Muscarinic receptors in the lower urinary tract

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Lambertus P.W. Witte

Colofon

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MUSCARINIC RECEPTORS IN THE LOWER URINARY TRACT

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Lower Urinary Tract Symptoms

Historically, the terms “prostatism” and “symptoms of benign prostatic hyperplasia (BPH)” were used to describe “lower urinary tract symptoms” (LUTS) in older men. Since LUTS are also common in women of similar age, these terms become less sensible [1]. Although the term prostatism implies a prostatic cause for symptoms, almost no direct evidence exists for such a cause. BPH is a histological diagnosis often associated with bladder outlet obstruction (BOO), due to benign prostatic enlargement (BPE), but most attempts to correlate either individual symptoms, or symptom groups with objective measures have failed to show any significant associations. For example, prostate size correlates poorly, if at all, with the severity of male LUTS, as measured with the international prostate symptom score (IPSS)[2]. On the other hand, female LUTS were traditionally attributed to detrusor overactivity (DO), and/or overactive bladder syndrome (OAB), but these typical findings may also be present in male patients [3]. Altogether, the term LUTS is nowadays more generally used to describe any combination of urinary symptoms in male and female patients, without any reference to specific underlying pathophysiological conditions. LUTS are often divided into three groups: storage, voiding, and postmicturition symptoms. Storage symptoms are daytime urinary frequency, nocturia, urinary urgency, stress urinary incontinence (SUI), and urgency urinary incontinence (UUI). Voiding symptoms are slow stream, splitting or spraying, intermittent stream, hesitancy, and straining. Postmicturition symptoms are described by the feeling of incomplete emptying and postmicturition dribble [4]. Urgency has been identified as one of the most bothersome symptom across both genders [5]. For women, SUI is one of the most bothersome symptoms, followed by UUI [5]. In contrast, men report postmicturition dribble, urgency and nocturia as most bothersome[6].

Overactive Bladder Syndrome

Typical storage LUTS are most often labeled as overactive bladder syndrome (OAB). OAB is defined by the presence of urinary urgency, usually accompanied by frequency and nocturia, with or without urge urinary incontinence, in the absence of urinary

tract infection or other obvious pathology [4]. OAB is a collection of symptoms, often used as an initial diagnosis, but it is not a disease entity on itself. Generally two types of OAB are described: neurogenic and idiopathic. Neurologic diseases can damage the central or peripheral pathways involved in control of the lower urinary tract and may result in DO and OAB. In all other cases, the cause for OAB is idiopathic, with or without presence of DO in urodynamic studies [7]. Idiopathic OAB is most common, and will be named OAB in this thesis.

Prevalence estimates of OAB from population-based studies in the United States and Europe range from 12%-16 %, increasing to even 40% in patients above 70 years [8;9]. OAB symptoms can have great impact on an individual's physical, mental, and social well-being and are associated with increased morbidity and mortality [10]. The estimated total national cost of OAB with UUI in 2007 in the United States was $65.9 billion, with projected costs of $76.2 billion in 2015 and $82.6 billion in 2020 [11].

Bladder Outlet Obstruction

"LUTS suggestive of bladder outlet obstruction (BOO)" is a term used in men predominately complaining of voiding symptoms in the absence of infection or obvious pathology other than possible causes of outlet obstruction [4]. A large population-based study in 5 western countries demonstrated that men experienced voiding symptoms suggestive of BOO in almost 26%, increasing with age [8]. Benign prostatic obstruction (BPO) can lead to BOO and may be diagnosed when the cause of outlet obstruction is known to be BPE, due to histologic BPH. In women, voiding symptoms are rare, but if present most often caused by detrusor underactivity, anatomical deformities or dysfunctional voiding [4].

Pharmacological Treatment of OAB: Muscarinic Receptor Antagonists

Muscarinic receptor antagonists (antimuscarinics) are the main drug class used to treat OAB [12]. While non-cholinergic neurotransmitters, most importantly ATP, contribute to the control of detrusor smooth muscle tone in many animal species, contraction of the healthy human detrusor is largely mediated by acetylcholine acting on muscarinic receptors. Non-cholinergic neurotransmitters can become more important in some pathological

conditions [13]. In the human bladder, M_2 muscarinic receptors are present in much greater numbers than M_3 muscarinic receptors, but bladder contraction is mediated primarily, if not exclusively by M_3 muscarinic receptors [14]. Data based on knockout animals or complex receptor subtype inactivation protocols have indicated that M_2 receptors may contribute primarily to modulation of relaxation responses [15;16].

In an updated review and a meta-analysis of all clinical studies of antimuscarinic treatment for OAB, all antimuscarinics demonstrated equal efficacy with subtle differences in responses to dosage, and tolerability (in favor of the newer drugs) [12]. Although epidemiological studies indicate that OAB is similarly present in both genders, most treatments are prescribed to women [12]. Accordingly, clinical studies on antimuscarinics were done in predominant female populations. While typical phase III studies were too small to allow robust comparison of treatment responses between genders, sub-analysis of an open label trial of 2250 male and female patients with OAB treated with tolterodine showed that age, but not gender has a significant impact on urgency, frequency, or urgency incontinence [17]. Nevertheless tolterodine, fesoterodine and solifenacin have been extensively and successfully tested as single agents in male patients with OAB, but without BOO [18;19].

Antimuscarinic monotherapy for male patients with BOO and OAB has been investigated, but with unfavorable results on voiding symptoms [20]. Men with BOO and urodynamically verified OAB treated with tolterodine 2 mg twice daily showed improvements in storage symptoms, but not on voiding symptoms [21]. In another study, where patients received tolterodine 4mg monotherapy, they showed significant improvement urge incontinence, but no improvement in urgency, IPSS, or overall benefit compared with placebo [22].

Although antimuscarinics are generally well tolerated, adverse events (AE's) are the main reason for patients to discontinue therapy. In controlled clinical studies, approximately 3-10% of patients withdraw treatment, which is not significantly different from placebo in most studies. Dry mouth (16%), dizziness (5%), constipation (4%), nasopharingitis (3%) and micturition difficulties (2%) are the most reported AE's [21]. Caution is advised in men with BOO treated with antimuscarinics, since there is a theoretical

decrease of bladder strength, which may lead to increase of post voiding residual (PVR) and AUR. A 12-week placebo controlled safety study of tolterodine vs. placebo in men with mild to moderate BOO demonstrated an increased PVR, but no increased events of AUR (3% in both arms), concluding the use of tolterodine in men with BOO is safe [21].

Pharmacological Treatment of OAB: β_3-Adrenoceptor Agonists

Recently, β_3-adrenoceptor agonists were introduced as a new therapeutic strategy for OAB. β-adrenoceptors are the primary mediators of relaxation in the urinary bladder, and in humans this occurs primarily, if not exclusively via the β_3-adrenoceptor subtype [23]. β-adrenoceptor agonists lack the typical antimuscarinic AE's, and are generally well tolerated [24].

In three phase III clinical trials with mirabegron, more than 6000 patients with OAB, treated with mirabegron at daily doses of 25, 50 and 100 mg demonstrated significant improvement of frequency, urgency and urgency incontinence at 4 weeks from the start and during the rest of the treatment [25-27]. A pooled analysis of these trials showed significant improvement with mirabegron 50 and 100 mg in terms of dry rates, $\geq$ 50% reduction in mean number of incontinence episodes/24hr, and the proportion of patients with $\leq$ 8 micturitions/24 hr at final visit [28]. These effects were also confirmed in patients aged above 65 years of age, treatment naïve patients and patient who had previously discontinued antimuscarinic treatment [24]. While mirabegron was the first β_3-agonist to enter clinical practice, other selective β_3-agonists are currently under development. Results of phase II clinical studies have been reported for solabegron in female patients with OAB and incontinence. Preclinical studies have been reported for ritobegron and TRK-380 [24].

Mirabegron was generally well tolerated, but most commonly reported AE's were hypertension (7.3%), nasopharyngitis (3.4%) and urinary tract infections (3.0%). Regarding safety, no effect on QT interval on 50 and 100 mg mirabegron was seen in the trials [24]. Dose dependent increase in heart rate was seen in healthy volunteers, but not in clinical studies. There were no clinical relevant effects on post void residual, acute urinary retention and voiding urodynamics [24].

Combination Therapy of OAB: Antimuscarinics and β_3-Adrenergic Agonists

Since treatment with antimuscarinics and β_3-agonists has different mechanisms of action, combining both agents may improve efficacy in OAB treatment. Combination therapy with reduced doses of antimuscarinics may deliver an improved tolerability profile with maximized efficacy, compared to antimuscarinics monotherapy.

Preclinical models have shown additive effects for augmented bladder storage function when different antimuscarinic drugs and β_3-agonists are combined [29;30]. In a phase II study (Symphony) combination therapy showed significant improvements compared with solifenacin 5 mg monotherapy in mean voided volume (MVV), a reduction of micturition frequency, incontinence episodes and urgency episodes [31].

AE's during treatment, especially regarding blood pressure, pulse rate, PVR volume, laboratory or ECG parameters were not seen in the monotherapy and combination groups. Patients treated with antimuscarinics reported AE's (dry mouth, constipation, blurred vision and dyspepsia) in a dose dependent manner. These AE's were not increased in the combination group [31].

Pharmacological Treatment of BOO

In the pathophysiology of BPH, androgens play a central role. The enzyme 5α-reductase converts the androgen testosterone to the more potent dihydrotestosterone, which stimulates cell proliferation, differentiation, inhibits cell death and thereby growth of the prostate [32]. Inhibition of this enzyme by 5α-reductase-inhibitors (5-ARI's) reduces prostate size, voiding symptoms and disease progression. Because 5-ARI's have a slow onset of action, they are best suitable for long-term treatment [33].

A treatment modality with a quicker onset of action is α_1-adrenoceptor blockade. This has become the most widely used drug treatment for male LUTS suggestive of BOO. All α_1-blockers are similarly effective in treating LUTS when used in appropriate doses, but can differ qualitatively and quantitatively in their tolerability [2]. Controlled studies demonstrate reduction of IPSS with approximately 30-40% and increase of maximal urinary flow (Q_{max}) by approximately 20-25%. The effect of treatment usually onsets after hours to days and appears to be effective for years, if

used daily. Prostate size and risk of acute urinary retention (AUR) were not decreased by α_1-blocker treatment [33].
It has long been assumed that α_1-blockers inhibit the effect of endogenously released noradrenaline on smooth muscle cells in the prostate, thereby reducing prostate tone and BOO. Contraction of the human prostate is mediated predominantly, if not exclusively, by α_{1A}-adrenoceptors [23]. However, it has been shown that α_1-blockers have little effect on urodynamically determined bladder outlet resistance [34] and that treatment-associated improvement of male LUTS is correlated only poorly with obstruction or obstruction relief [35]. Altogether, the mechanism of LUTS relief by α_1-blockade is not fully understood and its effect might be caused outside the prostate (e.g. urinary bladder and/or spinal cord); particularly in these other locations, other α_1-adrenoceptor subtypes (α_{1B}- or α_{1D}-adrenoceptors) may be mediators of the beneficial effects of α_1-blockers.

Combination Therapy of BOO: Antimuscarinics and α_1-Blockers

Undertreatment of storage symptoms in men with LUTS is highly prevalent [3;31]. Therapy with α_1-blockers together with a muscarinic antagonist combines the efficacy of both drug classes to achieve synergistic effects on voiding and storage symptoms. Several randomized controlled trials and prospective studies have evaluated the efficacy of this combination therapy, either as an initial treatment in men with OAB and presumed BPO, or as a sequential treatment (add-on approach) in men with persistent storage symptoms, despite treatment with α_1-blockers [31;33]. Persistent LUTS after α_1-blockers treatment, especially when detrusor overactivity has been demonstrated, is significantly reduced after addition of an antimuscarinic drug [20;36]. Initial combination treatment significantly reduced voiding frequency, nocturia, and IPSS compared to α_1-blockers or placebo alone. Combination therapy also significantly reduced urgency and UUI episodes, and increased quality of life (QoL) [22]. Three recent trials with fixed dose combinations of tamsulosin oral controlled absorption system (TOCAS) 0.4 mg with solifenacin (3, 6, and 9 mg) demonstrated the combination of TOCAS 0.4 mg and solifenacin 6 mg to be most effective and best tolerated [37]. In conclusion, both the add-on approach and initial combination

treatment are effective therapeutical strategies. Clinicians should make a choice for a therapeutical approach based on patient characteristics.
The most reported AE is a dry mouth, but some AE's (e.g. dry mouth or ejaculation failure) may appear with increased frequency and cannot simply be explained by adding together the frequencies of the adverse events of either drug. Combination studies of α_1-blockers and antimuscarinics showed an increase in PVR, but the overall risk of AUR seems to be low [33].

Other Pharmacotherapeutical Options

Next to the substances described above, other pharmacological options are available for the treatment of LUTS. These pharmacological therapies will not be described here, since none of these substances were used in any of our investigations. They involve PDE-5 inhibitors, desmopressin and botulinum toxin. For an overview of clinical efficacy and tolerability of these substances, the EAU guideline of male non-neurogenic LUTS is recommended [33].

Outline of Thesis

Part One: Muscarinic Receptors in the Prostate

As described above, muscarinic receptors are the most important mediators of bladder contraction and the primary target for the treatment of OAB. In recent years, focus of male LUTS has changed from prostate-only to the bladder and prostate, leading to a different therapeutical approach. Besides in the bladder, muscarinic receptors are abundantly present in the prostate. Little is known though about muscarinic innervation, function and therapeutic opportunities. In the first part of this thesis, the role of muscarinic cholinergic receptors in the prostate is explored.
In **chapter 2** the current knowledge of the distribution of cholinergic nerves and receptors in the prostate is described in a literature review. Different techniques to study cholinergic innervation, such as immunohistochemistry and electron microscopy are presented. Muscarinic receptors have been investigated both on mRNA and protein levels, the latter having been investigated by both radioligand binding and antibody-based approaches. Functional muscarinic responses are described at the cellular and tissue level, followed by the changes that occur in pathological conditions.

In **chapter 3**, the association of mRNA expression of all muscarinic receptor subtypes (M_1, M_2, M_3, M_4 and M_5) with patient age, prostate size, prostate-specific antigen (PSA) level, pathological diagnosis, and concomitant medication is studied. The only previous study on muscarinic mRNA expression was done in primary cell cultures of 3 patients with BPH [38]. Although, in general, the quantitative relationship between mRNA and functional protein (the receptor) remains uncertain, mRNA measurements of tissue samples have the advantage of being able to detect a given subtype with great specificity, without the risk of receptor changes in mRNA or receptor expression due to culturing. The current study is the largest study to date of regulation of muscarinic receptor mRNA expression in the human prostate of men with BOO (due to BPH or prostate cancer) and possibly interfering factors.

Part Two: Muscarinic and Adrenergic Signalling

Interaction between M_2, M_3 muscarinic and β_3-adrenergic receptors is studied in the first two chapters of the second part of this thesis. Preclinical models have shown that combination of antimuscarinic drugs and β_3-agonists increase bladder storage function [29;30]. In clinical studies, combination therapy showed significant improvements in several voiding parameters compared with monotherapy [31].

Exploring the interaction between muscarinic and β-adrenergic receptors is essential for the understanding of normal and pathological voiding behavior. Since muscarinic receptors mediate urinary bladder contraction, and β-adrenergic receptors relaxation, their coordinated interaction is crucial to normal urinary bladder functioning. Although the exact mechanism is unclear, the micturition cycle is the result of an accurate interplay of the autonomic (sympathetic and parasympathetic) and the somatic nervous system [39]. During the storage phase of the micturition cycle, the bladder slowly expands and low-level afferent signals are sent to the brain. If micturition is inappropriate, the brain sends an inhibitory signal to the pontine micturition center (PMC), which serves as the on/off switch for voiding. If set in “storage mode” the PMC activates sympathetic hypogastric nerves through the sympathetic nucleus of the thoraco-lumbar spinal cord, which induces relaxation of bladder smooth muscle by stimulating β_3-

adrenoceptors with noradrenaline (NA). Contraction of bladder outlet smooth muscle is also a result of NA stimulation, but on α_1-adrenoceptors. Parasympathetic outflow to the bladder via the pelvic nerve is inhibited resulting in contraction of the striated urethral sphincter. When the urge to void becomes strong enough, afferent signaling to the brain becomes stronger. If the brain decides voiding is appropriate, the PMC switches to the "micturition mode" and inhibits sympathetic action. Parasympathetic nerves are stimulated and release ACh, which stimulates M_2 and M_3 muscarinic receptors, leading to detrusor contraction. The somatic pudendal nerve is inhibited, allowing the striated urethral sphincter to relax [39].

Muscarinic antagonists competitively block muscarinic receptors of all subtypes, but not with similar selectivity. This subtype selectivity might account for different tolerability profiles amongst antimuscarinics, but all are supposed to be similarly effective [12]. In the treatment of OAB, muscarinic antagonists are believed to be mainly active during the storage phase of the micturition cycle by reducing "afferent noise", with little or no effect on voiding contraction [40]. The finding that very few patients develop acute urinary retention (AUR) after administration of antimuscarinics, suggests that afferent pathways and not efferent pathways are mainly influenced by antimuscarinics [12].

Interaction at Tissue Level

In the micturition cycle, β-adrenoceptor mediated relaxation is opposed by muscarinic receptor mediated contraction, or the other way around. A previous study demonstrated that presence of the non-selective β-agonist isoprenaline reduced the efficacy and potency of contraction by the non-selective muscarinic agonist carbachol in porcine bladder [41]. The effect on potency was even more pronounced in denuded bladder strips [42], suggesting that β-agonists may act, at least partially, on the urothelium.

The opposite experiment, of presence of a muscarinic agonist on β-adrenergic mediated relaxation, was studied in rat bladders. Isoprenaline relaxation was less potent and less efficacious against tone induced by carbachol than induced by KCl [43]. The reduction of relaxant responses was not seen against passive tension, bradykinin and serotonin [44]. Other β_3-selective agonists, such as KUC-7322 and TRK-380 also demonstrated weaker

relaxation responses against carbachol than against other stimuli [13].

In **chapter 4** we explore the contribution of M_2 and M_3 muscarinic subtypes in the attenuation of the isoprenaline induced relaxation using two novel subtype-selective receptor ligands; BZI, a M_3 sparing muscarinic agonist (M_2 stimulation) and THRX-182087, a highly M_2 selective antagonist in combination with carbachol (M_3 stimulation). The involvement of phospholipase C (PLC) and protein kinase C (PKC) intracellular pathways after M_3 stimulation was also investigated.

Intracellular Interaction

The intracellular signaling pathway of M_3 muscarinic receptors is stimulation of PLC, leading to formation of inositol phosphates and diacyglycerol, which in turn mobilizes Ca^{2+} from intracellular stores, and activates PKC, respectively [45]. PKC is additionally coupling to phospholipase D (PLD). As a result of the steps above, myosin light chain is phosphorylated and rho kinase is activated. Given the role of Ca^{2+} in initiating smooth muscle contraction, it seems plausible that the PLC activation is the molecular basis of muscarinic receptor mediated smooth muscle contraction. But surprisingly multiple studies in rat, mouse and human bladder have demonstrated that muscarinic agonists induce contraction largely independent of PLC and rather rely on the opening of L-type Ca^{2+} channels and the activation of rho kinase, indicating that influx of extracellular Ca^{2+} through such channels and Ca^{2+} sensitization of contractile filaments may be more important than mobilization of Ca^{2+} from internal stores [46]. However it should be noted that muscarinic receptor stimulation can not only directly cause smooth muscle contraction, largely via the M_3 subtype, but can also attenuate β-adrenergic mediated relaxation, at least partly via the M_2 subtype [13].

M_2 muscarinic receptors and β_3-adrenergic receptors both act on adenylyl cyclase, with the first having an inhibitory effect and the second having a stimulating effect on the enzyme. Stimulation of adenylyl cyclase leads to cAMP formation, which activates protein kinase A (PKA) [47]. If the β-adrenoceptors mediated relaxation is the result of cAMP, is currently under debate, since cAMP appears to play a minor role in bladder relaxation [46]. Whether muscarinic receptors mediate inhibition of cAMP accumulation in

the bladder has remained controversial, cAMP may alternatively activate the exchange protein activated by cAMP (Epac) [48]. β-adrenoceptors can also couple to activation of several potassium channels, mostly large conductance Ca^{2+} activated K^{+}-channels (BKCa). Collectively, these data demonstrate that muscarinic and β-adrenergic signaling oppose each other at multiple levels of their signaling cascade [13].
In order to study interaction of M_2 and M_3 muscarinic receptors and β_3-adrenoceptors in more detail we developed an in vitro model as described in **chapter 5**. Chinese hamster ovary (CHO) cells were double-transfected with human muscarinic and β_3-adrenergic receptors, producing clones with M_2/β_3 and M_3/β_3 combination. cAMP-, and Ca^{2+}-mobilization assays were performed to measure the effect of stimulation of both receptors on their prototypical signaling pathway.

In male LUTS suggestive of BOO α_1-adrenoceptor antagonists are used to improve voiding symptoms. A part of these patients also suffer from storage symptoms and are additionally treated with antimuscarinics. Propiverine is a non-selective muscarinic receptor antagonist possessing additional properties, i.e., block of L-type Ca^{2+} channels. Contraction of the prostate smooth muscle cells [49] and bladder smooth muscle cells [46] at least partly depends on these channels and blocking them might contribute to the therapeutic effect of propiverine in male LUTS with storage and voiding symptoms. In clinical studies, propiverine in combination with α_1-adrenoceptor antagonists demonstrated to be more effective on storage symptoms than α_1-adrenoceptor antagonists alone. This effect was seen with the non-subtype selective doxazosin [50], alfuzosin [51], and the α_{1A}-selective tamsulosin [52]. In a non-intervention study, treatment of OAB symptoms with propiverine extended release was equally effective in men with propiverine monotherapy or combination therapy with α_1-adrenoceptor antagonists, regardless of baseline Q_{max}. In men with reduced Q_{max}, IPSS improvement is significantly smaller with monotherapy, suggesting beneficial effects of addition of α_1-adrenoceptor antagonists in men with storage and voiding symptoms [53].
In **chapter 6** possible α_1-adrenoceptor antagonistic effects of propiverine and its metabolites M-5, M-6 and M-14 are studied,

which share the antimuscarinic and/or L-type Ca^{2+} channel blocking activity of propiverine [23;54]. Human prostate and porcine trigonal muscle strips were used to explore inhibition of α_1-adrenoceptor-mediated contractile responses. Chinese hamster ovary (CHO) cells expressing cloned human α_1-adrenoceptors were used to determine direct interactions with the receptor in radioligand binding and intracellular Ca^{2+}-elevation assays.

Part Three: Muscarinic Antagonists in Clinical Practice

While age is a major risk factor for UUI, female gender, obesity, obstructive airway disease and childbirth, particularly vaginal delivery, are risk factors for SUI [55]. The latter is attributed to anatomically damaging the bladder outflow tract and/or pelvic floor. Multiparity is also a risk factor for urinary incontinence in general [56], possibly in an ethnicity-dependent manner [57]. As these studies did not specify whether incontinence involved SUI, UUI or both, they raise the possibility that multiparity, and more specifically by vaginal delivery, may be a risk factor not only for SUI but also for UUI/OAB. Indeed, studies in Asian countries reported that multiparity [56] and childbirth through vaginal delivery [58] are risk factors for developing OAB. As vaginal delivery should not directly affect detrusor function, a link with UUI/OAB is not necessarily expected. On the other hand, prolapse surgery was reported to improve OAB symptoms [59], and the demonstration of reflex pathways between the urethra and the detrusor in both animals [60] and patients [61;62] could establish a mechanistic link between delivery-associated anatomical damage and detrusor dysfunction. Instead, males have a greater bladder outlet resistance based upon a longer urethra, but do not suffer less frequently from OAB [8;9;63]. Therefore, it appears important to determine whether the reported association of multiparity/vaginal deliveries with OAB can be confirmed, specifically in non-Asian countries with a predominantly Caucasian population. If indeed vaginal deliveries predispose for OAB, it also becomes important to know whether this affects the responsiveness of OAB to treatment.

In **chapter 7** a preplanned secondary analysis of an observational study of solifenacin in patients with OAB was performed. Of women without, and with one, two or more than two vaginal deliveries and men episode frequencies of OAB symptoms,

pad use and scores on OAB rating scales were documented and compared before and after 12-14 weeks of treatment with 5 or 10 mg of solifenacin.

Since OAB prevalence increases with age [8;9;63;64] and this patient group is more vulnerable for renal and hepatic impairment and the use of comedications, it is important to be aware of dose recommendations of antimuscarinic drugs in these special patient groups. Alterations in drug metabolism may cause greater exposure in the respective population and thereby increasing the risk of side-effects and non-compliance. Pharmacokinetic alterations can also be necessary in patients with genetic differences in drug metabolizing enzymes such as CYP 3A4 and CYP 2D6. In **chapter 8** the question if one muscarinic receptor antagonist suits the needs of all special patient groups is explored. The result is a literature review of the evidence and regulatory dosing recommendations in Europe and the USA for muscarinic receptor antagonists used in the treatment of overactive bladder symptom syndrome.

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PART ONE
MUSCARINIC RECEPTORS IN THE PROSTATE

Chapter 2 Cholinergic Innervation and Muscarinic Receptors in the Human Prostate

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Abstract

Purpose. In light of recent interest in the use of muscarinic receptor antagonists for the treatment of male lower urinary tract symptoms, an understanding how such drugs work not only on the bladder but also on the prostate is important.

Methods. A literature review was conducted to identify studies into the cholinergic innervation and presence and function of muscarinic acetylcholine receptors in the human prostate.

Results. The available studies demonstrate a dense cholinergic innervation within both stromal and epithelial compartments of the prostate. Concomitantly, the human prostate expresses muscarinic receptors at densities exceeding those of α_1-adrenoceptors. They mainly belong to the M_1 subtype and are found on epithelial cells, but a smaller population of M_2 receptors is found on stromal cells. Both populations have been shown to be functional in signal transduction assays. However, in line with the sparse receptor density on stromal smooth muscle cells, contractile responses of the prostate are only small. Data from prostate cancer cell lines and from botulinum toxin injections into the benign prostate raise the possibility that muscarinic receptors may promote prostatic growth. Animal data suggest that muscarinic receptors may be of primary importance in the genesis of prostatic secretions, but this needs to be confirmed in humans.

Conclusion. Taken together it appears that direct effects on the prostate need to be considered when using muscarinic receptor antagonists in male patients. They may primarily involve alterations of glandular secretion and prostatic growth.

1. Introduction

Muscarinic receptor antagonists are the standard of care to treat the overactive bladder (OAB) storage symptoms of urgency, frequency and nocturia [1]. These three symptoms also occur frequently in men presenting with lower urinary tract symptoms (LUTS) assumed to result from benign prostatic hyperplasia and are often the cause of presentation to a clinician. However, the package inserts of muscarinic receptor antagonists list a clinically relevant bladder outlet obstruction (BOO) as a caution or even a contra-indication for their use because of a perceived risk for urinary retention. On the other hand, muscarinic antagonists typically cause only little increase in post-voiding residual urine in OAB patients [2], and conversely muscarinic agonists have little effect in patients with an underactive detrusor [3]. More importantly, recent studies with muscarinic receptor antagonists in male LUTS patients, including some with proven BOO, observed only small if any increases in post-voiding residual urine or incidents of acute urinary retention [4,5], particularly when applied in combination with α_1-adrenoceptor antagonists [6,7,8,5,9]. These findings have led to a discussion whether muscarinic antagonists may actually be helpful in male LUTS patients, particularly in those with significant storage symptoms [10]. In a broader sense these findings raise the question to which extent the prostate and/or the bladder primarily contribute to male LUTS.

Cholinergic fibres innervate not only the bladder but also the prostate [11]. In either case they primarily release the transmitter acetylcholine which activates muscarinic receptors of which five subtypes exist [12]. Whilst the role of muscarinic receptors in the control of bladder function has been studied extensively [13,14] and very likely plays a major role in their beneficial effects, particularly at early time points, much less information is available regarding possible effects on prostate function. Direct effects of muscarinic antagonists on the prostate may contribute to potential beneficial effects and may also predict a low risk of urinary retention in LUTS patients. The presence of cholinergic nerves and muscarinic receptors along with the functions mediated by them in the prostate of several animal species has been reviewed previously [15,11]. While those studies indicate a physiological function for cholinergic mechanisms in glandular and, to a lesser extent, stromal parts of the mammalian prostate, they have also revealed considerable species heterogeneity which makes it

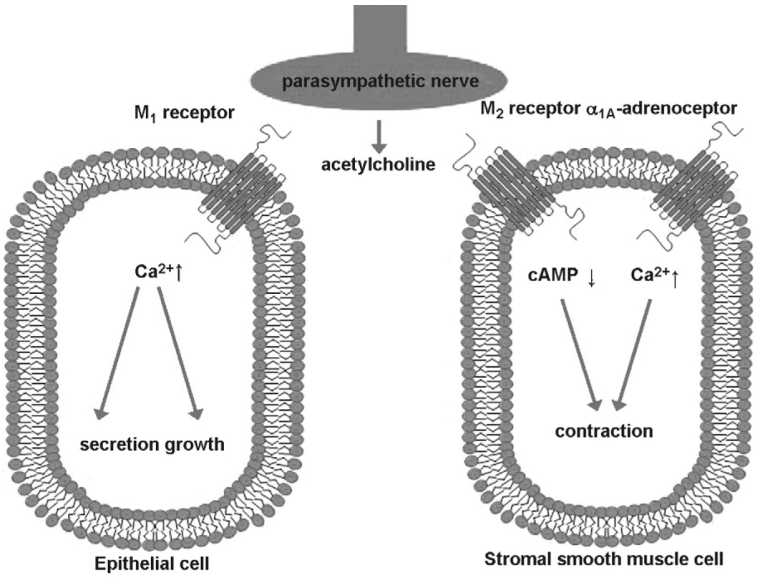

Figure 1. Schematic diagram of cholinergic nerves and muscarinic receptors in the human prostate. Note that M_2 receptors on stromal smooth muscle cells quantitatively play a smaller role than M_1 receptors on epithelial cells or α_{1A}-adrenoceptors on smooth muscle cells and that signal transduction and smooth muscle and epithelial cell function presently have been tested in few studies only (see text for details).

difficult to extrapolate from any of these species to the human situation. Therefore, the present manuscript will specifically review the presence and function of a cholinergic system in the human prostate. Studies in animal tissues will only be mentioned in cases where evidence for the human prostate is missing; in all other cases readers interested in the animal data are referred to two recent reviews [15,11]. A simplified summary of the available data is shown in figure 1.

2. Innervation with Cholinergic Fibres

The initial studies on the innervation of the human prostate did not discriminate between cholinergic, adrenergic and other nerve fibres [16,17]. Such discrimination became possible with the advent of more specific immunological markers for cholinergic nerves. In this regard most studies have relied on immunohistochemical staining for the presence of the acetylcholine-degrading enzyme acetylcholinesterase (AChE) as a marker of the cholinergic innervation of the human prostate. While expression of this

enzyme is typical for cholinergic nerves [18], it can also be observed in other structures including sensory nerves [11]. Moreover, both cholinergic and sympathetic preganglionic nerve fibres use acetylcholine as their neurotransmitter and hence may also express AChE [19]. Other markers such as choline acetyltransferase or vesicular acetylcholine transporters may be more specific but have rarely been applied in studies of the human prostate [20]. This limitation needs to be kept in mind when looking at studies investigating the cholinergic innervation of the human prostate.

While some early studies yielded negative results [21], various other histochemical studies have demonstrated the presence of AChE-positive nerves in the human prostate following the initial studies in the late 1970's [22,19]. Cholinergic fibres were found in various regions of the human prostate including the anterior capsule, peripheral zone, proximal central and distal central zone [18]. Some investigators have suggested that the density of cholinergic nerve fibres may exceed that of adrenergic fibres in the overall prostate [23,18]. In several cases, AChE-positive fibres were found to contain co-transmitters including vasoactive intestinal peptide [24,25,26], neuropeptide Y [20], or NO, as indicated by the presence of NO synthase [26,20], which are also cholinergic cotransmitters in many other tissues.

However, possibly due to methodological problems, early studies often failed to demonstrate close contacts between cholinergic fibres and target structures such as epithelial or smooth muscle cells within the human prostate, i.e. failed to detect cholinergic synapses [23,25]. This changed with the advent of ultrastructural techniques such as electronmicroscopy. Using this approach Gosling was the first to demonstrate cholinergic nerve terminals in association with smooth muscle cells and the acinar cells of the prostate; while the distance between nerve terminal and epithelial cell (0.5-1 μm) was relatively large as compared to such synapses in other tissues, these were nevertheless classified as a synapse [27]. At least in neonates and children, similar varicose connections between cholinergic nerves can be found with stromal and epithelial cells, and also with intraprostatic blood vessels [20].

3. Receptor Expression in the Prostate

The presence of muscarinic receptors and their subtypes in the human prostate has been investigated both at the mRNA and the protein level, the latter having been investigated by both radioligand binding and antibody-based approaches. In this regard the mRNA measurements have the advantage of being able to detect a given subtype with great specificity; on the other hand, their disadvantage is that the quantitative relationship between mRNA and functional protein remains unclear. While radioligand binding directly quantifies the functional receptor of interest, the specific labelling of muscarinic receptor subtypes is limited by the poor subtype-selectivity of most pharmacological tools which have been used in those studies. Antibody-based approaches can in principle combine the advantages of both techniques but most antibodies for G-protein-coupled receptors have been poorly validated for their specificity and many may also bind to proteins other than their specific receptor. These caveats need to be considered in the interpretation of the available data.

One study investigated the presence of mRNA encoding muscarinic receptors in the human prostate [28]. This study was primarily based upon primary culture of stromal and epithelial cells obtained from three patients with benign prostate enlargement. While mRNA for all five muscarinic receptor subtypes was detected in whole prostate using a polymerase chain reaction, primary stromal cultures only showed M_2, M_3 and M_4 receptors with M_2 apparently being most abundant. In contrast, primary epithelial cultures exhibited only M_1, M_2 and M_5 receptor mRNA. Northern blotting and *in situ* hybridization detected only the stromal M_2 but none of the other receptors [28]. However, it remains unclear how the results from the cultured cells relate to the *in vivo* situation, particularly at a quantitative level, when it is considered that the expression of many genes changes upon culturing. Moreover, no information on the non-enlarged prostate is available.

Several studies have investigated the presence of muscarinic receptor protein in the human prostate by radioligand binding using either [^{3}H]-methyl-quinuclidinyl benzilate (QNB) or [^{3}H] N-methylscopolamine (NMS) as the radioligand. In this regard QNB is a more lipophilic radioligand than NMS, and this may

lead to the detection of a greater number of muscarinic receptors in lower urinary and other tissues [29]. The initial study, based upon NMS saturation binding in three male patients with enlarged prostates, reported an average receptor density of 2.1 fmol/mg wet weight [30]. This was reported to be in the same range as values in rat, rabbit and pig prostate but somewhat lower than values in normal or enlarged canine prostate as measured by the same investigators. Later follow-up studies from that laboratory compared the density of NMS binding sites in samples from asymptomatic patients with those from symptomatic patients with enlarged prostates LUTS obtained either by open prostatectomy or transurethral resection [31,32]. The muscarinic receptor density did not differ significantly between groups (n = 8-10 in each group), all being 200-300 fmol/mg protein or 0.94 fmol/mg wet weight. Interestingly, these values were considerably higher than the densities of either α_1- or α_2-adrenoceptors (80-110 and 60-130 fmol/mg protein, respectively) as measured within these groups in the same study. Using QNB as the radioligand, other investigators reported a muscarinic receptor density of 158 and 123 fmol/mg protein in the capsule and adenomatous part of the human prostate, respectively (corresponding to 2.4 and 4.0 fmol/mg wet weight, respectively; not significantly different from each other) [33]. Also in this study, the density of muscarinic receptors was markedly higher than that of either α_1- or α_2-adrenoceptors (15-29 and 8-31 fmol/mg protein, respectively). Finally, one study using QNB reported a muscarinic receptor density of 46 fmol/mg protein in prostatic adenoma [34]. Thus, the density of muscarinic receptors in the human prostate is generally reported to be in the range of 50-300 fmol/mg protein or 1-4 fmol/mg wet weight, and in two direct comparative studies was considerably greater than those of either α_1- or α_2-adrenoceptors (Figure 2).

To better link the presence of muscarinic receptors in the human prostate to their possible function, autoradiographic studies have been performed which allow associating the receptors with specific anatomical structures. In this regard three independent studies report that most muscarinic receptors are found in the prostatic epithelium, whereas the stroma contains far fewer if any muscarinic receptors [30,33,35]. However, one study using primary cultures of human prostatic stromal smooth muscle cells reported a high density of muscarinic receptors (1250 fmol/

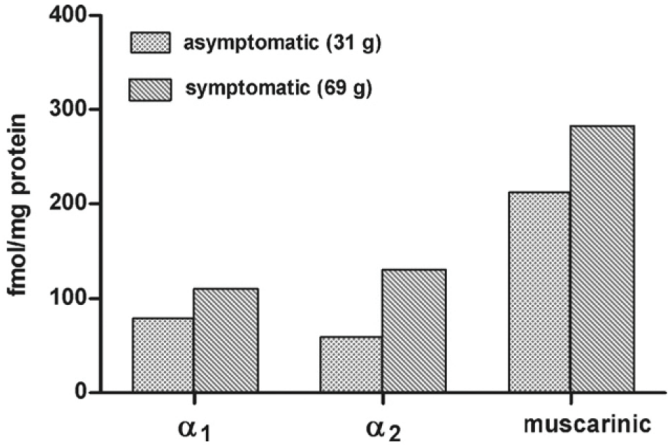

Figure 2. Presence of muscarinic receptors as compared to α_1- and α_2-adrenoceptors in the human prostate. Receptor expression was studied in two groups of men, referred to as ''asymptomatic benign prostatic hyperplasia'' and ''symptomatic benign prostatic hyperplasia'' by the original authors, with the former actually representing patients with invasive transitional cell carcinoma; the average prostate weight of the two groups is shown in parentheses. Note that the relative proportions of muscarinic receptors and α_1- and α_2-adrenoceptors were similar in both groups. Adapted from Gup et al. J Urol 1990;143:179–85.

mg protein) [36]; whether this reflects an up-regulation under the specific culture conditions used remains to be determined. Thus, muscarinic receptors in the human prostate are primarily found in the epithelial rather than the stromal cells. This differs considerably from the localization of α_1- and α_2-adrenoceptors, which are mainly found in stromal cells and intra-prostatic blood vessels, respectively [37]. This differential anatomical distribution of receptors within the prostate highlights the problems with interpreting data from tissue homogenates which are often difficult to interpret as a consequence of differences in cellular composition. In this regard it is important to note that all of the above autoradiographic studies were performed with tissues from patients with enlarged prostates, and little is known regarding the localization of muscarinic receptors in the normal prostate, which is known to have a smaller stroma/epithelium ratio.

Five subtypes of human muscarinic receptors exist [12]. One study in primary cultures of prostatic stromal cells has used competition binding experiments with several moderately subtype-selective compounds to identify the receptor subtype being present [36]. Based upon a rank order of potency of atropine (non-selective) > 4-DAMP ($M_{1/3}$-selective) > methoctramine (M_2-selective) > p-F-

HHSiD (M_3-selective) ≈ pirenzepine (M_1-selective) > AF-DX 116 (M_2-selective) these authors proposed that stromal cells in the prostate mainly express M_2 receptors; however, it should be noted that these compounds may allow a differentiation between M_1, M_2 and M_3 receptors but not from M_4 and M_5 receptors. Moreover, all of them have only moderate subtype-selectivity, and in some cases they are selective for a certain subtype but have lower overall affinity than other compounds, which influences the order of potency. Nevertheless, the proposal of a preponderance of M_2 receptors in prostatic stromal cells is in good agreement with the available mRNA data [28].

A more comprehensive assessment of muscarinic receptor subtypes in prostatic homogenates has been reported by other investigators [34]. In competition binding in prostate homogenates the rank order of potency was pirenzepine > HHSiD (selective for M_3 and M_1 relative to M_2 receptors) > p-f-HHSiD > methoctramine. These data indicate that the majority of receptors may belong to the M_1 subtype. However, the competition curves for pirenzepine were somewhat shallow and possibly biphasic indicating that M_1 may be the dominant but not the only subtype present in the prostate. In a second approach these authors have used immunoprecipitation of muscarinic receptors with subtype-selective antibodies for $M_{1\text{-}4}$ receptors. These data confirmed that M_1 receptors are more abundant than the other three subtypes combined. Using the M_1 antibody for immunohistochemistry yielded staining of the prostatic epithelium but not stroma. Taken together these data suggest that the human prostate mainly expresses M_1 receptors on epithelial cells at the protein level, whereas the much smaller population of receptors on the stromal cells may belong to the M_2 subtype. However, each of these conclusions is at present based upon a single study and needs to be confirmed by further research.

4. Muscarinic Responses in the Prostate

The functional role of muscarinic receptors and their subtypes in the human prostate has been assessed at multiple levels, which include signal transduction responses (e.g. elevation of intracellular Ca^{2+} or inhibition of cAMP formation), smooth muscle contraction and prostatic growth.

4.1 Responses at the Cellular Level

4.1.1. Calcium. An activation of a phospholipase C with

the subsequent release of Ca^{2+} from intracellular stores is a prototypical signalling pathway of M_1, M_3 and M_5 muscarinic receptors apparently involving $G_{q/11}$ proteins [12]. Moreover, M_3 receptors, e.g. in the human bladder, can also increase intracellular Ca^{2+} by stimulating its influx from the extracellular space via voltage-operated channels [38], and other receptors subtypes including M_2 receptors can also couple to elevations of intracellular Ca^{2+} [39].
Effects of muscarinic agents on human prostatic smooth muscle cell Ca^{2+} responses have been investigated in a single study reported in abstract form only [40]. These investigators reported Ca^{2+} elevations in response to carbachol and several other muscarinic receptor agonists; such responses were at least partly inhibited by several muscarinic antagonists but no quantitative data were presented which allow specific conclusions about the receptor subtypes which were involved.

4.1.2. cAMP. Inhibition of cAMP formation is a prototypical signalling response of M_2 and M_4 muscarinic receptors which occurs by activation of G_i proteins [12]. Accordingly, muscarinic receptor agonists such as carbachol and oxotremorine were found to inhibit cAMP formation in response to either the receptor-independent stimulus forskolin or the β-adrenoceptor agonist isoprenaline in cultured human prostatic stromal cells [36], which is in line with the proposed presence of M_2 receptors in stromal cells. As cAMP is known to inhibit prostatic contractility [41], muscarinic receptor-mediated reductions could in principle indirectly enhance prostatic tone.

4.2. Responses at the Tissue Level

4.2.1. Transmitter Release. Muscarinic receptors can exist prejunctionally on nerve endings where they can modulate transmitter release including that of acetylcholine (auto-receptors) and noradrenaline (hetero-receptors). In a single study in human prostate the muscarinic agonist carbachol and antagonist scopolamine had no consistent effects of noradrenaline release, whereas an α_2-adrenoceptor agonist and antagonist inhibited and enhanced it, respectively [33].

4.2.2. Contraction. In line with the prominent role of

muscarinic receptors in promoting urinary bladder contraction, various investigators have studied a possible contractile effect of muscarinic receptor agonists in human prostate. Many studies did not detect contractile responses [46,47,33], whereas others do report such effects [47,31,48]. While contractions were sensitive to muscarinic antagonists such as atropine [47,49], their magnitude was small (about 20%) as compared to the contractile effects of α_1-adrenoceptor agonists [49] and typically seen only with very high agonist concentrations. Therefore, it appears that muscarinic receptors contribute to human prostate contraction in a minor way only, which is in line with their primary expression on epithelial rather than stromal cells. Moreover, muscarinic agonists apparently do not modify α_1-adrenoceptor contractile responses to a relevant degree [49]. Some studies reported that muscarinic receptors can mediate contraction of the prostatic capsule but not adenoma tissue [47]. However, others did not find direct muscarinic agonist-induced contraction in either part, and if anything reported such agonists to selectively enhance α_1-adrenergic contraction in the adenoma part [33]. Therefore, it appears that muscarinic receptors contribute only in a limited way if at all to human prostatic contraction in a direct manner. However, prejunctional M_1 receptors may indirectly cause contraction in rats, guinea pigs and rabbits (but not in pigs) by facilitating the release of other transmitters which cause contraction [50,51]; whether a similar situation exists in the human prostate, is unknown. From a clinical point of view these data suggest that blockade of muscarinic receptors in the prostate is unlikely to lower the overall prostate tone, i.e. to lower the dynamic part of bladder outlet resistance in a clinically relevant manner.

4.2.3. Prostatic Secretion. Pivotal studies in dogs, which lack seminal vesicles and Cowper's glands and hence allow a pure assessment of the prostatic component, have demonstrated that parasympathetic stimulation increases the prostatic secretion [52]. Similar observations have subsequently been reported in other animal species such as rats [11]. While corresponding studies in humans are missing, a role for muscarinic receptors in the regulation of human prostatic secretion is likely, particularly as elevations of intracellular calcium (see above) are a typical stimulus for glandular secretion.

4.2.4. Prostatic Growth. Based upon the predominant muscarinic receptor expression in the prostatic epithelium, it could be expected that muscarinic receptors contribute to the regulation of prostatic growth. Indeed primary cultures from patients with either enlarged prostates or prostate cancer exhibited growth stimulation upon treatment with the muscarinic receptor agonist carbachol [42]. Although prostate cancer cell lines may not necessarily reflect the function of the normal prostatic epithelium, it is noteworthy that a muscarinic receptor agonist stimulated proliferation and related signal transduction pathways in three different prostate cancer cell lines, i.e. PC3, DU145 and LNCaP cells [43]. More importantly, and in line with rat experiments [44], a single injection of botulinum toxin into the human prostate was reported to reduce prostate volume by almost 20% [45]. As botulinum toxin is generally assumed to act by inhibiting the release of the endogenous muscarinic receptor agonist acetylcholine, these data provide evidence for a growth-promoting role of muscarinic receptors in the human prostate. Therefore, it would be of particular interest to obtain *in vivo* data on long term treatment of men with muscarinic antagonists and possible changes of prostate size.

5. Regulation of the Cholinergic System in the Prostate

Interestingly, the cholinergic innervation of the human prostate appears to undergo changes during development and in pathological conditions. Thus, the prepubertal prostate already displays dense AChE-positive innervation in the stroma but not yet in the epithelium [22], raising the possibility that the latter requires androgenic input. However, another study, relying on the more specific labelling of the acetylcholine transporter rather than AChE, reported innervation of both stroma and epithelium as early as the neonatal period [20]. During adulthood, as compared to the normal prostate, hyperplastic noduli are only sparsely innervated, and those nerve fibres are very thin and coiled [22,23,53]. Retrospectively, the description of thin corkscrew like nerve fibres in the hyperplastic prostate [17] supports these findings, although those early studies did not discriminate between cholinergic and other fibres. Such denervation similarly affects the epithelial and stromal parts of the prostate. A similar and possibly even greater

denervation has also been proposed for prostate cancer [17,22]. Unless local, non-neuronal acetylcholine formation is postulated, such partial denervation is likely to make the prostate less sensitive to *in vivo* effects of muscarinic receptor antagonists.
The expression of muscarinic receptors has been reported to be quantitatively similar in men with small asymptomatic and enlarged symptomatic prostates [31,32] or in normal, hyperplastic epithelium and in well to moderately differentiated carcinoma (however, it disappeared in poorly differentiated tumours) [54]. Similarly, muscarinic cholinoceptor density was not correlated with age or prostate size [31,32]. Whether potential growth inhibition by muscarinic antagonists is similarly effective in the normal and (benign or malignantly) enlarged prostate, remains to be studied.

6. Conclusion

In conclusion, the human prostate expresses a high density of muscarinic receptors, which are mostly associated with epithelial cells. Studies into the function of these receptors have only recently started to emerge and hence the limited available information must be interpreted with caution. Apart from a small effect on prostatic contraction these receptors are likely to be involved principally in glandular function and prostatic growth. These observations may contribute to the clinical effects of muscarinic receptor antagonists in male patients [55], but specifically the effects of long-term muscarinic antagonist use on glandular secretion and prostatic growth deserve further investigation.

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Chapter 3

Muscarinic Receptor Subtype mRNA Expression in the Human Prostate: Association with Age, Pathological Diagnosis, Prostate Size or Potentially Interfering Medications?

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Abstract

Purpose. As the prostate abundantly expresses muscarinic receptors and antagonists for such receptors are increasingly used in the treatment of men with voiding function and large prostates, we have explored an association of the mRNA expression of human M_1, M_2, M_3, M_4 and M_5 receptors in human prostate with patient age, prostate size, prostate-specific antigen level, pathological diagnosis and concomitant medication.

Methods. mRNA was isolated from prostate chips of 110 consecutive patients undergoing transurethral resection of the prostate for the treatment of benign prostatic hyperplasia or prostate cancer. Expression of each of the five muscarinic receptor subtype transcripts was assessed by real-time PCR and association with patient age, prostate size, prostate-specific antigen level, pathological diagnosis and concomitant medication were explored.

Results. M_1 and M_4 receptors were the most and least prevalently expressed subtypes in the human prostate, respectively. M_1 receptor mRNA expression was weakly but significantly associated with prostate size ($r = 0.0870$, $p = 0.0171$), but mRNA expression of none of the five subtypes was significantly associated with age, prostate-specific antigen level, pathological diagnosis (benign prostatic hyperplasia vs. prostate cancer) or concomitant medication (5α-reductase inhibitors, α_1- or β-adrenoceptor antagonists).

Conclusion. We conclude that human prostate muscarinic receptor subtype transcripts apparently undergo only a very limited regulation by a variety of physiological, pathophysiological or treatment factors. In light of the growing use of muscarinic receptor antagonists in men with voiding dysfunction including those with large prostates, the functional role of the weak association between M_1 receptor mRNA expression and prostate size merits further investigation.

Introduction

Muscarinic acetylcholine receptors and α_1-adrenoceptors are established drug targets for the treatment of voiding dysfunction. Within the human bladder muscarinic receptors, mainly their M_2 and M_3 subtypes, are consistently expressed at the mRNA and protein level, whereas α_1-adrenoceptors are detected in low abundance if at all at the protein level [1]. In contrast, α_1-adrenoceptors, specifically their α_{1A}-subtype, are consistently found in the human prostate, where they mediate smooth muscle contraction and perhaps also contribute to proliferation and the regulation of prostatic blood flow [2]. However, it is often ignored that the human prostate expresses more muscarinic than α_1-adrenoceptors at the protein level [3-5]. While both α_1-adrenoceptors and muscarinic receptors are found in stromal and epithelial cells, at the protein level the former are mainly found in stromal/smooth muscle and the latter mainly in epithelial/glandular cells [3;6;7]. At the mRNA level, all five muscarinic receptor subtypes were detected in whole prostate, but primary stromal cultures only showed M_2, M_3 and M_4 receptors with M_2 apparently being most abundant whereas primary epithelial cultures exhibited only M_1, M_2 and M_5 receptor mRNA [8]. In line with these mRNA data, the majority of muscarinic receptors at the protein level in whole prostate apparently belong to the M_1 subtype [9], whereas in stromal cells the M_2 subtype may be most abundant [10].

Only limited information is available on the function of muscarinic receptors in the prostate (for review see [11]), but some studies have explored their regulation. Thus, based on radioligand binding studies the total expression of muscarinic receptor protein appears to be quantitatively similar in men with small asymptomatic and enlarged symptomatic prostates [4;5] or in normal, hyperplastic epithelium and in well to moderately differentiated carcinoma (although it was not found in poorly differentiated tumors) [12]. Similarly, total muscarinic receptor protein density, as determined by radioligand binding studies, was not correlated with age or prostate size [4;5]. However, these studies have typically involved only small numbers of patients and did not take into account possible differences in the regulation of muscarinic receptor subtypes. Studies in other tissues have reported that muscarinic receptor subtypes undergo differential regulation and/or are regulated by different mechanisms [13-16]. Moreover, regulation of one of the muscarinic receptor subtypes can affect the density of

other muscarinic subtypes [17] and of adrenoceptors [18]; cross-regulation between muscarinic and adrenergic receptors typically occurs in opposite directions, with both types of receptors often increasing upon chronic treatment with an antagonist.
Against this background the present study was designed to explore associations of mRNA expression for each of the five muscarinic receptor subtypes with patient age, prostate size and its marker prostate-specific antigen (PSA), and pathological diagnosis (benign prostatic hyperplasia (BPH) vs. prostatic carcinoma (PC)). As a secondary objective, associations with medications potentially affecting muscarinic receptor expression were tested, i.e. 5α-reductase inhibitors (5-ARI's), muscarinic receptor antagonists, α_1-adrenoceptor antagonists and β-adrenoceptor antagonists.

Patients and Methods

Patients. The study protocol and patient consent form had been approved by the ethical committee of the Academic Medical Center and were in line with the Declaration of Helsinki. After having given informed written consent, 110 consecutive patients with lower urinary tract symptoms (median age 69 (62; 75) years) and undergoing mono- or bipolar transurethral resection of the prostate (TURP) participated in the study. RNA of acceptable quality was isolated from 95 patients. Based on histological examination, 82 of them had BPH and 13 PC. Fourteen patients were receiving 5-ARI's (7 dutasteride, 7 finasteride), 37 used an α_1-adrenoceptor antagonist (6 alfuzosin, 1 doxazosin, 1 prazosin, 29 tamsulosin), 13 a β-adrenoceptor antagonist (9 metoprolol, 2 propranolol, 2 atenolol), and 2 patients a muscarinic receptor antagonist (1 oxybutynin, 1 solifenacin). Patients had a median prostate volume, as assessed by transrectal ultrasound, of 73 (52; 110) ml and a median PSA of 5.1 (3.0; 8.9) ng/ml.

RNA Isolation. Prostate tissue chips with a total weight of approximately 2 g per patient (56-209 mg per chip) were collected during TURP. They were conserved in 10 ml RNA-later solution on crushed ice (4 °C) immediately after resection and then stored at -20°C. Prior to RNA extraction they were snap-frozen with liquid nitrogen. The tissue was ground into powder and homogenized in 1500 µl TRIzol. RNA was isolated according

to the manufacturer's protocol (Life Technologies, Bleiswijk, The Netherlands). The isolated product was quantified using the Nanodrop ND-1000 spectrometer (Isogen Life Science, IJsselstein, The Netherlands). RNA quality was tested using the Experion automated electrophoresis system (Bio-Rad Laboratories, Veenendaal, The Netherlands) and expressed as RNA Quality Indicator (RQI, ranging from 1–10). Samples with RQI > 7 and with specific fluorescence peaks in the electropherogram for 18S and 28S RNA were selected for cDNA synthesis. The selected mRNA was additionally treated with DNase I (Invitrogen, Bleiswijk, The Netherlands) to prevent genomic DNA contamination during cDNA synthesis. Total RNA (500 ng) was reverse transcribed using iScriptcDNA Synthesis Kit (Biorad) with RNAse inhibitor, based on the mix of oligo(dT) and random hexamer primers.

Diluted cDNA corresponding to 20 ng RNA input was used per individual real-time PCR (RT-PCR) performed in a custom-designed RT-PCR experiment. The genes analyzed with specific primers (Table 1) included all five muscarinic receptors, reference genes (ribosomal phosphoprotein P0 (RPLP0) and hypoxanthin phosphoribosyl transferase 1 (HPRT1)) and negative controls to control for selectivity and specificity of the reactions. Each of the primer sets met the criterion of ≥ 90% RT-PCR efficiency as tested. Per 96-well plate three samples each of 13 patients of the gene of interest and the reference gene were assayed, together with negative controls for reverse transcriptase and water control in a RT-PCR array using a MyiQ Single-Color RT-PCR Detection System (Bio-Rad Laboratories). The reaction conditions were as follows: initial activation 10 min at 95°C; 40 cycles of denaturation for 15 sec at 95°C; annealing/extension for 1 min at 60°C. Melting curve analysis confirmed a single PCR product formation for each individual primer set.

Data Analysis. Relative gene expression was analyzed for each well using the delta-delta cycle threshold ($\Delta\Delta C_t$) method, calculating the expression of each individual gene relative to that of the reference gene (ΔCt) and to the mean reference gene expression of all subjects ($\Delta\Delta Ct$). Based on the assay design and variability, all transcripts showing a C_t value higher than 35 were considered to be unexpressed (below detection limit); in cases

Table 1. Primer sequences used in PCR reactions

Gene	Reference sequence	Primers	Primer sequence	Length of product
RPLP0	NM_053275	Forward Reverse	GCTTCCTGGAGGGTGTCCGC TCCGTCTCCACAGACAAGGCCA	127
HPRT1	NM_000194	Forward Reverse	ACGAGCCCTCAGGCGAACCT AATCACGACGCCAGGGCTGC	160
CHRM1	NM_000738	Forward Reverse	CGGAACTCTGCAACAACAAAGCCTTCCG CTTGCGCCAGCGTCTCTTGT	80
CHRM2	NM_001006632	Forward Reverse	CGTACCCTGCTGTCACCTTTGGTACG CTTGCTGGCTCGGGATATGTG	98
CHRM3	NM_000740	Forward Reverse	TCCGGGTCACAGCACCATCCT GGCCTGCAGCTTGTCGGCTT	118
CHRM4	NM_000741	Forward Reverse	GCATTACGTCATCATCCCACAATCG CACGACAGTCACCAGGCTCAG	92
CHRM5	NM_012125	Forward Reverse	GACCTGGCTGACCTCCAGGGT GGTCGAGGACAGCGCAAGCA	101

where the C_t value exceeded 35 but nevertheless a single PCR product was identified, it was assumed to have a nominal of 35 association and group mean analysis.
In the direct comparison of eight pilot patients, RPLP0 showed a lower median C_t and less variability than HPRT1 (20 vs. 27; coefficient of variation, 4,3 vs. 4,7%, respectively). Moreover, in the total pool of samples RPLP0 expression also exhibited small variability (C_t 19.21 (18.17; 20.20); n = 393 experiments based on 95 patients). Therefore, RPLP0 was used as the reference gene for the calculation of all $\Delta\Delta C_t$ values.
During our quality checks we found that not all samples exhibited good melting curves and hence did not allow detection of a specific PCR product, most often when overall expression was low. Moreover, the triplicate C_t measurement from a given patient exhibited a range >1 in some cases, also most often in samples with low expression. All samples without an identifiable PCR product were excluded from the analysis. To balance data quality and sample size for the association analyses and group comparisons, three approaches were used in parallel. The broadest approach used median $\Delta\Delta C_t$ values of all patients who had at least one replicate with a specific PCR product. The intermediate approach also used median $\Delta\Delta C_t$ values but excluded all patients with >1 replicate without an identifiable PCR product. The most stringent approach was similar to the intermediate one but calculated the mean $\Delta\Delta C_t$ value only when the range within the triplicate was <1.
All group data are medians with interquartile ranges. Statistical significance of differences between two groups was tested by unpaired, two-tailed Mann-Whitney tests and that of associations by regression analysis for non-Gaussian data; in both cases $p < 0.05$ considered as significant and all statistical calculations were performed using the Prism program (v6.0, GraphPad Software, La Jolla, CA). Comparisons were not analyzed statistically if at least one of the groups being compared represented less than 10 patients.

Results

From the 110 consecutive patients, RNA of acceptable quality, i.e. RQI >7, could be isolated from 95 patients. The quality of the isolated mRNA was similar in samples obtained by mono- or bipolar TURP (RQI 8.0 (7.6; 8.8) vs. 8.0 (7.6; 8.4); n = 39 and 54,

respectively; p = 0.7167).
Using the broad, intermediate or most stringent approach allowed analysis of 74, 71 and 48 (M_1), 85, 82 and 42 (M_2), 63, 54 and 27 (M_3), 46, 33 and 33 (M_4) and 47, 36 and 10 (M_5) patients, respectively. For all correlation analyses and group comparisons the statistical analysis yielded very similar results for all three approaches; hence, all subsequent data are based on the broad approach unless specifically noted otherwise.
In 1, 3, 4, 16 and 9 cases of M_1, M_2, M_3, M_4 and M_5 receptor transcripts, respectively, a specific PCR product was detected but the raw C_t value was >35; in these cases it was set to 35 for analysis purposes. Median raw C_t values of 27.1 (25.6; 29.0), 29.0 (27.4; 30.8), 30.1 (28.8; 32.3), 33.6 (32.3; 34.5) and 31.1 (27.9; 33.2) for M_1, M_2, M_3, M_4 and M_5 receptor transcripts, respectively, indicated that M_1 and M_4 receptors were the most and least expressed subtypes, respectively, at the mRNA level in the human prostate.
The expression of none of the five muscarinic receptor subtype transcripts was associated with patient age (Figure 1). In contrast, the mRNA expression of the M_1 receptor was significantly associated with prostate size (Figure 2); this association was weak but consistently found to be statistically significant with all three approaches. Such association with prostate size was not detected for M_2, M_3, M_4 or M_5 receptor transcript expression (Figure 2).
On the other hand, mRNA expression of none of the muscarinic receptor subtypes was significantly associated with PSA levels (Figure 3).
Histological diagnosis (BPH vs. PC) or medication with 5-ARIs, α-blockers or β-blockers was not associated with a significant difference in the mRNA expression of any of the muscarinic receptor subtypes (Figure 4). A possible effect of muscarinic receptor treatment could not be analyzed as only two patients in our cohort received such treatment.

Discussion

The present study was designed to explore a subtype-selective regulation of the five muscarinic receptor subtype transcripts in association with patient age, prostate size and pathological diagnosis; association with potentially interfering medications was a secondary study aim.

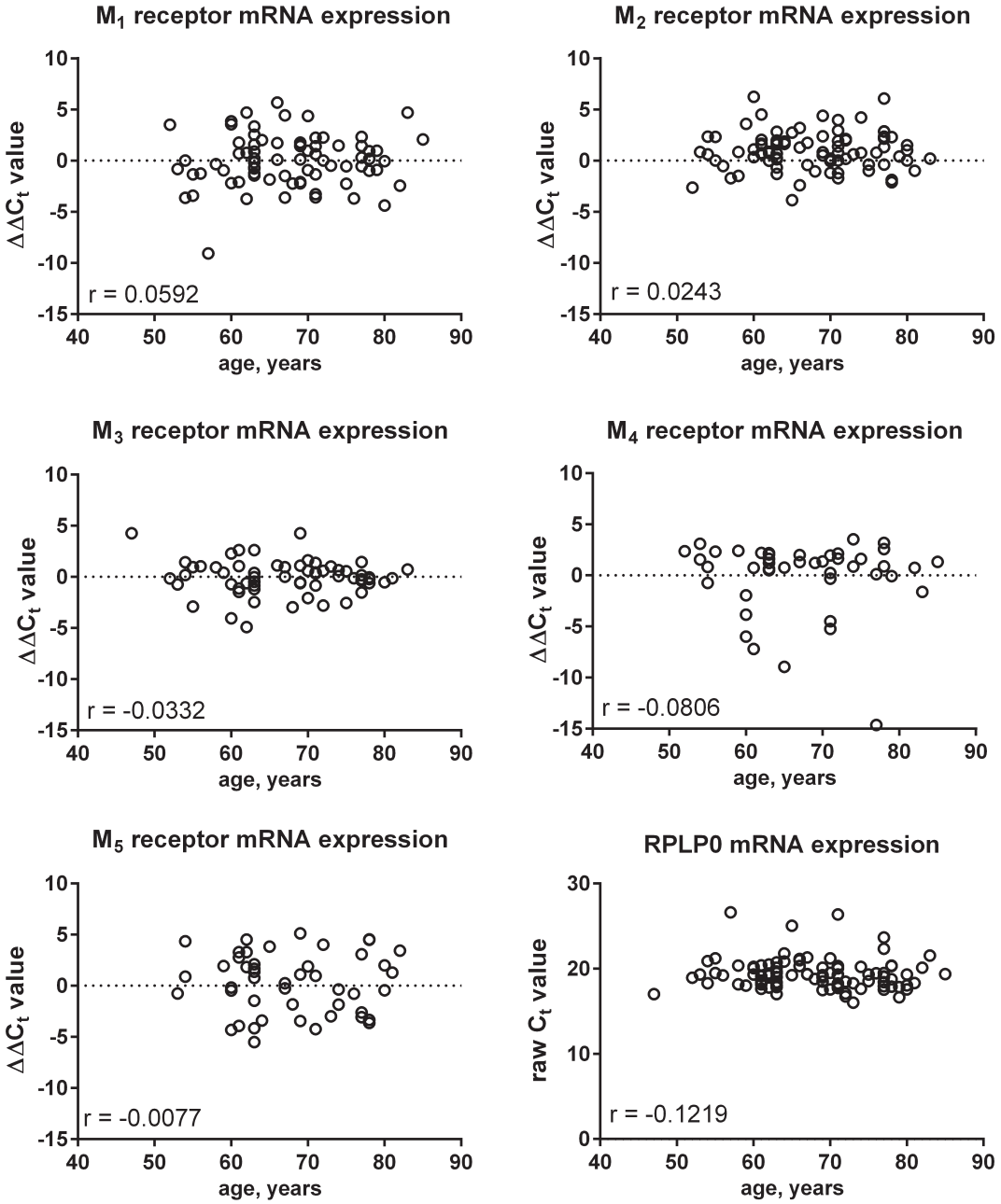

Figure 1. Association between patient age and mRNA expression of muscarinic receptor subtypes and the reference gene RPLP0. Note that none of these associations was statistically significant. The respective Spearman correlation coefficient is shown in each panel.

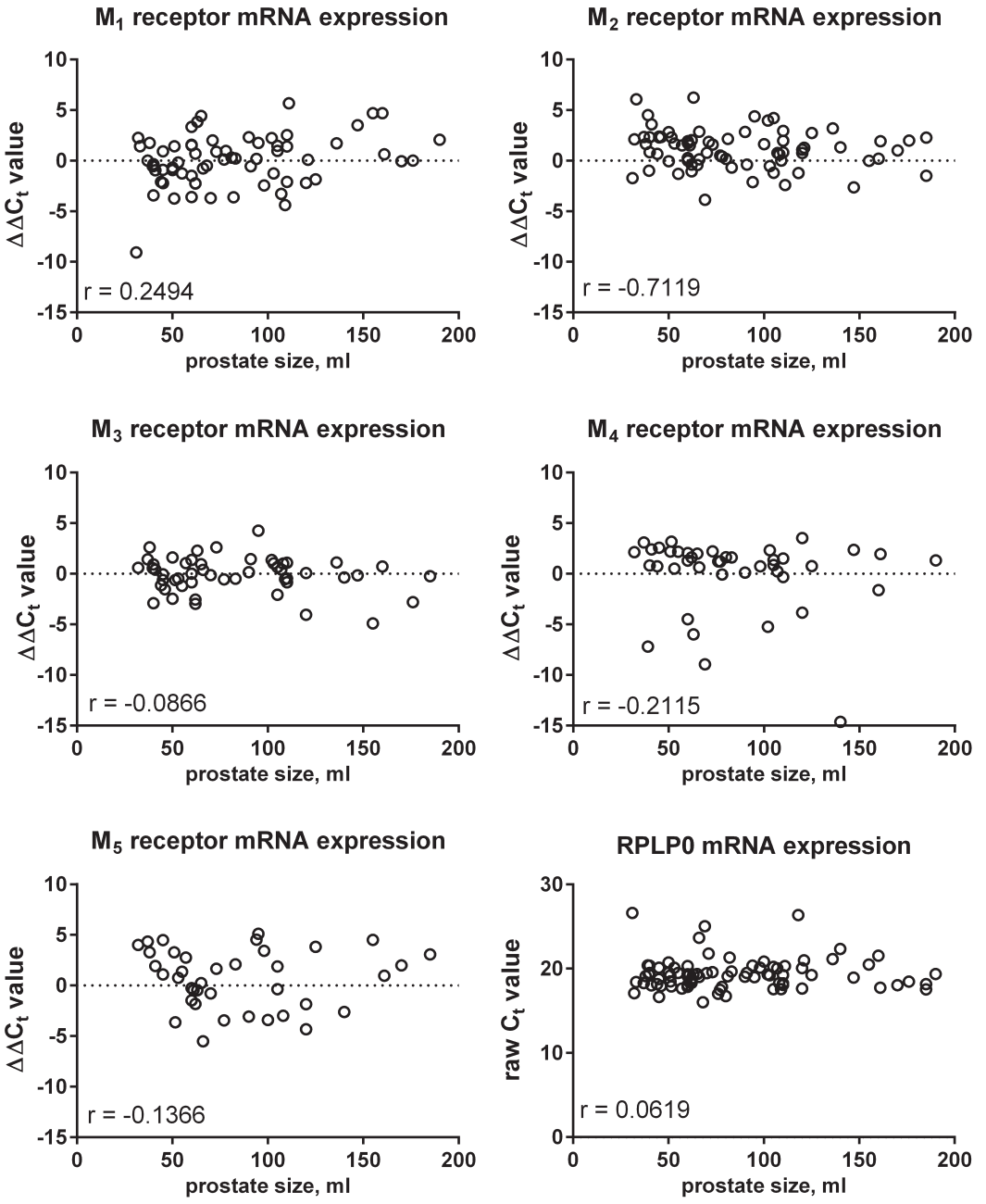

Figure 2. Association between prostate size and mRNA expression of muscarinic receptor subtypes and the reference gene RPLP0. The association of age with M1 receptor expression was weak but statistically significant ($p = 0.0451$; significance consistent across all three approaches), whereas that with expression the other four subtypes was not. The respective Spearman correlation coefficient is shown in each panel.

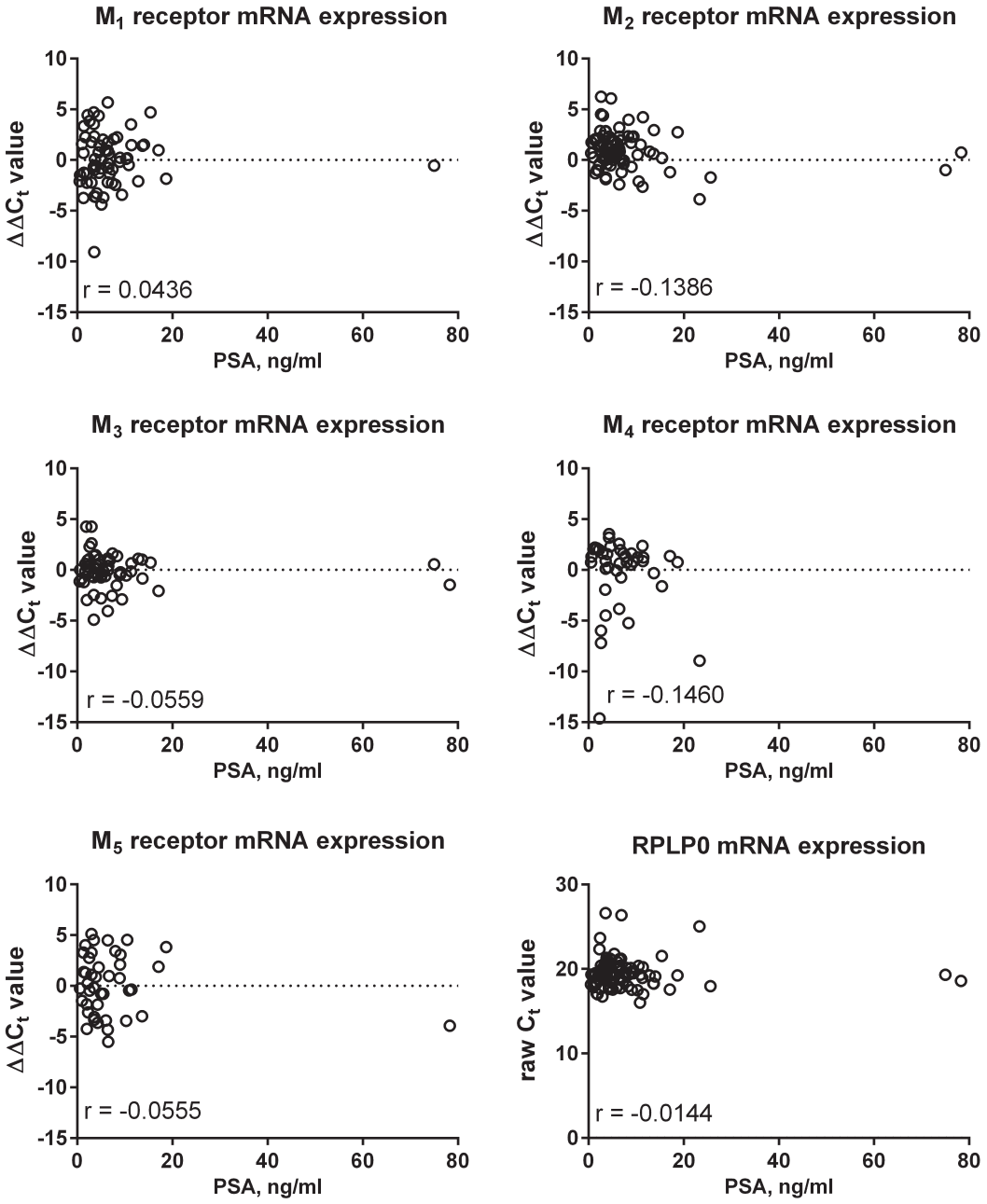

Figure 3. Association between PSA level and mRNA expression of muscarinic receptor subtypes and the reference gene RPLP0. Note that none of these associations was statistically significant. The respective Spearman correlation coefficient is shown in each panel.

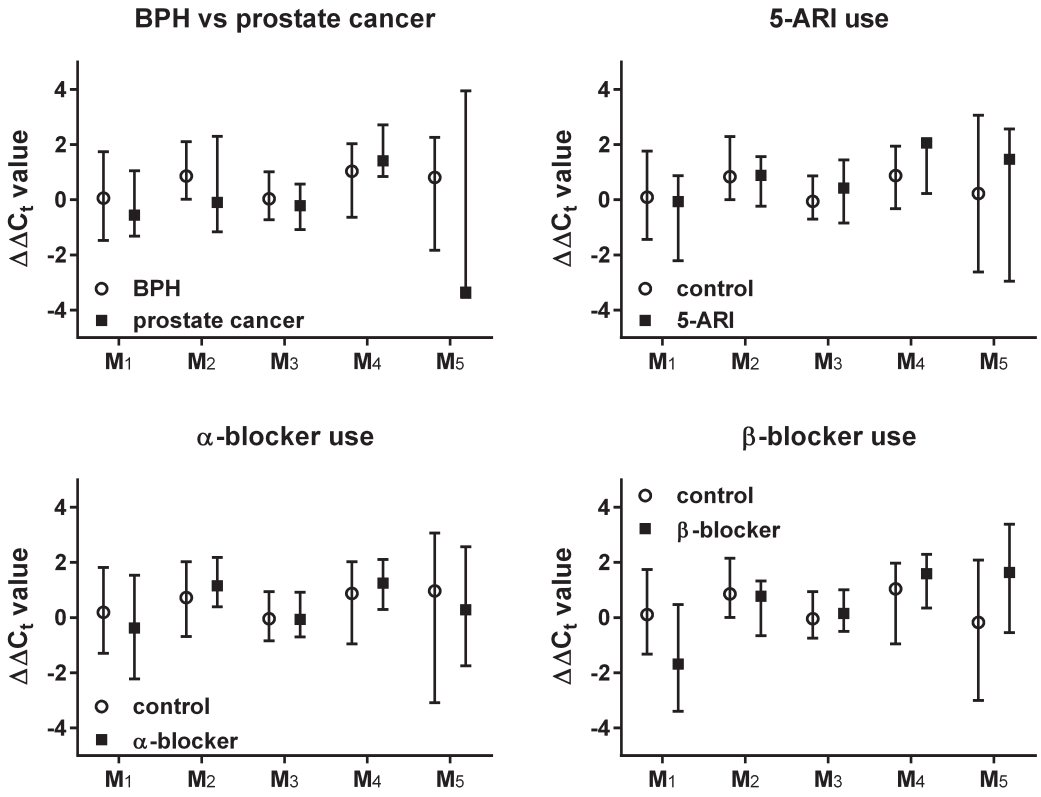

Figure 4. Effect of the diagnosis of prostate cancer or use of a 5α-reductase inhibitor (5-ARI), α-blocker or β-blocker as compared to the diagnosis of BPH or lack of such medication use on mRNA expression of muscarinic receptor subtypes. Note that none of these associations was statistically significant; however, statistical analysis was not performed for the following because at least one of the groups had <10 samples: M_3, M_4 and M_5 receptors for prostate cancer, M4 and M5 receptors for 5-ARI and β-blocker use, and M_2, M_3, M_4 and M_5 receptors for β-blocker use. Data are medians with interquartile ranges.

Critique of Methods. Previous studies have reported that the quality of RNA isolated from human prostate samples obtained by TURP may be poor [19]. Therefore, we have carefully checked the quality of our RNA isolation and accepted only samples with an RQI of ≥7 and with an identifiable specific PCR product as assessed by melting curves. Moreover, in our study RNA quality did not differ between chips obtained by mono- or bipolar TURP. A wide range of possible reference genes exist, but recent work highlights the problem that the expression of reference genes can be less stable than hoped for [20], which may affect the outcome of normalization for reference gene expression. Therefore, it is important that for any given study it is verified that the chosen reference gene is indeed stably expressed [21]. In our study RPLP0 was expressed to a greater extent and with less variability than HPRT1, and the variance of RPLP0 expression was also small in the overall population. Therefore, RPLP0 was chosen as the reference gene in our quantification of muscarinic receptor mRNA expression.

In a previous studies group size of 68-75 patients had been sufficient to detect associations between age or prostate size and mRNA expression of some α_1-adrenoceptor subtypes [22;23]. The present study had recruited 110 patients, but based on our stringent quality criteria not all patients could be used in all analyses; specifically for receptor subtypes with low expression and/or in subgroups with less frequent explanatory variables, e.g. the comedications, sample size was smaller. Accordingly, our study may have less power to detect associations with factors such as pathological diagnosis or concomitant medication.

mRNA expression is not necessarily predictive for the expression of functional protein, e.g. for the presence of some α_1-adrenoceptor subtypes in the human prostate [24]. A comprehensive validation of the predictive value of mRNA for muscarinic receptor expression at the protein level has not been reported to our knowledge, but the limited available evidence suggests that expression of muscarinic receptor subtype mRNA and protein is well correlated in human prostate and cell types isolated from it (see Introduction). In support of this idea, our study found that M_1 receptors exhibit the highest expression level among the five subtypes, which is in line with the finding that this subtype also is the most abundant one at the protein level in whole prostate

homogenates [9]. On the other hand, the same group reported that receptor mRNA changes correlated with those of receptor protein for M_2 but not M_3 receptors when a spectrum of pathologies associated with bladder hypertrophy and atrophy was investigated [16]. Thus, our findings on a possible regulation of muscarinic receptor subtypes at the mRNA level needs to be interpreted cautiously.

Finally, muscarinic receptor subtypes can be differentially expressed in prostate stroma and epithelium [8-10]. Nevertheless, our study was limited to whole prostate, as have been several previous studies at the protein level [4;5;10;12]. All of these potential limitations should be kept in mind in the interpretation of our data.

Association of Muscarinic Receptor Subtype mRNA with Potentially Regulating Factors. We found the M_1 receptor to be expressed highest at the mRNA level, and this subtype was also found to be most abundant at the protein level in whole prostate [9] and at the mRNA level in prostate epithelial cultures [8], thereby validating our findings and supporting the hypothesis that for muscarinic receptors of the prostate mRNA may be predictive for protein expression.

It has been reported that prostatic mRNA expression of α_{1A}- and α_{1D}-adrenoceptor subtypes and of total α_1-adrenoceptors is correlated with age [23]. In contrast, total muscarinic receptor density at the protein level was not associated with age in two previous studies [4;5], and our data at the mRNA level confirm that this is applicable to all five subtypes.

Two studies reported that prostatic α_1-adrenoceptor subtype mRNA expression is not associated with prostate size [22;23]. In contrast, we found a significant association of prostatic M_1 receptor expression with prostate size. This association was detected irrespective to the stringency of sample selection and specific for M_1 as compared to the other muscarinic receptor subtypes. While this association was weak and not reflected by a similar association with plasma PSA level, it has biological plausibility as M_1 receptors have been linked to the promotion of cellular growth in the prostate [11]. Of note, PSA levels in our study are not a pure indicator of prostate size as our study also includes PC patients. Exploring possible association of prostatic mRNA expression for muscarinic receptor subtypes with pathological diagnosis or

medication has been a secondary aim of our study. Studies in other tissues have implied M_3 muscarinic receptors in malignant cell growth [25]. However, a previous study at the protein level reported a similar total muscarinic receptor expression in normal epithelium, hyperplastic epithelium and well to moderately differentiated carcinoma [12]. Our study at the mRNA level demonstrates that this lack of expression change similarly applies to all five muscarinic receptor subtypes including the M_3 receptor. 5-ARI's reduce prostate size, primarily by affecting its epithelial component [26]. While such a shift between the epithelial and stromal component may theoretically affect the relative abundance of preferentially epithelial M_1 and stromal M_2 receptors, the mRNA expression of neither subtype was significantly affected by 5-ARI treatment. While changes in expression of α- or β-adrenoceptors, e.g. in the heart, may affect that of muscarinic receptors, mostly M_2 receptors [18], neither treatment was associated with an altered expression of any of the five muscarinic receptor subtype transcripts in the prostate. The number of patients being treated with a muscarinic receptor antagonist in our study was too low to allow conclusion, apparently reflecting the hesitancy of many physicians to apply this drug class in men with large prostates.

Conclusion

Our study demonstrates that high quality mRNA can be extracted from prostate chips obtained during TURP. While all five subtype transcripts are expressed in the human prostate, M_1 receptors are the most prevalently expressed muscarinic receptor subtype at the mRNA and protein level, and their expression is associated with prostate size. In contrast age, PSA level, pathological diagnosis or treatment with 5-ARI's or α- or β-blockers is not associated with alterations of mRNA expression for any of the five muscarinic receptor subtypes in the human prostate. In light of the growing role of muscarinic receptor antagonists in the treatment of men with voiding dysfunction [27], further studies of the functional role of prostatic muscarinic receptors are desirable, specifically with regard to M_1 receptors and prostate size.

Acknowledgements

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Chapter 4 **Muscarinic Receptor Subtypes and Signalling Involved in the Attenuation of Isoprenaline-Induced Rat Urinary Bladder Relaxation** 68

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PART TWO
MUSCARINIC AND ADRENERGIC SIGNALLING

Chapter 4

Muscarinic Receptor Subtypes and Signalling Involved in the Attenuation of Isoprenaline-Induced Rat Urinary Bladder Relaxation

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Abstract

Purpose. β-Adrenoceptors are important mediators of smooth muscle relaxation in the urinary bladder, but the concomitant presence of a muscarinic agonist, e.g. carbachol, can attenuate relaxation responses by reducing potency and/or efficacy of β-adrenoceptor agonists such as isoprenaline. Therefore, the present study was designed to explore the subtypes and signalling pathways of muscarinic receptors involved in the attenuation of isoprenaline-induced isolated rat detrusor preparations using novel subtype-selective receptor ligands.

Methods. In radioligand binding studies we characterized BZI to be a M_3 sparing muscarinic agonist, providing selective M_2 stimulation in rat bladder, and THRX-182087 as a highly M_2 selective antagonist. The use of BZI and of THRX-182087 in the presence of carbachol enabled experimental conditions with a selective stimulation of only M_2 or M_3 receptors, respectively.

Results. Confirming previous findings, carbachol attenuated isoprenaline-induced detrusor relaxation. M_2 selective stimulation partly mimicked this attenuation, indicating that both M_2 and M_3 receptors are involved. During M_3 selective stimulation, the attenuation of isoprenaline responses was reduced by the phospholipase C inhibitor U 73,122 but not by the protein kinase C inhibitor chelerythrine.

Conclusion. We conclude that both M_2 and M_3 receptors contribute to attenuation of β-adrenoceptor-mediated relaxation of rat urinary bladder; the signal transduction pathway involved in the M_3 component of this attenuation differs from that mediating direct contractile effects of M_3 receptors.

Introduction

Muscarinic receptors are the main mediator of physiological contraction of the urinary bladder. While M_2 and M_3 subtypes exist in an about 4:1 ratio in the bladder of humans and many other mammals, direct contraction responses to exogenous agonists or endogenous agonist as released by field stimulation are mediated predominantly if not exclusively by the minor fraction of M_3 receptors [1]. While it has been questioned on theoretical grounds that subtype-selective antagonists are sufficient to support this conclusion [2], it should be noted that it is consistent with findings from M_2 and M_3 receptor knock-out mice [3]. M_3 receptors, including those in the urinary bladder, couple to phospholipase C (PLC) stimulation, but the direct contractile effect via M_3-receptors occurs largely independent of PLC in the bladder [4]. On the other hand, bladder smooth muscle relaxation is mediated by β-adrenoceptors, in humans and many species mostly their β_3-subtype [5]. While β-adrenoceptors, including those in the bladder, couple to stimulation of an adenylyl cyclase, bladder relaxation by β-adrenergic agonists occurs largely independent of adenylyl cyclase stimulation [4]. Thus, bladder smooth muscle tone is under a dual control of M_3 muscarinic receptors and β-adrenoceptors. This is similar to smooth muscle tone regulation in many other tissues, e.g. the airways, except relaxation responses in most cases outside the bladder are mediated by the β_2-subtype [6].

The physiological control of bladder function involves activation of β-adrenoceptors by neuronally released noradrenaline during the storage phase of the micturition cycle, whereas neuronally released acetylcholine acting on M_3 receptors mediates detrusor contraction during the voiding phase [7]. However, under pathophysiological conditions non-neuronal acetylcholine release from the urothelium may also play a role and can be present not only during the voiding but also the storage phase [8]. The concomitant exposure of the detrusor to noradrenaline and acetylcholine during the storage phase may have important implications for bladder smooth muscle function. Thus, it has been shown that the potency and efficacy of a muscarinic agonist to elicit detrusor contraction is attenuated in the presence of a β-adrenoceptor agonist [9]. Perhaps even more important, muscarinic agonists can also attenuate the relaxing effects of β-adrenoceptor stimulation in bladder [10-12], airways [13-15] and ileum [15]. Such attenuation has been shown across multiple species, i.e. rat [11;12], mouse [10;15] and humans

[13;14], indicating that it is a general principle in the regulation of smooth muscle tone upon concomitant exposure to muscarinic and β-adrenoceptor agonists. While all studies agree on the existence of attenuation of β-adrenoceptor-mediated relaxation by muscarinic agonists, reduced potency and/or efficacy have been reported to underlie such attenuation. Of note, the degree of attenuation of β-adrenoceptor-mediated relaxation by muscarinic agonists exceeds that by other contractile stimuli in all cases where this has been studied, e.g. that by KCl, bradykinin or serotonin in the bladder [12] or that of histamine in the airways [13;16]. At least in rat bladder, this differential degree of attenuation cannot be explained by a comparison between weak and strong contractile agonists, indicating that it may involve a specific property of muscarinic receptors rather than purely reflecting functional antagonism.

The interaction between muscarinic receptors and β-adrenoceptors may also shed light on the physiological role of the numerous M_2 receptors in bladder and airways. Thus, M_2 receptor knock-out mice exhibited a mitigated attenuation of relaxation responses to isoprenaline [10;15] and receptor-independent elevation of cellular cAMP content such by the adenylyl cyclase activator forskolin [17]. As M_2 receptors largely signal via pertussis toxin-sensitive G-proteins [18], further support for a role of M_2 receptors in the attenuation response comes from studies in which pertussis toxin treatment enhanced the relaxant effects of forskolin on oxotremorine-M-mediated contractions in ileum and trachea [19;20]. In a similar vein it has been reported that under conditions of preferential alkylation of M_3 receptors the ability of a muscarinic agonist to reverse isoprenaline-induced relaxation is largely maintained [21;22]. Facing a lack of highly subtype-selective muscarinic antagonists, such alkylation protocols have been employed by several investigators to achieve reasonable subtype-selectivity. While this has been successful in some cases and very specific conditions [23], other alkylation protocols have proven to be poorly selective [24]. Moreover, in some cases M_3 responses have also been pertussis toxin-sensitive [25], making pertussis toxin an unreliable witness of M_2 involvement. While the knock-out mice circumvent selectivity problems, they are prone to other complications such as compensatory regulation of other receptors or signal transduction pathways.

Recently, an agonist with considerable selectivity for M_2 over M_3 receptors became available, but this compound exhibits a lower degree of selectivity, if any, over other subtypes; in the bladder, where muscarinic receptor subtypes other than M_2 and M_3 are largely absent, this compound produces selective M_2 stimulation [26-28]. Lacking a better term we refer to this compound as 'M_3 sparing' in the present manuscript. Moreover, we have recently discovered an M_2 receptor antagonist with unprecedented selectivity for this subtype, which we report here for the first time (Figure 1). These two tools have enabled us to explore the role of M_2 and M_3 receptors in the attenuation of isoprenaline-induced bladder relaxation. Moreover, we have used this approach to characterize the role of PLC and protein kinase C (PKC) activation in the attenuation response.

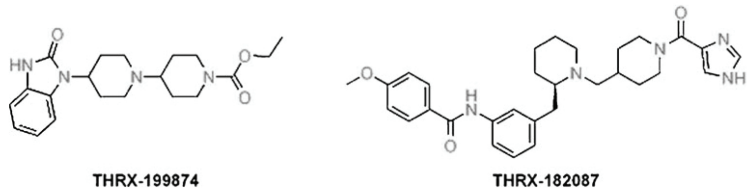


Figure 1. Structures of BZI (THRX-199,874) and THRX-182,087

Methods

Radioligand Binding Experiments. Radioligand binding assays were conducted with 1 nM [^{3}H]N-methyl scopolamine ([^{3}H]NMS; GE Healthcare, Piscataway, NJ, USA) in a buffer consisting of 10 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$ and 0.025% bovine serum albumin, pH 7.4 at 37°C. Nonspecific binding was defined in the presence of 10 µM atropine. Membrane fractions from CHO-K1 cells expressing human recombinant M_1, M_2, M_3, M_4 or M_5 muscarinic receptors were incubated with radioligand and unlabelled drugs for 1 hour at 37°C in a volume of 100 µl. Receptor expression levels (B_{max}) measured by saturation binding were determined to be 2.7, 2.5, 2.4, 2.0 and 3.2 pmol/mg protein for human recombinant M_1, M_2, M_3, M_4 or M_5 muscarinic receptors, respectively. After separation by vacuum filtration onto GF/B filter plates pre-soaked with 0.3% polyethyleneimine, the quantity of membrane bound radioligand was measured by scintillation counting.

Organ Bath Experiments

Tissue Preparation. Adult male Wistar rats weighing 300 ± 22 g were purchased from Charles River (Maastricht, The Netherlands). Animals were anesthetized using pentobarbital (75 mg/kg i.p.) and sacrificed by decapitation. The bladders were harvested and adipose and soft connective tissues were removed. After removal of the dome and the trigonum, the middle parts of the cleaned bladders (weight of 96 ± 15 mg) were cut transversally in four equal strips. The strips had a length of 19.9 ± 3.6 mm and a weight of 9.5 ± 2.7 mg (n =76). All experimental procedures were in line with European Union guidelines for the use of laboratory animals and approved by the Animal Care Committee of Academisch Medisch Centrum.

Relaxation Experiments. Experiments were performed as previously described [29] with minor modifications. Briefly, the bladder strips were mounted under a resting tension of 10 mN in organ baths containing 7 ml of Krebs-Henseleit buffer of the following composition: 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.025 mM Na_4EDTA, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 5.6 mM glucose at a temperature of 37°C, yielding a total potassium concentration of 5.9 mM. The organ baths were continually gassed 95% O2 /5% CO2 to maintain a pH of 7.4. The bladder strips were equilibrated for 120 min, during which the buffer solution was refreshed every 15 min until a steady contractile state had been reached.. Following the equilibration, the tissues were challenged with 50 mM KCl for 6 min (maintaining iso-osmolarity by reducing NaCl concentration from 116.8 mM to 68.5). After the first challenge we equilibrated the strips again at passive tension of 10 mN for 90 min and challenged the strips again with 50 mM KCl for 6 min. After 60 min equilibration at 10 mN after the second KCL challenge we added vehicle or substance of interest. Strips with a KCl response of < 20 mN were excluded. Carbachol was used at a concentration of 1 μM, BZI and THRX at 100 nM. The enzyme inhibitors U 73,122 (10 μM) and chelerythrine (1 μM) were added 5 min prior to the administration of THRX. Ten min later carbachol (1 μM) was added, which was given an incubation time of 15 min to reach steady contractile stadium. Thereafter, cumulative concentration-response curves

were generated for the β-adrenoceptor agonist isoprenaline. As isoprenaline-induced rat bladder relaxation can exhibit desensitization [30], only one isoprenaline concentration-response curve was constructed per bladder strip; however, conditions being compared were always tested in parallel using strips from the same animal, and those paired comparisons were the basis of our statistical analysis (see below).

Chemicals. The M_3 sparing agonist BZI (also known as THRX-199874, 4-(2-oxo-2,3-dihydro-benzimidazol-1-yl)-1,4'-bipiperidinyl-1'-carboxylic acid ethyl ester) was synthesized in house as described [31]. The M_2 selective antagonist THRX-182087 (N-(3-{(R)-1-[1-(1H-imidazole-4-carbonyl)-piperidin-4-ylmethyl]-piperidin-2-ylmethyl}-phenyl)-4-methoxy-benzamide) was synthesized in house as follows: Preparation of THRX-182087 was initiated from the condensation of 4-methoxyl benzoyl chloride and 3-bromo aniline. Metal halogen exchange with n-butyllithium in the presence of triethylchlorosilane and addition to (R)-2-formyl-1-Cbz-piperidine, followed by decarboxylation with palladium on carbon and potassium formate. Reductive alkylation with 4-formyl-1-Cbz-piperidine and Cbz removal with palladium on carbon, followed by acylation with 1H-imidazole-4-carbonyl chloride provided THRX-182087 (for detailed synthetic route see Stangeland, E.L., et al., manuscript in preparation). U 73,122 (1-(6-[([17ß]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino] hexyl)-1H-pyrrole-2,5-dione) was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and chelerythrine HCl from Calbiochem (via VWR, Amsterdam, The Netherlands).

Data Analysis. Bound radioactivity in counts-per-minute data was normalized to percent specific binding and analyzed using a four parameter logistic equation in Prism 3.0 (GraphPad Software, San Diego, CA, USA). Because Hill coefficients did not significantly differ from unity, IC_{50}'s were determined with slopes fixed to 1. Inhibition binding constants (K_I) for test compounds were calculated from the IC_{50} values using the Cheng and Prusoff correction [32] and reported as the mean negative logarithm of the inhibition binding constants (pK_I) ± SEM.
Non-linear regression was used to fit sigmoidal curves to the isoprenaline concentration-response curves to determine agonist

potency (pEC_{50}) and maximum effects (E_{max}) using Prism 5.0.
The force of contraction immediately prior to addition of the first isoprenaline concentration within a given experiment was defined as 0% relaxation, and a force of contraction of 0 mN was defined as 100% relaxation.
All data are expressed as mean ± SEM of n experiments.
Statistical significance of inhibitor effects on the Emax or pEC_{50} of isoprenaline was assessed by paired two-tailed t-tests as compared to the indicated control condition, with treated and control conditions being measured in paired strips prepared from the same bladder. All statistical analysis was calculated using the Prism program, and a $P < 0.05$ was considered statistically significant.

Results

The affinities of BZI and THRX-182087 for human recombinant M_1, M_2, M_3, M_4 and M_5 muscarinic receptors as determined in competition radioligand binding experiments are shown in Table 1, most notably demonstrating a >100-fold selectivity for M_2 over M_3 receptors for both compounds.
Five series of functional experiments were performed. In the first series, we re-investigated the role of muscarinic receptor stimulation vs. receptor-independent bladder contraction, induced by KCl, for isoprenaline-induced relaxation. Carbachol and KCl caused a comparable starting tension (Table 2). The maximum relaxation by isoprenaline was significantly smaller in carbachol- than KCl-pre-contracted strips whereas the potency of isoprenaline did not differ significantly between the two conditions (Figure 2).
The second series of experiments explored whether selective M_2 receptor stimulation mimics the effect of carbachol. Based upon previous data that BZI alone causes little bladder contraction [28], we compared BZI with passive tension and confirmed the lack of effect of BZI on detrusor tone (Table 2). While BZI did not affect maximum isoprenaline-induced relaxation, it significantly reduced its potency (Figure 3).
The third series addressed the reverse question, i.e. whether selective M_3 receptor stimulation (carbachol in presence of THRX-182087) mimics the carbachol effect. Both conditions caused comparable starting tension (Table 2). The potency and efficacy of isoprenaline were significantly greater upon M_3 selective as

Table 1. Affinity estimates of BZI and THRX-182,087 at human muscarinic receptor subtypes as determined in competition radioligand binding studies. Data are means ± SEM of 19-27 experiments and shown as pK_i values.

	BZI	THRX-182,087
M_1	7.27 ± 0.04	6.82 ± 0.02
M_2	8.59 ± 0.05	9.06 ± 0.02
M_3	<5	6.61 ± 0.02
M_4	8.01 ± 0.05	7.34 ± 0.02
M_5	6.11 ± 0.05	5.46 ± 0.02

Table 2. Starting tension of relaxation experiments in the abence and presence of muscarinic agonists and antagonists and/or signal transduction inhibitors. Data are means ± SEM of the indicated number of experiments and shown in mN/mg strip weight. *: p <.05 vs. test condition (KCl, carbachol or passive tension) in a paired, two-tailed t test.

Series 1: KCl vs. mixed muscarinic stimulation		
KCl 50 mM	2.86 ± 0.22	n=8
Carbachol 1 µM	2.46 ± 0.12	n=8
Series 2: passive tension vs. M_2-stimulation		
Passive tension	0.95 ± 0.15	n=6
BZI 100 nM	1.08 ± 0.10	n=6
Series 3: mixed vs. M_3 stimulation		
Carbachol 1 µM	2.60 ± 0.13	n=6
Carbachol 1 µM + THRX-182087 100 nM	2.14 ± 0.22	n=6
Series 4: M_3 stimulation in absence and presence of phospholipase C inhibitor		
Carbachol 1 µM + THRX-182087 100 nM	2.90 ± 0.21	n=6
Carbachol 1 µM + THRX-182087 100 nM + U 73,122 10 µM	2.33 ± 0.12*	n=6
Series 5: M_3 stimulation in absence and presence of protein kinase C inhibitor		
Carbachol 1 µM + THRX-182,087 100 nM	2.89 ± 0.51	n=6
Carbachol 1 µM + THRX-182,087 100 nM + chelerythrine 1 µM	2.90 ± 0.22	n=6

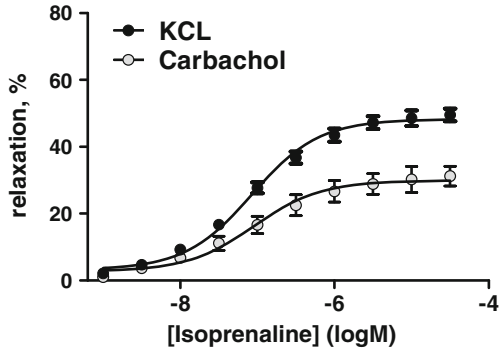
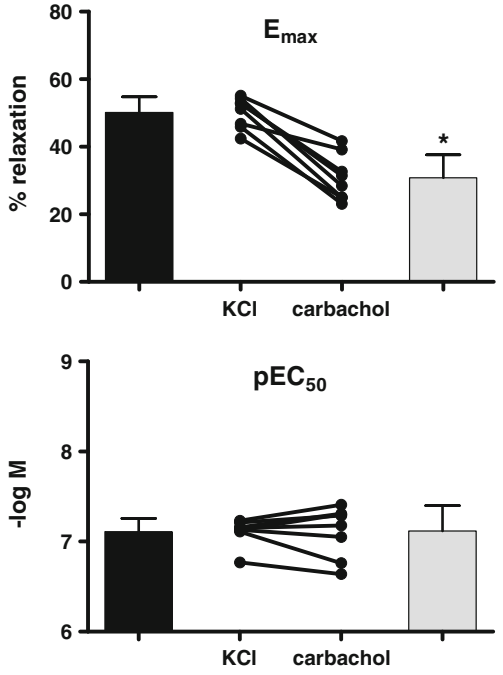
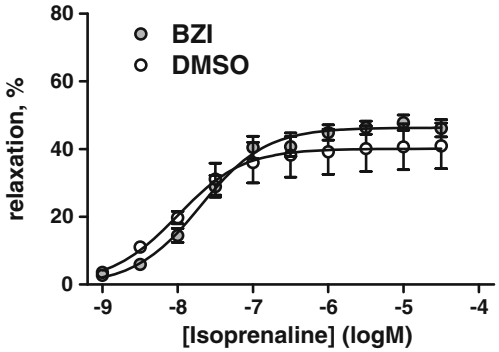


Figure 2. Comparison of isoprenaline-induced relaxation against tension induced by 50 mM KCl and 1 μM carbachol. Upper panel: Data are means ± SEM (n = 6).

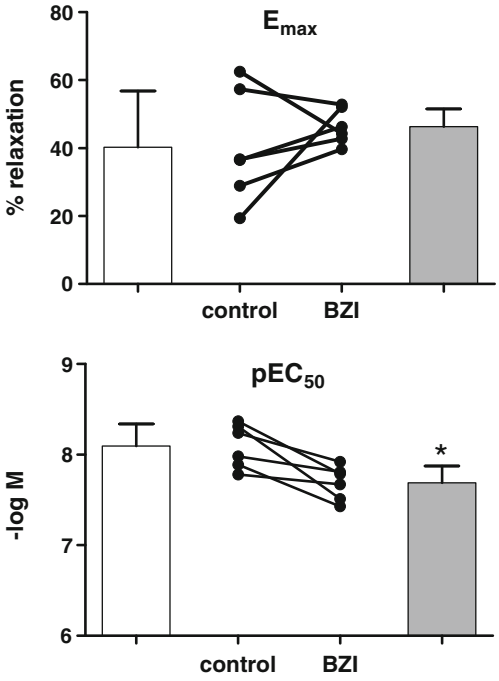


Middle and lower panel: Bars showing E_{max} and pEC_{50} as derived from the curves in upper panel indicate means ± SEM, whereas filled circles represent individual experiments. *: $p < 0.05$ vs. KCl in a paired t-test.

Figure 3. Comparison of passive tension and M_2 selective muscarinic stimulation by 100 nM BZI on isoprenaline-induced relaxation. Upper panel: Data are means ± SEM (n = 6).



Middle and lower panel: Bars showing E_{max} and pEC_{50} as derived from the curves in upper panel indicate means ± SEM, whereas filled circles represent individual experiments. *: $p < 0.05$ vs. passive tension in a paired t-test.



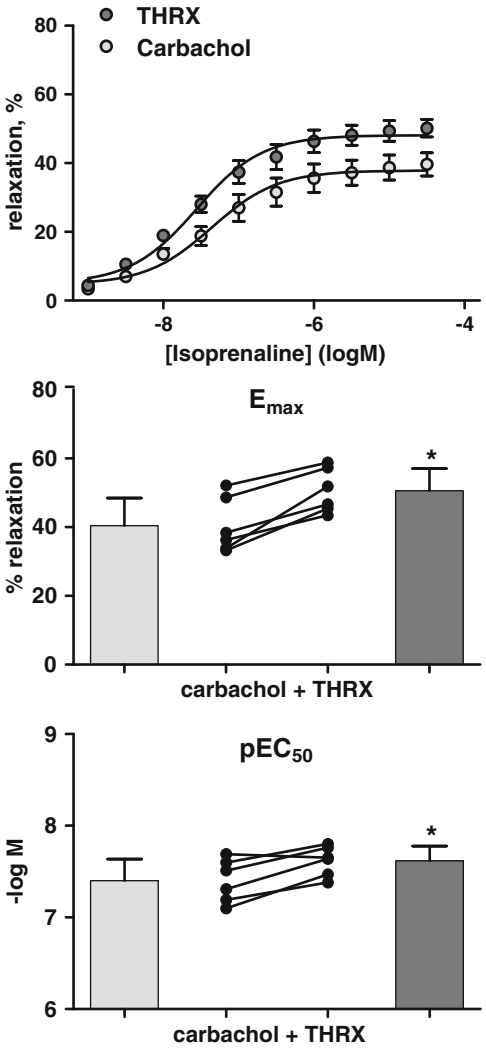
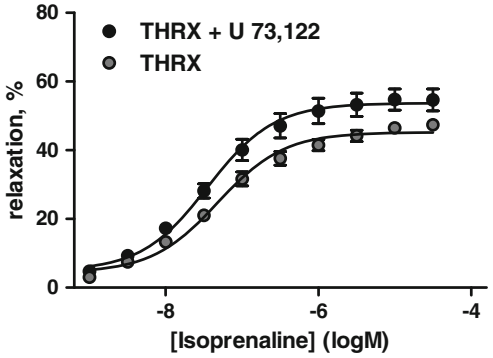


Figure 4. Comparison of mixed (1 μM carbachol) vs. M_3 selective muscarinic stimulation (1 μM carbachol + 100 nM THRX) on isoprenaline-induced relaxation. Upper panel: Data are means ± SEM (n = 6).

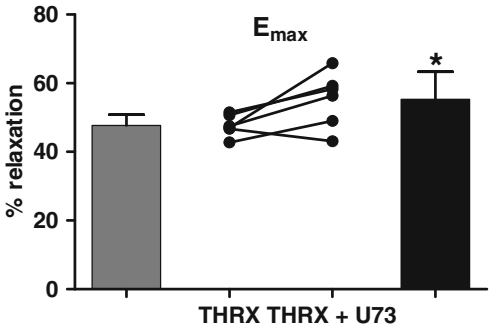
Middle and lower panel: Bars showing E_{max} and pEC_{50} as derived from the curves in upper panel indicate means ± SEM, whereas filled circles represent individual experiments.

*: $p < 0.05$ vs. carbachol alone in a paired t-test.

Figure 5. Effect of the phospholipase C inhibitor U 73,122 (10 μM) on isoprenaline-induced relaxation during M_3 selective stimulation (1 μM carbachol + 100 nM THRX). Upper panel: Data are means ± SEM (n = 6).



Middle and lower panel: Bars showing E_{max} and pEC_{50} as derived from the curves in upper panel indicate means ± SEM, whereas filled circles represent individual experiments. *: $p < 0.05$ vs. data in absence of signalling inhibitor in a paired t-test.



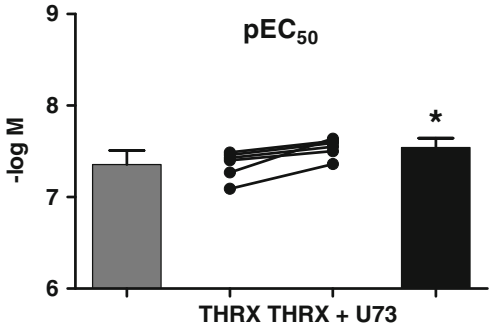
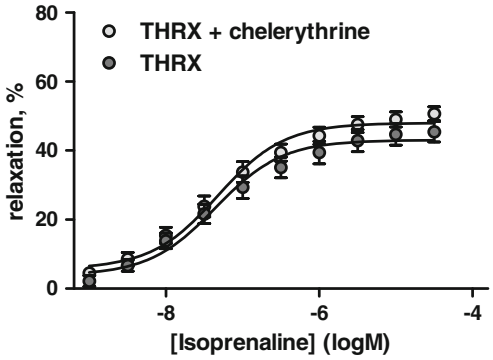
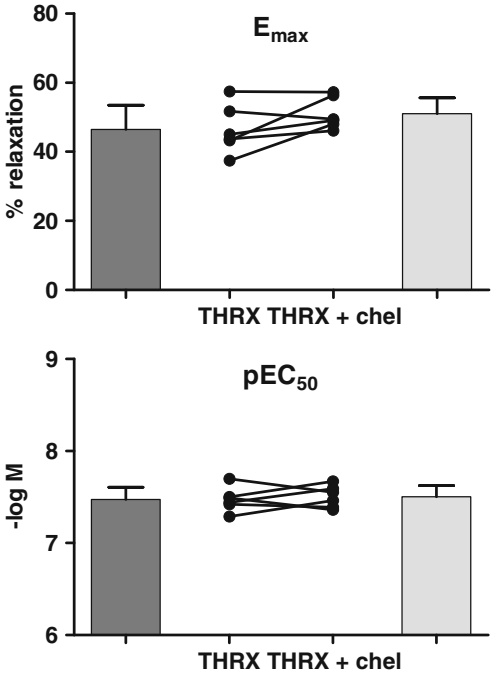


Figure 6. Effect of the the protein kinase C inhibitor chelerythrine (1 μM) on isoprenaline-induced relaxation during M_3 selective stimulation (1 μM carbachol + 100 nM THRX). Upper panel: Data are means ± SEM (n = 6).



Middle and lower panel: Bars showing E_{max} and pEC_{50} as derived from the curves in upper panel indicate means ± SEM, whereas filled circles represent individual experiments. Data in absence and presence of signalling inhibitor were not significantly different in a paired t-test ($p > 0.05$).



compared to general muscarinic receptor stimulation, confirming a contribution of M_2 receptors to the attenuation of the isoprenaline response (Figure 4). However, the enhancement of isoprenaline responses by M_2 blockade (Figure 4) was quantitatively less than the attenuation by carbachol (Figure 2), indicating that both subtypes contribute to the attenuation of isoprenaline responses by carbachol.

The fourth and fifth series of experiments explored whether PLC and/or PKC contribute to the attenuation of isoprenaline responses by M_3 selective stimulation. In the presence of the PLC inhibitor U 73,122 starting tension was significantly smaller than with M_3 selective stimulation alone (Table 2). Potency and efficacy of isoprenaline were significantly greater in presence of U 73,122 (Figure 5). In contrast, the PKC inhibitor chelerythrine affected neither starting tension (Table 2) nor potency or efficacy of isoprenaline-induced relaxation (Figure 6).

Discussion

Our study introduces a novel highly M_2 selective antagonist (THRX-182087) and uses it together with the M_3-sparing agonist BZI to explore the muscarinic receptor subtypes involved in the attenuation of isoprenaline effects in rat urinary bladder as well as the signalling pathways mediating such attenuation. According to our competition binding data, THRX-182087 is 281-fold selective for M_2 over M_3 receptors. Selectivity over other muscarinic receptors is 50-4000-fold. This compares favourably to the limited M_2 selectivity of other compounds which have been used in this field such as methoctramine which is only 30-fold selective [22]. While the selectivity of THRX-182087 over M_4 receptors is less pronounced, these receptors are of little importance in the regulation of bladder smooth muscle tone [1]. Hence, for practical purposes THRX-182087 is a highly selective M_2 antagonist in our experimental setting. The THRX-182087 concentration of 100 nM used in our studies produces an almost complete occupancy of M_2 receptors, and accordingly the combination of carbachol with THRX-182087 provides selective M_3 agonism.

BZI has been introduced as an M_3-sparing agonist [26;27], and our binding data show a more than 3000-fold selectivity for M_2 over M_3 receptors. Selectivity over other muscarinic receptors is 3-390-fold. Accordingly, BZI provides selective stimulation of M_2 receptors in bladder smooth muscle, where only M_2 and

M_3 receptors are functionally relevant [1]. The combined use of THRX-182087 and BZI has enabled us to explore the relative roles of M_2 and M_3 receptors in the attenuation of isoprenaline-induced relaxation.

Our data on starting tension demonstrate that inhibition of the M_2 receptors did not attenuate carbachol responses, whereas selective activation of M_2 receptors did not induce contraction as also observed in previous studies [28]. These data confirm a large body of evidence that despite the much larger presence of M_2 receptors in the urinary bladder, direct contractile responses are mediated predominantly if not exclusively by the M_3 receptor [1].

Studies in multiple tissues and species had demonstrated that muscarinic receptors can attenuate relaxation responses to the β-adrenoceptor agonist isoprenaline (see Introduction), and this is confirmed in the present data. While such attenuation was found very consistently, those previous studies had been inconsistent with regard to the question whether such attenuation affects the potency and/or efficacy of isoprenaline, and our data also are not fully consistent in this regard.

Studies based on knock-out mice had indicated that the M_2 subtype, which contributes little to direct detrusor contraction, plays a role in the attenuation of the relaxation response [15;17;21]. Using the complementary approach of selective pharmacological stimulation by BZI we confirm a role of M_2 receptors in the attenuation of relaxation. Our finding that selective inhibition of M_2 receptors by THRX-182,087 in the presence of carbachol enhances relaxation by isoprenaline further corroborates this idea. However, it should be noted that neither the effect of BZI nor that of THRX-182,087 can fully explain the attenuation obtained by mixed muscarinic stimulation using carbachol alone, suggesting that the attenuation response may also contain an M_3 component.

Stimulation of the PLC/PKC pathway is a prototypical signalling response of M_3 receptors [18;33], which also was detected in the bladder as being mediated predominantly if not exclusively via M_3 receptors [34;35]. Nevertheless, M_3 receptor-mediated bladder contraction has been shown to be insensitive to inhibition of PLC or PKC in rats, mice and humans [4]. In the present study we have used the PLC inhibitor U 73,122 in a concentration where it fully suppresses inositol phosphate formation in the bladder but does

not affect rat or human bladder contraction [36;37]. Interestingly, U 73,122 significantly enhanced isoprenaline-induced relaxation in the presence of M_3-selective stimulation. Thus, PLC may be involved in the M_3 component of attenuation of relaxation but not in direct bladder contraction mediated by the same receptor subtype. According to our data, PKC is not involved in either response, indicating that the involvement of PLC in the attenuation of relaxation occurs via a PKC-independent pathway.

Conclusion

In conclusion, we have introduced a novel antagonist with very high selectivity for M_2 over M_3 receptors, THRX-182,087. Using this compound as well as the M_3-sparing agonist BZI, we confirm a role for M_2 receptors in the attenuation of isoprenaline-induced bladder relaxation, which previously was mainly supported by genetic evidence, by a pharmacological approach. Our data also suggest involvement of M_3 receptors in this attenuation. Thus, muscarinic receptors cause direct contraction and inhibition of relaxation in the bladder, but the two responses involve different subtypes and, at least for M_3 receptors, different signalling pathways. This interaction may become clinically relevant under pathophysiological conditions when acetylcholine is being released in the bladder during the storage phase of the micturition cycle.

Acknowledgements

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Chapter 5

Effects of β_3-Adrenoceptor Stimulation on Signalling Responses of M_2 And M_3 Muscarinic Receptors upon Co-Transfection in Chinese Hamster Ovary Cells

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Article will only be published in this thesis.

Abstract

Purpose. In the human urinary bladder, contractile responses are mainly mediated by M_3 muscarinic receptors and relaxing responses by β_3-adrenoceptors. The role of the much larger number of M_2 receptors is not fully clear, but they can apparently attenuate relaxation responses. β-Adrenoceptors and M_2 muscarinic receptors prototypically signal by increasing and decreasing cAMP formation, respectively, whereas M_3 muscarinic receptors increase intracellular Ca^{2+}, but additional signaling pathways have been described for each of the three receptors. Interaction at the level of signaling is believed to account, at least partly, for functional antagonism between β_3-adrenoceptor and muscarinic receptor function in the bladder.

Methods. In order to study the underlying molecular mechanisms of such interactions, we developed a model of CHO-K1 cells double-transfected with M_2/β_3 or M_3/β_3 receptors. Receptor expression was characterized by saturation binding experiments and interaction was studied by functional signaling assays.

Results. All clones expressed muscarinic receptors in physiological densities, but receptor expression levels of β_3-adrenoceptors were too small to allow reliable estimates of receptor density. Nevertheless, β_3-adrenoceptors appeared to be functionally active, measured by an increase of cAMP after stimulation, but this did not affect intracellular Ca^{2+} concentrations. However, the achieved levels of cAMP apparently were insufficient to affect Ca^{2+} levels.

Conclusion. We have generated cell lines with either M_2 or M_3 muscarinic receptors co-expressing β_3-adrenoceptors. However, the model failed to exhibit co-expression of β_3-adrenoceptors at levels that enable studying of functional interaction. Thus, other model systems may be required to explore the interaction between β_3-adrenoceptors and either M_2 or M_3 muscarinic receptors.

Introduction

The sympathetic and parasympathetic nervous systems exert opposing effects on most tissue functions in the mammalian body. In the urinary bladder, smooth muscle contraction is induced by acetylcholine, acting on muscarinic receptors [1]. Similar to other mammalian species, human bladder expresses M_2 and M_3 muscarinic receptors at an approximate 4:1 ratio [2], but nonetheless direct contractile responses to a muscarinic receptor agonist are mediated predominantly, if not exclusively, by the minor populations of M_3 receptors [3]. The role of the much larger number of M_2 receptors is not fully clear, but they can apparently attenuate relaxation responses [1;4]. In the bladder, noradrenaline primarily acts on β-adrenoceptors to induce smooth muscle relaxation. While this occurs largely if not exclusively via the β_3-subtype in the human bladder, β_2- and perhaps even β_1-adrenoceptors can be involved in the relaxation responses of other species such as rats [5].

The β-adrenoceptor agonist isoprenaline induces rat bladder strip relaxation against all contractile stimuli [6], and this was also observed with the β_3-selective agonist KUC 7388 [7]. However, it was noted that β-adrenoceptor agonists caused weaker relaxation against contraction elicited by a muscarinic agonist than against passive tension or, pre-contraction by KCl, bradykinin, or serotonin [7-9]. Similar observations have been made in other organs including human lung and gut (for review see [10]). On the other hand, isoprenaline reduces the efficacy and potency of carbachol mediated contractions in the porcine bladder, with even stronger effect on potency in denuded bladder strips [11]. These findings have led to the idea of a privileged interaction between muscarinic receptors and β-adrenoceptors in smooth muscle, but the signaling mechanisms underlying this specific interaction have largely remained unclear (for review see [10]). We have previously reported that in rat bladder both the M_2 and the M_3 muscarinic receptor contribute to the attenuation of β-adrenoceptor mediated relaxation [4]. Moreover, we found in those experiments that the attenuation of relaxation by the M_3 component can be mitigated by inhibition of phospholipase C or protein kinase C.

Against this background, a study in cultured Chinese hamster ovary (CHO) cells co-transfected to express both human muscarinic M_2 or M_3 muscarinic receptors and β_3-adrenoceptors was designed to explore this interaction between the two receptor

systems in more detail.

Materials and Methods

Transfection. A two-stage transfection was done with CHO-K1 cells to obtain cells with concomitant expression of M_2/β_3 or M_3/β_3 receptors. The first transfection was done with vector pcDNA3.1 (Invitrogen, Life Technologies Europe BV, Bleiswijk, the Netherlands) containing cDNA of the coding region of the human muscarinic cholinergic receptor subtype 2 (CHRM2), subtype 3 (CHRM3), (obtained from Missouri S&T cDNA Resource Center, Rolla, MO, USA) a mock-construct (empty pcDNA3.1 vector) as a negative control, and green fluorescent protein (GFP) as a positive control, respectively. Transfection was done using the Nanofectin Kit (PAA Laboratories GmbH, Linz, Austria). After 30 minutes of incubation of Nanofectin with 5 µg of the construct, 500 µg of incubated mixture was added to a 10 cm culture dish with 60% confluent CHO-K1 cells. After 24 hours, cells were cultured in concentrations 1:200, 1:500 and 1:1000 with addition of 13 µl/ml of selection antibiotic G-418 (PAA Laboratories GmbH). Single growing clones of the cells transfected with CHMR2 (n=30) and CHRM3 (n=23) were selected and cultured to obtain stably transfected cells. All clones were screened for receptor expression levels using saturation-binding experiments using [^{3}H]-quinuclidinyl benzilate (QNB). Clones transfected with the M_2 receptor were functionally tested in cAMP accumulation experiments and M_3 clones in Ca^{2+} elevation assays.

Based on results of screening and functional tests, one clone of each cell line was selected, for a second transfection with vector pcDNA5.0/FRT/TO (Invitrogen) with cDNA of the coding region of the human β_3-adrenergic receptor (ADRB3 , Missouri S&T cDNA Resource Center)). Again mock- and GFP-constructs in vector pcDNA5.0/FRT/TO were used as negative and positive controls, respectively. After 24 hours, cells were cultured in concentrations 1:200, 1:500 and 1:1000 with addition of 13 µl/ml G-418 and 10 µl/ml hygromycine-B (Invitrogen). Single growing clones of cells with receptor combinations of M_2/β_3 (n=41), M_3/β_3 (n=33), mock/β_3 (n=38), M_2/mock (n=4), M_3/mock (n=4) and mock/mock (n=4) were selected and cultured to obtain stably

transfected cells. Functional testing in cAMP accumulation and Ca^{2+} elevation assays, combined with saturation binding experiments using radioligands QNB and [^{125}I]-iodocyanopindolol (ICYP) led to selection of four clones of M_2/β_3, M_3/β_3, M_2/mock, and M_3/mock cell lines.

Culturing of Cells. Cultured cells were passaged 1:10 every two or three days in Ham's F-12 Nutrient mixture, Gibco, Life Technologies Europe BV (Bleiswijk, the Netherlands) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% FCS and 1.2 g/l $NaHCO_3$. All cell lines were cultured at 37°C in humidified air containing 5% CO2. Selected clones were grown with selection antibiotics 13 µl/ml G-418 and 10 µl/ml hygromycine.

Radioligand Binding. Radioligand binding studies for muscarinic receptors [12;13] and β_3 –adrenoceptors [14] were performed as described earlier. Briefly, transfected CHO-K1 cells were harvested and membranes were prepared. Cells were washed twice by centrifugation at 200 g and homogenized in buffer (50 mM Tris, 10 mM $MgCl_2$, 0.5 mM EDTA at pH 7.5) at a temperature of 4°C using an Ultra-Turrax (Janke & Kinkel, Staufen, Germany) for 10 seconds at full speed and then for 20 seconds at 2/3 speed. The homogenates were centrifuged for 20 minutes at 50,000 g at 4°C. The final pellets were re-suspended and re-homogenized in binding buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4 at pH 7.4). Protein content was measured by the method of Bradford [15] using bovine immunoglobulin G as a standard. Frozen samples were thawed and homogenized for 10 seconds at full speed in buffer.
Saturation binding studies for muscarinic receptors were performed, using 200 µl QNB solution (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Non-specific binding was defined by 3 µM atropine. Binding experiments were performed in a total volume of 1,000 µl buffer containing 50–100 µg protein/assay. All experiments were performed in duplicate in 96-well plates, and incubations were performed for 60 minutes at 37°C. Incubation was terminated by rapid vacuum filtration over Whatman GF/C using a Filtermate harvester (Perkin Elmer, Zaventem, Belgium); each filter was washed with approximately

10 ml of buffer. Radioactivity adherent to the filter was quantified in a Topcount NXT (Perkin Elmer) using Microsint O (Perkin Elmer) scintillator.
Saturation binding studies for β_3-adrenoceptors were performed using ICYP (Amersham Biosciences) binding using 50-100 µg protein/assay, an assay volume of 250 µl and incubation for 90 minutes at 37°C; The concentration of ICYP used was up to 1000 pM. Non-specific binding was defined as binding in the presence of 100 µM isoprenaline. Termination of incubation, filtration and quantification of radioactivity was performed as described above.

cAMP Accumulation Assay. Transfected cells expressing M_2/mock, M_2/β_3, M_3/mock or M_3/β_3 were plated with 20,000 cells/well in 96-well plates two days before measurement. On the day of the experiment, following overnight serum starvation, cells were washed with stimulation buffer (HBSS containing 0.05% fatty-acid free BSA and 5 mM HEPES) and subsequently stimulated for 15 minutes with various concentrations of isoprenaline or forskolin in stimulation buffer with 0.5 mM IBMX at room temperature. After removal of the ligand, the cells were lysed in 50 µl 0.5% Triton X-100 in stimulation buffer with 0.5 mM IBMX, and 10 µl of the lysate was added to 384-well optiplates in triplicate. Detection of cAMP was performed with the LANCE cAMP 384 kit according to the manufacturer's protocol (Perkin Elmer). Measurements were carried out using a Wallac Victor 2 plate reader (Perkin Elmer), 3 hours after adding the detection buffer and antibody mixture.

Intracellular Ca^{2+}. Intracellular Ca^{2+} was measured as described earlier [16]. Cells were plated in black clear bottom 96-wells plates at 50,000 cells per well. After 24 hours of serum starvation, cells were loaded for 1 hour with 4 µM Fluo-4AM ester in buffer (HBSS containing 20 mM HEPES and 250 mM probenecid and 0.42% v/v pluronic acid). They were then washed twice and incubated for 45 minutes with buffer. Fluorescence was measured using an excitation filter at 485 nm and emission filter at 520 nm on a NOVOstar (BMG Labtech, via Isogen, IJsselstein, the Netherlands). After measuring the basal level for 10 seconds, carbachol was added and measured for 50 seconds, and then 5% v/v triton X-100 in basic buffer was added at 10% v/v to determine the maximal signal (F_{max}). After 20 seconds, 0.1 M EGTA in buffer

was added at 10% v/v to determine the minimal signal (F_{min}). The increase in free intracellular Ca^{2+} ($\Delta[Ca^{2+}]$) was calculated as the difference between $[Ca^{2+}]$ for the basal level and after addition of the ligand. $\Delta[Ca^{2+}]$ was calculated by the equation:

$$\Delta[Ca^{2+}]_i = K_d * ((F-F_{min})/(F_{max}-F))$$

K_d is the dissociation constant of the binding of Fluo-4 to Ca^{2+} (345 nM). Concentration-response curves for carbachol (10 nM–1 mM) were generated in duplicate in the absence and presence of 1 and 10 μM forskolin, 10 nM and 1 μM isoprenaline and 1 μM isoprenaline in absence and presence of IBMX.

Data Analysis. Saturation binding data were analyzed by fitting rectangular hyperbolic functions to the experimental data using iterative non-linear regression analysis. Percentages of non-specific binding were determined at the radioligand concentration of saturation binding experiments closest to the calculated K_d value within that experiment and previously obtained K_d values at the human β_3-adrenoceptor [14]. Data are presented as means ± SEM of n experiments. The statistical significance of inter-group differences was assessed for a comparison of K_d values of a given radioligand among subtypes and for percentage of non-specific binding at a given subtype among radioligands using unpaired, two-tailed t tests or one-way analysis of variance followed by Bonferroni-corrected t tests as appropriate; a p value of <0.05 was considered significant. All curve fitting and statistical calculations were performed with the Prism program (version 4.01, Graphpad Software, San Diego, California, USA).

Results

Radioligand Binding. Mock-cells showed no detectable specific binding, but were resistant to antibiotics G-418, indicating successful transfection with the empty vector pcDNA3.1. The four M_2 clones that were selected for QNB saturation binding experiments after the first transfection exhibited a B_{max} ranging from 30.0±1.6 to 70.9±12.4 fmol/mg protein and K_d from 8.1±1.2 to 13.6 ±1.6 pM. Functional testing of the clones revealed all clones to be functionally active in cAMP accumulation and Ca^{2+}-elevation assays (data not shown). Of these clones, one was selected for a second transfection with B_{max} of 36.8±1.8 fmol/mg

Table 1. Results of QNB saturation binding experiments in CHO cells stably transfected with M_2 or M_3 muscarinic receptors followed by transfection with β_3-adrenoceptors or mock transfection. All data are means ± SEM of 3 experiments.

Clone	K_d (pM)	B_{max} (fmol/mg protein)
M_2 receptor without β_3-adrenoceptor		
M_2/mock #1	14.2 ± 0.1	37.2 ± 2.0
M_2/mock #2	15.0 ± 1.0	31.2 ± 1.2
M_2/mock #3	31.5 ±16.2	57.1 ± 11.9
M_2/mock #4	12.7 ±0.1	52.5 ± 6.2
M_2 receptor with β_3-adrenoceptor		
M_2/β_3 #8	16.9 ± 2.2	85.1 ± 9.4
M_2/β_3 #12	18.6 ±1.5	70.5 ± 0.9
M_2/β_3 #24	14.2 ± 2.3	70.2 ± 13.1
M_2/β_3 #27	12.9 ± 0.9	115.1 ± 23.9
M_3 receptor without β_3-adrenoceptor		
M_3/mock #1	58.1 ± 34.8	135.5 ± 42.5
M_3/mock #2	29.3 ±6.5	75.1 ± 3.7
M_3/mock #3	21.9 ± 1.4	74.2 ± 7.7
M_3/mock #4	45.2 ± 22.2	122.1 ± 6.6
M_3 receptor with β_3-adrenoceptor		
M_3/β_3 #1	32.9 ± 10.7	17.3 ± 1.8
M_3/β_3 #4	31.5 ± 6.0	110.8 ± 7.8
M_3/β_3 #8	27.5 ± 4.7	94.3 ± 7.4
M_3/β_3 #9	17.2 ± 0.6	83.1 ± 1.8

Table 2. cAMP accumulation in response to isoprenaline or forskolin in CHO cells transfected with M_2 or M_3 receptors in the absence and presence of β_3-adrenoceptors (n=4)

Clone	Isoprenaline		Forskolin	
	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor without β_3-adrenoceptor				
M_2/mock #4	No response	No response	5.10 ± 0.13	20.45 ± 2.23
M_2 receptor with β_3-adrenoceptor				
M_2/β_3 #8	7.86 ± 0.12	14.90 ± 0.63	5.31 ± 0.13	30.98 ± 3.00
M_2/β_3 #12	7.28 ± 0.48	1.19 ± 0.21	5.45 ± 0.15	45.67 ± 4.67
M_2/β_3 #27	7.52 ± 0.31	6.18 ± 0.71	4.98 ± 0.08	20.36 ± 1.50
M_3 receptor without β_3-adrenoceptor				
M_3/mock #3	No response	No response	4.72 ± 0.13	22.26 ± 3.19
M_3 receptor with β_3-adrenoceptor				
M_3/β_3 #4	7.50 ± 0.36	1.10 ± 0.13	4.90 ± 0.11	28.55 ± 3.02
M_3/β_3 #8	7.73 ± 0.12	8.10 ± 0.35	5.41 ± 0.07	29.34 ± 1.41

protein and K_d of 9.5±1.6 pM (n=3).
The three M_3 clones made in the first transfection round exhibited a B_{max} ranging from 271.7±6.6 to 382.3±19.3 fmol/mg protein and K_d ranging from 28.7±2.2 to 40.1±0.4 pM. All clones were found functionally active in Ca^{2+}-elevation assays (data not shown). One M_3-clone with B_{max} of 382.3±19.3 fmol/mg protein and and K_d of 40.1±0.4 pM (n=3) was selected for the second transfection.
After the second transfection, again four clones of each stably transfected cell line were selected for QNB binding experiments (Table 1). M_2/mock clones demonstrated presence of muscarinic receptors at ranges of 31.2±1.2 to 57.1±111.9 fmol/mg protein and M_3-receptors in M_3/mock clones at 74.2±7.7 to 135.5±42.5 fmol/mg protein. Maximum saturable QNB binding for M_2-receptors in M_2/β_3 was 70.2±13.1 to 115.1±23.9 fmol/mg protein and for M_3-receptors was M_3/β_3 17.2±1.8 to 110.8±7.8 fmol/mg protein, respectively. Thus, co-transfection with β_3-adrenoceptors, as compared to mock transfection, did not consistently affect M_2 or M_3 receptor density.
For assessment of receptor expression levels of β_3-adrenoceptors the difference between total and non-specific ICYP binding was too small to allow reliable estimates of receptor density, as little, if any, saturable high-affinity ICYP binding was seen (data not shown). On the other hand, direct parallel experiments with previously transfected HEK cells [17] showed the expected density of high-affinity ICYP binding sites (data not shown), confirming the validity of the method. Thus, in all our clones β_3-adrenoceptor expression was <10 fmol/mg protein.

cAMP Accumulation. To assess the presence of β_3-adrenoceptors functionally, cAMP accumulation experiments were performed. In mock-transfected cells, i.e. those expressing only muscarinic receptors, isoprenaline did not stimulate cAMP accumulation whereas the direct adenylyl cyclase stimulator forskolin caused concentration-dependent stimulation (Table 2). In contrast, isoprenaline concentration-dependently enhanced cAMP accumulation in all cell clones transfected with β_3-adrenoceptors (table 2, figure 1), with largest cAMP responses for clones M_2/β_3 #8 and M_3/β_3 #8. However, in all cases maximum isoprenaline-stimulated cAMP accumulation was at least 50% smaller than that by forskolin (Table 2). While our study was not

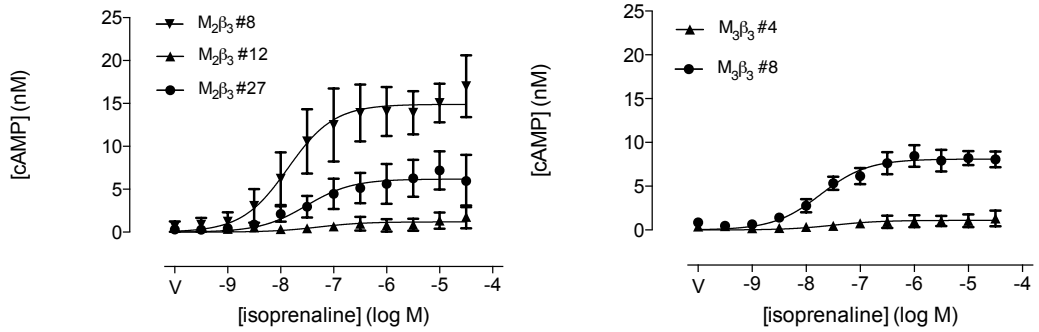

Figure 1. cAMP accumulation in response to isoproterenol in CHO cells expressing β_3-adrenoceptors in the presence of M_2 or M_3 receptors. n=4 experiments per clone, V= vehicle. A quantitative analysis of the data is shown in Table 2.

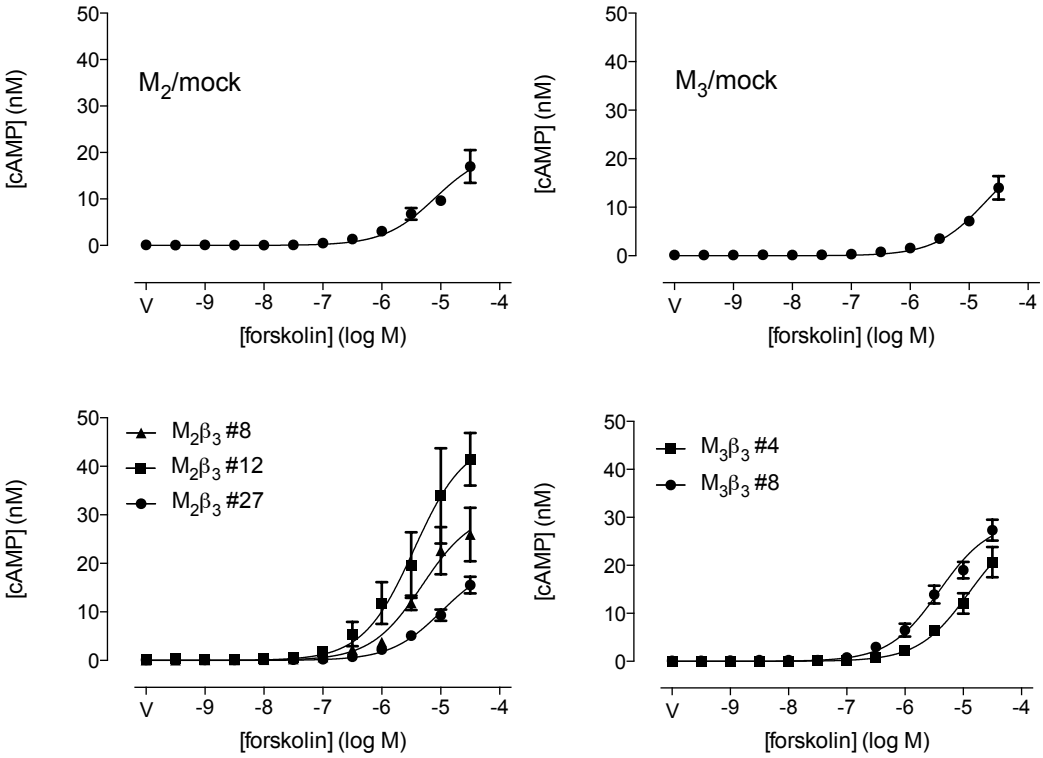

Figure 2: cAMP accumulation in response forskolin in CHO cells expressing M_2 or M_3 receptors in the absence and presence of β_3-adrenoceptors. n=4 experiments per clone, V= vehicle. A quantitative analysis of the data is shown in Table 2.

designed to compare forskolin responses across clones, forskolin-induced cAMP elevation appeared greater in cell lines with, than those without co-transfected β_3-adrenoceptors (Table 2, figure 2). Thus, the co-transfected clones clearly expressed functional β_3-adrenoceptors in both M_2 and M_3 cell lines.

Ca^{2+} Elevation. Clones with detectable isoprenaline-induced cAMP accumulation, indicative of presence of functionally active β_3-adrenoceptors, were selected for Ca^{2+} elevation experiments. In the absence of isoprenaline, carbachol concentration-dependently increased intracellular Ca^{2+} concentrations in all clones, but increases of intracellular Ca^{2+} appeared higher in M_3 than in M_2 cell lines (Table 3). However, there was no obvious relationship between muscarinic receptor density and maximum Ca^{2+} elevation (figure 3).

In contrast, isoprenaline (10 μM) alone did not induce changes in intracellular Ca^{2+} in cells with or without concomitant β_3-adrenoceptor expression (data not shown). Direct addition of isoprenaline (10 nM or 1 μM) concomitant with that of carbachol did not affect the potency or magnitude of the carbachol response in cells expressing M_2 or M_3 receptors (Figure 4, Table 3).

To test why isoprenaline did not attenuate carbachol-induced Ca^{2+} elevations, several types of experiments have been performed in cells co-expressing β_3-adrenoceptors and M_2 or M_3 receptors. Firstly, pre-incubation with isoprenaline (10 nM or 1 μM) for at least 15 minutes rather than concomitant administration was explored, but this also did not affect the carbachol-induced Ca^{2+} elevation (Figure 5, Table 4).

In a second approach, it was tested whether the phosphodiesterase inhibitor IBMX unveiled isoprenaline effects on the Ca^{2+} response. An IBMX concentration of 0.5 mM was chosen for this purpose. However, isoprenaline also failed to inhibit M_2- or M_3-receptor-mediated Ca^{2+} elevations in the presence of IBMX (Figure 6, Table 5).

To explore whether in our CHO cells cAMP elevation can attenuate carbachol-induced Ca^{2+} elevations at all, effects of 10 μM forskolin on carbachol-induced Ca^{2+} response were determined and found to be clearly reduced in M_2/mock and M_3/mock cells and also, albeit to a lesser extent, in M_2/β_3 and M_3/β_3 cells (Figure 7, Table 6).

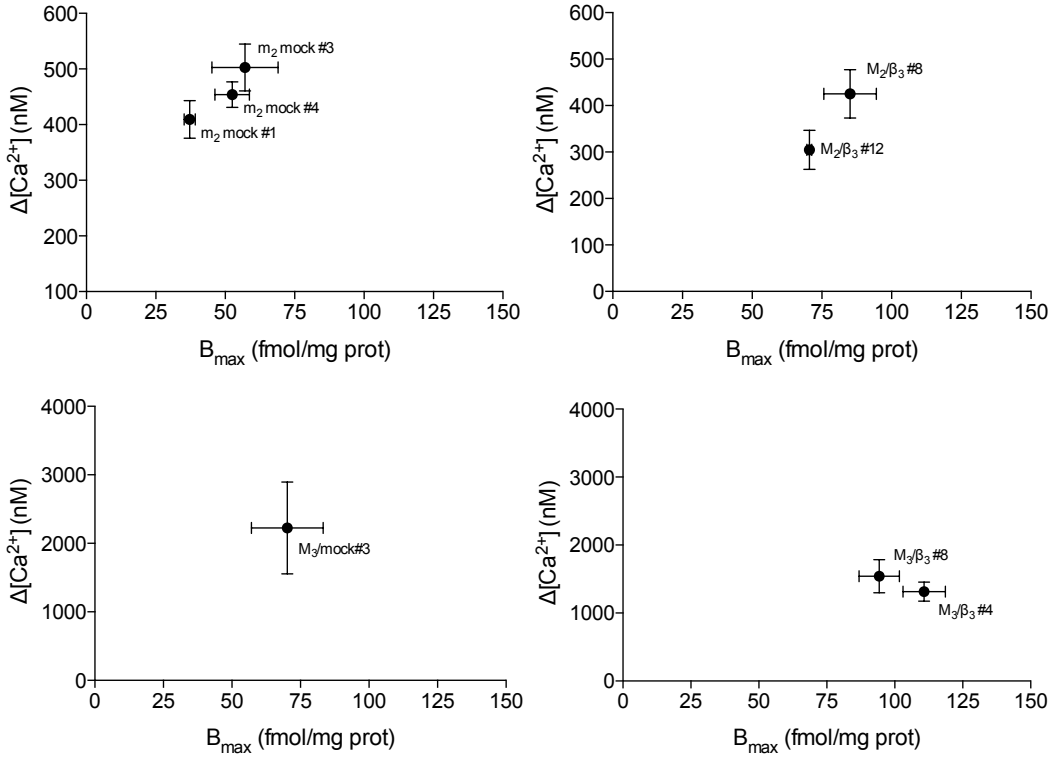

Figure 3. Association of muscarinic receptor expression and carbachol-induced Ca^{2+} elevation

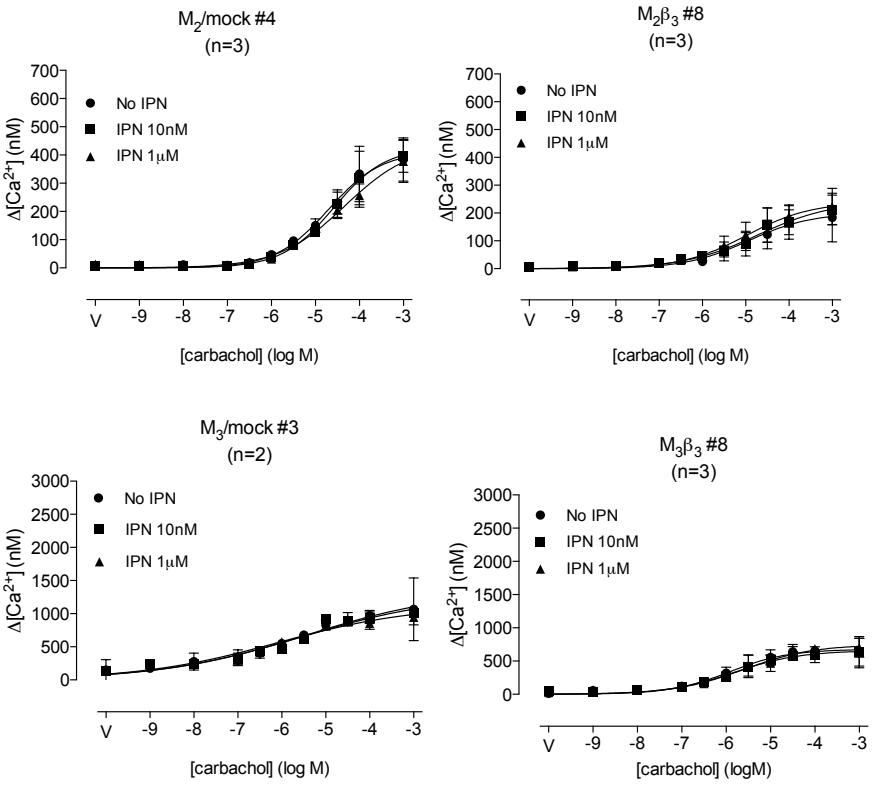

Figure 4. Effects of carbachol after simultaneous stimulation with isoprenaline (IPN) on intracellular Ca^{2+} concentrations in the absence and presence of β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment. A quantitative analysis of these data is provided in Table 3.

Table 3. Effect of carbachol in presence of no, 10 nM or 1 μM isoprenaline simultaneously administered on intracellular Ca^{2+} concentrations in the absence and presence of β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment

	no isoprenaline		10 nM isoprenaline		1 μM isoprenaline	
clone	pEC_{50}	E_{max} (nM)	pEC50	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor without β_3-adrenoceptor						
M_2/mock #4 (n=3)	4.71 ± 0.24	413 ± 53	4.58 ± 0.74	427 ± 51	4.33 ± 0.30	440 ± 66
M_2 receptor with β_3-adrenoceptor						
M_2/β_3 #8 (n=3)	4.93 ± 0.57	201 ± 53	4.72 ± 0.60	243 ± 63	4.98 ± 0.44	240 ± 48
M_3 receptor without β_3-adrenoceptor						
M_3/mock #3 (n=2)	6.08 ± 0.46	1129 ± 132	5.56 ± 0.60	1296 ± 198	5.43 ± 0.85	1357 ± 296
M_3 receptor with β_3-adrenoceptor						
M_3/β_3 #8 (n=3)	5.92 ± 0.18	678 ± 51	5.82 ± 0.18	658 ± 46	5.60 ± 0.19	747 ± 58

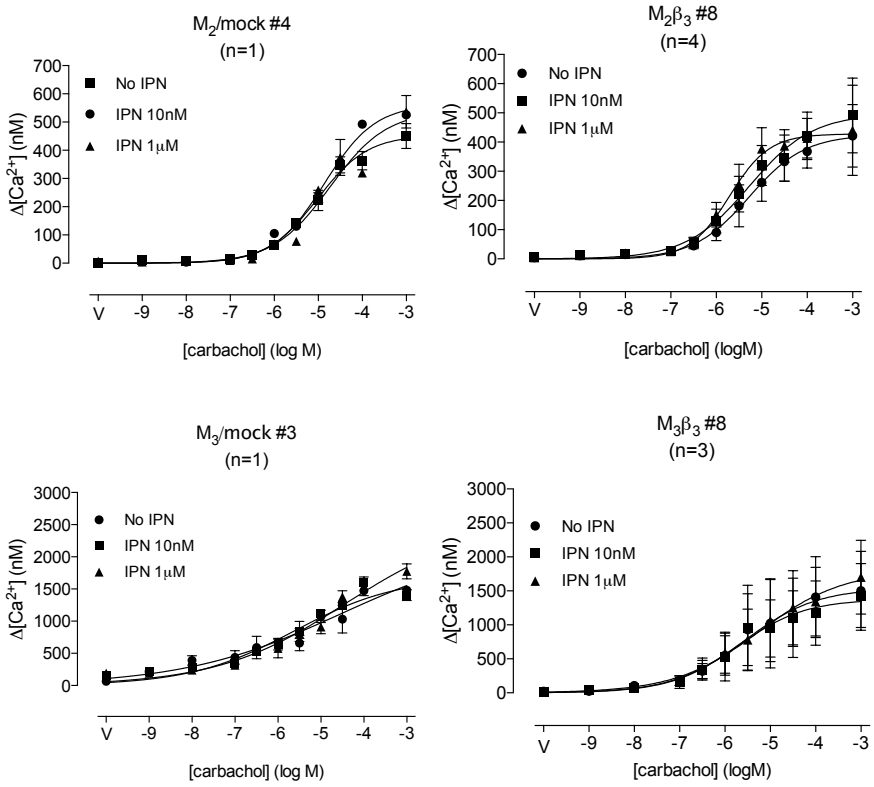

Figure 5. Effect of carbachol after 15 minutes of pre-stimulation with isoprenaline on intracellular Ca^{2+} concentrations in the absence and presence of β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment. A quantitative analysis of these data is provided in Table 4.

Table 4. Effect of carbachol after 15 minutes of pre-stimulation with 1 μM or 10 nM isoprenaline in clones with or without β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment.

	No isoprenaline		10 nM isoprenaline		1 μM isoprenaline	
Clone	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor without β_3-adrenoceptor						
M_2/mock #4 (n=1)	5.03 ± 0.09	454 ± 23	4.87 ± 0.06	563 ± 18	4.74 ± 0.20	540 ± 57
M_2 receptor with β_3-adrenoceptor						
M_2/β_3 #8 (n=4/3/3)	5.27 ± 0.25	425 ± 52	5.28 ± 0.30	497 ± 68	5.71 ± 0.18	428 ± 38
M_3 receptor without β_3-adrenoceptor						
M_3/mock #3 (n=1)	6.08 ± 0.46	1129 ± 132	4.45 ± 0.67	2530 ± 507	5.63 ± 0.34	1691 ± 181
M_3 receptor with β_3-adrenoceptor						
M_3/β_3 #8 (n=3)	5.57 ± 0.39	1543 ± 246	5.72 ± 0.36	1378 ± 204	5.24 ± 0.47	1806 ± 330

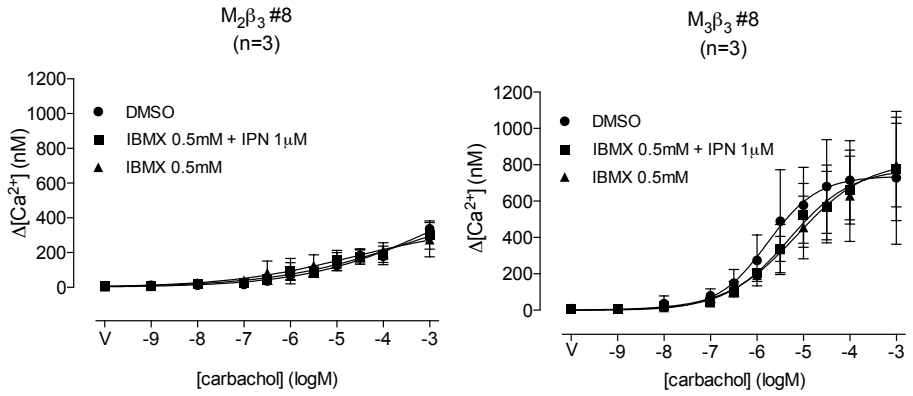

Figure 6. Effect of carbachol in presence of isoprenaline with or without IBMX on intracellular Ca^{2+} concentrations in presence of β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment. A quantitative analysis of the data is provided in Table 5.

Table 5. Effect of carbachol with or without 1 μM isoprenaline in presence of 0.5 mM IBMX on intracellular Ca^{2+} concentrations. All carbachol concentrations are measured in duplicates within one experiment.

	DMSO		0.5 mM IBMX		0.5 mM IBMX + 1 μM isoprenaline	
Clone	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor with β_3-adrenoceptor						
M_2/β_3 #8 (n=3)	3.02 ± 1.77	637 ± 465	4.67 ± 1.67	349 ±186	3.56 ± 2.19	493 ± 369
M_3 receptor with β_3-adrenoceptor						
M_3/β_3 #8 (n=3)	5.78 ± 0.23	738 ± 78	5.15 ± 0.28	822 ± 102	5.32 ± 0.22	779 ± 79

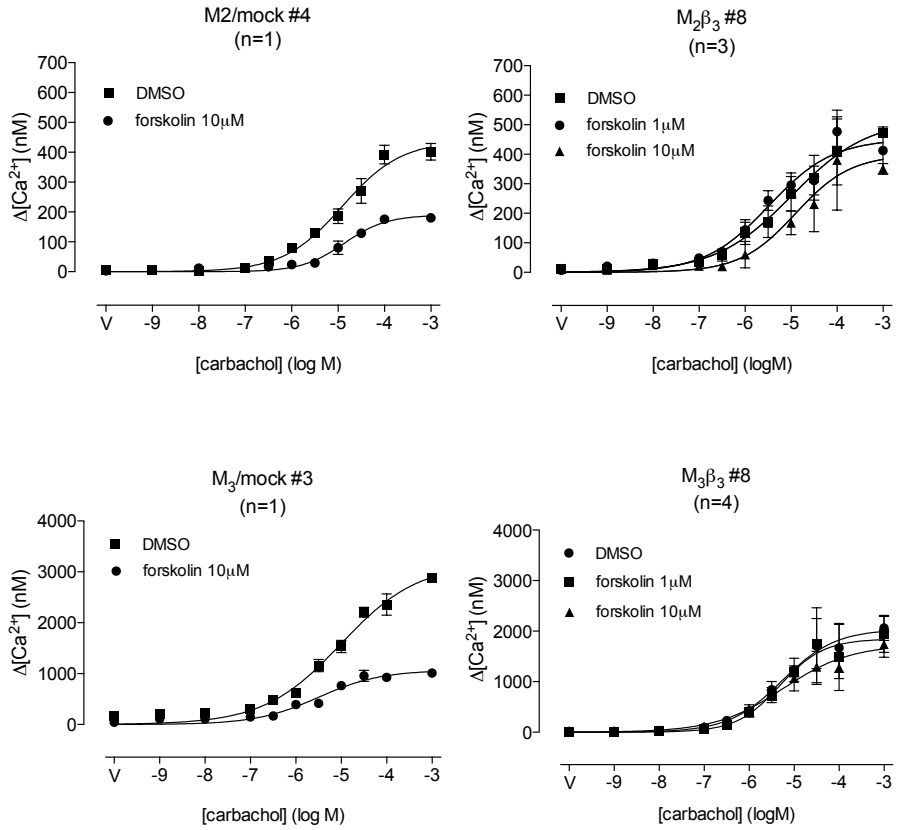

Figure 7. Effect of carbachol in presence of forskolin on intracellular Ca^{2+} concentrations in the absence and presence of β_3-adrenoceptors. All experiments with two comparisons were performed in triplicate, all others in duplicate within one experiment. A quantitative analysis of the data is provided in Table 6.

Table 6. Effect of carbachol in presence of 1 μM and 10 μM forskolin on intracellular Ca^{2+} concentrations. All experiments with three conditions are in duplicate, and two conditions in triplicate within one experiment.

	DMSO		1 μM forskolin		10 μM forskolin	
Clone	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor without β_3-adrenoceptor						
M_2/mock #4 (n=1)	4.91 ± 0.12	438 ± 27	No data	No data	4.87 ± 0.09	190 ± 10
M_2 receptor with β_3-adrenoceptor						
M_2/β_3 #8 (n=3)	4.99 ± 0.39	519 ± 91	5.48 ± 0.21	452 ± 42	4.90 ± 0.42	399 ± 91
M_3 receptor without β_3-adrenoceptor						
M_3/mock #3 (n=1)	5.01 ± 0.14	3166 ± 186	No data	No data	5.51 ± 0.13	1067 ± 63
M_3 receptor with β_3-adrenoceptor						
M_3/β_3 #8 (n=4)	5.27 ± 0.15	2039 ± 150	5.24 ± 0.15	1731 ± 113	5.32 ± 0.13	1847 ± 123

To further corroborate the hypothesis that inhibition of carbachol-induced Ca^{2+} elevations depended on the extent of cAMP elevation and not the stimulus used to achieve it, the effect of forskolin pre-incubation was explored in a concentration mimicking the maximum isoprenaline cAMP response within the same clone. Based upon the data shown in figure 1 and 2 and table 2, a forskolin concentration of 1 μM was considered equi-effective with 1 μM isoprenaline. In such experiments, both M_2 or M_3 receptor-mediated Ca^{2+} elevations were not affected by either forskolin or isoprenaline (Figure 8, Table 7).

Discussion

Many studies, largely performed with β_1- or β_2-adrenoceptors, have demonstrated that β-adrenoceptor agonists and other cAMP-elevating agents can inhibit responses to muscarinic receptor stimulation [10]. Based on our interest in β-adrenergic function in the human bladder, where relaxation is mediated predominantly, if not exclusively, by β_3-adrenoceptors [5], the present study had aimed to explore similar interactions for β_3-adrenoceptors. In order to develop model systems suitable for biochemical scrutiny of the underlying molecular mechanisms of such interactions, we had aimed to develop double-transfected CHO-K1 cells in which each receptor is present at densities assumed to reflect physiological values.

In a first round of transfection, M_2 or M_3 receptors were introduced into CHO-K1 cells. From these, clones were picked which exhibited receptor densities comparable to those found in the human bladder [18;19]. These clones underwent a second round of transfection to introduce the β_3-adrenoceptor. However, this did not yield any clone in which β_3-adrenoceptor expression was detectable at the protein level using saturation radioligand binding. In this regard false negatives due to inadequate binding techniques were excluded by parallel controls in which radioligand binding to previously transfected HEK cells was found. As stable β_3-adrenoceptor transfection into CHO cells does not generally cause problems [20;21] and our lab had successfully generated HEK cells stably expressing β_3-adrenoceptors [17], we assume that for unknown reasons the presence of muscarinic receptors limits the success of additionally inducing β_3-adrenoceptor expression at the protein level. Similar problems in generating double-transfected cells have also been observed by other investigators (personal

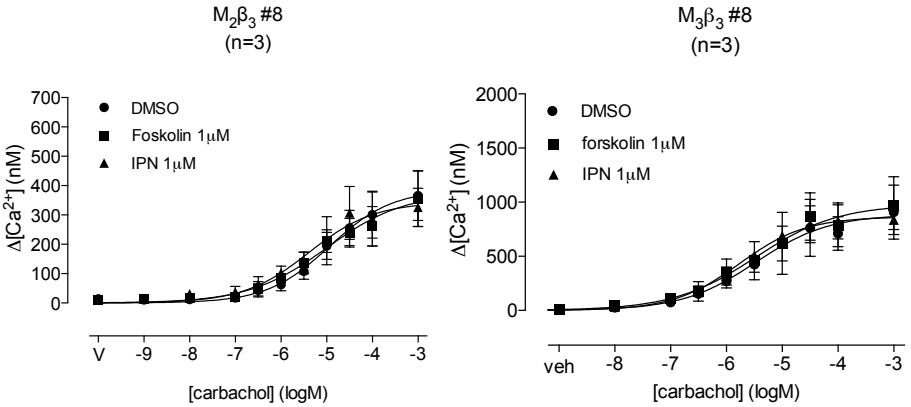

Figure 8. Effect of carbachol in presence of isoprenaline or forskolin on intracellular Ca^{2+} concentrations in presence of β_3-adrenoceptors. All carbachol concentrations are measured in duplicates within one experiment. A quantitative analysis of the data is provided in Table 7.

Table 7. Effect of carbachol in presence of 1 μM forskolin and 1 μM isoprenaline on intracellular Ca^{2+} concentrations. All carbachol concentrations are measured in duplicates within one experiment.

	DMSO		1 μM isoprenaline		1 μM forskolin	
Clone	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)	pEC_{50}	E_{max} (nM)
M_2 receptor with β_3-adrenoceptor						
M_2/β_3 #8 (n=3)	4.94 ± 0.30	385 ± 56	5.39 ± 0.35	344 ± 52	5.00 ± 0.5	376 ± 80
M_3 receptor with β_3-adrenoceptor						
M_3/β_3 #8 (n=3)	5.48 ± 0.22	887 ± 86	5.48 ± 0.22	980 ± 92	5.77 ± 0.10	865 ± 37

communication, Dr. J. Baker, University of Leicester, UK). Nevertheless, our co-transfection efforts were successful to some extent as clones transfected with β_3-adrenoceptors, but not mock-transfected clones, exhibited isoprenaline-induced cAMP accumulation. The potency of isoprenaline for such stimulation was within the range previously reported for CHO [20] and HEK cells [17], corroborating the idea that these cells indeed expressed β_3-adrenoceptors. All double-transfected clones exhibited cAMP accumulation in response to forskolin, which yielded maximum responses that were 2-40 times as large as those caused by isoprenaline. Being aware that small cAMP accumulation may limit the ability to test the effect of β_3-adrenoceptor co-transfection, further experiments were primarily based on the double-transfected M_2 and M_3 receptor clones exhibiting the largest isoprenaline-induced cAMP response. Thus, we had succeeded in generating cell lines co-expressing β_3-adrenoceptors together with either M_2 or M_3 receptors, where the muscarinic receptor was present at physiological expression levels and the β_3-adrenoceptor was present at low but functionally active expression levels. Muscarinic M_3 receptors prototypically couple to stimulation of phospholipase C, resulting in mobilization of Ca^{2+} from intracellular stores and activation of protein kinase C [22]. However, they can also couple to other signaling pathways, as urinary bladder contraction by muscarinic receptor agonists does not involve phospholipase C, but rather L-type Ca^{2+}-channels and rho kinase [23]. In contrast, the prototypical signaling pathway of M_2 receptors is inhibition of adenylyl cyclase leading to reduced cellular cAMP levels [22]. However, M_2 receptors can also couple to elevation of intracellular Ca^{2+} concentration, albeit less effectively and with lower potency than M_3 receptors [24;25]. Measurement of intracellular Ca^{2+} concentration was used for all of our subsequent studies as an indicator of function of both muscarinic receptor subtypes and its potential modulation by β_3-adrenoceptors for four reasons. First, intracellular Ca^{2+} measurements based on fluorescent indicator dyes [24] are easier to perform and less expensive than cAMP measurements. Second, a cAMP readout may yield misleading results for studies of the interaction of a cAMP lowering and cAMP increasing receptor as the two may cancel each other without any direct interaction. Third, inhibition of muscarinic agonist-induced intracellular Ca^{2+}

elevation by β-adrenoceptor agonists, or other cAMP-related agents, had repeatedly been described, e.g. airway smooth muscle of many species [10]. Fourth, Ca^{2+} plays a pivotal role in the control of smooth muscle contraction [26;27], including that of the urinary bladder [23], the organ in which we are primarily interested in.

In our experiments, carbachol concentration-dependently increased intracellular Ca^{2+} concentrations in all cell lines, in line with the prototypical signalling pathway of both receptors [22] and our previous observations [24]. However, carbachol was more potent and more effective in this regard at M_3 than at M_2 receptors. While the two cell lines co-expressing β_3-adrenoceptors exhibited smaller carbachol-induced Ca^{2+} elevations than those without, in the absence of isoprenaline, isoprenaline did not inhibit the M_2 or M_3 response, even in a concentration about 30 times its EC_{50} for cAMP accumulation. As this was an unexpected observation, further experiments were designed to elucidate this lack of inhibition.

First, we reasoned that cAMP accumulation may be too slow to affect the Ca^{2+} response, which peaks within less than 30 seconds [24]. Therefore, we performed experiments in which cells were pre-treated with isoprenaline for 15 minutes prior to carbachol addition. However, even with such pre-treatment Ca^{2+} elevations remained strong. Thus, lack of temporal linkage did not explain the lack of inhibition of the M_2 or M_3 Ca^{2+} elevations by β_3-adrenoceptor stimulation.

Second, we reasoned that isoprenaline-induced cAMP accumulation may be too weak to cause inhibition of Ca^{2+} elevations. To test this, experiments were performed in the presence of the phosphodiesterase inhibitor IBMX, which should amplify intracellular cAMP levels by inhibiting its breakdown [28]. However, even in the presence of IBMX a high isoprenaline concentration did not inhibit M_2 or M_3 mediated Ca^{2+} responses.

At this point, we became skeptical whether our cell lines were suitable at all to detect cAMP-dependent inhibition of Ca^{2+} elevations in response to a muscarinic agonist. Therefore, experiments were performed with the direct adenylyl cyclase stimulator forskolin [29], which indeed inhibited carbachol-induced Ca^{2+} elevations in cells transfected with M_2 or M_3 receptors, but no β_3-adrenoceptors. However, such inhibition was less pronounced in cells with concomitant β_3-adrenoceptor

expression. Therefore, we finally explored inhibition of carbachol-induced Ca^{2+} responses by forskolin in a concentration expected to raise cAMP to a similar levels as the maximally effective isoprenaline concentration. Under these conditions, neither isoprenaline nor the low forskolin concentration inhibited M_2 or M_3 mediated Ca^{2+} responses.

While the above data are not fully conclusive, they suggest that elevation of cAMP by forskolin can attenuate M_2 and M_3 receptor-mediated Ca^{2+} elevations. β_3-Adrenoceptors transfected into CHO cells already transfected with M_2 or M_3 receptors express at low density only, which leads to detectable, but only small cAMP accumulation in response to isoprenaline. This small increase in cAMP apparently is insufficient to inhibit M_2 or M_3 receptor-mediated Ca^{2+} elevations, even when isoprenaline is administered 15 minutes prior to carbachol, or when cAMP responses are amplified by IBMX. These findings are consistent with the hypothesis that major elevations of cAMP levels are required to inhibit Ca^{2+} elevations. However, the inhibitory effect of forskolin was much weaker in the cell lines co-expressing β_3-adrenoceptors than in those which did not. Therefore, we cannot exclude the alternative hypothesis that cAMP-induced inhibition of M_2 or M_3 receptor-mediated Ca^{2+} elevations is attenuated or even abolished upon co-transfection with β_3-adrenoceptors. Potential molecular mechanisms for such loss of inhibition remain unclear. Moreover, it should be noted that the two hypotheses to explain the observed lack of inhibition are not mutually exclusive. Despite not being fully conclusive, these data demonstrate clearly that the double-transfected CHO cells we have generated are not suitable as model systems to explore the molecular basis for the interaction between M_2 and M_3 muscarinic receptors on the one, and β_3-adrenoceptors on the other hand. Therefore, the project was discontinued at this point.

Conclusion

In conclusion, we have generated cell lines co-expressing β_3-adrenoceptors with either M_2- or M_3-muscarinic receptors. However, the few cell lines that could be established, despite considerable effort, expressed only very small numbers of β_3-adrenoceptors. While these small numbers yielded concentration-dependent stimulation of cAMP accumulation, they did not yield inhibition of M_2 or M_3 receptor-mediated Ca^{2+} elevations.

Therefore, other model systems may be required to explore the interaction between β_3-adrenoceptors and either M_2 or M_3 muscarinic receptors.

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Chapter 6

The Muscarinic Receptor Antagonist Propiverine Exhibits α_1–Adrenoceptor Antagonism in Human Prostate and Porcine Trigonum

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Abstract

Purpose. Combination therapy of male lower urinary tract symptoms with α_1-adrenoceptor and muscarinic receptor antagonists has recently attracted increasing interest. Propiverine is a muscarinic receptor antagonist possessing additional properties, i.e. block of L-type Ca^{2+}-channels. Here we have investigated whether propiverine and its metabolites can additionally antagonize α_1-adrenoceptors.

Methods. Human prostate and porcine trigone muscle strips were used to explore inhibition of α_1-adrenoceptor-mediated contractile responses. Chinese hamster ovary (CHO) cells expressing cloned human α_1-adrenoceptors were used to determine direct interactions with the receptor in radioligand binding and intracellular Ca^{2+}-elevation assays.

Results. Propiverine concentration-dependently reversed contraction of human prostate pre-contracted with 10 µM phenylephrine (–log EC_{50} [M]) 4.43 ± 0.08). Similar inhibition was observed in porcine trigone (–log EC_{50} 5.00 ± 0.05), and in additional experiments consisted mainly of reduced maximum phenylephrine responses. At concentrations ≥1 µM the propiverine metabolite M-14 also relaxed phenylephrine pre-contracted trigone strips, whereas metabolites M-5 and M-6 were ineffective. In radioligand binding experiments propiverine and M-14 exhibited similar affinity for the three α_1-adrenoceptor subtypes with -log K_i [M] values ranging from 4.72 to 4.94, whereas the M-5 and M-6 did not affect [^{3}H]-prazosin binding. In CHO cells propiverine inhibited α_1-adrenoceptor-mediated Ca^{2+}-elevations with similar potency as radioligand binding, again mainly by reducing maximum responses.

Conclusion. In contrast to other muscarinic receptor antagonists, propiverine exerts additional L-type Ca^{2+}-channel blocking and α_1-adrenoceptor antagonist effects. It remains to be determined clinically, how these additional properties contribute to the clinical effects of propiverine, particularly in male voiding dysfunction.

Introduction

The medical treatment of male lower urinary tract symptoms (LUTS) typically consists primarily of α_1-adrenoceptor antagonists, but in many patients this provides insufficient symptom relief. A combination of α_1-adrenoceptor and muscarinic receptor antagonists may be more effective relief of male LUTS, particularly against storage symptoms [1;2]. While most such studies have used tolterodine as the muscarinic antagonist, several studies have also been based on the muscarinic antagonist propiverine [1-4].

Propiverine is an antagonist with similar affinity for all muscarinic receptor subtypes [5;6]. In contrast to most other muscarinic antagonists, propiverine blocks not only muscarinic receptors but also voltage-gated L-type Ca^{2+}-channels [7-11]. As contractions of prostate [12] and bladder smooth muscle [13] at least partly depend on L-type Ca^{2+}-channels, their inhibition may contribute to the therapeutic effects of propiverine in LUTS suggestive of benign prostatic hyperplasia and overactive bladder (OAB), respectively.

Based upon the growing clinical interest in a combination of α_1-adrenoceptor and muscarinic receptor antagonists for the treatment of male LUTS, we have explored possible α_1-adrenoceptor antagonist effects of propiverine. As some propiverine metabolites share the anti-muscarinic and/or L-type Ca^{2+}-channel blocking activity of the parent compound [14;15], we have also included the metabolites M-5, M-6 and M-14 in our study.

Materials and methods

Prostate and Trigone Detrusor Contraction. Human prostate samples were obtained from six patients undergoing combined prostatectomy and radical cystectomy for invasive bladder cancer (65±3 years old). All patients had given informed written consent in accordance with the regulations of the local hospital ethical committee (permit EK 194092004). Urinary bladder trigone from juvenile and adult female pigs were obtained from a local abattoir. Prostate strips (10 mm long and 3-4 mm wide) and trigone strips (7-10 mm long and 2-4 mm wide) were prepared and mounted in organ baths as previously described [5;16] except for a resting load of 5 mN. During the equilibration period of 60 min the bath solution was changed twice. After this stabilization period the human prostate and porcine trigone detrusor strips were challenged with a single concentration of

phenylephrine (10 μM) to reach a stable α_1-adrenoceptor-mediated pre-contraction after 60 min. Then, increasing concentrations of test compounds were added in a cumulative manner. An equilibration period of 15 min was allowed before the next concentration step. At the end of each experiment, maximum relaxation of the preparations was induced with the adenylyl cyclase activator forskolin (10 μM). All experimental values were normalized to the forskolin effect (relaxation induced by forskolin = 100%) and were corrected for spontaneous decline of force in time-matched control experiments (TMC).
In a second set of experiments porcine trigone strips were exposed to cumulatively increasing concentrations of phenylephrine (30 nM – 100 μM) with 5 min of stabilisation between two subsequent additions of the α_1-adrenoceptor agonist. After maximum contraction was observed, phenylephrine was removed by washing 3 times with drug-free solution and re-equilibrating for 60 min. Then a single concentration of test compound was added and after additional 30 min, the second concentration-response curve for phenylephrine was generated.

Radioligand Binding. Binding of propiverine and its metabolites to α_1-adrenoceptor subtypes was analyzed in Chinese hamster ovary (CHO) cells, expressing approximately 2 pmol receptor/mg protein, by competition binding against [^{3}H]-prazosin as previously described [16;17]. For comparison the α_1-adrenoceptor antagonist phentolamine was used to determine binding on each α_1-adrenoceptor subtype. Radioactivity adherent to the filters was quantified in a Topcount NXT (Perkin Elmer, Zaventem, Belgium) using Microsint O (Perkin Elmer) scintillator.

Intracellular Ca^{2+}. Cells were plated in black, clear bottom 96 wells plates at 50,000 cells per well. 24 Hours prior to the experiment, cells were grown in serum-free medium. They were loaded for 1 hour with 4 μM Fluo-4 AM ester in basic buffer (HBSS containing 20 mM HEPES and 250 mM probenecid and 0.42% v/v pluronic acid). The cells were then washed twice and incubated for 45 min with basic buffer. Antagonist or vehicle was added to the cells 15 min prior to the experiment. Fluorescence was measured using an excitation filter at 485 nm and emission filter at 520 nm on a NOVOstar (BMG Labtech, via Isogen,

IJselstein, the Netherlands). A concentration-response curve was made with phenylephrine (100 pM - 10μM) at 10% v/v basic buffer with each condition measured in triplicates. After measuring the basal level for 10 seconds ligand was added and measured for 50 s, 5% v/v Triton X-100 in basic buffer was added at 10% v/v to determine the maximal signal (F_{max}). After 20 seconds 0.1M EGTA in basic buffer was added at 10% v/v to determine the minimal signal (Fmin). The increase in free intracellular Ca^{2+} ($\Delta [Ca^{2+}]_i$) upon ligand stimulation was calculated as the difference between the $[Ca^{2+}]_i$ for the basal level and after adding a ligand. $\Delta [Ca^{2+}]_i$ was calculated by the equation:

$$\Delta[Ca^{2+}]_i = K_d * ((F-F_{min})/(F_{max}-F))$$

Kd is the dissociation constant of the binding of Fluo-4 to Ca^{2+} (345 nM). Concentration-response curves for phenylephrine were generated in duplicate in the absence and presence of propiverine, its metabolites M-5, M-6 and M-14 and the reference antagonist phentolamine.

Data Analysis. Experimental data were analyzed by non-linear curve fitting of each individual experiment using GraphPad Prism® 4.00 (GraphPad Software, San Diego, CA, U.S.A.). Potencies of propiverine, tamsulosin and prazosin on phenylephrine pre-contracted human prostate and porcine trigone detrusor strips were determined as $-\log IC_{50}$ [M] values. The potency ($-\log EC_{50}$ [M]) and efficacy of phenylephrine-induced contractions and $[Ca^{2+}]_i$ elevations were determined in the absence and presence of the indicated test compounds. Maximum contraction during the second concentration-response curve for phenylephrine (E_{max}) was expressed as percent of the maximum effects during the first concentration-response curve (= 100%). Inhibitory potency as determined from competition binding experiments was transformed to $-\log K_i$ values using the Cheng and Prusoff equation. Statistical differences were tested by Student's t-test and were considered significant for $p<0.05$.

Chemicals. Propiverine hydrochloride, M-5 (2,2-diphenyl-2-propoxy-acetic acid [1-methyl-piperid-4-yl]-ester-N-oxide-trans), M-6 (2,2-diphenyl-2-hydroxy-acetic acid [1-methyl-piperid-4-

Figure 1A. Original recording of force of contraction in a human prostate tissue strip. The preparation was precontracted with phenylephrine (PE, 10 µM) (left arrow). After stabilization of force, propiverine was added in increasing concentrations (0.1–100 µM). Finally, forskolin (10 µM) was added for complete relaxation. The difference between force prior to addition of the test compound and force in the presence of forskolin was taken as maximum relaxation (=100%).

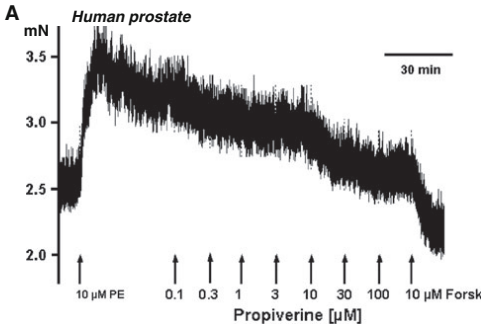


Figure 1B. Effects of tamsulosin, prazosin and propiverine on α1-adrenoceptor mediated contractions in the human prostate. Values were corrected for spontaneous relaxation during time-matched controls (TMC), normalized to percent relaxation by forskolin and expressed as means ± SEM.

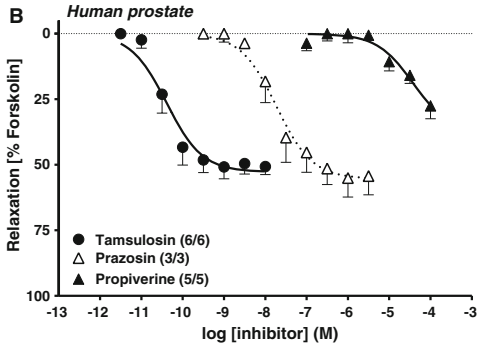


Table 1. -log IC_{50} [M] values for the antagonistic effects on phenylephrine-induced contraction of human prostate and juvenile porcine trigone. Data are means ± SEM of the number of experiments indicated in parentheses.

	human prostate	porcine trigone
Propiverine	4.43±0.08 [5]	5.01±0.05 [5]
Tamsulosin	10.39±0.04 [6]	10.98±0.04 [5]
Prazosin	7.73±0.04 [3]	7.83±0.01 [5]

yl]-ester-N-oxide-trans), M-14 (2,2-diphenyl-2-propoxy-acetic acid [piperid-4-yl]-ester) and tamsulosin were synthesized at APOGEPHA Arzneimittel GmbH. Phentolamine and phenylephrine were purchased from SIGMA-ALDRICH (Taufkirchen, Germany), prazosin from TOCRIS (Bristol, UK). Fluo-4 AM ester and pluronic acid were from Molecular Probes (via Invitrogen, Breda, the Netherlands. [^{3}H]-prazosin (specific activity 80 Ci/mmol) was purchased from Perkin Elmer (Zaventem, Belgium). All other chemicals were of analytical grade and purchased from SIGMA-ALDRICH.

Results

Effects on α_1-adrenoceptor mediated prostate contraction. At the end of the equilibration period, human prostate strips exhibited a passive tension of 0.06±0.01 mN/mg wet weight (n=16 strips/five patients). Addition of the α_1-adrenoceptor agonist phenylephrine (10 μM) increased force of contraction to a peak maximum of 0.16±0.02 mN/mg (n=19/6), that stabilized at steady-state values of 0.10 ± 0.02 mN/mg (n=16/5) within 45 min (Figure 1A). A maximum relaxation was achieved after addition of 10 μM forskolin amounting to 0.07±0.01 mN/mg (n=16/5). Propiverine, tamsulosin and prazosin relaxed phenylephrine-induced contractions in a concentration-dependent manner (Figure 1B, Table 1).

Effects on α_1-Adrenoceptor Mediated Trigone Contraction. Phenylephrine stimulated trigonal (but not bladder wall) contractions of adult porcine urinary bladders in a concentration-dependent manner. Maximum contractions of 1.24±0.23 mN/mg wet weight (n=6) were observed at 10 μM phenylephrine, the –log EC_{50} [M] value was 5.74±0.04. In juvenile tissues these contractions were concentration-dependently reversed by tamsulosin, prazosin and propiverine (Figure 2A). We have also investigated the metabolites M-14, M-5 and M-6 in trigone tissue, albeit from mature pigs. The overall relaxing effect of propiverine on contractions was similar in tissue from mature and juvenile animals, but the potency was lower in mature than in juvenile pigs (-log IC_{50} [M] 5.01±0.05 vs. 6.21±0.10, respectively; n=5-7; $p < 0.05$; Figure 2AB). M-14 relaxed trigone strips to the same extent as propiverine but with lower potency (4.84±0.08; n=8; Figure 2B) as compared to propiverine. M-5 and M-6 did not influence trigone

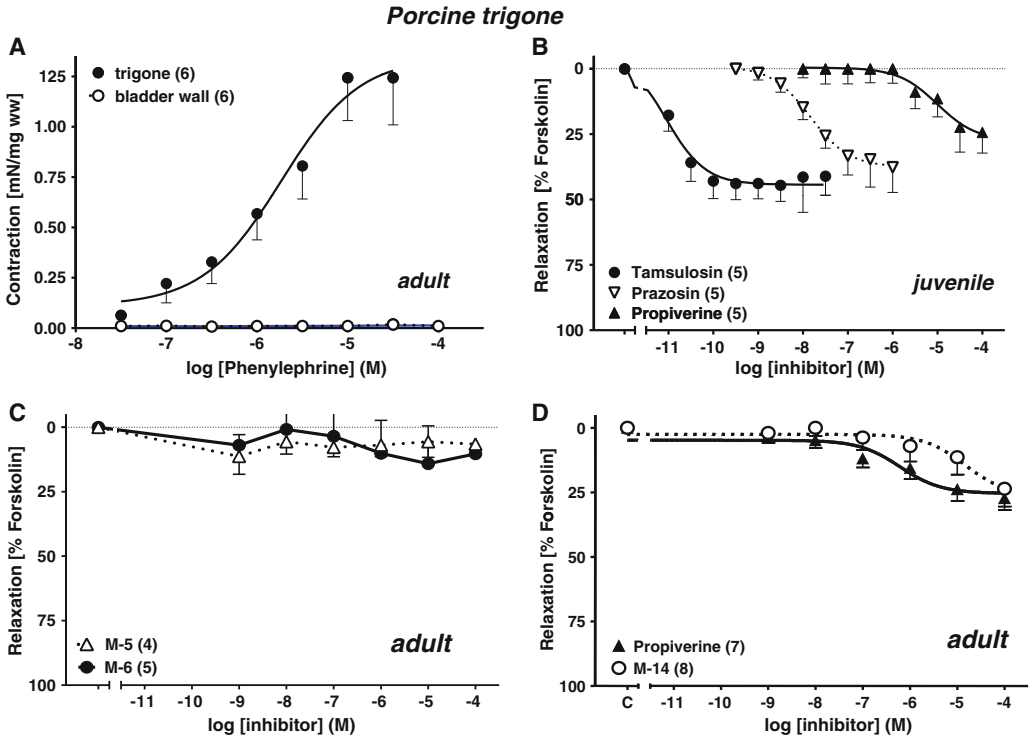


Figure 2A. Effects of cumulatively added concentrations of phenylephrine on detrusor tissue from the trigone and the dome area of the adult pig. Data were normalized to mN per mg wet weight of the detrusor strip.

Figure 2B. Effects of increasing concentrations of propiverine in comparison to those of the α_1-adrenoceptor antagonists tamsulosin and prazosin on juvenile porcine detrusor strips from the trigone area of the urinary bladder. Strips were pre-contracted with 10 µM of the α_1-adrenoceptor agonist phenylephrine. Data were normalized to % relaxation induced by 10 µM forskolin and corrected for time-matched controls (TMC).

Figure 2C. Effects of increasing concentrations of M-5 and M-6.

Figure 2D. Effects of propiverine and its metabolite M-14 on trigone detrusor strips from mature pigs pre-contracted with phenylephrine (10 µM). Data in B-D were corrected for spontaneous relaxation during time-matched controls (TMC) and normalized to percent relaxation by forskolin (10 µM). Means ± SEM.

contractions in concentrations up to 100 μM (data not shown). Concentration-response curves for phenylephrine in porcine trigone strips in the presence of increasing propiverine concentrations demonstrated that inhibition was largely insurmountable, i.e. mainly consisting of reduced maximum responses with only minor if any reductions in apparent potency (Figure 3, Table 2).

Binding to α_1-Adrenoceptor Subtypes. Direct α_1-adrenoceptor inhibition was evaluated in radioligand binding assays with CHO cells (Figure 4). Propiverine, M-14 and phentolamine competed for [^{3}H]-prazosin binding in similar concentration ranges at the three subtypes whereas the metabolites M-5 and M-6 were almost ineffective (Table 3).

α_1-Adrenoceptor-mediated Intracellular Ca^{2+} Elevation. The $[Ca^{2+}]_i$ increase with 10 μM phenylephrine was about 1600 nM, and all subsequent data values are normalized to that response as measured within a given experiment (= 100%). Propiverine and M-14 concentration-dependently inhibited the $[Ca^{2+}]_i$ elevations, but this inhibition largely consisted of reduced maximum responses with small if any effects on phenylephrine potency (Figure 5A, B). M-5 or M-6 had little effect (Figure 5C), whereas antagonism by phentolamine was surmountable (Figure 5D).

Discussion

Propiverine differs from most other OAB drugs as it is not only a muscarinic receptor antagonist [3;5] but also binds to and inhibits L-type Ca^{2+}-channels [7-11]. Based upon the increasing interest in a combination treatment with α_1-adrenoceptor and muscarinic receptor antagonists in men with LUTS [15;18;19], we have explored possible α_1-adrenoceptor antagonism of propiverine and three of its main metabolites. The α_1-adrenoceptor antagonists phentolamine, prazosin and tamsulosin were included in our experiments as reference compounds and exhibited the expected potency for interaction with α_1-adrenoceptors, thereby validating our model systems and techniques. Due to limited access to human prostate specimen suitable for organ bath experiments, we have used porcine trigone as a well-established animal model of α_1-adrenoceptor-mediated contraction of urological tissue.

Figure 3. Effect of increasing concentrations of propiverine on concentration-response curves for phenylephrine in adult porcine detrusor strips from the trigone area. Time-matched controls (TMC) without any test compound added are depicted for comparison. Values are normalized to the maximum force increase in response to phenylephrine during the control concentration-response curve (=100%). Means ± SEM from 5-8 experiments.

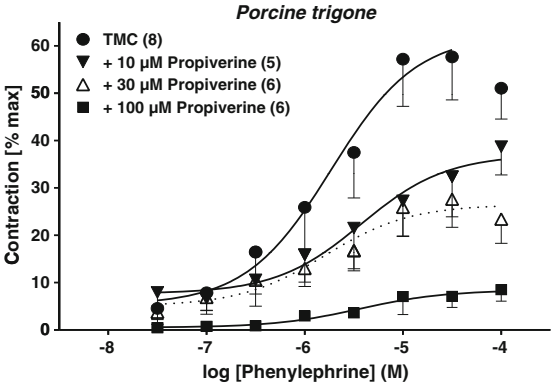


Table 2. –logEC_{50} [M], negative logarithm of phenylephrine for the half maximum effect during the 1st and 2nd CRC and E_{max} [%], maximum contraction during the 2nd CRC expressed in percent of the maximum effect during the 1st CRC (=100%); * $p < 0.05$ ** $p < 0.01$ (compared to time control).

	n	-logEC_{50} (1st CRC)	-logEC_{50} (2nd CRC)	E_{max} (2nd CRC)
time control	8	5.96±0.18	5.82±0.19	58±9
Propiverine				
10 µM	5	6.22±0.29	5.46±0.38	39±6
30 µM	6	6.02±0.16	5.48±0.28	28±6*
100 µM	6	5.82±0.10	5.90±0.22	9±2**

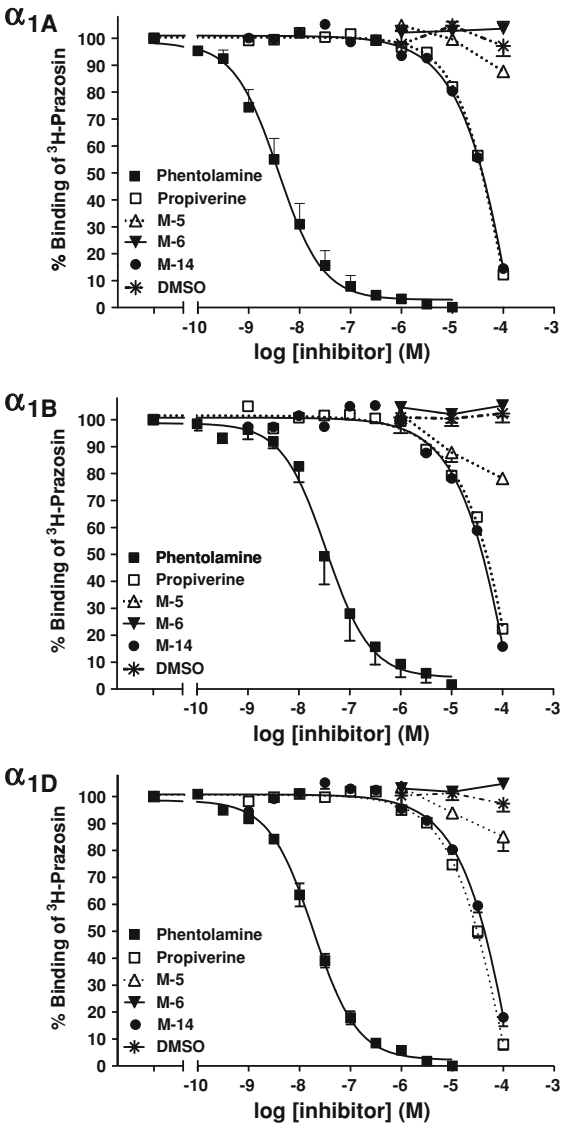
Table 3. -logK_i [M] values of propiverine and its metabolites at the three human α_1-adrenoceptor subtypes. Phentolamine was used as reference antagonist.

Compound	n	α_{1A}	α_{1B}	α_{1D}
Phentolamine	3	8.62±0.19	7.96±0.22	7.87±0.04
Propiverine	3	4.72±0.01	4.94±0.02	4.73±0.02
M-5	3	<4.00	<4.00	<4.00
M-6	3	<4.00	<4.00	<4.00
M-14	3	4.72±0.04	5.02±0.11	4.57±0.06

Our data demonstrate concentration-dependent relaxation of phenylephrine-induced tone of human prostate and porcine trigone. At least in trigone, similar relaxation was observed with the propiverine metabolite M-14 but not with M-5 or M-6. As phenylephrine-induced contraction in these two preparations is α_1-adrenoceptor-mediated [14], this relaxation provided initial evidence for α_1-adrenoceptor antagonism by propiverine and M-14. However, L-type Ca^{2+}-channels contribute to prostate and bladder contraction [12;13]. The direct interaction of propiverine and its metabolites with α_1-adrenoceptors was demonstrated in competition binding studies with cloned human α_1-adrenoceptor subtypes, yielding affinities in line with their potency to relax human prostate and porcine trigone. To verify that the inhibition of radioligand binding was indeed associated with receptor antagonism, concentration-dependent inhibition of phenylephrine-induced $[Ca^{2+}]_i$ elevation was demonstrated. In contrast to tissue relaxation experiments, inhibition of $[Ca^{2+}]_i$ elevation in CHO cells cannot be explained by Ca^{2+}-channel blocking properties of propiverine, because CHO cells do not express such L-type Ca^{2+}-channels. Taken together these experiments demonstrate that propiverine and its metabolite M-14 bind to human α_1-adrenoceptors, act as antagonists and accordingly can relax α_1-adrenoceptor-mediated contraction of human prostate and porcine trigone.

Propiverine is a competitive antagonist with similar affinity for all muscarinic receptor subtypes [3;5;9]. However, the antagonism of $[Ca^{2+}]_i$ elevations in CHO cells and of contraction in porcine trigone was insurmountable. Various molecular mechanisms could explain insurmountable antagonism including irreversible receptor binding and allosteric receptor modulation. While a detailed exploration of this molecular mechanism was beyond the scope of the present study, it should be noted that propiverine lacks alkylating or similar properties expected for an irreversible antagonist. Thus, the molecular interaction of propiverine with α_1-adrenoceptors and muscarinic receptors occurs in a different way despite the drug being an antagonist for both receptor families. This differential interaction is also supported by the fact that the propiverine metabolites M-5 and M-6 lacked α_1-adrenoceptor binding and antagonism but inhibited muscarinic receptor function in detrusor smooth muscle [5;8;16;20].

Figure 4. Effects of propiverine and its metabolites M-5, M-6 and M-14 on [^{3}H]-prazosin binding in human α_{1A}-, α_{1B}- and α_{1D}-adrenoceptor expressing CHO cells. The α_1-adrenoceptor antagonist phentolamine was used for comparison. Data in the presence of increasing concentrations of test compound are expressed in percent binding of [^{3}H]-prazosin. For control experiments only DMSO was added as a vehicle. Means ± SEM from 3 experiments.



The affinity of propiverine for α_1-adrenoceptors in the present study differs from that for human M_3 receptors by 50-100 fold [3;5;10] but is very similar to its potency for inhibition of L-type Ca^{2+}-channels [10]. As muscarinic antagonists typically are dosed to yield high receptor occupancy rates [17], it can be expected that therapeutic doses of propiverine exhibit some degree of α_1-adrenoceptor antagonism and L-type Ca^{2+}-channel blockade. Based upon reported plasma concentrations of propiverine (parent compound alone) [21] and our affinity estimates from the radioligand binding studies, therapeutic propiverine concentrations can be expected to occupy up to 10% of α_1-adrenoceptors. While this may be quantitatively less pronounced than the blockade of muscarinic receptors, it may nevertheless contribute to the clinical profile of propiverine and specifically may be beneficial for the treatment of male LUTS. Moreover, the insurmountable antagonism at α_1- (but not muscarinic) receptors raises the possibility that even limited α_1-adrenoceptor occupancy will yield considerable inhibition over time. Interseting, a large recent observational studyreported that the clinical effects of propiverine against OAB symptoms were quantitatively similar in men when administered alone or as add-on to an existing α-blocker treatment [22]. While these findings do not prove α1-antagonism of propiverine in vivo, they are in line with this proposal. However, dedicated studies will be required to determine the clinical relevance of such effects. They should also take into account that propiverine and its various metabolites differ in their in vivo plasma concentrations [23]. For each of them the relative contribution of the three molecular targets may differ in the generation of bladder selectivity and the overall relaxing effect in the lower urinary tract [24]. Even if propiverine itself turns out to have too little α_1-antagonism in vivo, it is an exciting starting point for the future synthesis of balanced α_1/muscarinic receptor antagonists.

Conclusion

In contrast to other drugs used for the treatment of OAB, propiverine is not only a muscarinic receptor antagonist but also has L-type Ca^{2+}-channel blocking and α_1-adrenoceptor antagonist effects. While each of these effects may be beneficial in the treatment of voiding dysfunction, including male LUTS, the relative contribution of these mechanisms and of the propiverine metabolites to the overall therapeutic effects upon oral administration of propiverine remains to be determined.

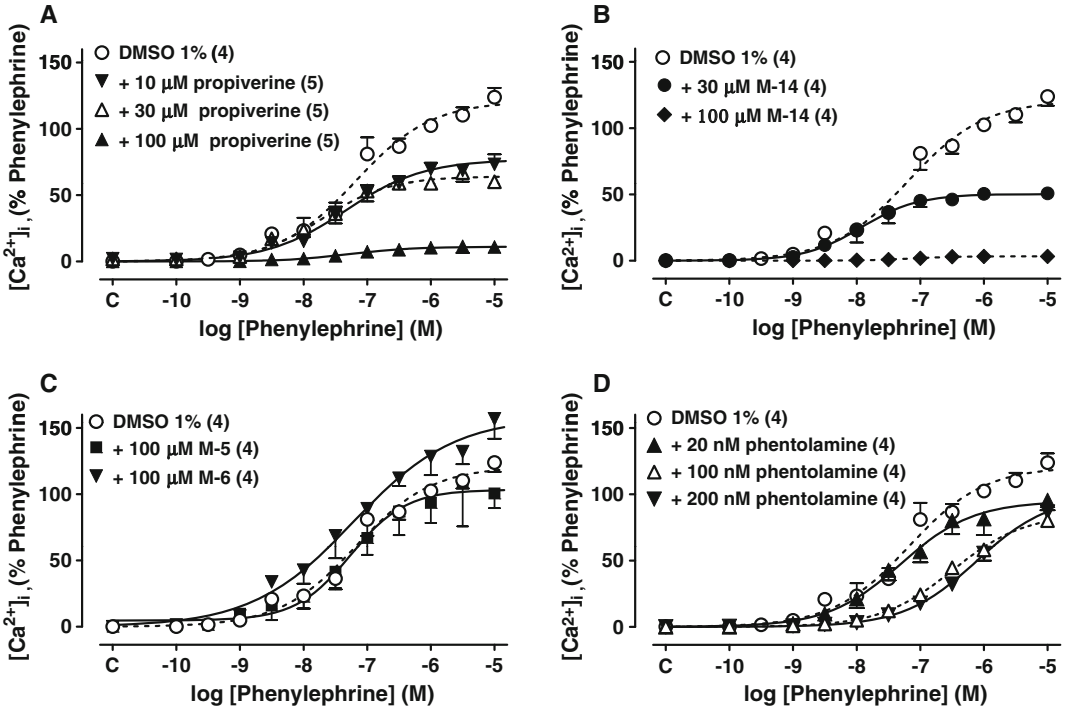


Figure 5. Phenylephrine-stimulated elevation of $[Ca^{2+}]_i$ in α_{1A}-adrenoceptor-expressing CHO cells.

A) Control concentration-response curves in the presence of DMSO, and responses to increasing concentrations of propiverine.

B) Responses to M-14.

C) responses to M-5 and M-6.

D) Shifts of concentration-response curves with the α1-adrenoceptor antagonist phentolamine. Data were normalized to $[Ca^{2+}]_i$ increase in the presence of 10 µM phenylephrine (= 100%). Means ± SEM from 4-5 experiments.

Acknowledgements

This study was funded in part by supported in part by the Bundesministerium für Wirtschaft, Germany (PRO INNO II, KA0558501UL7), Coordination Theme 1 (Health) of the European Community's FP7, Grant agreement number HEALTH-F2-2008-223234, and by APOGEPHA Arzneimittel GmbH, Dresden, Germany (M Wuest, S Propping, MP Wirth, U Ravens). MC Michel has received research support, lecturer and/or consultant honoraria in the anti-muscarinic field from Allergan, Astellas, Bayer, Boehringer Ingelheim, Pfizer, Schwarz Pharma, Theravance.

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PART THREE
MUSCARINIC ANTAGONISTS IN CLINICAL PRACTICE

Chapter 7

Does the Number of Previous Vaginal Deliveries Affect Overactive Bladder Symptoms and their Response to Treatment?

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Abstract

Purpose. To explore associations between the number of vaginal deliveries (primary aim) or gender (secondary aim) and overactive bladder (OAB) symptoms as well as their response to treatment with a muscarinic antagonist.

Methods. Pre-planned secondary analysis of an observational study of solifenacin in OAB patients. Episode frequencies of OAB symptoms, pad use and scores on OAB rating scales were documented in 4450 patients before and after a 12-14 week treatment with solifenacin 5 or 10 mg.

Results. Women without, 1, 2 or >2 vaginal deliveries and men were similar in their baseline characteristics. All groups also exhibited rather similar reductions in symptoms and improvements in rating scales upon treatment.

Conclusion. These data indicate that solifenacin, and perhaps other muscarinic receptor antagonists, are similarly suitable for the treatment of OAB symptoms in both genders and irrespective of previous vaginal deliveries.

Introduction

The two main types of incontinence are stress urinary incontinence (SUI) and urgency urinary incontinence (UUI), the latter mostly occurring in the context of the overactive bladder syndrome (OAB). They can exist in combination, but differ in pathophysiology and associated risk factors. While age is a major risk factor for UUI, female gender, obesity, obstructive airway disease and childbirth, particularly vaginal delivery, are risk factors for SUI [1]. The latter is attributed to anatomically damaging the bladder outflow tract and/or pelvic floor. Multiparity is also a risk factor for urinary incontinence in general [2], possibly in an ethnicity-dependent manner [3]. As these studies did not specify whether incontinence involved SUI, UUI or both, they raise the possibility that multiparity, and more specifically by vaginal delivery, may be a risk factor not only for SUI but also for UUI/OAB. Indeed, studies in Asian countries reported that multiparity [2] and childbirth through vaginal delivery [4] are risk factors for developing OAB. As vaginal delivery should not directly affect detrusor function, a link with UUI/OAB is not necessarily expected. On the other hand, prolapse surgery was reported to improve OAB symptoms [5], and the demonstration of reflex pathways between the urethra and the detrusor in both animals [6] and patients [7;8] could establish a mechanistic link between delivery-associated anatomical damage and detrusor dysfunction. On the other hand, males have a greater bladder outlet resistance based upon a longer urethra but do not suffer less frequently from OAB [9-11]. Therefore, it appears important to determine whether the reported association of multiparity/vaginal deliveries with OAB can be confirmed, specifically in non-Asian countries with a predominantly Caucasian population. If indeed vaginal deliveries predispose for OAB, it also becomes important to know whether this affects the responsiveness of OAB to treatment.

Therefore, we have performed a pre-planned analysis of the database of a previously published, large-scale observational study on OAB treatment [12] to explore associations between number of vaginal deliveries and OAB symptoms. Moreover, we have also explored a possible association between deliveries and the therapeutic response to antimuscarinic therapy. Finally, as a secondary aim, we explored whether OAB symptoms or their responsiveness to treatment differed between males and females without vaginal deliveries because very limited data comparing

genders are available from large OAB treatment studies.

Methods

Study Design. This manuscript describes a pre-planned secondary analysis of an open-label, observational study into the safety and efficacy of solifenacin in OAB patients [12]. That report also contains detailed information on study design, concomitant diseases and medications. Thus, no specific inclusion and exclusion criteria were applied other than a minimum age of 18 years and the recommendations from the Summary of Product Characteristics. Rather the participating 1316 office-based urologists were asked to systematically record their observation for patients receiving solifenacin based upon their medical judgment. Based upon the package insert, recommended initial solifenacin dose was 5 mg q.d. which could be increased to 10 mg q.d. The planned duration of treatment was 12 weeks. While ethnicity was not documented systematically, >95% of patients in Germany are Caucasians. Due its purely observational character, ethical committee approval or obtaining of informed consent was not recommended for this type of study in Germany at the time it was performed.

Patient Evaluation. Our analysis is based upon the visits before and at the end of treatment. In cases of premature discontinuation, the data from the last available visit were used in a last-observation-carried-forward manner. At the initial visit, a medical history including the number of previous vaginal deliveries was recorded. This was used to stratify the population for all further analyses. Other captured variables were episode frequencies of frequency, nocturia, urgency and incontinence [13], pad use, and various validated single-item OAB rating scales including the Indevus Urgency Severity Scale (IUSS) [14], the Urgency Perception Scale (UPS) [15;16], a visual analogue scale (VAS) [17] based upon the question “What is the severity of you urgency symptoms?”, two items from the King’s Health Questionnaire (“How would you describe your health at present?”(KHQ general) and “How much do you think your bladder problem affects you life?”(KHQ bladder) [18] and the quality of life question of the International Prostate Symptom Score (QoL) [19]. Finally, the

starting dose of solifenacin was recorded at the first visit, and alterations thereof were documented at the subsequent visits.

Statistical Analysis. Data handling was performed by Medidata (Konstanz, Germany). Note that in some cases reported percentages do not add up to 100% due to missing data. Baseline data are presented as means ± SD of absolute values of n patients. In line with previous similar analyses [20], treatment data are presented as % changes for OAB symptoms and pad use, and as absolute changes for the OAB rating scales. Descriptive statistical analysis was performed with the SAS program package (version 8.2) using women without vaginal deliveries were used as the reference group. Significance of differences was assessed by one-way analysis of variance with multiple comparison-corrected post-tests; a $p < 0.05$ was considered significant. As the initial descriptive analysis yielded rather similar values in all groups, no attempts were made for a more detailed multivariate analysis.

Results

Baseline Data. As compared to women without previous vaginal deliveries, males exhibited similar baseline characteristics with regard to voiding and other parameters (Table 1). Small but statistically significant differences were found with regard to age, nocturia and incontinence and pad use. Women without and with one, two or more than two vaginal deliveries also were similar in their baseline characteristics but greater numbers of deliveries were associated with slightly greater age, incontinence and pad use (Table 1).

Treatment Effects. In the overall group of patients, solifenacin treatment reduced the number of micturitions/24 h by 39%, the number of nocturia episodes/24 h by 59%, the number of urgency episodes/24 h by 63%, and the number of incontinence episodes/24 h by 73%; concomitantly, pad use/24 h decreased by 59%, and micturition volume increased by 67% (Figure 1). At the final visit the efficacy of solifenacin treatment was rated as very good, good, moderate and poor by 46.2%, 39.6%, 9.9% and 2.9% of patients, respectively. These results were obtained with 72% and 19% of patients receiving final doses of 5 and 10 mg q.d. solifenacin, respectively (other dose in 0.4%, no documented final dose for remaining patients). Final doses did not differ substantially between men and

Table 1. Baseline parameters in men and women, with women being divided into subgroups based upon their number of vaginal deliveries. Data are means ± SD of n patients. *: $p < 0.05$ vs. women without vaginal deliveries in a one-way analysis of variance followed by Dunnett's multiple comparison tests.

	Males	Females			
Deliveries		0	1	2	>2
N	713	673	694	1456	894
Age	66.3 ±11.5*	61.5 ± 16.0	61.8 ± 14.1	63.3 ± 12.3*	64.8 ± 11.6*
BMI	27.0 ± 3.3	26.6 ± 4.6	26.7 ± 4.2	26.9 ± 4.1	27.6 ± 3.9*
Micturitions	13.4 ± 5.1	13.1 ± 5.0	13.2 ± 5.0	13.3 ± 4.8	13.5 ± 4.7
Nocturia	4.3 ± 2.6*	3.6 ± 1.8	3.6 ± 1.9	3.7 ± 1.9	3.8 ± 1.7
Urgency	9.3 ± 5.7	9.1 ± 5.9	9.0 ± 6.4	9.2 ± 6.2	9.3 ± 5.9
Incontinence	4.1 ± 3.5	4.5 ± 3.2	4.7 ± 4.3	4.6 ± 3.2	5.0 ± 3.8*
Pad use	3.7 ± 2.8*	4.3 ± 3.1	4.3 ± 3.1	4.4 ± 3.6	4.9 ± 3.2*
IUS	2.2 ± 0.7*	2.3 ± 0.6	2.3 ± 0.6	2.3 ± 0.6	2.4 ± 0.6*
UPS	1.8 ± 0.6*	1.7 ± 0.6	1.7 ± 0.6	1.7 ± 0.6	1.6 ± 0.6*
VAS	69.5 ± 17.3	71.0 ± 17.8	70.0 ± 16.7	70.9 ± 16.2	73.5 ± 15.7*
KHQ general	2.2 ± 0.9*	2.0 ± 0.9	2.0 ± 0.9	2.1 ± 0.9	2.2 ± 0.9*
KHQ bladder	2.5 ± 0.6*	2.6 ± 0.6	2.6 ± 0.6	2.6 ± 0.6	2.6 ± 0.6
QoL	4.2 ± 1.4	4.2 ± 1.5	4.3 ± 1.3	4.1 ± 1.4	4.3 ± 1.5

Table 2. Treatment effects in men and women, with women being divided into subgroups based upon their number of vaginal deliveries. Data are means ± SD of n patients. *: $p < 0.05$ vs. women without vaginal deliveries in a one-way analysis of variance followed by Dunnett's multiple comparison tests.

	Males	Females			
deliveries		0	1	2	>2
n	713	673	694	1456	894
Final solifenacin dose (% 5/10 mg)	67.3 / 22.3 %	68.5 / 19.0 %	74.2 / 17.1%	76.0 / 17.0 %	72.0 / 21.7 %
Micturitions	-37.6 ± 19.0	-38.1± 17.2	-39.0 ± 16.2	-39.6 ± 16.3	-39.9 ± 16.5*
Nocturia	-56.3 ± 25.3	-56.7 ± 31.0	-61.1 ± 27.4*	-59.3 ± 27.0	-59.4 ± 27.3
Urgency	-60.9 ± 30.2	-63.6 ± 31.6	-63.9 ± 34.1	-63.2 ± 31.1	-63.3 ± 29.0
Incontinence	-72.8 ± 48.6	-72.0 ± 34.3	-75.0 ± 31.6	-73.6 ± 36.0	-73.2 ± 35.2
Pad use	-61.7 ± 34.9*	-55.3 ± 36.8	-58.2 ± 31.6	-59.7 ± 33.3*	-59.0 ± 30.7
IUS	-1.2 ± 0.8*	-1.3 ± 0.7	-1.3 ± 0.7	-1.3 ± 0.7	-1.4 ± 0.8*
UPS	0.8 ± 0.7*	0.9 ± 0.7	0.9 ± 0.7	1.0 ± 0.7*	1.0 ± 0.7*
VAS	-38.4 ± 20.1	-40.3 ± 21.6	-41.9 ± 21.4	-42.3 ± 20.9	-43.9 ± 21.4*
KHQ general	-1.0 ± 1.0*	-0.8 ± 0.9	-0.9 ± 0.9	-1.0 ± 1.0*	-1.1 ± 1.0*
KHQ bladder	-1.3 ± 0.9	-1.3 ± 0.9	-1.4 ± 0.9	-1.4 ± 0.8	-1.4 ± 0.9
QoL	-2.3 ± 1.6	-2.4 ± 1.7	-2.6 ± 1.6	-2.4 ± 1.6	-2.6 ± 1.7

the various groups of women (Table 2). While some small group differences in treatment responses yielded statistical significance with large patient numbers, the overall reductions in symptoms and improvements in rating scales were similar in women with and without deliveries and in males (Table 2).

Discussion

Critique of Methods. The present analysis is not a population-based epidemiological study but rather based upon patients seeking treatment of voiding dysfunction by a urologist and being diagnosed with OAB and considered for treatment with solifenacin [12]. This may have resulted in a somewhat selected population due to the type of treating physician (urologist) or the prescribed form of treatment (solifenacin). On the other hand, the involvement of board-certified specialists may yield more valid data, particularly with regard to the diagnosis of OAB, than symptom assessments based upon questionnaires or telephone interviews, which typically are the basis of epidemiological studies [9-11]. In this regard it should be noted that based upon their age it can be expected that at least some of the men in the study may have had their voiding symptoms mainly or at least partly due to benign prostatic hyperplasia. Therefore, it cannot be excluded with certainty that our male OAB group is somewhat contaminated with patients with voiding dysfunction due to factors other than OAB, but this caveat similarly applies to all previous studies comparing men and women with OAB.

Of note, the analysis of number of deliveries and gender was not retrospective but rather a pre-defined secondary aim of the overall study. We do not expect a major bias based upon the use of solifenacin, as all muscarinic receptor antagonists have similar clinical efficacy [21]. Moreover, a possible selection bias should similarly affect all subgroups of patients studied here and hence have limited impact on their comparison. Finally, based on previous studies on age-dependency, minor age differences between groups as seen in our study are not expected to have a major effects on baseline symptoms [9-11] or treatment responses [22]. Hence, we have not applied age-adjusted statistical comparisons. The potential effect of vaginal deliveries may relate to components including birth weight, mode of delivery or duration of the second stage. Based on age of most participants, delivery in most cases has been some 20-40 years ago, which makes it very

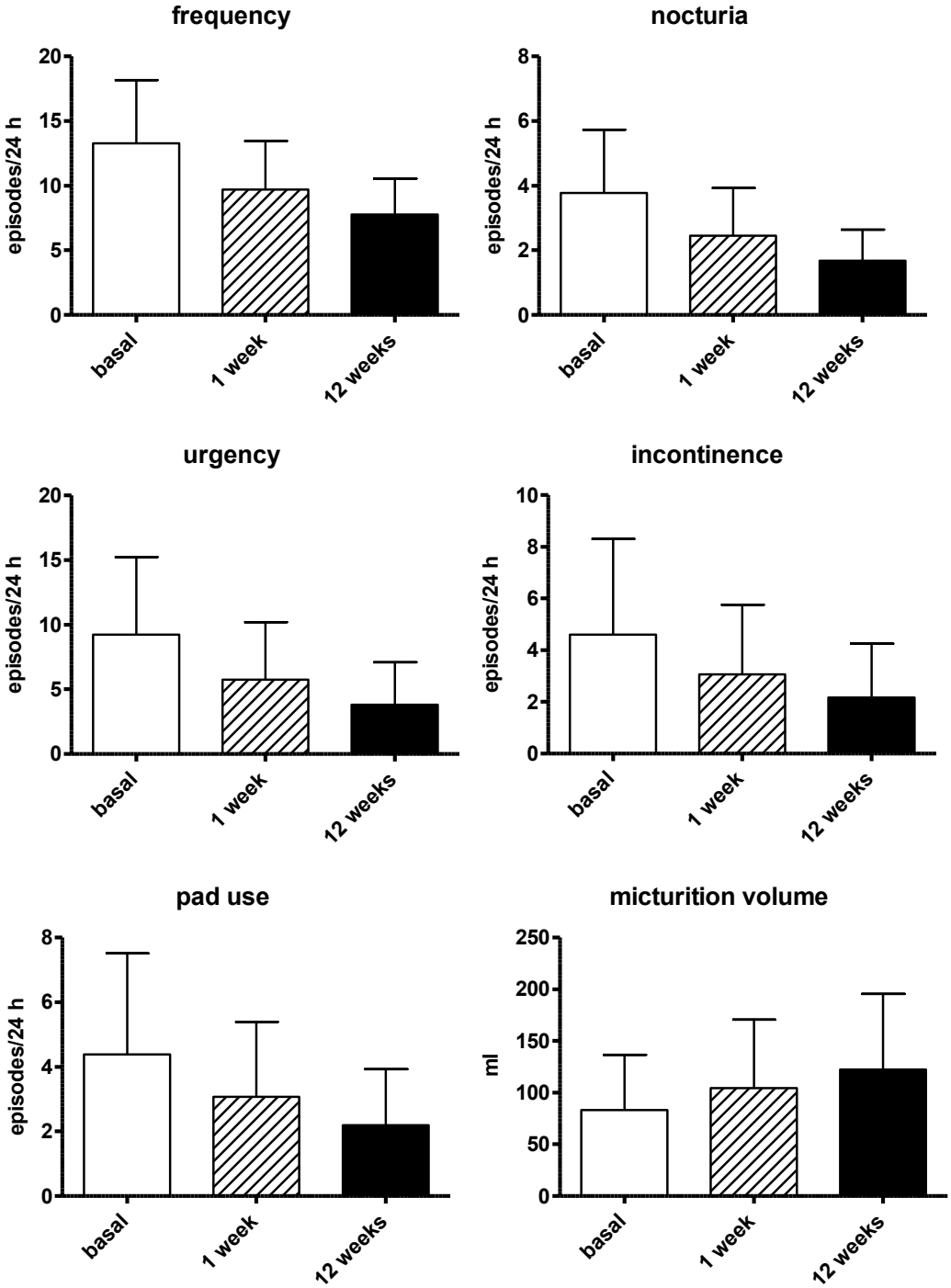

Figure 1. Treatment results upon 12 weeks of treatment with solifenacin. Data are for the entire study population. Data are means ± SD. All treatment effects are statistically significant vs. basal at the $p < 0.001$ level.

difficult or even impossible to obtain reliable information in this regard. In the absence of data on any of these factors, we assume that they will be randomly distributed across our large groups. The present data from an open-label, observational study do not allow conclusions about the absolute efficacy of solifenacin, but this has repeatedly been demonstrated in randomized, double-blind trials [23]. Of note, the use of the 10 mg solifenacin dose in this observational study was lower than in previously reported controlled trials [24]. On the other hand, the present data give an indication what can realistically be expected in a treatment situation in daily practice. Moreover, open-label data are suitable for the relative comparison among patient groups, and the large numbers of patients that typically are documented in post-marketing surveillance should increase the robustness of conclusions. While these limitations should be kept in mind in the interpretation of our data, we feel that the present data set is suitable for our research questions.

Baseline Data. The prevalence of OAB is roughly similar among men and women [9-11]. Accordingly, our data show a similar degree of symptoms and rating scale scores in both genders. We do not consider minor differences for some parameters to be of clinical relevance and feel that they can most likely be attributed to the concomitant presence of enlarged prostates in many elderly men [25].

Studies from Asian countries have reported an increased likelihood of OAB after vaginal delivery and/or multiparity [2;4]. Our study from Germany with its predominantly Caucasian population did not find a significant association between the number of vaginal deliveries and the number of micturitions, urgency or nocturia episodes but a slightly but significantly higher number of incontinence episodes, pad use and some rating scale scores in women with more than two vaginal deliveries. These studies differ in multiple ways: Firstly, our data are based upon patients seeking healthcare for their voiding symptoms. However, the two previous studies yielded similar conclusions despite one being based upon patients [4] and one being population-based [2]. Therefore, this is unlikely to explain differences. Secondly, the two previous studies have been performed in Asian countries whereas the present study was performed in a country with a predominantly Caucasian

population. The impact of this ethnic difference is difficult to appreciate based, but ethnic differences with regard to incontinence have been reported [3]. Thirdly, the previous studies have assessed the presence of OAB in a categorical manner [2;4] as compared to the quantification of OAB symptoms (present study). While it cannot be excluded that the number of deliveries has effects on the presence of OAB but not on its extent, we do not consider this to be likely. Fourthly, our study has only captured vaginal deliveries and not total parity. However, the only study comparing vaginal deliveries to Caesarean sections with regard to OAB has reported that the form of delivery does not significantly affect the association with OAB [2]. Finally, it needs to be considered that a relevant fraction of women with OAB concomitantly suffer from a SUI component [26;27], and this also applies to women with OAB receiving muscarinic receptor antagonist treatment [28]. Therefore, the possibility exists that an association between parity and OAB may be affected by a contamination with concomitant SUI. Arguments in favor of this possibility include the present finding that more than two vaginal deliveries were associated with more incontinence and pad use but not with more frequency, urgency and nocturia. Moreover, one of the Asian studies reported that multiparity was associated with a greater risk for OAB wet but not for OAB dry [2], whereas the other Asian study did not make this distinction and did not specifically exclude SUI [4]. Taken together the present data demonstrate that the number of vaginal deliveries has no major impact on the extent of OAB symptoms in a country with a predominantly Caucasian population. Associations of multiparity with more incontinence episodes or an increased risk for OAB wet most likely involve contamination with concomitant SUI.

Treatment Effects. Gender is not associated with major differences in expression or function of bladder muscarinic receptors in animals or humans [29]. Accordingly, post-hoc analyses of placebo-controlled studies with several muscarinic receptor antagonists [23;30] as well as large post-marketing studies [22] have consistently reported that such drugs are similarly effective in the treatment of male and female OAB. While the present study found some minor differences yielding statistical significance with large patient numbers, the overall picture

confirms with solifenacin that both genders respond similarly to treatment with a muscarinic receptor antagonist.
While the number of vaginal deliveries was associated with some small differences in treatment responses for some parameters, the overall data demonstrate a rather similar treatment response in all groups, i.e. the observed numerical differences were too small to be considered clinically relevant. As discussed above, the various patient groups may be contaminated to a different degree by concomitant stress incontinence. However, previous work with other muscarinic receptor antagonists has found that concomitant mild to moderate stress incontinence does not alter the efficacy of a muscarinic receptor antagonist in the treatment of OAB [28].

Conclusion

The present study did not find major differences in baseline symptom intensity among females and males or among females with and without previous vaginal deliveries. Moreover, despite some small differences reaching statistical significance with large patient numbers, all groups exhibited rather similar treatment responses to solifenacin. These data indicate that solifenacin, and perhaps other muscarinic receptor antagonists, are similarly suitable for the treatment of OAB symptoms in both genders and irrespective of previous vaginal deliveries.

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Chapter 8 Muscarinic Receptor Antagonists for Overactive Bladder Treatment: Does One Fit All?

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Abstract

Purpose. To review evidence and regulatory dosing recommendations for muscarinic receptor antagonists used in the treatment of overactive bladder symptom complex (darifenacin, fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine, trospium) in special patient populations.

Recent Findings. Growing evidence demonstrates effects of renal impairment, hepatic impairment, genetics and/or comedications on the pharmacokinetics of muscarinic antagonists. They may cause greater exposure in the respective population, which may translate into greater risks for side effects. These possible risks lead to drug-specific regulatory dosing recommendations or even contraindications in certain patient populations.

Conclusion. Physicians should be aware of pharmacokinetic alterations in special patient populations and possible associated risks. The evidence-based choice of a muscarinic antagonist in such patients should be guided by its specific pharmacokinetic profile.

Introduction

The overactive bladder symptom complex (OAB) is a prevalent condition in the general population, and muscarinic receptor antagonists are the primary form of its medical treatment. Their efficacy and safety have been evaluated in numerous large, randomized, double-blind controlled studies [1]. Increasingly, they are also being used in the treatment of male voiding dysfunction classically attributed to prostatic enlargement [2]. Across larger patient samples, all currently available muscarinic receptor antagonists appear to have comparable efficacy, but show some quantitative differences in tolerability. However, the randomized studies are typically performed in selected populations, and specifically patients at elevated risk for side effects are mostly under-represented as compared to the general population likely to receive such drugs. Side effect risks may be increased in some patient populations due to altered pharmacokinetic behavior of a given drug, e.g. in those with impaired renal or liver function or those with comedications. Moreover, genetic heterogeneity in drug metabolizing enzymes, namely cytochrome P450 (CYP) 2D6 [3], may additionally contribute to altered drug exposure in some patients. We estimate that about one third of all OAB patients have at least one of those risk factors. Against this background, we will review pharmacokinetic factors potentially increasing the risk for side effects and describe how this differentially affects specific drugs. We will focus on the roles of impaired renal or liver function, genetic differences in drug-metabolizing enzymes and comedications with relevance to pharmacokinetic drug-drug-interactions (DDI). A summary of all recommendations is given in table 1.

General Considerations

Most side effects of muscarinic antagonists are linked to their mechanism of action. Therefore, their incidence and/or severity are typically related to the exposure of the patient to the drug. The exposure can be assessed as peak concentrations (C_{max}) in plasma or serum, as total exposure typically measured as area under the curve (AUC) over the time course of drug action, and by duration of exposure typically assessed as terminal half-life ($t_{1/2}$). These pharmacokinetic parameters are routinely measured in special patient populations, but it often remains unknown which degree of

change of these parameters is clinically meaningful.
Apart from absorption and distribution, which typically do not vary much between patient populations, metabolism and excretion of a drug contribute strongly to the variability of overall exposure after ingestion of a given dose. The metabolism of OAB drugs occurs largely in the gut wall and the liver (fesoterodine possibly being an exception). This can generate inactive metabolites or metabolites with variable degrees of activity [4]. Therefore, metabolites may contribute to side effects in some but not in other cases. Ultimately, drugs need to be excreted, and the kidneys are a major route of elimination. This can occur in active (unchanged substance or active metabolites) or in inactive form. Renally excreted active forms may contribute to drug effects on the bladder from the luminal side [5]. The main routes of metabolism and excretion are summarized in table 2.

Methods

Our analysis includes the oral formulations of darifenacin, fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine and trospium because each of them is frequently used in at least some major countries. Fesoterodine and tolterodine will be discussed together as they share the common active metabolite 5-hydroxymethyl-tolterodine (5-HMT), previously also known as DD01 [6]. In order to avoid potential bias, our recommendations are primarily based on the Summary of Product Characteristics and/or package insert as approved by the regulatory authorities in the EU (assessed as the English version available for the UK) and US, respectively. In cases of incongruence, the more conservative view was adopted in the interest of patient safety. Apparently, the authorities have become stricter with their safety recommendations over time, i.e. a more recently registered drug may carry more serious warning labels than an older drug such as oxybutynin for a given safety profile. Moreover, the more recently launched drugs typically have been much better characterized with regard to potential pharmacokinetic alterations in special patient populations. Where recommendations have not been made by the authorities but appear prudent based upon available data, we add our personal interpretations and mark them as such.

Impaired Renal Function

The kidney excretes hydrophilic drugs or their metabolites, and impairments of renal function can cause elevated plasma concentrations of compounds with relevant renal excretion. Clinically this becomes relevant if the kidneys contribute in a major way to the excretion of parent drug or active metabolites. In this regard it should be considered that by average females have a smaller glomerular filtration rate (GFR) than males and that renal function typically declines with age in both genders. Therefore, in most cases no specific dosing recommendations are given for genders or age groups apart from those related to differences in renal function. Renal impairment can also indirectly affect pharmacokinetics by reducing plasma albumin concentrations and hence drug distribution and availability for excretion.

Darifenacin is mainly cleared by metabolism by CYP 2D6 and 3A4, and renal clearance is negligible [7]. A study in subjects with varying degrees of renal function (GFR ranging from >80 to <30 mL/min) confirmed the absence of clinically relevant effects on the pharmacokinetics of darifenacin [7]. Accordingly, the European [8] or US [9] label does not recommend dose adjustment in patients with impaired renal function. Nevertheless, caution should be exercised when treating this population [9].

Both fesoterodine and tolterodine are converted to the active metabolite 5-HMT which in turn is cleared partly by the kidneys and partly by the liver [6]. Whereas generation of 5-HMT from fesoterodine occurs by non-specific esterases, that from tolterodine is via CYP 2D6. A study in fesoterodine-treated subjects comparing patients with varying degrees of renal function (GFR >80 to <30 mL/min) reported 5-HMT peak plasma concentrations in the mild, moderate and severe renal impairment groups of 1.35, 1.48 and 2.03-fold greater, respectively, than those of healthy subjects; similar findings were obtained for the AUC values [6]. The standard dose of 4 mg/day of fesoterodine can be used, and can be titrated, with caution, to 8 mg/day in subjects with mild and moderate renal impairment; in patients with severe renal impairment a maximum dosage of 4 mg/day is advised [10]. Following tolterodine IR administration to subjects with a GFR of 10-30 mL/min, tolterodine and 5-HMT levels were 2-3 fold higher than in subjects without renal impairment [11]. The recommended

tolterodine dose in patients with significantly reduced renal function is 2 mg/day [12].

Oxybutynin is primarily cleared by metabolism by CYP 3A4, and oxybutynin or oxybutynin ER[1] studies in patients with renal impairment have not been reported to the best of our knowledge. The regulatory authorities recommend caution when using oxybutynin in patients with renal impairment [13].
Propiverine is primarily cleared by metabolism by CYP 3A4 and flavin monoxygenases, and serum levels of the parent compound and its main weakly active metabolite propiverine-N-oxide are not significantly altered by severe renal impairment. Accordingly, no dose adjustment is recommended as long as the total dose does not exceed 30 mg/day [14;15].

Solifenacin is partly cleared renally and has the longest half-life of all muscarinic antagonists used in OAB treatment. In subjects with mild, moderate and severe renal impairment the AUC increased by 44%, 28%, and 115%, respectively, as compared to healthy subjects [16]. Solifenacin should be used with caution in patients with reduced renal function [17]. Doses greater than the standard dose of 5 mg/day are not recommended in patients with a GFR <30 mL/min [17;18].

Trospium is a quarternary amine and thus has the highest hydrophilicity among all muscarinic antagonists for OAB treatment. It does not undergo major metabolism but rather is largely (approximately 60% of bioavailable drug) cleared by the kidneys in active form [4]. The renal elimination of trospium may lead to its presence in the bladder in sufficient concentrations to act from the luminal side, which may contribute to its pharmacological activity in vivo [5]. The trospium AUC and C_{max} were increased 4.5- and 2-fold, respectively, in patients with severe renal impairment, and an additional elimination phase with a long half-life (≈33 h) appeared [19]. Therefore, in patients with severe renal insufficiency trospium should be administered as 20 mg/day at

[1] Modified release drug formulations are identified in the general literature by multiple abbreviations with overlapping meanings. Within this manuscript, we refer to all of them as ER, although technically the release phase may not always be extended.

bedtime, with a possible additional extension of dosing interval to once per 2 days [19;20].

Impaired Hepatic Function

Many drugs are metabolized by the liver. While this causes inactivation in many cases, it may also generate active metabolites, and in some cases (although not for muscarinic receptor antagonists discussed in this paper) even turn inactive prodrugs into pharmacologically active compounds. In this context it is noteworthy that active drug metabolites may differ from the parent compound not only in potency but also in their qualitative profile, e.g. by altering the subtype selectivity or by acting on targets other than muscarinic receptors as observed with propiverine [4]. Therefore, alterations of hepatic function may affect drug action both quantitatively and, in some cases, qualitatively, but the latter has not specifically been studied clinically. For clinical purposes liver impairment is stratified as mild, moderate and severe impairment based upon the Child-Pugh classification (levels A, B and C, respectively). Liver impairment can also secondarily affect pharmacokinetics, e.g. by changing plasma albumin concentrations and hence drug distribution.

Darifenacin metabolism is primarily achieved by CYP 2D6 and 3A4 yielding extensive pre-systemic (first-pass) extraction. While mild hepatic impairment did not significantly affect the AUC or C_{max} of darifenacin 15 mg, steady state unbound darifenacin exposure after adjustment for plasma protein binding was estimated to be 300-370% higher with moderate hepatic impairment than in normal subjects [7]. Despite a risk of increased exposure, darifenacin requires no dose adjustment for subjects with mild hepatic impairment [8]. In patients with moderate hepatic impairment, the darifenacin dose should not exceed 7.5 mg/day [9]. Patients with severe hepatic impairment have not been studied, therefore darifenacin is not recommended for use in these patients due to lack of data [9].

Upon fesoterodine ingestion, 5-HMT C_{max} and AUC values were approximately twice those of healthy controls in patients with moderate impairment of liver function, whereas the terminal half-life was not significantly affected [6]. These data indicate that the starting dose of 4 mg/day can also be used in patients with mild

and moderate hepatic impairment and can be titrated, with caution, to 8 mg/day only in subjects with mild hepatic impairment [10]. Fesoterodine use is not recommended in patients with severe impairments of liver function due to lack of data. After administration of tolterodine, 5-HMT plasma concentrations are approximately 2-fold higher in patients with liver cirrhosis (Child-Pugh not specified) than those without [21]. The dosage of tolterodine and tolterodine ER should be reduced to 2 mg/day recommend for patients with significantly impaired liver function [12].

Oxybutynin is metabolized in the liver by CYP 3A4 to the pharmacologically active R- and S-enantiomers of N-desethyloxybutynin (DEOB). Oxybutynin is available in oral IR and ER as well as in a transdermal formulation, which differ in the ratio of plasma oxybutynin/DEOB [4]. While no major differences in efficacy exist between the formulations, a lower oxybutynin/DEOB ratio may lead to more adverse events (dry mouth) [4]. While studies on oxybutynin pharmacokinetics in patients with liver impairment have not been reported to our knowledge, most likely patients with hepatic impairment metabolize oxybutynin at a lower rate which could increase plasma concentration and/or elimination half-life of oxybutynin and also increase the oxybutynin/DEOB ratio. Caution is advised when using oxybutynin or oxybutynin ER in patients with hepatic insufficiency [13].

Propiverine and its main metabolite propiverine-N-oxide exhibited largely unchanged steady state pharmacokinetics (C_{max}, AUC and $t_{1/2}$) in patients with mild to moderate hepatic impairment [22]. No advice on dose adjustment is given in the package inserts for mild to moderate hepatic impairment. For patients with severe hepatic impairment no data are available [14;15].

Solifenacin did not exhibit a significantly altered C_{max} but a 60% higher AUC and a doubled half-life upon single dose (10 mg) administration in patients with moderate hepatic impairment [23]. Solifenacin should be used with caution in patients with reduced hepatic function [17]. Doses of solifenacin greater than 5 mg/day are not recommended in patients with moderate hepatic impairment [18]. Solifenacin is not recommended for patients with severe hepatic impairment [17].

Trospium C_{max} increased 12% and 63% in patients with mild and moderate hepatic impairment, respectively, compared to healthy subjects, whereas mean AUC was similar [19;24]. Trospium renal clearance is prolonged by 7% in patients with mild hepatic impairment and by 51% in patients with moderate/severe hepatic impairment [24]. Caution should be used but no dose adjustment is advised when administering trospium to patients with hepatic impairment [19;20].

Genetic Differences in Drug-metabolizing Enzymes

Members of the CYP family are important drug-metabolizing enzymes, and CYP 3A4 and CYP 2D6 are of specific relevance for the metabolism of muscarinic receptor antagonists. The activity of these enzymes varies between individuals, with both genetic and environmental factors (including comedications) contributing. The activity of CYP 2D6 is strongly influenced by genetics. The extensive metabolizer (EM) phenotype represents about 80% of Caucasian populations [3]; subjects with an intermediate metabolizer or poor metabolizer (PM, up to 7% of Caucasians) phenotype can generate much less if any metabolite. CYP 2D6 status has effects on the pharmacokinetics of some muscarinic antagonists which are of similar magnitude as those of liver impairment, but this is not reflected in the regulatory dosing recommendations for any of these drugs.

Darifenacin peak and total exposure is higher in PM than in EM subjects as thoroughly investigated in phase I studies and phase II and III population pharmacokinetic analyses [7]. Genotype was found to be a statistically significant covariate influencing darifenacin bioavailability (all analyses) and clearance (phase I only). Within the phase I analysis model clearance was 20% lower and bioavailability was 52% higher in PM subjects compared with EM subjects, who correspond to a longer terminal $t_{1/2}$ (3.8 hours in PM vs 3.1 hours in EM) and 92% higher in total exposure in PM subjects. Similarly, in the analysis of phase I data, relative bioavailability was 120% higher in PM than EM subjects, and within the models used for analyses of phase III data relative bioavailability was 47-66% higher in PM than EM subjects [7]. It appeared that the contribution of CYP 2D6 to darifenacin metabolism at steady state was inversely correlated to the dose and it is highest at low doses. One consequence is that the difference in

Table 1. Dosing recommendations in Europe and the US as based upon regulatory authority-approved summary of product characteristics/package insert. If recommendations differed between countries, the more conservative recommendation is listed. IR, immediate release; ER, extended release; max, maximally. All dosages refer to total daily doses. Unless otherwise noted, the recommendations relate to all available formulations of a given drug.

Drug (recommended daily dose)	Renal impairment	Hepatic impairment	Comedications
Darifenacin (7.5/15 mg)	caution	Child Pugh A: none, but risk of increased exposure Child Pugh B: max 7.5 mg, if benefit outweighs risk Child Pugh C: not recommended	CYP2D6 inhibitors: starting dose 7.5 mg, titrate to 15 mg if well tolerated Moderate CYP3A4 inhibitors: starting dose 7.5 mg Potent CYP3A4 inhibitors: do not use
Fesoterodine (4/8 mg)	Mild/moderate: start with 4 mg, increase dose cautiously to 8 mg Severe: 4 mg	Child Pugh A: start with 4 mg, increase to 8 mg with caution Child Pugh B: max 4 mg Child Pugh C: not recommended	CYP2D6 inhibitors: start with 4 mg Potent CYP3A4 inhibitors: max 4 mg
Oxybutynin (5-30 mg)	Caution	Caution	Potent and moderate CYP 3A4 inhibitors: caution
Propiverine (5/15 IR/30 mg ER)	Max dose 30 mg	Child Pugh A and B: no advice on dose adjustments Child Pugh C: not studied	Potent CYP 3A4 inhibitors: no studies available but in vitro data point to possible interactions
Solifenacin (5/10 mg)	Mild/moderate: no adjustment Severe: max 5 mg	Child Pugh A: caution Child Pugh B: max. 5 mg Child Pugh C: not recommended	Potent CYP 3A4 inhibitors: max 5 mg
Tolterodine (1/2/4 mg)	Mild/moderate: no data Severe: max 2x1 mg IR or 2 mg ER	max 2x1 mg IR or 2 mg ER	CYP2D6 inhibitor: none potent CYP3A4 inhibitors: max 2x1 mg IR or 2 mg ER
Trospium (40 mg)	Mild/moderate: caution Severe: max 20 mg	Child Pugh A, B and C: caution	-

darifenacin exposure between EM and PM subjects at steady state would be greater at lower doses [7].

Fesoterodine metabolism to 5-HMT does not involve CYP enzymes, whereas generation of 5-HMT from tolterodine is mediated by CYP 2D6. Moreover, CYP 2D6 and CYP 3A4 are involved in the inactivation of 5-HMT [6]. While tolterodine-treated subjects with the EM phenotype exhibit roughly similar serum concentrations of tolterodine and 5-HMT, PM subjects have considerably higher tolterodine and low or even undetectable 5-HMT plasma concentrations. Accordingly the mean C_{max} value and AUC of tolterodine can be 5 and 10 times higher, respectively, in subjects with the PM phenotype as compared to the EM phenotype, and the elimination half-life of tolterodine is longer in PM than in EM [21]. Given the similarity of the pharmacological profile of tolterodine and 5-HMT, the clinical relevance of these differences remains unclear. However, one recent study reported that tolterodine treatment interferes with sleeping patterns in PM but not EM, possibly related to the greater brain penetration of tolterodine as compared to 5-HMT [25]. In contrast, average plasma concentrations of 5-HMT following oral administration of fesoterodine differ by less than a factor of 2 in subjects with the PM and EM subjects [6].

Oxybutynin is metabolized in the liver by CYP 3A4, but not CYP 2D6 [4]. Propiverine pharmacokinetics and adverse event profiles were similar in EM and PM subjects [26]. Solifenacin is metabolized by CYP 3A4 into one active metabolite, 4R-hydroxy solifenacin, and 3 inactive metabolites, N-glucuronide, N-oxide and 4R-hydroxy-N-oxide, whereas CYP 2D6 does not contribute in a major way [27]. Trospium undergoes negligible metabolism by the CYP system [24], no metabolic interactions are expected [20]. Accordingly, CYP 2D6 EM/PM status appears to be of little relevance for the treatment with any of these four drugs.

Concomitant Medication/ Drug-drug-interactions

Pharmacokinetic DDI can occur at multiple levels. While clinically relevant interactions at the level of plasma protein binding are rare, those at the level of drug metabolizing enzymes are clinically most relevant. Particularly, comedications affecting the activity of CYP 2D6 or 3A4 can give rise to important DDI. Clinically relevant

Table 2. Half lives and routes of metabolism and excretion for muscarinic receptor antagonists. Statements largely based upon regulatory authority-approved summary of product characteristics/ package insert. For additional information see main text. Half lives are given for subjects with normal renal and liver function. Renal excretion percentages refer to those of active drug (including active metabolites). *: Based upon the low bioavailability, this represents about 60% of the systematically available drug.

Drug	Half life (h)	Role of renal excretion	Role metabolism	Comment
Darifenacin	13-19	3% as active drug	Involving CYP 2D6 and 3A4; metabolites considered not to contribute to clinical effects	-
Fesoterodine	7	16% as active drug (5-HMT)	5-HMT metabolized by CYP 2D6 and 3A4 yielding inactive secondary metabolites	Fesoterodine acts completely via its active metabolite 5-HMT
Oxybutynin	2-3	<1% as active drug (oxybutynin plus DEOB)	CYP 3A4, DEOB is active metabolite	Oxybutynin and DEOB are similarly active muscarinic antagonists but DEOB may have less favorable ratio of effects on bladder vs. salivary glands
Propiverine	14-22	<1% as unchanged compound	CYP 3A4 and flavin monoxygenases; several active metabolites	--
Solifenacin	45-68	<15% as unchanged compound	CYP 3A4; metabolites inactive or in low concentrations	-
Tolterodine	Tolterodine 2-3, 5-HMT 3-4	<1% as tolterodine, and 5-14% as 5-HMT (<2.5% and <1% in PM)	CYP 2D6 generates active metabolite 5-HMT, for further metabolism of 5-HMT see fesoterodine	Tolterodine and 5-HMT are similarly active muscarinic antagonists
Trospium	10-20	3.5% as unchanged compound*	Not fully defined, CYP apparently without major role	Oral bioavailability <10% and highly variable (4.0-16.1%), major of non-absorbed excretion in feces

CYP 2D6 inhibitors include bupropion, fluoxetine, paroxetine, terbinafine, quinidine and cimetidine (the latter also being a CYP 3A4 inhibitor). Subjects with a genetically low activity of CYP 2D6 are particularly at risk for DDI with corresponding inhibitors. Potent CYP3A4 inhibitors include ritonavir, ketoconazole, itraconazole, verapamil and cyclosporine, whereas moderate CYP3A4 inhibitors include erythromycin, clarithromycin, fluconazole and grapefruit juice.

Darifenacin in vitro studies have indicated that inhibition of CYP 2D6 has a smaller effect on metabolism than inhibition of CYP 3A4. Accordingly, coadministration of paroxetine 20 mg increased the AUC and C_{max} of darifenacin by only 33-36% and turns an EM into an apparent PM phenotype [7]. Coadministration of the mixed CYP inhibitor cimetidine (800 mg) increased the AUC and C_{max} of darifenacin with 34% and 42%, respectively [7]. On the other hand, a 6-day coadministration of ketoconazole increased the AUC and C_{max} of darifenacin 10.6 and 9.5-fold, respectively [7]. For concomitant use of potent CYP 2D6 or moderate 3A4 inhibitors, treatment should start with the 7.5 mg/day dose [8]. The darifenacin dosage may be titrated to 15 mg/day in such patients to obtain an improved clinical response provided the lower dose is well tolerated [8]. Darifenacin should not be used together with potent CYP3A4 inhibitors [8].

Fesoterodine yields 5-HMT C_{max} values upon coadministration with ketoconazole (200 mg) which are 2.2 and 1.5 times higher than in its absence in EM and PM, respectively; the 5-HMT AUC is similarly affected [6], reflecting the role of CYP 3A4 in the metabolism of 5-HMT. While tolterodine is primarily metabolized by CYP 2D6, CYP 3A4 may become important in PM subjects. In such subjects the terminal half-life of tolterodine increases from 10 to 15 h and the AUC doubles upon coadministration of 200 mg ketoconazole [28]. Comedication with fluoxetine increases tolterodine and slightly decreases in 5-HMT concentrations, whereas the serum concentration of unbound tolterodine plus 5-HMT increases only minimally [21]. Accordingly, the dose of fesoterodine should be restricted to 4 mg/day in patients concomitantly receiving potent CYP 3A4 inhibitors. No recommendation is advised for moderate CYP 3A4

inhibitors. However an increased exposure of the active metabolite of fesoterodine is expected, although a smaller increase than observed with a potent CYP 3A4 inhibitor. Co-administration of a potent CYP 2D6 inhibitor may result in increased exposure and adverse events. A dose limitation to 4 mg/day may be needed [10]. In contrast, the package inserts for tolterodine and tolterodine ER recommend the dose of 2 mg/day if potent CYP 3A4 inhibitors are coadministered [11;12;29;30]. Dose adjustments for coadministration of the CYP 2D6 inhibitor fluoxetine are not required for tolterodine [11].

Oxybutynin plasma concentrations are 3-4- fold higher upon coadministration with ketoconazole but the clinical relevance of this interaction is not known [13]. Coadministration of the CYP 3A4 inhibitor, itraconazol (200 mg/day), increased oxybutynin C_{max} (mean 89%) and AUC (mean 85%), but had no significant effect on oxybutynin t_{max} or terminal disposition half-life [31]. While the oxybutynin/DEOB ratio increased, there were no significant differences between the treatments in adverse event frequency or intensity. No dosing recommendations were given for the concomitant use of potent and moderate CYP 3A4 inhibitors. The clinical relevance is not known, but caution should be used when such drugs are coadministered [13].

Propiverine DDI based upon CYP 2D6 or 3A4 inhibitors have not been reported [31], but they are not unlikely as CYP 3A4 plays a role in the metabolism of propiverine [15]. Coadministration of ketoconazole (200 mg/day) increased the solifenacin C_{max} by 40%, doubled the AUC, and increased the $t_{1/2}$ by 56% [23]. Based on in vitro data, no clinically relevant interactions with the metabolism of trospium are expected. However, some drugs that are actively excreted may interact with trospium by competing for renal tubular secretion [19]. Accordingly, solifenacin dose is recommended not to exceed 5 mg/day upon coadministration of potent CYP 3A4 inhibitors [17;18], whereas no dose adjustments are required for propiverine or trospium.

Conclusion

Group averages from controlled clinical studies may not necessarily reflect the real life situation of special patient populations, which often have been excluded or at least under-represented in those studies. While each of these special populations may be small, their accumulated prevalence is considerable and merits medical attention to optimize the tolerability of muscarinic antagonists in the treatment of OAB. Patients concomitantly belonging to more than one special group, e.g. having low CYP 2D6 activity plus receiving a CYP inhibitor, may be particularly at risk with some drugs. The currently available muscarinic antagonists differ in their susceptibility to alterations of their pharmacokinetic behavior in various special groups. None of them is free of liabilities. Much more detailed information is available for newer than for older drugs such as oxybutynin, which makes their safety assessment more reliable.

Facing this situation, we advise two therapeutic strategies. If a specific complicating factor is known, a drug should be chosen which largely remains unaffected by this factor. For example, trospium may be the most vulnerable drug in patients with renal but the least vulnerable drug in patients with hepatic impairment. If no specific complicating factors are known but have not been excluded (a situation likely to occur frequently in daily practice), the presence of multiple inactivation/excretion mechanisms (renal plus CYP 2D6 plus CYP 3A4), e.g. with fesoterodine, may minimize overall tolerability risks, but this theoretical concept has not been tested in clinical studies. On the other hand, the reversible nature of most side effects of muscarinic antagonists does not necessitate to systematically screen for complicating factors by means other than a thorough patient history. Therefore, it is our key recommendations that prescribing physicians should be aware of risks for reduced tolerability due to pharmacokinetic factors and should specifically tailor medication to the situation of an individual patient.

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Chapter 9 Summary, Conclusions and Future Perspectives

Part One: Muscarinic Receptors in the Prostate

Muscarinic receptor antagonists are the main pharmacological treatment modality for the overactive bladder syndrome (OAB) in males and females. In recent years, the role of OAB in male voiding dysfunction has gained attention, leading to an increased usage of muscarinic antagonists in these patients. The increased clinical use demands a more precise understanding of how these drugs work to fully utilize their therapeutical potential.

In **chapter 2**, a literature review was conducted to identify studies on the cholinergic (muscarinic) innervation and the presence and function of muscarinic receptors in the human prostate.
In the current literature, dense cholinergic innervation is described within both stromal and epithelial compartments of the prostate. Interestingly, the human prostate expresses muscarinic receptors at densities exceeding those of α_1-adrenoceptors. The predominant muscarinic receptor in the prostate is the M_1 subtype and is mainly found on epithelial cells. Smaller amounts are found of the M_2 subtype, mainly on stromal cells. Both populations have been shown to be functional in signal transduction assays. However, in line with the sparse receptor density on stromal smooth muscle cells, contractile responses of the prostate are only small. Data from prostate cancer cell lines and from botulinum toxin injections into the benign prostate suggest that muscarinic receptors may promote prostatic growth. Animal data demonstrate that muscarinic receptors may be of primary importance in the genesis of prostatic secretions, but this finding needs to be confirmed in humans. Taken together, it appears that direct effects on the prostate need to be considered when using muscarinic receptor antagonists in men. They may primarily involve alterations of glandular secretion and prostatic growth.

The suggestion of direct effects of antimuscarinics on the prostate and sparse availability of studies describing regulation of muscarinic receptors in the prostate have encouraged us to further explore this matter. Some studies have investigated regulation of muscarinic receptors on the protein level in pathological conditions, but all in small numbers and without possibility to differentiate between subtypes.

In **chapter 3** we demonstrate that high quality mRNA can be extracted from the human prostate using transurethral resection of the prostate (TURP) without harm to specimens by thermal effects of the procedure. Real time-PCR showed presence of all five muscarinic receptor subtype transcripts, with M_1 and M_4 receptors as the most and least prevalent expressed subtypes, respectively. Of all subtypes, only M_1 receptor expression is weakly positively associated with prostate size. In contrast age, PSA level, pathological diagnosis or treatment with 5-ARI's or α- or β-blockers is not associated with alterations of mRNA expression for any of the five muscarinic receptor subtypes in the human prostate.
In conclusion, only regulation of M_1 muscarinic receptor subtype is weakly associated with prostate size. Further studies of the functional role of prostatic muscarinic receptor subtypes are needed, specifically with regard to M_1 receptors and prostate size.

Part Two: Muscarinic and Adrenergic Signalling

In the second part muscarinic and adrenergic signalling pathways in the bladder are explored using different models and techniques. M_3 muscarinic receptors and β-adrenoceptors have opposing actions on smooth muscle in the urinary bladder, namely contraction and relaxation, respectively. The role of the M_2 receptor is not fully clear. β-Adrenoceptor-mediated relaxation can be attenuated by muscarinic receptor agonist carbachol, but the precise contribution of muscarinic receptor subtypes is unknown.
In **chapter 4**, the role of signalling pathways of M_2 and M_3 receptors in the attenuation of β-adrenoceptor mediated relaxation is studied on the tissue level in isolated rat detrusor strips. We have introduced a novel antagonist with very high selectivity for M_2 over M_3 receptors, THRX-182,087. Using this compound as well as the M_3 sparing agonist BZI in the presence of carbachol enabled experimental conditions with a selective stimulation of only M_2 or M_3 receptors, respectively, which previously was mainly supported by genetic evidence.
Confirming previous findings, carbachol attenuated isoprenaline-induced detrusor relaxation. M_2 selective stimulation partly mimicked this attenuation, indicating that both M_2 and M_3 receptor subtypes are involved. During M_3-selective stimulation,

the attenuation of isoprenaline responses was reduced by the phospholipase C inhibitor U 73,122, but not by the protein kinase C inhibitor chelerythrine. The signal transduction pathway involved in the M_3 component of this attenuation differs from that mediating direct contractile effect of M_3 receptors.
Thus, muscarinic receptors cause direct contraction and inhibition of relaxation in the bladder, but the two responses involve different subtypes and, at least for M_3 receptors, different signalling pathways. This interaction may become clinically relevant under pathophysiological conditions when acetylcholine is being released in the bladder during the storage phase of the micturition cycle.

The previous chapter demonstrated that the interaction between muscarinic and β-adrenergic receptors on the tissue level is complex. To study interaction on the intracellular level, we developed a model of double-transfected CHO cells with human M_2/β_3 and M_3/β_3 receptors, which is described in **chapter 5**. Selected clones were subjected to saturation binding experiments and functional assays.
The prototypical second messenger for both the M_2 muscarinic and β_3-adrenergic receptors is cAMP, with cellular levels being lowered and raised by the former and the latter, respectively. cAMP is suspected to interact with other signaling pathways e.g. the prototypical M_3 muscarinic receptor second messenger Ca^{2+}. Although their precise role in bladder contraction and relaxation is under debate, muscarinic and β-adrenergic signaling opposes each other at multiple levels of their signaling cascade.
All clones expressed muscarinic receptors in physiological densities, but receptor expression levels of β_3-adrenoceptors were too small to allow reliable estimates of receptor density. Nevertheless, β_3-adrenoceptors appeared to be functionally active, measured by an increase of cAMP after stimulation, but this did not affect intracellular Ca^{2+} concentrations in our cells.
We have succeeded in generating cell lines with either M_2 or M_3 muscarinic receptors co-expressing β_3-adrenoceptors. However, the model failed to exhibit co-expression of β_3-adrenoceptors at levels that enable studying of functional interaction. Thus, other model systems may be required to explore the interaction between β_3-adrenoceptors and either M_2 or M_3 muscarinic receptors.

α_1–Adrenoceptors represent a different type of adrenergic receptors in the lower urinary tract and form an important target in the treatment of voiding dysfunction. α_1–Antagonists are mainstay of treatment of voiding dysfunction in men, especially those suggestive of benign prostatic obstruction (BPO). Since BPO and OAB very often coincide, combination therapy of α_1-antagonists and muscarinic receptor antagonists is a logic and proven concept. Propiverine is a muscarinic receptor antagonist possessing additional properties, i.e., blockade of L-type Ca^{2+}-channels. As contractions of prostate and bladder smooth muscle at least partly depend on such channels, their inhibition may contribute to the therapeutic effects of propiverine in voiding dysfunction in men.
In **chapter 6** we have explored possible α_1-adrenoceptor antagonist effects of propiverine and its metabolites M-5, M-6, and M-14, which share the antimuscarinic and/or L-type Ca^{2+}-channel-blocking activity of propiverine. We have investigated whether propiverine and its metabolites can additionally antagonize α_1-adrenoceptors. Human prostate and porcine trigone muscle strips were used to explore inhibition of α_1-adrenoceptor mediated contractile responses. Chinese hamster ovary (CHO) cells expressing cloned human α_1-adrenoceptors were used to determine direct interactions with the receptor in radioligand binding and intracellular Ca^{2+}-elevation assays.
Propiverine concentration-dependently reversed contraction of human prostate and porcine trigone strips. The propiverine metabolite M-14 also relaxed phenylephrine pre-contracted trigone strips, whereas metabolites M-5 and M-6 were ineffective. In radioligand binding experiments, propiverine and M-14 exhibited similar affinity for the three α_1-adrenoceptor subtypes, whereas the M-5 and M-6 did not affect [^{3}H]-prazosin binding. In CHO cells, propiverine and M-14 inhibited α_1-adrenoceptor-mediated Ca^{2+} elevations with similar potency as radioligand binding.
In contrast to other muscarinic receptor antagonists, propiverine exerts additional L-type Ca^{2+}-channel blocking and α_1-adrenoceptor antagonist effects. It remains to be determined clinically, how these additional properties contribute to the clinical effects of propiverine, particularly in male voiding dysfunction.

Part Three: Muscarinic Antagonists in Clinical Practice

In the third part of this thesis, clinical aspects of antimuscarinic treatment of OAB are further explored. Antimuscarinic therapy is the most widely used treatment for OAB. In female patients, OAB can occur in combination with incontinence. The two main types of incontinence are stress urinary incontinence (SUI) and urgency urinary incontinence (UUI). They can exist in combination, but differ in pathophysiology and associated risk factors. While age is a major risk factor for UUI, female gender, obesity, obstructive airway disease and childbirth, particularly vaginal delivery, are risk factors for SUI. The latter is attributed to anatomically damaging the bladder outflow tract and/or pelvic floor. Multiparity is also a risk factor for urinary incontinence in general, possibly in an ethnicity-dependent manner. As previous studies did not specify whether incontinence involved SUI, UUI or both, they raise the possibility that multiparity, and more specifically, vaginal delivery, may be a risk factor not only for SUI, but also for UUI/OAB.
In **chapter 7** we explored associations between the number of vaginal deliveries and OAB symptoms as well as the response to treatment with a muscarinic antagonist in a preplanned secondary analysis of an observational study of solifenacin in OAB patients. Episode frequencies of OAB symptoms, pad use and scores on OAB rating scales were documented in 4450 patients before and after a 12–14 week treatment period with solifenacin 5 or 10 mg. Women without, and women with one, two or more than two vaginal deliveries and men were similar in their baseline characteristics. All groups also exhibited rather similar reductions in symptoms and improvements in rating scales upon treatment. These data indicate that solifenacin, and perhaps other muscarinic receptor antagonists, are similarly suitable for the treatment of OAB symptoms in both genders, irrespective of previous vaginal deliveries.

In **chapter 8** we review evidence and regulatory dosing recommendations for muscarinic receptor antagonists used in the treatment of OAB.
Across larger patient samples, all currently available muscarinic receptor antagonists appear to have comparable efficacy, but show some quantitative differences in tolerability. However, the randomized studies are typically performed in selected populations, and specifically patients at elevated risk for side

effects are mostly under-represented as compared to the general population likely to receive such drugs. Side effect risks may be increased in some patient populations due to altered pharmacokinetic behavior of a given drug, e.g. in those with impaired renal or liver function or those with comedications. Moreover, genetic heterogeneity in drug metabolizing enzymes, namely cytochrome P450 (CYP) 2D6, may additionally contribute to altered drug exposure in some patients. We estimate that about one third of all OAB patients have at least one of those risk factors. Our analysis includes the oral formulations of darifenacin, fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine and trospium because each of them is frequently used in at least some major countries. Our recommendations are primarily based on the Summary of Product Characteristics and/or package insert as approved by the regulatory authorities in the EU and US, respectively.

We advise two therapeutic strategies. If a specific complicating factor is known, a drug should be chosen which largely remains unaffected by this factor. If no specific complicating factors are known but have not been excluded (a situation likely to occur frequently in daily practice), the presence of multiple inactivation/excretion mechanisms (renal plus CYP 2D6 plus CYP 3A4), e.g. with fesoterodine, may minimize overall tolerability risks, but this theoretical concept has not been tested in clinical studies. On the other hand, the reversible nature of most side effects of muscarinic antagonists does not necessitate to systematically screen for complicating factors by means other than a thorough patient history. Therefore, it is our key recommendations that prescribing physicians should be aware of risks for reduced tolerability due to pharmacokinetic factors and should specifically tailor medication to the situation of an individual patient.

Conclusions

This thesis, comprising of clinical studies, basic research as well as review of data, has aimed to provide further insight into the role and function of muscarinic receptors in the lower urinary tract. In light of increasing evidence of beneficial effects of combination therapies for LUTS, i.e. antimuscarinics with α_1–adrenoceptor antagonists and with β_3-adrenoceptor agonists, respectively, understanding mechanisms of interaction are crucial to maximize its therapeutic potency.

In the first part of this thesis we focus on muscarinic receptors in the prostate. As muscarinic receptors are abundantly present in the prostate, and antimuscarinic therapy is increasingly used in male patients with LUTS, direct effects of antimuscarinics on the prostate need to be considered.
The literature review in chapter 2 demonstrates that M_1 muscarinic receptors are mainly expressed on epithelial cells and exceed the M_2 subtype, which is present on stromal cells of the prostate. The direct effects on the prostate may involve alterations in glandular function and prostatic growth.
In chapter 3, we used high quality mRNA in real-time PCR experiments to show presence of all five muscarinic subtype transcripts, with M_1 and M_4 receptors as the most and least prevalently expressed subtypes, respectively. Of all subtypes, only M_1 receptor expression is positively associated with prostate size. In contrast, age, PSA level, pathological diagnosis or treatment with 5-ARI's or α- or β-blockers are not associated with alterations of mRNA expression for any of the five muscarinic receptor subtypes in the human prostate.

In the second part, muscarinic and adrenergic signalling pathways in the bladder are studied in further detail using different models and techniques. In chapter 4 we used a highly complex pharmacological approach to elucidate the role of M_2 and M_3 muscarinic subtypes in the attenuation of β-adrenoceptor-mediated relaxation in rat bladder. Muscarinic receptors cause direct contraction and inhibition of relaxation in the bladder, but the two responses involve different subtypes and, at least for M_3 receptors, different signalling pathways. This interaction may become clinically relevant under pathophysiological conditions when acetylcholine is being released in the bladder during the storage

phase of the micturition cycle.
In chapter 5, we described the development of cell lines with either M_2 or M_3 muscarinic receptors co-expressing β_3-adrenoceptors to study interaction on the intracellular level. Although both receptors were present in our cells, levels of co-expression of β_3-adrenoceptors was very low, and therefore this model was not suitable to study functional interaction. Thus, other model systems may be required to explore the interaction between β_3-adrenoceptors and either M_2 or M_3 muscarinic receptors.
The α_1-adrenoceptor antagonist effects of muscarinic antagonist propiverine are explored in chapter 6. Contractile experiments in human prostate and porcine trigone muscle strips, radioligand binding and intracellular Ca^{2+} experiments in Chinese hamster ovarium cells, demonstrated α_1-adrenoceptor antagonist effects. It remains to be determined clinically, how these additional properties contribute to the clinical effects of propiverine, particularly in male voiding dysfunction.

In the third part clinical aspects of antimuscarinic treatment of OAB are explored. In chapter 7 we explored associations between the number of vaginal deliveries and OAB symptoms, as well as the response to treatment with a muscarinic antagonist in a preplanned secondary analysis of an observational study of solifenacin in OAB patients. Women without, and women with one, two or more than two vaginal deliveries and men were similar in their baseline characteristics. All groups also exhibited rather similar reductions in symptoms and improvements in rating scales upon treatment. These data indicate that solifenacin, and perhaps other muscarinic receptor antagonists, are similarly suitable for the treatment of OAB symptoms in both genders, irrespective of previous vaginal deliveries.
In chapter 8 an overview is presented of antimuscarinics at risk for reduced tolerability in special patient populations, e.g. in those with impaired renal or liver function or those with comedications or genetic heterogeneity in drug metabolizing enzymes. We estimate that about one third of all OAB patients have at least one of those risk factors. Our analysis includes the oral formulations of darifenacin, fesoterodine, oxybutynin, propiverine, solifenacin, tolterodine and trospium because each of them is frequently

used in at least some major countries. Therefore, it is our key recommendations that prescribing physicians should be aware of risks for reduced tolerability due to pharmacokinetic factors and should specifically tailor medication to the situation of an individual patient.

Future Perspectives

The abundant presence of muscarinic receptors in the prostate and the increasing use of antimuscarinics in men with mainly storage symptoms, suggest potential direct effects of antimuscarinics on the prostate. Secretory function and benign and/or malignant growth of the prostate might be affected by antimuscarinic treatment (chapter 2).

Theoretically, semen volumes might decrease in men using antimuscarinics, since secretory function of many organs, including the prostate, is mediated by muscarinic receptors. Whether this is clinically relevant remains to be elucidated, but physicians should be aware of this possible side-effect when prescribing antimuscarinics, especially in cases of delayed parenthood.

Growth promoting effects of muscarinic receptor agonists are seen in primary cultures from patients with benign and malignant prostate disease and prostate cancer cell lines (chapter 2). Botulinum toxin injections in the prostate demonstrated volume reduction of almost 20% and are assumed to act by inhibiting the release of the endogenous acetylcholine (chapter 2). The role of receptor subtypes is particularly interesting, since M_1 and M_3 receptor subtypes may be related to benign and malignant prostatic cell growth (chapter 2 and 3), respectively, and antimuscarinics have different affinity profiles for these receptor subtypes. Therefore, it would be of particular interest to obtain in vivo data on long-term treatment of men with antimuscarinics and possible changes of prostate size and incidence of prostate cancer.

Since our study of mRNA expression of muscarinic receptors in the prostate (chapter 3) is the largest to date, but unique in its kind, it is desirable to confirm these data in another cohort, with special interest for antimuscarinic use and incidence of benign prostate enlargement or prostate cancer.

Although this thesis mainly focused on muscarinic and adrenergic receptors, many other receptor types are involved in bladder

functioning and the number of possible interactions of these receptors in normal and pathological conditions is even larger. The recent introduction of β_3-adrenoceptor agonists for the treatment of OAB, besides antimuscarinic therapy, supports the importance of further exploring downstream signaling and interaction to understand pathophysiology and optimize OAB treatment.
On the other hand, muscarinic and adrenergic receptors of the urothelium and on afferent nerves demand additional investigation, since these pathways may be more important than earlier suggested.
Different models allow studying of these mechanisms on the tissue level in vitro and in vivo in various species, including humans. Models to study receptor interaction at the cellular level are only scarcely available and development of these models is often accompanied by many difficulties (chapter 5). Although transfecting cells with multiple receptors is possible, factors such as controllable receptor expression, availability of signaling assays and the pharmacological tools, (i.e. specific agonists, antagonists (chapter 4 and 6) and radioligands (chapter 5)), define the success of such a model. Nevertheless, cell models have specific advantages, such as reduction of animal use, the almost unlimited possibility of culturing cells, and easy accessibility to test intracellular signaling. Therefore these models still have considerable potential for future research.

Clinical studies in large patient groups and special patient populations, after the introduction of a drug, contribute to knowledge of efficacy, tolerability and safety of a drug (chapter 7). Since antimuscarinics are well studied and prescribed for many years, the clinical experience is overly present, even in special patient groups (chapter 8). The recently introduced β_3-adrenoceptor agonist mirabegron and other β_3-adrenoceptor agonists in development have a different pharmacological profile and relatively little clinical experience. Because of the favorable tolerability profile of β_3-adrenoceptor agonists, compared to antimuscarinics, expectations are high, but clinical studies in large and special patient groups are needed for this new drug class to build up clinical experience. Phase III trials investigating combination therapy of β_3-adrenoceptor agonists and antimuscarinics for patients with severe OAB are

currently ongoing and results of these trials are to be expected soon. Whether addition of an α_1-adrenoceptor antagonist to the combination of antimuscarinics and β_3-adrenoceptor agonists men with OAB and BOO is effective remains uncertain, but it seems to be the next logical step.

Samenvatting

Voor de behandeling van het overactieve blaas syndroom (OAB) zijn muscarine-antagonisten ofwel anticholinergica de middelen van eerste keuze. OAB wordt gedefinieerd als verhoogde aandrang (urgency), meestal gepaard gaande met een verhoogde plasfrequentie (frequency) en nachtelijk plassen (nycturie) met of zonder urine-incontinentie. Hoewel in het verleden plasklachten bij vrouwen meestal werden geduid als OAB, werden plasklachten bij de man verklaard door een obstructieve prostaat. In de afgelopen jaren is echter het inzicht rondom mannelijke plasklachten veranderd en is de rol van de overactieve blaas, naast de obstructieve prostaat, meer onder de aandacht gekomen. Deze verandering heeft geleid heeft tot een toename van het gebruik van anticholinergica bij mannelijke patiënten. Omdat anticholinergica voor OAB in overwegend vrouwelijke patiënten getest zijn en tegelijkertijd de prostaat veel muscarine receptoren bevat is een beter begrip van de werking van anticholinergica in de lage urinewegen noodzakelijk om het therapeutisch potentieel volledig te kunnen benutten.

In het eerste deel van dit proefschrift worden de cholinerge innervatie, het voorkomen en de functioneren van muscarine-receptoren in de prostaat en de regulatie van expressie van muscarine-receptoren in de prostaat beschreven. In het tweede deel worden de signaalwegen bestudeerd die betrokken zijn bij muscarinerge, α-adrenerge, β-adrenerge gemedieerde contractie en relaxatie van de urineblaas. Hiervoor is gebruik gemaakt van technieken op weefsel-, cel- en intracellulair niveau. In het derde deel worden klinische data gepresenteerd met betrekking tot toepasbaarheid en tolerantie in verschillende patiëntengroepen.

Deel 1: Muscarine Receptoren in de Prostaat

In **hoofdstuk 2** wordt een literatuuroverzicht gepresenteerd met als doel een beter inzicht te krijgen van cholinerge innervatie en de verdeling en functie van muscarinerge receptoren in de prostaat. In de bestaande literatuur is cholinerge innervatie van de prostaat uitgebreid beschreven, zowel in de stromale als in de epitheliale cellen. Interessant genoeg blijkt de prostaat bij de mens veel meer muscarine-receptoren te bevatten dan α_1-adrenoceptoren. Deze laatstgenoemde receptoren spelen een belangrijke rol bij de behandeling van mannelijke plasklachten, veroorzaakt door een obstructieve prostaat. De meest voorkomende muscarine-receptor

in de prostaat is van het M_1-subtype en is voornamelijk aanwezig op epitheliale cellen. Van het M_2-subtype zijn kleinere aantallen aanwezig, voornamelijk op stromale cellen van de prostaat. Beide subtypen blijken functioneel actief in signaaltransductie-assays, maar zoals verwacht bij de lage receptordichtheid op stromale cellen, zijn contractiele responsen in de prostaat beperkt. Uit studies in prostaatkanker-cellijnen en uit studies waarbij de prostaat is geïnjecteerd met botulinum-toxine, blijkt dat muscarine-receptoren mogelijk een rol spelen bij groei van de prostaat. Daarnaast laten verschillende dierexperimenten zien dat muscarine-receptoren betrokken zijn bij de productie van prostaatvocht, dat de belangrijkste component is van sperma. Een vermindering van sperma-volume bij gebruik van anticholinergica is echter nooit aangetoond bij de mens.
Samenvattend, lijkt het aannemelijk dat er directe effecten zijn van muscarine-antagonisten op de prostaat. De meest voor de hand liggende effecten zijn remming van groei en remming van productie van prostaatvocht.

De mogelijke directe effecten van anticholinergica op de prostaat en het beperkte onderzoek op het gebied van regulatie van muscarine-receptoren in de prostaat geven aanleiding tot nadere verkenning. Hoewel er enkele studies zijn die de regulatie van muscarine-receptoren op eiwitniveau in pathologische situaties bestuderen, zijn de verschillende subtypen nooit afzonderlijk bestudeerd. Bovendien zijn de studies gedaan met zeer kleine aantallen patiënten.
In **hoofdstuk 3** hebben we de relatie onderzocht tussen de expressie van mRNA van de verschillende muscarine-receptoren in prostaatweefsel en verschillende factoren, zoals leeftijd, PSA-waarde, pathologische diagnose, prostaatvolume en co-medicatiegebruik. Met behulp van real time-PCR experimenten tonen we de aanwezigheid aan van mRNA van alle vijf subtypen van muscarine receptor in de prostaat. Hiermee tonen we aan dat het mogelijk is om hoge kwaliteit mRNA te verkrijgen uit prostaatweefsel na een transurethrale resectie van de prostaat (TURP), zonder schade aan het mRNA veroorzaakt door verhitting tijdens de procedure. In het prostaatweefsel van 110 mannen bleken mRNA transcripten van het M_1- en M_4-subtypen respectievelijk het meest en het minst aanwezig. Van alle subtypen,

is alleen de M_1-receptor mRNA expressie positief geassocieerd, zij het zwak, met het volume van de prostaat. Daarentegen zijn leeftijd, PSA niveau, pathologische diagnose of gebruik van 5-alfa-reductaseremmers, α- of β-adrenerge antagonisten niet geassocieerd met veranderingen in mRNA expressie van een van de vijf muscarine-receptoren in de prostaat.

Deel 2: Muscarinerge en Adrenerge Signaaloverdracht

In het tweede deel van dit proefschrift worden de muscarinerge en adrenerge signaalwegen in de urineblaas nader bestudeerd. In de blaas komen met name de M_2- en M_3-muscarine-receptoren voor, naast β-adrenerge receptoren. Hoewel M_3- en β-receptoren een grotendeels tegengestelde werking hebben in de blaas, namelijk contractie en relaxatie, is de rol van M_2-receptoren is niet geheel duidelijk. Eerdere experimenten hebben laten zien dat relaxatie van de blaas via β-adrenerge receptoren geremd kan worden door de muscarine-receptor agonist carbachol. De bijdrage van de M_2- en M_3-subtypen in dit proces is onduidelijk. In **hoofdstuk 4** beschrijven we de rol van M_2- en M_3-receptoren bij de remming van relaxatie door β-adrenerge receptoren na pre-contractie met carbachol in de urineblaas van ratten. Hiervoor hebben we gebruik kunnen maken van een nieuwe muscarine antagonist, THRX-182,087, die een hogere selectiviteit heeft voor M_2-, dan voor M_3-receptoren. Het gebruik van THRX-182,087 in combinatie met carbachol en de M_3-receptor sparende agonist BZI maakt selectieve stimulering van M_2- en M_3-receptoren mogelijk. Dit is tot heden nooit op functioneel niveau aangetoond. In onze experimenten geeft carbachol dezelfde remming van relaxatie door isoprenaline, een β-adrenoceptor agonist, zoals aangetoond in eerdere experimenten. Zowel M_2- als M_3- selectieve stimulatie geeft remming van relaxatie, hoewel dat effect bij M_3-stimulatie sterker is dan bij M_2-stimulatie. Tijdens M_3-selectieve stimulatie kan remming van relaxatie verminderd worden door de fosfolipase C inhibitor U73,122, maar niet door de proteïne kinase C inhibitor chelerythrine. Dit betekent dat de interne signaalwegen van de M_3-receptor die betrokken zijn bij contractie, vermoedelijk verschillend zijn van de signaalwegen betrokken bij remming van relaxatie. Mogelijk speelt bovengenoemd fenomeen een rol in situaties waarbij een verhoogde afgifte van acetylcholine tijdens de opslagfase van de mictiecyclus leidt tot een verminderd vermogen tot relaxatie van de blaas.

In het voorgaande hoofdstuk is gedemonstreerd dat de interactie tussen muscarinerge en β-adrenerge receptoren op weefselniveau complex is. Om deze interactie op intracellulair niveau te kunnen bestuderen, hebben we een model ontwikkeld van Chinese hamster ovarium (CHO) cellen waarin de menselijke genen coderend voor M_2- en β_3-receptoren getransfecteerd zijn. Daarnaast zijn ook cellijnen gemaakt met de combinatie M_3- en β_3-receptoren. In **hoofdstuk 5** wordt de ontwikkeling van de cellijnen beschreven, evenals de bindingsexperimenten en de functionele assays die met de cellijnen gedaan zijn. De typische intracellulaire signaalweg voor de M_2-receptor en β_3-receptor verloopt via het enzym adenylaat cyclase. De intracellulaire concentratie van cAMP daalt na stimulering van adenylaat cyclase via de M_2-receptor, maar stijgt door prikkeling van de β_3-receptor. cAMP interacteert met andere signaalwegen, zoals via het enzym fosfolipase C, wat kan lijden tot effecten op intracellulair calcium. Hoewel de precieze rollen van muscarinerge en β-adrenerge receptoren in contractie en relaxatie van de blaas onduidelijk zijn, zijn er waarschijnlijk interacties van deze signaalwegen op verschillende niveaus.
Alle klonen die gemaakt zijn, vertonen expressie van muscarine-receptoren op fysiologische niveaus. De expressie van β_3-adrenerge receptoren in de getransfecteerde klonen bleek echter te laag te zijn om een betrouwbare schatting van de receptordichtheid te maken met beschikbare radioligand. Desalniettemin is er wel receptoractiviteit van β_3-adrenerge receptoren aangetoond in een functionele assay middels een meetbare stijging van intracellulair cAMP na stimulatie met isoprenaline. Deze stijging van cAMP is echter zo klein dat het waarschijnlijk geen effect heeft op het intracellulaire calcium, althans niet in onze experimenten.
Samenvattend zijn we er in geslaagd om een cellijn te creëren van CHO cellen met daarin de combinaties van humane M_2/β_3 en M_3/β_3 receptoren. Het model is echter niet geschikt gebleken voor de bestudering van de functionele interactie tussen muscarinerge en β_3-adrenerge receptoren, waarschijnlijk omdat de receptorexpressie van de β_3-receptor te laag is. Andere modellen zijn mogelijk meer geschikt voor het bestuderen van deze interacties.

Een ander type adrenerge receptor dat veel voorkomt in de lage urinewegen, naast de β_3-receptor, is de α_1–adrenerge receptor. α_1–Adrenerge receptoren zijn aanwezig in de blaashals en in

de prostaat en zijn aangrijpingspunt voor de behandeling van mannelijke plasklachten veroorzaakt door een uitgangsobstructie van de blaas, meestal als gevolg van goedaardige prostaatvergroting. Omdat deze obstructieve plasklachten vaak gepaard gaan met overactiviteit van de blaas, is behandeling met een combinatie van een α_1-antagonist en muscarine-antagonist een bewezen en effectief concept. Propiverine is naast muscarine antagonist ook in staat tot blokkade van L-type-calciumkanalen. Omdat contracties in de blaas en prostaat voor een deel afhankelijk zijn van deze kanalen, kan remming hiervan bijdragen aan het therapeutisch effect van propiverine bij plasklachten bij de man. In **hoofdstuk 6** hebben we de mogelijke α_1-adrenerge receptor antagonerende effecten van propiverine en de metabolieten M-5, M-6 en M-14 onderzocht. Deze metabolieten van propiverine hebben zowel een anticholinerge als een L-type-calciumkanaal blokkerende werking. Wij hebben onderzocht of propiverine en zijn metabolieten ook een α_1-adrenoceptor antagonerend effect hebben. Voor de bestudering van een antagonerend effect van contractiele responsen, gemedieerd door de α_1-adrenoceptor, zijn weefselstrips gebruikt van de menselijke prostaat en van het trigonum van de varkensblaas. Daarnaast zijn CHO cellen gebruikt, die gekloneerde humane α_1-adrenerge receptoren tot expressie brengen. In radioligand-bindingsexperimenten en intracellulaire calcium assays zijn directe receptor interacties van propiverine en zijn metabolieten bestudeerd . Uit deze experimenten blijkt dat contractie van strips van menselijke prostaat en strips van het trigonum van een varkensblaas concentratie-afhankelijk geblokkeerd kan worden. De metaboliet van propiverine, M-14, is in staat trigonale strips te relaxeren die contractie vertonen na pre-stimulatie met α_1–agonist fenylefrine, terwijl metabolieten M-5 en M-6 dat niet doen. In radioligand-bindingsexperimenten met [^{3}H]-prazosine vertonen propiverine en M-14 dezelfde affiniteit voor de drie subtypen van de α_1-adrenerge receptor, terwijl M-5 en M-6 geen effect hebben op binding van [^{3}H]-prazosine. In CHO-cellen inhiberen propiverine en M-14 calciumverhoging door fenylefrine met dezelfde potentie als die van de radioligand-bindingsexperimenten. Samenvattend vertoont propiverine in tegenstelling tot andere anticholinergica naast anticholinerge ook L-type-calciumkanaal blokkerende en α_1-adrenoceptor antagonerende effecten. Of deze effecten bijdragen

aan de klinische effectiviteit van propiverine, in het bijzonder bij plasklachten bij de man, zal nog bewezen moeten worden.

Deel 3: Muscarine Antagonisten in de Klinische Praktijk

In het derde en laatste deel van dit proefschrift zullen enkele klinische aspecten van anticholinerge behandeling voor OAB beschreven worden. Zoals bekend, kan OAB gepaard gaan met urine-incontinentie. De twee meest voorkomende vormen van urine-incontinentie zijn stress urine-incontinentie (SUI) en urgency (aandrang) urine-incontinentie (UUI). Hoewel deze twee vormen van incontinentie verschillen qua risicofactoren en pathofysiologie, kunnen ze naast elkaar voorkomen (gemixte urine-continentie). Terwijl leeftijd een belangrijke risicofactor is voor UUI, zijn vrouwelijk geslacht, obesitas, obstructieve luchtwegklachten en vaginale bevallingen risicofactoren voor SUI. De laatstgenoemde risicofactor wordt geassocieerd met anatomische schade aan de blaasuitgang en/of de bekkenbodem. Uit eerdere studies waarin de relatie tussen urine-incontinentie en multipariteit is onderzocht, kwam naar voren dat multipariteit een risicofactor is voor urine-incontinentie in het algemeen, mogelijk afhankelijk van etnische achtergrond. Hoewel er in geen duidelijk onderscheid wordt gemaakt tussen SUI en UUI of gemixte incontinentie, werpen deze studies de mogelijkheid op dat multipariteit, en zelfs meer specifiek, een vaginale bevalling een risicofactor is voor niet alleen SUI, maar ook voor UUI en/of OAB. In **hoofdstuk 7** hebben we de relatie onderzocht tussen het aantal vaginale bevallingen en symptomen van OAB in een vooropgezette observationele studie naar het effect van solifenacine bij patiënten met OAB. Van 4450 patiënten werden episodes van frequency, gebruik van inleggers (pads) en OAB symptoom scorelijsten gedocumenteerd voor en na 12-14 weken van behandeling met solifenacine 5 of 10 mg. Basale karakteristieken bleken overeen te komen bij vrouwen zonder, vrouwen met één, twee of meer vaginale bevallingen en mannen kwamen overeen in. Alle groepen vertoonden daarnaast een vergelijkbare afname van klachten en verbetering van symptoomscores na behandeling. Deze data tonen aan dat solifenacine, en mogelijk ook andere muscarine antagonisten, geschikt zijn voor de behandeling van OAB symptomen bij zowel mannen als vrouwen, onafhankelijk van het aantal doorgemaakte vaginale bevallingen.

Hoofdstuk 8 geeft een overzicht van de aanbevolen doseringen van anticholinergica voor de behandeling van OAB. In grote patiëntengroepen hebben alle beschikbare muscarine-antagonisten vergelijkbare effectiviteit, maar verschillen ze met name op het gebied van tolerabiliteit en bijwerkingen. De meeste gerandomiseerde studies zijn gedaan bij geselecteerde patiëntengroepen, waarin specifieke patiënten met een verhoogd risico op bijwerkingen echter ondervertegenwoordigd zijn, ondanks dat deze laatste groep patiënten relatief vaker deze medicatie gebruikt. Het risico op bijwerkingen is groter bij bepaalde patiëntengroepen als gevolg van een veranderde farmacokinetiek van een geneesmiddel door nier- en/of leverfunctiestoornissen of comedicatiegebruik. Daarnaast kan genetische heterogeniteit van metabolische enzymen, zoals cytochroom P450 (CYP) 2D6 bijdragen aan verminderde afbraak van, en daarmee verhoogde blootstelling aan medicatie bij bepaalde patiënten. Wij schatten dat ongeveer een derde van alle patiënten met OAB tenminste één van deze risicofactoren heeft. In onze analyse hebben we de orale formuleringen van darifenacine, fesoterodine, oxybutynine, propiverine, solifenacine, tolterodine en trospium meegenomen, omdat deze in de meeste landen het meest gebruikt worden. Onze aanbevelingen zijn gebaseerd op de samenvattingen van de producteigenschappen zoals deze geaccepteerd en goedgekeurd zijn door de regulerende organen voor geneesmiddelen, zoals de European Medicines Agency (EMAE) in Europa en de Food and Drug Administration (FDA) in de Verenigde Staten. Samengevat adviseren wij twee mogelijke strategieën. Als een specifieke complicerende factoren, zoals nier- en/of leverfunctiestoornissen, comedicatie of genetische, metabole afwijkingen bekend zijn, bestaat de voorkeur voor een middel dat hierdoor zo min mogelijk wordt beïnvloed. Als complicerende factoren onbekend zijn, wat het meest voorkomt in de klinische praktijk, wordt geadviseerd om te kiezen voor een middel dat gemetaboliseerd wordt door verschillende mechanismen, zoals klaring door de nier, naast metabolisme door verschillende leverenzymen (CYP 2D6 en CYP 3A4), zoals bijvoorbeeld bij fesoterodine. Hoewel dit theoretische concept de tolerantie-risico's minimaliseert, is het niet getest in klinische studies. Aan de andere kant is het systematisch screenen op complicerende factoren wellicht overbodig, aangezien de meeste bijwerkingen

van antimuscarinerge middelen reversibel zijn na staken van toediening.
Concluderend is het belangrijkste advies voor voorschrijvende artsen om zich bewust te zijn van de risico's van verminderde tolerabiliteit als gevolg van farmacokinetische factoren. Bij het voorschrijven van anticholinerge medicatie moet maatwerk verricht worden, passend bij de situatie van de individuele patiënt.

Conclusies

Dit proefschrift bestaat uit zowel experimenteel farmacologisch onderzoek, klinische studies als enkele overzichtsartikelen en heeft als doel om het inzicht te vergroten in de rol en functie van muscarine receptoren in de lage urinewegen. In het kader van het toenemend bewijs voor de gecombineerde behandeling van plasklachten bij de man en vrouw, zoals bijvoorbeeld anticholinergica met α_1–adrenerge antagonisten en anticholinergica met β_3-adrenerge agonisten, is begrip van de werkingsmechanismen en interacties cruciaal voor het optimaliseren van de therapeutische potentie van deze behandelstrategieën.

In het eerste deel ligt de focus op muscarine-receptoren in de prostaat. In hoofdstuk 2 wordt beschreven dat M_1- en M_2-receptoren het meest voorkomen in de prostaat, respectievelijk op epitheliale en stromale cellen. Gezien de functie van muscarine-receptoren in de prostaat, kunnen muscarine-antagonisten directe effecten hebben op zowel de klierfunctie van de prostaat als op groei van de prostaat. In hoofdstuk 3 wordt in real-time PCR experimenten de aanwezigheid mRNA van alle vijf muscarine receptor-subtypes aangetoond in de prostaat van de mens, met M_1- en M_4- als de respectievelijk meest en minst voorkomende. Van alle subtypes is alleen de M_1-receptor positief geassocieerd met grootte van de prostaat. Daarentegen zijn leeftijd, PSA-waarde, pathologische diagnose of behandeling met 5-ARI's, α- of β-blokkers niet geassocieerd met veranderingen van mRNA expressie van een van de vijf subtypes.

In het tweede deel ligt de focus op de muscarinerge en adrenerge signaalwegen in de blaas. In hoofdstuk 4 hebben we een complexe farmacologische benadering gebruikt om de rol van M_2- en M_3-receptoren te onderzoeken bij de remming van relaxatie door β-adrenerge receptoren na pre-contractie met carbachol in de urineblaas van ratten. Stimulatie van muscarine-receptoren zorgt voor directe contractie en remming van relaxatie, maar bij deze twee responsen zijn verschillende subtypes betrokken en tenminste voor M_3-receptoren verschillende signaalwegen. In hoofdstuk 5 beschrijven we de ontwikkeling van een cellijn van CHO-cellen met daarin de combinaties van humane M_2/β_3- en M_3/β_3-receptoren. Het model is echter niet geschikt gebleken voor de bestudering van de functionele interactie tussen muscarine en β_3-adrenerge receptoren, waarschijnlijk omdat de receptorexpressie

van de β_3-receptor te laag is. In hoofdstuk 6 hebben wij aangetoond dat in tegenstelling tot andere anticholinergica, propiverine naast antimuscarinerge en L-type-calciumkanaal blokkerende ook α_1-adrenoceptor antagonerende effecten heeft. Of deze effecten bijdragen aan de klinische effectiviteit van propiverine, in het bijzonder bij plasklachten bij de man zal nog bewezen moeten worden.
In deel drie van dit proefschrift worden enkele klinische aspecten beschreven van de behandeling van OAB met anticholinergica. In hoofdstuk 7 wordt aangetoond dat solifenacine, en mogelijk ook andere antimuscarinerge antagonisten, geschikt zijn voor de behandeling van OAB symptomen bij zowel mannen als vrouwen, onafhankelijk van het aantal vaginale bevallingen van een vrouw. Tot slot wordt in hoofdstuk 8 een overzicht gepresenteerd over de aanbevolen doseringen van anticholinergica voor de behandeling van OAB in patiëntengroepen met veranderde farmacokinetiek als gevolg van nier- en of leverfunctiestoornissen, gebruik van comedicatie of genetische heterogeniteit van metabolische enzymen.

Toekomstperspectieven

De mogelijke directe effecten van anticholinergica op de prostaat zijn tweeledig: ten eerste afname van secretie-functie van de prostaat, ten tweede remming van groei van de prostaat. Een afgenomen secretie-functie van de prostaat kan mogelijk resulteren in een afname van spermaproductie en kan klinisch relevant zijn in patiënten met een onvervulde kinderwens die anticholinergica gebruiken. Tot heden is deze bijwerking slechts gebaseerd op theorie en zal relevantie moeten worden aangetoond in klinische studies. De groei-stimulerende effecten van muscarine-agonisten zijn beschreven in primaire celculturen van zowel benigne als maligne prostaatcellen van patiënten (hoofdstuk 2).
Daarnaast zorgen injecties met botulinum toxine in de prostaat voor een volumereductie van ongeveer 20%, vermoedelijk door remming van afgifte van endogeen acetylcholine (hoofdstuk 2). De M_1- en M_3-receptor subtypes zijn vermoedelijk betrokken bij respectievelijk benigne en maligne groei van prostaatcellen (hoofdstuk 2 en 3) en behandeling met muscarine-antagonisten kan derhalve mogelijk benigne prostaathyperplasie remmen en

de incidentie van prostaatkanker verlagen. Of dit daadwerkelijk het geval is, zal aangetoond kunnen worden door evaluatie van langdurige blootstelling aan anticholinergica, waarbij rekening gehouden moet worden met de verschillen in affiniteit voor de bekende receptor subtypes.

Ondanks dat onze studie over mRNA expressie van muscarine receptoren in de prostaat (hoofdstuk 3) de enige en grootste studie tot heden is, is het wenselijk om een dergelijke studie te herhalen om de uitkomsten te bevestigen, met name met het oog op de mogelijke klinische gevolgen voor benigne en maligne prostaataandoeningen.

Hoewel de focus van dit proefschrift hoofdzakelijk ligt bij muscarinerge en adrenerge receptoren, zijn er vele andere receptortypen die een rol spelen bij normale en pathologische fysiologie van de blaas. Daarom zijn de mogelijke interacties tussen deze systemen zeer complex. De recente introductie van β_3-adrenerge agonisten voor de behandeling van OAB, naast anticholinergica, bevestigt de noodzaak om de signaalwegen van deze receptoren en de mogelijke interacties tussen deze signaalwegen te onderzoeken om zo de behandeling van OAB te optimaliseren. Daarnaast is de rol van muscarinerge en adrenerge receptoren in het urothelium en op afferente zenuwen een uitdagend onderzoeksgebied, omdat hun rol belangrijker lijkt dan in het verleden gesuggereerd werd. Bestudering van deze mechanismen op het weefselniveau is mogelijk met behulp van verschillende modellen, zowel met dierlijk als menselijk materiaal, in vitro en in vivo. De mogelijkheden tot het bestuderen van intracellulaire signaalwegen zijn beperkter en ontwikkeling van dergelijke modellen gaat gepaard met veel uitdagingen (hoofdstuk 5). Hoewel het mogelijk is om meerdere humane receptoren te kloneren in bijvoorbeeld Chinese hamster ovarium-cellen (hoofdstuk 5), wordt het succes van een model bepaald door verschillende factoren, zoals controleerbare receptorexpressie en de juiste farmacologische middelen (bv radioliganden (hoofdstuk 5), selectieve agonisten en/of antagonisten (hoofdstuk 4 en 6). Desalniettemin hebben cel-modellen specifieke voordelen, zoals reductie van proefdiergebruik, onbeperkte voorraad van cellen in kweek en goede toegankelijkheid voor signaalweg-assays. Door deze voordelen zijn dergelijke modellen veelbelovend voor toekomstig onderzoek.

Klinische studies bij grote patiëntengroepen (hoofdstuk 7) en specifieke populaties, zoals patiënten met aandoeningen die metabolisme van geneesmiddelen beïnvloeden (hoofdstuk 8) dragen bij aan de kennis over effectiviteit en tolerantie van een middel. Omdat anticholinergica reeds jaren voorgeschreven worden, is de ervaring groot, ook in specifieke patiëntengroepen (hoofdstuk 7 en 8). Hoewel de recent geïntroduceerde β_3-adrenoceptor agonist mirabegron en de ontwikkeling van andere β_3-adrenoceptor agonisten voor de behandeling van OAB veelbelovend zijn, zal het enige tijd duren voordat dezelfde klinische ervaring is opgebouwd als bij de anticholinergica. Op dit moment zijn Fase III studies gaande waarin combinatietherapie bestudeerd wordt van β_3-adrenoceptor agonist mirabegron en muscarine antagonist solifenacine in patiënten met ernstige OAB. Of daar in de toekomst een α_1-adrenoceptor antagonist aan zal worden toegevoegd voor mannen met ernstige obstructieve en irritatieve plasklachten is de vraag, maar het klinkt als een logische volgende stap.

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Curriculum Vitae

Bart Witte was born on the 10th of july 1977 in Goirle, Noord-Brabant, the Netherlands. After graduation from high school in 1995 (Mill Hill College, Goirle) he studied medical biology at the University of Amsterdam, resulting in a Master's degree in 2001 with specialisation in neurobiology. From 2002-2004 he studied medicine at the University of Amsterdam, followed by clinical internships form 2004-2006. Early 2007 he started a collaborative PhD research project with the departments of Pharmacology & Pharmacotherapeutics (prof. dr. M.C. Michel) and Urology (prof. J.J.M.C.H. de la Rosette) of the University of Amsterdam. In 2010 he began his training in urology, starting with two years of general surgery in the "Westfriesgasthuis" in Hoorn (dr. J.W.D. de Waard), followed by two years of urological training in the "Sint Lucas Andreas Ziekenhuis" in Amsterdam (drs. E.A. Heldeweg). The last two years of his residency are in the "Academic Medical Center" in Amterdam (prof. dr. J.J.M.C.H. de la Rosette).
Bart lives together with Marianne Snijder and their son Willem.

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