CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOLOGICAL AND MEDICAL SCIENCES

DIPLOMA THESIS

Cyclophosphamide-induced cystitis in rats

An experimental study of the importance of muscarinic, nitrergic and purinergic mechanisms

Zánět močového měchýře u potkana vývolaný cyklofosfamidem

Experimentální studie o významu muskarinních, nitrergních a purinergních mechanismů

Vedoucí diplomové práce: PharmDr. Miloslav Hronek, Ph.D.

Školitel specialista: Prof. Gunnar Tobin

Hradec Králové 2011 **Zdeňka Niebauerová**

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Contents

1 Abstract

The present thesis is focused on studies of the importance of cholinergic, purinergic and nitrergic mechanisms in the healthy and inflamed rat urinary bladder, furthermore in which way specific pre-treatment affects the state of inflammation. Experiments were performed on prepared strips from normal healthy rats and cyclophosphamide-induced cystitis rats. In vitro contractile response of the strips to MeCh and ATP were investigated in the saline/CYP control group as well in the presence of five various antagonists and inhibitors. The rats were pre-treated by 4-DAMP (2mg/kg) as a M_3, M_5 muscarinic receptor antagonist; DPCPX (1mg/kg) as a $P1A_1$ purinoceptor antagonist; PSB1115 (1mg/kg) as a $P1A_{2B}$ purinoceptor antagonist; L-NAME (60mg/kg) as an inhibitor of eNOS and finally by suramin (10mg/kg) as a non-selective P2 purinergic receptor antagonist. Altered effects were observed via functional and morphological studies. The best results showed DPCPX and L-NAME pre-treated groups. It was found that DPCPX and L-NAME pre-treatments normalized contractile response to MeCh and ATP of the inflamed bladders and helped to reduce inflammatory signs. These in vitro functional findings evoke that $P1A_1$ receptor and nitric oxide are important pro-inflammatory factors since their inhibitions refine the state of inflammation. Morphological studies were foremost concerned on $P1A_1$ purinoceptor and M_5 muscarinic receptor expression and their alterations during cystitis. Immunohistochemical staining showed decreased number of both receptors in the inflamed bladders.

Tato práce je zaměřena na významnost cholinergních, purinergních a nitrergních mechanismů ve zdravém a zaníceném močovém měchýři potkana, a mj. jakým způsobem ovlivňuje konkrétní předléčení stav zánětu. Experimenty byly provedeny na předem připravených stripech ze zdravých potkanů a potkanů , u kterých byl vyvolán zánět močového měchýře cyklofosfamidem. In vitro funkční studie zaměřené na kontraktilní odpověď stripů na metacholin a adenosintrifosfát byly zkoumány u kontrolní skupiny a dále v přítomnosti pěti různých antagonistů a inhibitorů. Potkani byli předléčeni pomocí látek 4-DAMP (2 mg/kg), tj. antagonista M_3, M_5 muskarinních receptorů; DPCPX (1 mg/kg) jakožto antagonista $P1A_1$ receptoru; PSB1115 (1 mg/kg) působící jako antagonista $P1A_{2B}$ receptoru; L-NAME (60 mg/kg) jako inhibitor endoteliální NO syntázy a nakonec pomocí suraminu (10 mg/kg), který působí jako neselektivní antagonista P2 purinergních receptorů. Výsledné změny byly pozorovány pomocí funkčních a morfologických studií. Nejlepší výsledky ukázaly skupiny

předléčené látkami DPCPX a L-NAME. Ukázalo se, že předléčení pomocí DPCPX a L-NAME normalizovalo kontraktilní odpověď na metacholin a ATP v zanícených močových měchýřích a pomohlo redukovat zánětlivé projevy. Z těchto zjištění vyplývá, že $P1A_1$ purinergní receptory a oxid dusnatý jsou důležitými faktory zánětu, protože jejich inhibice zlepšuje stav zánětu. Morfologické studie byly zaměřeny především na receptory $P1A_1$ a M_5, resp. na změny v jejich expresi během cystitidy. V obou případech imunohistochemické barvení ukázalo snížení počtu receptorů v průběhu zánětu močového měchýře.

2 Introduction

The function of the urinary bladder is regulated by interaction between the autonomic nervous system and the voluntary nervous control. It is a complex and associated process. The parasympathetic nervous system is mainly responsible for the micturition contractions of the detrusor muscle. Stimulation of the parasympathetic causes the detrusor muscle to contract and relax the internal sphincter. This process leads to voiding. In addition to this a neuronal somatomotoric mechanism occurs – the voluntary control of the external sphincter, which has to be opened to void.

According to the previous studies, it seems to be not only the parasympathetic cholinergic system that affects contractions of the detrusor muscle. A non-cholinergic, non-adrenergic parasympathetic system exists also. This system is purinergic, which includes ATP and adenosine effects via purinergic receptors. Systematically it is possible to divide purinergic receptors into two groups: P1 and P2 receptors. P2 purinoceptors are activated by nucleotides such as ATP. It was discovered that ATP is not only a source of energy for the cells but it has also another function - ATP may act as a neurotransmitter, which is in focus in the current thesis. The released ATP is metabolized into another product – adenosine – which acts on the P1 purinoceptors. Nowadays we can thus say that there is a cholinergic and purinergic contractile response in the urinary bladder.

In the physiological regulation, urine remains in the urinary bladder until it starts to be uncomfortable full and the need to urinate becomes noticed by the individual. During inflammation in the urinary bladder (cystitis) the need to urinate occurs more frequently even if the urinary bladder is not filled. Also, the micturition is usually painful. This pathology is connected with different changes in the inflamed tissue such as increased number of blood

vessels, thickened wall of the urinary bladder, altered number and localization of receptors and last but not the least an up-regulation of nitric oxide synthase. Which is the connection between nitric oxide and inflammation and how important is the role of nitric oxide? These are some of the questions, which are tried to be answered in the current thesis.

A number of bladder dysfunctions exist such as overactive urinary bladder, interstitial cystitis and urinary incontinence which significantly reduce quality of life and where even modern medicine does not provide satisfying and efficient treatment. That is why it is necessary to continue the research of these unsolved problems and find the solution.

3 Aims (Task of the thesis)

The general aim of the thesis is to investigate the presence, function and importance of the specific cholinergic, purinergic and nitrergic mechanisms in the rat urinary bladder and characterize the responses in the healthy and inflamed bladder. The inflammation in the urinary bladder was induced by cyclophosphamide and the altered effects were observed via functional and morphological studies.

The main question was how the specific pre-treatment affects the bladder tissue. What does the pre-treatment do to the CYP-induced cystitis? The separate points of the task were to characterize the muscarinic and purinergic bladder contractile response affected by the pre-treatment and related NO releasing during the inflammation. In vitro functional studies confirmed that CYP pre-treatment affects contractile response by alteration the muscarinic and purinergic receptors and proved an important role of nitric oxide in the inflammation. The second part of the thesis is concerned in morphological and histological changes proved by immunohistochemical staining (IHC).

THEORETICAL PART

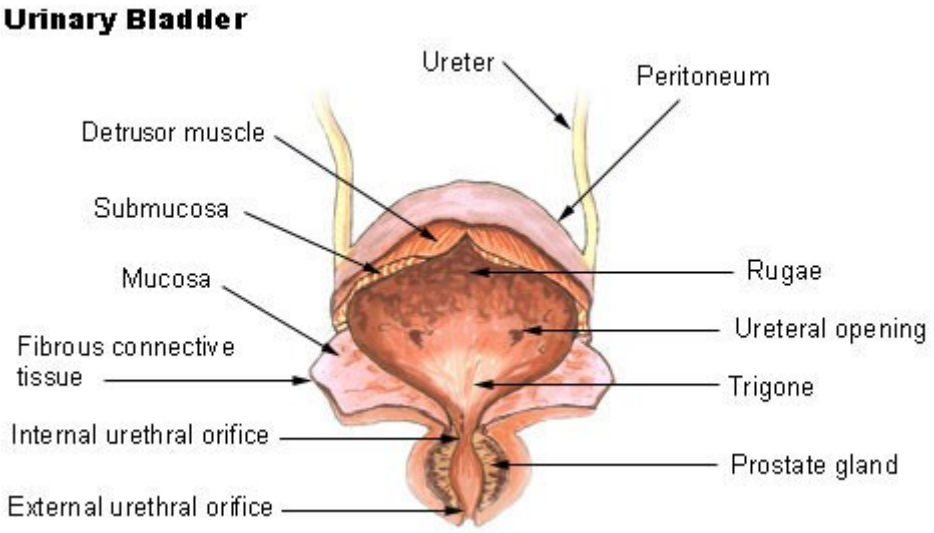
4 Review of literature

4.1 Anatomy of the urinary bladder

The urinary bladder is a hollow muscular organ situated in the lower abdomen, resting on the pelvic floor (Saito, Nakamura et al. 1997). The bladder is composed of two main parts. The *body* is the major part of the bladder into which the urine is drained. The *neck* is a funnel-shaped extension of the body. It is passing into the urogenital triangle and is connected with the urethra. The smooth muscle of the urinary bladder is called the detrusor muscle. Contractions of the detrusor are an important step in emptying the bladder.

On the posterior wall of the bladder is a small triangular area – *the trigone*. The bladder neck forms the lowest point of the trigone (apex). Conversely in the upper part of trigone two ureters enter the bladder. There is a sign how to recognize the trigone area because its mucosa is smooth in contrast to the remaining bladder mucosa, which is folded into rugae.

The muscle in the area of bladder neck is called *internal sphincter*. The wall is composed of detrusor muscle with a large amount of elastic tissue. Internal sphincter is involuntarily controlled and it contracts when the detrusor muscle relaxes. It prevents voiding until the pressure in the bladder rises above a critical threshold. Besides internal sphincter there is another layer of muscle in the urogenital diaphragm called *external sphincter*. It is a skeletal muscle, which is under voluntary nervous control. It can be used to consciously prevent micturition even when involuntary controls are attempting to empty the bladder (Guyton and Hall 2006).



Adapted by http://www.histology-world.com/factsheets/bladder1.htm

The empty bladder has a pyramidal shape, when it is filled an ovoid shape. The empty bladder has a superior side, two inferiolateral sides, a posterior base and an apex. The superior wall is covered with peritoneum (Saito, Nakamura et al. 1997; Campbell, Walsh et al. 2002).

In a female, the bladder contracts the anterior walls of the uterus and vagina. In a male, the bladder lies posteriorly against the rectum. The bladder passes into the urethra, a tube that lead away urine outside of the body. In a female, the urethra is about 4 centimeters long and it terminates with the external urethral orifice between the labia minora, situated anterior to the vaginal opening. In a male, the urethra can be divided into three sections: the *prostatic urethra,* which is about 2,5 centimeters long and passes through the prostate gland; the *membranous urethra*, which is about 2 centimeters long , passes through the urogenital diaphragm and it is surrounded by the fibers of the external urethral sphincter muscle; and the *penile urethra*, which is about 15 centimeters long and passes through the corpus spongiosum and terminates with the external urethral orifice at the tip of the penis (Hole, Shier et al. 2009)

4.2 Histology of the urinary bladder

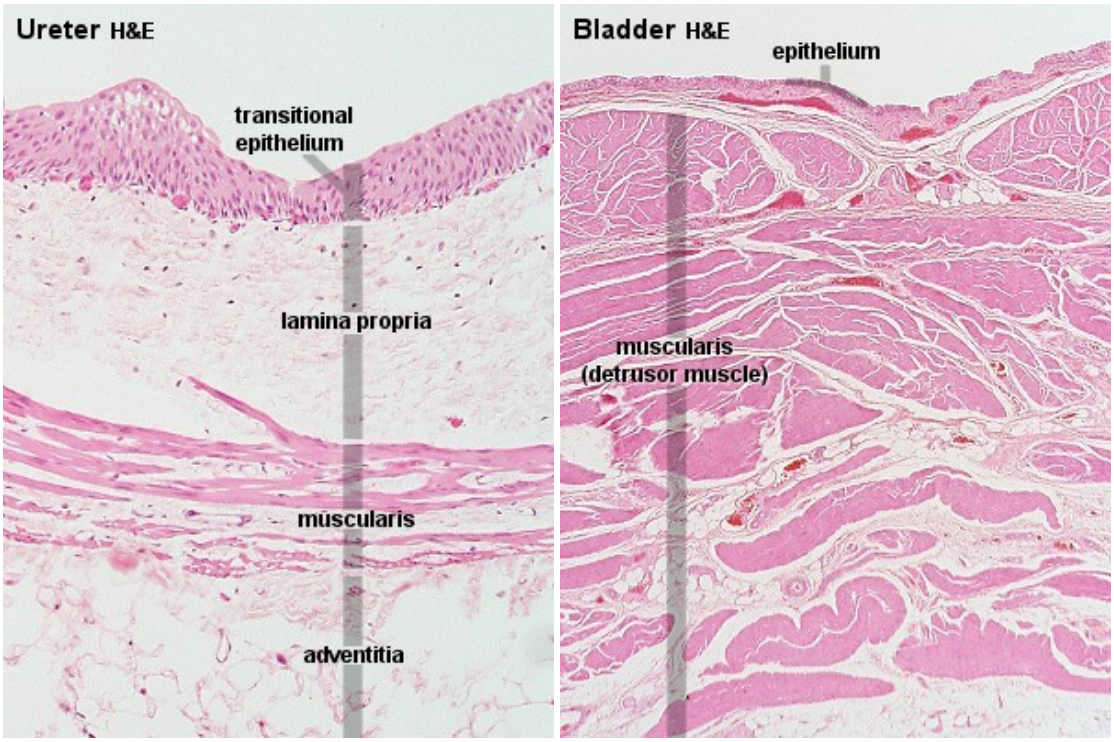
The wall of the urinary bladder consists of four basic layers: *mucosa*, *submucosa*, *muscularis* and *serosa* (Hole, Shier et al. 2009).

Mucosa, also known as the urothelium, is the inner layer of the bladder. The urothelium is a transitional epithelium consisting of 3-6 cell layers (Jost, Gosling et al. 1989). The thickness of the tissue changes as the bladder expands and contracts. During distension, the tissue appears to be only two or three cells thick but during contraction, it appears to be five or six cells thick (Hole, Shier et al. 2009). The size of the urothelial cells increase from the base to the surface. The largest surface urothelial cells are also known as umbrella cells due to their hexagonal umbrella-like morphology (Apodaca 2004). This epithelial layer is not vascularized nor directly innervated by the nervous system. Immediately beneath the urothelium is a thin basal lamina, separating the urothelium from the lamina propria. The lamina propria is often referred to as a part of the suburothelium.

Submucosa also known as the suburothelium consists of connective tissue (with many elastic fibers), muscular bundles, afferent and efferent nerve fibers and a numerous amount of capillaries (Wiseman, Fowler et al. 2003).

Muscularis, the third layer of the bladder wall, is formed by smooth muscle fibers, which together comprise the detrusor muscle. Three layers of smooth muscle have been described. The cells of the outer and inner sides tend to be oriented longitudinally, and those of the middle layer circularly. A circular muscle layer intercuts two longitudinal muscle layers (Andersson and Arner 2004).

Serosa is an outer layer of the bladder wall consisting of the parietal peritoneum. It is found only on the upper surface of the bladder. Elsewhere, the serosa is composed of fibrous connective tissue (Hole, Shier et al. 2009).



Adapted by http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Urinary/urinary.htm

4.3 Innervation of the urinary bladder

The principal innervation of the bladder is the pelvic nerves, which are connected with the spinal cord through the sacral plexus (mainly segment S-2 and S-3). Both, sensory nerve fibers and motor nerve fibers are mediated through the pelvic nerves. The sensory fibers detect the rate of stretch in the wall of the bladder. Parasympathetic fibers as the motor nerve fibers, terminate on ganglion cells located in the bladder wall. Subsequently short postganglionic nerves innervate the detrusor muscle, which causes its contractions.

But only the pelvic nerves are not responsible for the bladder innervation. There are two other types of innervation: skeletal motor fibers, which innervate and control the external urethral sphincter. The nerve supply is transmitted through the pudendal nerve. And the second, sympathetic innervation, which the bladder receives through the hypogastric nerves, mainly segment L-2 of the spinal cord (Guyton and Hall 2006).

4.4 Physiology of the urinary bladder

The urinary bladder has two basic functions, storage of urine and emptying of urine. The filling phase of the bladder can be divided into three distinct happenings: initial sensation of filling, first desire to void and strong desire to void. During the filling phase occurs increasing contraction of the bladder neck and urethra, and increasing relaxation of the detrusor. At the same time, muscles and connective fibers in the pelvic floor contribute maintain continence

(Sampselle and DeLancey 1998). However, the bladder cannot fill up forever and eventually the situation is reversed; the detrusor muscle contracts and internal urethral sphincter relaxes. At this moment comes the emptying phase – micturition.

4.4.1 Micturition

Micturition is the process by which the urinary bladder empties when it becomes filled. The bladder can accommodate up to about 300-400 ml urine with a little increase in tension. As volume increases, the wall tension rises.

There are two main steps in that process. First, the bladder fills progressively until the tension in its walls reaches a threshold level. And the second, induced by first step, which is a nervous reflex called the *micturition reflex*. This involves a coordinated relaxation of internal and external sphincter, contraction of the detrusor muscle and contraction of the abdominal wall and pelvic floor musculature (Guyton and Hall 2006; Petersen 2007).

Distension of the bladder wall as it fills with urine stimulates the urge to urinate. The wall expands, stimulating stretch receptors, which triggers the micturition reflex. The *micturition reflex center* is in sacral portion of the spinal cord. When sensory impulses from the stretch receptors signal the reflex center, parasympathetic motor impulses transmit it to the detrusor muscle, which contracts rhythmically in response. The desire to urinate usually begins when the bladder contains about 150 ml of urine. As urine volume increases up to 300ml or more, the sensation of fullness becomes uncomfortable. Once a micturition reflex begins, it is "self-regenerative". It is a single complete cycle, which is repeated until the bladder empties. As the bladder becomes more and more filled, micturition reflexes occur more and more often and more powerfully.

In micturition, the detrusor muscle contracts, internal urethral sphincter relaxes and external urethral sphincter has to be also opened to enable micturition. However, because the external

sphincter is composed of skeletal muscle, which is under conscious control, it usually remains contracted until a person decides to urinate. At this moment the external sphincter relaxes and inhibition of the micturition reflex lifts. The detrusor muscle contracts and urine is extracted through the urethra. Within a few moments, the detrusor muscle relaxes, and the bladder begin to fill with urine again (Guyton and Hall 2006; Hole, Shier et al. 2009).

4.5 Pathology of the urinary bladder

Among main pathological symptoms related to the act of urination belong incomplete emptying, frequency, intermittency, urgency, weak stream, straining and nocturia. These problems are generally called as lower urinary tract symptoms (LUTS) connected with pathological changes in bladder capacity, sensation and contractility. This symptom complex was traditionally associated with prostatic obstruction. However, the same symptoms can obviously occur in women as well. There are many conditions which can cause these symptoms (Reynard, Brewster et al. 2006).

Frequency might be caused by residual urine, which decreases the functional capacity of the organ. During infection the capacity of the organ decreases sharply. The decrease is due to the pain and the loss of bladder compliance. Usually it is needed to void also during night which is called nocturia. Dysuria is painful urination usually related to acute inflammation of the bladder, urethra or prostate. The pain is commonly described as burning on urination and disappears soon after the micturition is completed. Sometimes the pain occurs in the bladder just at the end of the voiding, suggesting it is likely the inflammation of the bladder. Enuresis means bedwetting at night. It is physiologic process during first two or three years of life. After that, it may be psychosocial or functional problem, but it may present as a symptom of organic disease (e.g. infection, distal urethral stenosis, neurogenic bladder). In adult life, enuresis may be replaced by nocturia. Straining in initiating the urinary stream is one of early symptoms of the bladder outlet obstruction. Prostate obstruction and urethral stricture are common causes of this symptom. Weak urinary stream, intermittency and terminal dribbling are other symptoms of bladder outlet obstruction (Tanagho, McAninch et al. 2004).

4.5.1 Overactive bladder (OAB)

OAB is a symptom syndrome including urgency, with or without incontinence, frequency and nocturia. These symptoms are usually caused by detrusor overactivity of the urinary bladder. 17% of the populations over 40 years old in Europe have symptoms of OAB according to a study (Milsom, Abrams et al. 2001).

Nowadays medication is based on anticholinergic drugs which inhibit bladder contractions and increase capacity (oxybutylin, tolterodine, trospium, propiverine). However regular long-term administration cause loss of function and compliance problems (Andersson 2009). Then further among medication belong tricyclic antidepressant (imipramine), which has a direct relaxation effect on bladder muscle, desmopressin (a synthetic vasopressin analogue) acting as an antidiuretic and baclofen (a GABA receptor agonist), which is used in patients with bladder dysfunction. Moreover there are some other options for failed conventional therapy as neuromodulation, surgery or intravesical pharmacology - it is a botulinum toxin A (BTX-A) injection therapy acts by inhibiting calcium-mediated release of Ach and reducing muscle contractility.

However the recent treatment is not satisfactory. Patients should be careful of their living style and behavior. That involves modifying fluid intake, avoiding stimulants (caffeine, alcohol) and bladder training for urgency (Reynard, Brewster et al. 2006).

4.5.2 Urinary incontinence (UI)

UI is defined as involuntary leakage of urine. It results from a failure to store urine during the filling phase due to abnormality of the detrusor or the urethral sphincter. It occurs often in women than men and there is increasing tendency with age (Reynard, Brewster et al. 2006).

There are many causes for incontinence and it exists classifacation:

- True incontinence – the patient may lose urine without warnings, it can be a constant or periodic symptom. There can be more or less obvious causes as radical prostatectomy, extrophy of the bladder, ectopic ureteral oriface, previous injury and other congenital or acquired diseases.

- Stress incontinence – when the sphincteric mechanisms are weak, urine may be lost in relation with physical strain as coughing, laughing, rising from a chair. This is

common in multiparous women and men have undergone radical prostatectomy. The patients stay dry while lying in the bed.

- Urge incontinence – urgency to urinate can be so acute and severe that there is no time to reach the toilet. Urge incontinence may occur with acute cystitis.

- Overflow incontinence – paradox of loss of urine due to chronic urinary retention or secondary to a flaccid bladder (Smith, Tanagho et al. 1995; Tanagho, McAninch et al. 2004).

4.5.3 Bladder outlet obstruction (BOO)

The principal cause of BOO in men is benign prostatic hyperplasia (BPH). BOO is less common in women because they obviosly have no prostate, even though it is possible to find women with symptoms of BOO (Pluta, Thompson et al.). There are several causes, which can evoke BOO including e.g. pelvic prolaps, prolapsing organ directly pressing the urethra, urethral stricture or in either sex, neurological diseases. Among main symptoms of bladder outlet obstruction belong incomplete emptying, frequency, intermittency, weak stream, straining and terminal dribbling (Reynard, Brewster et al. 2006).

4.5.4 Neuropathic bladder

A variety of neurological conditions are associated with abnormal bladder or sphincter function (e.g. spinal cord injury, spina bifida). The bladder or sphincter may be overactive or underactive, or it can be a combination. An overactive bladder intermittently contracts during bladder filling, when normally bladder pressure should be low. Underactive bladder manifests itself by low pressure during filling and voiding. The same priciple but in opposite way, can be observed in overactive and underactive sphincter. An underactive sphincter is unable to maintain enough pressure to prevent leakege of urine (Reynard, Brewster et al. 2006).

4.5.5 Interstitial cystitis (IC)

Cystitis is defined as an infection and/or an inflammation of the urinary bladder. IC is primarily a disease of middle-aged women and there does not appear infection as the cause of inflammation, because the urine is usually normal. It is characterized by fibrosis of the vesical

wall, with consequent loss of the bladder capacity. Frequency, urgency, incontinence and pelvic pain with bladder distension are the principal symptoms. Pathogenesis of IC is not well understood. Currently, it is believed that IC is a neuroimmunoendocrine disorder. It might be primarily a neurogenic inflammation that leads to the release of neuropeptides that activate the different secretion of potent mast cells mediators. It is thought that the mast cells, through their vasoactive and nociceptive secretion, have a major role in the etiology of IC. The signs of iflammation are apparent. The muscle is replaced by varying amounts of fibrous tissue. The lymphatics may be engorged, increased mast cells and lymphocytic infiltration are seen. Microscopically, the mucosa can be thinned or denuded. Nowadays there is no definitive treatment for IC. The therapy ussually affords partial relief, but it may be completely ineffective. It can be used hydraulic overdistention, vesical lavage, occasionally it can help instillation of 50 ml of 50% DMSO into the bladder every 2 weeks or corticiod treatment as symptomatic relief. But it does not treat the cause ((Smith, Tanagho et al. 1995; Tanagho, McAninch et al. 2004; Reynard, Brewster et al. 2006).

4.5.6 Bladder cancer

Bladder cancer is the second most common cancer of the genitourinary tract. It accounts for 7% of new cancer in men and 2% of new cancer in women. There is a positive social class gradient in both sexes. The average age at the diagnosis is 65 years. Approximately 75% of the cancers are localized to the bladder and 25% have spread to regional lymph nodes or distant sites. Majority of all the bladder cancers are epithelial malignancies having origin in the urothelium. Cigarette smoking increases the risk of the bladder cancer (Wynder and Goldsmith 1977). The association seems to be related to an agent alpha- and beta-naphtylamine, which are secreted into the urine of smokers. Oncologic patients who have recieved cyclophosphamide for the treatment of the various malignant diseases are also at increased risk (Fairchild, Spence et al. 1979). There are many other factors as genetic predisposition and other different carcinogens, which may increase the risk of the bladder cancer (Tanagho, McAninch et al. 2004).

4.6 Cyclophosphamide

Cyclophosphamide (CYP) belongs among alkylating agents evoking DNA damage. Alkylating agents are important in the treatment of various malignancies either as a single agent or as components of effective drug combination. There appears to be little or no cross-resistance of alkylating agents with other classes of drugs. The most susceptible region for alkylation of DNA is the N-7 position of guanine. CYP and other nitrogen mustards such as chlorambucil, ifosfamide ect. act in this way. They are classified as bifunctional agents. Beside this exists nonclassical, monofunctional alkylating agents such as procarbazine, which do not cross-link DNA but may produce single strand breaks.

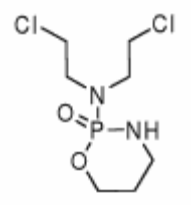
Cyclophosphamide was first described in 1958 and represents the results of an effort to develop drugs that are selectively activated at the tumor site. Nevertheless, subsequent studies have shown that the primary tissue of metabolic activation is the liver. CYP is a drug with wide spectrum of antitumor activity and it is also used as an immunosupressant (Carruthers and Melmon 2000).

Cyclophosphamide is an inactive substance, which is metabolized by the cytochrome P_{450} system, concretely by CYP_{2B}, in vivo to 4-hydroxycyclophosphamide, which is in stady state with the acyclic tautomer aldophosphamide. Besides the active metabolites are generated by other enzymes inactive substances carboxyphosphamide and 4-ketocyclophosphamide. The active metabolites are carried in the circulation to tumor cells where aldophosphamide cleaves spontaneously, genereting stechiometric amounts of phosphoramide mustard and acrolein. Phospshoramide mustard is responsible for antitumor effects, while acrolein causes hemorrhagic cystitis often seen during therapy with cyclophosphamide. The rate of metabolic activation of cyclophosphamide exhibits significant interpatient variability and increases with the successive doses. However, at the same time the high-dose regimens increase the risk of adverse effects, especially hemorrhagic cystitis (Brunton, Gilman et al. 2006).

CYP may be administered orally or intravenously. The drug is well absorbed orally and well tolerated in low doses. Nevertheless, higher oral doses may produce hematuria and other complications. During high-dose treatment is required vigorous hydratation to prevent hemorrhadic cystitis, which is caused by the metabolite acrolein, accumulating in the the urinary bladder. When the drug is administered intravenously, the risk of the bladder toxicity is minor. Another adverse effect, which is common for all alkylating agents, is bone marrow suppression. Mainly leukopenia is the dose-limiting factor of CYP. Further pulmonary and

cardiac toxicity may occur, especialy in high dose transplant regimens. Other uncommon toxicities include the syndrome of inappropriate secretion of antidiuretic hormone. It is important to aware of the possibility of water intoxication, since the patients are usually strongly hydrated to prevent bladder toxicity. Maximal concentration in plasma is achieved one hour after oral administration, the half-life of the parent drug in plasma is about 7 hours (Carruthers and Melmon 2000; Brunton, Gilman et al. 2006).

CYP is used in combanation modes to treat lymphoma, breast cancer, small-cell lung cancer and ovarian cancer. The drug has also an important use as part of high-dose regimens in bone marrow transplatation programs and it is widely used as an imunosuppressant. Reccommended doses vary according to the diagnosis and other factors (Carruthers and Melmon 2000).



Cyclophosphamide

4.7 Receptors of the urinary bladder

Receptors are proteins, which interact with neurotransmitters as a binding partner to induce an effect. Receptors have two main properties. They have a specific binding area, which is able to connect to a specific transmitter. And after the transmitter is bound, the receptor protein changes its conformation in an active state.

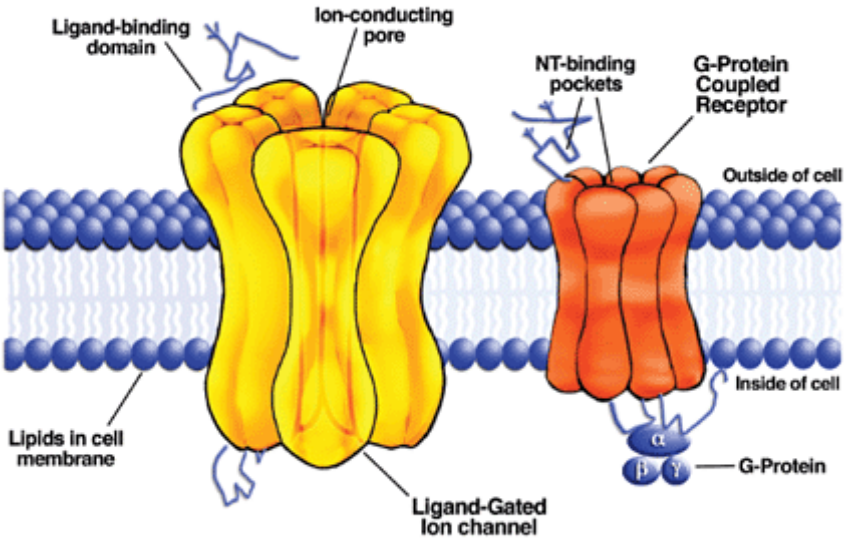
4.7.1 Types of the receptors

- *Ligand gated ion channel* – a type of ionotropic receptor, which is linked to transmembrane ion channels that are opened or closed in response to the binding of a chemical messenger generally named “ligand”. There is a direct link between ligand binding and opening or closing of the ion channel that is characteristic for ionotropic receptors in contrast with the indirect way of metabotropic receptors, which use

second messenger. An example of ligand gated channel is a nicotine-acetylcholine receptor, which is composed of a pentamer of glycoprotein subunits α, β, γ, δ. There are two binding sites (subunits α) for acetylcholine, which changes the conformation of the receptor after the transmitter is bound.

- *G-protein coupled receptors* – a type of metabotropic receptor, which acts in indirect way through second messenger that leads to open ion channel. Many receptors of the human body belong to this group, e.g. muscarinic, adrenergic, histamine receptors. There are two principal signal transductions - the cAMP and the phosphatidylinositol signal pathway. When a ligand binds to the G-protein coupled receptor it causes a conformational change in the receptor. The G-protein is composed of three subunits α, β, γ and during a resting phase guanosinediphosphate (GDP) is bound to the α subunit. After the transmitter is bound to the receptor, GDP is exchanged for GTP, α subunit with GTP dissociates and affects functional proteins.

Besides that other receptor types occur e.g. receptors regulating DNA transcription (Carruthers and Melmon 2000).



Adapted from
http://www.niaaa.nih.gov/Resources/GraphicsGallery/Neuroscience/Pages/ligand_gated.aspx

4.8 Cholinergic system

The muscarinic receptors belong to the family of G-protein coupled receptors and they appear in 5 subtypes (M_1-M_5). Neurotransmitter for all subtypes of muscarinic receptors is acetylcholine. M_3 stimulation principally induces contractile response by releasing of calcium. The detrusor smooth muscle is well innervated by cholinergic nerves but sparsely supplied with sympathetic nerve fibers. Also, the urethral internal sphincter is innervated by cholinergic and noradrenergic nerve fibers.

Muscarinic receptors in the human detrusor are mainly represented by M_2 (70%) and M_3 (20%) subtypes. Although in the minority M_3 receptors are mostly responsible for the cholinergic contractile response of the bladder. M_2 receptors have small direct contractile effects but mainly work in indirect way. That means they facilitate contractions by blocking relaxations induced by cAMP-coupled receptors. Also, in the urothelium occur a high density of muscarinic receptors – all subtypes, which is changed during inflammation (Giglio and Tobin 2009).

4.9 Adrenergic system

Adrenoceptors do not seem to be very important in a contractile response of the detrusor smooth muscle according to a fact there is a low number of adrenergic receptors in the detrusor. Several investigators demonstrate that the human detrusor is able to express α_{1A}, α_{1D}, β_1 and β_2 adrenoceptors. The response of the normal detrusor to noradrenaline is relaxation. The external urethral sphincter is innervated by noradrenergic nerve fibers and causes relaxation when the detrusor muscle contracts (Andersson and Arner 2004).

4.10 NANC system

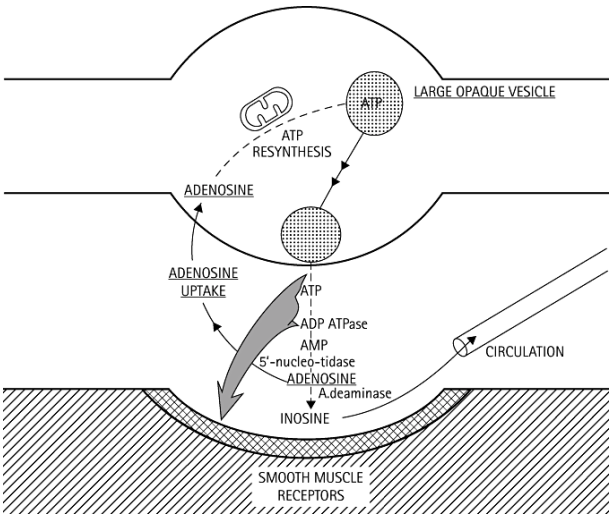
Muscarinic and adrenergic neurotransmission is not only the one which affects contraction and relaxation of the urinary bladder. There is another mechanism called non-cholinergic, non-adrenergic (NANC)-transmission which subsumes substances such as ATP, NO, neuropeptides, prostanoids,.. In the case of urinary bladder is the most important purinergic signaling.

4.10.1 Adenosine triphosphate

Adenosine triphosphate (ATP) is well known as an intracellular energy source for living cells. Less commonly known but no less essential role of ATP is done outside of the cells. Purinergic hypothesis was proposed in 1972 by Geoffrey Burnstock. The hypothesis is based on ATP as a neurotransmitter mediating non-adrenergic, non-cholinergic (NANC) neurotransmission. Despite a huge amount of data showing ATP release from neurons into muscle, gut and bladder tissue, many scientists remained sceptical about releasing ATP as a messenger. Mainly because they thought it is unlikely that such a ubiquitous substance could perform such a specific role. Moreover no specific receptors for ATP were known.

The need to identify receptors that respond to ATP was implicit. The first conceptual advance was made by Burnstock when he suggested two separate families of receptors for ATP/ADP named P2 receptors and for adenosine - a final ATP breakdown product - named P1 receptors. Further, in 1985, was proposed that the P2 receptors could be subdivided into P2X and P2Y receptor families. The large step in characterization of purinergic receptors has come in the early 1990s, when the molecular tools became available to recognize four subtypes of the P1 G-protein coupled receptors – A_1, A_{2A}, A_{2B} and A_3 seven subtypes of P2X ion channel receptors ($P2X_{1-7}$) and eight subtypes of P2Y G-protein-coupled receptors ($P2Y_{1,2,4,6,11,12,13,14}$).

It was established that ATP is a co-transmitter in every nerve type in both the peripheral nervous system and CNS. It acts in concert with other neurotransmitters such as noradrenaline or acetylcholine (Khakh and Burnstock 2009; Burnstock 2011).



Adapted from http://onlinelibrary.wiley.com/doi/10.1111/j.1464-410X.2010.09926.x/full

Table 1. P2X and P2Y receptors and their main distributions

Receptor	Main distribution
$P2X_1$	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurones
$P2X_2$	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia
$P2X_3$	Sensory neurones, NTS, some sympathetic neurones
$P2X_4$	CNS, testis, colon
$P2X_5$	Proliferating cells in skin, gut, bladder, thymus, spinal cord
$P2X_6$	CNS, motor neurones in spinal cord
$P2X_7$	Apoptotic cells in immune cells, pancreas, skin, etc.
$P2Y_1$	Epithelial and endothelial cells, platelets, immune cells, osteoclasts
$P2Y_2$	Immune, epithelial and endothelial cells, kidney tubules, osteoblasts
$P2Y_4$	Endothelial cells
$P2Y_6$	Some epithelial cells, placenta, T-cells, thymus
$P2Y_{11}$	Spleen, intestine, granulocytes
$P2Y_{12}$	Platelets, glial cells
$P2Y_{13}$	Spleen, brain, lymph nodes, bone marrow
$P2Y_{14}$	Placenta, adipose tissue, stomach, intestine, discrete brain regions

Adapted from http://onlinelibrary.wiley.com/doi/10.1111/j.1464-410X.2010.09926.x/full

ATP signaling: a brief history

1929 ATP discovered to be the energy source in muscle tissue.

1929 Albert Szent-Györgyi finds purines (ATP's chemical family) have potent effects on the heart.

1945 ATP structure confirmed.

1959 Pamela Holton shows ATP release from sensory nerves.

1962 Geoffrey Burnstock demonstrates message transmission from neurons to muscle by a new neurotransmitter.

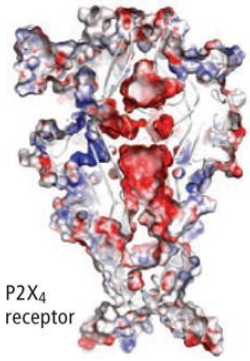
1972 Burnstock proposes the existence of nerves that signal using ATP.

1976 Burnstock proposes that ATP acts as a co-transmitter with other neurotransmitters.

1993 and 1994 P2X and P2Y receptors for ATP isolated from cells.

1998 Clopidogrel, a drug that acts on platelet P2Y receptors, introduced to prevent clot formation in blood vessels.

2009 Crystal structure of a P2X receptor revealed, which should aid drug discovery.



Adapted from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2877495/?tool=pubmed

4.10.2 Nitric oxide

The importance of nitric oxide (NO) has been emphasized in the regulation of many physiological functions as well as pathological processes of the human body (Mungrue, Bredt et al. 2003). NO is a small, labile, lipid-permeable free radical molecule and it is synthetized by NO synthase (NOS). NO is generated by three different isoenzymes of NOS. Inducible NOS (iNOS) is not normally expressed in healthy tissue but it can be induced during injury or exposure to pathogens. It plays a role in the immune system. NO generated by endothelial NOS (eNOS) is produced by endothelial cells to mediate blood vessel relaxation and inhibition of platelet aggregation. In the nervous system is NO expressed by neuronal NOS (nNOS) and it was first recognized as a messenger molecule that mediates increases in cyclic GMP (cGMP) levels. A role of NO as a biological messenger molecule altered concepts of neurotransmission (Kushner 2003). NO is generally accepted as one of the non-adrenergic, non-cholinergic (NANC)-transmitters affecting the urinary bladder (Bult, Boeckxstaens et al. 1990; Andersson and Persson 1994). During pathologic conditions such as

cyclophosphamide-induced cystitis, seems to be eNOS up-regulated (Giglio, Ryberg et al. 2005).

5 Immunohistochemistry

Immunohistochemistry (IHC) is a valuable tool for detecting specific antigens in tissues. Immunohistochemical staining enables the visualization of the tissue distribution of specific antigens. The process localizes protein targets by applying specific monoclonal or polyclonal antibodies. IHC is the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions. The visualization is provided by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. IHC combines anatomical, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label. Therefore, IHC has become a crucial technique and widely used in many medical research laboratories as well as clinical diagnostics.

The principle of immunohistochemistry has existed since the 1930s, but it was not until 1941 that the first immunohistochemistry study was reported. Coons and his colleagues used antibodies with a fluorescent dye to localize Pneumococcal antigens in infected tissues.

There are numerous immunohistochemistry methods that might be used to localize antigens. The main parameters to select suitable method are the type of specimen and the degree of sensitivity which is required. Important parts of IHC are the tissue preparation, fixation and sectioning since these steps often make the difference between staining and no staining. In general, many antigens can be successfully demonstrated in formalin-fixed and paraffin-embedded tissue sections. Certain cell antigens do not survive routine fixation and paraffin embedding. In this case frozen sections are used. However, the disadvantage of frozen sections includes poor morphology, poor resolution at higher magnifications, special storage needed, limited retrospective studies and cutting difficulty over paraffin sections. Sectioning is provided by a vibrating microtome. Microwave oven, pressure cooker and steamer are the most commonly used heating devices. The heating length of 20 minutes appears to be the most satisfactory and the cooling usually takes about 20 minutes. Citrate buffer is the most popularly used retrieval solution and is suitable for most of antibody applications.

Next step in IHC is a non-specific background blocking of endogenous peroxidase. The main cause of non-specific background staining is non-immunological binding of the specific immune sera to certain sites within tissue sections. This form of background staining is usually uniform.

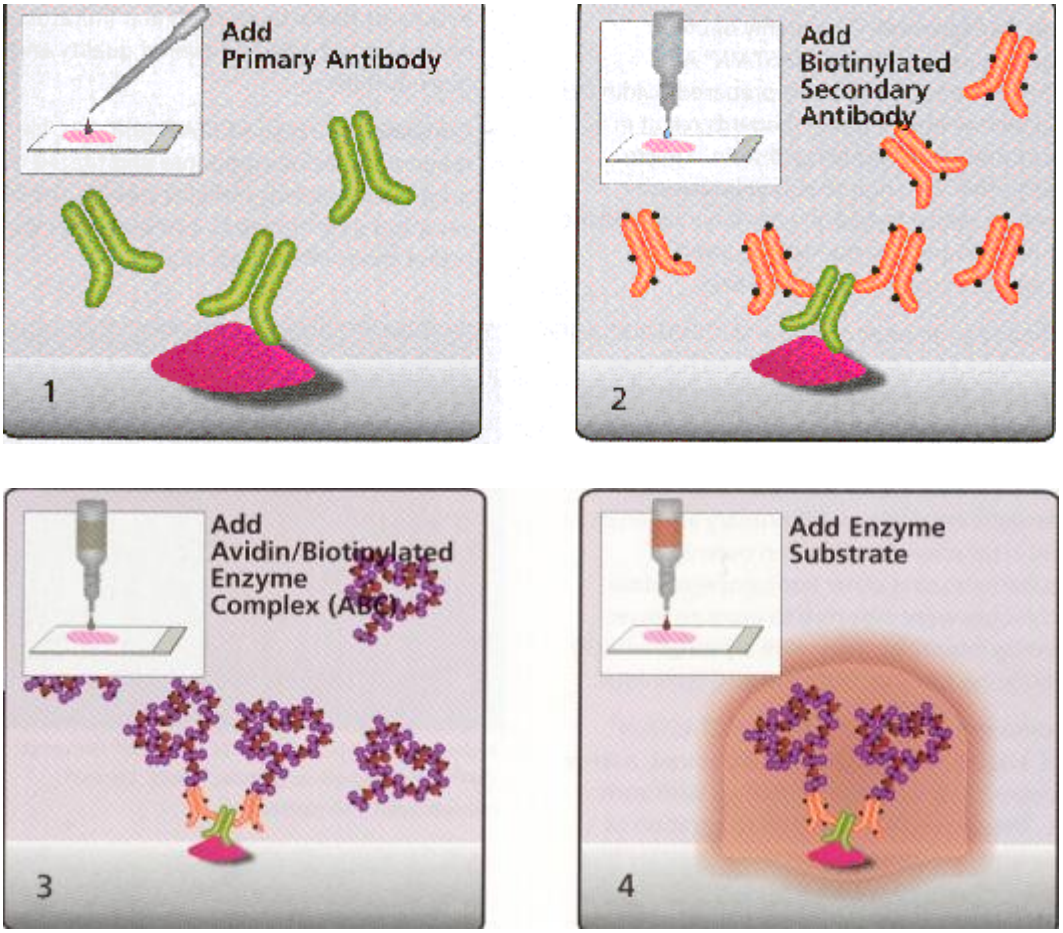
During the immunohistochemical staining control specimens are needed to be done. Positive control tests whether the procedure works correctly. Negative control tests specificity of an antibody. There are two main staining methods, direct and indirect. Direct method is one step staining method and involves a labelled antibody reacting directly with the antigen in tissue sections. This technique utilizes only one antibody and the procedure is short and quick. However, it is insensitive due to little signal amplification and rarely used since the introduction of indirect method. Indirect method involves an unlabeled primary antibody which reacts with tissue antigen, and a labelled secondary antibody reacts with primary antibody. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. Many indirect methods are used for the localization of proteins e.g. PAP (peroxidise anti-peroxidise) method, ABC (Avidin-Biotin Complex) method, LSAB (Labeled StreptAvidin Biotin) method. Besides that is often used multiple staining method, which is useful since it stains two or more antigens in one common tissue section. Disadvatage of this method is complexity of the staining and high risk of failure.

5.1 Avidin-Biotin Complex (ABC) Method

ABC method is a unique immunoperoxidase procedure for localizing a variety of histologically significant antigens and other markers. Nowadays it is one of widely used technique for immunohistochemical staining. Avidin, a large glycoprotein, has such an extraordinarily high affinity for biotin (over one million times higher than antibody for most antigens). Avidin has four binding sites for biotin, and most proteins including enzymes can be conjugated with several molecules of biotin. The binding of avidin to biotin is essentially irreversible. Avidin can be labelled with peroxidase or fluorescein. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies.

The first step of the procedure is to incubate the section with primary antiserum raised against the antigen such as rabbit antibody to a tumor-associated antigen. Further a biotin-labelled secondary antibody is added, which would be biotinylated anti-rabbit IgG. This

introduces many biotins into the section at the location of the primary antibody. The avidin biotinylated enzyme complex (ABC) is then added and binds to the biotinylated secondary antibody. In the last step of the procedure, the tissue antigen is localized by incubation with a substrate for the enzyme. In some cases it may be necessary to precede these steps with blocking procedures to eliminate any nonspecific reaction. The advantages of ABC system are foremost high selectivity, low background, efficiency and economy.



Adapted from http://www.reactolab.ch/Vector/ABC%20Method.htm

EXPERIMENTAL PART

6 Methods and materials

All experiments conducted during this thesis were approved by the local ethics committee at the University of Gothenburg. 39 male rats of the Sprague-Dawley (300-400 g) strain were used.

6.1 In vitro functional studies

6.1.1 Urinary bladder strip preparation

The rats were anaesthetized with an intraperitoneal injection of pentobarbital (60mg/kg). Before the rats were killed, the urine was picked up and the urinary bladder was removed and stored in Krebs bicarbonate solution at all times to keep it wet close to physiological conditions. The bladder was divided into two halves. One half was used for organ bath procedure and the second half for immunohistochemical staining. After a cut along the posterior surface from the urethral opening to the apex, the bladder was opened and two strips in the size of 2x6 mm were excised from the middle of the organ to avoid different distribution of the receptors in different parts of the bladder.

6.1.2 Organ bath experiment

For contraction experiments organ bath setup was used. It is an apparatus serving to measure contractions and relaxations of the strips according to the added substances. The strips were provided with a loop on one side and with a long tail of thread on the second side of the strip and fastened between two steel rods of the organ bath. One rod was adjustable to enable the measurement. Before beginning of any experiment a calibration is required. The organ bath with the strip was filled by Krebs bicarbonate solution of the following composition: deionised water, NaCl 118 mM, KCl 4.6 mM, KH_2PO_4 1.18 mM, $MgSO_4$ 1.16 mM, $NaHCO_3$ 25 mM, glucose 5.5 mM, the solution was gassed with 5% CO_2 in 95% O_2 for 45 minutes. Later $CaCl_2$ 1.27 mM was added and then it was bubbled by the same gas all the

time to keep the stable neutral pH. During the whole experiment the temperature was kept at 37 ºC by a thermostat.

At the beginning of the experiment the strips were pre-stretched to a tension of 15 mN for 45 minutes. The tension is reflected in the PC programme. The strips gradually got stabilised about 5 mN. After 45 minutes when all strips were stabilised, high potassium Krebs (HPK) 124 mM of composition 4.625g KCl in 0.5l of Krebs solution was added as a reference solution used in each experiment. When the maximum contractions were reached, the high potassium Krebs was washed out and the baths were refilled by the physiologic bicarbonate Krebs. Then the tension has to be stabilised on 5 mN again and let it be at least 20 minutes without any changes. After that it is possible to add next substance and repeat the process. All drugs used in the organ bath procedure were administered at a volume of 125 µl. Data were recorded using the Acquire software.

HPK - MeCh – ATP – HPK were added step by step during the organ bath experiment. Methacholine was administered in six increasing concentrations, ATP was administered in three increasing concentrations. All the experiments were ended by addition high potassium Krebs to compare reaction of the strips at the beginning and in the end of the experiment. It was necessary to observe at least 20 minutes intervals between additions of each substance and wash them throughly every time.

In the case of 4-DAMP, DPCPX, L-NAME and PSB1115 pre-treated groups were administered also adenosine as $P1A_1$ agonist causing relaxation. However, the results were not significant and they are not included in this thesis.

6.2 Immunohistochemistry procedure

This experiment takes two days. First day is focused on removing the paraffin, rupturing the membranes and binding the primary antibody. This is followed by overnight incubation and second day the incubating with antibody and staining is proceeded.

There are two specimens on each glass. One of them is marked as a negative control. To have a negative control is necessary because it shows selectivity of the primary antibody.

Day 1

1. The paraffinized sections are incubated at 60°C for 60 minutes.

2. Deparaffination.

   The glasses are rinsed for 30 minutes in xylene two times (after 30 minutes is the process repeated, meanwhile the glasses are emerged from the container and shaked up). Then follows 2 x 5 minutes in 99, 5% ethanol, 2 x 5 minutes in 95% ethanol, 1 x 5 minutes in 85% ethanol and finally 1 x 5 minutes in 70% ethanol. The glasses need to be checked if all paraffin is gone. If not, this step has to be repeated.

3. The glasses are rinsed in running deionised water for 10 minutes so residues are washed out. Follows washing in TBS for 2 x 5 minutes. It is supposed to wash out solutions used in previous step and inactivate the reactions which had been going on.

4. The glasses need to be rinsed in citrate buffer for 5 minutes. This is followed by boiling the samples in citrate buffer in a steamer at least for 20 min. Every time is the pot with the glasses refilled by the citrate buffer to prevent it from boiling away. The sections must not boil away but at the same time the sections must not be dry during cooking. The glasses are supposed to cool in citrate buffer for 30 minutes.

5. The glasses are washed in TBS 2 x 5 minutes.

6. Next step is non-specific background blocking by adding 0.03% H_2O_2 as a commonly used blocking agent to block endogenous peroxidise activity. It takes 30 minutes.

7. The glasses are washed again in TBS 2 x 5 minutes.

8. The area around each section was marked with a hydrophobic pen. Then the blocking of nonspecific background using 5% BSA in TBS for 30 minutes has to be done.

9. Incubation with primary antibody diluted in 1% BSA in TBS overnight. The dilution of the antibody starts on 1:100 but it can vary according to the manual of company and results. The box with the specimens during the incubation should be closed and filled with deionised water to prevent the sections from becoming dry. Only this step in protocol varies the positives and negatives samples from each other. That is why it needs special attention. The positives specimens are incubated overnight with primary antibody diluted in 1% BSA in TBS. The negative controls are incubated just in 1% BSA in TBS.

Day 2

1. The Santa Cruz ABC-kit was used for binding the secondary antibody and staining the slides. The ABC-kit has to be appropriate to the primary antibody which was used the previous day. According to the manual the secondary antibody, AB enzyme reagent and peroxidise substrate are mixed and prepared to use. Secondary antibody is a mixture of 75µl of blocking serum, 5 ml of TBS and 25 µl of biotinylated secondary antibody. AB enzyme reagent is mixed of 50 µl reagent A, 50 µl of reagent B and 2,5 ml of TBS. Peroxidase substrate is prepared by 1,6 ml of deionised water, 5 drops substrate buffer, 1 drop DAB chromogen and 1 drop of peroxidise substrate. The solutions work in the best way when they are used fresh.
2. The glasses are washed in TBS 2 x 5 minutes to wash them out.
3. Time for incubation with secondary antibody for 30 minutes.
4. Washing in TBS 2 x 5 minutes.
5. Another incubation with AB enzyme reagent for 30 min.
6. The glasses are washed in TBS 2 x 5 minutes again.
7. The sections are incubated in peroxidase substrate until they turn brown. Recommended time is 5 minutes. After 5 minutes is needed to check the slides and go on if it is not brown.
8. Rinse carefully in running deionised water for 10 minutes to wash the slides properly.
9. Next step is staining with haematoxyline until they get an intense colour, an approximate time is between 3-5 minutes.
10. The glasses are rinsed carefully in 37ºC running water until they become blue.
11. The samples are now needed to be dipped to the ethanol in increasing concentrations and finally in xylene for 10 minutes in each bath. Practically it is the same procedure as the deparaffination but in opposite way.
12. When the glasses are almost dry, the sections have to be sealed with a small drop of glue and covered by the cover glass while avoiding bubbles.

6.3 Materials

6.3.1 Immunohistochemical facilities

Xylene; ethanol 99,5%, 95%, 85%, 70%; deionised water; TBS (pH=7,5; mix 100ml A (85g NaCl in 1000 ml deionised water), 100 ml B (60,57 g Trizma Base in 1000 ml deionised water) and 800 ml deionized water); citrate buffer (pH= 6,0; mix 18ml A (0,1 M citric acid), 82ml B (0,1 M sodium citrate) and 900 ml deionized water); H_2O_2 (0,03%); 5% BSA (bovine serum albumin) in TBS; primary antibody diluted in 1% BSA in TBS; 1% BSA in TBS; Mayer´s haematoxyline; glue Pertex

Primary antibodies:

M5 muscarinic receptor antibody *(AS-3781S, Research & Diagnostic Antibodies)*

Anti-A1 adenosine receptor antibody *(A 268, Sigma-Aldrich)*

eNOS antibody *(610298, BD Transduction laboratories)*

ABC-kit:

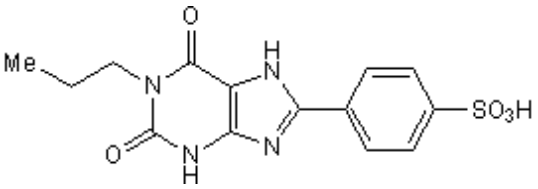
Rabbit ABC Staining system sc-2018 *(Santa Cruz Biotechnology, USA)* - blocking serum, biotinylated secondary antibody, AB enzyme reagent, peroxidase substrate

Storing of the bladders for IHC: phosphate buffer saline (PBS), formaldehyde 4%, ethanol 70%

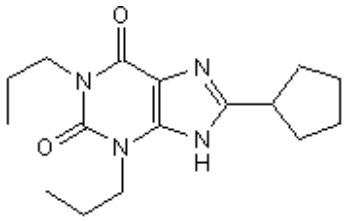
6.3.2 Drugs

The drugs employed in this thesis were buprenorphine hydrochloride (Temgesic 0,3mg/ml), cyclophosphamide monohydrate, pentobarbitalum natricum, acetyl-β-methylcholine, adenosine-5´-triphosphate, adenosine, 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME), 4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid (PSB1115), Suramin hexasodium salt, Dimethylsulfoxide (DMSO).

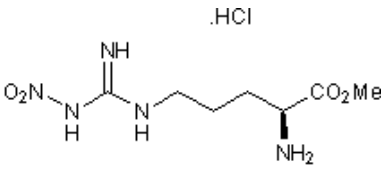
PSB1115



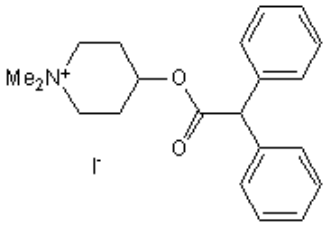
DPCPX



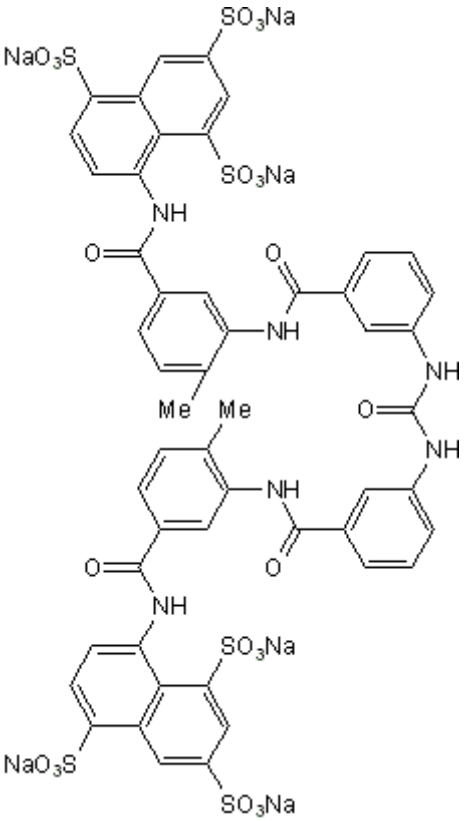
L-NAME



4-DAMP



SURAMIN



7 Results

7.1 Functional studies

Functional studies were done on a control group and five pre-treated groups of antagonists. The rats were pre-treated by 4-DAMP as a M_3, M_5 muscarinic antagonist; DPCPX as a $P1A_1$ purinergic receptor antagonist; PSB1115 as a $P1A_{2B}$ purinoceptor antagonist; L-NAME as an inhibitor of eNOS and suramin as a non-selective P2 receptor antagonist. The experiment was done simultaneously on healthy (saline) and CYP induced cystitis rats. The study is focused on functional effects of pre-treatment in normal and inflamed urinary bladder. Comparisons were made between raw data.

The five different pre-treatments affecting cholinergic, purinergic and nitrergic mechanisms were selected. The pre-treatment was proceeded 5 days and followed an exact schedule. Every day at the same time was intraperitoneally injected an antagonist L-NAME (60mg/kg), 4-DAMP (2mg/kg), DPCPX (1mg/kg), PSB1115 (1mg/kg) or suramin (10mg/kg) into the saline/CYP pre-treated rats. 60 hours after the saline/CYP pre-treatment was the organ bath experiment done. The cystitis after 60 hours is obviously dysplayed in the bladders. For examination of the potency of the antagonists, methacholine in six concentrations and ATP in three concentrations were administered. Methacholine in increasing concentrations of $5*10^{-8}$ M to $5*10^{-3}$ M evoked contractile response, maximal contractions were ussually observed in concentration $5*10^{-5}$ M, than the response was going slightly down since all receptors have been already occupied. ATP in increasing concentrations of $5*10^{-6}$ M to $5*10^{-4}$ M were administered, the contractile response were not so obvious as in the case of methacholine. The effects of antagonists with different selectivity for the receptor subtypes affected the contractile response.

7.1.1 Contractile response to methacholine

7.1.1.1 Control group

The contractile response was significantly larger in the normal than in the inflamed bladders in the saline/CYP control group (saline n=20, CYP n=24).

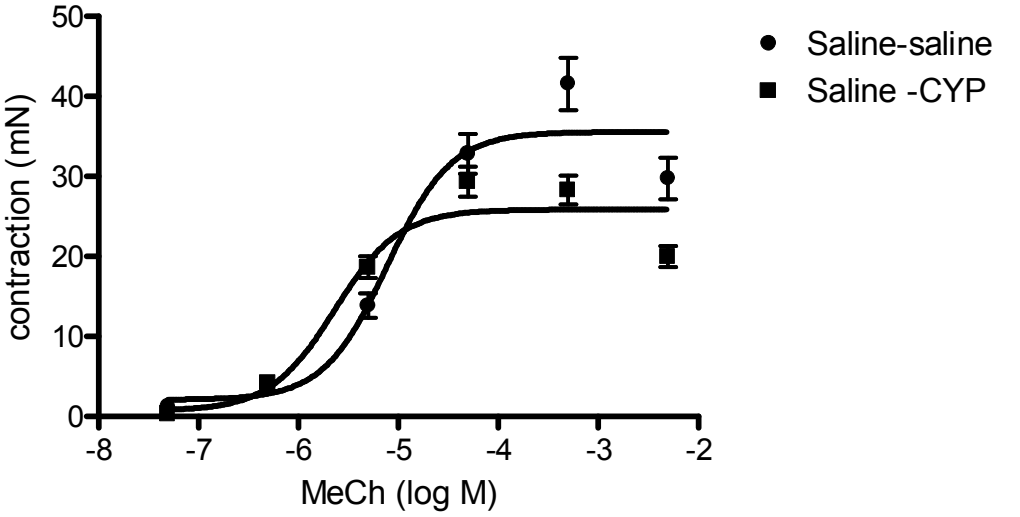


Fig.1 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders.

Functional studies have shown altered cholinergic mechanisms in the inflamed bladder which at least partly depend on muscarinic receptor-induced release of nitric oxide (NO).

7.1.1.2 Pre-treated groups

The pre-treatment affected the contractile responses. The best results showed DPCPX (DPCPX+SAL n=8, DPCPX+CYP n=12) and L-NAME (L-NAME+SAL n=6, L-NAME+CYP n=12) which obviously reduced state of inflammation. According to this findings we can say that purinergic $P1A_1$ receptor mechanisms and inhibition of eNOS are important factors of the inflammation. This implies that nitric oxide is responsible for the inflammatory changes in relation a larger releasing of nitric oxide causes worse stage of the inflammation. Therefore inhibition of eNOS disabling synthase of nitric oxide refines state of the inflammation. $P1A_1$ receptor seems to work as a pro-inflammatory factor, since the blocking of $P1A_1$ receptors normalized contractions most likely by opposing relaxation effect causing by adenosine, and consequently reduced the inflammatory signs.

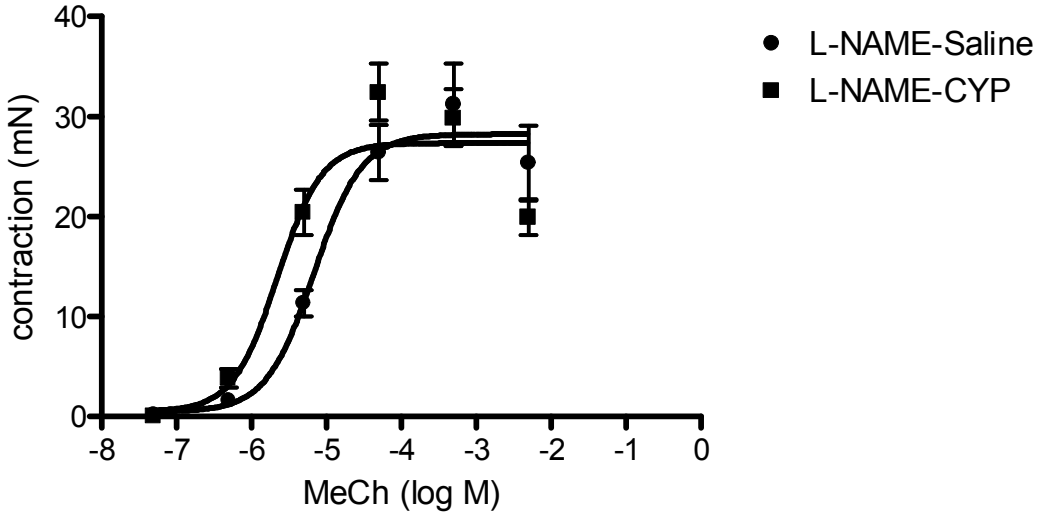


Fig.2 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of L-NAME (60 mg/kg).

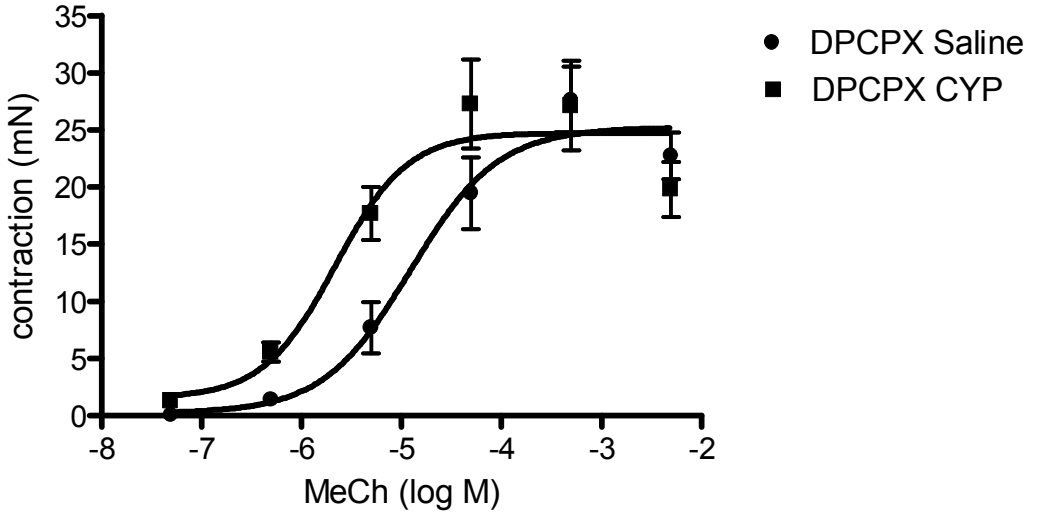


Fig.3 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of DPCPX (1mg/kg).

Conversely the presence of 4-DAMP (4-DAMP+SAL n=8, 4-DAMP+CYP n=12), PSB1115 (PSB1115+SAL n=8, PSB1115+CYP n=12) and suramin (SUR+SAL n=8, SUR+CYP n=8) did not affect contractile responses significantly. This evokes that inhibition of muscarinic M_3, M_5 receptors, purinergic $P1A_{2B}$ receptor and generally P2 mechanisms do not play an important role in the inflammatory process at least according to the functional studies done in this thesis.

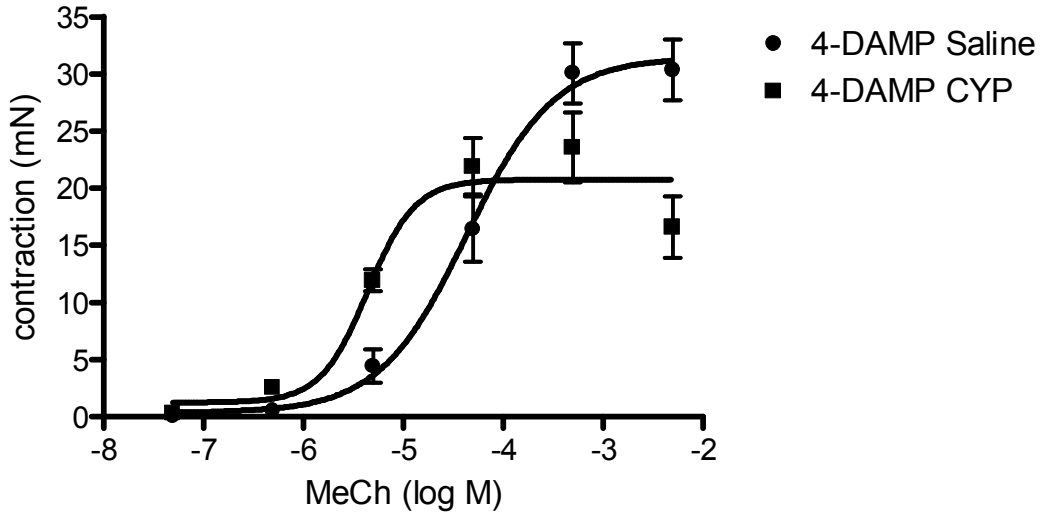


Fig.4 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of 4-DAMP (2mg/kg).

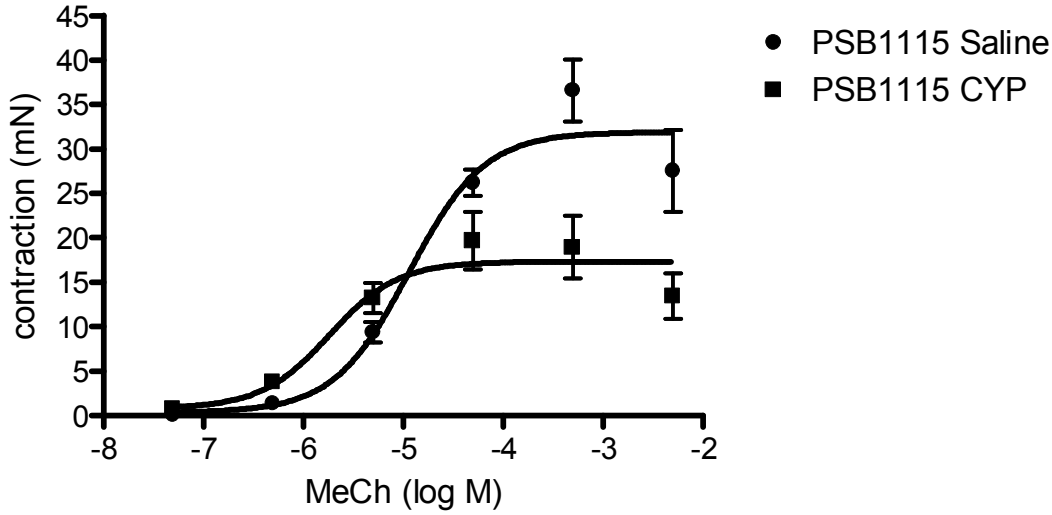


Fig.5 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of PSB1115 (1mg/kg).

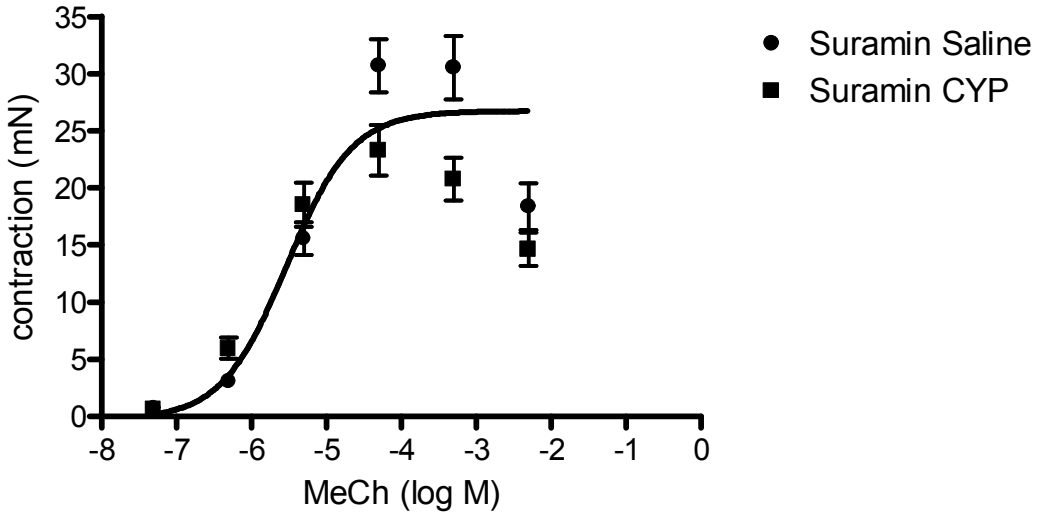


Fig.6 Contractile response to MeCh ($5*10^{-8}$ to $5*10^{-3}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of suramin (10 mg/kg).

7.1.2 Contractile response to ATP

The contractile response to ATP as a purinergic P2 receptor agonist is weaker and generally less obvious than MeCh functional results . The point which is deserved to be noticed, is higher contractile response of CYP pre-treated rats in the case of DPCPX, L-NAME and in some way also PSB1115 pre-treated group. However, the differences in the contractile response were not significant.

7.1.2.1 Control group

In the control group saline/CYP were the contractions larger in the healthy than in the inflamed tissue (saline n=20, CYP n=24). That means the contractile response was affected by the inflammation and there were alterations in the purinergic mechanisms during cystitis. The contractile response was probably affected through the ATP breakdown product, adenosine, which causes relaxation and indirectly reduces contraction.

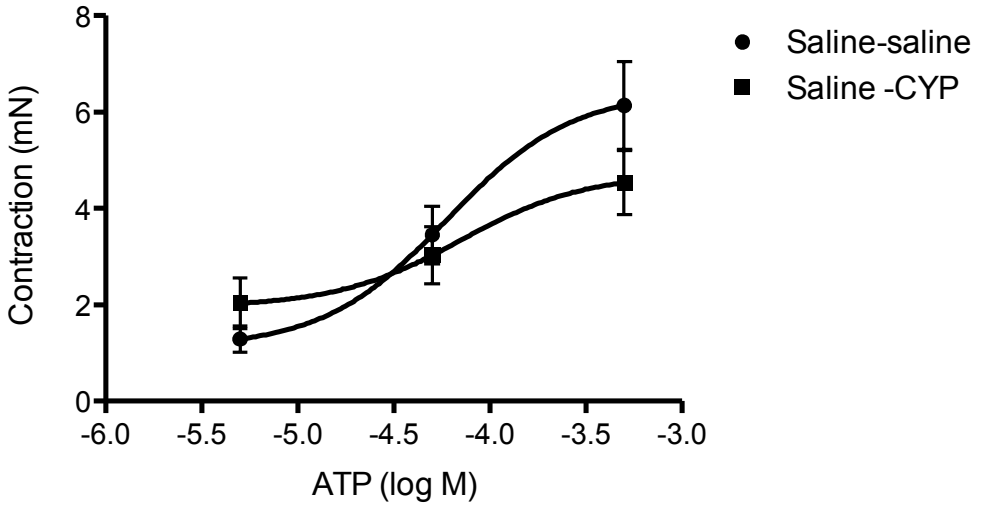


Fig.7 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders.

7.1.2.2 Pre-treated groups

The pre-treatment affected the contractile response foremost in DPCPX (DPCPX+SAL n=8, DPCPX+CYP n=12) and L-NAME (L-NAME+SAL n=6, L-NAME+CYP n=12) pre-treated groups. There the functional experiments showed reversed results to the control group. That means the contractile response to ATP was higher in the inflamed than in the healthy bladders. It could be really interesting point, since it implies that inhibiton of $P1A_1$ receptor and blocking of NO synthase lead to refine cystitis. Moreover the bladder condition became better than the healthy ones. However, the ATP findings are not really significant because the contractions were weak and badly readable. Anyway it is not a coincidence that MeCh experiments provided in principle similar findings. Based on these results it was found that inhibition of $P1A_1$ receptor and blocking of eNOS affect not only cholinergic but purinergic contractile response as well.

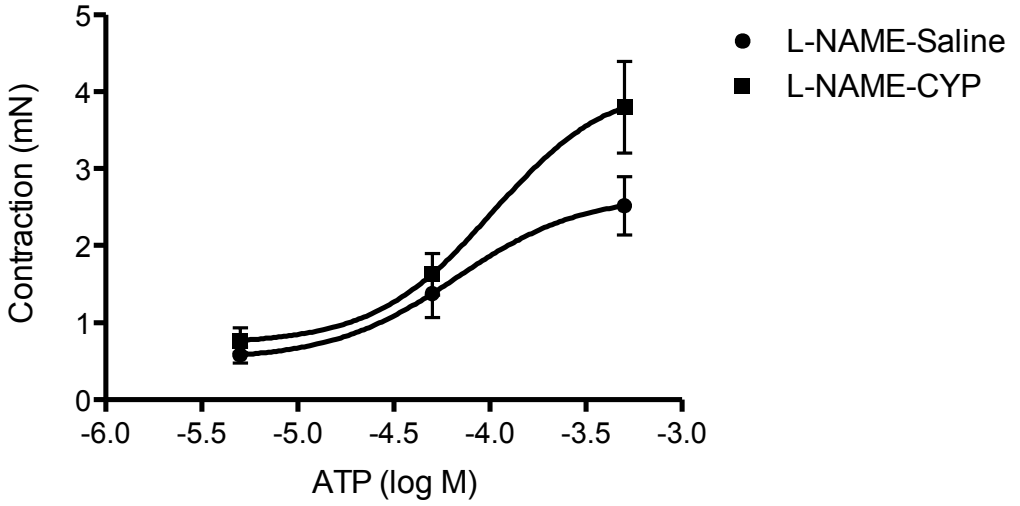


Fig.8 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of L-NAME (60 mg/kg).

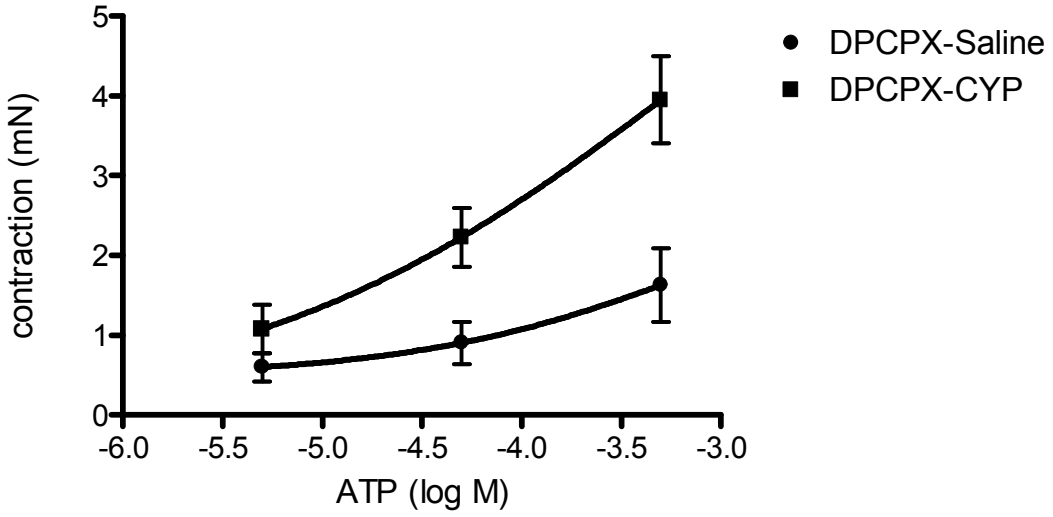


Fig.9 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of DPCPX (1mg/kg).

The same principle followed also PSB1115 pre-treated group (PSB1115+SAL n=8, PSB1115+CYP n=12). There higher contractions in the inflamed bladders than in the healthy ones can be observed as well. It could be explained as a correlation between inhibition $P1A_{2B}$ purinergic receptor and purinergic contractile response. However, the contractions were weaker and less apparent than in the case of DPCPX and L-NAME pre-treated groups.

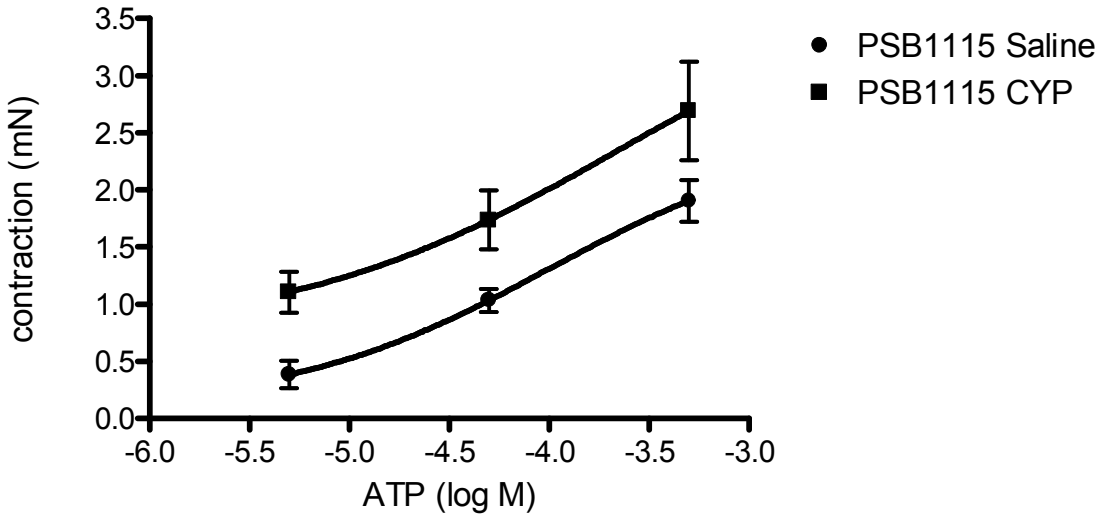


Fig.10 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of PSB1115 (1mg/kg).

Parallelly to the MeCh functional experiments, 4-DAMP (4-DAMP+SAL n=8, 4-DAMP+CYP n=12) and suramin (SUR+SAL n=8, SUR+CYP n=8) did not affect contractile responses significantly and showed resembling results as the saline/CYP controle group. It is remarkable that suramin as a non-selective P2 antagonist did not affect contractile response to ATP in significant way.

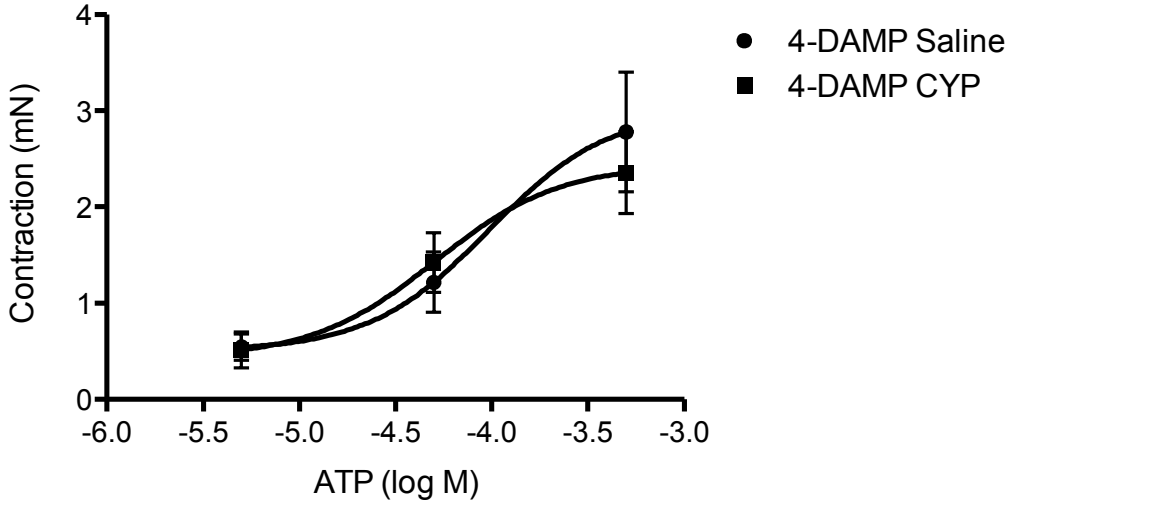


Fig.11 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of 4-DAMP (2mg/kg).

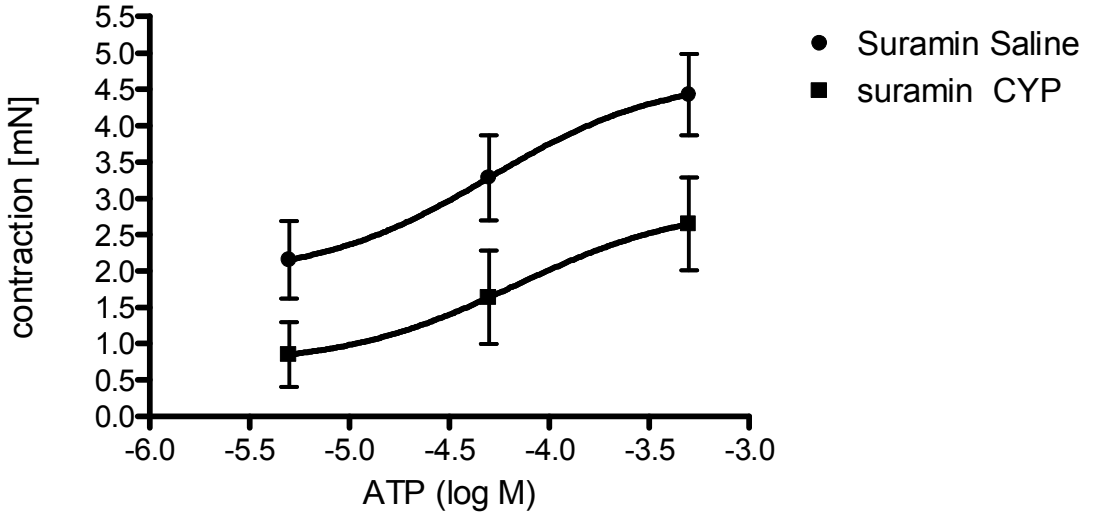


Fig.12 Contractile response to ATP ($5*10^{-6}$ to $5*10^{-4}$ M) of normal (saline) and inflamed (CYP) bladders in the presence of suramin (10 mg/kg).

7.2 Morphological studies

In general, the CYP pre-treated bladders show the signs of inflammation 60 hours after intraperitoneal injection. The urothelium is disrupted and an increased amount of vacuoles and blood vessels are observed, the bladder wall is thickened.

Immunohistochemical staining was done on five pre-treated groups and saline/CYP specimens as a control group. The results are based on 3 rounds of receptor staining in all cases.

7.2.1 $P1A_1$ receptor staining

7.2.1.1 Control group

The IHC staining for the $P1A_1$ purinoceptors showed the receptors are decreased during inflammation especially in the urothelium. The staining was weak or absent in CYP pre-treated bladders but particularly intense in the urothelium of healthy tissue. The bladder thickened wall of CYP-induced inflamed tissue is obvious.

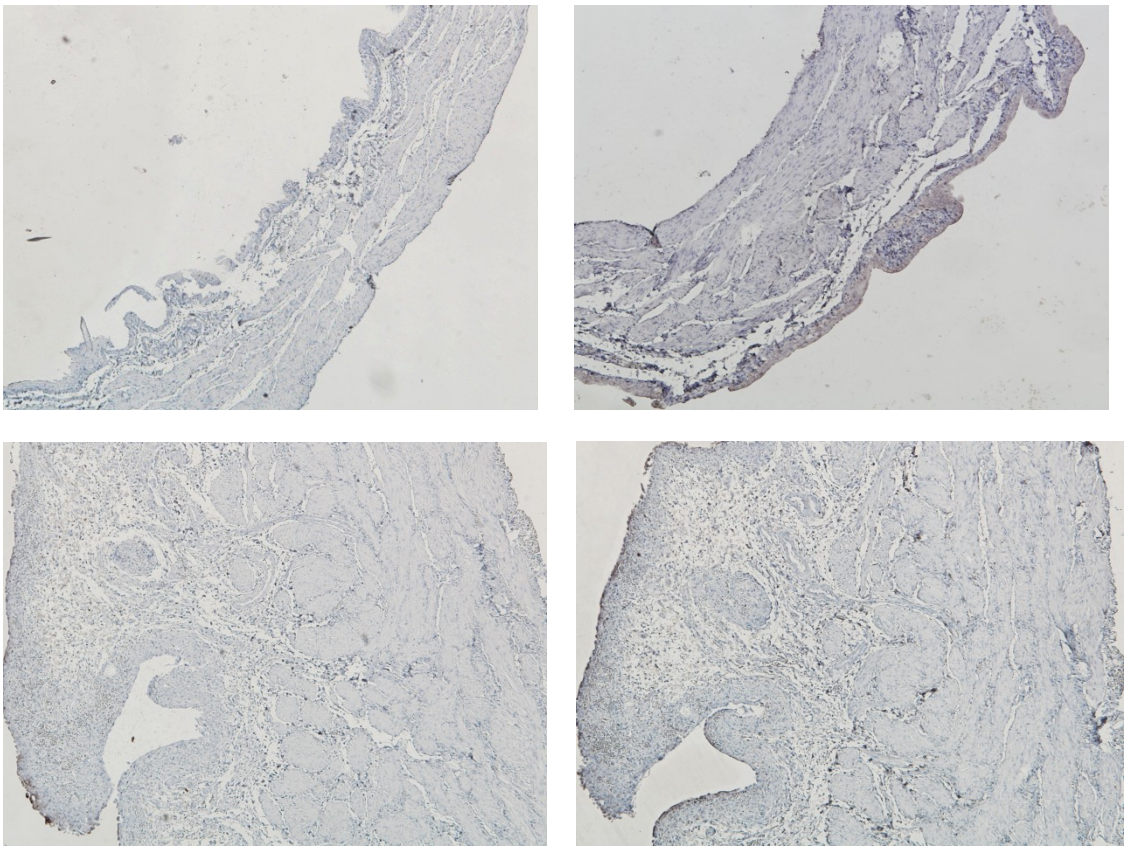


Fig.13 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder. The upper row shows healthy tissue (saline), while the row below inflamed (CYP-induced cystitis) tissue. On the left are the negative controls and the right side shows the staining for $P1A_1$ receptors. All sections were counterstained with Mayer's haematoxyline.

7.2.1.2 Pre-treated groups

The pre-treatment by antagonists affected the immunohistochemical staining. However, the number and localization of the receptors in the presence of antagonists did not differ from the control group significantly.

Expression of the $P1A_1$ receptor in the urothelium was slightly decreased in the pre-treated groups in comparison with healthy tissue of the control group. The presence of the receptor in the muscle was equal.

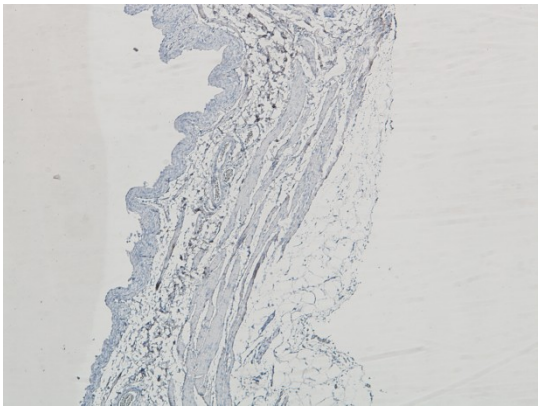
Table 2.

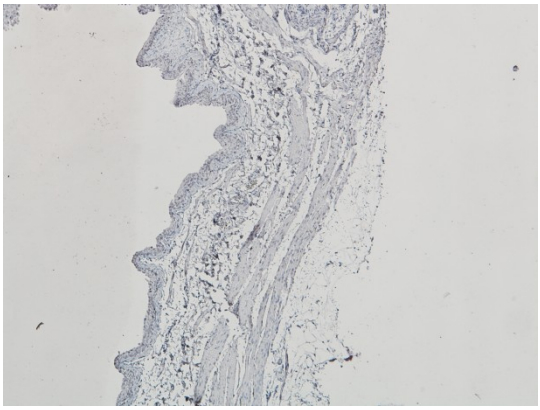
PRETREATMENT	**STAINING RESULTS**	
	UROTHELIUM	MUSCLE
SAL+SAL	++	+

SAL+CYP	(+)	(+)
L-NAME+SAL	+	(+)
L-NAME+CYP	(+)	(+)
DPCPX+SAL	+	(+)
DPCPX+CYP	+	(+)
PSB1115+SAL	+	(+)
PSB1115+CYP	(+)	(+)
4-DAMP+SAL	+(+)	+
4-DAMP+CYP	+	+
Suramin+ SAL	+	(+)
Suramin+CYP	(+)	(+)

- **L-NAME pre-treatment**

The results of immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by L-NAME as a non-selective eNOS inhibitor can be observed in the Fig.14. The $P1A_1$ receptors are mostly localized in the urothelium of the healthy tissue. The staining is barely appreciable in the CYP-induced cystitis tissue.





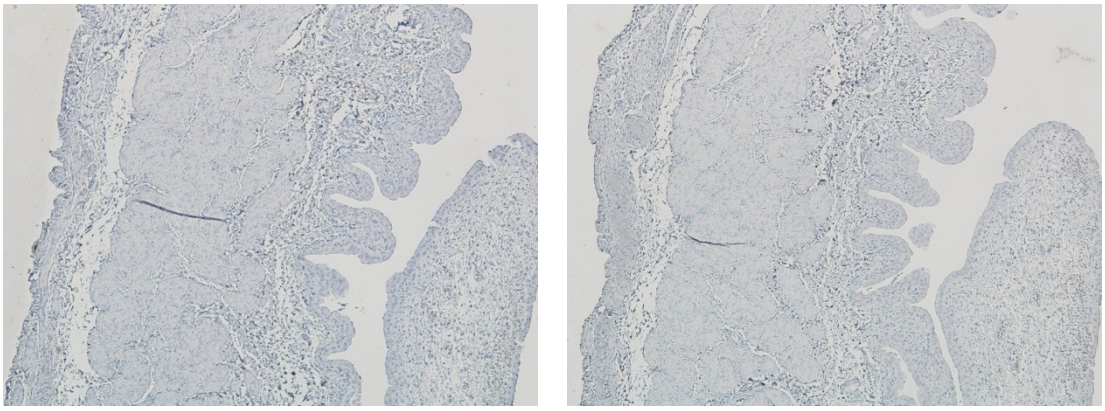
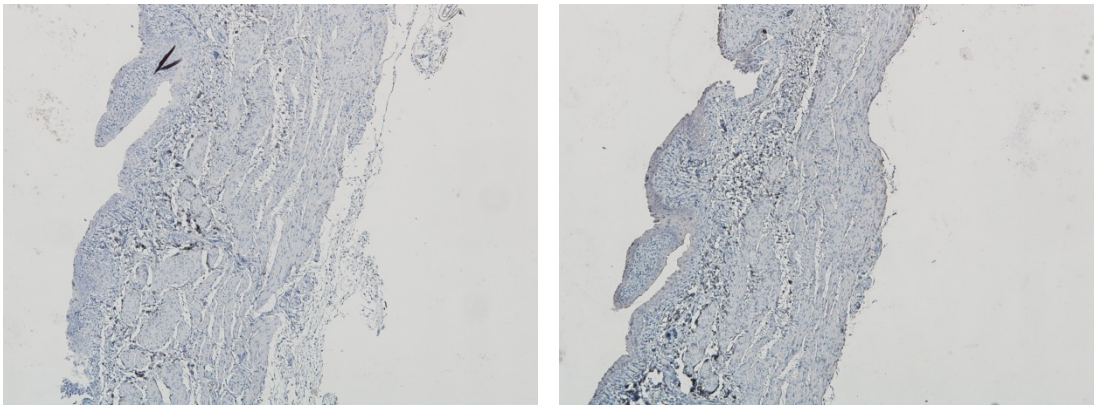


Fig.14 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by L-NAME (eNOS inhibitor). The upper row shows healthy tissue, the row below inflamed tissue. On the left are the negative controls, on the right side are the positive specimens. All sections were counterstained with Mayer's haematoxyline.

- **DPCPX pre-treatment**

Fig.15 shows results of immunohistochemical labelling of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by DPCPX as a $P1A_1$ receptor antagonist. The pictures on the right side show positive staining for the $P1A_1$ receptor. The number of $P1A_1$ receptors has decreasing tendency in the inflamed tissue. It follows the same staining pattern as the saline/CYP control group.



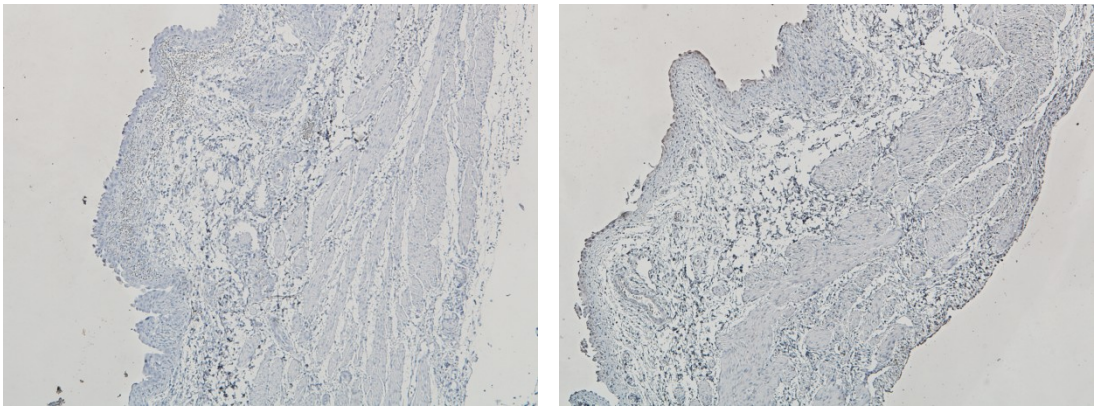


Fig.15 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by DPCPX ($P1A_1$ receptor antagonist). The upper row shows healthy tissue, while the row below inflamed tissue. On the left are the negative controls and the right side shows the staining for $P1A_1$ receptors. All sections were counterstained with Mayer's haematoxyline.

- **PSB1115 pre-treatment**

Fig.16 shows the IHC staining results of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by PSB1115 as a specific $P1A_{2B}$ receptor antagonist. Expression of $P1A_1$ receptor is decreased in both, healthy and inflamed bladders. The staining is not apparent.

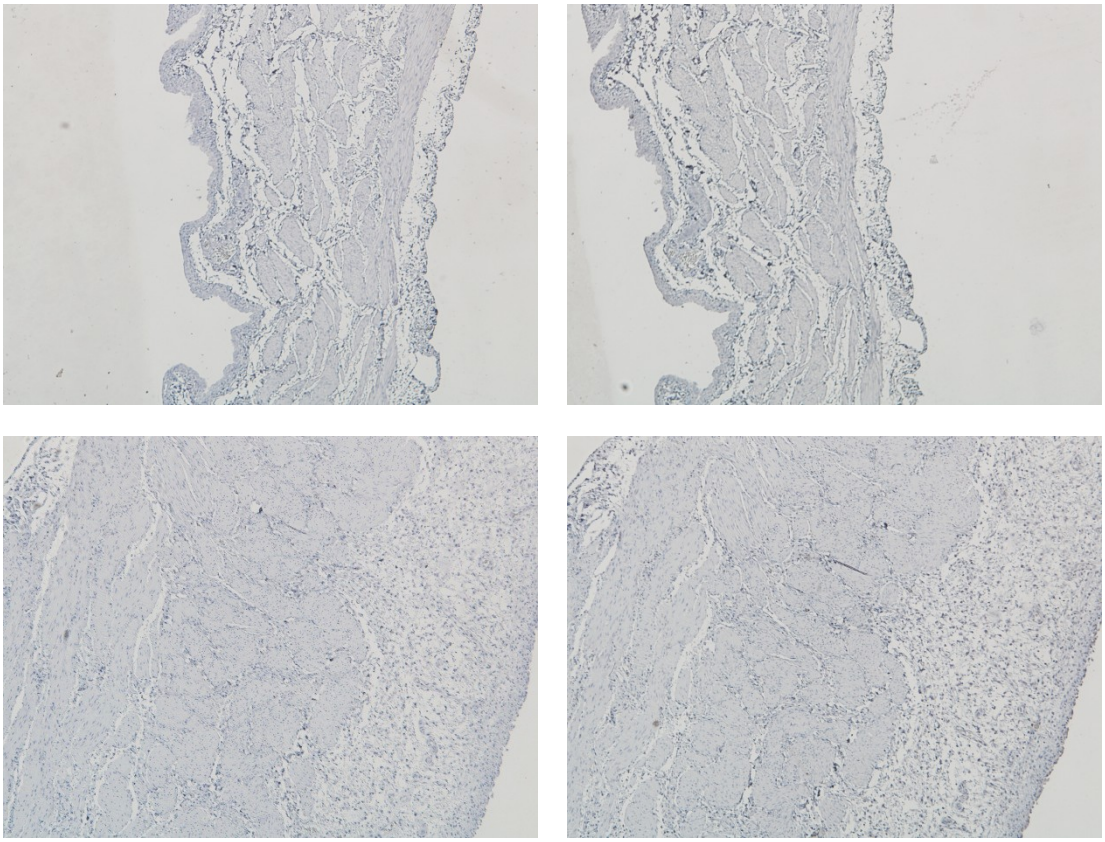


Fig.16 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by PSB1115 ($P1A_{2B}$ antagonist). The upper row shows healthy tissue, the lower row inflamed tissue. On the left are the negative controls, while on the right side are the positive specimens. All sections were counterstained with Mayer's haematoxyline.

- **4-DAMP pre-treatment**

The results of immunohistochemical labelling of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by 4-DAMP as a M_3, M_5 muscarinic receptor antagonist are demonstrated in the Fig.17. The same structures and staining pattern can be observed in the control group, although the staining in 4-DAMP pre-treated group is weaker, especially in the healthy urothelium.

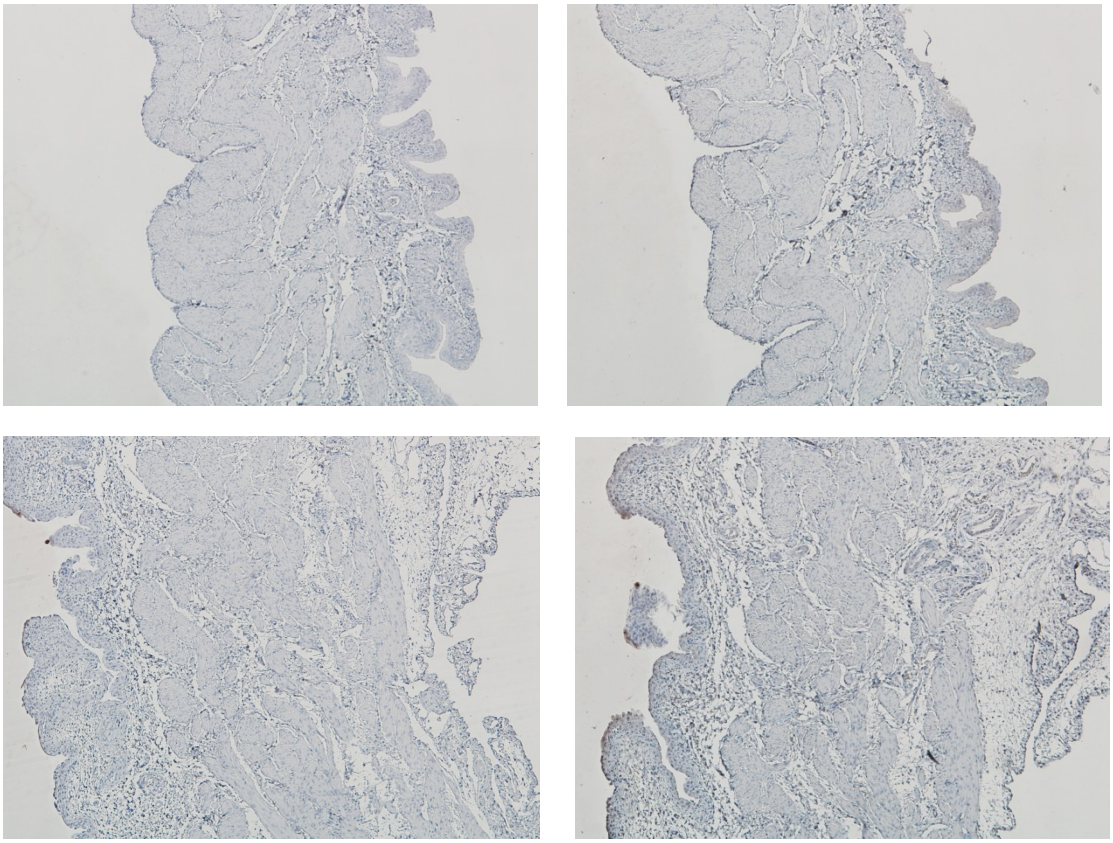


Fig.17 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by 4-DAMP (M_3, M_5 receptor antagonist). The upper row shows healthy tissue, the row below inflamed tissue. On the left are the negative controls and the right side shows the staining for $P1A_1$ receptors. All sections were counterstained with Mayer's haematoxyline.

- **Suramin pre-treatment**

Fig.18 shows the results of immunohistochemical labelling of the $P1A_1$ purinoceptors in the rat urinary bladder pre-treated by suramin as a nonspecific P2 purinoceptor antagonist. Similarly to the other IHC staining results, suramin did not affect $P1A_1$ receptor expression significantly.

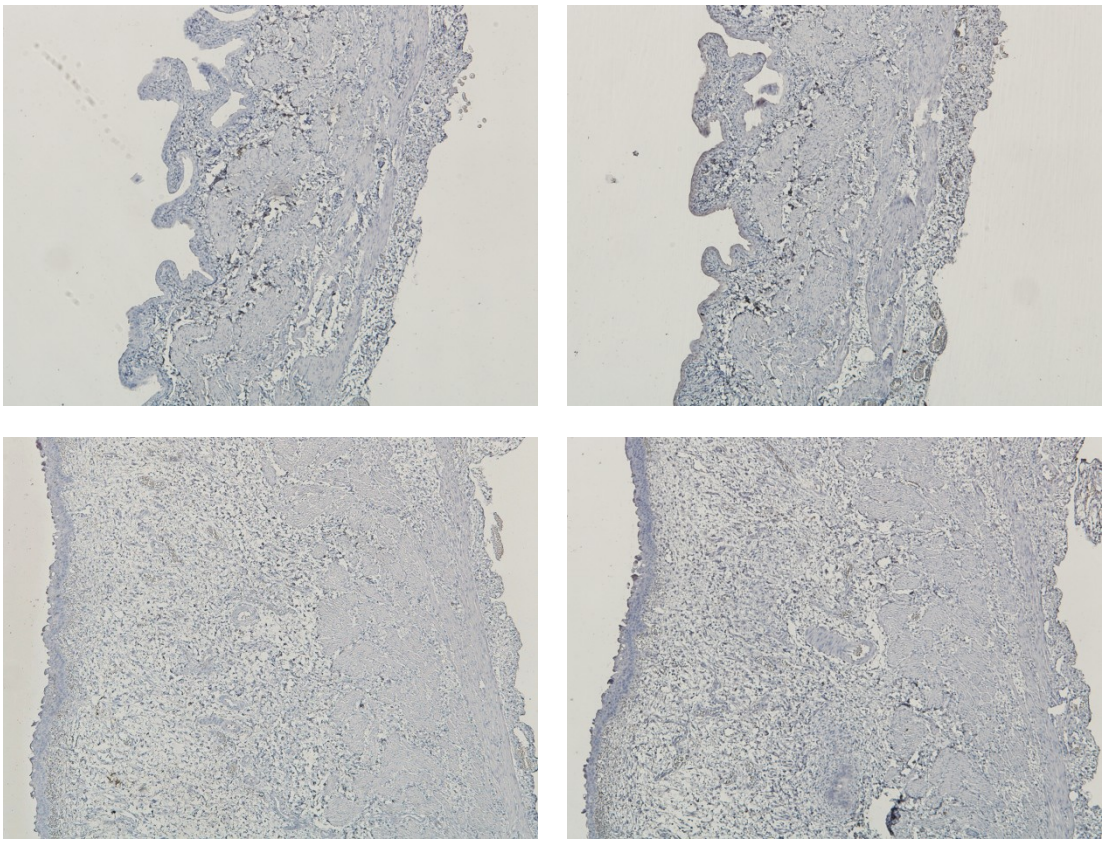


Fig.18 Immunohistochemical staining of the $P1A_1$ purinergic receptors in the rat urinary bladder pre-treated by suramin (P2 antagonist). The upper row shows healthy tissue, while the row below inflamed tissue. On the left are the negative controls, on the right are positive specimens. All sections were counterstained with Mayer's haematoxyline.

7.2.2 M_5 muscarinic receptor staining

7.2.2.1 Control group

The immunohistochemical staining for M_5 muscarinic receptor was successful and showed significant staining results. According to these results M_5 receptor expression is higher in normal (saline) tissue than in the CYP-induced cystitis tissue. Controversially IHC did not confirm organ bath findings reported previously which demonstrate correlation between M_5

receptors and NO releasing, resp. M_5 receptor indirect relaxation effect through nitric oxide. It implies an increased number of muscarinic M_5 receptors in inflamed tissue (Giglio 2006; Andersson 2010). However, the M_5 staining results done in this thesis did not follow this hypothesis.

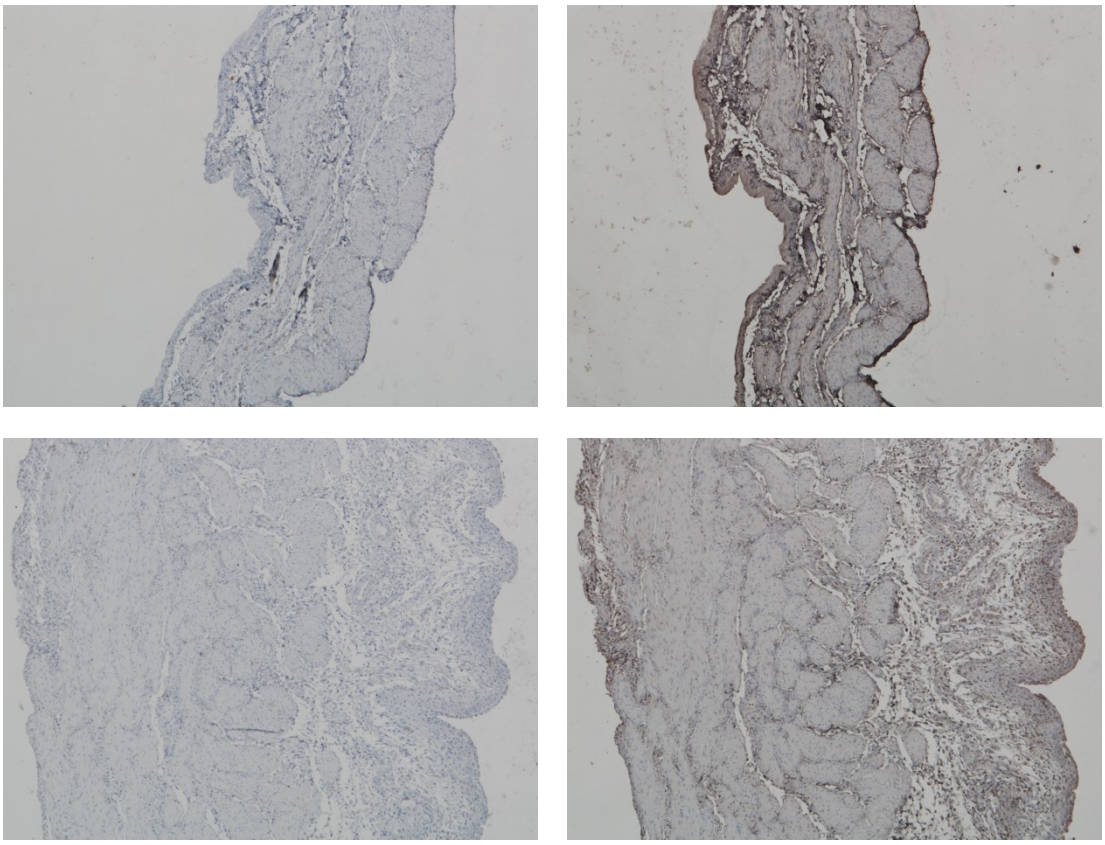


Fig.19 Immunohistochemical staining of the muscarinic M_5 receptor in the rat urinary bladder. The upper row shows healthy tissue (saline), while the row below CYP-induced cystitis tissue. On the left are the negative controls and the right side shows the staining for M_5 receptor. All sections were counterstained with Mayer's haematoxyline.

Fig.19 shows the results of IHC labelling of muscarinic M_5 receptor of the saline/CYP rat urinary bladder. The urothelium is more stained in the healthy tissue (an obvious brown line) than in the inflamed tissue (a weak brown line). The staining of the muscle is more or less equal.

7.2.2.2 Pre-treated groups

There are five pre-treated groups, which affected the immunohistochemical staining results. However, the staining of the pre-treated groups did not differ from the control group saline/CYP significantly.

Table 3.

PRETREATMENT	**STAINING RESULTS**	
	UROTHELIUM	MUSCLE
SAL+SAL	++(+)	+(+)
SAL+CYP	+(+)	+
L-NAME+SAL	++	+(+)
L-NAME+CYP	+	+(+)
DPCPX+SAL	++(+)	+(+)
DPCPX+CYP	+(+)	+
PSB1115+SAL	++(+)	+(+)
PSB1115+CYP	+(+)	+
4-DAMP+SAL	++	+
4-DAMP+CYP	+(+)	+(+)
Suramin+SAL	++	+(+)
Suramin+CYP	+	+

- **L-NAME pre-treatment**

The results of IHC staining of muscarinic M_5 receptor in the rat urinary bladder pre-treated by L-NAME shows Fig.20. The M_5 receptor expression is higher in the healthy tissue, especially in the healthy urothelium. Genarally the staining is weaker in comparison with the control group.

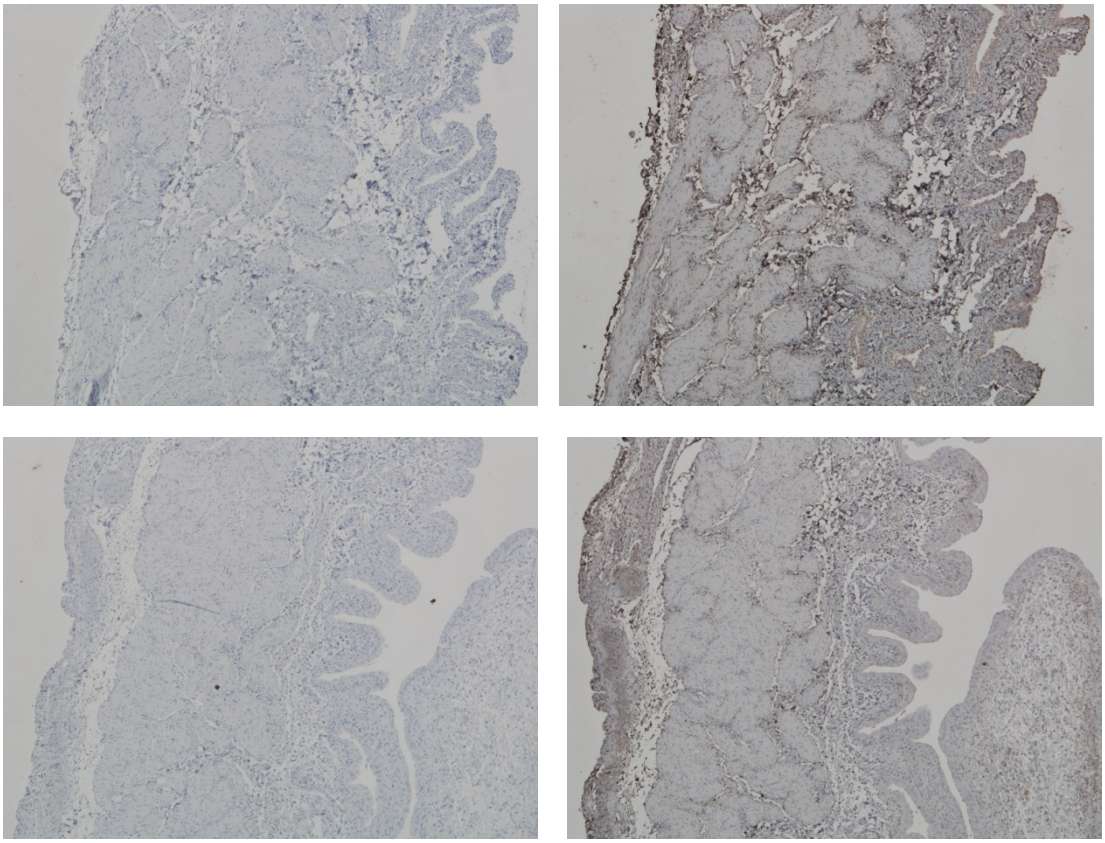


Fig.20 Immunohistochemical staining of the muscarinic M_5 receptor in the rat urinary bladder pre-treated by L-NAME (eNOS inhibitor). The upper row shows healthy tissue (saline), while the row below CYP induced cystitis tissue. On the left are the negative controls and the right side shows the staining for M_5 receptors. All sections were counterstained with Mayer's haematoxyline.

- **DPCPX pre-treatment**

Fig.21 shows IHC labelling of M_5 receptor in the rat urinary bladder pre-treated by DPCPX as a $P1A_1$ purinoceptor antagonist. The urothelium is significantly stained in the healthy tissue. The staining of the inflamed tissue does not show any obvious difference between the urothelium and the muscle. In comparison with the control group the pre-treatment affected the staining results especially of the inflamed tissue. There is no obvious M_5 receptor expression in the urothelium of the inflamed bladder.

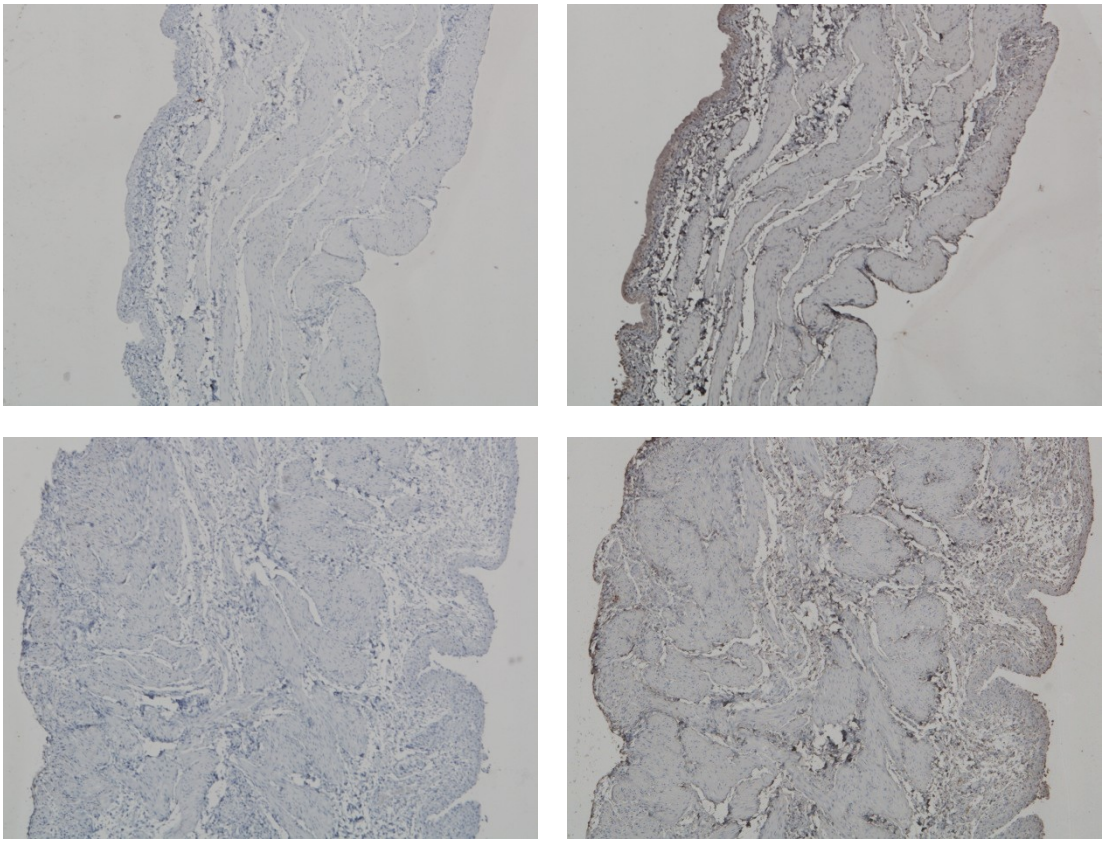


Fig.21 Immunohistochemical staining of the muscarinic M_5 receptor in the rat urinary bladder pre-treated by DPCPX ($P1A_1$ receptor antagonist). The upper row shows healthy tissue (saline), while the row below inflamed tissue. On the left are the negative controls and the right side shows the staining for M_5 receptor. All sections were counterstained with Mayer's haematoxyline.

- **PSB1115 pre-treatment**

The results of immunohistochemical staining of M_5 receptor in the rat urinary bladder pre-treated by PSB1115 as a $P1A_{2B}$ purinoceptor antagonist are presented in Fig.22. The muscarinic M_5 expression is significantly higher in healthy than in the inflamed urothelium. The pre-treatment did not affect the staining results significantly in comparison with the control group.

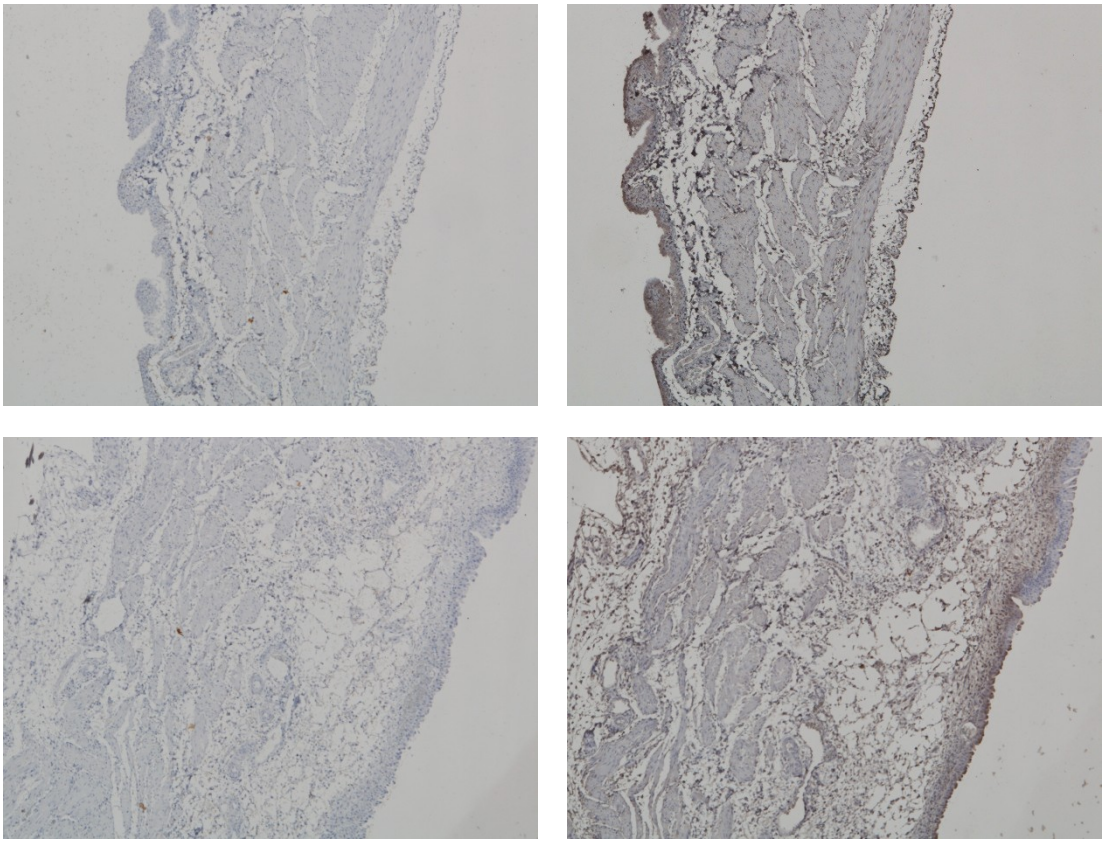


Fig.22 Immunohistochemical staining of the muscarinic M_5 receptor in the rat urinary bladder pre-treated by PSB1115 ($P1A_{2B}$ antagonist). The upper row shows healthy tissue (saline), while the row below inflamed tissue. On the left are the negative controls and the right side shows the staining for M_5 receptors. All sections were counterstained with Mayer's haematoxyline.

- **4-DAMP pre-treatment**

Fig.23 shows the immunohistochemical labelling of M_5 receptor in the rat urinary bladder pre-treated by 4-DAMP as a M_3, M_5 muscarinic receptor antagonist. The urothelium of the healthy tissue is more stained than the inflamed tissue. There is no significant difference between the muscle of healthy and inflamed tissue. 4-DAMP pre-treatment follows similar staining results as saline/CYP control group. Expression of M_5 receptor is strong in both, healthy and inflamed bladders as well.

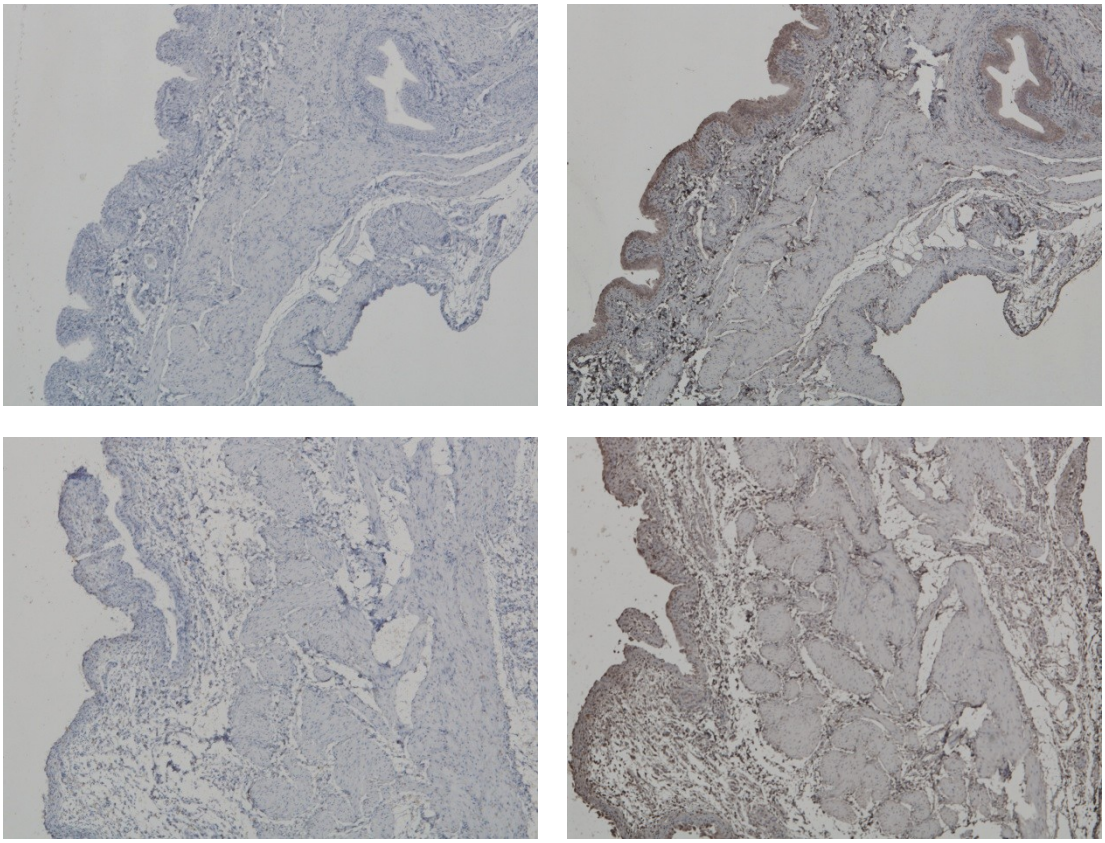


Fig.23 Immunohistochemical staining of the muscarinic M_5 receptors in the rat urinary bladder pre-treated by 4-DAMP (M_3, M_5 muscarinic antagonist). The upper row shows healthy tissue (saline), while the row below inflamed tissue. On the left are the negative controls, on the right are the positive specimens. All sections were counterstained with Mayer's haematoxyline.

- **Suramin pre-treatment**

Fig.24 shows the results of immunohistochemical labelling of M_5 receptor in the rat urinary bladder pre-treated by suramin as a non-selective P2 antagonist. The M_5 receptor expression is weaker in the healthy urothelium. The pre-treatment affected especially the normal bladder.

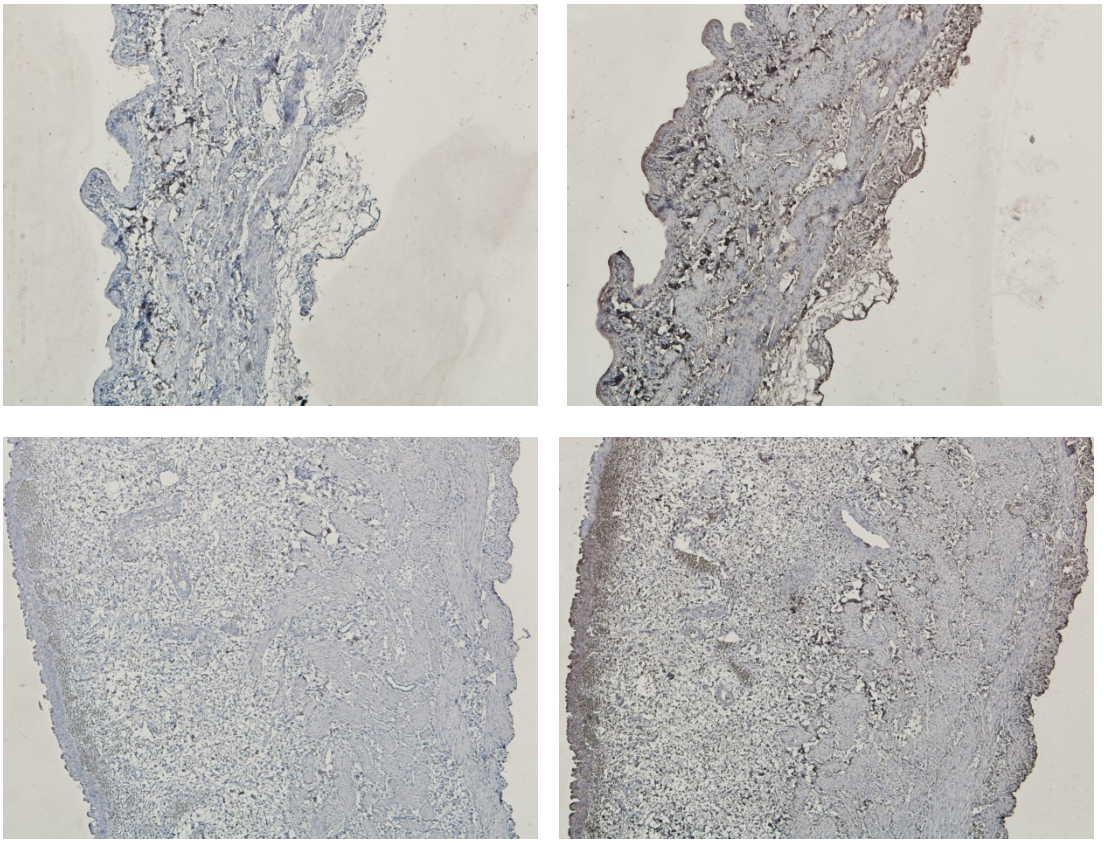


Fig.24 Immunohistochemical staining of the muscarinic M_5 receptor in the rat urinary bladder pre-treated by suramin (non-selective P2 antagonist). The upper row shows healthy tissue (saline), while the row below inflamed tissue. On the left are the negative controls and the right side shows the staining for M5 receptor. All sections were counterstained with Mayer's haematoxyline.

7.2.3 eNOS staining

Simultaneously immunohistochemical staining of eNOS was done. However, the procedure was not successful and the results were not significant. Therefore the findings are not included in this thesis.

8 Discussion

The present study is focused on cyclophosphamide-induced cystitis in the rat urinary bladder and specific pre-treatments altering the state of inflammation. The five pre-treated groups of antagonists affected muscarinic, purinergic and nitrergic mechanisms. In the presence of L-NAME as a non-selective inhibitor of eNOS and DPCPX as a purinergic $P1A_1$ receptor antagonist, altered effects were observed. Both, L-NAME and DPCPX pre-treatments, significantly reduced inflammation of the urinary bladder. The findings were

based on functional and morphological studies. Functional experiments measured contractile response to MeCh and ATP, which is significantly reduced in CYP-induced cystitis bladders. Pre-treatment by L-NAME and DPCPX normalized MeCh contractile response of inflamed tissue close to contractions of normal bladders and actually reversed contractile response to ATP in favour the inflamed tissue. To be exact, PSB1115 pre-treatment showed reversed contractile response to ATP as well but the results were less obvious and badly readable. 4-DAMP and suramin pre-treated groups did not affect functional examinations significantly.

These results evoke that nitric oxide and $P1A_1$ purinergic receptor are important factors of inflammation of the urinary bladder. It was found that NO releasing gets worse the cystitis. This hypothesis confirmed the functional studies since blocking of NO synthase reduced inflammation and normalized contractile response. According to the previous studies (Giglio 2006; Andersson 2010) was found a correlation between NO releasing and M_5 receptor up-regulation during cystitis. NO is being released upon activation of muscarinic M_5 receptors in the urothelium. However, morphological studies done in this thesis did not prove these findings.

The second pro-inflammatory factor, $P1A_1$ receptor, was observed. Adenosine evoked relaxation mainly depends on the activation of the $P1A_1$ purinoceptors. This finding confirmed normalized contractile response in the presence of DPCPX as a $P1A_1$ antagonist. However, IHC staining did not prove all the results. The labelling of $P1A_1$ receptors was particularly intense in the urothelium of healthy tissue but weak or absent in CYP-induced cystitis bladders.

For contractions of the urinary bladder is mainly responsible activation of the muscarinic receptors, namely M_2 and M_3 muscarinic receptors. M_3 receptors act in direct way causing contraction, M_2 receptors work indirectly opposing relaxation. Besides that purinergic NANC-system contributes and affects contractions of the urinary bladder. ATP principally causes contraction. Its breakdown product, adenosine, evokes a delayed relaxation response. According to the functional studies the specific pre-treatments did not affect ATP contractile response significantly. However, what is deserved to be noticed, were higher contractions in the inflamed bladders than in the healthy ones in the case of DPCPX and L-NAME.

Morphological studies were done by immunohistochemical staining on the three different antigens of tissue – M_5 muscarinic receptor, $P1A_1$ purinergic receptor and endothelial NO synthase. However, only the M_5 and $P1A_1$ staining results are presented in this thesis. eNOS

staining results were not really successful. The IHC staining results confirmed the previous findings (Vesela, Aronsson et al. 2011) which demonstrate down-regulation of $P1A_1$ receptors during cystitis. $P1A_1$ purinoceptors are widely distributed in the urothelium of normal bladders. Nevertheless, the staining is weak or absent in the inflamed ones. The pre-treatment did not affect $P1A_1$ staining results significantly. The $P1A_1$ immunohistochemical study revealed morphological correlates to the functional findings. Conversely M_5 receptor staining results did not confirm previous findings, resp. connection between NO releasing and M_5 receptor up-regulation in the inflamed tissue. The M_5 staining results done in this project showed higher number of M_5 receptors in the normal bladders than in the inflamed tissue, especially localized in the healthy urothelium. Anyway, to be honest, morphological studies belong to a complementary method and even though IHC procedure was done according to the exact schedule, we cannot rely on the staining results absolutely since the staining success is dependent on many factors. But that is the research.

9 Conclusion

The current study showed that muscarinic, purinergic and nitrergic mechanisms play an important role in inflammatory process of the rat urinary bladder. The thesis is focused on cyclophosphamide-induced cystitis and specific pre-treatments altering the state of inflammation. According to the functional and morphological studies, L-NAME as eNOS inhibitor and DPCPX as $P1A_1$ receptor antagonist, affected the state of inflammation, reduced cystitis and normalized contractile response. It implies that nitric oxide and $P1A_1$ purinoceptor are important pro-inflammatory factors. Morphological studies showed that expression of the P1A1 purinoceptor and M5 muscarinic receptor subtype are decreased during the cystitis.

10 Acknowledgements

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11 Abbreviations

4-DAMP, 4-diphenylacetoxy-N-methylpiperidine

ABC, Avidin-Biotin Complex

ATP, adenosine-5´-triphosphate

BTX-A, botulinum toxin A

BPH, benign prostatic hyperplasia

cAMP, cyclic adenosine monophosphate

CNS, central nervous system

CYP, cyclophosphamide

DMSO, dimethylsulfoxide

DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine

eNOS, endothelial nitric oxide synthase

GABA, gamma-aminobutyric acid

GDP, guanosindiphosphate

GTP, guanosintripohsphate

HPK, high potassium Krebs

IC, intersticial cystitis

IHC, immunohistochemistry

iNOS, inducible nitric oxide synthase

L-NAME, NG-Nitro-L-arginine methyl ester hydrochloride

LSAB, Labeled StreptAvidin Biotin

LUTS, lower urinary tract symptoms

MeCh, methacholine (acetyl-β-methylcholine)

NANC, non-adrenergic, non-cholinergic

NO, nitric oxide

nNOS, neuronal nitric oxide synthase

OAB, overactive bladder

PAP, peroxidise anti-peroxidise

PBS, phosphate buffered saline

PSB1115, 4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1H-purin-8-yl)-benzenesulfonic acid

SAL, saline

SUR, suramin

UI, urinary incontinence

TBS, Trizma base solution

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