Purinergic Effects in the Rat Urinary Bladder

Functional Studies of Cyclophosphamide Treatment on Afferent and Efferent Mechanisms

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Cover illustration: *Original recording of contractile responses of urinary bladder strip preparations to ATP (0.3, 1 and 5 mM), by the author.*

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ABSTRACT

Pathological conditions in the lower urinary tract are common and have a great impact on the quality of life for the patients suffering from such disorders. In this thesis cyclophosphamide (CYP)-induced cystitis, a well-established rat model of inflammatory bladder diseases such as bladder pain syndrome/interstitial cystitis (BPS/IC), has been employed to study the role of purinergic transmission in the normal and inflamed state. The main focus was to characterize purinergic functional contractile and relaxatory parameters, studied *in vitro*, *in vivo* and *in situ*, for which the latter a novel method was developed and validated. The P2X1 purinoceptor was, in concordance with previous studies, found to be the major contractile subtype, whereas P2Y purinoceptor(s) with different sensitivities to the purinergic agonists ADP/ATP and UDP/UTP were shown to be relaxatory. Furthermore, the adenosine $P1A_{2B}$ purinoceptor was demonstrated to play a functional relaxatory role.

Using the novel *in situ* experimental setup presented in this thesis it was concluded that stretch-evoked contralateral contractions, mediated by afferent nerve fibers, were increased during cystitis. This was in contrast to most other contractile studies, in which the response in the inflamed bladder was generally decreased. This enlargement to stretch stimulus was found to be due to both cholinergic and purinergic factors, of which the latter were more pronounced at lower stimulation intensities.

Since the purinoceptors are often mentioned in the context of inflammation, studies were also conducted to investigate the role of purinergic, as well as of cholinergic and nitrergic, blockade in the development of cystitis. It was concluded that blockade of the $P1A_1$ purinoceptor or inhibition of nitric oxide synthase can alleviate the change in contractile function to CYP-induced bladder inflammation, which was confirmed by the study of several inflammatory findings common in cystitis.

Taken together, the purinergic transmission is altered during cystitis, and the changes are likely predominantly on the afferent side of the micturition reflex arc. The novel *in situ* setup can be modified and used to study various afferent factors, without interfering with the contractility of the bladder. Future therapeutic drugs targeting purinoceptors on afferent neurons may provide a valuable addition to the currently used medicines. The fact that blockade of purinoceptors at the same time may have a beneficial impact on the inflammation itself may prove to be useful in the treatment of inflammatory conditions in the lower urinary tract.

Keywords: purinoceptor, cystitis prevention, detrusor, bladder function, ATP, adenosine, nitric oxide

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Sjukdomar som påverkar urinblåsan är vanligt förekommande och orsakar lidande och sänkt livskvalitet för de personer som drabbas. I många fall, såsom vid sjukdomen interstitiell cystit, är urinblåsan inflammerad, vilket leder till rubbningar i urinblåsans förmåga att dra ihop sig och slappna av. Dessa funktioner styrs genom en komplex samverkan mellan olika transmittorsubstanser (kemiska signalmolekyler).

Den viktigaste transmittorsubstansen för urinblåsans funktion är acetylkolin. Den utövar sin effekt genom att verka på mottagarprotein, muskarina receptorer, vilket generellt sett leder till att urinblåsemuskulaturen drar ihop sig. Dock har man sett att acetylkolin inte är ensam om att styra urinblåsan. Även signalmolekylen adenosintrifosfat (ATP) och dess nedbrytningsprodukt adenosin är av betydelse, speciellt vid sjukdom. Genom att verka på sina mottagarproteiner, purinoceptorerna, kan dessa ha effekt både på urinblåsans funktion och på själva sjukdomsförloppet. Även molekylen kväveoxid (NO) anses vara inblandad i inflammatoriska processer.

I denna avhandling använder vi oss av djurförsök, där friska råttor samt råttor som blivit behandlade för att utveckla inflammation i urinblåsan jämförs. För att besvara problemställningarna har vi använt oss av experiment både på små bitar av urinblåsevävnad (*in vitro*) och på sövda råttor (*in vivo*). Dessutom har vi utvecklat en ny metod för att studera reflexer som är viktiga för urinblåsans funktion hos sövda råttor (*in situ*). Vi har även behandlat råttor med substanser som blockerar olika purinoceptorer och muskarina receptorer samt ett ämne som hämmar produktionen av NO.

Vi fann att både ATP och adenosin, genom att binda till purinoceptorer av P2Y- och $P1A_{2B}$-typ, kan göra så att muskelvävnaden i urinblåsan slappnar av. Dessutom visade vi, vilket bekräftar resultat från tidigare studier, att purinoceptorer av typen P2X1 orsakar sammandragning av urinblåsan. Vidare såg vi att den afferenta delen (signaler in till det centrala nervsystemet) av den reflex vi studerade *in situ* ökar under inflammation, och att purinoceptorer av typen P2 är involverade. Slutligen kunde vi visa att vi kan påverka sjukdomsförloppet och lindra de funktionsnedsättningar som uppkommer vid inflammation genom att hämma produktionen av NO eller genom att blockera $P1A_1$-purinoceptorer. Detta bekräftade vi genom att undersöka ett antal inflammatoriska förändringar.

Våra fynd indikerar att framtida läkemedelsbehandling mot inflammatoriska tillstånd i urinblåsan skulle kunna riktas mot purinoceptorer. Dels då dessa är involverade i regleringen av urinblåsans funktion, framförallt då de verkar öka i betydelse i den afferenta delen av urinblåsans reflex. Dels då de, tillsammans med NO, kan påverka själva inflammationsförloppet. Trots att fler studier måste genomföras på detta område för att bekräfta dessa fynd, och för att undersöka om våra resultat stämmer även hos människor, är dessa upptäckter viktiga för vår förståelse av purinoceptorernas roll i urinblåsan vid sjukdom och hälsa.

LIST OF PAPERS

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

I. Aronsson P, Carlsson T, Winder M & Tobin G. A novel *in situ* urinary bladder model for studying afferent and efferent mechanisms in the micturition reflex in the rat. *Submitted.*

II. Aronsson P, Andersson M, Ericsson T & Giglio D (2010). Assessment and characterization of purinergic contractions and relaxations in the rat urinary bladder. *Basic & clinical pharmacology & toxicology* 107(1): 603-613.

III. Giglio D, Aronsson P, Eriksson L & Tobin G (2007). *In vitro* characterization of parasympathetic and sympathetic responses in cyclophosphamide-induced cystitis in the rat. *Basic & clinical pharmacology & toxicology* 100(2): 96-108.

IV. Aronsson P, Carlsson T, Winder M & Tobin G. Studies of the micturition reflex initiated by stretch stimulation of the urinary bladder wall in normal and cyclophosphamide-treated anaesthetized rats. *Manuscript*.

V. Aronsson P, Johnsson M, Vesela R, Winder M & Tobin G (2012). Adenosine receptor antagonism suppresses functional and histological inflammatory changes in the rat urinary bladder. *Autonomic neuroscience: basic & clinical* 171(1-2): 49-57.

VI. Aronsson P, Vesela R, Johnsson M, Tayem Y, Wsol V, Winder M & Tobin G. Inhibition of nitric oxide synthase prevents muscarinic and purinergic functional changes and development of cyclophosphamide-induced cystitis in the rat. *Submitted*.

TABLE OF CONTENTS

ABBREVIATIONS

ADP	adenosine 5’-diphosphate
ATP	adenosine 5’-triphosphate
BPS	bladder pain syndrome
CYP	cyclophosphamide
EFS	electrical field stimulation
IC	interstitial cystitis
i.p.	intraperitoneal
i.v.	intravenous
MIF	macrophage migration inhibitory factor
mN	millinewton
NANC	non-adrenergic, non-cholinergic
NO	nitric oxide
NOS	nitric oxide synthase
OAB	overactive bladder
UDP	uridine-5’-diphosphate
UTP	uridine-5’-triphosphate

1 INTRODUCTION

The urinary bladder has two basic functions; to store urine and to voluntarily expel urine when suitable. This may seem to be rather simple tasks and, indeed, most of the time in most people it all works well. Nevertheless, dysfunctions and diseases of the urinary bladder are relatively common and have large impact on the quality of life of individual patients, as well as great economic implications on society. The exact mechanisms leading to many of the diseases are, despite much scientific effort, not fully understood and further studies are required to increase the knowledge of the complex systems involved.

In this thesis different models for studying the impact of the purinergic system on the contractile and relaxatory function of the urinary bladder are addressed and a novel model for investigating the efferent and afferent parts of the micturition reflex is presented. Furthermore, the interplay between the purinergic system and the cholinergic and nitrergic systems in the functional regulation of the bladder and their respective importance in the development of inflammation are discussed and, thus, comparisons made between normal rats and ones with experimental cystitis.

Models useful in the studies of disorders in the lower urinary tract have been employed and compared in Paper I. In Paper II and III *in vitro* findings regarding aspects of purinergic mechanisms in the function of the normal and inflamed urinary bladder are presented, whereas we in Paper IV utilize the novel approach presented in Paper I to investigate differences in the micturition reflex in normal rats and ones with experimental cystitis. In the two final papers, Paper V and VI, ways of preventing or alleviating the development of urinary bladder inflammation by purinergic, cholinergic and nitrergic pre-treatments are explored and different signs of inflammation and loss of function discussed.

1.1 The urinary bladder

The urinary bladder is a hollow, smooth muscle organ serving the purpose of storing and, when suitable, releasing urine. Its regulation is governed by the autonomic nervous system.

1.1.1 Anatomy and morphology of the urinary bladder

The human urinary bladder is located in the pelvic cavity but can, when it is full, stretch also into the abdomen. The bladder base is formed like an inverted triangle and the ureters enter the bladder at the upper parts of its base and the urethra extends from its lowest point. The area between the two ureters and the urethra is known as the trigone (see Drake *et al.*, 2005). The bladder wall consists of a mucosa layer, which includes the urothelium and the lamina propria, the submucosa and the detrusor smooth muscle (Fig. 1). The latter is generally considered to consist of three layers of smooth muscle; an outer longitudinal, a middle circular and an inner longitudinal layer. Outermost a serosal covering is found (see Martini *et al.*, 2000). Even though the human urinary bladder can contain even larger volumes of urine, a first desire to void is usually felt at a volume of 150-200 ml and a sense of urgency at 400-450 ml (see Pocock *et al.*, 2004).

The rat urinary bladder has similar basic properties and weighs about 70 mg and the muscle layer is roughly 100 µm. Its inner diameters are approximately 14 x 9 mm and it can contain up to about 1.4 ml (Gabella *et al.*, 1990).

1.1.2 The urothelium

The inner epithelial lining of the urinary bladder, often referred to as the urothelium, consists of cells organized in three layers, as summarized in a review by Lewis (2000). A basal layer containing small cells, an intermediate cell layer made up of moderately-sized cells and outermost large umbrella cells interconnected by tight junctions are found. The latter ones form a barrier preventing flow of molecules in the urine through the bladder wall. The umbrella cells are covered by hexagonal particles of crystalline proteins that, together with the tight junctions, form a flexible and very impermeable barrier.

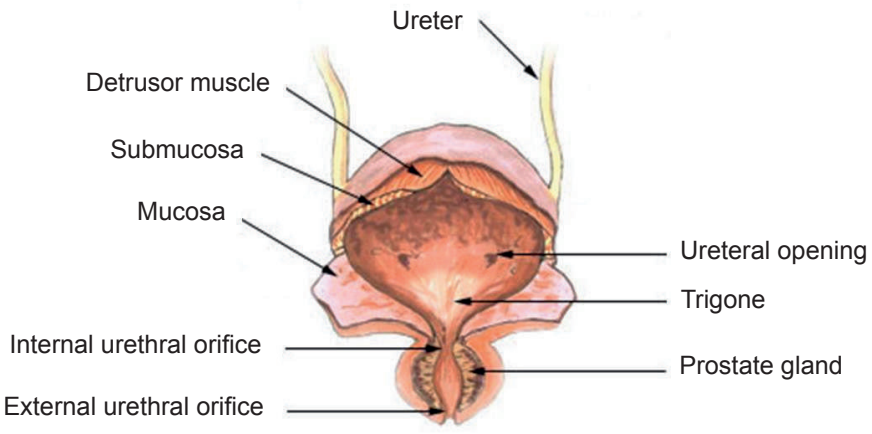

Figure 1. Structure of the (male) urinary bladder. Adapted from Wikipedia; http://commons.wikimedia.org/wiki/File:Illu_bladder.jpg

Worth mentioning is also the layer of glycosaminoglycane (GAG) lining the inner surface of the bladder. This layer prevents leakage of small molecules, such as urea and calcium, from the bladder contents into, and through, the wall of the urinary bladder. Disruption of the GAG layer, for instance by instillation of protamine sulfate, leads to a loss of these substances from the urine (Parsons *et al.*, 1990). In humans, disruption of the GAG layer has also been associated with urinary urgency and discomfort (Lilly *et al.*, 1990).

Previously the barrier function was considered to be the only property of the urothelium. More recent studies have, however, proven that the urothelium possesses also other qualities, including expressing receptors, releasing factors such as adenosine 5′-triphosphate (ATP) and nitric oxide (NO) (Birder *et al.*, 1998; Ferguson *et al.*, 1997). Afferent nerves have been found in the smooth muscle layers in the urinary bladder but also immediately adjacent to the urothelium with some of these nerve fibers extending into the urothelium (Birder *et al.*, 2001).

1.1.3 Innervation and the micturition reflex

Autonomic sympathetic (*e.g.* the hypogastric nerve) and parasympathetic (*e.g.* the pelvic nerve), as well as somatic (the pudendal nerve) nerves are

mediating the efferent stimuli to the bladder. The parasympathetic nerve originates from sacral parts of the spinal cord and forms ganglia in the pelvic plexus and bladder wall. Upon activation it releases acetylcholine, the neurotransmitter responsible for the major contractile response of the urinary bladder by acting on muscarinic M3 receptors in the detrusor (Longhurst *et al.*, 1995; Wang *et al.*, 1995). Furthermore, these nerves may co-release non-adrenergic, non-cholinergic (NANC)-transmitters such as ATP (mainly contractile) and NO (mainly relaxatory). The somatic nerve fibers also release acetylcholine acting on nicotinic cholinergic receptors mediating voluntary contraction of the striated muscle of the outer sphincter, see Yoshimura *et al.* (2008).

The sympathetic nerve fibers emerge from the thoracolumbar segments of the spinal cord and form the postganglionic hypogastric nerve. These nerves release noradrenaline which, in turn, activates β-adrenoceptors mediating relaxation of the detrusor smooth muscle, and α-adrenoceptors which mediate contraction of the "out-flow regions" of the urinary bladder, *i.e.* the bladder neck and urethra (Perlberg *et al.*, 1982; Yamaguchi, 2013). Thus, activation of sympathetic and somatic nerves puts the urinary bladder in the "filling phase", whereas stimulation of the parasympathetic nerves causes the opposite response, *i.e.* micturition/voiding. Furthermore, the autonomic nerves carry afferent signals of filling from the urinary bladder to the spinal cord and the pudendal and hypogastric nerve transmit similar signals from the urethra and bladder neck (Fowler *et al.*, 2008), a sensory feedback proven to be of importance for efficient voiding in the rat (Peng *et al.*, 2008). Despite many studies showing that afferent input from the bladder can arise in different parts of the bladder, *i.e.* detrusor, urothelium and lamina propria, the micturition reflex can be considered to originate mainly from activation of stretch receptors in the bladder wall, as a response to bladder filling (Kanai *et al.*, 2010).

Two types of neurons are of importance in the afferent signaling of the urinary bladder. Most important regarding bladder filling in the normal condition are the glutamatergic Aδ-fibers (de Groat *et al.*, 1990). These myelinated fibers mediate signals of bladder filling by responding to distention and contraction of the urinary bladder. The other neuron type is unmyelinated C-fibers, which transmit signals in response to cooling or chemical irritation, and in which substance P and calcitonin gene-related peptide are transmitters. Of interest in the perspective of the methods used in the current thesis, as discussed below, may be the fact that acrolein acting on the sensory nerve fiber TRPA1 receptor (belonging to the transient receptor potential superfamily) likely activates this latter type of fibers (Bautista *et al.*,

2006; Streng *et al.*, 2008). The C-fibers do, however, not seem to be a necessity for micturition (de Groat *et al.*, 1981). A receptor subtype expressed on afferent bladder nerves regarded to be of great importance in the micturition reflex is the P2X3 purinoceptor (Cockayne *et al.*, 2000). However, heteromeric purinoceptors containing the P2X2 subunit have also been implicated (Cockayne *et al.*, 2005).

The existence of a micturition controlling center in the brainstem was discovered almost a century ago when it was shown that, in cat, lesions in the dorsolateral pontine tegmentum inhibited the reflex involved in micturition (Barrington, 1925; Barrington, 1914). Electrical stimulation of this supraspinal center, called Barrington's Nucleus or the pontine micturition center (PMC), causes bladder contraction that seems to be gradually increased over time, *i.e.* the contractions were smaller just after a previous stimulation, compared to contractions after a longer period of resting (Noto *et al.*, 1989). Bladder relaxation is usually not accredited the PMC, but is rather believed to be exerted by a center in the ventrolateral part of the pontine tegmentum (Blok *et al.*, 1999) or the rostral part of the pontine reticular formation. For an excellent review on the subject of the PMC and micturition, see Sasaki (2005).

In the discussion of even higher level centers being of importance for the micturition reflex, the periaqueductal gray (PAG) in the midbrain is usually mentioned. It has been demonstrated that electrical stimulation of the PAG induces bladder contraction in the rat (Kruse *et al.*, 1990). Recent studies have, however, shown that, at least in the cat, PAG is not essential for evoking micturition, but that the Barrington's nucleus/PMC on the other hand is (Takasaki *et al.*, 2010). Most likely, the PAG is rather an important “relay station” in voluntary micturition, funneling signals from higher brain centers involved (Nour *et al.*, 2000). Interestingly, P2 purinoceptors have been shown to be of importance in both Barrington's nucleus and PAG in terms of generating activity in parasympathetic nerves supplying efferent input to the urinary bladder (Rocha *et al.*, 2001).

1.2 Diseases of the urinary bladder

Inflammation of the urinary bladder commonly referred to as "cystitis", and other more general disturbances such as overactive bladder are common disorders, which profoundly affects individual patients and are of great economical concern for society. Although several different diseases exist, the symptoms are often to a large extent overlapping, showing similar features such as functional disturbances, urinary urgency, frequency and pain or discomfort. Despite much effort the exact mechanisms underlying these diseases are still largely unknown.

The focus of this thesis is directed towards non-infectious inflammatory bladder diseases, and even though bladder pain syndrome/interstitial cystitis may seem to show the most resemblance with the animal models used, overactive bladder is also often studied in this way and, thus, discussed in the thesis.

1.2.1 Overactive bladder

Overactive bladder (OAB), sometimes called "detrusor overactivity", is defined by the International Urogynecological Association (IUGA) and the International Continence Society (ICS) as: "urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence, in the absence of urinary tract infection or other obvious pathology" (Haylen *et al.*, 2010). Usually, OAB is subdivided into two groups, OAB_{DRY} and OAB_{WET}, the latter describing patients with concomitant incontinence.

The prevalence of OAB has been estimated in numerous studies, but the numbers usually comes down to approximately 15% of the total population (Milsom *et al.*, 2001). OAB is about as common in women as in men, with some studies suggesting an increased prevalence in women, and the risk of developing OAB increases with age (Milsom *et al.*, 2001; Temml *et al.*, 2005; Wennberg *et al.*, 2009). The annual national cost for OAB treatment has in the United States been estimated to $65.9 billion (Ganz *et al.*, 2010). Assuming that most parameters are comparable between Sweden and USA, this would correspond to roughly 13 billion SEK in Sweden per year.

The mechanisms causing OAB are not fully understood, but both neurologic and myogenic factors are regarded to be of importance in the development of the disease, as summarized by Brading (1997), de Groat (1997) and Miller *et*

al. (2006). The pharmacological treatment of OAB consists mainly of anti-muscarinic drugs, whose main mechanism of action has been attributed to the blockade of contractile muscarinic receptors, but which may also be due to the inhibition of muscarinic receptors responsible for facilitating the release of ATP affecting afferent signaling (Nicholas *et al.*, 1996). Alternative treatments include injections of botulinum toxin which blocks the release of acetylcholine, and also the co-release of ATP, from efferent nerve fibers, thereby relaxing the bladder (Lawrence *et al.*, 2010).

1.2.2 Interstitial cystitis and Bladder pain syndrome

Among the earliest cases of interstitial cystitis (IC) found in the scientific literature is that of Alexander Skene, who described an inflammatory condition disrupting the mucous barrier, affecting also the muscular layers of the urinary bladder (Skene, 1887). Since then, this disease has been defined based on its resistance to conventional therapies (Hunner, 1915) and characterized based on its symptoms, which include a constant need to urinate (frequency) and pain (Bourque, 1951).

Today the term bladder pain syndrome (BPS) "largely replaces the older Interstitial Cystitis term, but the two are essentially interchangeable as there is no accepted definition that clearly delineates the interstitial cystitis syndrome from bladder pain syndrome" (Abrams *et al.*, 2009). The current definition of BPS, used by the International Continence Society, is "the complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and night-time frequency, in the absence of proven urinary infection or other obvious pathology" (Abrams *et al.*, 2002). More recently, the American Urological Association (AUA) used the guidelines developed by the Society for Urodynamics and Female Urology, stating "BPS/IC to be "an unpleasant sensation (pain, pressure, discomfort) perceived to be related to the urinary bladder, associated with lower urinary tract symptom(s) of more than 6 weeks duration, in the absence of infection or other identifiable causes" (Hanno *et al.*, 2011).

The prevalence of BPS/IC in the general population has been shown to be hard to estimate, in particular since different forms of questionnaires are being used. Two of the most commonly used forms have been compared, suggesting a prevalence of BPS/IC in women between 0.57-12.6% of the general population, most likely somewhere in between these two extremes

(Rosenberg *et al.*, 2005). Other studies have found the prevalence of clinically confirmed IC to be 300 per 100 000 in the female population (Leppilahti *et al.*, 2005), with women found to be about five times more likely than men to suffer from BPS/IC (Clemens *et al.*, 2005). These latter numbers are in line with the current opinion of the International Continence Society (Abrams *et al.*, 2009).

Even though much effort has been spent to understand the etiology of BPS/IC, the mechanisms leading to this condition are not yet fully understood, but the disease is regarded to be of multifactorial origin. Commonly mentioned proposed underlying mechanisms are inflammation, mast cell activation, changes in neuronal function, cross-talk between pelvic organs (regarding *e.g.* inflammation and pain) and autoimmune mechanisms (Abrams *et al.*, 2009; Vij *et al.*, 2012). Also, one feature in IC that may contribute to both the progression and the symptoms of the disease is the fact that the urothelium, including the GAG layer, often is damaged in patients suffering from this illness. The loss of barrier function is even greater in patients with ulcerative IC than in patients without ulcers, but also the latter group displays a pronounced increase in permeability to small molecules compared to healthy subjects (Parsons *et al.*, 1991).

As a consequence, no optimal treatment has been produced until this day, even though a number of pharmacological and non-pharmacological treatments have been evaluated. Among the non-pharmacological suggested are changes in diet, behavioral modification and physical therapy (Gish, 2011; Vij *et al.*, 2012). Often discussed pharmacological treatments include oral doses of the immunosuppressant cyclosporine A, the tricyclic antidepressant amitriptyline or pentosan polysulphate sodium. Also, intradetrusorial injections of botulinum toxin serotype A and intravesical treatment with dimethyl sulfoxide (DMSO) are established methods. Although a recent meta-analysis concluded the use of cyclosporine A to be of best proven use, the evidence are not clear-cut and much remains to be done in the pharmacological treatment of BPS/IC (Giannantoni *et al.*, 2012).

Regarding purinergic alterations in BPS/IC, it has been demonstrated that urinary ATP levels are elevated and that the release of ATP to stretch of urothelial cells is increased in patients with this disease (Sun *et al.*, 2001). Studies in the cat partly support this, showing that urothelial cells from animals with feline interstitial cystitis exhibit increased stretch-induced ATP release, although no difference was observed at a basal level, suggesting a mechanical hypersensitivity (Birder *et al.*, 2003). Other investigations have shown a reduced expression of, mainly, P2X1 purinoceptors in the bladder

smooth muscle and urothelium in this feline IC model (Birder *et al.*, 2004). Furthermore, *in vitro* examinations of bladder strip preparations from patients with and without BPS/IC have suggested that the purinergic signaling may be minute, or even absent, in healthy humans (Kinder *et al.*, 1985), but of markedly increased importance in patients with this disorder (Palea *et al.*, 1993).

1.2.3 Cyclophosphamide- induced cystitis

Cyclophosphamide (CYP) is an anti-neoplastic drug used in the treatment of certain forms of cancer, well known for its common side-effect of causing cystitis in patients. About 10% of the patients treated with high doses (>20 mg/kg) or for extended periods of time develop aseptic cystitis (Läkemedelsindustriföreningens Service AB, 2012). This side-effect has, however, led to the usage of CYP as an agent for causing experimental cystitis in animals, a method that is now widely established and used as a model for studying both BPS/IC and OAB. Two principal ways to induce cystitis by CYP exist; either by a single injection or by repeated injections of, usually, lower doses (as discussed in Boudes *et al.*, 2011).

The exact mechanism behind the induction of cystitis by CYP is not fully understood, but is regarded to be due to the contact of a metabolite of CYP with the urothelium. This has been demonstrated in studies in the dog, where cystitis has been induced by CYP-injection and the urine collected. The collected urine evoked, when instilled into another dog's urinary bladder, cystitis, unlike normal urine mixed with CYP which did so only to a minor extent (Philips *et al.*, 1961). The metabolite responsible has later been confirmed to be acrolein (Cox, 1979; Levy *et al.*, 1977).

Also, afferent nerves (capsaicin-sensitive primary afferent neurons) are believed to be of importance in the development of CYP-induced cystitis, indicating a possible mechanism for disorders in the lower urinary tract (Ahluwalia *et al.*, 1994). Interestingly, in CYP-induced cystitis, a substantial neurogenic inflammatory component has been suggested, as discussed by Geppetti *et al.* (2008). Furthermore, NO seems to have a significant impact in CYP-induced cystitis and the instillation of NO-donor into the bladder reduces the hyperactivity of the detrusor seen in this animal disease model, either by direct smooth muscle relaxation or by affecting afferent nerves (Ozawa *et al.*, 1999).

Alternative animal models to CYP-induced cystitis include feline spontaneous cystitis, neurogenic cystitis (due to stimulation of afferent nerves), and the use of other irritants besides CYP, such as lipopolysaccharide (LPS), turpentine or acid, as has been reviewed by Bjorling *et al.*(2011).

1.2.4 Markers for cystitis

Many markers have been suggested to be of potential clinical relevance in the diagnosis of diseases affecting the lower urinary tract. One of the more well-studied ones, see Mamedova *et al.* (2004) is antiproliferative factor (APF), which has been found to be produced by urothelial cells derived from BPS/IC patients, but not from healthy controls (Boyer *et al.*, 1998). This factor is suggested to inhibit the production of heparin-binding epidermal growth factor–like growth factor (HBEGF), which is significantly reduced in patients suffering from BPS/IC (Mamedova *et al.*, 2004). Interestingly, APF is increased only in the urine contained in the bladder, and not in urine samples collected directly from the renal pelvis (El-Tayeb *et al.*, 2006). Besides increased levels of APF and decreased levels of HBEGF, the expression of epidermal growth factor (EGF) is generally regarded to be elevated in urine from patients with BPS/IC (Mamedova *et al.*, 2004), and many other inflammation markers, such as tumor necrosis factor-alpha, insulin-like growth factor 1 (IGF-1), insulin-like growth factor-binding protein 3 (IGFBP3) and several interleukins have been suggested to be linked to IC/BPS (Crack *et al.*, 1994; Keay *et al.*, 1997).

More recently, macrophage migration inhibitory factor (MIF) has been identified as being of great interest when studying inflammation in the urinary bladder. MIF is a cytokine with proinflammatory properties expressed in the urinary bladder tissue, as well as in nerves supplying the bladder (Vera *et al.*, 2003). MIF has been shown to be increased during cystitis induced by LPS (Meyer-Siegler *et al.*, 2004a) or CYP in the rat (Vera *et al.*, 2008). Furthermore, inhibition of MIF-activity has been demonstrated to decrease the effects of CYP-induced cystitis in mice, strengthening the claim that this cytokine may be of importance in modulating and maintaining the inflammation process (Vera *et al.*, 2010).

1.3 Purinergic signaling

Although the main contractile transmitter of the urinary bladder is acetylcholine, it has long been known that a part of the contractile response of the urinary bladder is in many species insensitive to the muscarinic antagonist atropine (Langley *et al.*, 1895). This phenomenon, usually termed "atropine resistance", inspired other scientists into looking for still unknown non-adrenergic, non-cholinergic (NANC) transmitter systems. The results of these investigations included findings of adenine compounds (*i.e.* purines), especially adenosine, being present in the animal body and exerting significant cardiovascular effects such as bradycardia and hypotension, the latter partly due to arterial dilatation (Drury *et al.*, 1929). In the same year triphosphates, including ATP, were extracted and identified in muscle and liver (Fiske *et al.*, 1929; Lohmann, 1929). Efforts were later made to study the physiological effects of ATP on muscle preparations, showing direct contractile effects and implying a potentiating role of ATP to acetylcholine (Buchthal *et al.*, 1944a; Buchthal *et al.*, 1944b). The effects of ATP were however interpreted in different ways and it was, for instance, stated that "[...] it is not the ATP itself which produces the contraction, but that it releases from the muscle preparation acetylcholine or an acetylcholine-like substance which then in its turn elicits the contraction" (Beznak, 1951). Eventually, it was also demonstrated that ATP could be released from sensory nerves when stimulated (Holton, 1959).

1.3.1 A purinergic transmission?

What is usually described as a breakthrough in understanding the importance of purinergic signaling took place in the early 1960s when Geoffrey Burnstock and his colleagues performed a series of experiments in which they blocked both cholinergic and adrenergic receptors, as well as using bretylium to inhibit release of transmitters from sympathetic nerves (Burnstock *et al.*, 1963; Burnstock *et al.*, 1966). Contrary to what was expected, they found relaxation and rapid hyperpolarization to single stimuli. These new observations, combined with the earlier findings, led to the conclusions that only a part of the relaxation seen could be attributed to the classical autonomic nervous system and that the inhibitory responses still present after blockade were "mediated by intrinsic nerves which are distinct from the sympathetic and parasympathetic systems" (Burnstock *et al.*, 1964). By combining the earlier findings regarding physiological effects of ATP with new data, the term "purinergic" was finally coined (Burnstock, 1971)

and the concept of purinergic transmission was established (Burnstock, 1972).

1.3.2 ATP as a transmitter

In order to be recognized as a transmitter a substance has to fulfill certain requirements. The definitions vary, but they are usually adaptions of the one presented by Eccles (1964), even though they are sometimes expanded in more recent literature (Guarna *et al.*, 2005; Purves, 2012). Generally, it is said that in order to be classified as a neurotransmitter the substance and the enzymes necessary for its synthesis must be present in the neuron and the substance released from the terminal axon upon nerve activation (*i.e.* Ca^{2+}-dependent depolarization). Furthermore, a mechanism able to rapidly inactivate the response should be present in the synapse and it must be possible to mimic the physiological effects of the transmitter by administering it exogenously, allowing it to act on the same postsynaptic receptors on the effector tissue as the neurotransmitter usually does. All these requirements have now been shown to be fulfilled by ATP and will be dealt with in more depth below.

1.3.2.1 Purinergic co- transmission

One often used expression is that of "Dale's principle", which has been interpreted in several different ways. Eccles *et al*. (1954), who first coined this expression, used it in the sense implying the same chemical transmitter being released from all the synapses of a single neuron (*i.e.* stating that individual neurons are either adrenergic or cholinergic ones and that one nerve uses only one chemical transmitter). The original work he was referring to did, however, not explicitly claim this. It rather stated the chemical function of each neuron to be specific for this neuron as well as being "unchangeable" (Dale, 1935).

Even though there were clues in the literature, the formal proposal of the term "co-transmission", the concept of release of several chemical transmitters from one nerve simultaneously, was not published until 1976 (Burnstock, 1976). In this paper the "Dale's principle" (in the sense of one nerve – one transmitter) was openly challenged. It is, however, worth noticing that other researchers of that time had similar ideas. Silinsky, for instance, suggested ATP derived from cholinergic vesicles to be released from the motor nerve

ending (Silinsky, 1975), and also Eccles slightly but importantly rephrased his previous interpretation of Dale's principle, suggesting that "[…] at all the axonal branches of a neurone, there was liberation of the same transmitter substance or substances" (Eccles, 1976), thus opening up for the possibility of multiple chemical transmitters being released from the same nerve ending.

The concept of purinergic co-transmission has been suggested, and later proven, also in sympathetic nerves. Stimulation of sympathetic nerves in the guinea-pig vas deferens elicited chemical transmission that was shown to consist of more than just noradrenaline (Ambache *et al.*, 1971). Further studies made likely that ATP serves as a neuromodulator and/or co-transmitter to noradrenaline (Su, 1975; Westfall *et al.*, 1978), and by using the, at the time, novel purinoceptor desensitizer α,βMe-ATP this was proven to be accurate both in the guinea-pig (Sneddon *et al.*, 1984) as well as, later, in man (Banks *et al.*, 2006).

1.3.2.2 Formation and storage of neuronal ATP

Although mammalian cells are capable of *de novo* synthesis of adenine/adenosine, it is usually formed in various metabolic reactions, and can be absorbed by nerve terminals and further converted to ATP by oxidative phosphorylation (Sperlágh *et al.*, 1996). It has been shown that exogenous tritium-labeled adenosine can be converted to ATP and subsequently released from neurons in the taenia coli (Su *et al.*, 1971).

Evidence of ATP being stored in vesicles was first presented in a study of cholinergic vesicles from *Torpedo marmorata,* the marbled electric ray (Dowdall *et al.*, 1974). It was found that the vesicles located in the nerve terminals of the electric organ of this ray contained not only acetylcholine, but also considerable amounts of ATP. When enzymes were added in order to remove free substances, the ATP within the vesicles was found to remain intact. Furthermore, it was demonstrated that the ATP inside the vesicles was not just "trapped" there during their formation and that the ratio of vesicular acetylcholine to ATP was rather robust, about 5:1, in a series of experiments.

The vesicular uptake of ATP remained poorly characterized until a study of its kinetics was presented at the end of the millennium. Using florescent adenine nucleotide analogues, including labeled ATP, the presence of nucleotide transporters was confirmed in the rat brain (Gualix *et al.*, 1999).

1.3.2.3 Release of ATP

Studies on rat brain tissue have shown that stimulation with a secretagogue, for instance veratridine, releases the contents of synaptic vesicles, including ATP, in a Ca^{2+}-dependent manner (Pintor *et al.*, 1992). The same is true for chromaffin cells, modified postsynaptic sympathetic nerve cells able to release adrenaline and noradrenaline into the bloodstream instead of directly affecting an effector tissue (Pintor *et al.*, 1993). Recently, the neuronal release of ATP has been shown to be quantal in nature, suggesting exocytotic vesicular release (Pankratov *et al.*, 2007).

Non-neuronal release of ATP can be evoked in many different types of tissue by, for instance, stretch and shear stress. Urothelial cells release ATP upon stretch, which has been suggested to constitute a sensory mechanism for bladder filling (Ferguson *et al.*, 1997). It has also been demonstrated that the release of ATP from the urothelium occurs at both the serosal and mucosal sides (Wang *et al.*, 2005). Vascular endothelial cells release ATP when subjected to shear stress (Bodin *et al.*, 1991), by a mechanism involving exocytosis of ATP-containing vesicles, similar to those in neuronal synapses (Bodin *et al.*, 2001).

1.3.2.4 Breakdown and removal of neuronal ATP and adenosine

Once released into the synaptic cleft, ATP and its phosphorous metabolites are rapidly inactivated and removed. Several enzymes, both soluble and membrane bound, involved in these processes have been described. The main enzyme families responsible for these actions are currently being identified as the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), ecto-5'-nucleotidases/CD73, and alkaline phosphatases (Yegutkin, 2008).

Enzymes of the E-NTDPase, previously also known as ecto-ATPase, ATP-diphosphohydrolase, nucleoside diphosphatase etc., family are found intracellularly as well as bound to the cell membrane and can also be secreted extracellularly. In the context of ATP, the E-NTDPases metabolize nucleotide triphosphates (*e.g.* ATP to ADP) and nucleotide diphosphates (*e.g.* ADP to AMP), but do not further metabolize monophosphates, such as AMP (Zimmermann, 1996). The E-NPP family can hydrolyze triphosphates, but is mainly responsible for the breakdown of diphosphates in vertebrates (Vollmayer *et al.*, 2003). The E-NPPs have, as do the E-NTDPases, a rather

broad substrate specificity, sometimes making the discrimination between the two families somewhat difficult.

The ecto-5'-nucleotidases/CD73 are generally considered to be membrane bound, although some studies report possible soluble forms (Yegutkin *et al.*, 2000). This enzyme family is capable of forming adenosine by phosphohydrolysis of AMP (Colgan *et al.*, 2006).

Finally, the alkaline phosphatases are a family of abundant enzymes with broad range specificity, including dephosphorylation of ATP, ADP as well as AMP. The overall role of this family in purinergic signaling is, however, not that well investigated (Zimmermann, 2000). Thus, the alkaline phosphatases are, together with the ecto-5'-nucleotidases, responsible for the conversion of nucleoside phosphates to adenosine. It has been demonstrated that about 50% of the extracellular adenosine available is the result of the break-down of ATP (Smith, 1991) which is currently regarded to be the most important route of generating extracellular adenosine (Zhang *et al.*, 2012). *In vitro*, degradation studies of ATP in a guinea-pig detrusor strip organ bath setup showed that ATP was rapidly broken down within minutes to form mainly, but not exclusively, adenosine (Cusack *et al.*, 1984).

The break-down of adenosine is, in turn, facilitated by one of several mechanisms, of which the one most commonly discussed in the literature is adenosine deaminase (ADA). Adenosine deaminase is responsible for the catabolism of adenosine to inosine and the enzyme can be found both intra- and extracellularly (Spychala, 2000). Some studies also claim possible ADA expression on the surface of dendritic cells, constituting a regulatory mechanism in, for instance, the regulation of inflammation (Desrosiers *et al.*, 2007).

1.3.3 Purinergic receptors

The characterization of these receptor families, also known by the collective name “purinoceptors”, have been an ongoing work since the 1970’s when a seminal review was published, discussing a possible separation into P1 and P2 purinoceptors, based on four criteria (Burnstock, 1978). The first two were the relative potencies of the agonists, *i.e.* ATP, ADP, AMP and adenosine and the effects of selective antagonists, capable of inhibiting the response to adenosine, but not that evoked by ATP. The third and fourth criteria, the modulation of adenylate cyclase resulting in altered levels of

cyclic adenosine monophosphate (cAMP) by adenosine (but not ATP) and the generation of prostaglandin synthesis by ATP (but not by adenosine), were also investigated, as summarized by Abbracchio (1994). This view was maintained, and the P2 family was eventually further subdivided into the P2X and P2Y purinoceptors (Burnstock *et al.*, 1985).

To date, these receptors for purine and pyrimidine nucleotides are still divided into the adenosine receptors (also known as the P1 purinoceptors), the P2X purinoceptors and the P2Y purinoceptors. A somewhat aged, but still very comprehensive review article that provides a major part of the collective knowledge in the field of purinergic receptors was published at the end of the last century (Ralevic *et al.*, 1998).

1.3.3.1 Adenosine P1 purinoceptors

The adenosine receptors, or P1 purinoceptors, are G protein-coupled receptors of which four subtypes have currently been identified and whose naturally occurring ligand is adenosine (Fredholm *et al.*, 2012; Fredholm *et al.*, 2011). For long the P1 purinoceptors were thought to exist only in a monomeric state. This has however been disproved and, for instance, homomeric A_{2A}-A_{2A} receptors have been identified and suggested to be of functional importance on the cell surface (Canals *et al.*, 2004). Furthermore, oligomeric association of $P1A_1$ adenosine receptor subunits with non-adenosine receptor proteins, such as $P2Y_1$ purinoceptor subunits, has been described (Yoshioka *et al.*, 2001).

1.3.3.2 P2X purinoceptors

As of today, seven subtypes of the P2X purinoceptor family, termed P2X1; P2X2; P2X3; P2X4; P2X5; P2X6 and P2X7 have been identified, by cloning and pharmacological classification (Evans *et al.*, 2012). Notably these receptors are expressed either as homomeric (*e.g.* P2X1) or heteromeric trimers (*e.g.* P2X2/3), and seem to require binding of one, two or three molecules of their endogenous ligand ATP to be activated. An extensive recent review on the P2X subfamily of purinoceptors is available (Coddou *et al.*, 2011).

Being ionotropic ligand gated ion channels, the P2X purinoceptors today follow the same nomenclature as the rest of the members of this superfamily,

thus the use of subscripts in the subunit names has been abandoned for this family in accordance with the guidelines recently presented by The International Union of Basic and Clinical Pharmacology (IUPHAR) (Collingridge *et al.*, 2009).

1.3.3.3 P2Y purinoceptors

The P2Y purinoceptor superfamily currently contains eight mammal subtypes, namely the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ purinoceptors, all of which are G protein-coupled (metabotropic) receptors (Burnstock *et al.*, 2012). The first P2Y purinoceptor was cloned in 1993 (Webb *et al.*, 1993), but since then there has long been controversy regarding the classification and identification of the P2Y purinoceptors, leading to the use of many now unofficial names such as "platelet ADP receptor" ($P2Y_1$), "P_{2U}" ($P2Y_2$), "$P2Y_{ADP}$" and "$P2T_{AC}$", ($P2Y_{12}$) etc. (Burnstock *et al.*, 2012; Ralevic *et al.*, 1998).

Unlike the P2X purinoceptors, whose primary endogenous agonist is ATP, both ATP and ADP are regarded as naturally occurring agonists for the P2Y superfamily, even though the subtypes differ greatly in sensitivity. For instance, the human $P2Y_1$ purinoceptor has been shown to be stimulated by ADP, while ATP in some cases can act as an antagonist at this receptor (Leon *et al.*, 1997). Other P2Y purinoceptors, such as the $P2Y_2$, have ATP (or UTP) as endogenous agonist rather than the dinucleotides (*i.e.* ADP and UDP) (Nicholas *et al.*, 1996).

The current nomenclature of the mammal P2Y purinoceptor subtypes are, according to IUPHAR, to state the subtype number in subscript, *i.e.* $P2Y_{number}$ (Burnstock *et al.*, 2012).

1.3.3.4 Expression in the urinary bladder

Immunoreactivity for all seven subtypes of the P2X purinoceptor has been detected in the rat urinary bladder (Lee *et al.*, 2000). Also, mRNA for all subtypes of P2X purinoceptors has been identified in the rat bladder (Creed *et al.*, 2010). In mice the P2X3 purinoceptor has been shown to be expressed at sensory neurons in the bladder and this subtype is regarded to be of importance in the voiding reflex (Cockayne *et al.*, 2000).

In the human bladder, RNA for five of the P2X purinoceptor subtypes has been detected, showing the P2X1 subtype to be the predominant one whereas no transcripts were found for the P2X3 or P2X6 subtypes (O'Reilly *et al.*, 2001). Furthermore, other studies have shown expression of all seven P2X subtypes in immunohistochemical studies in man (Moore *et al.*, 2001). P2X3 has, together with P2X2, been shown to be expressed in the human bladder urothelium in increasing magnitude in patients with IC (Tempest *et al.*, 2004).

Regarding the P2Y purinoceptors, the $P2Y_1$, $P2Y_2$, and $P2Y_4$ subtypes have been identified in the feline urothelium (Birder *et al.*, 2004), and, in addition, the $P2Y_6$ subtype has been identified in the guinea-pig (Sui *et al.*, 2006). Transcripts for $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ purinoceptors have been found in human urothelial cell lines (Save *et al.*, 2010).

Additionally, western blot analysis has confirmed the expression of all four subtypes of adenosine receptors in the human urothelium (Yu *et al.*, 2006). Also, RT-PCR has successfully been used to identify mRNA for all subtypes in the rat urinary bladder (Dixon *et al.*, 1996).

1.3.3.5 Adenosine P1 purinoceptors in inflammation

It is no overstatement that the role of P1 purinoceptors in inflammation is complex, as has recently been thoroughly reviewed by Blackburn *et al.* (2009). Adenosine receptors are present on all types of immunological cells and all four subtypes have been shown to possess both anti- and pro-inflammatory properties in different cell types and under various conditions. Notably, inflammatory cells, such as mast cells, can also release adenosine (and ATP) when stimulated (Marquardt *et al.*, 1984).

1.3.4 Relevance of purinergic functional signaling in the urinary bladder

Administration of ATP to *in vitro* detrusor strip preparations causes a rapid contraction followed by a sustained relaxation. While the P2X purinoceptors are regarded to be mainly contractile, the P2Y purinoceptors are believed to be predominantly relaxatory (Bolego *et al.*, 1995; Inoue *et al.*, 1990). It has been suggested that ATP may be a key player in the initiation of voiding by giving rise to a fast contraction of the urinary bladder which in turn makes

possible the slower, more powerful and longer-lasting cholinergic contraction (Chancellor *et al.*, 1992; Streng *et al.*, 2004). Regarding actual voiding, it has in the rabbit been demonstrated that the response to ATP causes the urinary bladder to expel only about 15% of its contents, which is close to that of electric field stimulation in the presence of atropine (Levin *et al.*, 1986). It has been suggested that this swift low-volume purinergic voiding may be of great importance in territorial marking animals.

In man, as mentioned previously, the direct functional effects of the purinoceptors seem to be small in healthy humans, but the impact of purinergic signaling is elevated in certain diseases and medical conditions (Bayliss *et al.*, 1999; Kinder *et al.*, 1985; Palea *et al.*, 1993; Sjogren *et al.*, 1982). Furthermore, it has been demonstrated that the atropine-resistant component of electrical field stimulation-induced contractions increases and that the purinergic neurotransmission is elevated in the aged human bladder (>70 years of age), whereas cholinergic neurotransmission shows a negative correlation to age (Yoshida *et al.*, 2001). Possibly, the atropine-resistant parasympathetic response may have been underestimated because of underestimation of the complexity of transmitter interactions, as is indicated in this thesis.

1.3.5 Examples of practical current pharmacological modulation of the purinergic system

The worlds perhaps most common psychoactive drug, namely caffeine, acts as an antagonist on adenosine P1 purinoceptors, thereby promoting alertness. The purinoceptor subtypes being blocked are $P1A_1$ and $P1A_{2A}$, and the current opinion is that the latter is mediating the arousal effect (Huang *et al.*, 2005). Another drug inhibiting adenosine receptors of the P1A subtypes is theophylline (Daly *et al.*, 1987), which has in the past been used in the treatment of bronchial asthma, although its mechanism of action is not fully unraveled.

Regarding the P2 purinoceptors, clopidogrel is a well-known anticoagulatory drug (antiaggregant). Its mechanism of action is to block $P2Y_{12}$ purinoceptors on platelets, eventually inhibiting platelet aggregation and thus reducing the risk of blood clots (Geiger *et al.*, 1999).

1.4 Purinergic interactions with other transmitter and modulator systems

Apart from being a functional system on its own, the purinergic system also affects, and is affected by, other chemical transmitters and modulators. Many such substances have been implicated and two of them, namely acetylcholine and NO, are in the scope of this thesis important to discuss specifically.

1.4.1 Cholinergic systems

The discovery of acetylcholine as a major chemical transmitter in the body is usually accredited to Henry Dale and Otto Loewi. The former identified this substance in ergot extracts and investigated its pharmacological effects. Loewi later demonstrated that stimulation of the vagal nerve of the frog caused a reduction of the heart rate. When the stimulated heart was cannulated and rinsed with Ringers solution the resulting perfusate seemed to contain a substance, called "Vagusstoff", which in turn could lower the frequency of a separate frog heart (Loewi, 1921). This was a strong proof of chemical neurotransmission and the newly found substance, "Vagusstoff", was eventually identified as acetylcholine, an important parasympathetic transmitter. In turn, this revolutionized the understanding of the autonomous nervous system and the Nobel Prize in physiology or medicine was in 1936 awarded jointly to Henry Dale and Otto Loewi "for their discoveries relating to chemical transmission of nerve impulses" (Nobelstiftelsen, 1965).

Regarding the urinary bladder, all subtypes of the G-protein coupled muscarinic receptors (M1-M5) have been found to be expressed in the rat (Creed *et al.*, 2010; Giglio *et al.*, 2005; Giglio *et al.*, 2009). The muscarinic M3 receptor is the most important subtype for bladder contraction (Andersson *et al.*, 2012; Longhurst *et al.*, 1995; Tobin *et al.*, 1995), and studies have shown that knock-out mice for this subtype express voiding disturbances. It has been suggested that NANC transmitters may to some extent compensate for loss of cholinergic function (Igawa *et al.*, 2004; Matsui *et al.*, 2000).

Apart from having direct functional effects, acetylcholine also affects the purinergic system in the urinary bladder by facilitating the release of ATP in the urothelium. In the cat it has been demonstrated that acetylcholine not only releases ATP, but that it does so to a larger extent in animals suffering from feline IC (Birder *et al.*, 2003). Acetylcholine has also been suggested to be

able to induce the release of NO and prostanoids (Andersson *et al.*, 2008; Hanna-Mitchell *et al.*, 2007). Beside its effects on muscarinic receptors in the urothelium, acetylcholine may also increase the sensitivity of afferent nerve fibers in the lower urinary tract (Iijima *et al.*, 2007; Kullmann *et al.*, 2008).

Interactions between the cholinergic and purinergic systems must also be considered in the efferent part of the micturition arc. Parasympathetic nerve terminals exhibit facilitatory and inhibitory muscarinic receptors. While high intensity neuronal activity with relatively short endurance activates presynaptic muscarinic M1 receptors that facilitate transmitter release, low-intensity activity induces inhibition via muscarinic receptors, possibly of the M4 subtype (D'Agostino *et al.*, 2000; Somogyi *et al.*, 1994; Tobin *et al.*, 1995; Tobin *et al.*, 1998). Since presynaptic receptors have unspecific effects on the transmitter release (Ryberg *et al.*, 2009; Tobin, 1998), and also taking into account the co-transmission of ATP with acetylcholine (Silinsky, 1975), muscarinic receptors are likely to affect the purinergic transmission.

1.4.2 Nitrergic systems

NO was first found to be endogenously formed in man some thirty years ago (Green *et al.*, 1981) and a connection between this free radical gas and inflammation was soon suggested, as reviewed by Bredt *et al.* (1992). The amino acid L-arginine was found to be a source of endogenous NO (Hibbs *et al.*, 1987) produced by enzymes collectively known as nitric oxide synthases (NOS) (Palmer *et al.*, 1989). Regarding smooth muscle, NO is commonly regarded to produce relaxation by binding to iron in the heme part of guanylyl cyclase, thereby stimulating formation of cGMP which acts on protein kinases phosphorylating myosin, giving rise to relaxation (Bredt *et al.*, 1992). In the urinary bladder, direct relaxatory effects of NO have been suggested (Chen *et al.*, 1996), while questioned by other studies where alternative NO mechanisms, such as actions on nerves and stromal cells, have been implied (Fujiwara *et al.*, 2000).

Inducible and endothelial forms of NOS have been shown to be markedly increased in the urothelium of rats treated with LPS or CYP, thereby inducing inflammation, which suggests an important role of NO during pathological conditions (Andersson *et al.*, 2008; Haleen *et al.*, 1987; Longhurst *et al.*, 1995). While acetylcholine may induce release of NO which counteracts the cholinergic contraction (Andersson *et al.*, 2008; Bruns *et al.*, 1987; von Kugelgen, 2006), the interaction of ATP and NO in the contractile

response appears to be small (Muller *et al.*, 1998; Vesela *et al.*, 2012b). This is in contrast to the relaxatory purinergic response, which seems to involve NO (Muller *et al.*, 1998).

Furthermore, the purinergic and nitrergic systems may interact during inflammation. In endothelial cells, extracellular ATP has been shown to increase the production of NO through stimulation of P2X purinoceptors (Dunn *et al.*, 1988). Also, opposing interactions have been identified, namely that NO and ATP are linked so that the ratio of ATP/NO is high in the overactive- but low in the underactive bladder (Soto *et al.*, 1997).

1.5 Common models for studying the function of the rat urinary bladder

Traditionally, a common method to investigate functional responses to agonists and antagonists in the urinary bladder has been *in vitro* studies in organ baths (tissue baths). The use of this method has many advantages. It is a reliable and robust setup, which makes possible the examination of direct effects of water soluble drugs on detrusor contraction and relaxation. In many cases drugs are metabolized in the liver, gut or bloodstream and may produce confounding factors which can be disregarded when using the organ baths, although one must bear in mind that hydrolysis and metabolization of pharmacologically active substances by enzymes present in the tissue can still occur. Ethically, one must consider the need to euthanize the animal before the study, although no, or very few, experimental procedures causing discomfort must be performed before sacrifice. This also means that the same animal cannot be used in further studies, *e.g.* before and after treatment.

Like many other *in vitro* methods studies executed in the organ bath cannot, however, answer questions regarding whole body physiological and pharmacological effects. For this purpose one must resort to *in vivo* studies, which include investigation of the micturition pattern using an awake, freely moving animal in a metabolism cage. Here, the composite impact of factors affecting the micturition pattern can be evaluated and also urine can be collected for further analysis. Cystometry is another method which can be performed in many ways, all using the same basic principle. The urinary bladder is filled using either a catheter through the urethra, or in some cases through the bladder wall. Alternatively, the animal can be injected with saline increasing the production of urine. Thereafter the intravesical, and sometimes the rectal/abdominal, pressure is recorded when experimental stimulation is performed. Also, the *in vivo* methodology enables the studies of the influence of the nervous system and reflexes which are not possible *in vitro*.

True for these *in vivo* models is the fact that its greatest strength is also its greatest weakness; the whole body is studied, giving the response most likely to be of clinical interest but, at the same time, the picture can be obscured by the many unknown, or uncontrolled, factors affecting the results.

Many of the studies in this thesis are based on *in vitro* organ bath studies. We did, however, identify a need to develop a novel method to further make possible investigations of the influence of cystitis on both efferent and afferent nerves, as well as the micturition reflex, at a level between *in vitro* and *in vivo*. The novel *in situ* method should render a controlled setup with

the possibility to measure direct effects, while having access to the complexity of the whole animal. A key issue was also the relative ease by which the new model would be comparable to previously established *in vitro* and *in vivo* methods.

1.6 Aims

The overall aims of this thesis were to characterize the purinergic functional effects in the urinary bladder and to investigate the role of purines and NO in the development of cystitis. Furthermore, a novel method to study functional responses to afferent and efferent nerve stimuli *in situ* was developed and evaluated.

1.6.1 Specific aims

Several methods used in studies of the function of the rat urinary bladder were employed and compared in Paper I. Contractile and relaxatory purinergic responses were investigated *in vitro* in the normal rat urinary bladder and conclusions were drawn regarding which purinoceptor subtype(s) that are of importance in bladder function (Paper II). Furthermore, the purinergic part of the contractile response to electric field stimulation was characterized in both healthy rats and in ones with CYP-induced cystitis (Paper III).

The study of the alterations in the inflamed rat urinary bladder, compared to the normal case, was further expanded using our novel *in situ* setup. Changes in contractility and reflexly evoked contractions to stretch stimulation were investigated in Paper IV, in which the significances of purinergic, as well as cholinergic, signaling were studied.

Lastly, the roles of the purinergic, nitrergic and, to some extent, cholinergic systems in the development of inflammation were investigated in Paper V and VI, using pre-treatment with antagonists for these systems. The impact of the pre-treatments on not only functional response, but also on several other inflammation parameters, such as bladder morphology, mast cell localization and expression of receptors (*e.g.* muscarinic M5 receptors) and MIF, were thus investigated.

2 METHODS AND MATERIALS

The methods and materials employed in the current studies are fully described in the respective papers. Here follows an overview of the most relevant aspects of the methods and materials used in this thesis.

In all experiments male Sprague-Dawley rats were used and all studies were approved by the local ethics committee at the University of Gothenburg (# 8-2006, 315-2007, 410-2008, 279-2009, 282-2010, 362-2011).

2.1 *In vitro* organ bath studies (Paper I- III, V- VI)

Rats were anesthetized and sacrificed using an overdose of carbon dioxide. Full thickness bladder strip preparations (approximately 2x6 mm) were cut from the excised urinary bladders and mounted in 25 ml organ baths containing Krebs solution gassed with 5% carbon dioxide and 95% oxygen, kept at 37^{o}C (see Fig. 2 for a schematic representation). The specimens were gently stretched and left to equilibrate for 45-60 min before the experiment was commenced. Viability was confirmed by addition of a high potassium Krebs solution (124 mM) obtained by exchanging Na^+ for equimolar amounts of K^+.

Agonists were administered cumulatively in the volume of 125 µl and the change in tension was recorded. Antagonists used were administered in the same volume and left to equilibrate for about 20 min before agonists were employed. When relaxations were studied the bladder strip preparations were pre-contracted using a 50 mM potassium Krebs solution obtained by exchanging Na^+ for equimolar amounts of K^+. Electric field stimulation was employed at increasing frequencies, delivered as square wave pulses with 0.8 ms duration at supra-maximal voltage, continuously until the peak response was obtained.

Removal of urothelium was in some experiments performed by filling the excised bladder with collagenase I (0.1% w/v in Hank's Balanced Salt Solution) for 30 min and then gently scraping the urothelium with a wet cotton swab (Paper II).

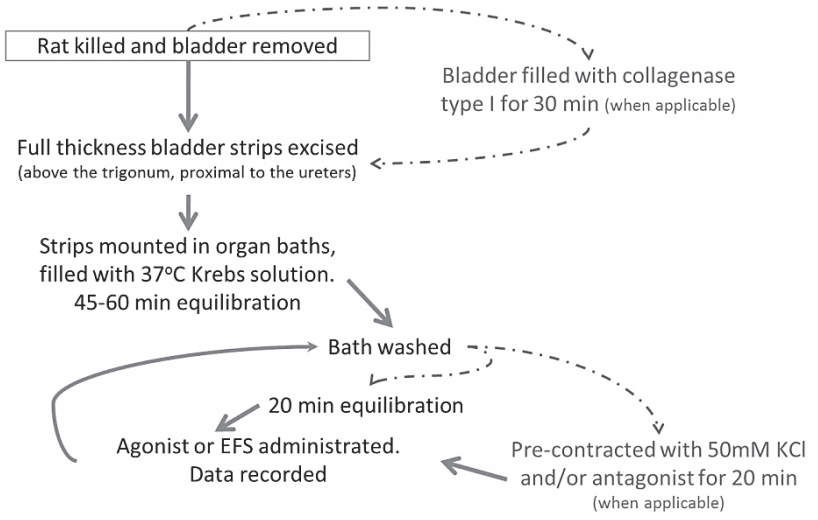

Figure 2. Schematic representation of the preparation and execution of in vitro organ bath experiments.

2.2 *In vivo* whole bladder preparation (Paper I)

Rats were anesthetized and the femoral artery and vein were catheterized in order to make possible blood pressure recordings and injections of pharmacologically active substances, respectively (see Fig. 3). Surgery was performed on a thermoregulated blanket to preserve body temperature and the trachea was cannulated to maintain a free airway.

When the urinary bladder had been exposed, a small incision was made at the top and a catheter was inserted, thus enabling a bladder pressure recording. The change in bladder pressure to intravenous (i.v.) administration of agonists, *i.e.* methacholine and ATP, in the absence or presence of antagonists was then studied. Also, responses to electric nerve stimulation (approximately 30 s; 10-15 V square wave, 0.8 ms pulse width) were investigated.

2.3 *In situ* half bladder preparation (Paper I, IV)

The general procedure was as described above (*In vivo* whole bladder preparation, Fig. 3). The urinary bladder was, however, exposed and split into two completely separated halves via a midline incision. Great care was taken not to damage any major blood vessels or nerves. The lower part of one of the two bladder halves was then fixated with ligatures to the underlying tissue and the upper ligature was connected to an adjustable isometric force transducer. The lower part of the other bladder half was also fixated and the upper part was used for stretching the preparation using weights of 3, 5 or, occasionally, 8 grams, thus evoking a stretch stimulus. Electrical and pharmacological stimulation was also performed in the same manner described previously.

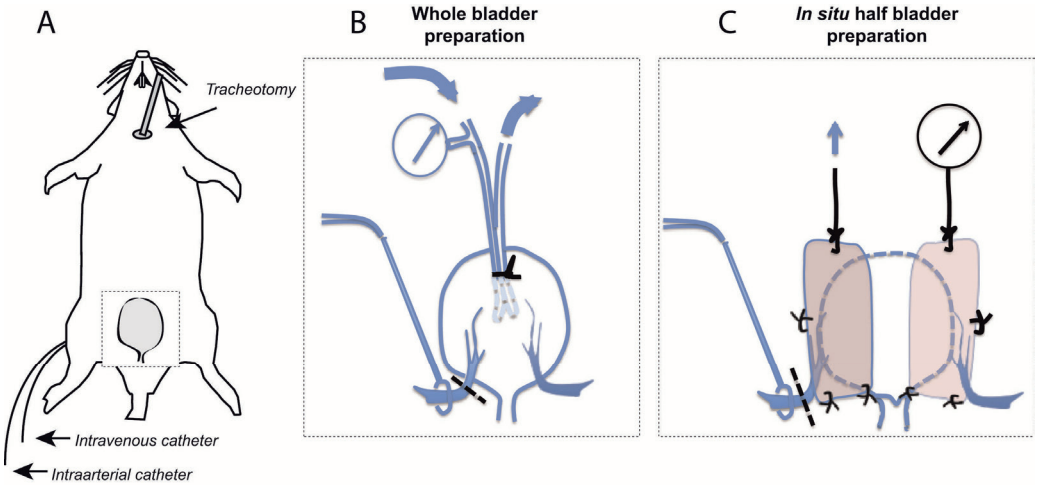

Figure 3. Schematic illustration of whole bladder and in situ half bladder preparations in the rat. Catheters were in anesthetized rats inserted in the femoral vein and artery (A). Thus, drug administration and blood pressure monitoring were made possible. Also, tracheotomy was performed (A). Subsequently, preparations were made for either whole bladder recordings (B), by inserting a dual catheter through the top of the bladder, or in situ half bladder recordings (C), by splitting the bladder into two separate halves where one could be stretched and the other was left free to contract. In both cases, preparations for electric nerve stimulation of the pelvic nerve were made, as depicted by the electrode in the bottom left corner of (B) and (C). Recording of pressure (B) or contractile force (C) are symbolized by the encircled arrow. From Paper I.

2.4 CYP- treatment (Paper III- VI)

Experimental cystitis was induced by a single injection of CYP (100 mg/kg; i.p.) in combination with the analgesic buprenorphine (10 µg/kg s.c.) 60 hours before the experiment. Controls instead received an injection of saline (0.9%; 1 ml/kg).

2.5 Pre- treatment (Paper V- VI)

Rats were pre-treated with intraperitoneal (i.p.) injections of purinergic antagonist (DPCPX, 1 mg/kg; PSB1115, 1 mg/kg or suramin, 10 mg/kg), muscarinic antagonist 4-DAMP (1 mg/kg), NOS inhibitor L-NAME (30 mg/kg), or saline (1 ml/kg) as control once daily for five consecutive days (Fig. 4). 60 hours before the experiment the rats were treated with either CYP or saline (as described above).

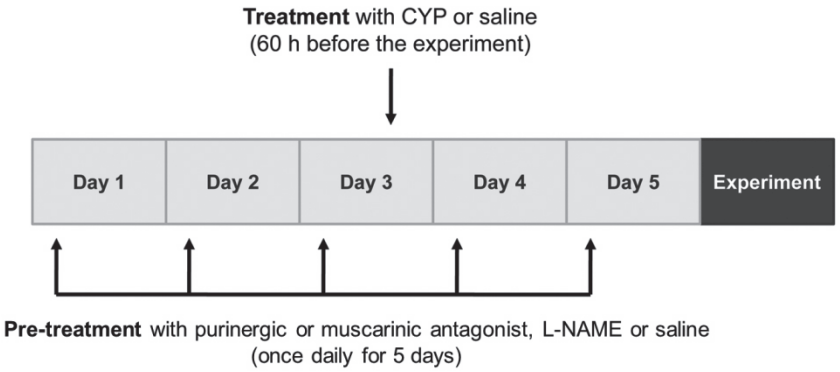

Figure 4. Schematic representation of the pre-treatment/treatment regimen used in Paper V and VI. Purinergic antagonist (suramin, DPCPX or PSB1115), muscarinic antagonist (4-DAMP), nitric oxide synthase inhibitor (L-NAME) or saline (as control) was administered by intraperitoneal injections once daily for five consecutive days. Sixty hours before the functional experiment treatment with either cyclophosphamide (CYP) or saline (as control) was administered. Adapted from Paper V.

2.6 Mast cell count (Paper V- VI)

Sections of paraformaldehyde fixed paraffin embedded rat urinary bladders (4 μm) were de-paraffinized, rehydrated and stained with toluidine blue working solution for 2.5 min. The sections were then cleared in ethanol and xylene and covered using a resinous mounting medium. Toluidine blue positive cells in the sections, excluding the adventitia, were counted using a light microscope.

2.7 Hematoxylin & eosin staining (Paper V)

Four μm sections of paraformaldehyde fixed paraffin embedded bladders were run in a Leica Autostainer XL programmed for hematoxylin and eosin staining according to a standard protocol. The preparations were then investigated using a light microscope.

2.8 Immunohistochemistry (Paper V- VI)

Sections of paraformaldehyde fixed paraffin embedded bladders (4 μm) were de-paraffinized, rehydrated and treated with citrate buffer and phosphate buffered saline to break any protein-cross linking and non-specific staining, respectively. The sections were subjected to primary polyclonal antibodies overnight and, subsequently, secondary antibodies and rhodamine conjugated phalloidin. After dehydration in ethanol, sections were mounted with Prolong gold antifade reagent with DAPI and viewed by microscopy. Negative controls were treated in the same way without addition of primary antibody.

2.9 Substances (Paper I- VI)

All substances were purchased from Sigma-Aldrich, St Louis, MO, except for those marked with numbers as follows; [1] Tocris Bioscience, Bristol, United Kingdom, Stockholm, Sweden, [2] Santa Cruz Biotechnology, Santa Cruz, USA, [3] Molecular Probes, Eugene, USA, [4] Research Diagnostic Antibodies, Berkley, USA, [5] Invitrogen, Burlington, Canada, [6] Histolab, Gothenburg, Sweden, [7] Kemetyl, Stockholm, Sweden, [8] Apoteket Farmaci, and [9] Apoteket Produktion & Laboratorier AB, Gothenburg, Sweden.

Key substances used are summarized in Tab. 1-3.

2.9.1 Substances acting on purinoceptors

Adenosine, adenosine 5´-diphosphate (ADP), adenosine 5´-triphosphate (ATP), N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)]thio urea [1] (MRS 2578), 8-(3-chlorostyryl)caffeine (CSC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt (MRS 2179), α,β-methylene adenosine 5´-triphosphate (α,β-meATP), 3-(2-oxo-2-phenylethyl)-uridine-5'-diphosphate [1] (PSB0474), pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), reactive blue 2 (RB-2), 8-p-sulfophenyltheophylline (8-SPT), suramin, 4-(2,3,6,7-tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid [1] (PSB1115), 2-thiouridine 5'-triphosphate tetrasodium salt [1] (2-ThioUTP), uridine-5´-diphosphate (UDP), uridine-5´-triphosphate (UTP).

2.9.2 Substances acting on muscarinic receptors

Acetyl-β-methylcholine (methacholine), atropine, carbamylcholine (carbachol), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), methoctramine, pirenzepine.

2.9.3 Substances acting on adrenoceptors

Isoprenaline, phentolamine methansulphate, phenylephrine, propranolol.

2.9.4 Substances used for immunohistochemistry

ABC staining system sc-2018 [2], Alexa Fluor 488 goat anti-rabbit IgG [3], anti-M5 produced in rabbit [4], anti-MIF produced in rabbit, anti-$P1A_1$ produced in rabbit, citric acid, goat serum, methanol, rhodamine conjugated phalloidin, sodium citrate tribasic dehydrate, phosphate buffered saline tablets [5], Prolong gold antifade reagent with DAPI [5].

2.9.5 Other substances

6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5-triphosphate trisodium salt (ARL 67156 trisodium salt), $CaCl_2$, collagenase I, cyclophosphamide monohydrate (CYP), dimethyl sulfoxide (DMSO), eosin [6], ethanol [7], [8] glibenclamide, glucose, HCl, KCl, KH_2PO_4, lidocaine hydrochloride

(Xylocain®; AstraZeneca, Södertälje, Sweden), N_{ω}-nitro-L-arginine (L-NNA), N_{ω}-nitro-L-arginine methyl ester (L-NAME), Meyers HTX [6], $MgSO_4$, NaCl, $NaHCO_3$, pertex [6], phosphate buffered formalin (4%) [9], toluidine blue O, tetrodotoxin (TTX), xylene [6].

2.9.6 Anesthetics and analgesics

Buprenorphine [8] (Temgesic®; Schering-Plough, Brussels, Belgium), Medetomidine [8] (Domitor®Vet; Orion Pharma, Espoo, Finland), Pentobarbitone [8] (Pentobarbitalnatrium vet.; APL, Stockholm, Sweden).

2.10 Statistics and calculations (Paper I-VI)

Statistical significance was determined by Student's t-test for paired or unpaired data. For multiple comparisons, statistical significance was determined by one-way analysis of variance (ANOVA) or repeated measurements one-way analysis of variance (MANOVA) followed by the Bonferroni multiple comparison test or Tukey's HSD post hoc test. P-values <0.05 were regarded as statistically significant. Graphs were generated and parameters computed using GraphPad Prism (GraphPad Software, Inc., San Diego, US). Data is presented in the form of mean±S.E.M.

Table 1. Key substances affecting mainly P1 purinoceptors

Name	**Type of substance**	**Acting on receptor**	**Reference**
Adenosine	Agonist	P1	(Burnstock, 1978; Ralevic *et al.*, 1998)
CSC	Antagonist	$P1A_{2A}$	(Jacobson *et al.*, 1993)
DPCPX	Antagonist	$P1A_{1}$	(Bruns *et al.*, 1987; Haleen *et al.*, 1987)
PSB1115	Antagonist	$P1A_{2B}$	(Muller *et al.*, 1998)
8-SPT	Antagonist	P1	(Daly *et al.*, 1985)

Table 2. Key substances affecting mainly P2 purinoceptors

Name	Type of substance	Acting on receptor	Other information	Reference
ADP	Agonist	P2Y	Mainly $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$	(von Kugelgen, 2006)
ATP	Agonist	P2X, P2Y		(Burnstock, 1978; Ralevic *et al.*, 1998)
PSB0474	Agonist	$P2Y_6$		(El-Tayeb *et al.*, 2006)
2-thio-UTP	Agonist	$P2Y_2$		(El-Tayeb *et al.*, 2006)
UDP	Agonist	P2Y	Mainly $P2Y_6$	(Nicholas *et al.*, 1996)
UTP	Agonist	P2Y	Mainly $P2Y_2$, $P2Y_4$	(Chen *et al.*, 1996; von Kugelgen, 2006)
MRS 2179	Antagonist	$P2Y_1$		(Boyer *et al.*, 1998)
MRS 2578	Antagonist	$P2Y_6$		(Mamedova *et al.*, 2004)
PPADS	Antagonist	P2	Not P2X4, P2X6	(Ralevic *et al.*, 1998; Soto *et al.*, 1997; Ziganshin *et al.*, 1993)
RB-2	Antagonist	P2		(Nakazawa *et al.*, 1991; Ralevic *et al.*, 1998)
Suramin	Antagonist	P2	Not $P2Y_4$. Has ecto-nucleotidase inhibiting properties	(Crack *et al.*, 1994; Dunn *et al.*, 1988; Soto *et al.*, 1997)

α,β-meATP	Desensitizer /Agonist	P2X1, P2X3	Generally not P2Y. Functionally used as antagonist	(Ralevic *et al.*, 1998)
ARL 67156	Ectonucleo-tidase inhibitor	-	Blocks break-down of ATP/UTP	(Crack *et al.*, 1995)
Gliben-clamide	Antagonist	-	Blocks ATP sensitive K-channels	(Gribble *et al.*, 2003)

Table 3. Key substances affecting cholinergic, nitrergic and adrenergic receptors

Name	Type of substance	Acting on receptor	Other information	Reference
Carbachol	Agonist	Cholinergic receptors	Both muscarinic and nicotinic	(Goodman *et al.*, 2001; Sellers *et al.*, 2012)
Methacholine	Agonist	Muscarinic M1-M5	Only minor nicotinic activity	(Goodman *et al.*, 2001)
4-DAMP	Antagonist	Muscarinic M1/M3/M5	Possibly most effective at M1	(Eglen *et al.*, 2000)
Atropine	Antagonist	Muscarinic M1-M5		(Goodman *et al.*, 2001; Hegde *et al.*, 1997)
Methoctramine	Antagonist	Muscarinic	M2 > other muscarinic subtypes	(Melchiorre *et al.*, 1987; Wess *et al.*, 1988)
Pirenzepine	Antagonist	Muscarinic	M1 > other muscarinic subtypes	(Caulfield, 1993)
L-NAME	NOS inhibitor	-	Is rapidly metabolized to L-NNA	(Brouillet *et al.*, 1995; Pfeiffer *et al.*, 1996)
L-NNA	NOS inhibitor	-	More potent & less water soluble than L-NAME	(Dwyer *et al.*, 1991; Kopincova *et al.*, 2012)

Isoprenaline	Agonist	β-adrenoceptors	Affects all β-subtypes	(Emorine *et al.*, 1994; Lotti *et al.*, 1982)
Phenylephrine	Agonist	α1-adrenoceptors		(Minneman *et al.*, 1994)
Phentolamine	Antagonist	α-adrenoceptors	Similar affinity for α1 and α2	(Goodman *et al.*, 2001)
Propranolol	Antagonist	β-adrenoceptors	More effective at β1 and β2 than β3	(Cohen *et al.*, 1995; Cohen *et al.*, 1980)

3 RESULTS AND DISCUSSION

The primary endpoint of all medical experiments is to find the underlying causes for morbidity. The extrapolation of preclinical findings requires at some stage *in vivo* experiments to be performed in order to gain knowledge needed to verify the significance of other experimental findings.

However, functional responses in the urinary bladder can be studied in several ways, all with their respective pros and cons. Among the most common approaches are *in vitro* examinations of contractions and relaxations using bladder strip organ bath preparations, which have been used in Paper I-III, V-VI. This method is considered to generate robust data and allows for the study of selected receptors or systems by administration of agonists, antagonists or other substances such as enzyme inhibitors or, alternatively, electric field stimulation. Also, concentrations of pharmacologically active substances being toxic, or causing unwanted side-effects, to a living animal can be used, thereby obtaining concentration-response data allowing for mathematical assessment.

This *in vitro* technique does, however, not necessarily provide all information required to fully understand the complex nature of the living organism. It is, for instance, in isolated bladder strips not possible to look at reflexes, or other involvement of the central nervous system, changes in blood pressure, hormonal factors etc. Therefore, *in vivo* studies on changes in intravesical pressure in anesthetized animals, as have been employed in Paper I, are usually performed. This methodology, while adding more complexity to the performance and interpretation of the study, makes possible investigations of the impact of pharmacologically active substances or electric nerve stimulation on the bladder pressure, taking into account many of the intricate systems involved in the functional regulation of the lower urinary tract.

Alternatively, in order to study the wake, free-moving animal, we have previously used metabolism cages (Andersson *et al.*, 2011). While this methodology may arguably present the “true” nature of the systems studied, it also adds further complexity, compensating mechanisms and confounding factors, such as water intake etc., to take into consideration, which may obstruct the possibility to draw conclusions from such studies.

3.1 Experimental setups for functional studies

In this thesis, the *in vitro* organ bath preparation and *in vivo* bladder pressure studies on anesthetized rats have been compared to a third, novel *in situ* half bladder setup, used in Paper I and IV. This novel model allows for studies of the stretch-evoked micturition reflex arc, while at the same time make possible the use of pharmacological stimulation and also afferent and efferent nerve stimulation. The three functional methodologies mentioned were compared based on contractile responses to administration of agonists and electricity.

3.1.1 Comparisons of response to agonists

The respective responses to the administration of agonists were studied in the aforementioned three experimental setups. Since the contractile responses are measured in different units in the whole bladder preparation (mmHg) compared to the *in situ* half bladder preparation and *in vitro* organ bath setup (mN), the magnitude of the figures will of course differ. Important conclusions can, however, be drawn from analyzing the response patterns.

In the whole bladder preparation (Paper I), the contractions to methacholine (5 μg/kg) and ATP (100 μg/kg) were of similar sizes (7.3±1.7 and 7.9±2.0 mmHg, respectively, Fig. 5). Comparable responses were found in the novel *in situ* half bladder experimental setup, where the same doses as above of agonists evoked contractions of 7.0±2.3 mN (methacholine) and 8.9±2.0 mN (ATP). The maximal contractile response seemed to be reached at higher doses of methacholine in the whole bladder model, compared to in the *in situ* setup. This may possibly be due to the difference in muscle organization, where the whole bladder can contract in the *in vivo* setup whereas only longitudinal contractions are possible *in situ* (Gabella et al., 1990).

On a molar basis, the differences in contraction between methacholine and ATP were rather small in the *in situ* setup, with 18 nmol/kg ATP yielding a contraction of a magnitude between those evoked by 10 and 26 nmol/kg methacholine (4.0 compared to 2.8 and 7.0 mN, respectively). Atropine (1 mg/kg) abolished contractions to methacholine and reduced ATP-evoked contractions (100 μg/kg) to 1.7±0.2 mN.

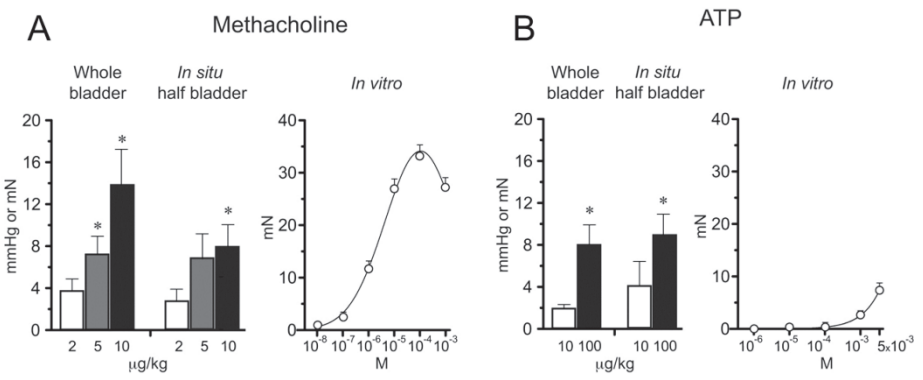

*Figure 5. Mean contractile responses to methacholine (A) and ATP (B) in the whole bladder in vivo model, the in situ half bladder setup and the organ bath in vitro method. * denotes $p<0.05$ compared to lowest agonist dose. Functional response is measured in mmHg in the whole bladder model and in mN in the half bladder setup and in vitro studies. n=4-9, vertical bars represent the S.E.M. Adapted from Paper I.*

Interestingly, administration of methacholine and ATP to the *in vitro* organ bath preparation (Paper I) evoked much larger contractions in the former case than did ATP (33.0±2.1 mN to 0.1 mM methacholine and 7.3±1.4 mN to 5 mM ATP). The relation between the ATP- and methacholine-evoked responses thus varied under different conditions. The discrepancies elucidate the composite effects in the response in the whole animal, which may enable synergistic, as well as counteracting, influences, by factors such as blood flow reductions etc. This underlines the complexity of possible interactions previously shown, namely that the muscarinic M2 receptor plays a inhibitory role on purine-evoked contractions *in vitro* (Giglio *et al.*, 2001) as well as recent findings of possible negative interactions between P2X purinoceptors and cholinergic receptors in rat muscle cells (Heppner *et al.*, 2009; Jenes *et al.*, 2012). Also, an ATP-induced release of ATP has been described in several types of tissue (Bodin *et al.*, 1996; Sperlagh *et al.*, 1991), including the urinary bladder (Sun *et al.*, 2006). Previous studies have also described an ATP-induced release of acetylcholine (Northway *et al.*, 1980) in the dog intestine, even though others have found an inhibitory relationship (Barajas-Lopez *et al.*, 1995), which puts further emphasis on the complex nature of the interactions of these systems.

3.1.2 Comparisons of response to electrical stimulation

Electrical efferent stimulation of the parasympathetic pelvic nerve evoked similar, but nevertheless slightly different, patterns in the *in vivo* and *in situ* preparations (Paper I, Fig. 6). The former setup displayed its peak response at 10-20 Hz (13.3±1.2 mmHg at 20 Hz), whereas the latter, *in situ*, preparation showed no absolute maximum to efferent simulation, although occasional observations showed no further increase above 40 Hz (19.8±3.6 mN at 40 Hz). *In vitro*, the maximal response to electric field stimulation was observed at 40 Hz (Paper II and III).

Also the contractile responses to afferent electrical stimulation showed great resemblance *in vivo* and *in situ* (Paper I). Maximal responses were detected around 40 Hz (6.7±0.7 mmHg *in vivo* and 4.4±0.5 mN *in situ*), even though no absolute maximum contraction could be determined in the whole bladder preparation, whereas it seemed to occur at 20-40 Hz in the *in situ* half bladder preparation. Additional pentobarbitone or transection of the pelvic nerve on the contralateral side abolished the functional response to afferent stimulation.

Furthermore, the threshold frequencies were similar in the *in vivo* and *in situ* setups studied to both efferent (<2 Hz) and afferent electrical nerve stimulation (5-10 Hz, Paper I). *In vitro*, the electric field stimulation threshold frequency was found to be <2 Hz (Paper II and III).

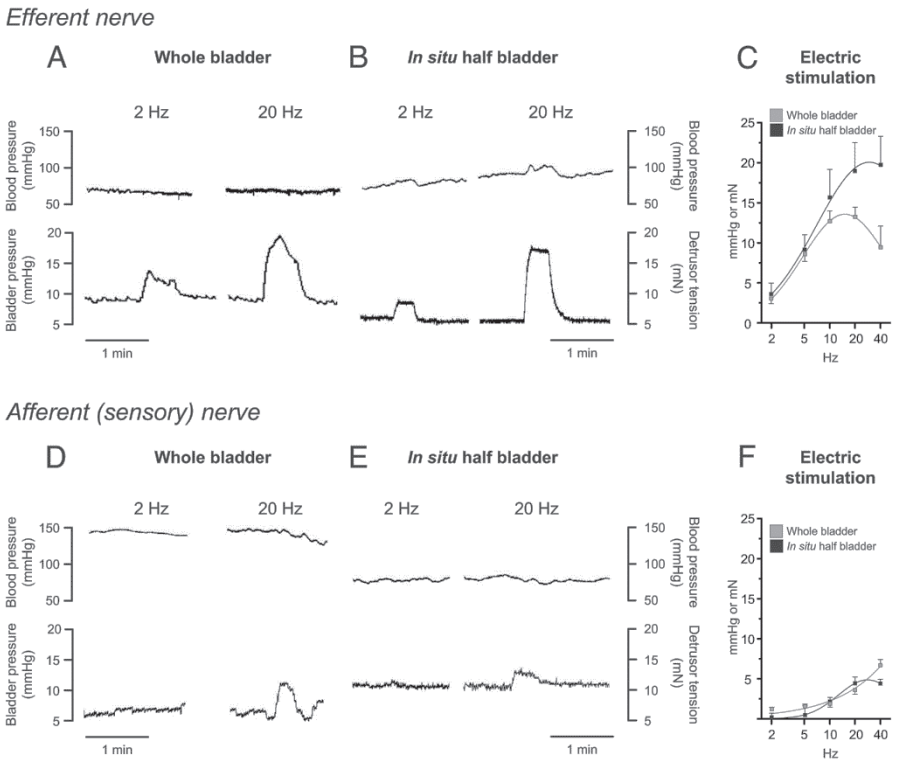

Figure 6. Representative recordings of electric stimulation of efferent (upper row) and afferent (bottom row) pelvic nerve fibers in the conventional in vivo whole bladder model (A, D) and in situ half bladder model (B, E). Mean contractions to 2-40 Hz are also presented (C, F). Functional response is measured in mmHg in the whole bladder model and in mN in the half bladder setup. n=4-5, vertical bars represent the S.E.M. From Paper I.

3.1.3 Functional response to mechanical stretch

Mechanical stretch of 30, 50 or 80 mN to one half of the bladder caused contractions on the contralateral side that were close to the threshold in the first case, and well above in the following (Paper I and IV), where the responses to 50 and 80 mN stretch stimulation were of similar magnitudes (Paper IV). Furthermore, the size of the stretch-evoked response was in the same order as the contraction to afferent nerve stimulation (5.4±1.4 and 4.4±0.5 mN, respectively, in Paper I). The response to these stimulations was found to be well preserved over time (up to 60 min) and could be abolished by administration of additional pentobarbitone or by cutting the contralateral pelvic nerve.

3.1.4 Conclusion of experimental setup comparisons

A large degree of similarity between the novel *in situ* half bladder setup and, predominantly, the traditional *in vivo* urinary bladder pressure model was seen. Overall, the agonist- and EFS-evoked responses of the various models studied correlated well and were found to be comparable. The major difference observed was compared to the *in vitro* organ bath setup, where the purinergic response was much smaller compared to the cholinergic, which indicates a complex interaction between purinergic and cholinergic systems. The novel method was proven to be reliable for a rather long period of time (at least one hour) and to yield reproducible data. Furthermore, it offers the significant advantage of making possible the study of stretch-induced contractile responses of the urinary bladder, mediated via the reflex arc, a feature which is likely altered during cystitis (see Paper IV). Afferent mechanisms and stretch sensitivity have previously been studied. In those experiments, however, the focus has often been on neuronal activity, rather than on the functional outcome of this parameter, *i.e.* bladder contraction (Ishii *et al.*, 2012; Zagorodnyuk *et al.*, 2007). Alternatively, micturitions have been allowed, producing spikes in bladder pressure depending on the level of bladder filling (Mallory *et al.*, 1989; Yu *et al.*, 2013). The novel *in situ* setup provides an alternative to this, enabling graded measurements of bladder contraction and studies of lateral cross-transmission without interference of the volume of bladder contents.

It may be argued that the afferent stimulation evoked substantially smaller contractions than did the efferent stimulation. However, one ought to bear in mind that the responses were measured on the contralateral side to the nerve being stimulated, and that only one side was stimulated. It has previously been demonstrated in the cat that contralateral neuronal activity occurs, however with less intensity than in the ipsilateral side (Mazieres *et al.*, 1998). Also, in the normal micturition reflex the whole bladder is stretched, and by that more impulses may reach the central nervous system.

By studying the stretch-evoked contralateral contractions, one can gain important knowledge of this physiologically important mechanism. Also, the risk of any antidromic transmitter release is markedly reduced by using the novel setup for stretch-stimulation instead of electrical nerve stimulation. Additionally, compared to the *in vivo* whole bladder model, one does not have to take into consideration the confounding factors of saline in- and outflow or the extent of bladder filling. Also, it would, in future experiments, be possible to manipulate the half of the bladder being stretched, *e.g.* by

removal of the urothelium, while keeping the contralateral half intact, thereby avoiding to cause any damage on the contractile half. Furthermore, experiments could be performed, in which the direct functional role of the different types of stretch receptors can be characterized (Zagorodnyuk *et al.*, 2007).

Thus, the novel *in situ* half bladder method provides an important complement to the well-established *in vivo* and *in vitro* methods presented.

3.2 Purinergic *in vitro* functional effects in the normal urinary bladder

3.2.1 Agonist- evoked responses

In contrast to the EFS-evoked response (see next section), aiming to activate transmitter release from nerve fibers, contractions and relaxations to administration of exogenous ATP were not affected by addition of TTX (1.25 μM, Paper II), suggesting the purinergic response to be mediated through post-synaptic receptors.

ATP gave rise to more pronounced bladder contractions than did ADP, UTP and UDP (Fig. 7A), which were employed in order to characterize the involvement of different P2 purinoceptor subtypes. The relaxatory responses to the nucleotides were, however, clearly divided into two separate groups, where ADP=ATP > UDP=UTP (Fig. 7B). At 300 μM the relaxations to ADP and ATP were -71±12 and -68±9%, respectively, whereas the relaxations to UDP and UTP were -35±5 and -33±3%, respectively.

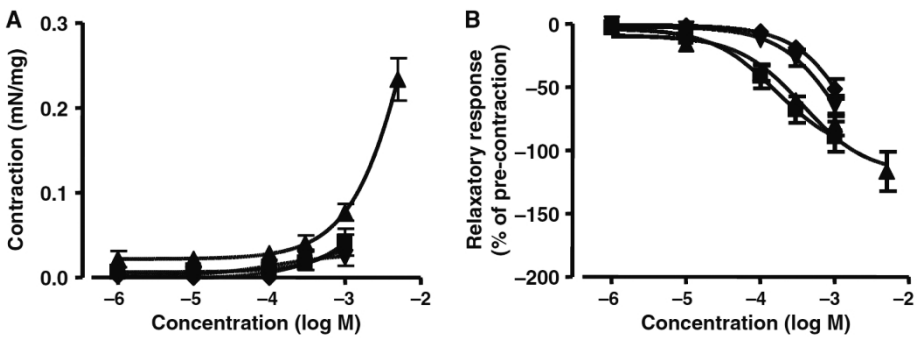

Figure 7. Mean contractions (A; n=5-12) and relaxations (B; n=6-9) in urinary bladder strip preparations to the purinergic agonists ADP (■), ATP (▲), UDP (▼) and UTP (◆). Vertical bars represent the S.E.M. From Paper II.

Addition of the ectonucleotidase inhibitor ARL67156 did, in Paper II, not seem to cause any major over-all alterations in the relaxatory response to ATP or UTP. Interestingly, the relaxations to the lowest dose (1 μM) of both ATP and UTP were attenuated (from -19 to -2% and -7 to -2% $p<0.05$,

respectively), suggesting the break-down into di- or monophosphates to be of importance to the relaxatory response. Further studies, perhaps using higher doses of the ectonucleotidase inhibitor, should be performed to investigate if this is accurate.

The P2 purinoceptor antagonist PPADS concentration-dependently (0.3-30 μM; Paper II) attenuated the ATP-evoked contractions whereas the P2 purinoceptor antagonist suramin did not have any significant effect (Fig. 8). Furthermore, the unselective P1 purinoceptor antagonist 8-SPT, the $P1A_{2A}$ purinoceptor antagonist CSC and the unselective P2Y antagonist RB-2 did not affect the contractile response. One ought to bear in mind that PPADS, and perhaps to an even greater extent suramin, are "dirty drugs" and thus may exert a number of actions, as reviewed by Ralevic *et al.* (1998) and Soto *et al.* (1997). However, taking into account the fact that PPADS and suramin do not have identical affinities for the P2X1 purinoceptor, together with the results from the studies with α,β-meATP may support the conclusion that P2X1 is the purinoceptor mainly, but not solely, responsible for the purinergic contraction of the urinary bladder (Kennedy *et al.*, 2007).

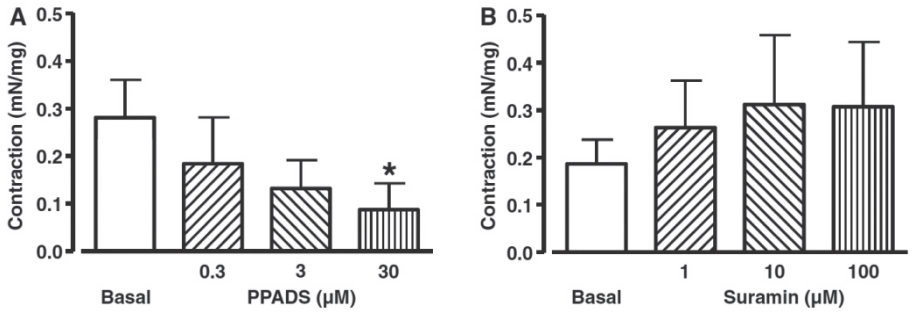

*Figure 8. ATP-evoked contractions in the absence of antagonist (basal) and in the presence of increasing concentrations of PPADS (A) or suramin (B). n=5-6, vertical bars represent the S.E.M. *p<0.05 vs. basal. From Paper II.*

The general P1 purinoceptor antagonist (8-SPT) did, however, lower the relaxatory response to ATP, which was unaffected by the $P1A_{2A}$ purinoceptor antagonist CSC, as well as by suramin and PPADS. To further investigate the relaxatory role of the P1 receptors, adenosine was administered, which caused relaxations that could to some extent be blocked by the $P1A_{2B}$ purinoceptor antagonist PSB1115. Thus, purinergic relaxation seems to be, at

least partly, depending on the $P1A_{2B}$, but not the $P1A_{2A}$, purinoceptor. These findings in Paper II were confirmed, and the understanding expanded, in one of our later studies, where the relaxatory response to a standard dose of adenosine (50 μM) was investigated in the presence of various antagonists (Vesela *et al.*, 2011). In the normal rat (but not in animals with CYP-induced cystitis), the $P1A_1$ purinoceptor antagonist DPCPX was found to decrease the major part of the relaxatory response to adenosine, thus the $P1A_1$ purinoceptor was suggested to be responsible for this relaxation. Furthermore, another selective $P1A_{2A}$ purinoceptor antagonist (SCH 58261 instead of CSC) was administered, but also failed to affect the adenosine-evoked relaxation, whereas the $P1A_3$ purinoceptors, in contrast to the other P1 purinoceptors, were found to inhibit the relaxatory responses to adenosine. It is thus likely that the DPCPX-resistant part of the relaxation is due to the $P1A_{2B}$ purinoceptor.

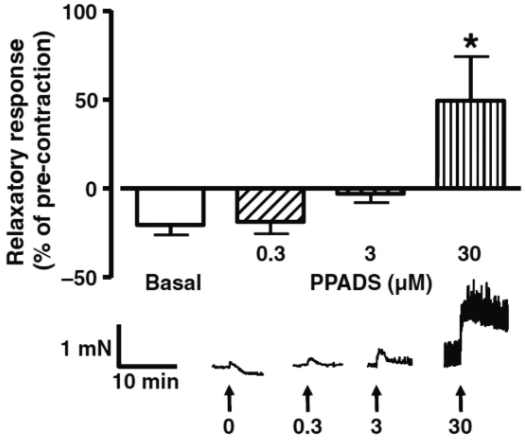

*Figure 9. Mean relaxations/contractions to UTP (0.3 mM) in the absence or presence of increasing concentrations of PPADS. n=5, vertical bars represent the S.E.M. * denotes p<0.05 compared to absence of antagonist. Lower panel shows representative recordings of relaxations/contractions to UTP (0.3 mM) in the absence (0) or presence of PPADS (0.3-30 μM). From Paper II.*

In Paper II the relaxatory response to UDP was reduced by the addition of suramin and tended to be blocked by PPADS. The relaxation was not affected by the $P2Y_6$ purinoceptor antagonist MRS 2578. On the other hand,

relaxations to UTP were reduced by PPADS (but not suramin), which even caused contractions, instead of relaxations, at the highest antagonist concentration (Fig. 9). Relaxations to ATP and ADP were unaffected by both PPADS and suramin.

This further strengthens the hypothesis that relaxations to ADP/ATP and UDP/UTP are mediated via two separate (groups of) receptors in the rat urinary bladder. Based on the data presented, one cannot be certain which receptor subtype that is responsible for the relaxations to UDP/UTP, but judged by the pharmacological profile of UDP/UTP, the P2Y purinoceptor family is most likely involved. One candidate may be a $P2Y_6$-like purinoceptor (as suggested to be important in other tissues). Possibly, the $P2Y_4$-purinoceptor may be involved as well.

Regarding urothelium-denuded full thickness urinary bladder strip preparations investigated in Paper II, the contraction to the muscarinic receptor agonist carbachol was unaffected, whereas the ATP-evoked relaxatory response was attenuated by removal of the urothelium. This indicates that the urothelium has a relaxatory role, to some extent affecting the relaxatory response to ATP, which further strengthens the theory of the urothelium being important in bladder signaling (Ferguson *et al.*, 1997).

3.2.2 Electric field stimulation- evoked responses

Frequency-dependent contractions to electric field stimulation were obtained, showing maximal contraction at 40 Hz. These contractions were totally abolished by the addition of TTX (Paper II) or lidocaine (Paper III), demonstrating that the effect seen was strictly due to neuronal release of transmitter and not because of any direct effect on the smooth muscle.

Frequency-dependent contractions can be sub-divided into the fast, transient "phasic" phase and the longer lasting "tonic" phase (Maggi *et al.*, 1985). The phasic phase is to a large extent atropine-resistant (Paper III, Fig. 10A) but was found to be attenuated by the repeated addition of the P2X1/P2X3 desensitizer α,β-meATP (10 μM), with no further decrease caused by the general P2 antagonist PPADS (Paper II, Fig. 10C). In contrast, the tonic phase was found to be sensitive to atropine (Paper III, Fig. 10B), which completely abolished the tonic component of the EFS-evoked response when added after addition of α,β-meATP and PPADS (Paper II, Fig. 10D).

Interestingly, the tonic component seemed to be unaffected by atropine at low frequencies (<5 Hz) but lowered by purinergic blockade, whereas the opposite was seen at higher frequencies (Paper II and III, Fig. 10B and Fig. 10D), suggesting a relatively large purinergic involvement at low frequencies. The effects of atropine and α,β-meATP in the normal (*i.e.* not inflamed) bladder are in line with previous studies in the guinea-pig (Brading *et al.*, 1989; Kasakov *et al.*, 1982), and also in the rat (Brading *et al.*, 1990), where this blockade abolished the EFS-evoked contractile response. The phasic part of the contraction, however, could at higher frequencies not currently be abolished by cholinergic and purinergic antagonists suggesting other NANC transmitters to be of some importance in the response to EFS, which has been previously indicated (Luheshi *et al.*, 1990).

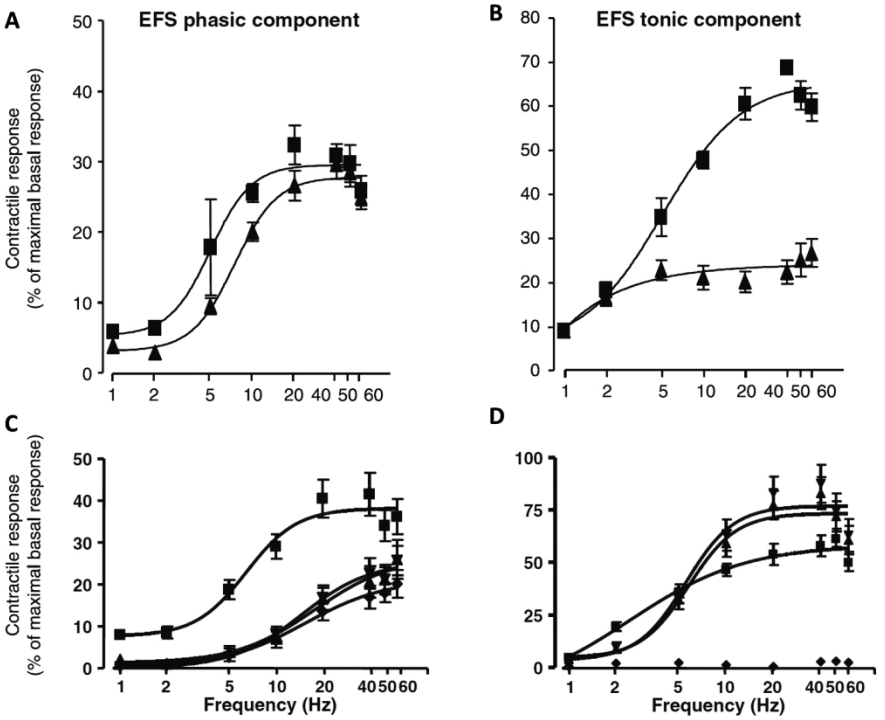

Figure 10. Mean phasic (A, C) and tonic (B, D) contractile responses to electrical field stimulation (1-60 Hz). The upper row (A, B) shows responses in the absence (■) and presence of atropine (▲; 5 μM). The lower row (C, D) depicts contractions in the absence (■) and presence of the P2X1/P2X3 desensitizer α-βmeATP (▲; 10 μM, repeated 3-4 times), α-βmeATP (10 μM, repeated 3-4 times) and the P2 purinoceptor antagonist PPADS (▼; 30 μM) and α-βmeATP (10 μM, repeated 3-4 times), PPADS (30 μM) and the muscarinic receptor antagonist atropine (◆; 5 μM). n = 8-10, vertical bars represent the S.E.M. Adapted from Paper II and III.

This confirms the notion that purinergic transmitters are of the greatest importance in the rapid phasic contraction, whereas the cholinergic system is mainly responsible for the tonic component of the contraction (Fig. 11). In analogy, further studies (Paper III) revealed that the muscarinic M1/M3/M5-receptor antagonist 4-DAMP caused a concentration-dependent decrease in tonic contraction, while affecting the phasic contraction only to a minor extent. In this context it may be worth considering that the cholinergic bladder response may include urothelial inhibitory factor(s) (Andersson *et al.*, 2012; Fovaeus *et al.*, 1999; Giglio *et al.*, 2008) that may partly mask the real reduction of the muscarinic M3 receptor mediated contraction. Furthermore, since the composite EFS-evoked response was found to be composed of both cholinergic and purinergic responses, the adrenergic system was also investigated by the administration of the adrenergic antagonists propranolol (1 μM) and phentolamine (1 μM) in the presence of atropine. This did, however, not affect the atropine-resistant EFS-response (Paper III), suggesting that the non-cholinergic contractile response to EFS does not depend on adrenergic transmission to any large extent.

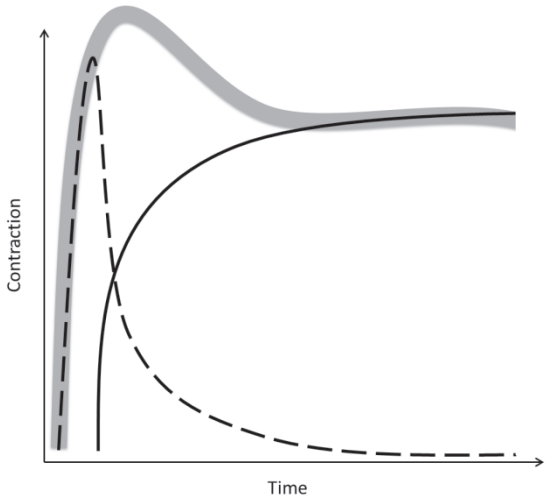

Figure 11. Schematic representation of possible interplay between purinergic (black dotted line) and cholinergic (black solid line) responses to electric field stimulation. Tentatively, the purinergic part of the contraction constitutes a swift initial contraction, while the onset of the cholinergic part of the response is slower, but the response longer lasting. The composite response (grey thick line, corresponding to that of electric field stimulation at 2 Hz) consists of both these contractions, where the purinergic response is of greater importance in the initial, phasic, phase and the cholinergic in the tonic phase.

In separate experiments, the effect of the ectonucleotidase-inhibitor ARL67156 on the EFS-evoked contraction was investigated (Paper II). While the tonic component of the response was unchanged, the phasic component was decreased by 23-53% ($p<0.01$-0.05, $n=7$). This is in contrast to previous studies in other species (Westfall *et al.*, 1997), and this discrepancy may perhaps be due to lowered stimulation of purinoceptors sensitive to metabolites of ATP (*i.e.* ADP, AMP or, possibly, adenosine) involved in mediation of contractile response or pre-synaptic positive feedback of transmitter release. In this context it is worth considering the rather common occurrence of for instance $P1A_1$ purinoceptors known to modulate transmitter release in the central nervous system (Van Dort *et al.*, 2009), in the neuromuscular junction (Veggetti *et al.*, 2008) and also suggested in the urinary bladder (Fry *et al.*, 2004). This could possibly provide an explanation to the opposite responses seen in Paper II.

3.2.3 Conclusion of purinergic *in vitro* functional effects in the normal urinary bladder

A number of different purinoceptor subtypes are expressed in the lower urinary tract. Since their effects vary markedly, the functional response is likely the composite result from synergistic, as well as counteracting, effects. The current results suggest, in accordance with other studies (Kennedy *et al.*, 2007), the purinergic contractions to be mediated mainly by the P2X1 purinoceptor. While ATP evoked larger contractions than the other agonists used, the relaxatory response was divided into two groups, where ADP and ATP evoked larger relaxations than UDP and UTP. Based on the pharmacological profile of the latter two, and the fact that the relaxation was sensitive to P2 purinoceptor antagonism, purinoceptors of the P2Y family are likely to play a relaxatory role in the rat urinary bladder.

The picture gets even more complicated by the possible interactions with other transmitter systems, as underlined in the studies in different experimental setups (Paper I) where, for instance, ATP evoked markedly larger responses compared to methacholine, *in situ* than *in vitro*. Also, these responses to ATP were reduced by atropine. In the canine intestine, ATP has previously been shown to cause neuronal release of acetylcholine (Northway *et al.*, 1980). In unpublished experiments in the rat, the removal of the urothelium did not affect the magnitude of the ATP response, thus ATP-evoked release of acetylcholine from rat bladder neurons seems feasible. *In*

vitro this did not seem to occur since the ATP response was much smaller than the cholinergic and since TTX did not affect the response.

Adenosine was presently shown to play a relaxatory role in the function of the urinary bladder, with the $P1A_{2B}$ purinoceptor subtype being partly responsible for the purinergic relaxation.

Furthermore, the phases of the EFS-evoked contraction were studied, and the initial, rapid phasic part, as well as contractile responses to low frequencies, was found to be largely mediated by the purinergic system, whereas the long-lasting tonic part was affected to a much greater extent by atropine and other cholinergic antagonists. The results also indicate that the phasic part of the contraction to some extent depends on other NANC transmitters than the purinergic. Thus, purinergic and cholinergic systems interact in a number of ways.

3.3 Changes in cyclophosphamide- induced cystitis

Macroscopically, bladders from CYP-treated rats were clearly affected, showing reddening and thickening of the bladder wall (Paper III-VI).

Regarding general contractility, the contractile response by KCl (124 mM) was larger in control than in inflamed bladder strips. Data normalized to the maximal KCl response show twice the contractile response per mg in control strips compared to preparations from rats with CYP-induced cystitis (1.9±0.2 and 0.9±0.1 mN/mg, respectively, $p<0.001$, Paper III).

3.3.1 Changes in response to electric field stimulation

Contractile responses to EFS (1-60 Hz, Fig. 12) were the same in control and inflamed strip preparations (100±5 and 101±7% at 40 Hz) when normalized by the maximal contraction to KCl. This indicates that the reduction in response observed in inflamed bladder strip preparations largely depends on a hampered muscle contractile capacity.

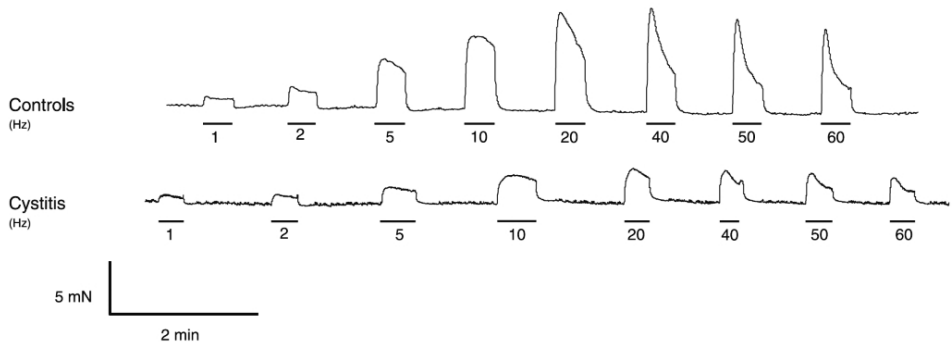

Figure 12. Representative recordings of contractions evoked by electrical field stimulation (1-60 Hz) in urinary bladder strip preparations from controls (first row) and rats with CYP-induced cystitis (60 hours before the experiment; second row). Horizontal bars below the recordings indicate stimulation periods.

The time to maximal EFS-evoked contraction was similar in control and inflamed strips, with the time to peak response increasing with frequency from 7-8 sec at 1 Hz to 14-16 sec at 10 Hz (Paper III). However, the contractile velocity (expressed in the vector-like form of "% of maximal basal response per sec") increased with higher frequencies, probably due to the much higher maximal response at these frequencies. The increase in time to maximal contractile response, in contrast to contractile velocity, with frequency may well, however, be due to a combination of the increased maximal contraction and that the fast purinergic transmission may be more prominent at lower frequencies (<5 Hz).

The relative atropine-sensitivity of the rapid "phasic" phase, previously described in this thesis, was similar in both normal and inflamed strip preparations. However, the muscarinic antagonists used were shown to be less potent in inhibiting the tonic component in inflamed bladders, compared to controls (Paper III), suggesting the NANC efferent contractile transmission to be of greater importance in the tonic contraction in cystitis than in the normal case. However, as indicated in Fig. 13, the atropine-resistant part of the EFS-evoked response may not necessarily only reflect contractions evoked by ATP. Other NANC transmitters, such as tachykinins also contribute, see Vesela (2012a). Nevertheless, taken together this shows that the normalization of contractions to potassium-evoked responses do not show the complete picture. Namely, in CYP-induced cystitis, compensatory NANC mechanisms may mask the reduced cholinergic response. Also, direct comparisons of the relative size of the different parts of the contraction, *e.g.* comparing the magnitude of the NANC part under different conditions, have proven to be hard to perform. Positive and negative interactions on pre- and post-synaptic levels may occur (Giglio *et al.*, 2001; Tobin *et al.*, 1995; Tobin *et al.*, 1998). For instance, if the cholinergic part of the contraction is reduced, the NANC part may increase because of less negative feed-back.

Further studies were carried out (in Paper III) to investigate the differences in the atropine-resistant part of the EFS-evoked contraction between normal and inflamed bladder strip preparations. The fact that this part was, to a large extent, sensitive to the addition of α,β-meATP (10 μM; applied 3-4 times), suggests an involvement of P2X1 purinoceptors (Fig. 13). This purinergic contraction was not further decreased by the subsequent addition of suramin, and tended to be somewhat larger in the controls, compared to preparations from CYP-treated rats, as indicated in gray in the figure.

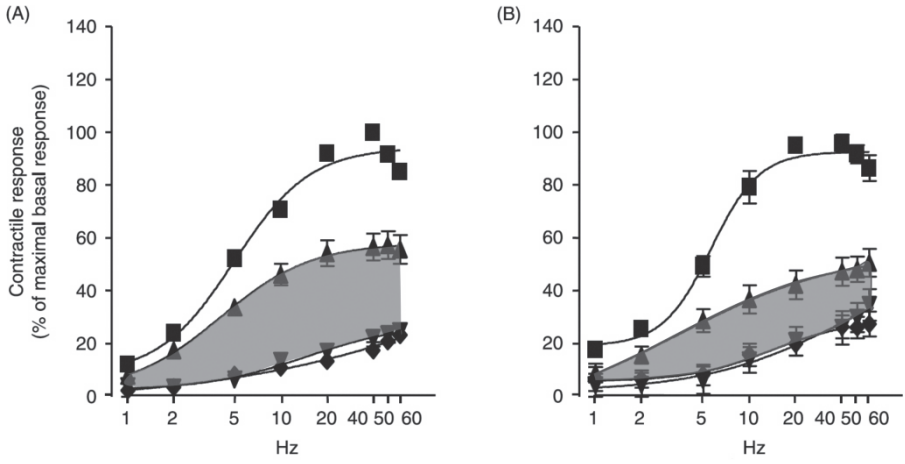

Figure 13. Contractile responses to electrical field stimulation (1-60 Hz) in urinary bladder strip preparations from control rats (A) and rats with CYP-induced cystitis (B). Mean responses in the absence (■), and in the presence of atropine, propranolol and phentolamine (▲; 5 μM, 1 μM and 1 μM, respectively) and the subsequent additional administration of α-βmeATP (▼; 10 μM, repeated 3-4 times) and α-βmeATP and suramin (◆; 10 μM, repeated 3-4 times and 0.1 mM, respectively). The gray area represents part of the response suggested to be mediated by P2X1 purinoceptors. n=7 in both groups, vertical bars represent the S.E.M. Adapted from Paper III.

Further modeling of the raw data used in Paper III combined with previously unpublished data of the effect of PPADS more clearly show the EFS-evoked contractions to be smaller in cystitis than in control (Tab. 4). Also, it is evident that the addition of PPADS (30 μM) significantly inhibits the composite (*i.e.* not separated into phasic and tonic) contraction to EFS-stimulation in bladder strip preparations from normal, but not CYP-treated, rats, as indicated by the large difference in EF_{50} between the normal, but not inflamed, preparations.

Table 4. Modeling of in vitro EFS-evoked contractions in normal and inflamed urinary bladder strip preparations. Pharmacodynamic modeling was performed by applying built-in models for nonlinear regression (curve fit according to either agonist vs. response (variable slope and four parameters), bell-shaped or biphasic concentration-response curves). E_{max} and Hill coefficients (n_H) were generated. In the model fitting procedure, comparisons were based on visual inspection of observed vs. predicted plots, value of the objective function and assessment of parameter correlation precision (R^2). Numbers within parenthesis are modeled E_{max} values. The data used for modeling are previously presented in percentage values in Fig. 4 in Paper III, except for the data regarding PPADS which is unpublished material from pilot experiments.

	E_{max} (mN)	EF_{50} (Hz)	n_H	n	R^2
Normal	16±2	4.8	1.5	10	0.79
CYP	10±2	5.5	1.1	8	0.49
Normal with atropine (5 μM)	7±1	3.7	1.2	10	0.52
CYP with atropine (5 μM)	5±1	6.8	0.8	8	0.39
Normal with PPADS (30 μM)	(18±2)	7.0	1.1	6	0.67
CYP with PPADS (30 μM)	(8±1)	6.2	1.0	6	0.81

3.3.2 Cyclophosphamide- induced cystitis in the *in situ* experimental setup

In the *in situ* half bladder preparation (Paper IV) the contractions to methacholine were lowered in rats with CYP-induced cystitis, compared to control rats (2.1±0.2 and 3.1±0.5 mN, respectively to 5µg/kg methacholine). The response to ATP was, however, not significantly altered (Fig. 14). Transection of the pelvic nerve did not affect the exogenously administered agonist-evoked contractions. Interestingly, addition of atropine did not only abolish the contraction to methacholine, but did also attenuate the response to ATP in both normal and CYP-treated rats.

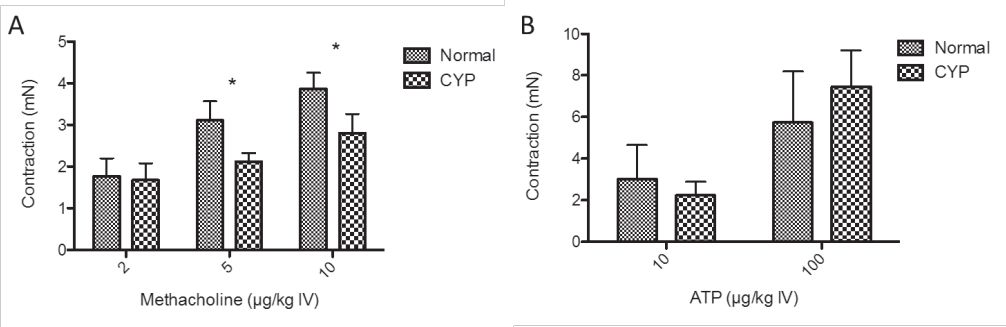

*Figure 14. Mean contractile responses to intravenously administered methacholine (A) and ATP (B) in control (normal) rats and rats with cyclophosphamide-induced cystitis (CYP), using the novel in situ half bladder setup. n=5-8, vertical bars represent the S.E.M. * denotes p<0.05. From Paper IV.*

The contractile response to electric nerve stimulation was lowered in CYP-treated rats. Even though no absolute maximum was observed, a plateau seemed to be reached at around 40 Hz, which is consistent with what is reported in Paper I. The reduction in contractile response was evident at frequencies above 5 Hz, and at 40 Hz the maximal contraction was reduced by approximately 50% in the CYP-treated animals.

In contrast to the methacholine- and electric nerve stimulation-evoked responses which were reduced, and the ATP-evoked contraction which was unchanged, the contractile response to stretch-stimulation was significantly elevated in CYP-treated rats. Stretch of one bladder half with 50 or 80 mN produced statistically significant increases in contralateral bladder half

contraction (Fig. 15), and application of 30 mN showed a convincing tendency of doing the same.

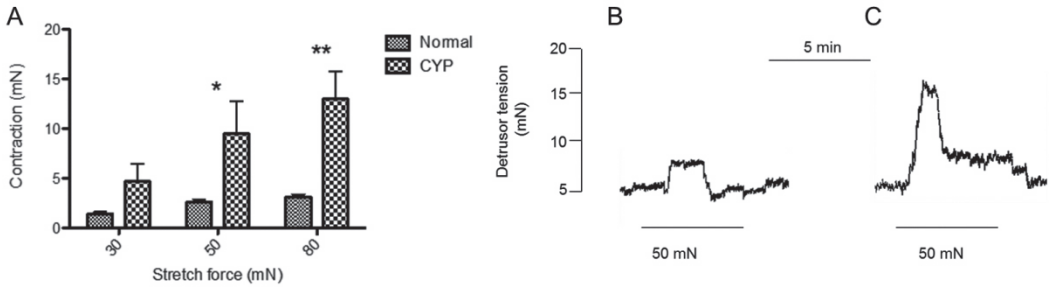

*Figure 15. Mean in situ contractile responses to contralateral stretch-stimulation (A) in control (normal) rats and rats with cyclophosphamide-induced cystitis (CYP). n=6, vertical bars represent the S.E.M. * denotes p<0.05 and ** denotes p<0.01. Also shown are representative recordings of contralateral stretch-evoked (50 mN) in situ bladder half contractions in normal (B) and CYP-treated (C) rats. Horizontal bars represent duration of stretch-stimulation. Adapted from Paper IV.*

In separate experiments, where the aforementioned observations were reproduced, atropine (1 mg/kg i.v.) reduced the stretch-induced contralateral contractions by about 50% in control rats and by about 75% in rats with CYP-induced cystitis. The subsequent addition of PPADS (2 mg/kg i.v.) lowered the contractions by a total of about 80% in the normal rats and >90% in CYP-treated rats (Fig. 16). Noteworthy is that stretch-induced contraction, by application of 30 mN, was not affected to any large extent by atropine, while the subsequent addition of PPADS almost completely removed the reflexly-evoked contractions in both normal and CYP-treated rats. This indicates that this “low-intensity stimulation” to a larger extent is mediated by purinergic transmission and that the part of the response being mediated by cholinergic receptors requires a more powerful stimulus.

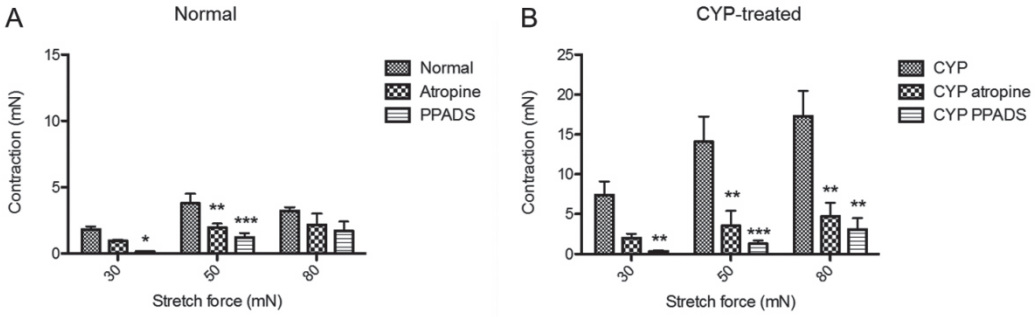

*Figure 16. Mean in situ contractions to contralateral bladder half stretch-stimulation (30-80 mN) in control (normal; A) rats and rats with cyclophosphamide-induced cystitis (CYP-treated; B) in the absence and presence of atropine (1 mg/kg) and the subsequent (following atropine) administration of PPADS (2 mg/kg) n=7, vertical bars represent the S.E.M. * denotes p<0.05, ** p<0.01 and *** p<0.001. Adapted from Paper IV.*

Bearing in mind that it in Paper I was demonstrated that the *in situ* half bladder model generates responses of the same, or if anything larger, magnitude after 30-60 minutes, and that any issues with the anesthesia wearing off would most likely cause an increase in reflexly-evoked contractions, the present results according to the effects of the antagonists are convincing.

3.3.3 Conclusion of changes in cyclophosphamide- induced cystitis

Besides the obvious macroscopic signs of inflammation, urinary bladders from CYP-treated rats showed a decrease in contractility. Even though a large part of the loss of function likely can be accredited to general muscle impairment, a change in the respective transmitter systems was also observed. *In vitro*, the rapid, phasic phase of the contraction was not found to be affected to any large extent by the inflammation, whereas the tonic phase was seen to become less sensitive to cholinergic antagonism during this condition (Paper III). In all, the purinergic part of the parasympathetic response was smaller, or at least not increased, in the CYP-treated rats, particularly at low stimulation frequencies. At certain stimulation frequencies, the cholinergic relative response remained unaffected in CYP-treated rats, while at others it seemed to be reduced. This may reflect an

interaction between muscarinic and nitrergic systems (Andersson *et al.*, 2011).

Generally, changes observed in the micturition reflex in patients with lower urinary tract disorders have been ascribed to the afferent part of the micturition, as reviewed by Daly *et al*. (2011). Furthermore, afferent nerves have been shown to be important in the development of CYP-induced cystitis (Ahluwalia *et al.*, 1994; Maggi *et al.*, 1992). In contrast to the *in vitro* findings in this thesis, the contractile response to methacholine, but not that to ATP, was reduced in rats with CYP-induced cystitis in the *in situ* setup (Paper IV). Interestingly, the contractions to ATP were atropine sensitive, which further stresses the intricate phenomenon of interaction between the purinergic and cholinergic systems. Furthermore, the stretch-induced response was elevated in cystitis and could to a large extent be blocked by purinergic antagonists, especially at stimulation of a lower magnitude.

Previous observations have showed an increase in the release of ATP in animal models of BPS/IC (Birder *et al.*, 2003) and in patients with painful bladder syndrome (Kumar *et al.*, 2007). Increased levels of ATP have in turn been coupled to increased urinary frequency (Pandita *et al.*, 2002), increased desire in OAB patients to void (Cheng *et al.*, 2010) and sensitization of sensory P2X purinoceptors on afferent nerves in rats with CYP-induced cystitis (Dang *et al.*, 2008). No findings in the current observations contradict this. On the contrary, the substantial difference between the stretch-induced responses in the normal and inflamed bladder is likely to be explained by this mechanism. Likely, other factors, such as NO, causing an increment of detrusor sensitivity or reactivity may also contribute to the large differences observed.

3.4 Impact of pre- treatments on cyclophosphamide- induced cystitis

In the present thesis, urinary bladders from rats with CYP-induced cystitis and saline-treated control rats, pre-treated with either saline (as control) or one of the following substances; DPCPX ($P1A_1$ purinoceptor antagonist, Paper V), PSB1115 ($P1A_{2B}$ purinoceptor antagonist, Paper V), suramin (P2 purinoceptor antagonist, Paper V), 4-DAMP (muscarinic receptor antagonist, Paper VI) or L-NAME (NOS inhibitor, Paper VI) were compared.

Bladders from CYP-treated rats were clearly affected, showing reddening and thickening of the bladder wall compared to control urinary bladders. There were, however, great variations in the macroscopic appearances, especially in inflamed bladders. Bladder preparations from CYP-treated rats were over all thicker, and also slightly heavier. There were, however, no statistically significant differences in bladder strip weight within the two groups (saline- and CYP-treated rats, respectively; Paper V).

Functionally, contractions to both methacholine and ATP were investigated in Paper V and VI and although the response to methacholine proved to be somewhat more robust, also the ATP-evoked contractions provided valuable insights. Also, the relaxatory response to adenosine was studied (Paper VI).

3.4.1 Functional studies

The unspecific muscarinic agonist methacholine (50 nM - 5 mM) evoked significantly larger contractions in control rats pre-treated with saline than in CYP-treated, saline pre-treated rats in both studies (Paper V and VI). The contractions to methacholine invariably caused bell shaped concentration-response curves which were modeled using a traditional E_{max} equation, excluding the highest concentration (Paper V) or a non-linear bi-phasic equation (Paper VI). This relation between the contractions to methacholine in saline- and CYP-treated rats was similar also in urinary bladder strip preparations from rats pre-treated with suramin or 4-DAMP (Paper V and VI).

Pre-treatment with PSB1115 maintained, or if anything tended to further increase the observed difference between control rats and rats with cystitis (Paper V). Notably, the functional responses in specimens from rats pre-treated with the $P1A_1$ antagonist DPCPX were almost identical in controls

and rats with cystitis, suggesting a normalizing effect of this drug (Paper V; Fig. 17). Also preparations from rats pre-treated with L-NAME showed a normalized cholinergic response in CYP-treated rats compared to control (Paper VI).

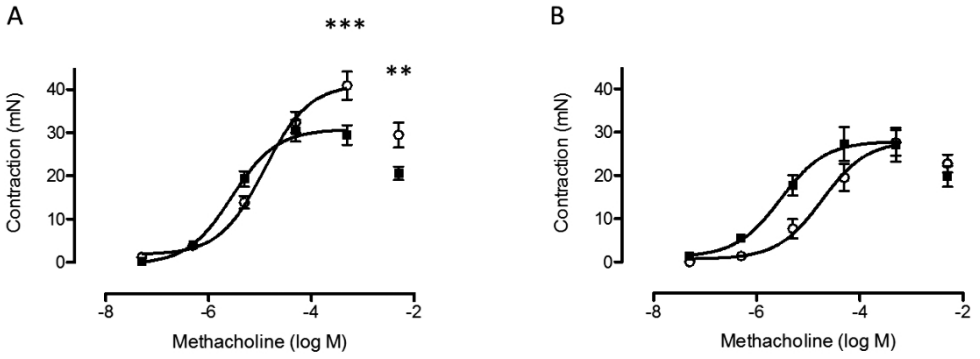

*Figure 17. Mean contractile responses to methacholine in urinary bladder strip preparations from rats treated with saline (○) or CYP (■) and pre-treated with (A) saline; n=14-16 or (B) DPCPX; n=8-12. ** p<0.01 and *** p<0.001. Vertical bars represent the S.E.M. Adapted from Paper V.*

Similarly to what was observed in the response to methacholine, the unspecific P2 purinoceptor agonist ATP (5 – 500 μM) caused (Paper V) or tended to cause (Paper VI) larger contractions in saline pre-treated control rats than in saline pre-treated rats with CYP-induced cystitis. Pre-treatment with either suramin, PSB1115 (Paper V) or 4-DAMP (Paper VI) caused a decrement in contractile response to ATP in both saline-treated and CYP-treated rats and no difference between the groups could be statistically proven.

In the case of PSB1115 (Paper V) and L-NAME (Paper VI) a shift where inflamed bladder strips responded with greater magnitude than controls was seen, but the differences were minor and not statistically significant. DPCPX-pretreatment, however, caused the ATP-evoked contractile response to be significantly higher in CYP-treated rats than in controls pre-treated with this antagonist, thus producing a response different than that evoked by methacholine.

The relaxations to adenosine (50 μM, Paper VI) were significantly larger in control specimens than in inflamed bladder strip preparations (-2.4±0.1 (n=10) vs. -0.7±0.1 (n=12) mN; $p<0.001$). This was similar in the 4-DAMP pre-treatment group, whereas the difference in relaxation to adenosine was abolished in L-NAME pre-treated rats (Fig. 18). Even though the maximal relaxation in saline-treated, L-NAME pre-treated rats was smaller than in control specimens, the inflamed L-NAME pre-treated bladder strip preparations tended to respond to a larger extent than did the inflamed control strips.

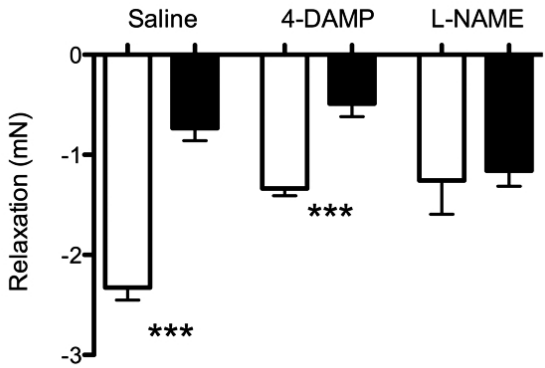

*Figure 18. Mean relaxatory responses to adenosine (50 μM) of isolated urinary bladder strip preparations from control (saline-treated; white) rats and rats with CYP-induced cystitis (black), pre-treated with saline (n=10 and 12), 4-DAMP (n=8 and 10) or L-NAME (n=8 in each group). The vertical bars represent the S.E.M. *** denotes p<0.001. From Paper VI.*

3.4.2 Bladder histology

Microscopically, both the total bladder wall and the mucosa/submucosa were thicker in the preparations from CYP-treated rats (Paper V and VI). Also, the detrusor layer seemed more disorganized in CYP-treated bladders, and the mucosal and submucosal part of the bladder wall was found to be significantly smaller in normal bladder specimen (25.7 and 20.4% in saline pre-treated, saline-treated rats in Paper V and VI, respectively) compared to in rats with CYP-induced cystitis (46.3 and 42.6%, in Paper V and VI, respectively). Neither of the pre-treatments caused significant changes in mucosa and submucosa thickness in the saline-treated rats, even though DPCPX and possibly 4-DAMP and L-NAME tended to lower this parameter. In the CYP-treated animals on the other hand, pre-treatment with DPCPX

(Paper V, Fig. 19) and L-NAME (Paper VI) caused a significant reduction in mucosa and submucosa thickness, compared to preparations from rats with CYP-induced cystitis pre-treated with saline.

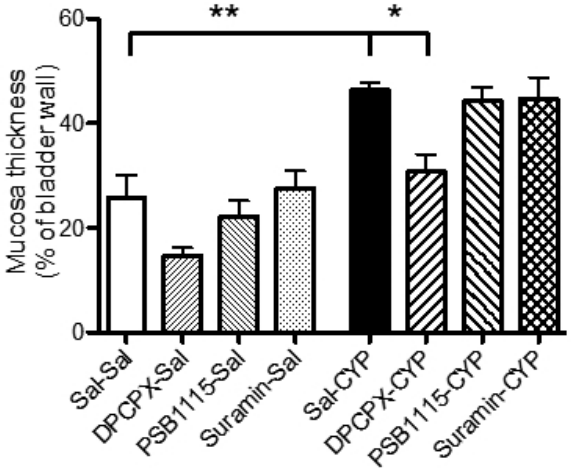

*Figure 19. Mean mucosa and submucosa thickness, expressed as percent of total bladder wall, in urinary bladders from rats pre-treated with saline (Sal), DPCPX, PSB1115 or suramin and treated with either saline (Sal) or cyclophosphamide (CYP). n=5 in each group, * denotes p<0.05 (between Sal-CYP and DPCPX-CYP) and ** denotes p<0.01 (between saline-treated and CYP-treated bladders). Vertical bars represent the S.E.M. From Paper V.*

3.4.3 Mast cells

No significant difference was observed in the total number of mast cells (colored by toluidine blue), but the percentage of mast cells found in the detrusor *vs.* the total mast cell count was over all greater in CYP-treated rats, compared to control (Paper V and VI). Interestingly, L-NAME pre-treatment (Paper VI) abolished any statistically significant difference between the two groups (Fig. 20), whereas DPCPX, and possibly also PSB1115, tended to cause a reduction of this ratio in bladders with CYP-induced cystitis. Specimens treated with saline did not appear to undergo any notable changes. This is in line with previous studies where an increase of mast cells in the detrusor in patients have been suggested to be an early marker of BPS/IC (Frazer *et al.*, 1990), and has even been shown to be a possible factor discriminating between different forms of BPS/IC (Peeker *et al.*, 2000). Even though some studies claim toluidine blue staining and fixation in

formaldehyde to cause underestimation of the degree of mastocytosis (Aldenborg *et al.*, 1986; Theoharides *et al.*, 2001), this should not be a major concern in the current studies, since all preparations were treated in the same fashion.

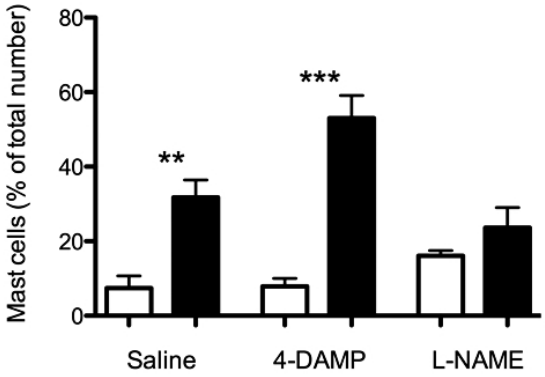

*Figure 20. Ratio of toluidine blue stained mast cell occurrence in the detrusor compared to in the whole bladder wall in urinary bladders from rats pre-treated with saline,4-DAMP or L-NAME, treated with either saline (white) or CYP (black). n=3 in each group, ** denotes p<0.01 and *** denotes p<0.001. Vertical bars represent the S.E.M. From Paper VI.*

3.4.4 Immunohistochemistry

MIF- and muscarinic M5 receptor-like immunoreactivity were detected in CYP-treated, as well as control, bladder preparations (Fig. 21 and 22). The MIF-like staining showed a large variation in the treatment groups, and no certain conclusions could be drawn. There seemed, however, to be a difference in the distribution of the MIF-like expression; in urinary bladders from saline treated rats it was localized to the basal part of the urothelium, whereas in CYP-treated specimens the staining seemed to be more widely spread across the urothelium (Paper V and VI), which corresponds to other reports where changed levels of urothelial MIF and increased MIF-levels in the urine have been observed during inflammation (Meyer-Siegler *et al.*, 2004b; Vera *et al.*, 2008). Pre-treatment with DPCPX, but not L-NAME, 4-DAMP, PSB1115 or suramin, seemed to reduce the more diffuse immunoreactivity in CYP-treated rats although, as previously mentioned, these results should be interpreted with caution. The $P1A_1$ purinoceptor-like immunoreactivity (Paper VI) seemed slightly lowered in inflamed bladder

specimens, and may have been slightly elevated after L-NAME pre-treatment, but these results should be interpreted with care.

Regarding the muscarinic M5 receptor-like expression, it was shown to be elevated during CYP-induced cystitis (Paper V and VI), as has been reported previously (Giglio *et al.*, 2005). This increase in immunoreactivity in CYP-treated rats, compared to control rats treated with saline, was inhibited by pre-treatment with DPCPX (Paper V) or L-NAME (Paper VI). Also pre-treatment with suramin seemed to lower the muscarinic M5 receptor-like expression in CYP-treated rats, however to a much lower degree than did DPCPX. PSB1115 pre-treatment appeared to possibly decrease this staining in preparations from rats treated with either CYP or saline, although to a lower extent, whereas 4-DAMP caused no changes in muscarinic M5 receptor-like immunoreactivity.

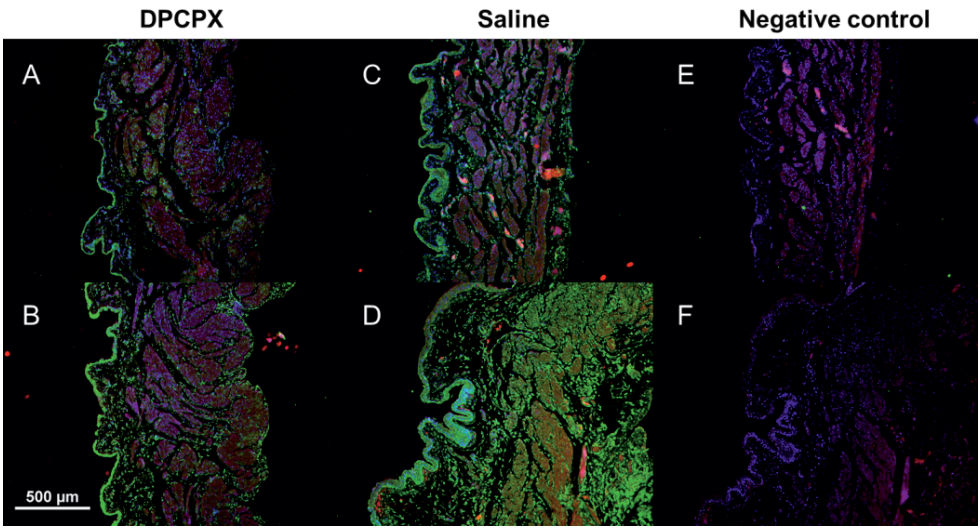

Figure 21. Sections of urinary bladder stained with antibodies targeting the muscarinic M5 receptor from rats pre-treated with DPCPX (A, B) or saline (C, D) and treated with saline (upper row A, C, E) or CYP (bottom row B, D, F). Also shown are negative controls of sections C and D (E and F, respectively). Green color represents muscarinic M5 receptor-like expression, red and blue color represents phalloidin and DAPI, respectively. Adapted from Paper V.

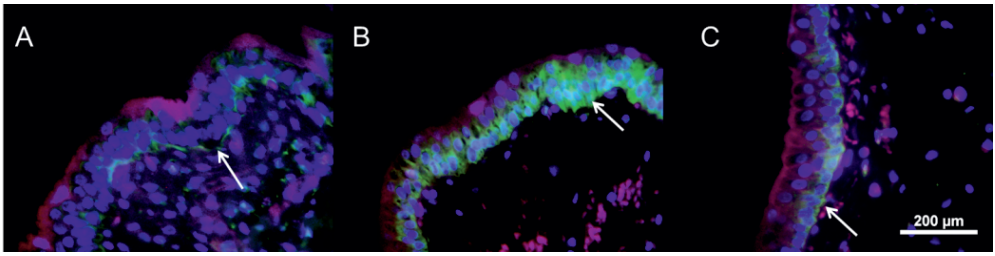

Figure 22. Sections of urinary bladder stained with antibodies targeting macrophage migration inhibitory factor (MIF) from rats pre-treated with saline (A, B) or DPCPX (C) and treated with saline (A) or CYP (B, C). Green color represents MIF-like distribution, red and blue color represents phalloidin and DAPI, respectively. Arrows indicate the basal part of the urothelium. Adapted from Paper V.

3.4.5 Conclusion of impact of pre- treatments on cyclophosphamide- induced cystitis

Even though the main scope of the studies (Paper V and VI) was to characterize the functional contractile changes in the treatment and pre-treatment groups, other parameters regarded to be of interest in the evaluation of the development of cystitis were also looked upon. The studies were designed and performed to first and foremost investigate changes between controls and CYP-treated rats within each pre-treatment group, rather than to the saline pre-treatment. Thus, comparisons of individual concentration-response curves between the pre-treatment groups must be made with caution, even though being discussed to some extent in this thesis.

Taken together, the normalized contractile response to methacholine seen after pre-treatment with DPCPX and L-NAME (Paper V and VI) might have to some extent been due to a decreased contractile function in saline-treated rats. However, a decreased inflammatory damage to the urinary bladder in these animals undoubtedly played a significant role since a number of parameters regarded to occur in CYP-induced cystitis were affected. Normalizing changes in relaxatory response, histological examinations, muscarinic M5 receptor expression, mast cell count etc. support the statement that the inflammation was alleviated, suggesting both NO and the $P1A_1$ adenosine receptor to be of importance in the regulation of bladder inflammation. A prominent alteration in CYP-induced cystitis is the bladder wall thickening, to a large part caused by edema (Cox, 1979; Crocitto *et al.*, 1996; Terado *et al.*, 2005). This may be an important factor hampering the contractile capacity, possibly by just mechanically counteracting the strength of the muscle contraction. This may also, just by passive stretch, cause the bladder to persist in a state at which it is more close to the threshold for a stimulus to evoke a reflex response. DPCPX and L-NAME reduced the mucosa thickness, which might make them suitable in the treatment of bladder inflammation.

Since a link has been seen between adenosine acting on $P1A_1$ purinoceptors and the production and release of NO in endothelial cells (Ray *et al.*, 2002), and since NO has previously been investigated in the context of inflammation, the effect of L-NAME on CYP-induced cystitis was also studied. Even though intravesical administration of a NO-donor has been shown to suppress CYP-induced bladder hyperactivity (Ozawa *et al.*, 1999), blockade of iNOS with the inhibitor aminoguanidine has been described to prevent some of the fibrotic changes occurring in rodents during bladder outlet obstruction (Felsen *et al.*, 2003). The administration of L-NAME and

other NOS inhibitors to rats with CYP-induced cystitis also has been studied previously (Oter *et al.*, 2004; Souza-Fiho *et al.*, 1997). In these studies, however, other non-functional parameters, such as extravasation, were studied and the experiments were conducted over a shorter period of time. The conclusion in this thesis regarding a protective role of NOS blockade thus seems feasible.

In a very recent study, administration of the $P1A_1$ purinoceptor agonist CCPA, which effects can be inhibited by DPCPX, was shown to worsen the bladder hyperactivity symptoms in rats with CYP-induced cystitis (Prakasam *et al.*, 2012). This is in concordance with our current findings that the $P1A_1$ purinoceptor is a proinflammatory receptor which also seems to mediate functional effects in both healthy and inflamed urinary bladders. However, since the expression of the $P1A_1$ purinoceptor shows great plasticity over time in inflammation (Save *et al.*, 2009), the duration of the inflammation ought to be addressed in future studies.

4 GENERAL DISCUSSION

The concept of ATP being an important chemical transmitter is now widely accepted (Burnstock, 2013). This was, however, not the case for many years after this had first been suggested. Initial criticism was directed towards the notion that it "seemed odd" that the body would "waste" precious energy in this manner to transmit signals and that it was a much too common molecule in the body to produce specific responses (every day the human body is estimated to turn over an amount of ATP equal of its body weight (Tornroth-Horsefield *et al.*, 2008)). However, the discovery and characterization of specific receptors and interactions with other transmitter systems etc. have provided solid evidence of ATP being, if not a classical, a true transmitter indeed. *In vitro,* relatively high concentrations of ATP are often needed to elicit a functional response. The P2X1 purinoceptor, which is responsible for the major part of the purinergic direct contractile response of the urinary bladder, has been shown to be readily desensitized by low concentrations (in the range of nM) of ATP, which may mask the true potency of this agonist (Rettinger *et al.*, 2004).

The fact that ATP is a common molecule, capable of acting on many receptor subtypes, that also yields active metabolites further contributes to the complexity of the purinergic signaling. Furthermore, many steps in the release and breakdown of ATP, as well as in the expression of purinoceptors and interactions with other transmitter systems can be altered under pathological conditions, such as during the course of inflammation. This thesis pinpoints the significance of interactions between signaling systems in the regulation of urodynamic functions in the urinary bladder. It shows that these interactions may occur on a neuronal or non-neuronal level, and may affect both efferent and afferent signaling. Furthermore, disease, *e.g.* inflammation of the urinary bladder, may substantially alter any single component with great impact on the composite functional response.

The complexity of the bladder responses may be one reason for the unclear situation in patients, leading to a pharmacotherapy with often suboptimal outcome and less than well understood mechanisms of action. It may also explain some of the difficulties in establishing the role of NANC transmitters in the human situation. For instance, anticholinergic drugs have for a long time been used in the treatment of incontinence and overactive bladder. Very recently, mirabegron, a new beta-3 adrenoceptor agonist (Igawa *et al.*, 2012) has been developed as a bladder relaxant used in the treatment of overactive bladder. Currently, no drug acting on purinoceptors is available for the treatment of diseases in the lower urinary tract, even though work is carried

out to identify substances acting on purinoceptors on sensory neurons in the bladder (Gever *et al.*, 2010). The results in this thesis, suggesting an increased role of afferent, but not necessarily efferent, purinergic signaling in CYP-induced cystitis, supports the idea that a purinergic tool aimed at afferent purinoceptors would seem to be the preferable option when treating symptoms in the lower urinary tract.

If the role of the purinoceptors in direct bladder function is, or has been, considered to be a bit unorthodox, the fact that ATP and adenosine, by acting on their respective purinoceptors, are able to affect the course of inflammation is less controversial. Different purinoceptor subtypes are known to act in both pro- and anti-inflammatory manners, depending on the situation (*e.g.* level of inflammation) and tissue (Kruse *et al.*, 2012; Wilson *et al.*, 2009). Their involvement in these processes is complex. However, by interacting with other systems, the purinoceptors are likely to have a large impact on the development of inflammatory conditions.

In this thesis, the relevance of the adenosine $P1A_1$ purinoceptor in the development of CYP-induced urinary bladder inflammation is highlighted. Beneficial effects to blockade of this purinoceptor subtype, as well as of the synthesis of NO, were in the current studies seen during cystitis. Antagonism of muscarinic receptors was also investigated due to possible interactions with the purinergic system and well-established clinical use, but no impact on the course of inflammation was seen. Certainly, more studies need to be conducted in order to confirm these findings; other lower urinary tract disease models should be used to study the generalizability of this mechanism, other doses and antagonists with similar, but not identical, pharmacological profiles ought to be tested and other routes of administration should be tried to optimize clinical effect and minimize the risk of unwanted side effects occurring. Moreover, the impact of $P1A_1$ purinoceptor- or NOS blockade after the induction of cystitis, *i.e.* when the inflammation already is a fact, ought to be addressed to further increase clinical relevance. Nonetheless, the current findings regarding pre-treatment present valuable insights about the role of purinoceptors and NO in the development of cystitis.

The principal role of purines must be discussed. The results in this thesis, together with many other studies, suggest a direct functional significance of ATP and adenosine in the rat, both in the normal state and during urinary bladder inflammation. Currently, both contractile and relaxatory functional purinoceptors have been characterized and the impact of purinergic signaling in the normal state and during cystitis scrutinized. Undoubtedly, purinoceptors play an important role in many species, including territory

marking animals, and most likely also in man during pathological conditions. Regarding efferent functional contractility, ATP should be regarded not as the main player, but as a modulating transmitter besides acetylcholine and, to some extent, noradrenaline. In the pathological state, alterations regarding purinergic function are seen predominantly on the afferent side. The ability of the purinoceptors to affect both the course of inflammation, altered afferent signaling and bladder smooth muscle contraction make them a very interesting target for pharmacological treatment of symptoms in the lower urinary tract, for instance in BPS/IC.

5 CONCLUDING REMARKS

In this thesis, several methods have been employed to study the purinergic impact on rat urinary bladder function in the normal state and during CYP-induced cystitis, and a novel *in situ* experimental setup has been described. Also, the impact of purinergic, cholinergic and nitrergic pre-treatment on the development of inflammation has been addressed.

Functionally, the contractile purinergic function was, as is in concordance with previous findings, found to be mediated by the P2X1 purinoceptor. Relaxatory purinergic responses were in the normal rat found to be elicited by P2Y purinoceptors with different sensitivities for ADP/ATP and UDP/UTP, as well as by the $P1A_{2B}$ purinoceptor. A purinergic phase of EFS-evoked contractions was clearly identifiable, which did not seem to increase in importance during cystitis.

A novel *in situ* experimental setup, enabling the study of contralateral contractile responses to stretch-evoked afferent stimuli, was designed and employed. In contrast to *in vitro*, purinergic contractions were not reduced during cystitis *in situ* and the stretch-induced contractions were markedly increased. This reflex was found to exert a composite response mediated by both muscarinic receptors and P2 purinoceptors, where the latter are of greater importance at lower stimulation intensities. Generally, when efferent responses (agonist- or nerve-evoked) are studied, they are reduced, or at least not increased, in CYP-treated rats. The enhancement in the reflexly evoked contractions *in situ* is thus likely to occur in either the afferent part of the reflex, or in the transmission in the central nervous system. The novel *in situ* model offers many interesting possibilities for future studies.

Pre-treatment with either $P1A_1$ purinoceptor antagonist or NOS inhibitor was shown to alleviate the change in contractile function to CYP-induced bladder inflammation, which was confirmed by the study of several inflammatory findings common in cystitis.

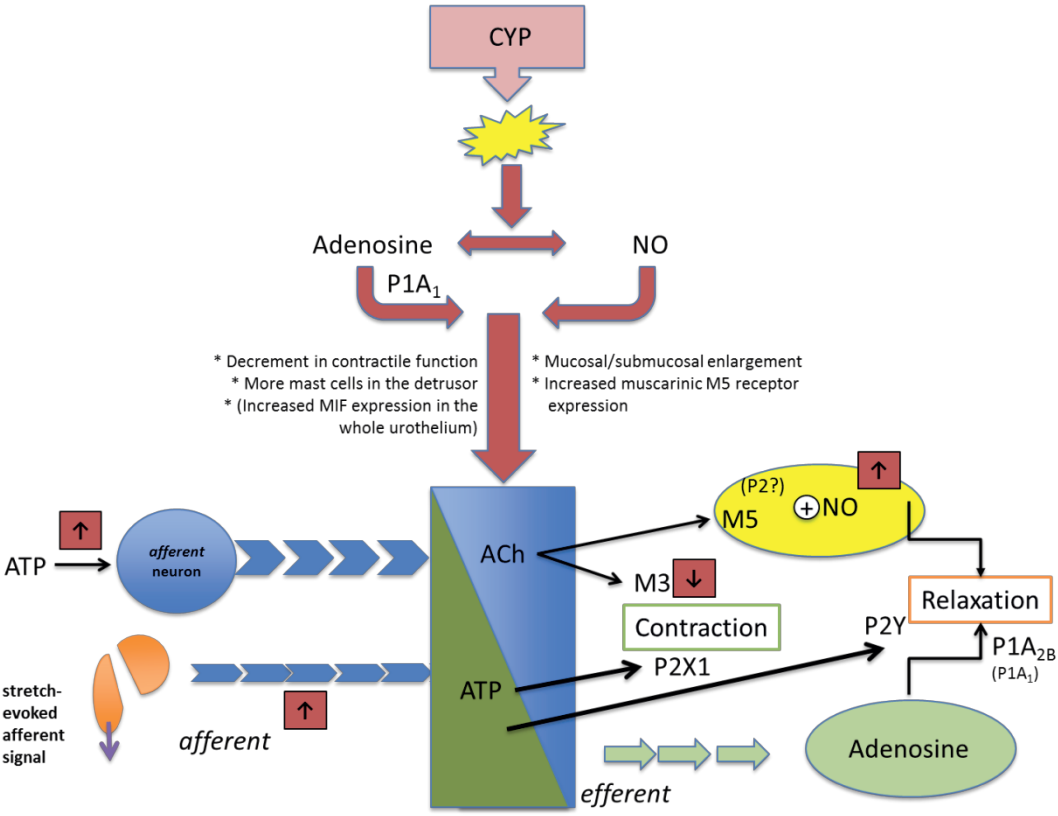

Figure 23. Tentative overview of purinergic, cholinergic and nitrergic interplay in the afferent and efferent control of the detrusor discussed in this thesis. CYP-treatment causes cystitis with many observable effects in the urinary bladder. Many of these changes can be alleviated with $P1A_1$ or NOS-blockade. Arrows within red boxes indicate changes during CYP-induced cystitis. The afferent purinergic signaling, such as during stretch, seems to be of greater importance during cystitis. Muscarinic M3 receptors and P2X1 purinoceptors are considered to be functionally contractile, whereas muscarinic M5 receptors and urothelial P2 purinoceptors are mediating relaxation, probably by an NO-coupled mechanism. Adenosine $P1A_{2B}$ (and $P1A_1$) purinoceptors also mediate relaxation, as does receptors of the P2Y family.

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