent nerve fibres in both the bladder and urethra (see Andersson & Persson, 1994), and capsaicin has been shown to stimulate the release of NO from rat bladder by activation of a population of small diameter afferent nerves (Birder *et al.*, 1998). Both functional (Maggi *et al.*, 1993) and immunohistochemical (Su *et al.*, 1986) studies have identified the presence of capsaicin sensitive primary afferents in the rat urethra, suggesting that nicotinic agonists could cause the release of NO, and possibly other substances, from sensory nerve terminals. The release of transmitters from the peripheral nerve endings of sensory nerves has been termed the 'efferent function of sensory nerves' (see Maggi, 1991), and in this respect, nicotine has been shown to evoke the release of tachykinins in the guinea pig isolated bronchi (Kizawa & Takayanagi, 1985) and perfused lung and heart (Franco-Cereceda, Saria & Lundberg, 1989). Functional experiments have also implicated transmitter release from sensory nerves in the contraction produced by nicotinic receptor stimulation in the rabbit isolated iris sphincter muscle (Hisayama *et al.*, 1988a) and urinary bladder (Kizawa *et al.*, 1988). Thus, the

activation of neuronal nicotinic receptors on sensory nerve terminals in the urethra could activate the 'efferent function' of these nerves, causing the release of NO with subsequent tissue relaxation. In this respect, the release of other neurotransmitters and regulatory substances contained within the afferent nerve terminals may be co-released with NO, and this could account for the L-NAME resistant component of nicotinic agonist-evoked urethral relaxations. For example, the release of substance P and related peptides from afferent nerve terminals could influence urethral contractility to evoke a relaxation (see Steers, 1995). Further studies to investigate this mechanism of action of nicotinic agonist-evoked urethral relaxations includes examination of the effects of these agents in the urethra following desensitisation of the sensory afferents by chronic capsaicin treatment. Furthermore, investigating the effects of nicotinic agonists on the urethra following bilateral removal of the pelvic ganglia would prove useful in determining if the neuronal nicotinic receptors involved in these responses are located pre-synaptically on parasympathetic or sensory nerve terminals, or at other non-neuronal sites (see below). Interestingly, Ayajiki *et al.* (1998) have recently shown that neosurugatoxin, a selective nicotinic receptor antagonist, is more effective at inhibiting the presynaptic actions of nicotine at sensory than motor nerve terminals. Therefore, experiments using this antagonist may prove useful in delineating some of these issues.

Alternatively, nicotine, epibatidine and DMPP may cause the release of NO, and possibly other neurotransmitters and regulatory substances, from non-neuronal sites within the urethra. In this respect, constitutive endothelial NOS

immunoreactivity has been detected in urothelial cells of the hamster proximal urethra (Pinna *et al.*, 1999), rat bladder (Birder, Kanai & de Groat, 1997), and human intestinal epithelial cells (Salzman *et al.*, 1996). Furthermore, NO has been shown to be released from rat bladder urothelium in response to capsaicin and adrenergic agonists (Birder *et al.*, 1998). Therefore, it is possible that neuronal nicotinic receptors are located on the urethral urothelium, and their stimulation promotes the release of substances from the urothelial cells by mechanisms independent of action potential generation (Figure 5.10). Indeed, the presence of nicotinic receptors has been demonstrated in a wide variety of non-neuronal non-excitabile cells such as bronchial epithelial cells, endothelial cells of blood vessels and skin keratinocytes (see Conti-Fine *et al.*, 2000). Furthermore, human and rat bronchial epithelial cells have been shown to express messenger RNA encoding all of the subunits that constitute neuronal nicotinic receptors (e.g.  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 2$  and  $\beta 4$  subunits), and possess specific binding sites for [<sup>3</sup>H] epibatidine (Maus *et al.*, 1998). Interestingly, the findings that bronchial epithelial cells and vascular endothelial cells express neuronal nicotinic receptors has led to the suggestion that the activation of these receptors by nicotine is involved in the pathogenesis of bronchitis in smokers (Conti-Fine *et al.*, 2000). Further experiments to investigate this possible mechanism of action of nicotinic agonists include examining the effects of these agents in rat urethral preparations with the urothelium removed. However, a role for neuronal nAChRs located on the endothelium of blood vessels present in the isolated rat urethral preparation cannot be excluded, and removal of the urethral urothelium may not necessarily

provide evidence against an involvement of epithelial neuronal nicotinic receptors.



**Figure 5.10.** Schematic representation of the possible involvement of urothelial nicotinic receptors in nicotinic agonist-evoked relaxations of the isolated female rat urethra. (1) Nicotinic agonists stimulate nicotinic receptors located on urethral urothelial cells. (2)  $Ca^{2+}$  enters the urothelial cell directly via the nicotinic channel. (3)  $Ca^{2+}$  dependent nNOS is activated promoting the formation of NO which diffuses to the urethral smooth muscle to evoke a relaxation (4).

A further explanation for the phenomenon of TTX resistance in the urethral responses to nicotinic agonists is that these effects involve the activation of TTX resistant sodium channels. Sensory endings of C fibres have been shown to have TTX resistant conductances (Kirchhoff, Reeh & Waddell, 1989), and have been suggested to play an important role in pain pathways (Jeftinija, 1994). Indeed, bradykinin has been shown to excite TTX resistant primary afferent fibres in dorsal horn neurones (Jeftinija, 1994), raising the possibility that, in some way, neuronal nAChR receptor agonists activate the 'efferent function' of sensory nerves by an interaction with these channels. Experiments involving the use of sodium-free Krebs solution and experimental elimination of mechanisms of action involving activation of presynaptic or urothelial neuronal nAChRs, outlined previously, would provide further evidence for a role for TTX resistant sodium channels in the urethral responses to nicotinic agonists.

*Neuronal nicotinic receptors in the female rat urethra: possible subunit composition*

Neuronal nicotinic receptors comprise both heteromeric combinations of  $\alpha 2$ -,  $\alpha 3$ -,  $\alpha 4$  and  $\alpha 6$ -subunits with  $\beta 2$ - or  $\beta 4$ -subunits, and homomeric types comprising  $\alpha 7$ -,  $\alpha 8$ - or  $\alpha 9$ -subunits (Linstrom, 1997). These numerous neuronal nicotinic receptor combinations of  $\alpha$  and  $\beta$  subunits, and in the case of  $\alpha 7$ - $\alpha 9$ , the  $\alpha$  subunits alone, can be expressed in oocytes or other expression systems, where they form functional ion channels with diverse pharmacological properties (for examples see Elgoyhen *et al.*, 1994; Gopalakrishnan *et al.*, 1995; McGehee

& Role, 1995). In this respect, there is now a considerable amount of evidence that both the  $\alpha$  and  $\beta$  subunits influence the pharmacology of the derived neuronal nicotinic receptor channels (Hussy, Ballivet & Bertrand, 1994; Cachelin & Rust, 1995). The question then arises as to which subtypes of either presynaptic, urothelial or other neuronal nAChRs, the nicotinic agonists used in this study stimulate to evoke these urethral relaxations. A number of studies have examined the affinities of the nicotinic agonists, nicotine and epibatidine, at neuronal nicotinic receptors that are inhibited by hexamethonium, and can be successfully expressed in oocytes (Table 5.1). Conversely, studies measuring the affinities of DMPP for these subtypes are limited and those studies examining the effects of this agonist at neuronal nicotinic receptors have shown it does not discriminate between these receptor types. Moreover, few studies have examined the affinities of these agonists specifically at rat neuronal nicotinic receptors. Despite these limitations, both nicotine and epibatidine display high affinities at the combinations of neuronal  $\alpha 4$  and  $\beta 2$  subunits (Table 5.1). One of the limited studies that has employed rat tissues has also shown that nicotine and epibatidine display a higher affinity at the combination of  $\alpha 4$  and  $\beta 2$  subunits than  $\alpha 3$  and  $\beta 4$  subunits (Xiao *et al.*, 1998; these studies are not shown in table 5.1, as these authors have expressed the affinities of nicotinic agonists at neuronal nAChR subunits as  $K_i$  values rather than  $EC_{50}$  values, which comprise the data in the table). Interestingly, most of the neuronal nAChR binding sites so far identified in rat nervous tissue are composed of  $\alpha 4$  and  $\beta 2$  subunits (Flores *et al.*, 1992; Whiting *et al.*, 1987), suggesting that if presynaptic neuronal nAChRs are involved in the nicotinic agonist-evoked urethral relaxations described in the

present study, then these receptors are likely to comprise these subunits. In addition, mice lacking the  $\alpha 3$  and  $\beta 4$  subunits have enlarged bladders with dribbling urination, suggesting that nAChRs containing these subunits are necessary for normal bladder function (de Biasi, Nigro & Xu, 2000). Therefore, it is possible that these subunits are also present in urethral tissue, and may play a role in these responses. If these nicotinic agonist-evoked urethral relaxations are specifically mediated by an interaction with sensory nerve terminals, then the possible subunits involved include  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and/or  $\beta 2$ , which have been identified in rat trigeminal ganglia (Flores *et al.*, 1996). Alternatively, if these responses involve neuronal nAChRs located on urethral urothelial cells, then in this respect, bronchial epithelial cells and endothelial cells of the blood vessels express neuronal nAChRs composed of  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 2$  and  $\beta 4$  subunits (see Conti-Fine *et al.*, 2000). The wide variety of neuronal nicotinic receptor subunits located in different neuronal and non-neuronal sites within the urethra may be activated to different degrees by nicotine and epibatidine, and this may account for the different sensitivities of the urethral relaxations evoked by these agonists to L-NAME. Experiments to determine the exact neuronal nicotinic receptor subunits that are present in the urethra would prove useful in delineating the exact composition of the receptors that mediate these responses. Furthermore, if these receptors are shown to comprise neuronal nicotinic receptor subunits that are distinct from 'classical' neuronal and neuromuscular nicotinic receptors, then they may represent an attractive therapeutic target in the treatment of LUT dysfunction.

*Neuronal nicotinic receptors in the female rat urethra: physiological relevance*

There are a number of possible physiological roles for the neuronal nicotinic receptors in the isolated female rat urethra that have been described in the present studies, which are dependent on the exact location of these receptors in this tissue. Presynaptically, nicotinic receptors on acetylcholine-containing nerve terminals present the most straightforward concept of physiological presynaptic modulation (see Wonnacott, 1997). However, stimulation of nicotinic receptors in the female rat urethra has been shown to cause the release of NO, suggesting that if these receptors are presynaptic, then they are likely to be located on parasympathetic nerve terminals (see previous sections of this discussion). In this respect, these receptors may play a role in the control of the release of NO, or other transmitters, from parasympathetic nerve terminals in the female rat urethra. However, a great many peripheral nerve endings, both sensory and motor, possess a variety of receptors, including nicotinic receptors, that in many instances seem to have no physiological relevance (see Wonnacott, 1997). Indeed, these nicotinic receptors in the female rat urethra may only be present because they have survived evolutionarily, and possess no physiological roles in this tissue. These possibilities require further investigation. If these receptors are located at non-neuronal sites in the urethra, for example on the urethral urothelium, then additional potential roles such as cell proliferation and differentiation in the maintenance of urothelial function must also be considered (see Conti-Fine *et al.*, 2000). A large amount of attention has recently been directed to the potential role of neuronal nicotinic receptors in disease and

therapy (see Holladay, Dart & Lynch, 1997). In this respect, a number of studies have suggested that women who smoke are more likely to develop stress incontinence than non-smokers (Bump & McClish, 1994; Tampakoudis *et al.*, 1995). Although there are several plausible explanations for this relationship between smoking and incontinence, including sphincteric, neurologic and anatomic damage caused by violent coughing, to the adverse effects of smoking on collagen synthesis and oestrogen levels (Tampakoudis *et al.*, 1995), a role for the neuronal nicotinic receptors in the female rat urethra described in the present studies cannot be excluded. Indeed, a number of bladder disorders including interstitial cystitis are associated with abnormalities in NO production and changes in urothelial permeability, suggesting that nicotinic agonist-evoked NO release from the urethral urothelium may also be relevant in the aetiology of smoking-related incontinence (Birder *et al.*, 1988). Determination of the exact roles of these receptors in the physiology and pathophysiology of the urethra may lead to the development of novel therapeutic targets in the therapy of LUT dysfunction.

*Contractile urethral responses to DMPP: pharmacology and possible mechanisms of action*

In addition to relaxatory responses, DMPP also evoked a delayed contractile response of the isolated female rat urethra. These DMPP-evoked urethral contractions were insensitive to hexamethonium, chlorisondamine and TTX, indicating that they are not mediated by the activation of neuronal nicotinic receptors, or involve action potential generation. Furthermore, these DMPP-evoked urethral contractions were also evident after DMPP-evoked urethral relaxations had been inhibited with hexamethonium, showing that they occur independently of these DMPP-evoked inhibitory urethral responses. DMPP-evoked responses were also insensitive to a high concentration of d-tubocurarine, which is an antagonist of neuromuscular and neuronal nicotinic receptors (see Broadley, 1996), showing that they are not the result of neuromuscular nicotinic receptor activation. Indeed, contractile effects of DMPP that are not mediated by activation of neuronal or neuromuscular nAChRs have also been observed in the bovine retractor penis muscle (Klinge, Arlanta & Sjostrand, 1988). It has been suggested that DMPP causes the release of noradrenaline in a number of tissues, via a tyramine-like action, by displacing this transmitter from intraneuronal stores and by inhibiting its re-uptake (Holbach, Lindmar & Loffelholz, 1977). Furthermore, in the guinea pig heart, these sympathomimetic effects of DMPP have been shown to be unaffected by hexamethonium, and to be slow in onset and termination (Bhagat, Robinson & West, 1967). Indeed, in the present experiments, DMPP-evoked urethral contractions were unaffected by

hexamethonium and chlorisondamine, slow in onset and maintained. Therefore, it is possible that in the rat urethra, DMPP evokes the release of noradrenaline by nAChR-independent mechanisms, which in turn stimulates urethral  $\alpha_1$ -adrenoceptors to produce contractile responses (Chess-Williams, Aston & Couldwell, 1994). However, in the present study, prazosin, a selective  $\alpha_1$ -adrenoceptor antagonist, had no effect on DMPP-evoked contractions, in tissues pre-treated with L-NAME to inhibit DMPP-evoked urethral relaxations. However, the use of this antagonist *in vitro* is limited by the maximum concentration in the bath that can be achieved, as it is difficult to dissolve this agent at stock concentrations above 1 mg ml<sup>-1</sup>. Therefore it is possible that the amount of noradrenaline released by DMPP is sufficient to overcome the degree of  $\alpha_1$ -AR blockade that is achieved with this concentration of prazosin. Furthermore,  $\alpha_2$ -adrenoceptor stimulation has been shown to produce contractile responses of the rat (Willette, Sauermelch & Hieble, 1989) and rabbit (Morita *et al.*, 1987) urethra, raising the possibility that DMPP-evoked noradrenaline release also stimulates  $\alpha_2$ -adrenoceptors to produce contractile urethral responses. In addition, presynaptic  $\alpha_1$ - and  $\alpha_2$ -adrenoceptors may also play a role in these responses. Indeed, presynaptic facilitatory  $\alpha_{1A}$ -adrenoceptors have recently been identified in the rat bladder (Szell *et al.*, 2000), and  $\alpha_2$ -adrenoceptor antagonists have been shown to enhance urethral responses to hypogastric nerve stimulation in the rat (Willette, Sauermelch & Hieble, 1990). Therefore, prazosin may not produce sufficient blockade of these presynaptic receptors that play a role in the modulation of the effects of noradrenaline in the urethra. Interestingly, Seshita *et al.* (2000) have recently identified the presence

of inhibitory and facilitatory  $\alpha_1$ - and  $\alpha_2$ -adrenoceptors, respectively, on nitrenergic nerve terminals in the rabbit urethra, that modulate the release of NO. Therefore, an interaction between the DMPP-evoked release of NO and DMPP-evoked release of noradrenaline may also contribute to the urethral responses evoked by this agent. Experiments involving the measurement of noradrenaline release in urethral tissues in response to DMPP, and the use of selective  $\alpha_1$ - and  $\alpha_2$ -adrenoceptor antagonists would be useful in further investigating the mechanism(s) of action of this drug. Alternatively, DMPP could evoke the release of other excitatory transmitters in the urethra, by nAChR-independent mechanisms. Indeed, a DMPP-evoked relaxation of the guinea pig bladder, that is unaffected by hexamethonium, TTX, and adrenoceptor antagonists, has previously been reported (Kuwahara, 1980). In this respect, studies in the rat (Maggi *et al.*, 1989), rabbit (Mutoh *et al.*, 1997) and cat (Hassouna *et al.*, 1983), have demonstrated a strong contractile urethral response to cholinergic stimulation, raising the possibility that DMPP causes the release of acetylcholine in the isolated rat urethra to evoke contractile responses. However, in the present study, atropine, at a high concentration, had no effect on DMPP-evoked urethral contractions. Other excitatory transmitters and substances may also be released following exposure to DMPP. For example, 5-hydroxytryptamine (5-HT) produces contractile urethral responses in a number of species, acting through 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors (see Steers, 1995). The use of selective antagonists for a number of excitatory transmitters would prove useful in determining the exact identity of this transmitter(s) or regulatory substance(s). Finally, a direct action of DMPP on the smooth or striated muscles of the urethra (Kuwahara, 1980),

urethral neurones (Sorenson & Gallagher, 1996) or effects on novel nicotinic receptors that are distinct from neuronal and neuromuscular subtypes (Rao, Correa & Lloyd, 1997) cannot be excluded.

### *Conclusions*

In conclusion, the present study has demonstrated that the nicotinic receptor agonists, nicotine, epibatidine and DMPP, evoke relaxations of the isolated female rat urethra by stimulation of neuronal nicotinic receptor subtypes. Surprisingly, a neuromuscular nicotinic receptor-mediated contractile response of the striated muscle of this tissue could not be observed, although the reasons for this remain unclear. Nicotinic agonist-evoked urethral relaxations were also found to be unaffected by TTX, providing evidence against these receptors comprising neuronal nicotinic receptors of the ganglionic subtype. These receptors may therefore be located at a variety of neuronal and non-neuronal sites within the urethra, for example, pre-synaptically on nerve terminals and/or on the urethral urothelium. The urethral relaxations evoked by the nicotinic agonists employed in the present study appear to be mediated by the release of NO, although an involvement of other inhibitory neurotransmitters or regulatory substances cannot be excluded. Further, the release of these substances may be dependent on the location of these neuronal nicotinic receptors. The alternate mechanisms by which DMPP produces contractile responses in the isolated female rat urethra remain to be determined.

Overall, the results of the present study provide evidence against a role for neuronal nicotinic receptors of the ganglionic subtype at the level of the urethra in the chlorisondamine- sensitive interactions between urethral smooth and striated muscles previously described in this thesis (see chapter two). However, an involvement of non-ganglionic neuronal nicotinic receptors at the level of the urethra in this reflex cannot be excluded. Furthermore, an involvement of central nicotinic receptors remains a distinct possibility. Indeed, NO released from afferent nerve fibres in response to distension of the isolated rabbit stomach has recently been shown to modulate the central nicotinic activation of coeliac plexus neurones in the regulation of gastroduodenal inhibitory reflexes (Quinson, Niel & Miolan, 2000). Further studies are therefore required to determine the exact mechanisms of action of this reflex. In addition, elucidation of the exact mechanisms of action of nicotinic agonists in the isolated female rat urethra, and the subunit composition of the nicotinic receptors that mediate these responses, would prove useful in determining if these receptors possess alternative physiological roles in urethral control, and possibly provide novel therapeutic targets in the treatment of LUT dysfunction.

## **Chapter Six**

### **General Discussion**

### **6.1. General overview of the present study**

The primary objective of the present study was to investigate the pharmacological control of the female rat urethra using a combination of *in vivo* and *in vitro* studies. Although certain aspects of LUT function differ from humans, experiments in this thesis were performed on female rats, as the urethral response during micturition in the female rat and human is a relaxation, whereas in the male rat, urethral contractions are commonly observed. Therefore, the results of studies in the female rat urethra may be more closely correlated to the physiological and pharmacological control of the human urethra, with possible implications for the pharmacological treatment of LUT dysfunction.

### **6.2. The isolated female rat urethra: histological and functional characterisation**

The histological studies in this thesis have demonstrated the presence of both longitudinal and circular smooth and striated muscles in the isolated female rat urethral preparation (see chapter three). The question then arises as to whether the urethral striated musculature is functional in these experimental conditions. Indeed, Brading (1999) has previously noted that the properties of the striated muscle are difficult to study *in vitro*, as damage to the fibres and subsequent depolarisation of their membranes inactivates the action potential mechanism. In the present experiments, succinylcholine, a neuromuscular depolarising agent, failed to produce the classical rapid fasciculations associated with contraction of

this musculature that were observed in isolated female rat diaphragmatic preparations (see chapter three). Furthermore, a contractile response of the striated muscle to the nicotinic agonists, nicotine, epibatidine and DMPP, could not be demonstrated in the isolated female rat urethra (see chapter five). However, these agonists display selectivity for neuronal nicotinic receptors over neuromuscular nicotinic receptors, and the concentrations used in the present studies would be insufficient to interact with neuromuscular nicotinic receptors on the basis of previously published literature. Moreover, a contractile urethral response to succinylcholine and nicotinic agonists may not be observed because of the orientation of the striated muscle fibres, in that this region of musculature in the female rat urethra is circular, C-shaped and deficient posteriorly (as shown by the histological studies in chapter three), and therefore the mounting of this preparation longitudinally may not record an increase in tension in this plane. Examination of the effects of succinylcholine and nicotinic agonists on urethral preparations comprising only the mid-part of the whole female rat urethra, where striated muscle dominates, and mounted in the 'plane' of the striated muscle fibres may prove useful in delineating some of these issues. Alternatively, the striated muscle fibres may be damaged in this preparation, as a result of the dissection techniques or the experimental conditions, or the neuromuscular nicotinic receptors in the urethral striated muscle may be distinct from those of the rat diaphragm and therefore not respond to succinylcholine or nicotinic receptor agonists. These possibilities require further investigation. Despite the surprising lack of effects of these agents on the urethral striated musculature, the observation that electrical stimulation evoked urethral contractions at the same

frequencies that produced contractions of the isolated rat diaphragm, and the fact that this musculature can be visualised histologically, implies that a role for the urethral striated muscle in the effects of agents used in the *in vitro* studies in this thesis must be considered. In addition, an involvement of both the urethral smooth and striated muscles must be considered when correlating the results of *in vitro* and *in vivo* investigations, and when determining the relevance of these findings to the pharmacological control of the female rat urethra during urine storage and voiding.

### **6.3. NO in the female rat urethra**

The present studies have demonstrated that NO mediates urethral smooth muscle relaxations evoked by the micturition reflex with bladder distension and DMPP in the anaesthetised female rat (see chapter two). These data also indicate a role for this neurotransmitter in urethral relaxations evoked by electrical stimulation of the isolated female rat urethra (see chapter four). These relaxations are assumed to be mediated by relaxation of the urethral smooth muscle on the basis of previous *in vitro* investigations (Andersson, Malgren & Uvelius, 1990). Evidence for a physiological role for NO in relaxation of the smooth muscle of the urethra during voiding has been provided from a large number of previous *in vivo* and *in vitro* studies (see Andersson & Persson, 1994). However, it was important in the present studies to confirm a role for NO in these responses to be able to further investigate the roles of cGMP transduction in this tissue. Interestingly, relaxations of the female rat urethra evoked by the nicotinic

receptor agonists, nicotine, epibatidine and DMPP were attenuated by L-NAME, a NOS inhibitor, indicating a further role for NO in these responses. To the author's knowledge, these effects of nicotinic receptor agonists on this tissue have not been reported previously. These receptors may be located pre-synaptically, on the urethral urothelium or at other urethral sites. The relevance of these findings to the *in vivo* investigations in this thesis are considered in later sections of this discussion.

In addition to an important physiological role in relaxation of the urethral smooth muscle during voiding, the present studies have suggested a role for NO in the control of the urethral striated muscle. Indeed, L-NAME increased urethral striated muscle activity *in vivo*, suggesting that there is a tonic release of NO to this musculature (see chapter two). Moreover, L-NAME also increased baseline urethral tension *in vitro*, suggesting that the effects of this agent on urethral tone are due to a direct action on this tissue (see chapter four). Whether these L-NAME-evoked increases in tone in the isolated female rat urethra are also mediated by an effect on the urethral striated muscle, as suggested from these *in vivo* experiments, remains to be determined. Indeed, a role for the urethral smooth muscle, lamina propria and/or urethral urothelium in the effects of NOS inhibition in this tissue cannot be excluded, and further studies would prove useful in delineating the exact sites of action. The aforementioned effects of NO may be physiologically relevant in the prevention of excessive increases in urethral tone during periods of increased urethral tension, that have previously been suggested from the results of *in vitro* studies (Persson *et al.*, 1992).

#### 6.4. NO/cGMP signalling in the female rat urethra

Preliminary studies investigating the effects of PDE inhibition in the female rat urethra *in vivo*, showed that zaprinast, an inhibitor of PDE types 1, 5, 6 and 9, potentiated reflex- and DMPP-evoked urethral relaxations (see chapter two). These results suggested that these responses were mediated by increases in cGMP levels in this tissue. Furthermore, the earlier findings that reflex- and DMPP-evoked urethral relaxations are effected by the urethral smooth muscle, led to the suggestion that these effects of zaprinast would most likely be mediated by an increase in cGMP levels in this urethral layer, following inhibition of PDE 5. However, in further experiments in which the urethral striated muscle was paralysed with  $\alpha$ -Bungarotoxin, a potentiating effect of this PDE inhibitor could not be demonstrated. In fact these responses were now actually attenuated. These surprising observations are likely to be related to the ability of zaprinast in these experiments to increase both baseline and reflex-evoked urethral striated muscle activity. In this respect, potentiations of reflex- and DMPP-evoked urethral relaxations are thereby the result of an increase in urethral tone. There are a number of possible explanations for the ability of this zaprinast to produce this increase in urethral striated muscle activity, including inhibition of PDEs other than PDE 5, for example PDE 1, or a direct effect of this antagonist on striated muscle via modulation of glycogen synthesis, glucose oxidation or modulation of  $\text{Ca}^{2+}$ .

### **6.5. Reflex interactions between urethral smooth and striated muscles and the role of neuronal nicotinic receptors**

An alternative mechanism of action for the ability of zaprinast to increase urethral striated muscle activity was suggested from the effects of this agent on baseline urethral tone both *in vitro* and *in vivo*. In the isolated female rat urethra, zaprinast caused a decrease in baseline urethral tension and attenuated electrically-evoked urethral relaxations (see chapter four). Furthermore, in the anaesthetised neuromuscular-blocked female rat, zaprinast attenuated both reflex-evoked urethral relaxations and the increase in urethral tone that occurred over the period of the reflex, suggesting that this agent also lowered urethral smooth muscle tone *in vivo*. Therefore, it is possible that the effects of zaprinast on the urethral striated muscle are indirect, and the result of a zaprinast-evoked decrease in urethral smooth muscle tone. Indeed, this possible mechanism of action of zaprinast is supported by the fact that intraurethral administration of SNP and isoprenaline also caused a decrease in urethral tone and similarly increased urethral striated muscle activity (see chapter two). Furthermore, in the presence of chlorisondamine, a selective neuronal nicotinic receptor antagonist, an effect of SNP and isoprenaline on the urethral striated musculature was not observed, and a greater drug-evoked decrease in urethral tone was produced. As these agents are assumed to decrease urethral smooth muscle tone, these findings suggest that these agents evoke a reflex increase in urethral striated muscle activity in response to urethral smooth muscle relaxation, via the activation of neuronal nicotinic receptors. To the author's knowledge, the aforementioned

reflex between the urethral smooth and striated muscles has not been previously described. The exact mechanisms and sites of action involved in this reflex remain to be elucidated, although an involvement of ganglionic neuronal nicotinic receptors at the level of the urethra can be excluded on the basis of *in vitro* studies in this thesis showing that nicotinic receptor agonist-evoked urethral responses are resistant to TTX, and therefore independent of action potential propagation (see chapter five). However, an involvement of non-ganglionic neuronal nicotinic receptors at the level of the urethra, or nicotinic receptors at other peripheral and central sites cannot be excluded. The physiological relevance of these interactions remains to be determined, although a role in the maintenance of urethral tone, and thereby continence, can be envisaged. Further experiments to determine the exact mechanisms of action of these reflex interactions between the urethral smooth and striated muscles, and their physiological importance in the control of the female rat urethra may give rise to novel pharmacological approaches in the treatment of LUT dysfunction.

## 6.6. Does NO/cGMP signalling play a physiological role in the female rat urethra?

The surprising complex effects of zaprinast on the urethral striated muscle and overall urethral tone *in vivo* complicate investigations regarding the physiological roles of NO/cGMP signalling in the female rat urethra, and as a result, an unequivocal role for this transduction pathway in this tissue *in vivo* could not be demonstrated. However, experiments in the isolated female rat urethra found that a potentiating effect of zaprinast on the duration of electrically-evoked urethral relaxations was unmasked when the decrease in urethral tension that this agent evoked was 'compensated' for by re-tensioning urethral preparations (see chapter four). Therefore, it appears that cGMP plays a role in NO-mediated field stimulation-evoked urethral relaxations *in vitro*, but the complex effects of zaprinast on urethral tone are a limiting factor in the use of this drug in experiments to determine the role of NO/cGMP signalling in the female rat urethra in both *in vitro* and *in vivo* studies. Experiments investigating the effects of other cGMP-selective PDE inhibitors would prove useful to determine if these effects of zaprinast on the urethral striated muscle and urethral tone represent a generalised effect of this class of drugs, or are unique to this agent. Furthermore, alternative pharmacological approaches in the manipulation of the NO/cGMP transduction pathway, for example inhibition of guanylate cyclase or inhibition of one or more of the effector systems for the actions of cGMP in the urethra, for example, protein kinases, may be more appropriate. Indeed, Persson *et al.* (2000) have recently reported that mice lacking the gene for cGMP protein kinase type I

exhibit hyperactive voiding. Furthermore, urethral strips from these mice show an impaired relaxant response to NO and cGMP analogs. Alternatively, measurement of cGMP levels in the urethra may provide more definitive evidence for a role for this transduction system in NO-mediated relaxations of the female rat urethra during voiding.

## 6.6. Concluding remarks

The recent development of sildenafil, a selective PDE 5 inhibitor, for the treatment of male erectile dysfunction (Boolell *et al.*, 1996) has prompted interest in the use of PDE inhibitors to treat various other disorders. Amongst these is the use of PDE inhibitors to treat voiding disorders, where inhibition of cGMP-selective PDEs would be expected to potentiate NO-mediated urethral smooth muscle relaxations and thereby enhance urine flow in the treatment of urinary retention. However, the present results have significant implications for the manipulation of the NO-cGMP pathway in the treatment of voiding disorders. The potential for PDE inhibitors to produce an increase in urethral striated muscle activity, possibly mediated by reflex changes in response to urethral smooth muscle relaxation, must be taken into consideration in the development of novel therapeutic strategies for voiding dysfunction. In this respect, determination of the exact mechanisms of action of zaprinast to increase urethral striated muscle activity may lead to the development of novel therapies in the treatment of stress incontinence by increasing urethral striated muscle activity, and thereby urethral tone.

# **Chapter Seven**

## **References**

## A

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## APPENDIX

### PUBLICATIONS ARISING FROM THIS THESIS

WIBBERLEY, A., NUNN, P.A., NAYLOR, A.M. and RAMAGE, A.G. (2000). Investigation of the role of nicotinic receptors in the isolated female rat urethra. *British Journal of Pharmacology*, In Press.

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