Functional Models in the Search for Pharmacological Treatment of Urinary Incontinence

The Role of Adrenergic, Cholinergic, and Serotonergic Receptors

BY

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Abstract


Stress incontinence and overactive bladder are disorders with a common symptom, urinary incontinence, which is a serious medical and social handicap. Several neurotransmitters regulate the function of the lower urinary tract, including noradrenaline, acetylcholine, and serotonin.

The present study is part of the search for pharmacological incontinence drugs. The aims of this thesis were to improve the existing pharmacological treatments of urinary incontinence and to look for alternative treatments: i) an \( \alpha_1 \)-adrenergic agonist that preferentially affects urethral over blood pressure was tested \textit{in vivo}; ii) a modified cystometry model was developed for screening of muscarinic antagonists, by construction of a complete dose-response curve in each individual animal; iii) a new muscarinic antagonist, PNU-171990, was pharmacologically characterized \textit{in vitro} and \textit{in vivo}; iv) functional differences of the isomers of the muscarinic agonist BM-5 were characterized in the urinary bladder and ileum, \textit{in vitro} and \textit{in vivo}; v) the role of serotonin 5-HT\(_2\)A, 5-HT\(_3\) and 5-HT\(_4\) receptors were characterized on urinary bladder contractions \textit{in vivo}.

In the search for urethra selective compounds, the \( \alpha_1 \)-adrenoceptor agonists phenylephrine and phenylpropanolamine selectively enhanced blood pressure as compared to the urethral pressure in rabbit. This is in contrast to the effect of oxymetazoline and NS-49. Muscarinic antagonists produced a dose-dependent inhibition of the volume-induced micturition pressure in the rat. PNU-171990, a non-selective muscarinic antagonist, revealed selectivity for urinary bladder pressure over salivation (P<0.05). (R)-BM-5 induced bladder contraction and saliva secretion in cats. The selective serotonin 5-HT\(_2\)A and 5-HT\(_3\) receptor antagonists, ketanserin and tropisetron, both inhibited the effect of chemically induced bladder contraction in the anaesthetized cat.

In conclusion, an urethral-selective \( \alpha_1 \)-adrenoceptor agonist may be a good treatment of stress incontinence. A bladder-selective competitive muscarinic antagonist is considered a good pharmacotherapy for overactive bladder. In addition, the 5-HT\(_2\)A and 5-HT\(_3\) receptor antagonist may improve lower urinary tract symptoms.

Key words: urinary bladder, urethral, overactive bladder, stress incontinence, \( \alpha_1 \)-adrenergic, muscarinic, 5-HT\(_2\)A, 5-HT\(_3\), 5-HT\(_4\), agonist, antagonist.

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To my Family
All human beings are in truth akin;
All in creation share one origin.

When fate allots a member pangs and pains;
No ease for other members then remains.

If, unperturbed, another’s grief canst scan;
Thou are not worthy of the name of man.

The Iranian poet & mystic, Sadee,
born in Shiraz lived about 1207-1291
1 LIST OF ARTICLES

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


V. Ali-Reza Modiri, Jan Svartengren, Per-Göran Gillberg, The serotonin 5-HT$_{2A}$ and 5-HT$_{3}$ receptors mediate cat urinary bladder contraction *in vivo*. (Manuscript)
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### 3 ABBREVIATIONS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2-M-5-HT</td>
<td>2-methyl 5-hydroxytryptamine</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin, 5-hydroxytryptamine</td>
</tr>
<tr>
<td>5-MeOT</td>
<td>5-methoxytryptamine</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>CHO-cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>DD 01</td>
<td>PNU-200597, 5-hydroxymethyl metabolite of tolterodine, (+)-N,N-diisopropyl-3-(2-hydroxy-5-hydroxymethyl-phenyl)-3-phenylpropylamine</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dose required to produce 50% effect</td>
</tr>
<tr>
<td>&lt;sup&gt;3&lt;/sup&gt;H-QNB</td>
<td>(l)-(−)-quinuclidinyl (phenyl-4,4'-3H) benzilate</td>
</tr>
<tr>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dose required to produce 50% inhibition</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>m&lt;sub&gt;1&lt;/sub&gt;-m&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Muscarinic receptor genes 1 to 5</td>
</tr>
<tr>
<td>M&lt;sub&gt;1&lt;/sub&gt;-M&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Muscarinic receptor subtypes 1 to 5</td>
</tr>
<tr>
<td>PE</td>
<td>Poly ethylene catheter</td>
</tr>
<tr>
<td>PNU-171990</td>
<td>2-diisopropyl aminoethyl 1-phenylcyclopentane carboxylate hydrochloride</td>
</tr>
<tr>
<td>PVC</td>
<td>Poly vinyl catheter</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal pressure (contraction)</td>
</tr>
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</table>
4 INTRODUCTION

Urinary incontinence is a serious medical and social handicap, which affects more than 50 million people in the developed world (Abrams et al., 2000) and the cost is estimated to about 2-3% of the total health care budget in Denmark, US, and Sweden (the cost is estimated as about 5 billion SEK) (Milsom, 2000; Moller et al., 2001). It is particularly prevalent in older people, affects women (10%) at approximately twice the frequency of men (5%), and is often the reason an elderly person is forced to abandon independent living and enter a nursing home.

This condition is characterized by dysfunction of bladder muscle contractility and/or bladder sphincter muscle function (Wein, 2001). The function of these two organs is controlled by interplay between the central and peripheral nervous system and local regulatory factors (Andersson, 1993; Andersson et al., 1999). Injuries or diseases of the nervous system, and disorders of the peripheral organs, can produce voiding dysfunctions such as urinary frequency, urgency, and incontinence or inefficient voiding and urinary retention (de Groat et al., 2001).

Urinary incontinence is a symptom of bladder disorders such as urge, stress, mixed, and overflow incontinence. However, the therapeutic problem is not only urine leakage, but also associated symptoms, such as urgency and frequency (Andersson, 2000a). Abrams and Wein illustrated the symptomatology of overactive bladder and incontinence as shown in figure 1.

Stress incontinence is an involuntary loss of urine caused by an opening pull on the bladder orifice as in straining or coughing. Stress incontinence is a result of urethra sphincter incompetence and many factors such as urethral smooth muscle, the external urethral sphincter, the inner urethral factors, the pressure transmission to bladder and urethra, the pelvic floor muscle, hormones, connective tissue, and nerves may contribute to the symptom. The urethral smooth muscle and the urethral sphincter muscle contribute to the intraurethral pressure (Andersson, 2000a).

Overactive bladder describes the lower urinary tract symptoms of urgency and frequency of micturition with or without urinary incontinence (Abrams et al., 2000). Urgency incontinence is characterized by urinary incontinence preceded by sudden, uncontrollable impulse to discharge urine. The condition is due to detrusor instability or hyperreflexia (as a consequence of neurological disease) (Wein, 2001). Bladder overactivity may be the result of several different mechanisms, both myogenic (Brading, 1997) and neurogenic (de Groat, 1997). Most likely, mechanisms of both types contribute to the genesis of the hyperactive bladder.
Figure 1. Illustration shows the symptomatology of overactive bladder and incontinence (Abrams et al., 2000). The latter is represented by shaded area.

4.1 Normal voiding mechanisms
The function of the lower urinary tract is to store urine at low intravesical pressure and periodically eliminate urine by activation of the urinary bladder, urethra, and external urethral sphincter muscle. The neural circuits in the brain and spinal cord co-ordinate the activity of these components (de Groat et al., 1998). The pelvic, hypogastric and pudendal nerves innervate the lower urinary tract. Storage and voiding are dependent on efferent and afferent signaling in parasympathetic, sympathetic and somatic nerves (Figure 2).

Storage of urine is in part dependent upon spinal reflex mechanisms that activate sympathetic and somatic pathways to the urethral outlet as well as tonic inhibitory systems in the brain that suppress the parasympathetic outflow to the urinary bladder (de Groat, 1998).

Micturition occurs when the bladder volume reaches the threshold level. Just at that level, the mechanoreceptors increase the sensory signaling via Aδ-fibers to dorsal root ganglia and further to the lumbosacral spinal cord. These Aδ afferents are connected to an ascending limb to the pontine micturition center that is located in the rostral brain stem, and a descending limb from the micturition center to the parasympathetic nucleus in the lumbosacral spinal cord (Andersson, 2000a). The parasympathetic nerves reach the bladder through the pelvic ganglia and mainly release of acetylcholine, and activation of muscarinic
receptors, cause the bladder contraction (Andersson, 1993). Simultaneously, sympathetic and somatic reflex pathways are inhibited (de Groat, 1998), leading to evacuation of urine.

There are various neurotransmitters regulating the lower urinary tract such as acetylcholine, norepinephrine, serotonin, dopamine, excitatory and inhibitory amino acids, adenosine triphosphate, nitric oxide, and neuropeptides (de Groat et al., 2001). In this thesis, the focus has been on the role of $\alpha$-adrenergic mechanisms in urethra, and muscarinic and serotonergic mechanisms in urinary bladder.

### 4.2 The role of $\alpha$-adrenoceptors

In the human, urethral tone is largely maintained by activation of postsynaptic $\alpha$-adrenoceptors (Andersson, 1993). The $\alpha$-adrenoceptor antagonists phentolamine and phenoxybenzamine decrease the resistance to urinary flow (Caine et al., 1976). Phentolamine decreases the urethral pressure in normal women (Donker et al., 1972) and in patients with lower urinary tract obstruction
or incontinence (Abel et al., 1974; Awad et al., 1976; Whitfield et al., 1975). Noradrenaline increases resistance to flow in the isolated human fetal urethra and the increase is blocked by phenoxybenzamine (Andersson et al., 1978). Intravenous infusion of noradrenaline causes a dose-dependent increase in the maximum urethral pressure in both women and men (Mattisson et al., 1984) and α-adrenoceptor agonists cause contraction of isolated human urethral muscle (Ek et al., 1977) and can cause urinary obstruction (Boston, 1928). Consequently, α-adrenoceptor agonists, such as midodrine (Jonas, 1977), ephedrine (Diokno et al., 1975), phenylpropanolamine (Gillberg et al., 1998a) and methoxamine (Radley et al., 2001) have been used for treatment of stress incontinence. The most widely used drugs are ephedrine and norephedrine (Andersson, 1988; Wein, 1995b). However, the developed drugs have side effects and poor treatment efficacy and there is no good pharmacotherapy for stress incontinence (Andersson, 2000b; Kelleher et al., 1997; Radley et al., 2001).

It has been suggested that contraction of rodent urethra is caused by both postsynaptic α1- and α2-adrenoceptors (Andersson et al., 1984; Larsson et al., 1986; Yablonsky et al., 1986) and that the presynaptic autoreceptor in the guinea-pig urethra is of the α2A-adrenoceptor subtype (Alberts, 1992; Alberts, 1995b; Trendelenburg et al., 1997). In vitro, the α1A-adrenoceptor has been suggested to be the functional receptor subtype in the rat (Chess-Williams et al., 1994), rabbit (Auguet et al., 1995) and pig (Lefevre-Borg et al., 1993) urethra. In vivo, however, the functional urethral α-adrenoceptor does not appear to be unambiguously identified (Andersson, 1993; Lefevre-Borg et al., 1993; Martin et al., 1995; Shibasaki et al., 1992; Van der Graaf et al., 1997).

4.3 The role of muscarinic receptors
Muscarinic receptors mediate the bladder contraction of normal voiding and the main part of contraction in bladder overactivity associated with urge and urge incontinence (Andersson, 1993). Parasympathetic neurons release the neurotransmitters acetylcholine (ACh) and non-adrenergic non-cholinergic transmitters, possibly adenosine-5’-triphosphate (ATP) (Andersson, 1993) which act at muscarinic (de Groat et al., 1976) and P2X3-purinergic (Bo et al., 1990; Hoyle et al., 1989; Yiangou et al., 2001) receptors respectively to elicit contraction.

The muscarinic receptor family includes five molecularly distinct subtype receptors, all widely distributed in the body (M1-M5) (Caulfield et al., 1998; Hegde et al., 1999) and coded by five genes (m1-m5) (Bonner, 1989; Caulfield, 1993; Hulme, 1990). The postsynaptic muscarinic receptor of the rat urinary bladder in binding studies has been claimed to be a combination of both M2 and M3 subtypes (Monferini et al., 1988). Muscarinic m2 and m3 receptors have been
identified in the rat urinary bladder using subtype-selective antisera (Wall et al., 1991). In rat urinary bladder, mRNA coding for m3 muscarinic receptors has been identified, but the presence of mRNA for m1 and m2 muscarinic receptors were not tested (Maeda et al., 1988). In the pig, urinary bladder mRNA coding for m2 and m3 muscarinic receptors have been identified using blot hybridization (Maeda et al., 1988). Functional studies suggest that contraction of guinea-pig urinary bladder is mediated by receptors pharmacologically different from cardiac receptors (Noronha-Blob et al., 1989). The ratio of m2 to m3 immunoprecipitated solubilized muscarinic receptors is 3:1, 9:1, 3:1 and 3:1 in the human, rat, rabbit and guinea-pig urinary bladder respectively (Wang et al., 1995) (subtypes m1, m4 and m5 did not precipitate). M1, M2, and M3 receptor subtypes, with a distinct predominance of M3 receptors, were detected in the human detrusor muscle by receptor binding (Kondo et al., 1995). However, M2 receptor mediated effects can be observed after selective M3 receptor inactivation (Yamanishi et al., 2002).

The presynaptic autoreceptor in urinary bladder has been classified as the M4 subtype in the guinea pig (Alberts, 1995a), rat (D'Agostino et al., 1997; Shen et al., 2001), and man (D'Agostino et al., 2000) and M2 subtype in rabbit (Tobin et al., 1995) and rat (Somogyi et al., 1992). The facilitatory pre-junctional muscarinic receptor has been suggested to be M1 in rat and rabbit urinary bladder (Somogyi et al., 1994; Tobin et al., 1998). In addition, M1 and M3 receptors appear to be localized in salivary glands (Tobin, 1995).

In smooth muscle, activation of the M3 subtype appears to mediate direct contraction and activation of the M2 subtype can elicit an indirect contraction by counteracting cyclic AMP-mediated relaxation (Hegde et al., 1997). Thus, agents with affinity to muscarinic receptor subtypes M2 and M3 have therapeutic potential for treatment of disorders associated with altered smooth muscle contractility or tone such as overactive bladder.

4.4 The role of serotonergic receptors

Serotonin (5-hydroxytryptamine: 5-HT), one of the several natural neurotransmitters, has profound effects on the function of the lower urinary tract (de Groat et al., 1993). Serotonergic control of lower urinary tract is complex and most experimental studies have been done in the cat. The serotonin receptor system has 14 different subtypes.

The 5-HT1A autoreceptor in the raphe nucleus provides an inhibitory control over raphe-spinal activity (de Groat et al., 1993; Testa et al., 2001). In addition, at the spinal level, 5-HT has inhibitory action on bladder function. Activation of postsynaptic 5-HT receptors in the spinal cord inhibits on-going bladder contraction (Thor et al., 1990).
It has been shown that intrathecal administration of 5-HT increased sympathetic activity, which can cause relaxation of urinary bladder muscle (Espey et al., 1992) and increased micturition volume threshold in cat mediated by 5-HT₃ receptor activation (Espey et al., 1995). Espey and co-workers have shown that activation of 5-HT₃ receptor has an inhibitory effect on ascending activity and also facilitates spinal reflex activity caused by activation of bladder afferents in anaesthetized cat (Espey et al., 1998). In addition, the serotonin 5-HT₁C (5-HT₂C) receptor is involved in parasympathetic inhibitory mechanisms (de Groat et al., 1993).

In vitro, serotonin produces marked contraction in cat (Cohen et al., 1989), dog (Cohen, 1990), pig (Sellers et al., 2000) and human (Klarskov et al., 1986) urinary bladder strips. In contrast, direct contractile effects of 5-HT are absent in the rat, guinea pig (Cohen et al., 1989) and monkey (Waikar et al., 1994) urinary bladder. In vivo, 5-HT-induced bladder contraction has been indicated to be mediated by serotonin 5-HT₂ and M (=5-HT₃) receptors (Saxena et al., 1985).

In human urinary bladder 5-HT has multiple effects on bladder contractility by e.g. direct 5-HT evoked contraction (Klarskov et al., 1986) or modification of neuronal release of the ACh (Corsi et al., 1991; Tonini et al., 1994). In pig (Khan et al., 2001; Sellers et al., 2000) and human (Corsi et al., 1991) bladder strips presynaptic 5-HT₄ receptors have been reported to be responsible for potentiation of electrical field stimulation. Therefore, a 5-HT₄ receptor antagonist may have therapeutics potential for treatment of overactive bladder.
4.5 Objectives
The present study is part of the search for pharmacological compounds for treatment of urinary incontinence. The aims of this thesis were to improve the existing pharmacological treatments of urinary incontinence and to look for alternative treatments, and also to establish and/or to develop models that can identify new and potential compounds for further development in the treatment of stress incontinence and overactive bladder.

The aims of the present studies have been:

- To test adrenergic agonists for urethra selectivity in the anaesthetized female rabbit urethra (paper I).
- To establish an in vivo model to screen new muscarinic antagonists effect on micturition pressure in normal, conscious rats (paper II).
- To characterize a new compound, PNU-171990, with the aim of identifying a candidate drug with urinary bladder selectivity at least as high as tolterodine, which is the “golden standard” for treatment of overactive bladder (paper III).
- To explain the observed diversity in functional responses of racemic BM-5 by studying the affinity, efficacy and muscarinic receptors subtype selectivity (paper IV).
- To pharmacologically characterize the role of 5-HT$_2$A, 5-HT$_3$ and 5-HT$_4$ receptors in contraction of the cat urinary bladder in vivo (paper V).
5 MATERIALS AND METHODS

All the experiments were approved by the local animal ethical committee (Uppsala Djurförsöksetiska Nämnd), Tierps district court.

5.1 In vivo

5.1.1 Urethra and blood pressure (I)

Adrenoceptor agonist effect on urethral pressure, blood pressure and heart rate was determined in the anaesthetized New Zealand White female rabbits (2.2-4.0 kg, Estuna AB and Lidköpings Rabbit Farm, Sweden). Animals were anaesthetized by using pentobarbital (0.17 mg/kg/min, 30 mg/ml in physiological saline).

A ureteric PVC catheter with one 'eye' about 5 cm from the tip was inserted in the urinary bladder through the external urethral meatus. The urethral catheter was continuously perfused with physiological saline at 4 ml/h, connected to a pressure transducer and the pressure was recorded on a Grass Polygraph. The urethral pressure profile was obtained by withdrawing the urethral catheter from the urinary bladder. The catheter was fixed at the place of maximum pressure (Gillberg et al., 1998a).

Noradrenaline (25 µg/kg) in 1 ml physiological saline was injected in the catheterized right femoral vein in 1 min with a pump. Noradrenaline injections were made to ascertain that the agonist caused an increase in urethral and blood pressure. Test compounds and physiological saline were infused in a volume of about 1 ml in about 1 min. The urethral pressure, the blood pressure in the left femoral artery and the electrocardiogram were recorded continuously on a Grass Polygraph.

The effect of drugs on urethral pressure, blood pressure and heart rate was related to the basal level before administration of drug. The dose-response curves were characterised by an equation describing a hyperbolic function containing two constants, \( y = \frac{P_1 \times x}{P_2 + x} \), where \( y \) is the measured effect, \( x \) is the dose, \( P_1 \) is the maximal effect asymptotically approached at 'infinitely' high dose, i.e. the 'maximal enhancement' or 'maximal inhibition', and \( P_2 \) is the dose yielding half the maximal effect 'ED_{50}' or 'ID_{50}'. The constants, 'maximal enhancement', 'maximal inhibition', ED_{50} and ID_{50}, were calculated using an iterative non-linear regression computer program (Fig.P for Windows, Biosoft, UK) (Alberts, 1992).

The following chemical compounds were used: 2-((2,6-dimethoxy-phenoxethyl) aminomethyl)-1,4-benzodioxane (WB-4101), oxymetazoline hydrochloride, L-phenylephrine hydrochloride (Sigma/Aldrich), clonidine
hydrochloride (Boehringer Ingelheim), rauwolscine hydrochloride, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304) (Research Biochemicals Inc.), (±)-phenylpropanolamine hydrochloride (norephedrine) (Alps Pharmaceuticals, Furukawa-Cho, Gifu Prefecture, Japan), (±)-3´-(2-amino-1-hydroxyethyl)-4´-fluoromethane-sulfonanilide hydrochloride ((±)-NS-49, Pharmacia & Upjohn, Uppsala). All other chemicals were obtained from general sources and were of analytical grade.

5.1.2 Cystometry (II)
Muscarinic antagonist potency of tolterodine, its major metabolite and reference substances were determined by using volume-induced contraction in the urinary bladder of conscious female Sprague-Dawley rats (200-560 g, Charles River, Germany). The animals were anaesthetized intraperitoneally (1.5-2.0 ml/kg) with Dormicum® (1.25 mg/ml; Roche, Basel/Schweiz) and Hypnorm® (fluanisonum 2.5 mg/ml; fentanylum 0.05 mg/ml, Janssen, Belgium).

Two days after the surgical procedure, the pressure transducer was connected to a Grass Polygraph (7 DAG, USA) and the micturition pressure determined graphically. A fluid collector was connected to a Grass force displacement transducer (FT03C) to measure micturition volume (Grass Polygraph). Saline (1 ml/kg) was injected intravenously and the micturition registration was continued for at least 20 min for the rat to adapt to the test situation. The rat was then allowed to rest for 3-5 hours.

After the resting period, continuous intravesical saline infusion (12 ml/h) was restarted. A first intravenous saline (1 ml/kg) injection was given and a cystometry profile was recorded for 20 min. This control cystometry was directly followed by administration of test compound or saline and recordings were continued for 20 min. Cumulative dose response curves of muscarinic antagonists or saline using 3-4 different doses were generated for each compound. This protocol ensured stability in micturition pressure, micturition number, micturition volume, bladder capacity and residual volume.

Stability and reproducibility of the volume-induced contraction were monitored in a parallel control group of animals to each test group. The control animals were injected with intravenous saline 4 consecutive times. The inhibition of spontaneous volume-induced bladder contraction (ID50) values was calculated using a non-linear regression computer program (Fig. P).

The following chemical compounds were used: Tolterodine hydrogentartrate, PNU-200577 mandelate (DD 01; (+)-N, N-diisopropyl-3-(2-hydroxy-5-hydroxymethyl-pheny)-3-phenylpropylamine), N-desethyl-oxybutynin, (±)-terodiline hydrochloride, darifenacin fumarate, propiverine (Pharmacia), and atropine sulfate, carbamylcholine chloride (carbachol),
oxybutynin hydrochloride (Sigma). All other chemicals were obtained from general sources and were of analytical grade.

5.1.3 ACh-induced bladder contraction and electrically stimulated salivation (III)
The muscarinic antagonist effect of PNU-171990, 2-diisopropyl aminoethyl 1-phenylcyclopentane carboxylate hydrochloride (Pharmacia) was studied in female European short-haired cats (Nilvebrant et al., 1997a). Initially anaesthesia was induced by injection of pentobarbital (30–39 mg/kg) and infusion of α-Chloralose (25 mg/min in 5% sodium tetraborate; 25 mg/kg) in a saphenous vein throughout the experiment. Bladder contraction, measured isovolumetrically, was evoked by intra-arterial injection of submaximal doses of acetylcholine (1-4 µg/kg) 1 min before and about 9 min after administration of each test compound dose. Salivation was induced by submaximal stimulation of the lingual nerve (6V, 2 ms, 5 Hz, 2.0 min periods) with a bipolar platinum ring electrode, 7 min before and approximately 7 min after administration of each chemical compound dose. PNU-171990 (0.03-3 mg/kg) or saline was given at 1 ml/kg/min.

The ID50 values for PNU-171990 affecting the urinary bladder pressure and the salivary secretion were determined from each individual dose-response curve by linear regression (Nilvebrant et al., 1997a). Statistical analysis was performed using Student’s t-test. All chemicals were obtained from general sources and were of analytical grade.

5.1.4 (R)-BM-5-induced bladder contraction and salivation (IV)
The in vivo effects of (R)-BM-5 were studied in the anaesthetized cat. Female European short-haired cats (2.4-3.5 kg, 9±1 months of age) were used in the experiments. They were initially anaesthetized with an intraperitoneal injection of pentobarbital sodium (30–39 mg/kg) followed by intravenous administration of α-chloralose (25 mg/min in 5% sodium tetraborate; 25 mg/kg). For bladder pressure recordings, a catheter (PE 240) was inserted into the urinary bladder through an incision of the proximal urethra. The catheter was connected to a pressure transducer (Statham p23A) for recording of intravesical pressure before and 7 min after dose of saline or compound. For measurement of salivation the duct of the submandibular gland was exposed in the neck and catheterized (PE 10). Saliva was collected for 2 min (during 4-6 min after each dose) and weighed. (R)-BM-5 (0.3-300 µg/kg) was administered by intravenous infusion in the right femoral vein at a rate of 1 ml/kg/min. IC50 values were calculated in each individual experiment by linear regression, using data from the linear part of the concentration-response curve.
The following chemical compounds were used: pentobarbital sodium (Apoteksbolaget, Sweden); α-chloralose (Merck, Germany). R-BM-5 and S-BM-5 (both oxalate salts) (Department of Organic Pharmaceutical Chemistry, Uppsala University, Uppsala, Sweden). The R-enantiomer was formulated as (R)-BM-5(COOH)\textsubscript{0.1}((C\textsubscript{2}H\textsubscript{5})\textsubscript{2}O) (MW 373 g/mol) and the (S)-enantiomer as (S)-BM-5(COOH)\textsubscript{2} (MW 366 g/mol). All other chemicals were obtained from general sources and were of analytical grade.

5.1.5 Intravesical electrical stimulation and chemically induced bladder contraction (V)

Nineteen European shorthaired female cats 7-9 months old, 2.7-3.5 kg, from Laboratory Cat Farm, Halmstad, Sweden, were used.

The cats were initially anesthetized with Saffan (alphaxanol-alphadolone mixture, Glaxovet) 20-25 mg/kg intramuscularly, followed by intravenous infusion of α-chloralose (25 mg/ml, 260-310 µl/kg/h) throughout the experiment.

The animal was tracheotomized and the left femoral artery was catheterized with a polyethylene tube (PE 50, Clay Adams, NJ, USA) in order to measure blood pressure and heart rate by a Gould P23 ID transducer connected to a Grass EKG Tachograph Model 7P4F and a Grass Polygraph Model 7D (Statham, Boston, Mass., USA).

The right femoral artery was catheterized with a polyethylene tube (PE 50), which was inserted about 7 cm from the femoral triangle towards the heart and used for administration of agonists and collection of blood samples.

The right femoral vein was catheterized with a polyethylene tube (PE 90) and used for infusion of the Ringer solution at 15-30 ml/h (Sage model 355 pump, Cambridge, Mass., USA), and antagonists and physiological saline at 1 ml/kg/min (CMA/100, Carnegie Medicine, Stockholm, Sweden).

An incision was made along the mid-line, linea alba, of the abdomen. In order to denervate the urinary bladder, the hypogastric nerve in the intestinal mesenterium and both right and left pelvic nerves dorsal to the urinary bladder were identified and cut. Catheters (PE 10) were inserted into both ureters so that urine could not pass into the urinary bladder. The mid-urethra was opened and a catheter PE 240 with or without an unipolar silver electrode (0.6 mm diameter) was inserted through the mid-urethra into the urinary bladder. This catheter was connected to CMA/100 microinjection pump and grass P23 ID pressure transducer.

In animals with intact urinary bladder innervation, 2-3 cystometry cycles were performed by intravesical infusion of warm saline; each cystometry caused
a spontaneous micturition cycle with spontaneous relaxation after the maximal contraction. Bladder volume was adjusted to 90% of bladder capacity. The threshold volume was 13.1±2.8 ml.

An intravesical electrode was used as cathode and a copper plate wrapped in a saline soaked gauze-pad under the abdominal skin at the level of the bladder as anode (Ebner et al., 1992). The electrodes were connected to a Grass stimulator. The urinary bladder was continually stimulated until the spontaneous inhibition occurred, at the optimal stimulation frequency 20 Hz, 20-35 V with 0.2 millisecond pulse duration.

The selective 5-HT4 agonists (cisapride, S-zacopride, SC53116) in doses of 0.001-0.1 µg/kg caused no bladder contraction, measured isovolumetrically (n=1-2).

In the animals with intact bladder innervations, 5-MeOT (10 µg/kg, i.a.) followed by two IVES and 2-M-5-HT (10-30 µg/kg, i.a) for 2, 6, 12, 16 min respectively, evoked bladder pressure. This schedule was used repeatedly after administration of each test compound dose or physiological saline. The cumulative doses of tropisetron (0.0001 -1 mg/kg, n=2), SB204070 (0.0001-1 mg/kg, n=2), and control (time control saline, n=2) were given intravenously at intervals of 20-24 min.

Other selective 5-HT4 antagonists, SB207710 (n=1), GR 125487 (n=1), GR 113808 (n=1), were also tested in doses of 0.1-1 mg/kg.

In animals with denervated urinary bladder, the bladder was filled by warm saline during 15 min (1 ml/min). Agonists, 5-MeOT (0.3, 1, 3, 10 and 30 µg/kg), 5-HT (0.3, 1, 3, 10 and 30 µg/kg), ACh (0.5, 1, 2, and 4 µg/kg) and 2-M-5-HT (0.3, 1, 3, 10 and 30 µg/kg), in 0.5 ml physiological saline were injected in the right femoral artery. In each experiment a control dose-response curve for agonists-induced urinary bladder contraction was constructed. These agonists, at the present concentration, exerted no prolonged effect on homeostasis condition.

A submaximal dose of the agonists, 5-MeOT (10 µg/kg), 5-HT (1-3 µg/kg), ACh (2-4 µg/kg) and 2-M-5-HT (10-30 µg/kg) was injected before and 2, 6, 10, 15 min, after administration of each test compound dose or physiological saline. Using this schedule (n=4), cumulative doses of antagonists, tropisetron (0.0003-0.01 mg/kg) followed by ketanserin (0.003-0.3 mg/kg) and atropine (0.03-0.3 mg/kg) were administrated in a 20-22 min interval. The stability of this model was tested by repeated administration of intravenous saline (time control saline, n=4).

The shape of both the agonist and antagonist dose-response curves was hyperbolic. The 'ID50' and 'EC50' were calculated as previously described (Alberts, 1992). The constants were calculated using a non-linear regression
computer program (Fig. P). The graphs shown in the figures are drawn using the fitted constants. All data are expressed as mean ± SEM, the number of animals used and dose-response curves made (n) is shown.

The following chemical compounds were used: 5-hydroxytryptamin hydrochloride (5-HT), 5-methoxytryptamine hydrochloride (5-MeOT), acetylcholine chloride (ACh), 2-methyl-5-hydroxytryptamine maleate (2-M-5-HT), tropisetron (3-tropanyl-indol-3-carboxylate HCl), ketanserin tartrate, mesulergine hydrochloride, and SC-53116 hydrochlorid (Sigma-RBI, Saint Louis, USA), GR 125487, and GR 113808 (Glaxo, UK), SB204070 and SB207710 (SmithKline Beecham, UK), S-zacopride (Synthelabo Recherche, France), and cisapride (Janssen Research Foundation, Belgium). All other chemicals were obtained from general sources and were of analytical grade.

5.2 In vitro

5.2.1 Functional studies in isolated tissues (II, III, IV)

Muscarinic antagonist potency was determined in urinary bladder of rat and guinea pig (Nilvebrant et al., 1997a) and in ileum of guinea pig and human. Longitudinal muscle preparations were mounted in organ baths (physiological Krebs-Henseleit solution, containing (mM): NaCl 119, KCl 4.6, CaCl₂ 1.5, MgCl₂ 1.2, NaHCO₃ 20, NaH₂PO₄ 1.2, D-glucose 11, and hexamethonium 0.1 (Nordström et al., 1983)) under a passive resting tension of 5 mN (human ileum, rat and guinea pig bladder) and 10 mN (guinea pig ileum) equivalent to 0.5 and 1 g weight, and allowed to contract by the muscarinic agonist carbachol (1 µM). The solution was aerated with 5 % CO₂ in O₂ to give pH 7.4 at 37 °C.

Concentration-response curve was performed by cumulative addition of carbachol to the organ bath until the contraction did not increase any further. Muscle strips were washed before adding any compound. Antagonist was incubated for 15 min (ileum) and 60 min (bladder) (Nilvebrant et al., 1983) and the effect on agonist concentration-response curves was determined.

EC₅₀ values were calculated from each concentration-response curve using non-linear analysis (Fig.P) were used to calculate pA₂ and pKₐ values (Alberts et al., 1999) and a Kₐ value was determined by linear regression (Kenakin, 1997).

The following chemical compounds were used: carbamylcholine chloride (carbachol), hexamethonium bromide (Sigma/Aldrich, St. Louis, MO, USA), PNU-171990. All other chemicals were obtained from general sources and were of analytical grade.
5.2.2 Radioligand binding studies (III, IV)

Competition binding at muscarinic receptors was determined with (l)-(−)-quinuclidinyl (phenyl-4,4'-3H) benzilate (1 nM, ³H-QNB) in male guinea pig (Dunkin Hartley, 300-500g, Charles River, and National Veterinary Institute, Uppsala) tissue homogenates and in Chinese hamster ovary (CHO) cells expressing human muscarinic receptor subtypes (m₁-m₅) (Nilvebrant et al., 1997a). Incubation (25 °C) under equilibrium conditions was 60 min for urinary bladder, 210 min for parotid gland, 80-100 min for heart, 80 min for cortex, and 330 min (Nilvebrant et al., 1988) for CHO cell homogenates at 37 °C.

Non-specific binding was defined in the presence of atropine (10 µM). Incubations were done in 24 and 96-well microtiter plates and rapidly filtered on GF/B plates. Radioactivity was measured in a Packard TopCount™ scintillation spectrometer.

The antagonist concentration that inhibited ³H-QNB binding by 50% (IC₅₀) was calculated by non-linear regression analysis (“Add-in” to Microsoft Excel, XL-fit, ID Business Solutions, Surrey, UK). Kᵢ values were calculated from IC₅₀, Kᵢ=IC₅₀/(1+(S/Kₐ₅₀)), assuming competitive inhibition (Cheng et al., 1973).

The following chemical compounds were used: ³H-QNB (Amersham Pharmacia Biotech, and Du Pont NEN Research Products, Boston, MA, USA), phenylmethylsulphonyl fluoride (Sigma, St Louis, MO, USA); Dulbecco’s modified Eagle’s medium and HAM’s F12 (National Veterinary Institute, Uppsala, Sweden); fetal bovine serum (HyClone Lab, Logan, UT, USA); L-glutamine, penicillin and streptomycin (ICN Biomedicals, Costa Mesa, CA, USA). All other chemicals were obtained from general sources and were of analytical grade.
6 RESULTS

6.1 The effect of $\alpha_1$-adrenoceptor agonist (I)

The basal urethral pressure was recorded in anaesthetized rabbits, and was 12.3 ± 0.84 cm H$_2$O ($n=51$, $N=51$). In control experiments, six repeated infusions of physiological saline at intervals of minimum 20 min did not change the basal urethral pressure; the mean basal urethral pressure in these experiments was 12.3 ± 1.0 cm H$_2$O ($n=30$, $N=5$).

Oxymetazoline, NS-49, phenylephrine and phenylpropanolamine enhanced the urethral pressure in the anaesthetized rabbit in a dose-dependent manner. The urethral pressure obtained at the highest dose was 23 ± 3 cm H$_2$O (oxymetazoline, 0.03 mg/kg, $N=7$), 25 ± 6 cm H$_2$O (NS-49, 0.3 mg/kg, $N=3$), 43 ± 11 cm H$_2$O (phenylephrine, 0.3 mg/kg, $N=4$), and 33 ± 9 cm H$_2$O (phenylpropanolamine, 10 mg/kg, $N=5$). Clonidine (0.0003 - 0.3 mg/kg) and UK 14,304 (0.0003 – 0.1 mg/kg) had no effect on the urethral pressure at the doses tested.

Repeated infusion of oxymetazoline (0.003 mg/kg; $N=5$) increased the urethral pressure. Infusion of physiological saline had no effect on subsequent oxymetazoline-evoked increase in urethral pressure. The increase in urethral pressure caused by oxymetazoline (0.003 mg/kg) was inhibited in a dose-dependent manner by rauwolscine and WB-4101.

The basal blood pressure and heart rate was recorded in anaesthetized rabbits. In control experiments, six repeated infusions of physiological saline did not change the basal blood pressure and heart rate. The basal systolic blood pressure was 86.1 ± 3.7 mmHg ($n=30$, $N=5$), the basal diastolic blood pressure was 60.6 ± 3.0 mmHg ($n=30$, $N=5$), and the basal heart rate was 243 ± 9 beats per min ($n=24$, $N=4$).

Oxymetazoline, NS-49, phenylephrine and phenylpropanolamine increased the systolic (by 40-61%) and diastolic (by 52-80%) blood pressure and decreased the heart rate (by 10-24%). Clonidine and UK 14,304 had no effect on the blood pressure and decreased the heart rate by about 35 % at 0.1 mg/kg.

6.2 The effect of muscarinic antagonists (II)

The basal cystometrical values after the first saline injection were for micturition pressure (132.1±4.7 cm H$_2$O); micturition number (4.58±0.23); micturition volume (0.89±0.036 ml); bladder capacity (0.87±0.035 ml); and residual volume (-0.016±0.012 ml) (n=76).

*In vivo* intravenous infusion of atropine, PNU-200577, tolterodine, oxybutynin, darifenacin, desethyl-oxybutynin, propiverine, and terodiline all
produced a dose-dependent inhibition of the intravesical volume-induced rat urinary bladder contraction measured as the micturition pressure. The parallel control group of animals, showed no changes in micturition pressure, micturition number, micturition volume, bladder capacity and residual volume after four intravenous injections of saline (n=25).

The rank order of ID$_{50}$ values (nmol/kg) of the muscarinic antagonists was atropine (14±4), PNU-200577 (22±12), tolterodine (94±20), oxybutynin (175±89), darifenacin (236±144), desethyloxybutynin (313±209), propiverine (4561±2079), and terodiline (18339±5348). The compounds did not cause changes in micturition number, micturition volume, residual volume or bladder capacity (data not shown).

The concentration-response curve for the muscarinic agonist carbachol was determined in the rat urinary bladder in vitro. The calculated EC$_{50}$ value was 4.2±0.5 µM (n=16). The contractile response to carbachol (6 µM) was inhibited in a time-dependent manner by tolterodine, PNU-200577, atropine, oxybutynin and (±)-terodiline. A standard antagonist incubation time of 60 min was chosen, i.e. a time 2.6-6.6-fold, that which caused half-maximal inhibition.

The in vivo pID$_{50}$ values correlated significantly with the present rat in vitro pK$_{B}$ values (p<0.01, n=5, correlation coefficient = 0.98) and with anaesthetized cat in vivo pID$_{50}$ values (p<0.05, n=7, correlation coefficient = 0.99 (Gillberg et al., 1997; Gillberg et al., 1998b; Nilvebrant et al., 1997a; Nilvebrant et al., 1997b; Nilvebrant et al., 1996) including cat terodiline pID$_{50}$ = 5.06; Modiri et al., unpublished). The present rat in vitro pK$_{B}$ values correlated significantly with guinea pig in vitro pK$_{B}$ values (p<0.01, n=5, correlation coefficient = 0.98 (Gillberg et al., 1997; Gillberg et al., 1998b; Nilvebrant et al., 1997a; Nilvebrant et al., 1997b; Nilvebrant et al., 1996) including guinea pig terodiline pID$_{50}$ = 6.69; Modiri et al., unpublished).

Tolterodine, PNU-200577, atropine, oxybutynin and (±)-terodiline (the latter at concentrations not exceeding 1 µM) caused a parallel shift of the concentration-response curve to the right and did not alter the maximal contraction. The Schild plots of all antagonists were linear with slopes close to unity. The pK$_{B}$ (n, Schild plot slope) values were for atropine 9.1±0.03 (10, 1.01), PNU-200577 9.04±0.05 (12, 1.22), tolterodine 8.71±0.02 (12, 1.01), oxybutynin 7.95±0.04 (24, 1.18), and terodiline 6.53±0.04 (10, 1.16).

6.3 The effect of a new muscarinic antagonist (III)

In radioligand binding studies, PNU-171990 and atropine inhibited the specific binding of $^{3}$H-QNB in homogenates of guinea pig urinary bladder, cerebral cortex, heart, and parotid gland, and in cloned human receptors in a concentration dependent manner. In guinea pig tissue homogenates, the affinity
appeared to be highest for receptors in cerebral cortex (2.3 nM), lowest in bladder (42.7 nM) and intermediate in heart (14.6 nM) and parotid gland (12.5 nM). In human cloned receptors expressed in CHO-cells, the dissociation constants (Ki nM) values were 19, 64, 25, 25, and 20 for M₁ to M₅ receptors, respectively.

In functional *in vitro* studies, concentration-response curves using carbachol were constructed. The calculated EC₅₀ values for carbachol were in the guinea pig ileum (0.31±0.043 µM, n=46, N=16) and human ileum (0.95±0.084 µM, n=51, N=12) longitudinal muscle, where (n) is number of preparations and (N) is number of guinea pigs and patients.

In the guinea pig and human ileum, and the guinea pig urinary bladder, PNU-171990 and atropine caused a parallel shift in the carbachol concentration-response curve. The Schild-plots were linear in the concentration-range tested with slopes not significantly different from negative unity. The dissociation constants (Ki nM) values were 3.3 and 20 guinea pig ileum and urinary bladder and 79 for human ileum.

In the anaesthetized cat, PNU-171990A inhibited both the acetylcholine-induced contraction on the urinary bladder intravesical pressure and the electrically induced salivary secretion in a dose dependent manner. PNU-171990 inhibited urinary bladder pressure and salivation with ID₅₀ values of 0.25±0.04 mg/kg and 0.73±0.13 mg/kg, respectively. The effect on urinary bladder contractions occurred at significantly lower doses than effect on saliva secretion achieving a selectivity index of 2.9 at ID₅₀. Administration of saline did not alter bladder contraction or salivation.

### 6.4 The effect of muscarinic partial agonist, BM-5 (IV)

In the functional studies, both enantiomers of BM-5 induced contractions of the isolated urinary bladder and ileum (I). Compared with the ileum, the maximal agonist effect was very low in the bladder for (R)- and (S)-BM-5. (R)-BM-5 was the most potent enantiomer. (R)-BM-5 was significantly (*P* < 0.05) more potent in the ileum as compared to the bladder, while (S)-BM-5 showed similar potency in both tissues.

The contractions induced by (R)-BM-5 and (S)-BM-5 were completely inhibited by 1 µM atropine. Carbachol-induced contractions of the bladder were competitively inhibited by both enantiomers of BM-5, giving Schild plots with slopes that were close to unity.

Both (R)- and (S)-BM-5 inhibited the specific binding of (−)³H-QNB in a concentration-dependent manner. Hill coefficients were not significantly different from unity, and no separation of high and low affinity states of the receptors could be detected. (R)-BM-5 showed about 40-times higher affinity...
than (S)-BM-5 at M₁–M₅ receptors. The enantiomers bound with similar affinities to all muscarinic subtypes. The selectivity for one subtype over another was less than 5-fold.

The specific binding of (–)³H-QNB in ileum and bladder was inhibited by (R)- and (S)-BM-5 in a concentration-dependent manner. (R)-BM-5 was 43-60-times more potent than the (S)-enantiomer in cerebral cortex, heart, parotid gland and longitudinal ileum. In the bladder, a smaller difference (15-fold) was noted. The selectivity for one tissue over another was less than 5-fold. Hill coefficients were close to unity, except for (S)-BM-5 in ileum where a significantly ($P < 0.05$) better fit was obtained with a two-site model. The estimated fraction of high affinity sites was 58 ± 8%. The affinities ($pK_i$) were estimated to be 5.76 ± 0.12 and 4.22 ± 0.09, respectively.

(R)-BM-5 induced both bladder contraction and saliva secretion in a dose-dependent manner (0.3–30 µg/kg) in anaesthetized cats. At higher doses (100–300 µg/kg) the effect declined to 44% and 74% respectively of the maximal effect on bladder contraction and salivation. The dose-response curves for bladder contraction and salivation were bell-shaped for the dose interval studied but they did not reach a level below baseline. ED₅₀ values were similar for bladder contraction and salivation (4.1 ± 1.1 and 6.2 ± 1.7 µg/kg respectively; $N=4$). The maximal effect on bladder contraction and salivation was reached at the same dose, 30 µg/kg.

6.5 The effect of serotonin 5-HT₂A, 5-HT₃ and 5-HT₄ receptor antagonists (V)

The mean basal values for urinary bladder contraction induced by intravesical electrical stimulation (IVES) were 111-113 cm H₂O. For different 5-HT receptor agonists in the innervated and denervated urinary bladder, the values were 11-32 cm H₂O.

In the innervated cat urinary bladder experiments, intra-arterial administration of the selective 5-HT₃ receptor agonist (2-M-5-HT) induced bladder contraction. This response was dose dependently inhibited by the selective 5-HT₃ receptor antagonist (tropisetron). Tropisetron did not alter the urinary bladder contraction induced by 5-HT₄ receptor agonist, 5-MeOT, and IVES up to 1 mg/kg.

The selective 5-HT₄ receptor antagonists, SB207710, GR 125487, GR 113808 in doses of 0.1-1 mg/kg were unable to influence the IVES or chemically induced bladder contraction (data not shown). However, SB204070 decreased chemically induced bladder pressure at 0.3 and 1 mg/kg with 17-61%. In addition, the maximal inhibition of IVES by SB204070 (1 mg/kg) was 74%.
Administration of saline did not alter the IVES and chemically induced bladder contraction.

In the denervated cat urinary bladder intra-arterial injection of 5-MeOT, 5-HT, ACh and 2-M-5-HT increased intravesical pressure in a dose-dependent manner \( (n=8) \). The calculated ED\(_{50} \) (µg/kg) and V\(_{\text{max}} \) (cm H\(_2\)O) values were 5.7 ± 2.0 and 55.6 ± 6.6 for 5-MeOT; 2.6 ± 0.9 and 55.8 ± 6.8 for 5-HT; 1.4 ± 0.5 and 30.5 ± 5.2 for ACh and 50.1 ± 40.4 and 91.4 ± 48.1 for 2-M-5-HT.

Cumulative doses of the 5-HT\(_3\) receptor antagonist tropisetron \((0.0003-0.01\, \text{mg/kg})\) specifically blocked bladder contraction induced by the selective 5-HT\(_3\) receptor agonist, 2-M-5-HT \((\text{ID}_{50} \, 0.00044 \pm 0.00018 \, \text{mg/kg}, \, n=4)\). Tropisetron did not inhibit the urinary bladder contraction induced by agonists 5-MeOT, 5-HT and Ach. Consequently, the selective 5 HT\(_{2A}\) receptor antagonist ketanserin \((0.003-0.3 \, \text{mg/kg})\) inhibited 5-MeOT and 5-HT induced bladder contraction with ID\(_{50}\) values of 0.033 ± 0.013 and 0.019 ± 0.007 mg/kg, respectively. However, ketanserin at 0.3 mg/kg also inhibited ACh induced bladder contractions by 25%. Finally, ACh-induced bladder contraction was inhibited by atropine \((0.03-0.3 \, \text{mg/kg})\) by 94.8 ± 4.5% of basal response. Administration of saline did not alter the chemically induced bladder contraction \((n=4)\).
7 DISCUSSION

7.1 \( \alpha_1 \)-adrenoceptors

Stress incontinence can be improved by increasing outlet resistance (Andersson, 1993; Andersson et al., 1999). \( \alpha_1 \)-Adrenoceptor agonists have been used for the treatment of stress incontinence, acting to increase urethral tone by contracting urethral smooth muscle. The most widely used compounds are ephedrine and norephedrine (Andersson, 1988; Wein, 1995b). However, there is no good pharmacotherapy for stress incontinence and developed drugs have side effects and poor treatment efficacy (Andersson, 2000b; Kelleher et al., 1997; Radley et al., 2001). In paper I, we established an in vivo model for measurement of urethral pressure in anaesthetized rabbit, for future development of drugs for treatment of stress incontinence.

The \( \alpha \) -adrenoceptor agonists oxymetazoline, NS-49, phenylephrine and phenylpropanolamine enhanced the urethral pressure in the anaesthetized rabbit in a dose-dependent manner. The putative \( \alpha_2 \)-adrenoceptor partial agonists clonidine and UK 14,304 had no effect at the doses tested, suggesting the increase in urethral pressure was caused by activation of an \( \alpha_1 \)-adrenoceptor.

Oxymetazoline, NS-49, phenylephrine and phenylpropanolamine also increased the blood pressure. Interestingly, the ED\( _{50} \) values for the effect on blood pressure were lower than those for the effect on urethral pressure of phenylephrine (P<0.001) and phenylpropanolamine (P<0.05). These two compounds were selective for cardiovascular effects, as compared to urethral effects. In contrast, the urethral ED\( _{50} \) values for oxymetazoline and NS-49 were lower than the values for the cardiovascular parameters. This finding support that oxymetazoline and NS-49 were relatively tissue-selective for the urethra.

The \( \alpha_1 \)-adrenoceptor subtype selectivity for some agonists has been evaluated (Minneman et al., 1994). Thus, oxymetazoline has some selectivity for \( \alpha_{1A} \) versus \( \alpha_{1B} \) and \( \alpha_{1D} \)-adrenoceptors. Phenylephrine, in contrast, has some selectivity for the \( \alpha_{1D} \)-adrenoceptor. Pharmacological receptor classification takes advantage of drug selectivity and the fact that most adrenergic compounds, agonists and antagonists, bind to both \( \alpha_1 \) - and \( \alpha_2 \)-adrenoceptors. Selective compounds have higher \( pK_B \) values for some receptor(s) (Wilson et al., 1991), e.g. the putative \( \alpha_2 \)-adrenoceptor agonists oxymetazoline and clonidine have been shown to bind also to all three \( \alpha_1 \)-adrenoceptor subtypes (Minneman et al., 1994).

Since the \( \alpha_1 \)-adrenoceptor antagonist WB-4101 had a lower ID\( _{50} \) value than the \( \alpha_2 \)-adrenoceptor antagonist rauwolscine it can be suggested that the urethral pressure enhancement was caused by activation of an \( \alpha_1 \)-adrenoceptor, in
agreement with the suggestion that the \( \alpha_{1A} \)-adrenoceptor is the functional receptor subtype in the rat (Chess-Williams et al., 1994), rabbit (Auguet et al., 1995) pig (Alberts et al., 1999), and human (Nishimatsu et al., 1999) urethra \textit{in vitro}. The apparent urethra-selectivity of the \( \alpha_{1A} \)-adrenoceptor selective compound oxymetazoline found in the present study may thus reflect a functional role of the \( \alpha_{1A} \)-adrenoceptor also \textit{in vivo}.

Three human \( \alpha_1 \)- and three human \( \alpha_2 \)-adrenoceptor subtypes have been cloned (Bylund et al., 1998). The existence of a fourth \( \alpha_1 \) subtype, designated \( \alpha_{1L} \), has been postulated; however, it has not been cloned and may represent a particular conformational state of the \( \alpha_{1A} \)-adrenoceptor (Alberts et al., 1999; Bylund et al., 1998; Ford et al., 1997). In the human female urethra, different levels of \( \alpha_{1A} \)-, \( \alpha_{1B} \)- and \( \alpha_{1D} \)-adrenoceptor expression have been suggested using RNase protection assay and \textit{in situ} hybridization (Nasu et al., 1998).

Our results suggest that in the female rabbit \textit{in vivo} activation of \( \alpha_1 \)-adrenoceptors increased the urethral pressure. Phenylephrine and phenylpropanolamine, in contrast to oxymetazoline and NS-49, selectively enhanced blood pressure as compared to the urethral pressure. Provided that the present results have validity also in humans, it seems possible to develop urethra selective drugs for treatment of stress incontinence with little or no cardiovascular side effects.

### 7.2 Muscarinic receptors

Bladder overactivity is traditionally treated by drugs mainly influencing the efferent neurotransmission or the detrusor muscle itself (Andersson, 1999a). Consequently, muscarinic antagonists are the most widely used treatment for bladder overactivity (Wein, 1995a). With the exception of tolterodine, however, most drugs lack functional bladder selectivity and side effects may limit their usefulness (Andersson et al., 1999; Nilvebrant, 2000). Therefore, new and more bladder-selective muscarinic antagonists are needed.

In the paper II, we modified an \textit{in vivo} rat cystometry model (Malmgren et al., 1987) to screen new muscarinic antagonists, to calculate the antagonist \textit{in vivo} ID\(_{50}\) values, to lower inter-individual variation by making each animal its own control, and to minimize the number of animals used.

All muscarinic antagonists tested were able to inhibit volume-induced spontaneous micturition pressure in a dose-dependent manner in conscious rats. The compounds did not cause changes in micturition number, micturition volume, residual volume, and bladder capacity (Guarneri et al., 1991; Igawa et al., 1993). Muscarinic antagonists, e.g. oxybutynin and terodiline, have been shown to decrease micturition pressure without changing bladder volume capacity (Guarneri et al., 1991) in rat cystometry. However, using muscarinic
antagonists alter bladder capacity in man. In addition, recent work has shown that intracerebroventricular administration of atropine, tolterodine, but not darifenacin, increased bladder capacity in conscious rats indicating the differences in the supraspinal effect on micturition reflex by muscarinic antagonists (Ishizuka et al., 2001). It may be suggested that alteration of bladder capacity depends on the importance of central reflex mechanisms. However, investigation of this possibility is beyond the scope of the present study.

The present rat in vivo pID_{50} values correlated significantly with the present rat in vitro pK_{B} values and with anaesthetized cat in vivo pID_{50} values. The present rat in vitro pK_{B} values correlated significantly with guinea pig in vitro pK_{B} values. Therefore, the present rat in vivo cystometry model appears to be valid for testing muscarinic antagonist effects on micturition pressure. In addition, the present rat in vivo pID_{50} values correlated significantly with human in vitro bladder pA_{2} values (p<0.01)(Yono et al., 1999). Tolterodine, a potent muscarinic receptor antagonist (Nilvebrant, 2001; Nilvebrant et al., 1997a; Nilvebrant et al., 1997c) that has shown a good clinical effect for the treatment of overactive urinary bladder (Andersson, 2000a; Nilvebrant, 2001), inhibited bladder contractions in the rat cystometry model. Thus, this in vivo model is a useful tool as an initial screening method for new anti-muscarinic compounds.

Tolterodine, a non-selective muscarinic antagonist, is developed specifically for treatment of overactive bladder (Nilvebrant, 2001; Nilvebrant et al., 1997a). Clinical data indicate that tolterodine has a significantly better tolerability (less dry mouth) than oxybutynin in patients with overactive bladder (Nilvebrant, 2001). However, dry mouth, a known side effect for muscarinic antagonist, has been reported to be the most commonly adverse events (40% for tolterodine (Appell, 1997), and 80% for oxybutynin (Yarker et al., 1995)). Therefore, we explored the potential for a better and more selective bladder selective muscarinic antagonist.

The aim of the paper III was to pharmacologically characterize a new compound for better functional smooth muscle selectivity than tolterodine for treatment of diseases where a muscarinic antagonist effect is beneficial.

The present results show that the new muscarinic antagonist PNU-171990 showed no subtype selectivity for muscarinic receptors from isolated guinea pig tissues or cloned human receptors. In functional in vitro studies, PNU-171990 antagonized carbachol-induced contractions in smooth muscle from guinea pig urinary bladder, and guinea pig and human ileum, yielding pK_{B} values similar to the pK_{i}. PNU-171990 caused a parallel shift in the concentration-response curve for carbachol. Schild plot slopes were not significantly different from unity, suggesting that PNU-171990 bound to a single receptor site in a competitive manner.
Our *in vivo* cat model has previously been shown to be predictive for tissue selectivity of a muscarinic antagonists, such as tolterodine, in man (Nilvebrant, 2001; Nilvebrant et al., 1997a). PNU-171990, like tolterodine, has no selectivity for any muscarinic receptor subtype. In addition, both compounds show functional tissue selectivity for urinary bladder contraction over salivation in the anaesthetized cat *in vivo*. Thus, PNU-171990 showed significant tissue selectivity for bladder contraction over salivary secretion yielding a numerically higher selectivity ratio than tolterodine.

In a recent clinical paper (Van Kerrebroeck et al., 2001), an improved bladder selectivity for tolterodine was demonstrated with an extended release formulation. The prevalence of dry mouth have been reported to be 23% for extended-release and 30% for immediate release. This is probably due to lower serum peak concentrations giving even less incidence of dry mouth. Accordingly, pharmacokinetic parameters may contribute to the *in vivo* PNU-171990 tissue selectivity. However, the mechanism of tissue selectivity for both tolterodine and PNU-171990 remain to be explained.

The racemic molecule BM-5 is potentially a good example for describing a non-selective muscarinic compound that exerts different tissue efficacy. From the literature, racemic BM-5 is known to be a muscarinic agent with different effects depending on the test system. Racemic BM-5 acts as a muscarinic antagonist in the bladder but as a muscarinic partial agonist in the ileum (Ringdahl, 1987). The different functional effects of (R)- and (S)-BM-5 cannot be attributed to a difference in affinity for the various muscarinic receptor subtypes (Baumgold et al., 1989; Engstrom et al., 1987). In paper IV, we tried to find a pharmacological explanation of this selectivity and investigate if there was a different effect on bladder contraction and salivary secretion *in vivo* in cat.

In a radioligand binding study, both enantiomers lacked binding selectivity in cell lines expressing human muscarinic receptor subtypes and in various guinea pig tissues. However, the binding affinity of (R)-BM-5 was 40-60 fold higher than the affinity of (S)-BM-5, except in the bladder where a smaller difference (15-fold) was found.

However, (S)-BM-5, but not (R)-BM-5, bound to a heterogeneous receptor population in the ileum. Such heterogeneous binding profiles have been reported for racemic BM-5 in rat tissues (Engstrom et al., 1987), but not in cell lines (Baumgold et al., 1989). This cannot be due to interaction with several receptor subtypes as the subtype selectivity is too low. The longitudinal ileum contains 80% M₂ and 20% M₃ receptors (Michel et al., 1990). With a selectivity ratio of about 2 between M₂ and M₃, a Hill coefficient of approximately 0.98 can be expected.
In addition, if the heterogeneous binding observed in the ileum with (S)-BM-5 was a result of different receptor subtypes, (R)-BM-5 would also bind to more than one binding site, as the selectivity profiles of (R) and (S)-BM-5 are similar. Furthermore, heterogeneous binding would also be expected in the bladder, as the distribution of muscarinic receptor subtypes in the bladder (Wang et al., 1995) is very similar to the distribution in ileum (Michel et al., 1990), i.e. M₂ dominating over M₃. A positive correlation between the ratio of low and high affinities and efficacy has been reported both for muscarinic ligands (Birdsall et al., 1978) and for β-adrenergic agonists (Lefkowitz et al., 1982). The binding data indicates differences in the efficacy of the enantiomers and in receptor-effector coupling between bladder and ileum.

In the isolated bladder and ileum, (S)-BM-5 showed higher maximal agonist effect but lower potency than (R)-BM-5. This has previously been reported for the ileum (Dahlbom et al., 1982; Messer et al., 1992; Ringdahl, 1984). Both the enantiomers of BM-5 were partial agonists in the isolated bladder and ileum. Complete inhibition of the (R) and (S)-BM-5 induced contractions by atropine indicates that they were mediated by muscarinic receptors. As predicted from binding data, the maximal agonist effect was much smaller in the bladder compared with the ileum. The effects on isolated ileum were in good agreement with a previously reported study (Dahlbom et al., 1982). The present study showed that the partial agonist effect of (R) and (S)-BM-5 in the bladder was weak while Ringdahl (Ringdahl, 1987; Ringdahl et al., 1987) reported racemic BM-5 to be an antagonist in the guinea pig bladder. A possible explanation for the different results is that the maximal agonist effect of racemic BM-5 is very low and difficult to measure. Baumgold & Drobnick (Baumgold et al., 1989) reported racemic BM-5 to be a pure antagonist for receptors coupled to phosphoinositide (PI) turnover. However, this seems to be a less likely explanation as both (R)- and (S)-BM-5 can stimulate contraction in ileum and bladder, effects that have been reported to be coupled to PI turnover (Best et al., 1985; Noronha-Blob et al., 1987).

In vivo, racemic BM-5 has been reported to behave as an agonist for salivation and for centrally mediated responses in mouse (Engstrom et al., 1987; Ringdahl et al., 1987) and rat (Casamenti et al., 1986). Antagonist properties have been reported for oxotremorine-induced tremor in mouse (Resul et al. 1982) and for inducing acetylcholine release (Nordstrom et al., 1983). In our studies, we found (R)-BM-5 to act as an agonist in the anaesthetized cat, as bladder contraction and saliva secretion were observed. The dose-response curves were bell-shaped, which may indicate that multiple dosages of (R)-BM-5 induce desensitization of receptors. As partial agonists do not have a receptor reserve, the response declines if there is a desensitization.
Overall, the affinity and efficacy data suggests that the functional effects of racemic BM-5 are most probably mediated by the (R)-enantiomer. As (R)-BM-5 has about 40-times higher affinity than (S)-BM-5 for muscarinic receptors; the (R)-enantiomer in a racemic mixture will occupy more than 95% of the receptors. Although the (S)-enantiomer has a maximal agonist effect that is about 2.5-times higher, this difference is too small to compensate for its much lower affinity. The diversity in effects of racemic BM-5 between different test systems seems to be regulated by variances in receptor-effector coupling.

Despite the lack of receptor selectivity, (R)- and (S)-BM-5 showed differences in functional response \textit{in vitro}. A higher maximal agonist effect was demonstrated for both (R)- and (S)-BM-5 in the ileum as compared to the urinary bladder. A difference in the receptor-effector coupling seems to regulate the effect. Both enantiomers of BM-5 act as partial agonists at muscarinic receptors, while functional effects of racemic BM-5 are mediated by (R)-BM-5. The enantiomers of BM-5 provide a good example of opposed enantiomeric-selectivity for affinity and efficacy.

### 7.3 Serotonergic receptors

The mechanisms responsible for bladder relaxation during storage phase are unknown (Andersson, 1999b). However, compounds that can relax the bladder muscle directly and/or prevent the release of excitatory mediators may improve storage ability and increase bladder capacity (Andersson, 1997). Serotonin has profound effects on the function of the lower urinary tract and activation of 5-HT\textsubscript{4} receptor modulates cholinergic/purinergic transmission in human urinary bladder (Corsi et al., 1991; Tonini et al., 1994). Using a serotonergic 5-HT\textsubscript{4} antagonist approach may have advantages such as reduction of bladder overactivity without affecting micturition pressure, which can cause urinary retention.

In paper V, the roles of 5-HT\textsubscript{2A}, 5-HT\textsubscript{3}, and 5-HT\textsubscript{4} in intravesical electrical stimulation (IVES) and chemically induced contraction in intact and denervated urinary bladder in anaesthetized cats were determined.

We conducted preliminary experiments with several 5-HT receptor agonists to evoke chemically induced bladder contraction. The natural transmitter 5-HT, or 5-MeOT, S-zacopride, SC 53116, cisapride and 2-M-5-HT were injected intra-arterially. None of the selective 5-HT\textsubscript{4} agonists induced bladder muscle contraction. In contrast, 5-HT, 5-MeOT (a non-selective 5HT receptor agonist) and 2-M-5-HT (a 5HT\textsubscript{3} receptor agonist) induced bladder contraction in a dose dependent manner.

In the present study, we observed one very sharp and short-lasting peak in bladder contraction after intra-arterial administration of a selective 5-HT\textsubscript{3}
agonist (2-M-5-HT). The 5-HT and non-selective 5-HT receptor agonist (5-MeOT) have almost similar receptor profiles and showed a similar dose response curve with very similar calculated ED\textsubscript{50} and V\text{max} values. Both substances generated a broad and long lasting peak.

IVES activation of A\text{δ}-fibers caused a recruitment of detrusor muscle unit contraction leading to increased intravesical bladder pressure as previously described by Ebner and co-workers (Ebner et al., 1992). After IVES, the innervated bladder showed lower maximal bladder pressure induced by chemical stimulation than the denervated bladder. This effect could be interpreted as tachyphylaxis of the receptors involved in the micturition reflex. The results of Jiang and Lindstrom (Jiang et al., 1996) who found an increase in the micturition threshold volume in rat induced by IVES could also be explained by tachyphylaxis.

The selective 5-HT\textsubscript{4} receptor antagonist, SB204070, inhibited the IVES and chemically induced bladder contraction by 17-74% at doses 0.3-1 mg/kg. This indicates that a 5-HT\textsubscript{4} receptor might be involved in micturition reflex. Nevertheless, the 5-HT\textsubscript{4} receptor antagonists, SB207710, GR 125487, GR 113808 had no influence either on chemical induced or IVES induced contractions at doses tested. It is unlikely that the effect of SB204070 is a specific 5-HT\textsubscript{4} receptor inhibitory effect in cat urinary bladder. In contrast, the selective 5-HT\textsubscript{3} antagonist tropisetron already at a very low dose completely blocked the selective 5-HT\textsubscript{3} receptors agonist 2-M-5-HT-induced contraction without influencing the effect of 5-MeOT or IVES. These results clearly demonstrate that the 5-HT\textsubscript{3} receptor plays an important role in the cat urinary bladder contraction.

In denervated urinary bladder, intraarterial injection of 5-MeOT, 5-HT, 2-M-5-HT generated a dose dependent bladder contraction. The selective 5-HT\textsubscript{3} receptor antagonist tropisetron inhibited the effect of 2-M-5-HT, suggesting that the 5-HT\textsubscript{3} receptor is located postsynaptically or at the level of the motor neuron and involved in influencing bladder contraction in cat. In addition, tropisetron had no effect on 5-HT and 5-MeOT induced contraction. Furthermore, ketanserin, generally accepted as a selective 5-HT\textsubscript{2A} receptor antagonist (Sleight et al., 1996), inhibited both 5-HT and 5-MeOT induced contractions at almost identical ID\textsubscript{50} values indicating that 5-HT\textsubscript{2A} is involved in the mediation of bladder contraction in anaesthetized cat (Saxena et al., 1985).

Interestingly, tropisetron had no inhibitory effect on ACh induced contractions, which indicated that this receptor might be located post-junctionally at bladder smooth muscle. This contrasts with ketanserin that could block about 25 % of the ACh induced contractions. This indicates that the 5-HT\textsubscript{3} and 5-HT\textsubscript{2A} receptors might be located at different anatomical sites in the cholinergic transmission pathway. One fraction of the 5-HT\textsubscript{2A} receptors seems to
be located on the post-ganglionic neuron in the bladder wall. Another important fraction is probably located on the bladder smooth muscle, since the effect of both 5-HT and 5-MeOT could be totally blocked by ketanserin. As expected the cholinergic mediated effect could be totally blocked by atropine.

Our results demonstrate that 5-HT$_{2A}$ and 5-HT$_3$ receptors are involved in bladder muscle contraction in cat. Thus, the 5-HT$_{2A}$ and 5-HT$_3$ receptor antagonist may improve lower urinary tract symptoms. The role of serotonergic 5-HT$_4$ receptors seems to be less important.

### 7.4 General discussion

The aims of this thesis were to improve the existing pharmacological treatments of urinary incontinence and to look for alternative treatments. The focus has been on three different pharmacological approaches, the role of $\alpha$-adrenergic mechanisms in urethra, and the role of muscarinic and serotonergic mechanisms in urinary bladder. Some notable factors in each mechanism will be discussed below:

1. Tissue selectivity of a $\alpha_{1A}$-adrenoceptor agonist might be due to the functional receptor subtype in the target organ (urethral) and the tissue distribution of different $\alpha$-adrenoceptor receptor subtypes. In the human female urethra, different levels of $\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-adrenoceptor expression have been suggested using RNase protection assays and in situ hybridization (Nasu et al., 1998). Evidence also indicates that the $\alpha_{1A}$-adrenoceptor activation enhances intraurethral pressure both in rabbit and human (Abel et al., 1974; Awad et al., 1976; Donker et al., 1972; Nishimatsu et al., 1999; Whitfield et al., 1975). In addition, it has been asserted that the $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors are not predominant in any vascular bed (de Mey, 1999). On this basis, it is reasonable to hypothesize that $\alpha_{1A}$-adrenoceptor activation might enhance urethral pressure with less pronounced cardiovascular side effects. Thus, an urethral-selective $\alpha_{1A}$-adrenoceptor agonist may be a good treatment of stress incontinence.

2. In a conscious rat cystometry model and in ACh-induced contraction in anaesthetized cat model, muscarinic antagonists (e.g. atropine, tolterodine, oxybutynin, darifenacinc, and terodiline) were found to inhibit urinary bladder contraction. Moreover, these muscarinic antagonists have been clinically used for treatment of bladder overactivity and exert clear effect on bladder muscle contraction in human (reviewed in (Andersson, 2000a)). Therefore it will be reasonable to assume that rat cystometry model is of value to screen new drugs for bladder overactivity treatment.

3. The tissue-selectivity for muscarinic antagonist has been discussed previously by Nilvebrant et al and also by Gillberg et al (Gillberg et al., 1998b;
Nilvebrant et al., 1997a). The *in vivo* functional selectivity for tolterodine was not attributed by selectivity for a single receptor subtype. As can be seen in Table 1, darifenacin and AQ-RA 741, in contrast to PNU-171990, tolterodine and atropine, have high affinity for M₃ and M₂ receptor subtypes, respectively, using expressed human muscarinic receptor in cell lines. However, like atropine and oxybutynin, these selective compounds show no tissue-selectivity *in vivo*.

Table 1. *In vitro* affinity (Kᵢ) ratio and *in vivo* efficacy (ID₅₀) ratio of different muscarinic antagonists.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Affinity ratio of Ki values in CHO-cells</th>
<th>Affinity ratio of Ki values in CHO-cells</th>
<th>Affinity ratio of Ki values in guinea pig tissues</th>
<th>ID₅₀ ratio in vivo in cat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₂/M₃</td>
<td>M₁/M₃</td>
<td>Bladder/Parotid gland</td>
<td>Salivation/contraction</td>
</tr>
<tr>
<td>PNU-171990</td>
<td>2.3</td>
<td>0.8</td>
<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Tolterodine ⁹</td>
<td>1.1</td>
<td>0.9</td>
<td>0.6</td>
<td>2.5</td>
</tr>
<tr>
<td>AQ-RA 741 (M2) ⁶</td>
<td>0.1</td>
<td>1.1</td>
<td>0.07</td>
<td>1.4</td>
</tr>
<tr>
<td>Atropine ⁹</td>
<td>2.9</td>
<td>1.9</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Darifenacin (M3) ⁶</td>
<td>47.0</td>
<td>29.2</td>
<td>46.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Oxybutynin ⁹</td>
<td>10</td>
<td>3.6</td>
<td>6.5</td>
<td>0.5</td>
</tr>
<tr>
<td>UH-AH 37 ⁶</td>
<td>6.8</td>
<td>0.3</td>
<td>3.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

(⁹ Nilvebrant et al., 1997a) and ⁶ Gillberg et al., 1998b).

However, this selectivity has been proposed to be due to the higher affinity for bladder smooth muscle than parotid gland. PNU-171990, in contrast to tolterodine, showed higher affinity ratio to parotid gland (M₃) compared to urinary bladder muscle homogenates (M₂ and M₃) in the present study. However, PNU-171990 showed a numerically better selectivity ratio for the bladder contraction over salivation than tolterodine *in vivo*.

The affinity ratio of these compounds for M₁/M₃ muscarinic receptor subtypes is shown in Table 1. As can be seen both tolterodine and PNU-171990 have almost the same affinity for M₁ and M₃ receptor subtypes. In contrast, atropine, darifenacin, and oxybutynin show higher affinity for M₁ than for M₃, and also have a lower tissue-selectivity *in vivo*. For treatment of overactive bladder, a new approach has recently been suggested, using a muscarinic antagonist for M₂/M₃ receptor subtypes, lu 25-109, which is also a partial M₁ receptor agonist (Waldeck et al., 2002). Our data indicate that the affinity of
both M₂ and M₃ receptors for muscarinic antagonists is important for inhibition of bladder contraction. Also, a lower affinity for M₁ than M₃ receptor subtypes might be the explanation for lower effect on salivary secretion.

In addition, in a recent clinical paper (Van Kerrebroeck et al., 2001), improved bladder selectivity for tolterodine was demonstrated with an extended release formulation. The prevalence of dry mouth has been reported to be 23% for extended-release and 30% for immediate-release. This is probably due to lower serum peak concentrations giving reduced dry mouth effects. Accordingly, pharmacokinetic parameters may also contribute to the in vivo tissue selectivity. The mechanism of tissue selectivity for both tolterodine and PNU-171990 remains to be fully explained. However, a bladder-selective competitive muscarinic antagonist is considered a good pharmacotherapy for overactive bladder.

4. The role of 5-HT in urinary urge incontinence has been reported. Our results using denervated urinary bladder in cat demonstrate that 5-HT and 5-MeOT increased bladder contraction in a dose dependent manner. We also showed that ketanserin, generally accepted as a selective 5-HT₂ₐ receptor antagonist (Sleight et al., 1996), inhibited both 5-HT and 5-MeOT induced contractions indicating that 5-HT₂ₐ receptors play an important role in urinary contraction. These results would suggest that 5-HT₂ₐ receptor antagonists might be an alternative for treating symptoms of incontinence. In addition, it has also been reported that patients with diabetes complain of urinary frequency, a condition that often does not respond to treatment with muscarinic antagonists. Clinical data obtained with the 5-HT₂ₐ antagonist sarpogrelate, suggest that this compound may improve urinary frequency in diabetic patients (Takimoto et al., 1999). In diabetic rats, bladder overactivity is related to smooth muscle hypertrophy and/or hyperplasia, and 5-HT₂ₐ mediates the contractility effect of 5-HT-induced contraction (Kodama et al., 2000). Thus, 5-HT₂ₐ antagonist might be useful in the management of bladder overactivity. Other benefits of serotonergic drugs in treatment of incontinence might include their lack of effect on saliva secretion and bladder voiding contraction.
8 CONCLUSIONS

- It seems possible to develop urethra selective drugs for treatment of stress incontinence with little or no cardiovascular side effects.
- The modified rat model can be used as an initial screening method for new muscarinic antagonists.
- PNU-171990 is a competitive and potent muscarinic antagonist in vitro with a numerically better selectivity ratio for the bladder contraction over salivation in vivo than tolterodine.
- Despite the lack of receptor selectivity, (R)- and (S)-BM-5 showed differences in functional response. The enantiomers of BM-5 provide a good example of opposed enantioselectivity for affinity and efficacy.
- The 5-HT\textsubscript{2A} and 5-HT\textsubscript{3} receptors are involved in bladder muscle contraction in cat. 5-HT\textsubscript{2A} and 5-HT\textsubscript{3} receptor antagonists may improve lower urinary tract symptoms.
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