Thesis for the degree of Doctor of Medicine

Muscarinic Receptors in the Urinary Bladder

The role of the urothelium regarding cholinergic and nitrergic effects in inflammation

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2010

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Printed by Intellecta Infolog AB, Gothenburg, Sweden, 2010

ISBN 978-91-628-8198-6

Till min familj

Abstract

Inflammation alters the functional properties of the urinary bladder. Interstitial cystitis (IC) is a chronic inflammatory syndrome in man that is characterized by urgency, frequency and visceral pain. The overall aim of this study was to investigate how the rat urinary bladder is affected by inflammation, and what specific part the urothelium plays in this.

Methods: Cystitis was induced in rats by a single injection of cyclophosphamide (CYP; 100 mg/kg). This treatment causes a disease state which is highly comparable to IC. Data comparing the properties of the healthy and inflamed bladder were gathered from (1) contraction experiments *in vitro* in an organ bath setup, (2) cystometrical studies *in vivo* in anaesthetized rats and (3) wake, freely moving rats in a metabolism cage. Cell cultures were also cultivated in order to investigate if proliferation of urothelial cells is influenced by receptor activation.

Key findings: Induction of cystitis by CYP altered the cholinergic response of the urinary bladder. *In vitro* studies showed a significantly lower response to carbachol in the inflamed bladder. Both *in vitro* and *in vivo,* the altered cholinergic response could be normalized by either removal of the urothelium, blockade of nitric oxide (NO) synthase or blockade of muscarinic M1/M3/M5 receptors. These findings indicate that during CYP-induced cystitis, NO is released from the urothelium upon muscarinic receptor activation. Further characterization *in vitro* revealed the M5 receptor as the most likely candidate for mediating this release.

In vivo experiments in the metabolism cage showed that micturition parameters are affected by CYP-induced cystitis. Increasing doses of a muscarinic antagonist eliminated these differences, and a connection between the effects of antimuscarinic and antinitrergic

drugs was indicated. These findings underline the importance of muscarinic receptors and NO in the alterations seen during cystitis.

Proliferation experiments indicated that adrenergic, but not muscarinic, nicotinic or EGF receptors, are involved in the regulation of urothelial cell proliferation.

Conclusions: In CYP-induced cystitis in the rat, the urothelium exerts an inhibitory influence on the cholinergic response of the urinary bladder. We conclude that this is caused by the release of NO upon activation of urothelial muscarinic M5 receptors.

Keywords: urinary bladder, inflammation, cyclophosphamide-induced cystitis, muscarinic receptor, urothelium, nitric oxide, M5 receptor, rat, proliferation, micturition

Populärvetenskaplig sammanfattning

Urinblåsan utgör en central del av urinvägarna. Dess uppgifter består av lagring och tömning av urin. Så länge urinblåsan fungerar som den ska är man omedveten om dess existens, men när den krånglar kan man inte låta bli att tänka på den. I Sverige lider runt 400.000 personer av inkontinens, och 4,5% av all diagnostiserad cancer har sitt ursprung i urinblåsan. Flera av de behandlingar som finns idag är otillräckliga, vilket visar på behovet av mer kunskap om urinblåsan och dess sjukdomar. I denna avhandling presenteras ny kunskap om de förändringar som sker i urinblåsan vid inflammation.

Att urinblåsan blir inflammerad är mycket vanligt och kan bero på flera orsaker. Till exempel kan inflammation i urinblåsan, s.k. cystit, orsakas av en bakterieinfektion eller närvaro av kemiska ämnen i urinen som irriterar urinblåsans insida till den grad att en inflammation uppstår. Urinblåsans insida täcks av en typ av celler som kallas urotelceller. Dessa trodde man ända fram till för 10-15 år sedan var passiva celler med ett enda syfte: att skydda urinblåsan och resten av kroppen från toxiska ämnen i urinen. Senare forskning har dock visat att dessa celler har flera viktiga uppgifter, inte minst vid diverse sjukdomstillstånd. Interstitiell cystit (IC) är ett av dessa sjukdomstillstånd, och kännetecknas av kronisk (långvarig) inflammation, smärta, överaktivitet samt förtjockning av urinblåsan. Kunskaper om vad som orsakar IC är begränsade, men man vet att IC inte orsakas av en infektion. Flera studier har visat att urinblåsans funktionalitet förändras vid cystit. Det övergripande målet med denna avhandling var att förstå vilka skillnader som uppstår vid inflammation i urinblåsan, och hur dessa påverkar urinblåsans funktion. Fokus lades till stor del på urotelceller och hur dessa förändras vid cystit. Känt sedan tidigare var att urotelceller har förmågan att frisätta kvävemonoxid (NO). Avhandlingsarbetet syftade till att klargöra hur denna frisättning påverkas vid cystit, och vad NO-frisättningen har för påverkan på urinblåsan. Det var också

känt sedan tidigare att en speciell sorts receptor, den muskarina M5 receptorn, uttrycks mer i urotelcellerna vid cystit. Vi ville ta reda på om detta ökade receptoruttryck påverkar urinblåsans funktion.

För att framkalla ett tillstånd som efterliknar IC användes en etablerad metod i vilken råttor injicerades med substansen cyklofosfamid (CYP). Denna substans används inom sjukvården för att behandla vissa former av cancer och svåra varianter av multipel skleros (MS). En känd biverkning av denna behandling är uppkomsten av cystit, och två dygn efter injektion med CYP uppvisade 100% av försöksdjuren inflammation i urinblåsan. Denna cystit är på flera sätt mycket lik den som finns hos patienter med IC. Under studierna noterades konsekvent att lagret av urotelceller var tjockare i inflammerade urinblåsor. Detta ledde till ett intresse att undersöka hur celldelningen av urotelceller är reglerad. Specifikt ville vi undersöka om det fanns någon receptor som var extra viktig för denna celldelning. Dessa undersökningar gjordes på både friska urotelceller och cancerurotelceller från människa.

Våra djurförsök visade att inflammation (cystit) orsakad av CYP påverkar urinblåsans förmåga att kontrahera. Eftersom blockad av frisättningen av NO från urotelceller vid cystit konsekvent förbättrade urinblåsans förmåga att kontrahera drar vi slutsatsen att NO utövar en dämpande effekt på funktionen i den inflammerade urinblåsan. En minskad frisättning av NO var möjlig att åstadkomma genom att antingen blockera bildningen av NO, blockera muskarina receptorer eller helt enkelt ta bort urotelcellerna. Resultaten från dessa studier bekräftade därmed att NO frisätts från urotelceller och visade att denna frisättning kunde minskas genom att blockera muskarina receptorer. Utifrån experimenten konstaterade vi att denna muskarina receptor med ganska hög sannolikhet är av typen M5. Om våra resultat är giltiga även för människa kan det innebära en möjlighet att behandla den överaktivitet som uppstår hos den inflammerade urinblåsan med ett läkemedel som selektivt påverkar

den muskarina M5 receptorn, och därigenom påverkar frisättningen av NO.

Våra försök på urotelceller indikerade att s.k. adrenoceptorer är viktiga för regleringen av celldelning. Vi kunde se att aktivering av en typ av adrenoceptor (β) ledde till ökad celldelning, medan aktivering av en annan typ (α) ledde till minskad celldelning. Kolinerg stimulering, t.ex. via muskarina receptorer, tycktes inte påverka celldelningen.

En intressant fråga som ännu inte besvarats är exakt vilken roll (eller roller) NO spelar under utvecklingen av cystit. Det kan vara så att NO enbart är en produkt av inflammationen, eller så är NO en av de molekyler som driver på utvecklingen av inflammationen. Det kan till och med vara så att det är en kombination av båda dessa. Om NO är med och driver på utvecklingen av inflammationen skulle det teoretiskt sett vara möjligt att förhindra uppkomsten av cystit genom att hämma bildningen av NO. Detta skulle visserligen kräva att man utvecklar ett test för att upptäcka inflammationen när den är i sin linda, men skulle potentiellt sett kunna minska lidandet för miljontals människor världen över.

Sammanfattningsvis visar denna avhandling att råttans urinblåsa förändras vid inflammation. Betydelsen av NO frisatt från urotelceller ökar, och detta NO minskar urinblåsans förmåga att kontrahera. Frisättningen sker, åtminstone till viss del, som svar på aktivering av muskarina receptorer i urotelcellerna. Dessa muskarina receptorer är med stor sannolikhet av typen M5. Framtida studier bör göras för att avgöra om våra fynd är tillämpbara även på människa. Framtida experiment bör också göras för att undersöka betydelsen av NO vid utvecklingen av cystit.

List of Publications

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

I. **Andersson MC.**, G. Tobin & D. Giglio, 2007

Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide-induced cystitis of the anaesthetized rat

Br J Pharm 2008 Apr;153(7):1438-44. Epub 2008 Feb 4

II. **Andersson M.**, P. Aronsson, D. Doufish, A. Lampert & G. Tobin

The muscarinic M5 receptor is the primary mediator of urothelium-derived nitric oxide effects in the rat urinary bladder

Manuscript

III. **Andersson M.**, P. Aronsson, D. Giglio, A. Wilhelmson, P. Jeřábek & G. Tobin, 2010

Pharmacological modulation of the micturition pattern in normal and cyclophosphamide-pretreated conscious rats

Auton Neurosci 2010. Epub 2010 Sep 17

IV. **Andersson M.**, P. Aronsson, D. Eskandari, D. Giglio & G. Tobin

Characterization of receptor-mediated proliferation in the human bladder urothelial UROtsa and T24 cell line

Manuscript

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List of Abbreviations

4-DAMP	4-diphenylacetoxy-*N*-methylpiperidine methobromide
AC	adenylate cyclase
ACh	acetylcholine
ANOVA	analysis of variance
APF	anti-proliferative factor
ATP	adenosine 5'-triphosphate
BPH	benign prostatic hyperplasia
BPS	bladder pain syndrome
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CYP	cyclophosphamide
DAG	diacylglycerol
DMEM	Dulbecco´s modified Eagle medium
DO	detrusor overactivity
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
HB-EGF	heparin-binding epidermal growth factor-like growth factor
IC	interstitial cystitis
IL-6	interleukin-6
IP_3	inositol triphosphate
L-748,337	*N*-[[3-[(2S)-2-Hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino] propoxy]phenyl]methyl]-acetamide
L-NAME	N^{ω}-nitro-L-arginine methyl ester hydrochloride
L-NNA	N^{ω}-nitro-L-arginine
LUTD	lower urinary tract dysfunctions
MeCh	methacholine
MS	multiple schlerosis
NA	noradrenaline
NANC	non-adrenergic, non-cholinergic

NO	nitric oxide
NOS	nitric oxide synthase
OAB	overactive bladder
PBS	phosphate buffered saline
PEST	penicillin/streptomycin
pFHHSiD	*p*-fluoro-hexahydro-sila-diphenidol hydrochloride
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
SR59230A	3-(2-Ethylphenoxy)-1-[[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxalate salt
UTP	uridine 5'-triphosphate

Introduction

Imagine life without the urinary bladder. Day in and day out it enables us to store and dispose of numerous waste products filtered from the blood. Without it, the urinary system would be dysfunctional. For most people, the actions of the urinary bladder passes unnoticed, until it malfunctions. In Sweden more than 400 000 people have daily symptoms of urinary incontinence, and annually approximately 4.5% of all diagnosed cancer has its origin in the urinary bladder. Many of the treatments for diseases affecting the urinary bladder are suboptimal, rendering the need for greater knowledge, in order to be able to generate improved treatments.

Anatomy of the urinary bladder

The urinary bladder is a hollow muscular organ situated in the anterior part of the lower abdomen, resting on the pelvic floor (Figure 1). Its basic functions are to store and void urine. The maximum capacity of the urinary bladder varies interindividually, but various sources say somewhere between 0.5 – 1 L in the healthy bladder. Urine produced in the kidneys enters the bladder via the two ureters. The entrances for these are situated on each side of the posterior wall of the bladder, also known as the bladder base. The empty bladder has a pyramidal shape, giving it, apart from the posterior base, a superior side, two inferiolateral sides and an apex (Figure 1).

Voiding is performed by contraction of the large smooth muscular layer known as the detrusor. Urine thereby exits the bladder via the urethra, which is situated in the most inferior part of the bladder (Figure 2a). The involuntarily controlled internal sphincter keeps the urine from leaking into the urethra and remains closed until the detrusor contracts and the pressure inside the bladder, the intravesical pressure, rises. The area surrounding the internal

sphincter is known as the bladder neck, and its contractile properties differ slightly from the rest of the detrusor, relaxing while the rest of the detrusor contracts. Eventually, urine will be voided once the external urethral sphincter, a circular band of skeletal muscle which is under voluntary control, is opened.

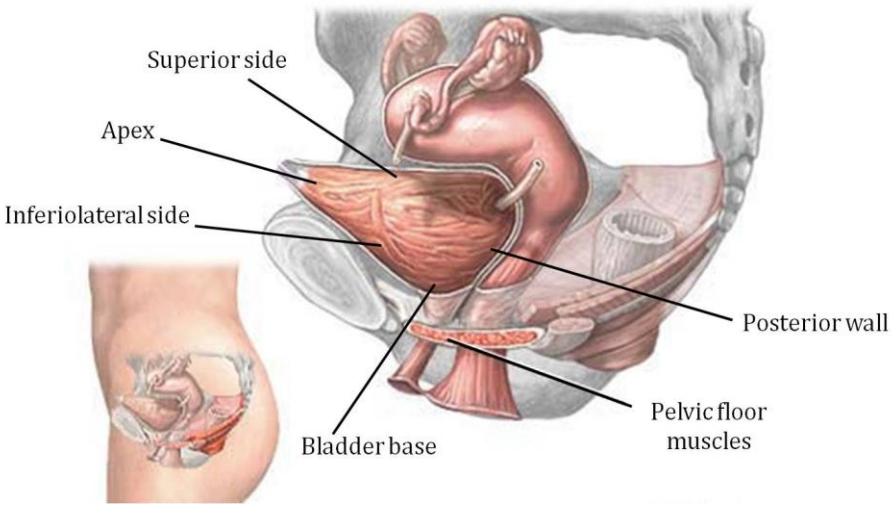

Figure 1. Lateral view of the female lower abdomen. The pyramidal shaped urinary bladder is situated in the anterior part of the lower abdomen, resting on the pelvic floor. Adapted from http://en.wikipedia.org/wiki/Abdomen

The inner surface of the detrusor, the mucosa, is folded into rugae when the bladder is empty. As the bladder fills up, the surface turns smooth. However, one area remains smooth at all times, the trigone. This is a triangular shaped area of the detrusor, situated between the openings of the two ureters and the urethral opening. The mucosa in this area is thicker, and the population of receptors in the smooth muscle has been shown to slightly differ from the rest of the detrusor (Saito *et al.*, 1997).

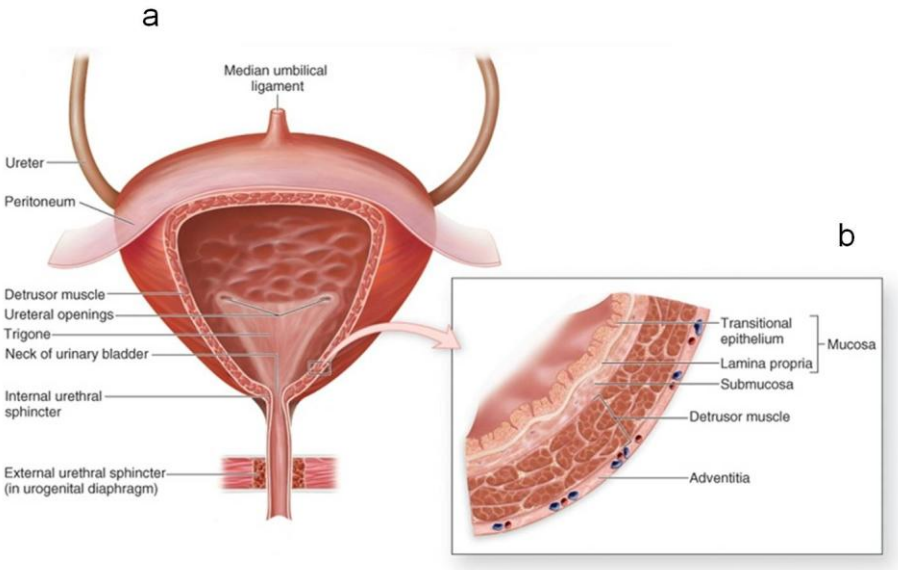

Figure 2. Anatomy of the urinary bladder. (a) Anterior view of the urinary bladder. Urine enters the urinary bladder via the ureters, and exits via the urethra. (b) Cross-section of the bladder wall. Reprinted with permission (Absolute Medical, 2010).

Urothelial morphology

From the inside and outwards, the bladder consists of a mucosal layer known as the urothelium, a suburothelium, three muscular layers and a cover of serosa (Figure 2b). The three smooth muscle layers consist of a circular muscle layer interlaced between two longitudinal muscle layers. The urothelium is a stratified epithelium consisting of 3-6 cell layers; a basal layer, one or more intermediate layers and a surface layer (Jost *et al.*, 1989). The size of the urothelial cells increase from the base to the surface; the smallest being the basal cells, intermediate cells being intermediate also in size, and surface cells being the largest. Surface urothelial cells, often referred to as umbrella cells due to their hexagonal umbrella-like morphology, are laterally connected by tight junctions (Apodaca, 2004). Together with a cover of glycosaminoglycan and crystalline proteins, uroplakins, these tight junctions maintain a barrier

between the urine and the bloodstream (Parsons *et al.*, 1990; Hu *et al.*, 2002; Born *et al.*, 2003). Immediately beneath the urothelium one finds a thin basal lamina, separating the urothelium from the lamina propria. The lamina propria is today often referred to as part of the suburothelium. The suburothelium consists of connective tissue (bundles of collagen and elastic fibers), muscular bundles, afferent and efferent nerve fibers and a numerous amount of capillaries. Thus, the urothelium is not vascularized nor directly innervated by the nervous system, but is still in close vicinity to capillaries and nerve endings. Within the suburothelium one also finds myofibroblasts. While the function of these cells has not been entirely established, it has been suggested that they function much in the same way as cells of Cajal do in the gut, modulating neurotransmission and bladder function (Wiseman *et al.*, 2003; Tobin *et al.*, 2009).

Normal bladder function

The urinary bladder has two basic functions; storage of urine and emptying of urine. Since the production of urine is constantly active, at all given times one of these functions are active. One often refers to the urinary bladder being in either the filling or the emptying phase. The sensations during the filling phase can be divided into three distinct happenings; initial sensation of filling, first desire to void and strong desire to void. During the filling phase, there is a concomitant increasing contraction of the bladder outlet region (bladder neck and urethra), and increasing relaxation of the detrusor. At the same time, muscles and connective fibers in the pelvic floor help maintain continence (Sampselle & DeLancey, 1998). The increasing relaxation of the detrusor as the bladder fills up yields a situation where despite bladder filling, the intravesical pressure remains rather constant. However, the bladder cannot fill up forever and eventually the

intravesical pressure will increase. As the bladder continues to fill up, the balance is shifted towards decreased contraction of the bladder outlet region, and increased contraction of the detrusor. Afferent pressure sensing systems (stretch receptors) are activated, rendering awareness to us that the bladder needs to be emptied. Before we can empty the bladder the outer sphincter has to be opened. This sphincter is via the pudendal nerve under our voluntary control, at least in healthy individuals. Our voluntary opening of the outer sphincter initiates the micturition reflex, causing the bladder outlet region to relax and the detrusor to contract.

Like most organs in the human body, the urinary bladder is innervated by both sympathetic and parasympathetic nerve fibers. Activation of these nerve fibers leads to activation of muscarinic and adrenergic receptors in the detrusor, respectively. The muscarinic receptor population in the urinary bladder consists of five subtypes, denoted M1-M5 (Caulfield & Birdsall, 1998; Giglio & Tobin, 2009). Several studies have shown that M2 is the most abundantly expressed subtype in the detrusor, with roughly a 3:1 ratio over the functionally most important muscarinic receptor, the M3 receptor (Eglen *et al.*, 1994; Wang *et al.*, 1995; Yamaguchi *et al.*, 1996; Hegde & Eglen, 1999; Sigala *et al.*, 2002). The overall distribution of muscarinic receptors varies throughout the urinary bladder, with a generally lower concentration in the bladder neck (Saito *et al.*, 1997). Three of the muscarinic receptor subtypes, namely the M1, M3 and M5 receptor, are $G_{q/11}$ coupled receptors whose activation induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol triphosphate (IP_3) by phospholipase C (PLC; Figure 3). Both products (DAG and IP_3) increase the amount of intracellular calcium (Ca^{2+}). In the detrusor this results in an increased tonus. The other two subtypes are G_i coupled, and even if several pathways have been suggested (Bolton & Zholos, 1997; Togashi *et al.*, 1998), in man they predominantly inhibit adenylate cyclase (AC) upon activation (Figure 3).

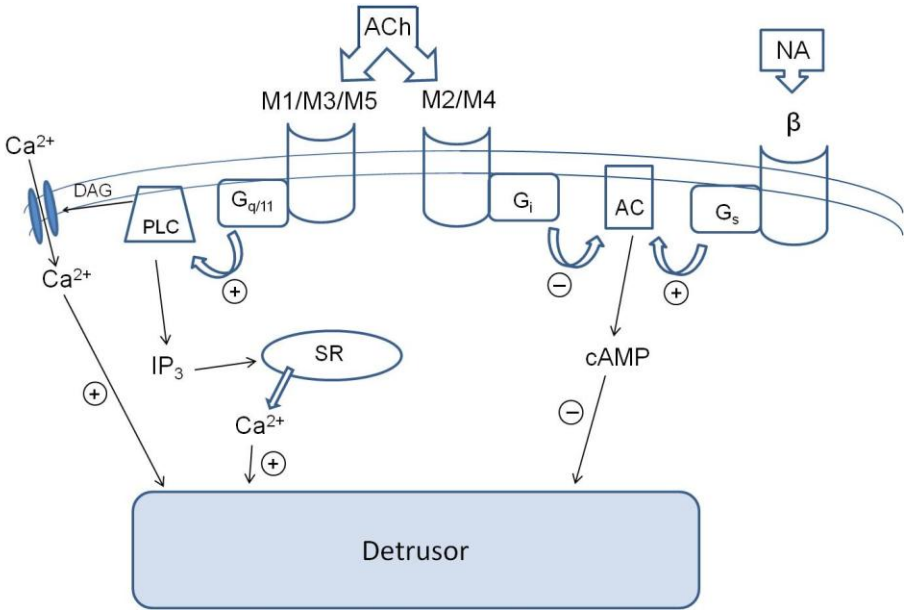

Figure 3. Main intracellular pathways of muscarinic and β-adrenergic receptors in the urinary bladder. An increase of intracellular calcium causes an increase in detrusor tonus, while an increase of intracellular cAMP causes a decrease in detrusor tonus. The increase of intracellular calcium upon muscarinic M1/M3/M5 receptor activation is achieved through two separate mechanisms; (1) diacylglycerol (DAG) in the cell membrane causes an influx via L-type Ca^{2+} channels and (2) calcium is released from the sarcoplasmatic reticulum (SR) upon inositol triphosphate (IP_3) stimulation.

The adrenergic receptors, or adrenoceptors, which are widespread throughout the lower urinary tract, are divided into the α- and β-adrenoceptors. The α-adrenoceptors are further subdivided into the α_1- (α_{1A}, α_{1B} and α_{1D}) and α_2-($\alpha_{2A,}$ α_{2B} and α_{2C})adrenoceptors. The β-adrenoceptors are divided in to three subtypes; the β_1-, β_2- and β_3-adrenoceptor. All subtypes are found in the urinary bladder of both man and rat, predominantly in the bladder body (Levin *et al.*, 1988; Seguchi *et al.*, 1998). One study determined the β_3 subtype to be the most abundant in the human bladder in regard to mRNA expression (Nomiya & Yamaguchi, 2003). Activation of β-adrenoceptors mainly

leads to the formation of cyclic adenosine phosphate (cAMP), and the subsequent lowering of detrusor tonus (Figure 3).

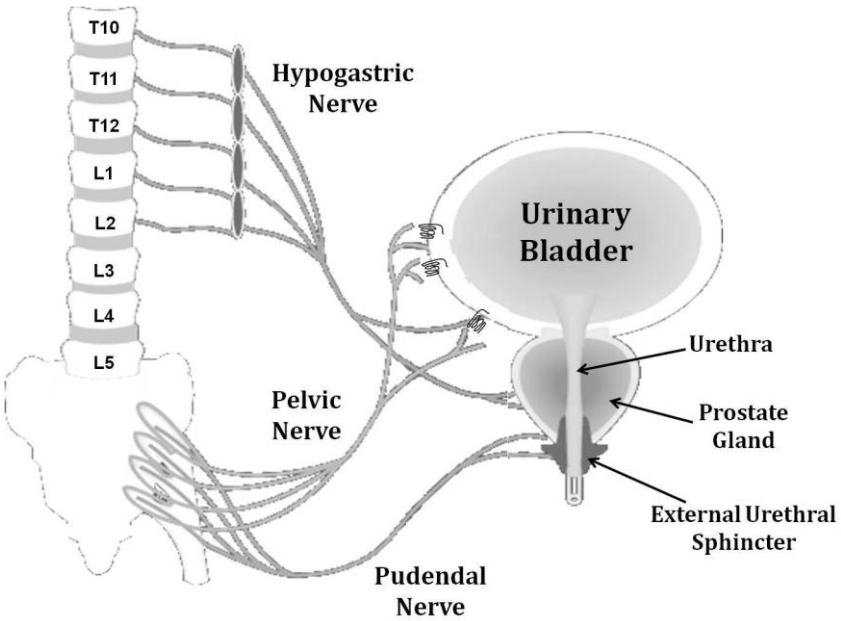

Figure 4. Autonomic innervation of the male urinary bladder, prostate gland and urethra. The sympathetic nervous system innervates the urinary bladder and prostate gland mainly via the hypogastric nerve. The parasympathetic nervous system innervates the urinary bladder mainly via the pelvic nerve. The external urethral sphincter, which is under voluntary control, is primarily innervated by the pudendal nerve.

The efferent nervous system

Sympathetic and parasympathetic nerve fibers reach the urinary bladder mainly via the hypogastric and pelvic nerve, respectively (Figure 4). During the filling phase, it is predominantly the sympathetic nerve fibers in the hypogastric nerve that are active, maintaining tonus in the bladder neck to avoid unwilling leakage of urine. At the same time, centers in the brain and brainstem act to

suppress the parasympathetic activity. When the threshold is reached, and we have willingly opened the outer sphincter, the balance between sympathetic and parasympathetic activation is reversed. Then the pelvic nerve signals dominate, causing a contraction of the detrusor and a relaxation of the bladder neck and urethra.

During the filling phase, several receptor subtypes are involved in the maintaining of constant intravesical pressure. Adrenergic α_1-receptors, particularly of the α_{1A}-subtype, are dominant in the bladder neck region, causing contraction of the smooth muscle (Caine *et al.*, 1975; Andersson *et al.*, 1984; Walden *et al.*, 1997; Chang *et al.*, 2000). Partly therefore, α_1 antagonists are used to relieve outlet obstruction symptoms in elderly men with benign prostatic hyperplasia (BPH). However, α_1-adrenoceptors are poorly expressed in the rest of the detrusor (Goepel *et al.*, 1997; Malloy *et al.*, 1998; Sigala *et al.*, 2004), and their physiological relevance there seems to be of low importance (Ueda *et al.*, 1984; Lluel *et al.*, 2003). Instead, β-adrenoceptors are responsible for relaxation of the detrusor during the filling phase (Figure 5; Elmer, 1974; Nergardh *et al.*, 1977; Abdel-Rahman *et al.*, 1983). In man, it is predominantly the β_3-adrenoceptor that mediates relaxation, while in rat it is a combination of β_2- and β_3-adrenoceptor activation (Oshita *et al.*, 1997; Igawa *et al.*, 1998; Yamazaki *et al.*, 1998; Igawa *et al.*, 2001). Studies have shown that β-adrenergic tone increases with increasing intravesical pressure (Lecci *et al.*, 1998) and that the nerve fibers that mediate this relaxation are sensitive to both prostaglandins, neurokinin A and capsaicin (Tucci *et al.*, 2002).

Noradrenaline (NA) is the key signal substance of efferent sympathetic innervation. Upon activation, the release of NA can be modulated by several mechanisms, including negative feedback on pre-junctional α_2-adrenoceptors (Mattiasson *et al.*, 1987; Somogyi & de Groat, 1990). However, in some species such as pig, cat and

guinea-pig, bladder smooth muscle seems to lack expression of α_2-adrenoceptors, and it is unclear whether α_2 activation affects bladder function other than via blood flow regulation (Ueda *et al.*, 1984; Monneron *et al.*, 2000). In rat, the release of NA can also be facilitated by both activation of muscarinic M1 receptors as well as α_1-adrenoceptors (Somogyi *et al.*, 1995; Somogyi *et al.*, 1996).

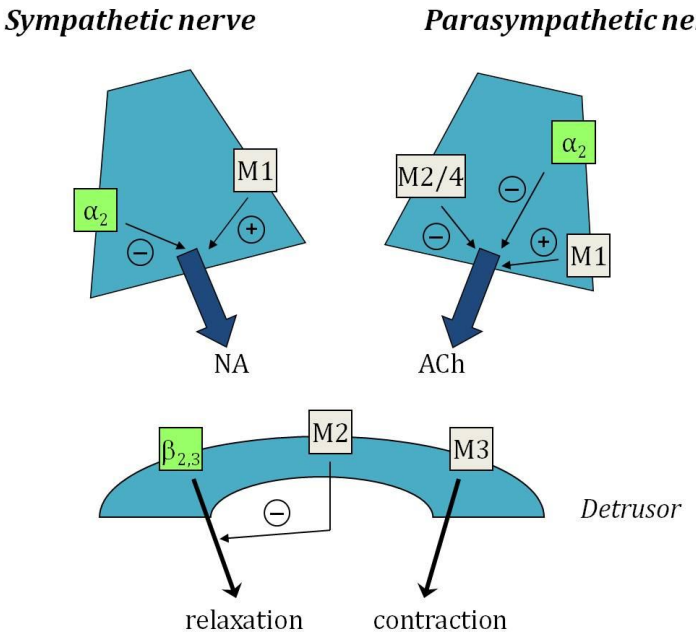

Figure 5. Depiction of the nervous control of detrusor tonus. Activation of the sympathetic nervous system leads to relaxation of the detrusor, which is mediated mainly via activation of β-adrenergic receptors. Activation of the parasympathetic nervous system leads to contraction (increased tonus) of the detrusor, which is mediated directly via muscarinic M3 receptors and indirectly via muscarinic M2 receptors.

The primary signal substance released by efferent parasympathetic nerves, activating muscarinic receptors throughout the urinary

bladder, is acetylcholine (ACh). Binding of ACh to muscarinic receptors in the detrusor causes it to contract, rendering the bladder to empty. Even though it is the M3 receptor that is considered the functionally most important subtype for detrusor contraction, it is thought that there is a dual effect between the activation of M3 and M2 receptors (Figure 5; Hegde & Eglen, 1999). In the healthy human bladder, the M3 subtype has been shown in functional studies to be responsible for direct contraction (Chess-Williams *et al.*, 2001; Fetscher *et al.*, 2002). Second messenger studies have confirmed these results, showing an increased formation of IP_3 upon M3 activation (Noronha-Blob *et al.*, 1989; Andersson *et al.*, 1991). Also, studies on M3 knock-out mice revealed a 95% reduction in bladder contractile response to carbachol (Matsui *et al.*, 2000).

Activation of M2 receptors has been suggested to yield an indirect contraction by inhibiting β-adrenoceptor and purinergic receptor mediated relaxation (Hegde *et al.*, 1997; Giglio *et al.*, 2001; Chess-Williams, 2002; Giglio *et al.*, 2005a). These findings are supported by second messenger studies that show a decrease of cAMP upon carbachol stimulation (Noronha-Blob *et al.*, 1989; Harriss *et al.*, 1995). Even though it has never been shown in man that M2 plays a significant role in the direct contraction of the healthy urinary bladder, some studies indicate an increased role of M2-mediated detrusor contraction during disease (Eglen *et al.*, 1994; Braverman *et al.*, 1999).

The release of ACh from parasympathetic nerve endings can be both inhibited and facilitated via activation of different muscarinic receptors. Prejunctional M1 receptors have been shown to facilitate the release of ACh in several species (Somogyi *et al.*, 1994; Tobin & Sjogren, 1995; Inadome *et al.*, 1998). It has also been shown that this facilitatory effect can increase during hyperreactive disease states (Somogyi *et al.*, 1998). Which prejunctional muscarinic receptor that inhibits ACh release via negative feedback seems to vary among

species (for review see Somogyi *et al*. (1999)). Prejunctional M2/M4 receptors have been found to inhibit the release of ACh in rat and rabbit (Tobin, 1995; Tobin & Sjogren, 1995; Braverman *et al.*, 1998), and later studies indicated that M4 receptors do the same in rat and man (D'Agostino *et al.*, 1997; D'Agostino *et al.*, 2000). Apart from muscarinic receptors, also the activation of α_1- and α_2-adrenoceptors has been shown to facilitate and inhibit the release of ACh, respectively (Nakamura *et al.*, 1984; Somogyi *et al.*, 1995; Tobin & Sjogren, 1998; Szell *et al.*, 2000).

In several species it has been shown that there can be other signal substances than ACh that are involved in bladder emptying. In cat and rat for instance, adenosine 5'-triphosphate (ATP) is co-released together with ACh from parasympathetic nerve fibers (MacKenzie *et al.*, 1982; Chancellor *et al.*, 1992; Palea *et al.*, 1993; Theobald, 1995). In these species ATP acts upon $P2X_1$ receptors (Aronsson *et al.*, 2010), causing a fast transient contraction which possibly is the initiator of the detrusor contraction (for review see Burnstock, 2009). The co-release of ATP makes up the most important part of the so called atropine-resistant component of parasympathetically induced bladder contraction, a part that hence cannot be blocked by the non-selective muscarinic antagonist atropine. In man, even though it has been hard to show that the atropine-resistant component is of any great importance in the healthy bladder, ATP can play a role in the activation of the detrusor during certain disease states and in the aged bladder (Sjogren *et al.*, 1982; Bayliss *et al.*, 1999; Yoshida *et al.*, 2001). On the other hand, the atropine-resistant component in the healthy bladder has been shown to be of greater importance in both rabbit (Longhurst *et al.*, 1984), guinea-pig (Chesher & Thorp, 1965), cat (Theobald, 1996) and rat (Giglio *et al.*, 2007).

The afferent nervous system

Several afferent systems in the urinary bladder work together in order to maintain optimal bladder function. Afferent, or sensory, nerve fibers are widespread throughout the detrusor, some which extend into the basal layer of the urothelium (Andersson, 2002a). These nerve fibers are mainly conveyed via the pelvic nerves, but also via the hypogastric and pudendal nerve (Andersson, 2002a). The sensory nerves involved are both of the myelinated Aδ-fiber and unmyelinated C-fiber type. Roughly, the Aδ-fibers convey sensation of filling while the C-fibers mediate thermal stimulus and relay information of chemical irritation (Janig & Morrison, 1986; Fall *et al.*, 1990; Habler *et al.*, 1990). Stretch receptors in the bladder wall are stimulated by increased intravesical pressure. These receptors activate the Aδ afferent nerve fibers in the pelvic nerve that carry the signal to the sacral spinal cord. The afferent signaling can be modulated by several transmitters, including ATP (Andersson, 2002b).

Adrenergic α_{1A}-receptors found in the urothelium have been shown to modulate bladder afferent activity in rats, shortening the intercontraction interval (Yanase *et al.*, 2008). The physiological relevance of this finding is debatable, but perhaps valid during certain patophysiological conditions that can give increased concentrations of NA in the urine. Several other substances have also been shown to be able to modulate afferent activity including neutrophins, prostaglandins and tachykinins (Yoshimura & de Groat, 1997). While prostaglandins mainly seem to act as sensitizers of afferents, intravesically administered tachykinins are able to cause direct contraction of the detrusor (Ishizuka *et al.*, 1995a, b).

The urothelium

For a long time the urothelium was considered solely a protective barrier, shielding the urinary bladder, as well as the rest of the body, from the toxic and corrosive urine. But during the last decade several studies have showed that the urothelium does much more than so. For instance, the urothelium has been shown to respond to its environment in regard to luminal pressure and urine composition by releasing diffusible agents such as acetylcholine (ACh), ATP and nitric oxide (NO) (Ferguson *et al.*, 1997; Klapproth *et al.*, 1997; de Groat, 2004). To exemplify, the mechanism of stretch-induced ATP release from the basolateral side of urothelial cells is well established (Ferguson *et al.*, 1997). It has also been shown that the release of ATP can increase upon infection (Save & Persson, 2010). This release, and that of other diffusible agents, can in turn affect afferent nerves and smooth muscle bundles in the suburothelium, thereby assigning the urothelium modulatory properties. Within the suburothelium one finds nerve fibers reactive to cholinergic, adrenergic, purinergic (P2X) and peptidergic stimuli (Jen *et al.*, 1995). Since receptor stimulation on some of these nerves has been shown to evoke exocytosis, they could be both of an afferent and paracrine/modulatory nature at the same time. Interestingly, the expression and function of the diffusible agents from the urothelium that can affect these nerves can be altered by urinary bladder diseases such as cystitis (Sun *et al.*, 2001; Giglio *et al.*, 2005b; Smith *et al.*, 2005). Further, the urothelium is capable of directly responding to various stresses, such as ischemia and urinary tract infections (Mysorekar *et al.*, 2002).

Receptor	Activator	Method of detection	References
Muscarinic (M1-M5)	ACh	IHC, IB RT-PCR	Giglio *et al.*, 2005 Tyagi *et al.*, 2006
Nicotinic (subunits alpha 3, 5, 7, 9, 10 and beta 3, 4)	ACh	RT-PCR	Beckel *et al.*, 2006 Bschleipfer *et al.*, 2007
Purinergic ($P1_{A1}$, $P1_{A2A}$, $P1_{A2B}$, $P1_{A3}$)	adenosine	IB	Yu *et al.*, 2006 Säve *et al.*, 2009
Purinergic ($P2X_{2,3}$ and $P2Y_{1,2,4}$)	ATP	ICC, IB, PCR RT-PCR	Chopra *et al.*, 2008 Tempest *et al.*, 2004
Adrenergic (α_{1A}, α_{1D} and β_{1-3})	A/NA	IB, IHC RT-PCR ISH	Ishihama *et al.*, 2006 Otsuka *et al.*, 2008 Walden *et al.*, 1997
PAC1, VPAC1, VPAC2	VIP/PACAP	ICC RT-PCR	Braas *et al.*, 2006 Girard *et al.*, 2008
EGFR	EGF	IHC	Røtterud *et al.*, 2005
Tachykinin (NK1)	substance P	RT-PCR, IB IHC	Sanchez Freire *et al.*, 2010
Vanilloid (TRPV; VR1)	pH, heat, capsaicin	RT-PCR, ICC	Birder *et al.*, 2001
Bradykinin (B2)	bradykinin	RT-PCR, ICC	Chopra *et al.*, 2005

Table 1. Receptor expression in urothelial cells. ACh - acetylcholine; ATP - adenosine 5'-triphosphate; A - adrenaline; NA - noradrenaline; VIP - vasointestinal peptide; PACAP - pituitary adenylate cyclase activating polypeptide; EGF - epidermal growth factor; RT-PCR - reverse transcriptase polymerase chain reaction; IHC - immunohistochemistry; IB - immunoblotting; ICC - immunocytochemistry; ISH - in situ hybridization.

All five muscarinic receptor subtypes have been shown to be present in both human and rat urothelium (Giglio *et al.*, 2005b; Tyagi *et al.*, 2006). Activation of these has been shown to lead to a number of events, showing that they possess functional properties. For instance, in both man and pig an unidentified relaxant factor has been shown to be released from the urothelium upon muscarinic receptor activation (Hawthorn *et al.*, 2000; Chaiyaprasithi *et al.*, 2003). Others have shown that activation of urothelial muscarinic receptors can lead to the release of ATP, which possibly modifies the bladders afferent response (Kullmann *et al.*, 2008a; Kullmann *et al.*, 2008b; Munoz *et al.*, 2010). In turn, ATP and uridine 5'-triphosphate (UTP) have been shown to yield larger contractions in intact than urothelium-denuded strips of guinea-pig detrusor (Sui *et al.*, 2008), further demonstrating the presence of functional urothelial receptors. The most likely candidates for this functional response are of the P2X subtype, but also the presence of P1 and P2Y receptors has been shown in the urothelium (Tempest *et al.*, 2004; Yu *et al.*, 2006; Chopra *et al.*, 2008). Apart from muscarinic and purinergic receptors, urothelial cells express a vast array of receptors (see Table 1).

Nitric oxide

Nitric oxide (NO) is generally accepted as one of the non-adrenergic, non-cholinergic (NANC)-transmittors affecting the bladder (Bult *et al.*, 1990; Andersson & Persson, 1994). Studies using a NO scavenger have shown that NO can be involved in normal bladder activity, and that the removal of NO can cause a condition similar to overactive bladder (Pandita *et al.*, 2000). During conditions such as cyclophosphamide (CYP)-induced cystitis, nitric oxide synthase (NOS) has been shown to be up-regulated (Alfieri & Cubeddu, 2000; Giglio *et al.*, 2005b), and NO has been suggested to not only affect the detrusor, but also act directly on afferent pathways (Ozawa *et al.*, 1999; Yoshimura *et al.*, 2001).

NO can be released in the bladder in several ways. Studies have shown the presence of nitric oxide containing nerve fibers in the suburothelium, but their exact function is still unclear (Smet *et al.*, 1996). Others have seen release of NO from cultured urothelial cells in response to muscarinic agonists (Kullmann *et al.*, 2008b), and from urinary bladder strips in response to adrenergic agonists (Birder *et al.*, 1998). A recent study by Munoz *et al*. (2010) could show the release of NO from the urothelium upon muscarinic receptor stimulation, but also other parts of the rat urinary bladder could release NO, most likely the suburothelium.

Urinary tract disorders – diseases and treatments

OAB - Overactive bladder

One of many lower urinary tract dysfunctions (LUTD) is overactive bladder (OAB), defined as *urgency, with or without incontinence, usually accompanied by increased frequency and nocturia* (Abrams *et al.*, 2002). For a patient to be diagnosed with OAB, a concomitant infection has to be ruled out. The prevalence of OAB is about 17% (Milsom *et al.*, 2001), but few prospective studies have been performed. The cause of OAB is not well understood, but the general risk of developing OAB increases with age. One possible cause of this has been shown in a study by Li *et al.* (2003) where they found the amount of binding sites for β-adrenoceptor agonists to decrease with age. Since it has been shown that the most important function of β-adrenoceptors is mediating relaxation of the detrusor during bladder filling, it would be logical that a decrease of sympathetic activity yields an increase in detrusor sensitivity. However, the most widespread theory is increased afferent activity, either depending on increased sensitivity or an increased number of afferents (Drake, 2008).

β_3-agonists, due to their hampering of bladder contraction, have been suggested to be a possible treatment for OAB, but to date no such drug has been successfully developed. During the testing of experimental β_3-agonists on rats, induced cystitis and renal tubular necrosis was reported (Waghe *et al.*, 1999). Since the beneficial site of action very well may be at the spinal level (Durant *et al.*, 1988), intrathecal administration could be a possible route of administration, but that is something that most patients would probably wish to avoid. However, the adverse effects seem to vary considerably between different substances, and some promising oral β_3 agonist candidates are currently in phase II or phase III trials (for review see Drake, 2008).

Despite the promising future, the drug treatments available today are few. Patients are advised to manage their fluid intake and avoid certain drinks, for instance those containing caffeine. The by far most common drug treatment is antimuscarinic drugs, which give good clinical effect initially, but regular long-term loss of function and compliance problems (Andersson, 2009). Apart from the antimuscarinic drugs and β_3-adrenoceptor agonists, Ca^{2+}-antagonists and K^{+}-channel openers have been suggested as possible inhibitors of detrusor activity (Guarneri *et al.*, 1991; Pandita *et al.*, 2006). Also, drugs that reduce afferent activity and/or affect NK-1 receptors at the spinal level are under development and are currently in clinical phase II or III trials (for review see Drake, 2008).

Acute and chronic incontinence

Urinary incontinence, or enuresis, is defined as *involuntary leakage of urine* (Abrams *et al.*, 2002). This involuntary leakage can either be a constant dribbling or large emptying of the bladder. The large emptying can be caused by stresses such as sneezing or coughing, or be so called urge incontinence, a condition where the patient feels the urge to micturate, but does not have time to reach the toilet

before the expel of urine. While stress incontinence is by far the most common form in women (80%), in men urge incontinence is the most widespread form (Hampel *et al.*, 2004). Incontinence in any form is a very common condition, and prevalence increases with increasing age. It has been estimated that 250 million people worldwide suffer from incontinence, and in Sweden the prevalence among 80-year olds is as high as 25% (Fall, 1999; Milsom, 2009).

Treatment of acute and chronic incontinence has, apart from lifestyle changes, focused on antimuscarinic drugs. Older drugs, such as oxybutynin, are basically unselective, whereas the development of new treatments has focused on finding M_3-selective compounds. However, no drug has yet been launched that does not cause the most common adverse effects; dry mouth and gastrointestinal disturbances. The main reason for that is surely due to the fact that these adverse effects *per se* are M_3 receptor-mediated (Tobin, 1995; Barras *et al.*, 1999). The mechanism of action of antimuscarinic drugs was first thought to be solely by blocking efferent muscarinic receptors in the detrusor. But further studies have shown that both the urothelium and afferent nerve fibers are possible sites of action (Kim *et al.*, 2005; Abrams *et al.*, 2006; Yamaguchi, 2010).

Other possible treatments include β agonists. One study found that in bladder strips taken from patients with urge incontinence, clenbuterol, a fairly β_2-selective agonist, gave a significantly greater relaxation than in bladder strips taken from patients without any history of incontinence (Hudman *et al.*, 2001). This finding in itself is interesting, but as previously discussed, it is mainly the β_3-adrenoceptor that mediates relaxation in man.

Interstitial cystitis

Interstitial cystitis (IC), which is also known as bladder pain syndrome (BPS), is a non-infectious chronic pain syndrome that is

characterized by painful urination, urgency, frequency, inflammation, stiffening of the bladder wall and visceral pain. In its classic form one finds ulcers upon cytoscopic examination, but in most cases (~80%) the disease is ulcer-free (Koziol *et al.*, 1996). As the symptoms are similar to other LUTD, and the objective diagnostic criteria are few, the epidemiology of IC is difficult to determine (Hanno, 2002). Estimations vary between 10 per 100 000 to 70 per 100 000 (Oravisto, 1975; Curhan *et al.*, 1999). The etiology of IC is mostly unknown, and treatment regimens are mainly symptomatic. However, sensitization of afferent pathways, as well as altered release of ATP and NO, has been linked to the condition (Sun *et al.*, 2001; Logadottir *et al.*, 2004). Further, the expression of muscarinic receptors has been shown to be up-regulated, and studies have linked the increase of certain inflammatory markers (mainly IL-6) and changes in the levels of proliferative factors such as anti-proliferative factor (APF), epidermal growth factor (EGF) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) in urine to the condition (Keay *et al.*, 1997; Erickson *et al.*, 2002; Sanchez Freire *et al.*, 2010).

Urinary bladder cancer

Urinary bladder cancer accounts for 4-5% of all diagnosed cancer in Sweden, making it the 6th most common form of cancer (Cancer Incidence in Sweden 2007, Socialstyrelsen, 2008; IARC Globocan, 2008). Worldwide it is the 11th most common form of cancer, accounting for about 3% of all reported cancers (IARC Globocan, 2008). However, the worldwide estimation is probably somewhat on the small side as several developing countries, with a lower probability of reporting, have high incidences of Bilharzia (also known as Schistosomiasis) infections, a known cause of bladder cancer (Elsebai, 1977; Schwartz, 1981).

The major causative agents for bladder cancer are various chemical

carcinogens, with different metabolites from cigarette smoking accounting for about half of the cases (Shirai, 1993). More particularly, these carcinogenic metabolites are thought to accumulate in the urine, irritate certain urothelial cells to the degree that they develop point mutations, and eventually cause them to transform (for review see Brandt, 2009). As a result of this, most urinary bladder cancer (~90%) has its origin in the urothelium (Cordon-Cardo, 2008). Chemical carcinogens aside, also chronic inflammation, caused by infections, indwelling catheters or pharmacological treatment, has been shown to increase the risk of developing bladder cancer (for review see Michaud, 2007). Studies on Bilharzia, both clinical and pre-clinical, have shown a chain of events starting with infection, continuing with chronic inflammation and eventually leading to bladder cancer (Samaras *et al.*, 2010). The coupling between inflammation and cancer is well-established and has been shown in several other tissues (Coussens & Werb, 2002; Ohshima *et al.*, 2003). Apart from Bilharzia infection, it has also been shown that long-term treatment (>10 years) with CYP, generating a state similar to chronic inflammation, increases the risk of developing bladder cancer (Travis *et al.*, 1995; Talar-Williams *et al.*, 1996).

Presently, the exact mechanisms of transformation of urothelial cells from healthy to malignant are incompletely understood, and therefore we today only have treatment options for already arisen tumors. In the future we hopefully will have greater knowledge of the transformational mechanisms, giving us the opportunity to develop treatment strategies that prevent the origination of bladder cancer.

Aims

The main aim of this thesis was to investigate the effects of CYP pre-treatment in rats, with special interest in the release of NO from the urothelium.

Specific aims

In vivo in the anaesthetized rat (Paper I), we wondered how CYP pre-treatment affected the muscarinic bladder contractile responses and if NO might be released from the urothelium by cholinergic stimuli. Our studies confirmed that the muscarinic bladder contractile responses were altered by CYP pre-treatment, and our data indicated that one component of this alteration was regulated by the cholinergic release of NO from the urothelium. This led us to a study *in vitro* on bladder strips (Paper II) where we wanted to characterize the muscarinic changes involved in the alterations during CYP-induced cystitis. This study confirmed the results found *in vivo* and raised the question whether or not CYP pre-treatment altered the micturition parameters in the conscious rat and, further, if muscarinic, purinergic or NOS blockade could alter these changes (Paper III). In all studies on CYP pre-treated rats, we could see morphological changes, most prominently an increase in the thickness of the bladder wall. Several studies have shown a coupling between chronic CYP-induced cystitis, including bladder wall thickening, and the development of urothelial bladder cancer. This led us to wonder how the proliferation of urothelial cells is regulated (Paper IV); more specifically, if the proliferation rate of healthy and malignant urothelial cells is receptor mediated and if there are any changes in this regulation after transformation.

Methods and Materials

All experiments conducted during this thesis were approved by the local ethics committee at the University of Gothenburg. Consistently, male rats of the Sprague-Dawley (300 – 400 g) strain were used.

Cyclophosphamide-induced cystitis

Cyclophosphamide (CYP)-induced cystitis is a common and well documented method for inducing experimental cystitis (deVries & Freiha, 1990). CYP is a DNA alkylating agent used in man to treat neoplastic diseases and multiple schlerosis. Its metabolite acrolein accumulates in the bladder lumen, where it causes severe irritation of the urothelium, subsequently inducing haemorrhagic cystitis (Cox, 1979). To induce cystitis we have given a single intraperitoneal dose of CYP (100 mg/kg) in combination with the analgesic buprenophinum (Temgesic®; 10 μg/kg). As previously seen in our lab, the peak inflammatory state arises after 60 h (Giglio *et al.*, 2005b). Depending on which type of experiment was to be carried out, we waited for an appropriate time before we conducted the experiments. I.e., for cystometrical studies and *in vitro* functional studies in the organ bath, experiments were carried out 60 h after induction of cystitis. For *in vivo* functional studies in the metabolism cage, in which the experiments were conducted during a 24 h period, the experiments were started 48 h after induction of cystitis and thus continued until 72 h post induction, comprising the 60 h peak inflammatory time point.

Removal of urothelium

To study the direct impact of the urothelium on bladder function we conducted both *in vivo* and *in vitro* experiments where the urothelium was removed. In order to achieve this, we injected

collagenase (0.1% in Hank's balanced salt solution) directly into the bladder of an anaesthetized rat, either via a secured catheter or needle. The volume injected varied between 0.2 - 0.5 mL, depending on the size of the bladder. Careful notice was taken to first empty the bladder of urine, and then properly fill it without distending it extensively. Once injected, the collagenase was left in the bladder for 30 min, and then removed via the catheter/needle. Thereafter, the serosal side of the bladder was gently rubbed, and loose urothelial cells were removed by rinsing the bladder with saline. Regularly after the conduction of the experiments, the bladder was microscopically examined in order to ensure that the urothelium was properly removed.

Cystometrical studies

Rats (300 - 350 g) were anaesthetized with an intraperitoneal injection of pentobarbitone (45 mg/kg). During the remainder of the experiment supplementary doses were given intravenously when needed. The rats were placed on a thermostatically controlled blanket, keeping their body temperature at 38°C. Airways were kept free by tracheotomy and placement of a cannula in their trachea. Catheters were placed in both the femoral artery and femoral vein, in order to monitor blood pressure and administer drugs, respectively. Two cannulas were inserted through a small incision at the apex of the bladder, where they also were fixed to the bladder with a ligature. One of the bladder cannulas was used to maintain bladder pressure at 10 - 15 mmHg at all times by injection of small volumes of saline (0.05 - 0.20 mL), and the other was used to monitor intravesical pressure. When needed, the same cannula that was used to maintain intravesical pressure was also used to instill collagenase. Both the catheter in the femoral artery and the pressure sensing cannula in the urinary bladder were connected to pressure transducers, as part of the MP100WSW data acquisition system. All data was recorded using the Acquire software (Biopac, Goleta, USA).

Adrenergic effects were blocked in all experiments by administration of the phentolamine (1 mg/kg i.v.) and propranolol (1 mg/kg i.v.). At the beginning of each experiment, regardless of series, the unselective muscarinic agonist methacholine (MeCh) was given in increasing doses (0.5 – 5 μg/kg) in order to establish a dose-response curve. Dose-response curves were therefore generated for untreated control rats and CYP pre-treated rats, with and without urothelium. In one experimental series, dose-response curves were also generated for CYP pre-treated rats, before and after removal of the urothelium, in the presence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; 30 mg/kg i.v.). In another experimental series, L-NAME (30 mg/kg i.v.) or saline (0.9% i.v.) was given to normal rats with an intact urothelium and CYP pre-treated rats with or without urothelium. Thereafter, repeated doses of MeCh (2 μg/kg i.v.) were given after increasing doses of the muscarinic M1/M3/M5-selective antagonist 4-diphenylacetoxy-N-methyl-piperidine methobromide (4-DAMP; 0.1 – 1000 μg/kg i.v.). After each experiment, rats were killed with an overdose pentobarbitone and their urinary bladders were examined macroscopically and microscopically.

In vitro functional studies – the organ bath

Experiments on detrusor contractility were conducted in an organ bath setup. Rats (300 - 350 g; n=52), either CYP(100 mg/kg i.p.) or saline (0.9%; 1 mL/kg i.p.; serving as controls) pre-treated, were anaesthetized with an intraperitoneal injection of medetomidine (Domitor®; 1 mg/kg). Thereafter, they were killed with an overdose of carbon dioxide and the urinary bladder, with an intact urothelium or after removal of the urothelium, was removed and placed in Krebs bicarbonate solution. Via a cut along the posterior surface from the urethral opening to the apex the bladder was opened, and two or three full thickness strips (6 x 2 mm) were excised from the detrusor

medially to the orifices of the two ureters. The detrusor strips were mounted between two steel rods, of which one was fixed and the other was adjustable and connected to an isometric force transducer (Linton). Thereafter, they were immersed in 25 mL organ baths containing Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 25, and glucose 5.5. The baths were kept at 37°C and gassed with 5% CO_2 in O_2. The strips were repeatedly stretched for 45 min, in order to achieve a stable tension of about 5 mN. At the beginning of each experiment, a high K^+ Krebs solution (containing 124 mM K^+; obtained by exchanging Na^+ for equimolar amounts of K^+) was administered in order to assess the viability of each strip preparation. All antagonists, as well as the NOS inhibitor N^ω-nitro-L-arginine (L-NNA), were administered 20 min before each series of the unselective muscarinic agonist carbachol. All drugs were administered to the organ baths at a volume of 125 µl. Data were recorded using a MP100WSW data acquisition system and the Acquire software (Biopac, Goleta, USA).

In vivo functional studies – the metabolism cage

For these experiments, rats (310 - 400 g; n=100) were pre-treated with either saline (0.9%; 1 mL/kg i.p.; serving as controls) or CYP (100 mg/kg i.p.), both in combination with the analgesic buprenorphinum (10 µg/kg i.p.). Forty-eight hours later, they were injected intraperitoneally with either saline (0.9%), 4-DAMP (1 mg/kg), the purinergic P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 10 mg/kg) or L-NAME (30 mg/kg) at a volume of 1 mL/kg. Thereafter, they were placed in a metabolic cage with free supply of water. During the following 24 h observation period, the rats were kept in a light-dark-light cycle of 6, 12 and 6 h. Expelled urine was gathered in a graded bottle below the cage and a laser Doppler registered each drop of urine continuously. This allowed the urine

output, number of micturitions per hour and voided volume per micturition to be calculated. Total water consumption was also measured after each experiment. All data were recorded using a MP100WSW data acquisition system and the Acquire software (BioPac, Goleta, USA).

Cell cultivation

Two human urothelial cell lines, the UROtsa and T24 cell lines, were cultivated, both growing as a monolayer. The UROtsa cell line is an immortalized urothelial cell line derived from the ureter urothelial lining (Petzoldt *et al.*, 1995). It has been shown to have several characteristics that closely resemble the intermediate layers of normal human urothelium (Rossi *et al.*, 2001). The T24 cell line is a transitional carcinoma cell line that has been shown to be non-tumorigenic (Flatow *et al.*, 1987), and is frequently used as a cell culture model to study urothelial cancer cell proliferation (Chen *et al.*, 2008; Teng *et al.*, 2008; Nakanishi *et al.*, 2009). Both cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2. The UROtsa cell line was cultured in Dulbecco´s Modified Eagle Medium (DMEM; Sigma-Aldrich, St.Louis, USA) while the T24 cell line was cultured in McCoy's 5a medium (LGC Promochem, Boras, Sweden). Both mediums were supplemented with 10% fetal bovine serum (Sigma-Aldrich, St.Louis, USA) and 1% penicillin/ streptomycin (PEST; PAA Laboratories, Pasching, Austria). Medium was renewed every other day, and when the cells reached 90% confluency they were split to a 1:3 ratio.

Measurement of proliferation

In order to measure the proliferation rate of the UROtsa and T24 cell lines, the MTT Cell Proliferation Assay was utilized. In this assay, confluent (90-95%) cells were trypsinized and resuspended in growth medium at a concentration of 400 000 cells per mL. This

concentration was determined, by a standard procedure, to yield a linear relationship between the number of cells and absorbance for both cell lines. The cells were subsequently pipetted (100 µL) into separate wells. Two hours later, drugs were added to the wells at a volume of 10 µL, and the plate was incubated overnight at 37°C in a 5% CO_2-atmosphere. Untreated cells and medium not containing cells were used as control and blank, respectively. The following day, MTT Reagent (10 µL), which enabled a spectrophotometrical quantitative measurement of the proliferation rate, was added to each well. The plate was then returned to the incubator for approximately three hours. In order for the cells to lysate, a detergent reagent (100 µL) was added to each well and the plate was put in dark, at room temperature, overnight. The proliferation rate (i.e. number of cells), equivalent to the amount of absorbance at 570 nm, was measured the following day.

Materials

Drugs

The drugs employed in this thesis were buprenorphine hydrochloride (Temgesic®, Apoteket AB, Stockholm, Sweden), cyclophosphamide monohydrate (CYP), collagenase type I (Invitrogen, Paisley, UK), acetyl-β-methylcholine chloride (methacholine; MeCh), carbamylcholine chloride (carbachol), nicotine hemisulfate salt (nicotine), 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP), p-fluoro-hexahydro-sila-diphenidol hydrochloride (pFHHSiD), pirenzepine dihydrochloride (pirenzepine), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), N^{ω}-nitro-L-arginine (L-NNA), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), chlorbenzoyl-5-methoxy-2-methylindole-3-acetic acid (indomethacin), phenylephrine hydrochloride (phenylephrine), isoprenaline hydrochloride

(isoprenaline), phentolamine methansulphate (phentolamine), doxazosin mesylate (doxazosin), propranolol hydrochloride (propranolol; ICI Pharmaceuticals, London, UK), N-[[3-[(2S)-2-Hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino]-propoxy]phenyl]methyl]-acetamide (L-748,337; Tocris, Ellisville, USA), 3-(2-Ethylphenoxy)-1-[[(1S)-1,2,3,4-tetrahydronaphth-1-yl]-amino]-(2S)-2-propanol oxalate salt (SR59230A), epidermal growth factor (EGF) and heparin-binding EGF-like growth factor (HB-EGF). Unless otherwise stated, all drugs were purchased from Sigma-Aldrich (St Louis, USA).

Solutions and reagents

The solutions and reagents used in this thesis for cell cultivation and cell proliferation assays were phosphate buffered saline (PBS; Sigma-Aldrich, St Louis, USA), Dulbecco´s Modified Eagle Medium (DMEM; Sigma-Aldrich, St.Louis, USA), McCoy's 5a medium (LGC Promochem, Boras, Sweden), fetal bovine serum (Sigma-Aldrich, St.Louis, USA), penicillin/streptomycin (PEST; PAA Laboratories, Pasching, Austria), MTT reagent (ATCC, Manassas, VA, USA), MTT detergent (ATCC, Manassas, VA, USA) and trypsin (PAA laboratories, Pasching, Austria).

Statistical calculations

Paper I

Comparisons were made between raw data or between relative data. Raw data comparisons were made when increases in bladder pressure (Δ bladder pressure) are compared between two groups. Relative data comparisons were made when comparing the effect that 4-DAMP had (% decrease) on basal contraction to MeCh.

Statistical significance was determined by Student's t-test for paired or unpaired data.

Paper II

Statistical comparisons using one-way analysis of variance (ANOVA), followed by the Bonferroni correction test, was made between raw data of mean contractile responses to carbachol in repetitive rounds. When comparing raw data (of mean contractile responses to carbachol) between control and CYP pre-treated bladder preparations, Student's t-test for unpaired data was used to determine statistical significance. Schild plots and pA2-values were estimated by obtaining dose ratios of full carbachol response curves from three antagonist concentrations (Arunlakshana & Schild, 1959). The estimated Schild slopes were compared to unity utilizing a built-in test in the GraphPad software (GraphPad Software Inc., San Diego, USA). Likewise, pharmacodynamic modeling was performed by applying built-in models for nonlinear regression.

Paper III

One- or two-way ANOVA, followed by the Bonferroni correction test, was used to determine statistical significance when comparing either (1) data measured at several time points in the saline pre-treated (control) group to data measured at several time points in the CYP pre-treated group, (2) data from saline treated (control) rats to data from rats treated with an antagonist or L-NAME, (3) data from one treatment group to combined data from two treatment groups or (4) data from saline pre-treated rats to data from CYP pre-treated rats where both groups also received equivalent treatment with 4-DAMP.

Paper IV

In this paper three types of comparisons were made. Either (1) the

proliferation rate of UROtsa cells treated with an agonist or antagonist was compared to the proliferation rate of T24 cells treated with the same agonist/antagonist, (2) the proliferation rate of cells treated with an agonist or antagonist was compared to the proliferation rate of untreated (control) cells or (3) the proliferation rate of cells treated with both an antagonist and an agonist was compared to the proliferation rate of cells treated with solely the agonist. Statistical significance was determined by Student's t-test for paired or unpaired data.

Results and Discussion

Paper I

Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide-induced cystitis of the anaesthetized rat

In anaesthetized rats, MeCh (0.5 – 5 μg/kg i.v.) generated almost similar bladder contractions in saline and CYP pre-treated rats with an intact urothelium. This finding was in contrast to previous findings *in vitro*, in which significant differences have been reported (Giglio *et al.*, 2005b; Giglio *et al.*, 2007). However, this discrepancy might be explained by a combination of several factors. Firstly, *in vivo* the intravesical pressure can only reach a certain level before the micturition reflex is initiated. This may have prevented the expected differences in maximal detrusor contractility. Secondly, the largest dose of MeCh led to a dramatic drop in blood pressure which could have affected bladder pressure (i.e. lowered the maximal response). Thirdly, *in vivo* MeCh reaches primarily the detrusor, as it is introduced to the bladder via the blood stream, while *in vitro* carbachol has equal access to detrusor and urothelium. The inhibitory CYP-induced effects seen *in vitro* might therefore be imperceptible *in vivo*.

When the response to MeCh (0.5 – 5 μg/kg i.v.) was tested in rats in the presence and absence of the urothelium, larger contractions were observed in CYP pre-treated bladders ($p < 0.05$; $n = 5$) in the absence of the urothelium. Meanwhile, the contractions were unaffected by removal of the urothelium in normal bladders. When examining the dose dependent effects of MeCh on CYP pre-treated bladders in the presence of L-NAME, no contractile alterations arose

by removal of the urothelium. These results indicate that there is a relaxatory factor present in the urothelium of CYP pre-treated bladders, and that this factor disappears upon NOS blockade.

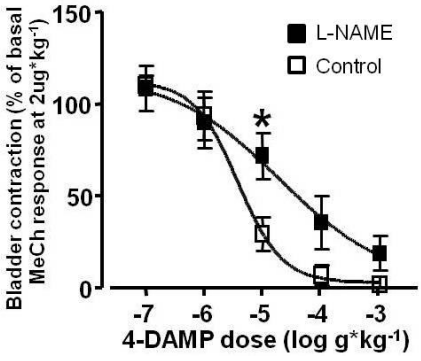

Figure 6. Contraction of intact inflamed bladders after successively larger doses 4-DAMP (0.1 – 1000 µg/kg). Mean contractile responses to MeCh (2 µg/kg) in the absence (□) and presence (■) of L-NAME (30 mg/kg). Contraction is expressed as percentage of basal response. Vertical bars represent the SEM. * $p<0.05$

Increasing doses of 4-DAMP (0.1 - 1000 µg/kg i.v.) could dose dependently inhibit bladder contractions evoked by MeCh (2 µg/kg i.v.) in both saline and CYP pre-treated bladders. This inhibition was unaffected by the presence of L-NAME as well as the removal of the urothelium in saline pre-treated rats. However, in intact CYP pre-treated bladders, blockade of NOS yielded larger contractions (Figure 6). These results showed that blockade of NOS affected bladder contractions in cystitic but not in normal bladders. Since NOS-blockade yielded larger contractions in cystitic bladders, the presence of a relaxatory NO-component was indicated. However, the source of this relaxatory NO was not revealed. For instance, it could be present in both the urothelium, suburothelial vessels and parasympathetic neurons. To examine this we ran experiments on

urothelium-denuded CYP pre-treated bladders in the presence and absence of L-NAME (Figure 7). The increased contractions to MeCh seen in cystitic bladders in the presence of L-NAME were abolished by the removal of the urothelium. This showed the presence of a relaxatory factor in the urothelium which was released upon muscarinic receptor activation.

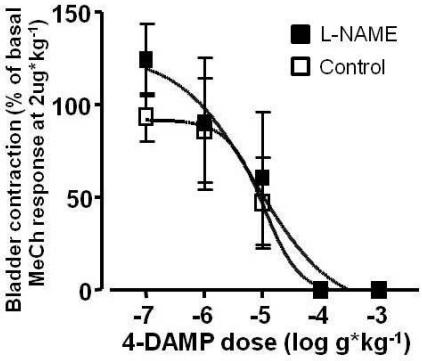

Figure 7. Contraction of urothelium-denuded inflamed bladders after successively larger doses 4-DAMP (0.1 – 1000 µg/kg). Mean contractile responses to MeCh (2 µg/kg) in the absence (□) and presence (■) of L-NAME (30 mg/kg). Contraction is expressed as percentage of basal response. Vertical bars represent the SEM.

Pre-treatment with CYP is a well established method for inducing hemorrhagic cystitis (deVries & Freiha, 1990). The macroscopical signs of successful CYP pre-treatment, i.e. bladder wall thickening/edema and hemorrhages, were present in all CYP pre-treated bladders. Removal of the urothelium by treatment with collagenase could potentially damage the suburothelium, affecting the results in an unknown fashion. However, immunohistochemical examinations revealed that the suburothelium remained intact in all

bladders and that 80-90% of the urothelium was removed by the procedure. Further, damages caused by urothelial denudation should render a lower contractile response, which was not the case.

Detrusor overactivity (DO) and increased frequency are two of the most common features of CYP-induced cystitis in both humans and animals. During our experiments, we observed small spontaneous contractions that were more frequent in cystitic bladders, especially after treatment with L-NAME. In the state of anaesthesia, these spontaneous contractions were too small to elicit a micturition. However, it cannot be excluded that these contractions could yield micturition events in the conscious rat, thereby linking the DO to increased frequency. Many patients with DO do not respond to antimuscarinic treatment. Minaglia *et al*. (2005) showed a link between patients with DO that are refractory to antimuscarinic drugs and concomitant IC. This non-responsiveness to antimuscarinic treatment could perhaps in part be explained by our findings.

The current investigation shows that CYP pre-treatment alters the cholinergic response of the urinary bladder. We show that NO is present in the urothelium during CYP-induced cystitis and that this NO exerts an inhibitory effect on detrusor contractility. Further, we suggest that NO is released upon cholinergic stimulation, possibly through activation of M5 and/or M3 receptors.

Paper II

The muscarinic M5 receptor is the primary mediator of urothelium-derived nitric oxide effects in the rat urinary bladder

In bladder strips from CYP pre-treated rats, the maximal contractile response to carbachol (0.01 - 100 μM) was significantly reduced in comparison to normal bladder strips (Figure 8; 19.3 ± 1.0 mN and 14.3 ± 1.2 mN in saline and CYP pre-treated strips, respectively; $p < 0.01$).

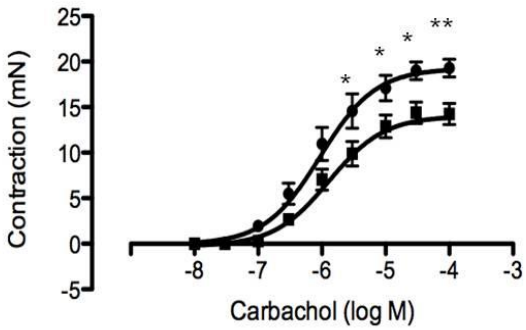

Figure 8. Contraction of intact urinary bladders. Mean contractile responses to carbachol (0.01 - 100 μM) of isolated detrusor strip preparations from saline (●) and CYP (■) pre-treated rats. Vertical bars represent the SEM. * $p<0.05$; ** $p<0.01$

A comparison of the EC_{50}-values also showed a significant right-shift of the concentration-response curve in CYP pre-treated bladder strips ($logEC_{50}$ = -6.1 and -5.4 in saline and CYP pre-treated rats, respectively; $p < 0.001$). The right shift, as well as the difference in maximal contractile response, was abolished by removal of the urothelium (Figure 9). Administration of the NOS inhibitor L-NNA

also normalized the contractile differences between normal and cystitic bladder strips (Figure 10). The same normalization of contractile responses to carbachol appeared when comparing normal and cystitic urothelium-denuded bladder strips in the presence of L-NNA.

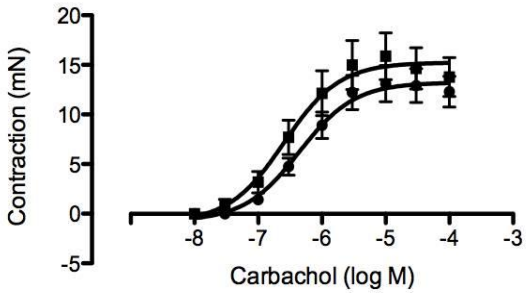

Figure 9. Contraction of urothelium-denuded urinary bladders. Mean contractile responses to carbachol (0.01 - 100 μM) of isolated detrusor strip preparations from saline (●) and CYP (■) pre-treated rats. Vertical bars represent the SEM.

These findings further strengthen the idea that in CYP-induced cystitis the urothelium exerts a relaxatory influence on the detrusor. Further, since it was possible to block this influence by administration of L-NNA, NO is likely to be the primary inhibitory factor. Other possible relaxatory factors include prostaglandins and adenosine. Particular interest should be taken to the latter, as it has been shown to exert a relaxatory influence on the detrusor (Aronsson *et al.*, 2010). However, we could not see any effect of indomethacin, an inhibitor of COX, on the contractile responses, and studies have shown that the relaxatory effect of adenosine decreases in cystitis (Vesela *et al.*, 2010).

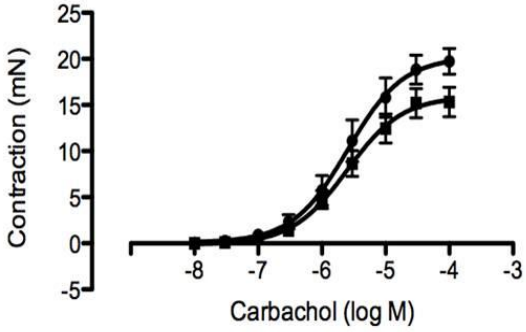

Figure 10. Contraction of intact urinary bladders in the presence of L-NNA (100 μM). Mean contractile responses to carbachol (0.01 - 100 μM) of isolated detrusor strip preparations from saline (●) and CYP (■) pre-treated rats. Vertical bars represent the SEM.

The carbachol induced contractions were concentration-dependently inhibited by both 4-DAMP, the muscarinic M3 antagonist pFHHSiD and the muscarinic M1 antagonist pirenzepine. While pFHHSiD and pirenzepine inhibited the contractile responses of saline and CYP pre-treated rats in a similar fashion, 4-DAMP inhibited the contractile responses of CYP pre-treated bladder strips less potently than saline pre-treated (pA2 value = 9.9 and 9.5 in saline and CYP pre-treated bladders, respectively; $p < 0.01$). In the CYP pre-treated bladders, the lowest concentration of 4-DAMP (10^{-10} M) even tended to generate a left-shift of the contraction curve. By either removing the urothelium or running the experiments in the presence of L-NNA, the difference in inhibitory potency of 4-DAMP in CYP pre-treated bladders was abolished. Analysis of the Schild slopes for all three antagonists revealed that they were all significantly different from unity in CYP pre-treated bladder strips, but not in the normal preparations. This is in favor of the notion that a change in contractility occurs during cystitis. Removal of the urothelium eliminated this difference, indicating that the contractility is altered due to changes mediated via the urothelium. Similarly, the difference

from unity of the Schild slopes was abolished by the presence of L-NNA.

The antagonistic profile is different for the three examined antagonists. While 4-DAMP inhibits M1, M3 and M5 in a similar fashion, pFHHSiD and pirenzepine are considered mainly a M3- and M1-antagonist, respectively (Eglen & Nahorski, 2000). The antagonistic potency of 4-DAMP is reduced in cystitic bladder strips, indicating that either the M1, M3 or M5 receptor (or more than one of them) is involved in the altered contractile responses seen during cystitis. Since neither the potency of pFHHSiD nor pirenzepine is changed during cystitis, M5 seems to be the primary receptor involved in this alteration. Taken together, our findings indicate that changes occur during CYP-induced cystitis that alter NO-mediated effects via urothelial M5 receptors.

Correlation plots for the pA2-values obtained in the study versus $-K_i$ values reported in the literature for the five muscarinic receptors support the generally accepted idea that detrusor contraction is mediated mainly by stimulation of muscarinic M3 receptors, both in normal and inflamed bladders (r^2 for M3 = 0.98 and 0.93 in normal and inflamed bladder strips, respectively). Thus, as expected, these plots do not support a notion that M5 receptors are directly involved in the contractile response of the detrusor. However, the correlation plots show that during CYP-induced cystitis, the M3-mediated contraction seems to be less dominant, indicating the influence of some other receptor or factor on contraction.

It is important to keep in mind that there may be differences between CYP-induced and naturally occurring cystitis, as well as inter-species disparities. Studies on bladder strips from cats have shown that during naturally occurring cystitis, activation of urothelial muscarinic receptors may actually lead to increased contractions at low agonist doses (Ikeda *et al.*, 2009). Therefore,

extrapolation of the interpretation of the data in this study to man must be performed with caution. Further, one also has to keep in mind that these are studies performed in an organ bath setup, thereby excluding the possibility for compensatory effects via the nervous system or changes in blood pressure.

To summarize, this study shows that the contractile changes seen in CYP-induced cystitis are mediated via stimulation of muscarinic M5 receptors and the following release of NO. Further, this study agrees with several previous studies that conclude that detrusor contraction in the healthy rat bladder is mediated almost entirely via muscarinic M3 receptors.

Paper III

Pharmacological modulation of the micturition pattern in normal and cyclophosphamide-pretreated conscious rats

The micturition pattern of conscious rats was assessed during 24 h, while the rat was placed in a metabolism cage. In the saline treated rats, pre-treatment with CYP (100 mg/kg) significantly reduced the volume per micturition over 24 h (Figure 11; 0.57±0.19 vs. 0.85±0.11 mL in CYP and saline pre-treated rats, respectively; $p < 0.01$; $n = 6$ and 10, respectively). At the same time, the water intake was not significantly different between these groups, or between any other groups.

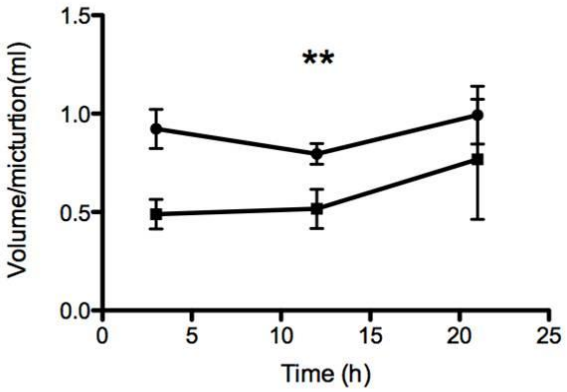

Figure 11. Volume of urine produced at each micturition event in saline (●; n=10) and CYP (■; n=6) pre-treated rats. Values are given as means ± SEM. Points indicate time periods 0 – 6 h, 6 – 18 h and 18 – 24 h. ** $p<0.01$ (overall difference between the groups (ANOVA))

Thus, changes occurred during CYP-induced cystitis that reduced the micturition volume per event. This could depend on alterations in

the contractile threshold of the detrusor, or solely be a result of a decreased detrusor contractile ability. The latter would increase the amount of residual urine, which has been shown to occur during CYP-induced cystitis (Ito *et al.*, 2007). We could also see a slight, but not significant, increase in micturition frequency in CYP pre-treated rats. Since the volume per micturition is lower in CYP pre-treated rats, at the same time as the water intake is unchanged, and other studies have shown an increase in frequency during CYP-induced cystitis (Smaldone *et al.*, 2009), we would have expected a significant increase in micturition frequency. However, in many other studies observations have been made over shorter time periods (< 12 h). In our study, measurements were made over a 24 h period, which may conceal temporary changes occurring during shorter periods.

Others have shown that CYP-induced cystitis can cause bladder overactivity (Borvendeg *et al.*, 2003). During CYP-induced cystitis, an increase in both the release of urothelial ATP and of the expression of eNOS and muscarinic receptors has been shown to occur (Giglio *et al.*, 2005b; Smith *et al.*, 2005). In the current study, in CYP pre-treated rats, treatment with L-NAME (30 mg/kg) led to an increase in micturition frequency (Figure 12a; 2.53 ± 0.36 vs. 1.25 ± 0.17 events/h in L-NAME and saline treated rats, respectively; $p < 0.05$; $n = 6$ in both groups). In saline pre-treated rats, L-NAME did not affect any micturition parameter. This is in line with findings in other studies that show that intravesically administered NO-donors can abolish the increased frequency seen in CYP pre-treated rats (Ozawa *et al.*, 1999). Our results, and those of others, show that in CYP-induced cystitis, NO exerts an inhibitory (relaxatory) effect on the detrusor.

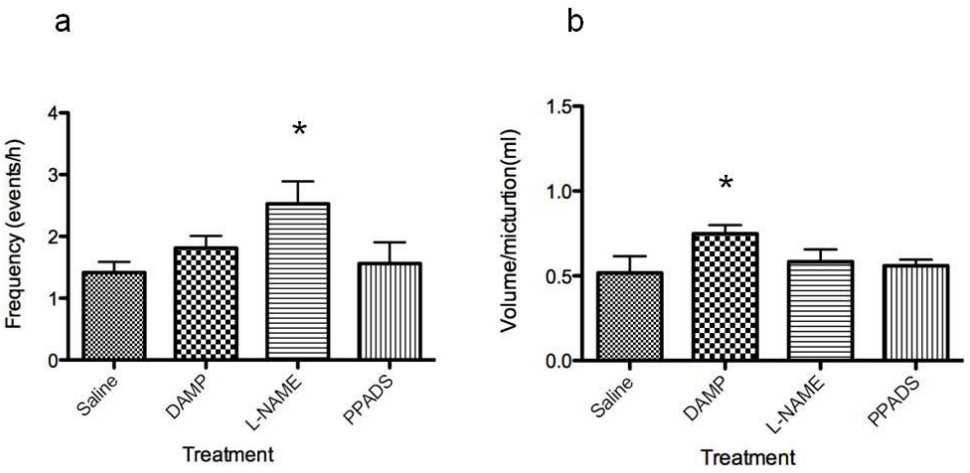

Figure 12. Effects of treatments on micturition parameters in cystitis. Frequency of micturition events (a) and volume of urine produced at each micturition event (b) of CYP pre-treated rats during the 6 – 18 h dark period. Forty-eight hours after CYP pre-treatment, saline (1 mL/kg i.p.; n=10), 4-DAMP (1 mg/kg i.p.; n=6), L-NAME (30 mg/kg i.p.; n=6) or PPADS (10 mg/kg i.p.; n=6) was administered. Values are given as means ± SEM. * $p<0.05$

The muscarinic antagonist 4-DAMP affected micturition parameters of CYP but not saline pre-treated rats. The volume per micturition of CYP pre-treated rats during the 6 – 18 h dark period was significantly increased by treatment with 4-DAMP (Figure 12b; 1 mg/kg; 0.75 ± 0.05 and 0.51 ± 0.09 mL in 4-DAMP and saline treated rats, respectively; $p < 0.05$; $n = 6$ and 10, respectively). Similarly, in paired comparisons, treatment with different doses of 4-DAMP (10 - 1000 µg/kg) eliminated the significant difference in volume per micturition between saline and CYP pre-treated rats. Even though non-significant, the frequency of CYP pre-treated rats was increased, rather than decreased, by treatment with 4-DAMP (Figure 12a). This finding is in line with studies in man that report that patients with DO are non-responsive to antimuscarinic treatment (Minaglia *et al.*, 2005). It is evident that treatment with

antimuscarinic drugs has limited effects in the healthy bladder of rats, which is in line with previous studies (Modiri *et al.*, 2002). However, in the cystitic bladder, 4-DAMP increases the volume per micturition, perhaps via an increase of detrusor contractility. The absence of effect of an antimuscarinic drug in the normal rat bladder is likely, at least partly, due to compensatory effects from the purinergic system.

Treatment with a combination of 4-DAMP (1 mg/kg) and L-NAME (30 mg/kg) did not affect any of the micturition parameters differently than separate administration of the two drugs, in neither saline nor CYP pre-treated rats. However, when combining 4-DAMP (1 mg/kg) with PPADS (10mg/kg), a synergistic effect was seen on the voiding frequency in CYP pre-treated rats during the 6 – 18 h dark period. This could indicate that at least part of the effects of muscarinic antagonists and those of NOS blockers are mediated via the same pathway, while the effects of purinergic antagonists are mediated via a separate pathway. The reason for why a synergistic effect is only seen during the dark period is likely due to the fact that it is during this period that the rats are awake, and thus the micturition activity is the highest and most likely to reveal differences in micturition parameters.

When given separately to CYP pre-treated rats, treatment with 4-DAMP leads to increased volumes per micturition, while treatment with L-NAME leads to increased micturition frequency. Even though these two parameters are not totally interlinked, they indicate an opposite effect of 4-DAMP and L-NAME in CYP pre-treated rats. To understand the discrepancies between a combinatory treatment and that of 4-DAMP and L-NAME alone, one has to remember that 4-DAMP may exert its effects via two separate pathways; (a) via blockade of muscarinic receptors in the efferent and/or afferent pathways, leading to decreased detrusor activity, and (b) via blockade of urothelial muscarinic receptors, which according to our

studies *in vivo* and *in vitro* leads to a decreased relaxatory influence of NO on the detrusor. This kind of dual effect of 4-DAMP would explain our findings.

Experiments on wake, freely moving rats present certain problems when examining drugs that have a relatively small impact on micturition parameters. More specifically, the interindividual variations among rats are sometimes larger than the effects of the studied drugs, making it impossible to statistically detect minute changes. However, this observable fact strengthens the correctness in those significant differences one does find.

The current study indicates that changes occur during CYP-induced cystitis that not only affects the micturition parameters, but also the effects of antimuscarinic, antipurinergic and antinitrergic drugs. It is possible to reverse the altered micturition parameters in the inflamed rat bladder by treatment with an antimuscarinic drug, indicating the strong involvement of these receptors in the alterations occurring during cystitis. Further, this study shows that there is a connection between the effect of antimuscarinic and antinitrergic drugs. Thus, this study strengthens the previously proposed idea of an increased importance of NO, which is released upon muscarinic receptor stimulation, during CYP-induced cystitis.

Paper IV

Characterization of receptor mediated proliferation in the human bladder urothelial UROtsa and T24 cell lines

Studies on various types of gastrointestinal inflammation have shown that chronic inflammation of the bowel together with increased proliferation of the mucosa is strongly associated with the development of cancer (Ament, 1975; MacDonald, 1992). Treatment with CYP causes similar proliferative changes in the urothelium (Zupancic *et al.*, 2009). Further, more than 90% of all urinary bladder cancer originates from the urothelium. When studying this disease, others have reported a link between chronic inflammation and urothelial transformation (Talar-Williams *et al.*, 1996; Michaud, 2007). It has also been shown that the expression and/or sensitivity of certain receptors in the urothelium can be altered in the state of inflammation and transformation (Giglio *et al.*, 2005b; MacLaine *et al.*, 2008).

The proliferative effects of adrenergic, muscarinic, nicotinic and EGF receptor agonists were examined in an immortalized normal human bladder urothelial cell line, designated UROtsa, and a malignant human bladder urothelial cell line, designated T24. Regarding the cell culturing, all experiments were performed during passage 4 – 10. This should be well within the time frame where one would expect cell properties to stay consistent.

The non-selective β-adrenoceptor agonist isoprenaline concentration-dependently increased the proliferation rate of both the UROtsa and T24 cell line (Figure 13a; pEC_{50} = 3.0 and 3.1, respectively). This proliferative effect could not be antagonized by the β_1/β_2-selective antagonist propranolol (2×10^{-5} M) in neither the UROtsa nor T24 cell line. However, the selective β_3-adrenoceptor

antagonist L-748,337 (1×10^{-6} – 5×10^{-5} M) showed a concentration-dependent inhibition of the proliferative effect of isoprenaline in both the UROtsa and T24 cell line (at 2×10^{-5} M $p < 0.01$ and 0.05 in the UROtsa and T24 cell line, respectively; n = 6 in both groups). SR59230A (2×10^{-5} M) had no effect in the UROtsa cell line, but significantly decreased the proliferative response to isoprenaline in the T24 cell line ($p < 0.05$; n = 6). SR59230A was first believed to be a β_3-selective antagonist, but later studies have shown that it also can function as both an α_1-adrenoceptor antagonist and an unselective β-adrenoceptor antagonist (Hoffmann *et al.*, 2004; Bexis & Docherty, 2009). Further, when tested separately, SR59230A significantly decreased the basal proliferation rate of T24, but not UROtsa, cells ($p < 0.0001$; n = 6). Therefore, the inhibitory effect of SR59230A on the T24 cell line is likely to be the result of SR59230A acting as a dirty drug.

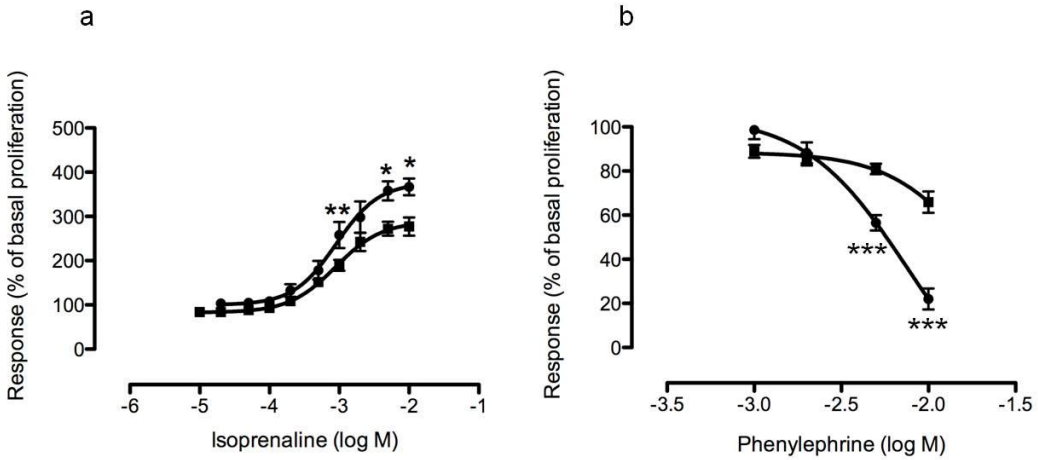

Figure 13. Proliferative response to (a) isoprenaline and (b) phenylephrine of the UROtsa (●; n=10) and T24 (■; n=7) cell lines. Vertical bars represent the SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

These results indicate that the increased proliferative response to isoprenaline in the UROtsa and T24 cell lines is mediated mainly via the β_3-adrenoceptor. Interestingly, β_3-agonists, which have been suggested as treatment for OAB, have been shown to induce cystitis in rats (Waghe *et al.*, 1999). Based on our finding in urothelial cells, we would expect such treatment to yield thickening of the bladder mucosa, one of the characteristic symptoms of cystitis. The reason for why it was not possible to block the entire proliferative effect of isoprenaline with L-748,337 could depend on one of two things (or both of these). Either the effect is partly mediated via other pathways than activation of the β_3-adrenoceptor, or the concentrations of L-748,337 chosen for the experiments ($1 \times 10^{-6} – 5 \times 10^{-5}$ M) were not high enough to block the entire proliferative effect. The range of concentrations of L-748,337, and that of all other agonists and antagonists, was based on pilot experiments, and doses showing signs of toxic effects were excluded. Unfortunately, it was therefore not possible to test higher concentrations of L-748,337.

In the presence of the α_1-adrenoceptor agonist phenylephrine, a decrease of the proliferation rate was seen in both the UROtsa and T24 cell line (Figure 13b; pIC_{50} = 2.2 and 1.7, respectively; n = 6). At the highest concentration (1×10^{-2} M), there was a significantly greater anti-proliferative effect in the UROtsa cell line (Figure 13b; $p < 0.0001$; n = 6). When it comes to anti-proliferative effects, one cannot exclude that the response is not simply a toxic effect. This is further highlighted by the fact that neither the unselective α-adrenergic antagonist phentolamine ($10^{-9} – 10^{-4}$ M) nor the α_1-selective adrenoceptor antagonist doxazosin ($10^{-9} – 10^{-4}$ M) could block the effect of phenylephrine (at 5×10^{-3} M). However, if the anti-proliferative effect of phenylephrine was not mediated via receptor activation, the impact should be equal in the two cell lines. Further, the concentrations of α-adrenergic antagonists used in this study could have been too low to inhibit the effect of phenylephrine at

5 x 10^{-3} M. Unfortunately, it was not possible to use higher concentrations of antagonists due to toxic effects.

The expression of muscarinic receptors seems to be different in the two studied cell lines. While all subtypes (M1 - M5) have been shown to be present in the UROtsa cell line (Tyagi *et al.*, 2006), another study could only detect mRNA for the M2 and M5 receptors in the T24 cell line (Tully *et al.*, 2009). As we have shown in a previous study on rats, the expression of muscarinic receptors, in particular the M5 subtype, was altered in the state of CYP-induced cystitis (Giglio *et al.*, 2005b). Thus, one of the possible links between inflammation and bladder cancer could be alterations in the expression of muscarinic receptors. We could, however, not see any functional effect of this altered expression, as the non-selective muscarinic agonist methacholine did not affect the proliferation rate of neither the UROtsa nor T24 cell line. One plausible explanation for the lack of proliferative effects upon activation of muscarinic receptors could be due to two or more effects cancelling each other out. I.e., by employing a non-selective agonist, activation of one subtype might increase proliferation, while activation of another subtype might decrease the rate of cell division. Future experiments should be designed to elucidate this possibility.

Nicotine, and the activation of the nicotinic receptor, has been associated with the development of bladder cancer and modulation of the proliferation of malignant urothelial cells (Chen *et al.*, 2008). However, we could not see any effect of nicotine (1 x 10^{-9} – 1 x 10^{-7} M) in neither the UROtsa nor T24 cell line. This could be due to toxicity problems, which only rendered the possibility to use very low concentrations.

Activation of the EGF receptor by EGF or HB-EGF is closely linked to increased cell proliferation, and their involvement in the transformation of urothelial cells has been suggested (Adam *et al.*,

2003). Several other studies have shown an increased proliferation rate upon EGF receptor activation, but despite using wide ranges of concentrations for both EGF (5 – 500 ng/mL) and HB-EGF (1 – 200 ng/mL), we could not see this effect in neither the UROtsa nor T24 cell line. This could possibly be explained by studies that have shown that only the autocrine release, and not the exocrine addition, of EGF or HB-EGF can affect the proliferation rate of urothelial cells (Southgate *et al.*, 1994; Rebel *et al.*, 1995; Freeman *et al.*, 1997; Sterle *et al.*, 1997). Further, other studies have suggested that activation of the EGF receptor only increases the proliferation rate of urothelial cells during challenges such as wound repair (Varley *et al.*, 2005).

Studies on cell cultures are beneficial in the sense that they offer an experimental setup that allows the study of one specific target in one specific cell type. However, one must always draw conclusions from cell culture studies with caution. The proliferation of urothelial cells is most likely an interplay between not only the urothelial cells themselves, but also other cell types. Findings in cell cultures should therefore, when possible, be verified *in vivo*.

The results in this report show the importance of adrenergic receptors in the regulation of urothelial cell proliferation, both in a normal and a malignant cell line. Further, since the UROtsa cell line is affected to a greater extent than the T24 cell line by both α- and β-adrenoceptor stimulation, we show that the impact of adrenergic receptor stimulation on cell proliferation is decreased in the malignant state. This report also examines the impact of muscarinic, nicotinic and EGF receptor stimulation, but the current results do not support the involvement of any of these in urothelial cell proliferation during unchallenged conditions.

Concluding remarks

A primary aim of this thesis was to elucidate the cause of the difference in contractility between the normal and CYP-inflamed urinary bladder. By piecing together several indirect and direct findings, we show that during CYP-induced cystitis there is an increased influence of NO on bladder contractility. Further, our accumulated findings point to this NO being released upon activation of muscarinic M5 receptors in the urothelium.

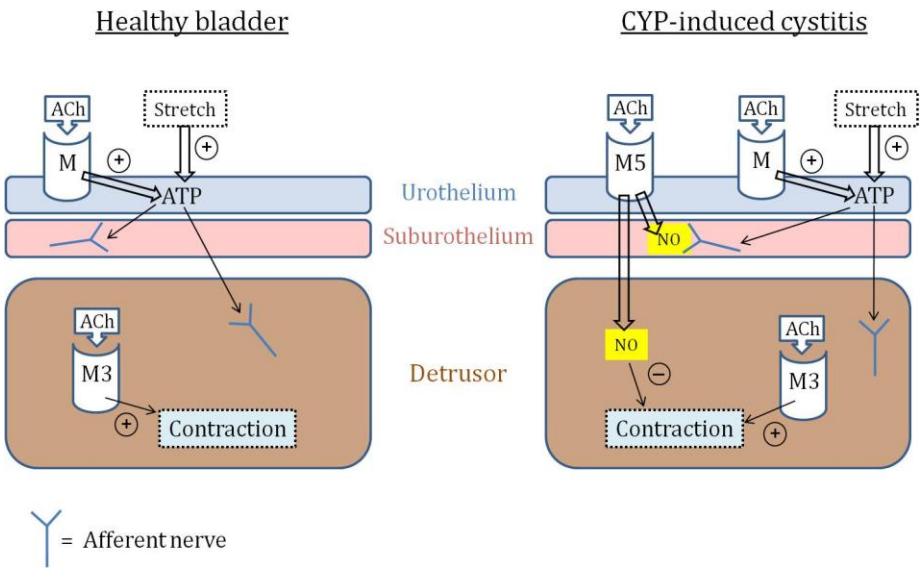

Figure 14. Influence of the urothelium on the healthy (left) and inflamed (right) urinary bladder. In both, contraction of the detrusor is mediated mainly by activation of muscarinic M3 receptors. During CYP-induced cystitis, a significant amount of nitric oxide (NO) is released from the urothelium upon activation of muscarinic M5 receptors, thereby dampening the contractile properties of the detrusor. In the inflamed bladder, NO has also been suggested to act on and modulate the activity of afferent nerve fibers in the suburothelium. Apart from NO, ATP is released from the urothelium upon stretch and/or muscarinic receptor activation, affecting afferent nerve fibers in the suburothelium and detrusor. The impact of ATP in man has been suggested to be greater in the state of cystitis. M - muscarinic receptor; M3 - muscarinic receptor of the M3 subtype; M5 - muscarinic receptor of the M5 subtype; NO - nitric oxide; ACh - acetylcholine.

Much of the research on the urothelium has focused on its role as an integrator of afferent signaling. This led to that early studies on the involvement of the urothelium during cystitis mainly examined the effects that factors released from the urothelium had on afferent nerves. However, the results presented in this thesis show that urothelial factors have impact on not only afferent, but also efferent mechanisms. It is also currently shown that the significance of this impact can be altered during disease states such as cystitis. This knowledge might highlight numerous new targets for future pharmacological treatments. For instance, there is a debate whether up-regulation of NO during cystitis is solely a result of the inflammation, or if it is actually part of the cause of the inflammation (Souza-Fiho *et al.*, 1997). This is a matter of great interest, particularly regarding the development of new pharmacological therapies for IC, which are needed. A major problem today is the uncertainty in the diagnosis of IC. Several factors have been suggested as early markers for IC, but so far no diagnostic tests have been developed. If an early marker for IC was found, and NO plays an important part in the development of inflammation, it might be possible to prevent IC by inhibiting the increased impact of NO during cystitis. Future experiments should be conducted in order to elucidate the development of inflammation, and if it is possible to influence this development.

A key concern for our studies was the proper induction of cystitis. In order for our data to be translatable, it was relevant for us that the cystitis we studied was similar to IC as it appears in man. Several animal models have been established to study experimental cystitis, of which induction with CYP is the most common (deVries & Freiha, 1990). There are many similarities between CYP-induced cystitis and IC. For one, CYP-induced cystitis gives symptoms that are very similar to IC. Secondly, none of them give rise to inflammation due to infection. Thirdly, CYP-induced cystitis, just as IC, has been reported to sensitize afferent pathways (Yu & de Groat, 2008). Further, IC is

associated with an altered release of NO and ATP. During CYP-induced cystitis, an up-regulation of both endothelial nitric oxide synthase (eNOS) and muscarinic receptors, mainly of the M5 subtype, has been shown (Giglio *et al.*, 2005b). Also, both altered release of ATP and increased frequency have been shown to arise during CYP-induced cystitis (Wood *et al.*, 2001; Smith *et al.*, 2005). However, there are also certain differences between IC and CYP-induced cystitis. For instance, ultrastructural studies of bladder specimens from IC patients has shown a disrupted barrier function of the urothelium (Elbadawi & Light, 1996). This is not equally apparent in CYP-induced cystitis, where the common observation is a thickening of the bladder mucosa. There may also be differences in the initiating causes of inflammation. In CYP-induced cystitis the inflammatory response is evoked by the presence of chemical irritants in the urine, while the exact cause for IC remains unclear.

The results presented in this thesis may also be applicable to other bladder disturbances than IC. In man, the classic symptom of DO is frequency. Treatment for patients suffering from frequency is today focused on antimuscarinic drugs. However, several studies have shown limited long-term clinical effects of these drugs (Andersson, 2009). A possible goal for the future treatment of symptoms of frequency could be via regulation of NO release. If our data are translatable to man, a compound selective for the muscarinic M5 receptor might improve quality of life for millions of patients suffering from inflammation-induced DO.

As mentioned previously, a recurrent observation during our studies was an increased proliferation of the urothelium upon CYP-treatment. Concomitantly, an increase of the expression of muscarinic M5 receptors occurs in the urothelium (Giglio *et al.*, 2005b). This same link, between increased proliferation and expression of M5 receptors, is observed in other diseases such as Sjögrens syndrome (Ryberg *et al.*, 2008). Despite these studies

showing a potential link between proliferation and increased expression of M5 receptors we could not show any effect of a muscarinic agonist on urothelial cell proliferation. The expression of all five muscarinic receptors has been shown in normal human urothelium (Table 1). However, in order to validate these findings, future studies should be performed to elucidate receptor expression in the UROtsa and T24 urothelial cell lines. Furthermore, future proliferation experiments should be performed in order to investigate if other results could have been obtained by the use of selective agonists and antagonists for these receptors.

Acknowledgements

My sincere gratitude goes out to all of you whom have helped me complete this thesis. You know who you are. However, some of you I would like to thank in particular.

First and foremost I would like to thank my supervisor, Gunnar Tobin. Without your help and encouragement I would have never even begun my PhD-studies, let alone completed them. I owe you one.

My co-supervisor, Daniel Giglio. Thank you for the many pointers and good scientific discussions.

My bladder friend, Patrik Aronsson. Thank you for your company, friendship and all our great discussions. And thank you for putting up with me during our trips.

My fellow PhD-student in our group, Anders Ryberg. Thank you for teaching me immunological methods in the lab and that comic strips can be considered fine art.

Group members Hanna Asklund, Martin Johnsson and Christine Ahlström. Thank you for fun lunches and help with my papers.

Co-authors Anna Wilhelmson, Davoud Eskandari, Deya Doufish and Anette Lampert. Thank you for your help in gathering data for my papers and for you good company.

My pharmafriends from the Czech Republic, Renata Vesela, Ondrej Soukup and Pavel Jeřábek. Thank you for helping me with my papers. And thank you for teaching me that dumplings are an essential part of life.

Professor Scott Garrett, Univeristy of South Dakota, for kindly providing me with the UROtsa cell line without any request for compensation.

To Hans Nissbrandt and Christer Nilsson at the department of pharmacology. Thank you for discussing sports, my favorite interest, with me. I need that sometimes.

To all other co-workers at the department of pharmacology. Thank you for proving such a stimulating and positive surrounding.

To my friends at other parts of the University, Johan Bourghardt, Louise Strandberg and Marie Kalm. Thank you for numerous fun lunches

To former and present members of the PhD committee, in particular Malin von Otter and Karin Hultman. Thank you for your commitment.

To my parents, for guiding me to where I am today. Thank you for your endless support.

To my son, William. You bring a smile to my face each and every day. Thank you for teaching me that one should not take the voluntary control of the bladder for granted.

Finally, but mostly, to my beloved Elin. Thank you for always being there, for always listening to me (even when I ramble) and for showing me what is most important in life. I love you with all my heart.

This thesis was supported by grants from *Anders Otto Swärd Foundation/Ulrika Eklund Foundation, Ferrings och Svenska Enures Akademin, Lars Hierta Foundation, Wilhelm och Martina Lundgrens Vetenskapsfond, Karl och Annie Leons Minnesfond* and the *Magn. Bergvall Foundation*

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