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On purinoceptors in the urinary bladder of the rat: altered effects of purinergic $P1A_1$ receptors in cystitis

Purinové receptory močového měchýře potkana:
funkční změny purinergních $P1A_1$ receptorů při zánětu močového měchýře

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I declare that my diploma thesis is my original publication which I have written by myself. All used literature and other sources are named in References and cited properly.

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V Hradci Králové Renata Veselá

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Contents

Abbreviations

α,βme-ATP, α,β-methylene ATP

ATP, adenosinetriphosphate

BzATP, 2′,3′-O-(4-benzoyl-benzoyl) ATP

cAMP, cyclic adenosinemonophosphate

CCPA, 2-chloro-N 6-cyclopentyladenosine

CPA,N 6-cyclopentyladenosine

CPX, 8-cyclopentyl-1,3-dipropylxanthine

CNS, central nervous system

4-DAMP, 4-Diphenylacetoxy-N-methylpiperidine methiodide

DBX RM, 1,3-dibutylxanthine-1-riboside-5′-N-methylcarboxamide

DPCPX, 1,3-Dipropyl-8-cyclopentylxanthine

GDP, guanosindiphosphate

GTP, guanosintripohsphate

Ins(1,4,5)P3, inositol (1,4,5)-trisphosphate

IP5I, diinosine pentaphosphate

LUTS, lower urinary tract symptoms

NECA, N-ethylcarboxamidoadenosine

NO, nitric oxide

NANC, nonadrenergic, noncholinergic

OAB, overactive bladder

PPADS , Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate

2meSATP, 2-methylthioATP

RB2, Reactive blue 2

SUI, stress urinary incontinence

TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate

XAC, xanthine amine congener

Abstract

This project was focused on studies of the location and function of selected purinergic receptors in the rat urinary bladder. Its special concerns are on adenosine $P1A_1$ purinoceptors and on their expression in healthy and inflamed urinary bladders of the rat.

It was found that both $P2X_1$ and $P1A_1$ receptors are expressed in urinary bladder. The quantity of $P1A_1$ receptors is decreased in the inflamed bladder. Functional studies based on an agonist stimulation (adenosine) showed importance of $P1A_1$ receptors for relaxation of the tissue. During the cystitis this effect was reduced. Electric field stimulation (EFS) pointed out the difference between the response to a stimulus affecting just postsynaptic receptors (adenosine stimulation) and responses to nerve stimulation including both post- and presynaptic effects.

Abstrakt

Tato práce je zaměřena na určení výskytu a funkce vybraných purinergních receptorů v močovém měchýři potkana. Zabývá se zejména adenosinovými $P1A_1$ purinoceptory a jejich expresí ve zdravém a zaníceném močovém měchýři.

Studie ukázala, že jak $P2X_1$, tak $P1A_1$ purinoceptory jsou přítomny v močovém měchýři potkana. U $P1A_1$ receptorů je jejich exprese během zánětlivého onemocnění signifikantně snížena. Funkční studie založené na stimulaci agonistou (adenosinem) ukázaly na význam $P1A_1$ receptorů během relaxace studované tkáně. Během zánětu je tento vliv částečně potlačen. Stimulace vzorku elektrickým proudem (Electric field stimulation) pak poukázala na rozdíl ve funkční odpovědi receptorů stimulovaných jen postsynapticky (adenosinem) a stimulovaných post- a zároveň presynapticky.

“Overactive bladder (OAB) is a common, disabling condition associated with considerable negative impact on quality of life, quality of sleep, and mental health. The age-specific prevalence of OAB is similar among men and women. Urge incontinence affects only a portion of the OAB population: 33% of patients have OAB with urge incontinence ("OAB wet"), while 66% have OAB without urge incontinence ("OAB dry"). The symptoms of OAB can affect social, psychological, occupational, domestic, physical, and sexual aspects of life. OAB can also lead to depression and low self-esteem.” (Tubaro, 2004)

1 Introduction

Impulses in parasympathetic nervous system elicit the contractions of detrusor muscle of the urinary bladder which lead to voiding. However, it has been proved that the parasympathetic stimulation of the urinary bladder through the muscarinic receptors is not the only way of affecting it. There are also responses of the tissue that are resistant to atropine (Langley & Anderson, 1895). Consequently there must be other transmitters than sympathetic noradrenaline and parasympathetic acetylcholine affecting the urinary bladder. As one of these transmitters ATP was proposed (Burnstock *et al.*, 1970). It generates a contraction by acting on P2X receptors and this contraction is followed by a relaxation which is believed to be generated by adenosine, the ATP breakdown product, acting on the P1 (adenosine) receptors. The adenosine effect via P1 receptors is thus inhibition of contractile mechanisms (Giglio *et al.*, 2001).

During the inflammatory process in the urinary bladder the expressions of muscarinic and P2 receptors are increased. This higher appearance can cause a disordered state of urinary bladder, which is called an overactive urinary bladder. The imbalance in the vegetative innervation leads to several bladder dysfunctions, which the patient apprehends as uncomfortable, such as frequency of voidings caused by insufficient contraction and over-dilatation of the bladder or very strong and sharp pain, both the result from increased expression of the receptors. It is suggested that the P1 receptors are involved in this pathological process also.

2 Review of literature

2.1 Urinary bladder

2.1.1 Clinical aspects of the urinary bladder

The lower urinary tract is composed of the bladder and the urethra—the two functional units for storage (the bladder body, or reservoir) and elimination (the bladder neck and urethra, or outlet) of urine (Chancellor & Yoshimura, 2004).

The urinary bladder is a concave, muscular organ, which is located in pelvic cavity under the peritoneum and behind the symphysis pubic. The urinary bladder consists of three parts- the fundus (lower part), the corpus (middle part) and the apex (top). Its septum is composed of mucous membrane, submucousa and muscle. The ureters enter and the urethra drains the bladder in the fundus.

The function of the urinary bladder is to store the urine incoming from the kidneys and to expel the urine through the urethra out of the body. It works almost as a ball. Incoming urine increase the pressure in the bladder, the muscle fibres conform to a volume of urine and the bladder relaxes until the wall is stretched.

First urge to micturition in human body is experienced when the bladder is filled to about 150 ml, and a distinct urge when it is about 300-400 ml. Rejection of micturition is possible till the bladder is filled up to 700-750 ml.

2.1.2 Anatomy of the urinary bladder

The bladder is extraperitoneal and roughly pyramidal (Fig. 2.1). From the superior side it is covered by the pelvic peritoneum with parts of small intestine and the sigmoid loop lying against it. The bladder can be divided into two main components: the bladder body, which is located above the ureteral orifices, and the base, consisting of the trigone, urethrovesical junction, deep detrusor, and the anterior bladder wall. Anteriorly, the bladder lies behind the pubis with its apex attached by a fibrous strand, the median umbilical ligament, to the umbilicus. This represents the remains of the fetal urachus. Posteriorly in the male, the base of the

bladder relates to the rectum, the vasa deferentia and the seminal vesicles; in the female, to the vagina and the supravaginal cervix. It receives the ureters at its upper lateral angles. Laterally, the inferolateral surfaces relate to the levator ani and obturator internus muscles on each side. The bladder neck fuses with the male prostate; in the female it rests directly on the pelvic fascia.

As the bladder distends, during the filling with urine, it becomes spherical and projects into the lower abdomen, pushing the peritoneum upwards from the anterior abdominal wall. This may allow a safe extraperitoneal puncture of the distended bladder to be performed.

In the infant, the pelvis is comparatively small and therefore the bladder neck is in level with the upper part of the symphysis and the bladder itself, although still extraperitoneal, is in contact with the anterior abdominal wall.

The interior of the bladder is readily visualized through the cystoscope. The ureteric orifices are seen as a slit on either side. Between them, a raised fold of mucosa, the inter-ureteric ridge, is constantly visible, produced by an underlying bar of muscle. Between the ureteric orifices and the urethral orifice is the smooth triangular area of the trigone. The smooth muscle of the bladder wall is formed by a criss-cross of fibres. When these undergo hypertrophy as a result of chronic urethral obstruction, they produce the characteristic trabeculated appearance at cystoscopy. Blood is supplied from the superior and inferior vesical branches of the internal iliac artery. The vesical veins form a plexus which drains into the internal iliac vein. Lymphatics drain along the vesical blood vessels to the iliac and then the para-aortic nodes.

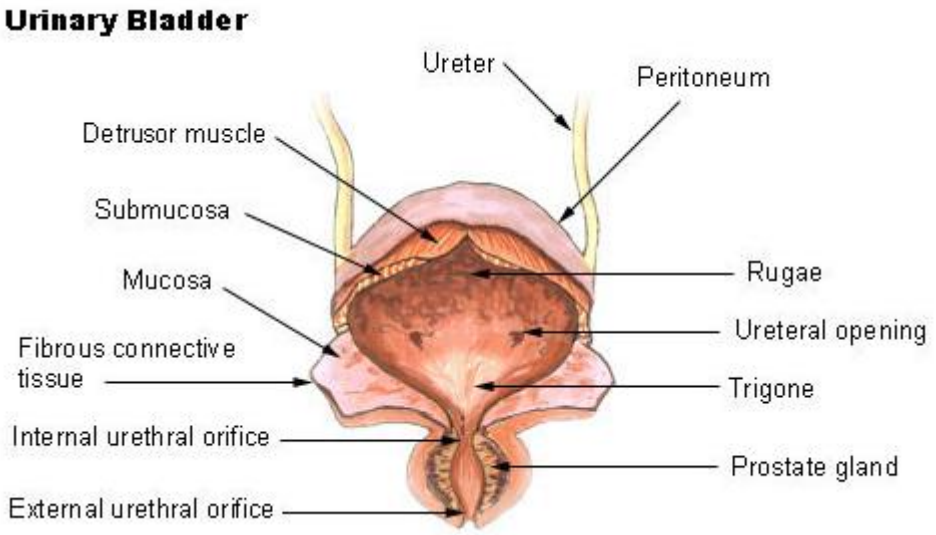


Fig. 2.1: Anatomy of human urinary bladder
(adapted from: http://www.daviddarling.info/images/urinary_bladder.jpg)

In males, the **urethra** is 18- 20 cm long and consists of three parts: i., prostatic – 3-4 cm long and it goes through the prostate; ii.,membranous – 2 cm long, the narrowest part of the urethra, traverses the external urethral sphincter; iii., spongy – 15 cm long and traverses the corpus spongiosum of the penis (Ellis, 2005). In females, the urethra is approximately only 4 cm long, extending from the anterior wall to the external meatus.

2.1.3 Histology of the bladder

The tissue layers of the whole of the lower urinary tract, thus including the renal pelvic and the urethers, are the same. The layers are mucosa, muscularis and serosa/ adventina (Fig. 2.2, Fig. 2.3).

A) Histology of mucosa (urothelium): The mucosa is the innermost portion of the urinary bladder. It is composed of transitional epithelium and connective tissue. The transitional epithelium, also called the urothelium, is a stratified epithelium lining the distensible walls of the urinary tract. The name "transitional" derives from this tissue's ability to change its shape from cuboidal to squamous when stretched. It is observed that the transitional epithelium consists of domed shaped cells on the

apical surface. The epithelial layer contains no blood vessels or lymphatic vessels. The basement membrane is composed of a single layer of cells and separates the epithelium and the connective tissue. The epithelium located on the connective tissue is called the lamina propria. The lamina propria is composed of areolar connective tissue and it contains blood vessels, nerves and in some regions even glands.

B) Histology of muscularis (smooth muscle- detrusor): The bladder muscular wall is formed by smooth muscle cells, which comprise the detrusor muscle. The detrusor is structurally and functionally different from, e.g., trigonal and urethral smooth muscle. Three layers of smooth muscle have been described. The cells of the outer and inner layers tend to be oriented longitudinally, and those of the middle layer circularly. In the human detrusor, bundles of muscle cells of various sizes are surrounded by connective tissue rich in collagen. Cells with long dendritic processes can be found in parallel to the smooth muscle fibers. These cells contain vimentin, an intermediate filament protein expressed by cells of mesenchymal origin, and of nonmuscle myosin (Andersson & Arner, 2004).

C) Histology of serosa: There is a serosal covering on the upper region of the bladder. On a histology slide, it is apparent that the serosa is composed of a simple squamous epithelium overlying a small bit of connective tissue. The serosa is part of the peritoneum. In all other regions of the bladder, the outer layer of the bladder is formed by the adventitia. The adventitia is composed of connective tissue. Beyond the serosa/adventitia covering of the bladder perivesical fat occurs, which surrounds the bladder.

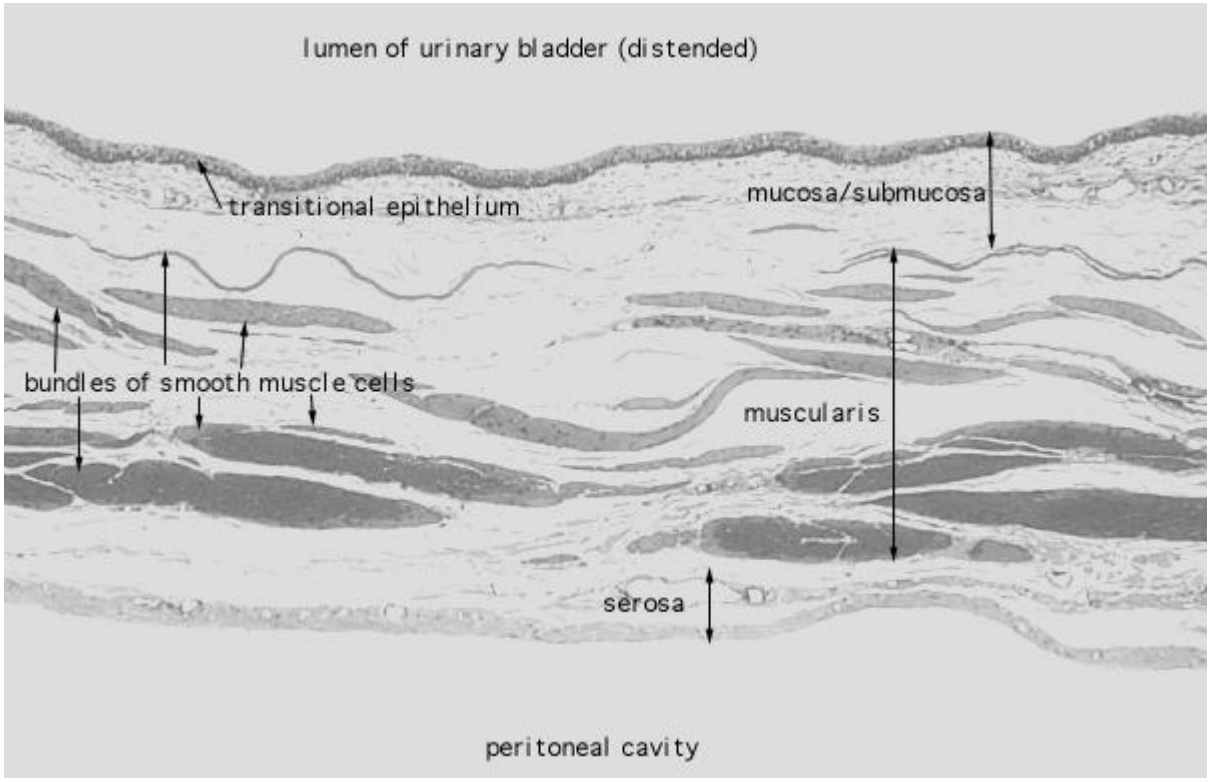


Fig. 2.2: Histology of distended urinary bladder
(adapted from: http://www.bu.edu/histology/p/16604loa.htm)

A muscularis mucosae is present in all regions of the human urinary bladder. This tissue is discontinuous and it consists of irregularly-arranged muscle bundles composed of relatively small smooth muscle cells. These cells are different morphologically and histochemically from the detrusor muscle cells from which they originate. They are rich in non-specific cholinesterase and glycogen. The muscularis mucosae also contain acetylcholinesterase-positive nerve fibres. The constituent smooth muscle cells contain an extensive sarcoplasmatic reticulum and large peripheral clusters of dense glycogen granules. The myofilaments are concentrated in the center of the cells. Between adjoining cells, many intercellular junctions occur where presumptive cholinergic nerve terminals, which lie close to the muscle cell surface and contain small agranular and large granulated vesicles (Dixon & Gosling, 1983).

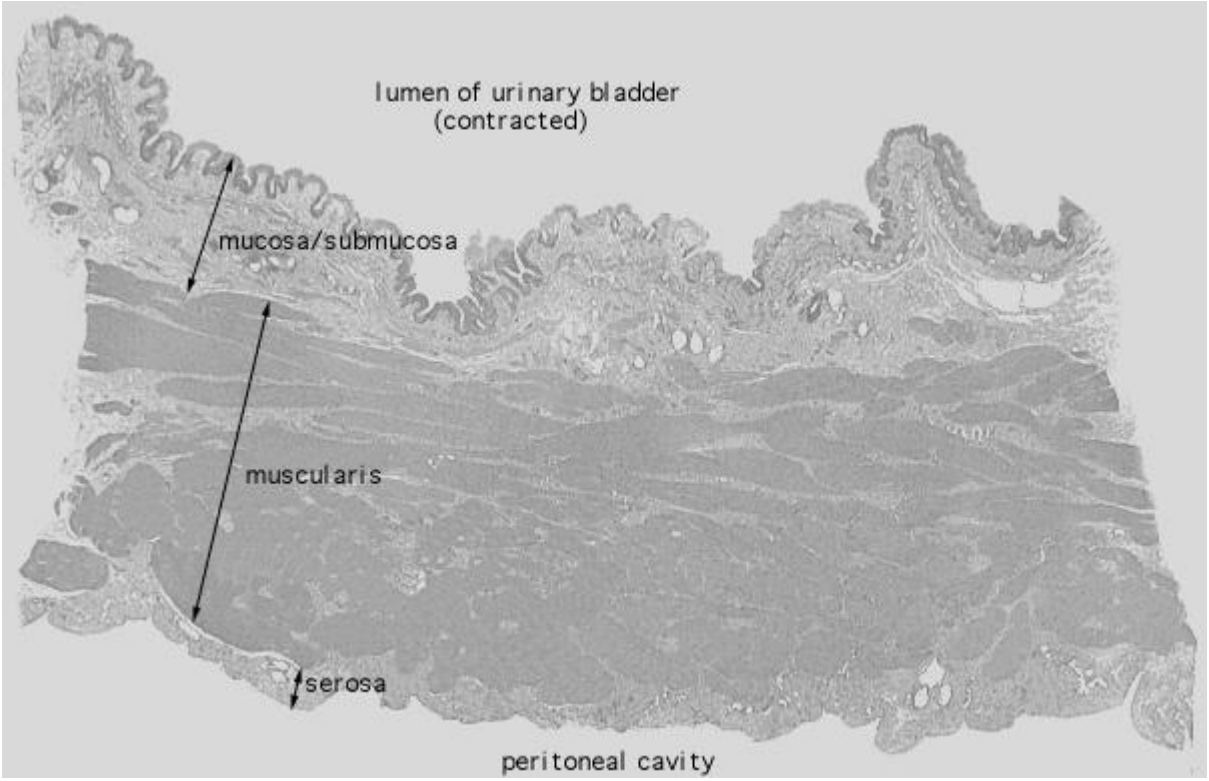


Fig. 2.3: Histology of contracted urinary bladder
(adapted from http://www.bu.edu/histology/p/16501lca.htm)

2.2 Incontinence

Incontinence is one of the most common chronic diseases in women and it is found in 17-60% of the whole population (Starczewski *et al.*, 2008). Continence and micturition involve a balance between urethral closure and detrusor muscle activity. Urethral pressure is normally higher than the bladder pressure, which maintains continence. Both the proximal urethra and the bladder are located in the pelvis. Intraabdominal pressure increases (from coughing and sneezing) are equally transmited to both the urethra and the bladder. However the pressure difference may be changed towards a larger bladder pressure, which thus may result in incontinence. Normal voiding is the result of changes in both of these pressure factors: urethral pressure falls and bladder pressure rises. Incontinence is a significant health issue in that it has physical, social and economic implications for women and men of all ages as well as children, families and the community.

Types of incontinence:

1. Stress urinary incontinence (SUI): involves involuntary leakage of urine in response to abdominal pressure caused by activities such as sneezing and coughing (Chancellor & Yoshimura, 2004). Also physical changes resulting from pregnancy, childbirth and menopause often cause stress incontinence. In men this type of incontinence often follow the prostatectomy. It is the most common form of incontinence in women and it is often treatable.

2. Urge incontinence: is an involuntary loss of urine occurring for no apparent reason often combined with a sudden feeling of the need to urinate. The most common cause of urge incontinence is involuntary and inappropriate detrusor muscle contraction.

Idiopathic detrusor overactivity: local or surrounding infection, inflammation or irritation of the bladder.

Neurogenic detrusor overactivity: defective CNS inhibitory response. The bladder is described to be unstable, plastic or overactive. Urge incontinence may also be called reflex incontinence if it is the result from overactive nerves controlling the bladder. Patients with this type of incontinence may suffer during sleep, after drinking small amounts of water or even when hearing it running (for example during washing dishes or if somebody else is taking a shower). The reasons for the involuntary actions of bladder muscles can be damages of the nerves to the bladder, of the central nervous system (spinal cord and brain) or the muscles themselves. Bladder nerves or muscles can be harmed by diseases such as Parkinson´s, Alzheimer´s, Sclerosis Multiplex, stroke or injury including the ones which can occur after surgery.

3. Functional incontinence: it occurs when a person does not recognize the need to go to the toilet in time. The urine loss may be large. The reasons of functional incontinence include confusion, dementia, poor eyesight, poor mobility, poor dexterity or unwillingness to toilet because of depression, anxiety or anger. There are also environmental factors, which may contribute to this type of incontinence, such as poor lighting, low chairs that are difficult to get out of, and toilets that are difficult to access (Australian Government, 2008).

4. Overflow incontinence: is the type of incontinence where the bladder becomes too full because it cannot be fully emptied (Starczewski *et al.*, 2008). It can occur either because the outflow of the urine is blocked or because the bladder becomes underactive. The blockage can be caused by an enlarged prostatic gland, constipation or narrowing of the urethra. Symptoms may include frequency, urgency, nocturia, in-completed bladder emptying or frequent urinary tract infections. An underactive bladder may be the result from spinal injuries, a complications of diabetes, sclerosis multiplex or tumors.

5. Bedwetting: is an episodic urinary incontinence while asleep. It is normal in young children. But it can also be connected to sleep-disordered breathing such obstructive sleep apnea (Umlauf & Chasens, 2003).

6. Other types of incontinence: Are mainly considered as a "mixed incontinence". Quite often a combination of stress and urge incontinence occurs in women but may also occur in elderly patients irrespective of sex.

2.3 Overactive bladder (OAB) and other LUTS

A symptom complex with urgency, with or without incontinence, and usually accompanied by urinary frequency and nocturia has been designated "overactive bladder" (OAB) (Michel & Barendrecht, 2008). OAB is a symptom, which is very common, especially for the elderly. The prevalence increases with the increasing age (it occures in 17% older than 18 years, at the age of 75 years: 31% women, 42% men) (Mašata, 2007).

The definition of overactive bladder according to International Continence Society (ICS) is a complex of symptoms of a dysfunction of the low urinary tract. The typical symptom of OAB is urgency, which can be connected with incontinence. Other symptoms are frequency and nocturia. The OAB has a huge negative impact on quality of life of the patients.

The International Continence Society (ICS) has been trying since 1976 to uniform the terminology regarding function and dysfunction of the lower urinary

tract. Last Report from the Standardization Sub-committee was published in 2002 and this report can be considered as a golden standard. This thesis follows by this Report standardized lower urinary tract symptoms (LUTS) and other symptoms which are related to this issue of classification and description of the symptoms in very detail.

LUTS – symptoms as subjective indicator of disease or as a change of state noticed by patient, career or partner. LUTS is devided into three main groups:

1. storage of the symptoms
2. voiding symptoms – symptoms during voiding
3. post micturition symptoms,

There are four additional categories of classification:

i. symptoms associated with the sexual intercourse
ii. symptoms associated with pelvic organ prolapse
iii. with genital and lower urinary tract pain
iv. symptom syndromes.

The LUTS are mainly defined according the individual (mainly patients) perspective (Homma, 2008).

Urgency – The report defines urgency as a complain to sudden invincible feeling for micturition which is difficult to overcome. Urgency is then "strong desire to void with an abrupt onset". Healthy people would have similar feeling to void when they would not be able to go to the bathroom for a very long time.

Frequency – The report defines "increased daytime frequency" as a complain of patient who thinks he voids very often during the day. This is also called pollakisuria. **Nocturia (nycturia)** is trouble, when the patient has to get up once or more during the night to go to the bathroom to void. In this context the night is defined as a space of time between when the patient goes to bed until he wakes up in the morning.

"The increased daytime frequency" is the complain of the patient and that is why a voiding five times a day is considered as a symptom when the patient feels that way, while the voiding fifteen times a day is not considered as a symptom when the patient does not mind it is too often. Contrary to this nocturia is a symptom even when there is just one occasion per night when the patient goes to the toilet.

The definition can be then explained in other words that voiding before going to the bed or voiding early in the morning which hinder the patient to fall asleep again are not considered as the nocturia. Also the waking up during the night by other reason than the feeling for voiding (for example noise) but followed by a toilet visit, is not considered as nocturia.

These voidings which occur during the night, but not defined as a nocturia, are called "the nighttime frequency". Consequently nocturia does not "cover" either voiding between going to the bed and falling asleep nor waking up and getting out of the bed (Homma, 2008).

The principal symptom of OAB is the urgency. Contrary to the physiological need to mictate, the urgency comes suddenly and forces the patient to an immediate emptying. The time between the first feelings for micturition to the eventual outflow of the urine is usually getting shorter, the frequency is rising and the volume of each voiding is lowering.

2.4 Neurophysiology of micturition

Both central (supraspinal and spinal) and peripheral (pelvic parasympathetic, lumbar sympathetic and somatic pudendal nerve) pathways of the nervous system are activated regulating the lower urinary tract function. The center for micturition (pons and periaqueductal grey matter of midbrain) is organizing the micturition operational reflex functions between the higher centers of the brain and the urinary tract (Kennelly & Devoe, 2008). The basal ganglia, cerebellum, cerebral cortex, thalamus, and hypothalamus have influence on the micturition center.

The peripheral innervation for the bladder involves the sympathetic, parasympathetic, and also somatic nerves. The bladder receives the sympathetic innervation via the hypogastric nerve and parasympathetic innervation mainly by the pelvic nerve. The detrusor muscle relaxation and bladder neck contraction is mediated by activation of sympathetic verves from thoracolumbal spinal cord T12-L2 segments. This results in gradual distention of the bladder without any pronounced increase in bladder pressure. When threshold volume is reached, afferents are activated and via the central nervous system sympathetic impulses are

inhibited and parasympathetic activated. By activation of the parasympathetic nerves from sacral cord S2-S4 segments, detrusor is stimulated to contract and this promotes voiding. Axons of Onuf´s nucleus (an area in the sacral spinal cord involved in the maintenance of urinary and defecatory continence, as well as a muscular contraction, *e.g.*, during an orgasm) contains motor neurons, and is also the origin of the pudendal nerve in the sacral cord segment (S2-S4). The external urethral sphincter is innervated by the pudendal nerve.

The voluntary voiding is normally achieved by voluntary relaxation of the external urethral sphincter, allowing release of urine (Blaivas, 1982).

During the filling of the urinary bladder, the smooth muscle cells have to relax and to elongate the wall over a very large length interval. During micturition, the force generation and shortening must be initiated comparatively fast and to occur over a large length range. These activities thus require both regulation of contraction and regulation of relaxation. To respond to the nervous and hormonal control systems, each part of the urinary tract muscles has to have specific receptors for the transmitters/modulators, released from nerves, endocrine glands or generated locally, and a distinct receptor coupling to the associated cellular pathways for initiating contraction and relaxation (Andersson & Arner, 2004).

2.5 Neural control

2.5.1 Receptors

Neurotransmitter needs a reaction partner to bind to so it could induce an effect. These transmitters binding partners are the receptor proteins, also called receptors.

Receptors have got two main properties:

-they have a specific binding area, which is able to connect to just a specific transmitter

-after the transmitter is bound, the receptor protein changes its conformation, resulting in a functional state.

2.5.2 Types of the receptors

Ligand gated ion channel

One example of ligand gated channels (Fig. 2.4) is the nicotine-acetylcholine receptors situated in the nerve-muscle plate belong to this group. The receptor is composed of four glycoprotein subunits (α, β, γ, δ) of molecular mass 40-60 kDa. It is inserted in the membrane and there is a trans-membrane channel going through the center of these five member group of molecules. Every subunit is composed of a protein fibre constituting an α- helix. Two of five subunits are identical and each of them carries one binding spot for acetylcholine. The general name for the receptor binding substance is "ligand".

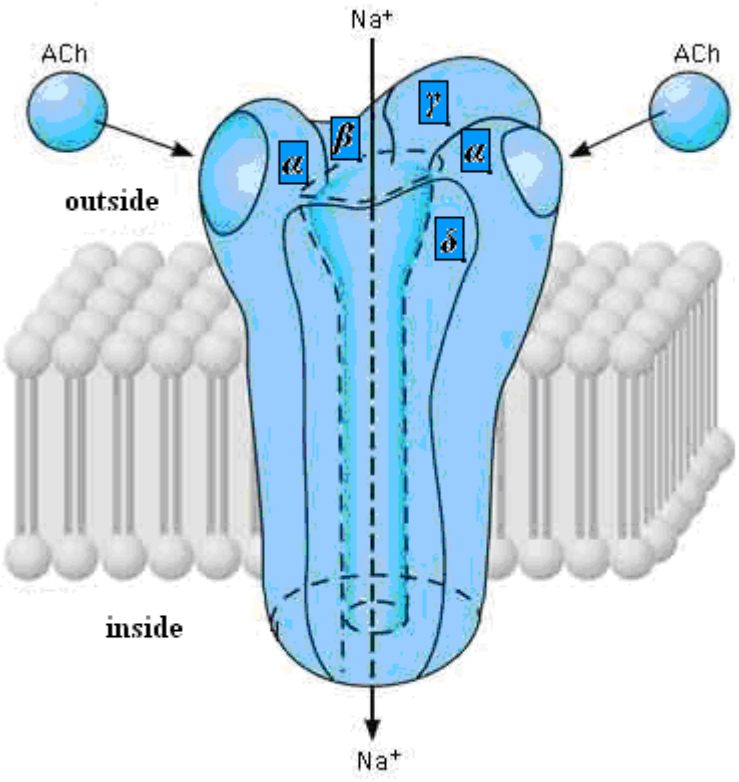


Fig. 2.4: Ligand gated ion channel *(adapted from: http://www-hsc.usc.edu/~bolger/ced/autonomic/images/N1-Nicotinic.jpg)*

When released from the neuronal terminal of the motoric nerve, two of the acetylcholine molecules occupy the binding spots of the receptor and open the channel. The influx of sodium is functionally most important. It causes a depolarization of nerve-muscle plate and the depolarization is then spread.

The acetylcholine molecule does not bind to the receptor for very long. It is in short loosened and it meets an acetylcholinesterase and is by the enzyme dissociated. This whole machinery takes just few milliseconds. This is how a fast skeletal muscle movement is regulated.

G proteins coupled receptors

Many receptors of the human body belong to the G protein coupled group of receptors (Fig.2.5). The examples of receptors belonging to this group are numerous and include muscarinic receptors, adrenoceptors and histamine receptors.

The typical receptor protein of this group is a peptidic chain of ca 500 amino-acids having a molecule mass of approximately 60 kDa. It is an α- helix which transmigrate through the membrane seven times. It does seem as it is organized in a circle and somehow it creates a kind of cavern – a perfect place for transmitter binding.

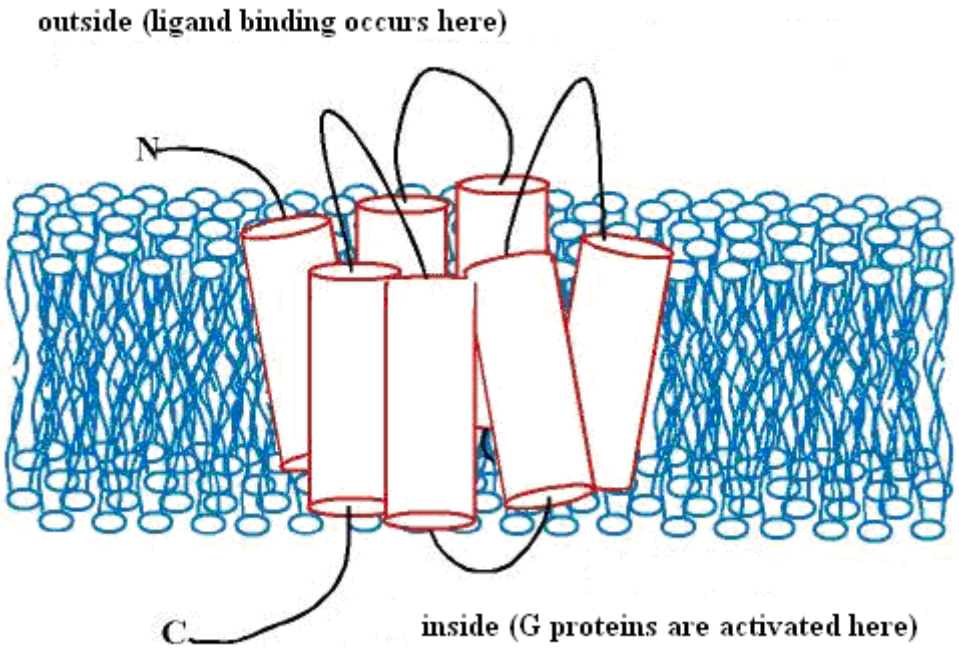


Fig. 2.5: G protein coupled receptor
(adapted from: http://structbio.vanderbilt.edu/sanders/Gpcr.jpg)

Signal transduction (Fig. 2.6) is provided by a guanyl-nucleotide – the G protein. This protein is located at the inner side of the membrane and it is able to move laterally. The G protein is composed of three subunits α (5kDa), β (35kDa) and γ (7kDa). During a state of resting, a molecule of guanosindiphosphate (GDP) is bound to the α subunit.

When the transmitter connects to the receptor, GDP is released from the α subunit and the α subunit is then free to bind to the guanosintripohsphate (GTP) molecule and move away of the other units of the G protein. By that it can reach the "effector proteins" and change their functional state.

Another important effector protein is an adenylatecyclas. It catalyses a production of cyclic adenosinemonophosphate (cAMP), which is an intracellular messenger.

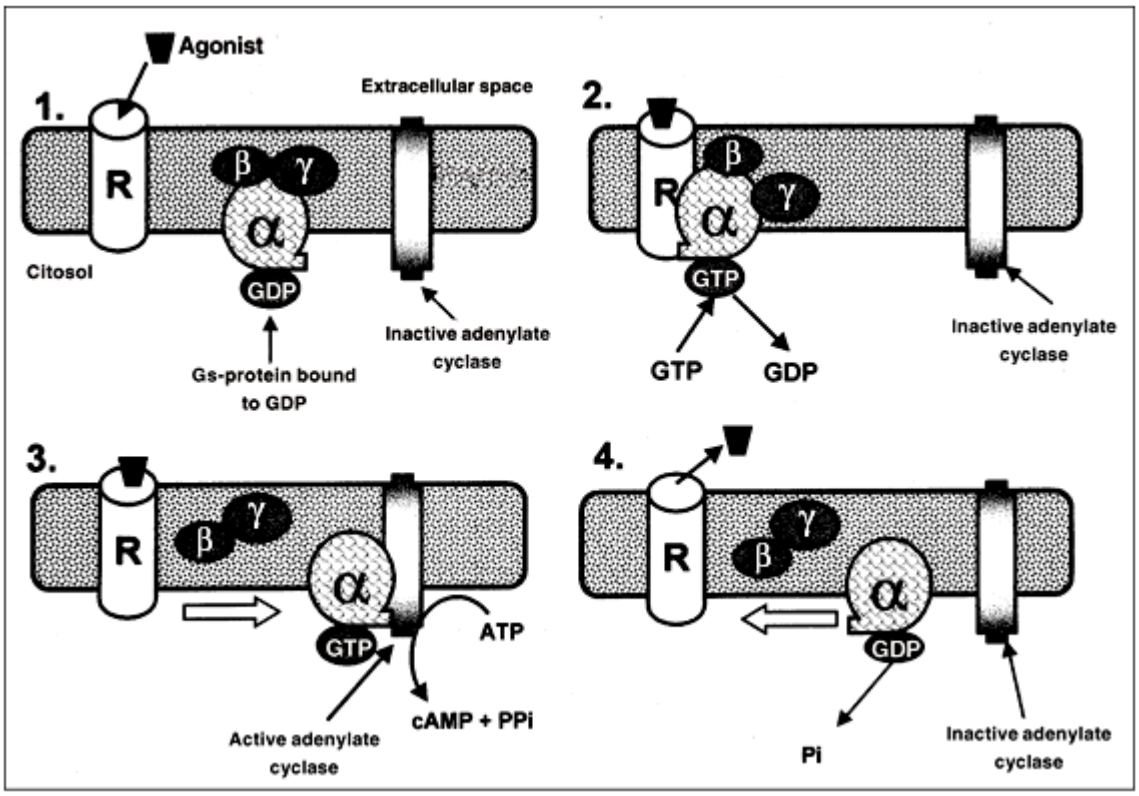


Fig. 2.6: Signal transduction
(adapted from: http://www.scielo.br/img/fbpe/abc/v72n2/a09fig01.gif)

Besides the receptor groups mentioned above, other receptor types occur. These groups generally regulate long-term effects and exert their effect after a longer latency. Examples of effects that can exerted **are enzyme activity** and **DNA transcription** (Lüllmann *et al.*, 2003).

2.6 Urinary bladder and its receptors

2.6.1 Cholinergic Mechanisms

2.6.1.1 Muscarinic receptors

In most animal species, bladder contraction is mediated by both cholinergic and nonadrenergic, noncholinergic (NANC) mechanisms. However, the cholinergic response seems to be the functionally most important in more or less all species. It has been showed that acetylcholine produces a slight depolarization and causes a contraction of the muscle. These contractions are extended by cholinesterase inhibitors and abolished by atropine. Muscarinic receptors (M_1 – M_5) mediate metabotropic activity of acetylcholine in nerve systems. An overwhelming amount of data shows that they also interpose autocrine function of the molecule (Eglen, 2006).

The detrusor smooth muscle contains muscarinic receptors mainly of the M_2 and M_3 subtypes but it has been observed that mRNAs for all muscarinic receptor subtypes occur in the tissue. Muscarinic receptors are G protein coupled as mentioned previously and regarding the contraction, this is their functional connection. But the signal transduction varies according to other effects in the bladder.

In the human bladder the muscarinic receptors are believed to cause a direct smooth muscle contraction through phosphoinositide hydrolysis (Andersson *et al.*, 1991). Bladder smooth muscle might have a difference in the localization of muscarinic receptors, in that junctional receptors may be M_2 receptors, and while extrajunctional receptors are M_3 receptors (Hashitani *et al.*, 2000). Nevertheless, there is general agreement that M_3 receptors are mainly responsible for the normal micturition contraction (Andersson & Arner, 2004).

The contraction of the detrusor muscle of the bladder is thus caused by activation of M_3 muscarinic receptors. But there are actually more of the M_2 receptors in the urinary bladder but the physiological function is not properly known yet. One of the suggestions is that it oppose the relaxant stimuli of the β-

adrenoceptors which is supposed to be important for the physiological way of the micturition. But some functional studies also showed M_2 receptors have direct and small contractile effects on the urinary bladder.

2.6.1.2 Therapeutic use of drugs acting on M receptors

The problem with drugs acting on muscarinic receptors has been the frequent occurrence of adverse effects, since muscarinic receptors regulate a number of mechanisms in almost every part of the body. And even though cholinergically acting drugs with different selectivity for the muscarinic subtypes exist, the selectivity window is too small to be clinically useful. However, different approaches have been tried in order to overcome the problem. According to cognitive disorders, selective M_1 agonists or combination of M_1 agonists and M_2 antagonists have been suggested to be one of the possible ways how to cure the disorders, and M_3 antagonists or M_2/M_3 antagonists are accepted for treatment of contractile diseases including overactive bladder and chronic obstructive pulmonary disease. Practical data shows selective antagonists for M_4 receptors will act as a new anti-nociceptive agens. Therapeutic effective antagonists and agonists on M_5 receptors are currently missing (Eglen, 2006).

2.6.2 Adrenergic Mechanisms

2.6.2.1 α-Adrenoceptors

There is a big question about the importance of the adrenergic receptors according to the fact there is just few of them in the bladder detrusor. Drugs selectively stimulating α-adrenoceptors produce a small and variable contractile effect. It has been found that only $\alpha1_A$ and $\alpha1_D$ mRNAs are expressed in the human urinary bladder (Malloy *et al.*, 1998).

2.6.2.2 β-Adrenoceptors

Noradrenaline is released by electrical stimulation of the adrenergic nerves in the detrusor tissue. Because β-ARs have been shown to predominate over α-ARs, the response of the normal detrusor to noradrenaline is relaxation. Also, several investigators have been able to demonstrate that the human detrusor is able to express β_1-, β_2-, as well as β_3-ARs (Andersson & Arner, 2004).

2.6.3 NANC Mechanisms

Muscarinic and adrenergic agonists are not the only ones which affect contraction or relaxation of the urinary bladder. In the last group of stimuli, which cause a bladder response and which we call non-adrenergic, non-cholinergic transmission (NANC), we can find substances such as ATP, nitric oxide, neuropeptides (vasoactive intestinal polypeptide, endothelins, tachykinins, angiotensins) and prostanoids.

In the current project, the investigations focus on the purinergic responses. For this purpose, agonists and antagonists of the purinergic receptors, i.e., purines and pyrimidines, are used.

2.6.3.1 ATP on the way to become a pop-star

Adenosine triphosphate has got a very important role as a source of energy for living cell. The proofs showing that ATP is actually released to the extracellular areas and that specific receptors for ATP exist on the target cells were very important findings for the definition of extracellular ATP physiological activity.

Today, ATP is considered as a valid transmitter in both types of the nerve system - centrally and peripherally. Information about the release of ATP by exocytosis from the nerve endings cell also exists. However, in non-neuronal cells we can probably find different ways of revieling the release of ATP to the extracellular areas, respective ATP could be carrier-mediated, by using ATP-binding cassette-protein (ABC), an omnipresent family of ATPase.

In 1929 adenosine-5'-phosphate (ATP) (a molecule, which is part of the purine family and which is composed of heterocyclic base adenine, saccharide ribose and a chain of three ionized phosphate groups connected to the saccharide) was identified in muscle extracts.

It was firstly thought that this molecule is responsible for the muscle contraction. After few years became obvious three chemically and functionally different parts if this bio-molecule were synthetised by independent metabolic ways and that the main regulator ATP synthesis was adenosine-5'-diphosphate. Then it was demonstrated that ATP is produced during the break down of glucose to lactic acid (anaerobic glycolysis) within the citrate cycle and then mainly during oxidative phosphorilation. This is of course correlated to the finding that ATP is a source of energy, which Lippman later proved by his hypothesis about energy transfer in the living cells.

Very soon ATP was considered as the most important of all the biomolecules, according to the new discoveries, since it is an universal source of chemical energy for the living cells. And because of this role of ATP is so important, it was then thought for many years that this was the only role of ATP. Nobody was thinking that there could be other extracellular physiological functions of ATP. The main argument why ATP could not have any extracellular function was that a cell would never release anything that is so important for their survival. Also the scientists considered the size of the molecule by saying that such a big molecule would not be able to get through the cell membrane by simple diffusion. However it was soon demonstrated that the purines have real extracellular effects on various organs and tissues.

In 1929 Drury and Szent-Gyorgyi lectured for first time about extracellular purines being responsible for negative chronotropic effects on the heart, dilatation of the heart vessels and of inhibition of spontaneous activity of the intestinal smooth muscle.

Later on, the effects of purines on gut, bladder, vagina and other visceral organs and vascular bed were introduced. It was thought that all these effects are caused just by adenosine or adenosine-5'-monophosphate (both breakdown products of ATP) but other studies showed that a length of the phosphate chain influences activity. Studies also proved that ATP has the largest action of all the purinergic compounds.

In 1978 Burnstock introduced the thesis, based on his experimental work, that purinoceptors concists of two main groups: P1 adenosine selective receptors and P2 ADP and ATP selective receptors. The presence of the extracellular receptors for ATP occurs widely. This kind of receptors is possible to find on neuronal as well as on non-neuronal cells. It brings out the question if the ATP is present even in a blood. An answer to this question came during the Second World War. When injured, cell destruction leads to efflux of the cytoplasmatic adenine compounds to the vessel bed, where the really high concentrations of ATP are found. The authors then enounced a premise that ATP is part of the biochemical process of the trauma shock development. They explained the similarities between shocks caused by low blood pressure and ATP.

Later the effect of high ATP concentrations was explained by fact that while is this purine is present extracellulary it causes real changes in permeability of the membrane of erythrocytes, which results in huge efflux ions and nucleotides together with ATP itself. Soon the same mechanism was described for the smooth muscle cells and endothelium (Bodin & Burnstock, 2001).

2.6.3.2 ATP as a neurotransmitter

For many years, ATP was thought to be the only source of the energy for cell and just this role in the metabolism of the cell was known. But in 1972, Burnstock published a theory that ATP is a neurotransmitter. In 1978, he showed the specific extracellular receptors for nucleosides (P1) and nucletides (P2) to mediate physiological effects of adenosine (P1) and ATP (P2).

These compounds are very strong signals for exocrine as well as for endocrine secretory cells. They also have a specific function as short-term co-transmitters in both the central and peripheral nervous systems. It is not just cell growth, differentiation and proliferation that the purines and pyrimidines take care of, but also for instance the release of hormones, neurotransmitters and cytokines are examples of their properties. Now it is also known that they are involved in pathological processes such as of the immune system disease, infections and pain, neurodegenerative diseases and osteoporosis. There also exist studies about a

protective function of purines and pyrimidines in various pathological states for example in cancer, ischemia diseases, organ toxicity caused by drugs or radiation, trauma, stress and in hemorrhagic shock. These findings about the protective functions used to be neglected since all saving procedures were appropriated to nonspecific metabolic processes.

The knowledge of purinergic receptors led us to the issue from the other point of view, where we can see effects concerning a lot of different specific mechanisms. It helped us A) to understand the mechanisms which are the basics of many physiological processes and B) to approximate the principles of different kinds of illnesses and contributed to the pharmacological localizing of the right goal for treatments (Abbracchio & Burnstock, 1998).

2.6.3.3 Release of ATP

In 1954 Holton and Holton noticed that the venous efflux from a perfused rabbit ear showed increased optical density value (at 260 nm) during antidromic stimulation of the major auricular nerve. A few years later the same authors came up with the conclusion that ATP is released while the same nerve is stimulated. And this made them say that ATP is released from the sensory nerve ending and probably it has a role in a chemical transmission (Holton, 1959).

In 60's was the release of ATP observed when a muscle or nerve of a frog was stimulated by electric field stimulation and this release of ATP was connected with the depolarization of the tissue (Abood *et al.*, 1962). Later it was illustrated that ATP is synthesized, stored and released from non-adrenergic, non-cholinergic (NANC) nerves supplying the intestinal smooth muscle, and the fact the adding of ATP actually simulated an effect of nerve stimulation in these muscles, led to the suggestion that ATP is a transmitter in non-adrenergic inhibitory nerves in the gut (Burnstock *et al.*, 1970).

Subsequently, it was suggested that ATP could be released as a primary neurotransmitter by NANC nerves supplying various vessel beds and visceral organs and consequently these nerves were called purinergic nerves. Burnstock also tried to find out what the mechanism of the release of ATP out of the cell was.

First it was demonstrated in the *taenia coli* that the molecule of ATP can be released by sympathetic nerves together with nor-adrenaline. Later on, in parasympathetic nerves, the ATP was proved to work as a co-transmitter with acetylcholine in the urinary bladder (Burnstock *et al.*, 1978) and with nor-adrenaline in rats in vas deferens, in the tail artery and the aorta. Lastly, ATP was found to be a co- transmitter widely used by both nervous systems, centrally in the same way as peripherally (Bodin & Burnstock, 2001).

2.7 Purinergic receptors

The classical autonomic transmitters acetylcholine and nor-adrenaline are not the only transmitters causing contraction (acetylcholine) and relaxation (nor-adrenaline), since other transmitters have been shown to take part in urinary bladder functional regulation.

One of these "other" transmitters is ATP. It contributes to the atropine resistant contraction by acting on P2X receptor and this contractile response is followed by a slower relaxation of the detrusor muscle. There is also a relaxatory response to adenosine, a breakdown product of ATP acting on the adenosine $P1A_2$ receptors (Giglio *et al.*, 2005a).

However, the interaction between the different detrusor receptors may be elucidated by how muscarinic receptor activation can inhibit the purinergic relaxatory effect. In the case of the need of a prolonged contractile stimulus, the breakdown of ATP could oppose the contraction. The relaxatory effect, however, caused by exogenous adenosine acting on the adenosine receptor is also inhibited by cholinergic stimuli acting on the muscarinic M_2 receptor of the detrusor muscle (Giglio *et al.*, 2001). This may thus be of functional significance in cases of the need of prolonged contractile stimulation.

During last years, there is an exponential growth of interest of purinergic receptors. Mediation of effects by purinergic receptors was first described in 1929 and the purineceptors were defined in 1978. The difference between P1 (adenosine purineceptor) and P2 (ATP, ADP purineceptors) were discovered also around this year, and later A1 and A2 and also P2X and P2Y subclasses of P1 and P2 purineceptors were characterized.

New subclasses have subsequently been defined, particularly of the nucleotide receptors concerning P2T, P2Z, P2U and P2D. Consequently there is confusion how to sort out the new findings related to different tissues and purins.

Studies defining the molecular structures of P2 purinoceptor subtypes are constantly appearing. These studies will probably contribute to a clearer picture regarding the receptors. They will possibly bring forward some systematic pattern according to function and they will most likely be of importance for describing pharmacologic properties of drugs with purinergic effects (Abbracchio & Burnstock, 1994).

Purines and pyrimidines (ATP, ADP, adenosine and UTP) have specific extracellular signalling activity in regulation of various functions in different kinds of tissues, both in invertebra as in vertebra. It shows that they seem to have a key role in growing, proliferation and gifferenciation of the cell.

Thus, the activity of extracellular nucleotides and nucleosides (adenosine and ATP) was as mentioned previously, first observed in 1929 in a thesis about effect on adenosine on mammal heart (Drury & Szent-Gyorgyi, 1929). Thirty years later it was found that ATP is released during anti-dromic stimulation of sensoric nerves of rabbit ear artery. The NANC compound supplying both the gut and the bladder was thus observed and later claimed to be ATP. This suggestion resulted in classification of effects by post- junctional receptors for ATP.

Primary classification of P1 and P2 receptors was based on 4 criterions:

i. relative effect of ATP, ADP, AMP and adenosine

ii. selectivity of antagonists, especially methylxantines (these completely antagonise an activity of adenosine but not the activity of ATP)

iii. the modulation of adenylate cyclase with the resulting changes at the levels of cAMP inside the cell caused by adenosine, but not by ATP

iv. an increased production of prostaglandins caused by ATP which adenosine does not evoke.

This classification has been accepted by the International Union of Pharmacology (IUPHAR) Subcommitee for the Nomenclature and Classification of Purinoceptors.

It was clear that P1 and P2 are two huge receptor families. Now we know that there are at least 4 different subfamilies of receptors coupled to G protein- P1 receptors (adenosine) A_1,A_{2a},A_{2b},A_3 and at least 13 subfamilies of P2 receptors.

Therefore it was needed to set up other rules defining how to divide the fast growing group of P2 receptors. Now the group is divided to two smaller- P2Y and P2X subtypes. These can cause vasoconstriction, vasodilatation but they respond differently to synthetic analogs of ATP. Subtypes have been found, which do not match any of these groups. Since so many of these appeared, Abbracchio and Burnstock came with a new nomenclature (Abbracchio & Burnstock, 1998).

1. Two purinoceptors families are determined: i. P2X ligand gated family and ii. P2Y coupled to G protein
2. 4 subclasses of P2X were identified: $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$
3. Different receptors coupled to G protein and named P2 should be included in subclass of P2Y family.

These are:

i. $P2Y_1$ represents the recently cloned P2Y receptor (clone 803) from chick brain

ii. $P2Y_2$ represents the recently cloned P2U (or P2N) receptor from neuroblastoma, human epithelial and rat heart cells

iii. $P2Y_3$ represents the recently cloned P2Y receptor (clone 103) from chick brain that resembles the former $P2_t$ receptor

iv. $P2Y_4$–$P2Y_6$ represent subclasses based on agonist potencies of newly synthesised analogues

v. $P2Y_7$ represents the former P2D receptor for dinucleotides

This new classification of P2 purinoceptors is very similar to the one developing for the receptors of other major transmitters, such as acetylcholine, γ-aminobutyric acid, glutamate and serotonin, where two main families are recognized

– one connected to the fast transmission (receptors directly linked to the ion channel) and the second one mediating slower responses by G protein coupled receptors.

A scientific discussion is mots likely to occur on the matter of the numbering of the different receptor subtypes within the P2X and P2Y families. But many authors believe that this new way of defining the receptors for nucleotides according to agonist potency, transduction mechanism and molecular structure will give a more order and logical approach to adjustment new findings (Abbracchio & Burnstock, 1994).

2.7.1 Purinoceptors subtypes

2.7.1.1 P1 purinoceptors

Four subtypes of P1 receptors have been cloned, A_1, $A_{2A,}$ A_{2B} and A_3 and all of these P1 receptors are G protein coupled. They have seven putative transmembrane domains of hydrophobic amino acids. Each of these domains is composed of 21-28 amino acids and makes a structure of an α-helix. This long protein has two ends - the N- terminal and the C- terminal. The N-terminal is situated on the extracellular side and the C- terminal on the cytoplasmatic side of the membrane. The intracellular part of the receptor connects to the G protein. When this happens, the transduction of the signal is performed.

The parts in the transmembrane regions are of great importance for the ligand binding. This part is also the determinant for the receptor specifity. On the other hand, the extracellular loops, the C-terminal and the N- terminal do not seem to be involved in ligand recognition (Burnstock, 2006b).

A_1 and A_3 receptors preferably interact with members of the G_i family and inactivate adenylate cyclase to decrease the production of cAMP, whereas A_{2a} and A_{2b} receptors are coupled to G_s and stimulate cAMP production. All four receptors activate phospholipase C (PLC), resulting in inositol 3,4,5-trisphosphate (IP_3) production and increased cytoplasmic Ca^{2+}, and can also stimulate mitogen-activated protein kinase cascades (Schulte & Fredholm, 2003).

2.7.1.1.1 Review of main distribution, agonists and antagonists for the P1 receptors (Tab. 2.1: adapted from Burnstock, 2006b)

Receptor	Main distribution	Agonists	Antagonists
A_1	Brain, spinal cord, testis, heart, Autonomic nerve terminals, bladder	CCPA, CPA	DPCPX, CPX, XAC
A_{2A}	Brain, heart, lungs, spleen	CGS 21680	KF17837, SCH58251
A_{2B}	Large intestine, bladder	NECA	Enprofylline
A_3	Lung, liver, brain, testis, heart	DB-MECA,DBXMR	MRS1222, L-268,605

Tab. 2.1: CCPA, 2-chloro-N 6-cyclopentyladenosine; CPA,N 6-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, xanthine amine congener;NECA, N-ethylcarboxamidoadenosine;DBX RM, 1,3-dibutylxanthine-1-riboside-5′-N-methylcarboxamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine;

An investigation to explore the role of A1-purinegic receptors according to the adenosine-5'-triphosphate (ATP) and diadenosine tetraphosphate (AP4A)-induced contractions was performed by Khattab et al. The presence of A1-purinoceptors mediating contraction in response to selective A1-purinoceptor agonists was then demonstrated on isolated rings of rat urinary bladder (Khattab *et al.*, 2007). On the other hand there are some studies indicating an opposite view in comparison with Khattab's statement. Rubino showed that P1- A_{2B} subtype lowers the vascular tone in the rat isolated mesenteric arterial bed. In preparations at basal tone, adenosine lacked vasoconstrictor actions, while ATP elicited dose-dependent vasoconstrictor responses. When the tone of preparations was raised by adding methoxamine to the perfusate, adenosine and its stable analogue, 2-chloroadenosine (2-CADO) elicited dose-dependent vasodilation (Rubino *et al.*, 1995).

Also an experiment done by Mathie supported the suggestion about relaxation. This study was carried out in order to identify the receptor being responsible for adenosine-induced dilatation of the hepatic arterial vascular bed. They found, by adding adenosine and its more stable analogues, you actually get a dilatation of the rabbit hepatic arterial bed as a result from activation of P1-purinoceptors of the A2 subtype. They showed that there is significant attenuation of the vasodilatation to adenosine and analogues in the presence of the P1- purinoceptor antagonist 8-phenyltheophylline (Mathie *et al.*, 1991). That adenosine causes a relaxation has been also suggested in study about purinergic responses in urinary bladder (Giglio *et al.*, 2001). It seems then that we cannot aver that we get a relaxatory response by stimulating generally P1 adenosine receptors but we can claim some subtypes of P1 adenosine receptors give us a relaxation.

Western blot analysis of urinary bladder tissues has established that all four adenosine receptors (A_1, A_{2a}, A_{2b}, and A_3) are expressed in the urothelium. A_1 receptors are prominently localized to the apical membrane of the umbrella cell layer, whereas A_{2a}, A_{2b}, and A_3 receptors are localized intracellularly or on the basolateral membrane of umbrella cells and the plasma membrane of the underlying cell layers. Adenosine is released from the urothelium, which is potentiated 10-fold by stretching the tissue. The urothelium is responsive to adenosine and adenosine may increase exocytosis in the urothelium.

The agonist/antagonist studies have indicated that the activity of A_1 receptors may dominate at the mucosal surface, with some contribution from A_{2A} receptors. In contrast, A_{2A} receptors may dominate at the serosal surface, but with a significant contribution by A_1 and possibly A_{2B} receptors (Yu *et al.*, 2006).

2.7.1.1.2 Adenosine

ATP generates contraction. This contraction is followed by relaxation, which has been suggested to depend on the metabolite of ATP, adenosine, stimulating inhibitory P1 purinoceptors (Giglio *et al.*, 2001).

Adenosine is generated within the cell from the hydrolysis of *S*-adenosyl-L-homocysteine and is also formed both extracellularly and intracellularly from the

hydrolysis of ATP, ADP, AMP, or cAMP (Jackson & Raghvendra, 2004). Also it is a potent vasodilator in most vascular beds. It has been shown in many studies that this nucleoside plays a significant role in the local, metabolic regulation of blood flow in various organs. The dilatation induced by adenosine is mediated by the activation of A2A receptors and the subsequent production of NO and opening of K^+-ATP channels (Hein *et al.*, 2005).

In the bladder, adenosine, acting through cell surface receptors, may also act in an autocrine/paracrine manner to regulate exocytosis in the umbrella cell layer both in response to, and independently of, bladder filling. One particular function for adenosine has been identified in the urothelium, namely the modulation of exocytic traffic. However, it is likely that adenosine regulates other functions of the urothelium and the bladder including ion transport, urothelial-afferent nerve signaling, and bladder contraction (Yu *et al.*, 2006).

2.7.1.2 P2X purinoceptors

P2X receptors are honourable members of ligand gated ion channel receptor family (Fig.2.7). P2X receptor subtypes are characterized by two transmembrane domains, one short intracellular N- and C-termini and an extensive extracellular loop with conservation of ten cysteines (Burnstock, 2001).

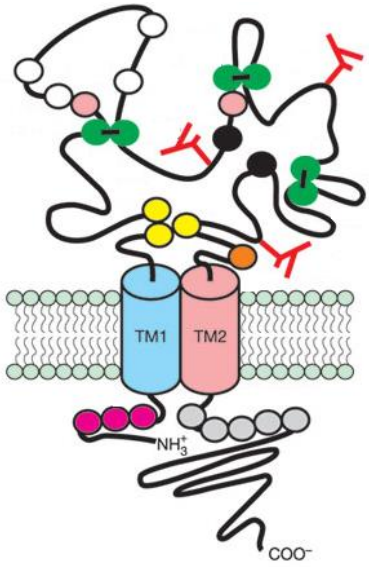


Fig 2.7: Ligand gated ion channel receptor *(adapted from: http://www.nature.com/nature/journal/v442/n7102/images/nature04886-f2.2.jpg*)

Changes in the sensory input from the bladder (i.e., bladder hypersensitivity) play an important role in the development of interstitial cystitis (IC). IC is associated with an increased release of adenosine triphosphate (ATP) from bladder urothelial cells and ATP is also released from the bladder urothelium in response to stretch or bladder distension (Vlaskovska *et al.*, 2001). Interactions between the urothelium and bladder nerve terminals are thought to regulate micturition and nociception and ATP and P2X receptors have been implicated in bladder nociception (Ford *et al.*, 2006).

The bladder inflammation produces hyperexcitability and sensitizes bladder neurons. This hypersensitivity is associated with an increased expression and/or properties of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in lumbosacral and thoracolumbar bladder neurons, respectively. The altered P2X receptor expression accounts for greater current density in response to purinergic agonists, and is likely to contribute to the slowed desensitization kinetics of the slow desensitizing current after bladder inflammation. Findings also suggest that the altered expression of P2X receptors contribute to the enhanced responses of bladder neurons during cystitis (Dang *et al.*, 2008).

2.7.1.2.1 Review of main distribution, agonists and antagonists for the P2X receptors (Tab. 2.2: adapted from Burnstock, 2006b)

Receptor	Main distribution	Agonists	Antagonists
$P2X_1$	Smooth muscle, platelets, cerebellum, dorsal horn spinal, neurons	α,βmeATP = ATP = 2meSATP (rapid desensitization)	TNP-ATP, IP5I, NF023
$P2X_2$	Smooth muscle, CNS, retina, autonomic and sensory	ATP ≥ ATPγS ≥ 2meSATP >> α,βmeATP (pH + zinc sensitive)	Suramin, PPADS

	ganglia		
P2 X_3	Sensory neurons, sympathetic neurons	2meSATP ≥ ATP ≥ α,βmeATP (pH + zinc sensitive)	TNP-ATP, suramin, PPADS
P2 X_4	CNS, testis, colon	ATP >> α,βmeATP	-
P2 X_5	Proliferating cells in skin, gut, bladder, thymus, spinal cord	ATP >> α,βmeATP	Suramin, PPADS
P2 X_6	CNS, motor neurons in spinal cord	(does not function as – Intrinsic ion channel homomultimer)	-
P2 X_7	Apoptotic cells in immune cells, pancreas, skin	BzATP > ATP ≥ 2meSATP >> α,βmeATP	KN62, KN04, Coomassie brilliant blue

Tab. 2.2: α,βme-ATP, α,β-methylene ATP; 2meSATP, 2-methylthioATP; BzATP, 2′,3′-O-(4-benzoyl-benzoyl) ATP; Ins(1,4,5)P3, inositol (1,4,5)-trisphosphate; IP5I, diinosine pentaphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RB2, Reactive blue 2; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate;

2.7.1.3 P2Y purinoceptors

P2Y receptors, which, in common with other G protein coupled receptors (Fig. 2.8), have seven transmembrane domains, have an extracellular N-terminus and intracellular C-terminus. The conservation between the different subtypes is greatest in the transmembrane domains, while the C-terminus shows the greatest diversity (Burnstock, 2001).

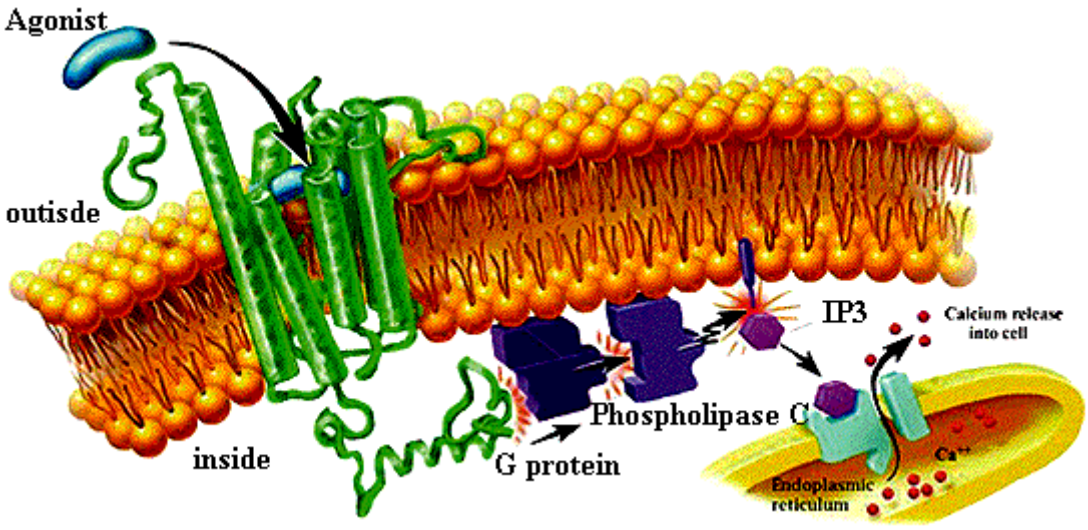


Fig. 2.8: G protein coupled receptor *(adapted from: http://journals.prous.com/journals/dof/19992407/html/df240759/images/Yerxa2.gif)*

2.7.1.3.1 Review of main distribution, agonists and antagonists for the P2Y receptors (Tab. 2.3: adapted from Burnstock, 2006b)

Receptor	Main distribution	Agonists	Antagonists
$P2Y_1$	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	2meSADP > 2meSATP = ADP > ATP	MRS2279, MRS2179
$P2Y_2$	Immune cells, epithelial and endothelial cells,	UTP = ATP	Suramin

	kidney tubules, osteoblasts		
P2 Y_4	Endothelial cells	UTP = ATP	RB2, PPADS
P2 Y_6	Some epithelial cells, placenta, T cells, thymus	UDP > UTP >> ATP	RB2, PPADS, suramin
P2 Y_{11}	Spleen, intestine, granulocytes	ARC67085MX > BzATP ≥ ATPγS > ATP	Suramin, RB2
P2 Y_{12}	Platelets	ADP	ARC67085MX, ARC69931MX
P2 Y_{13}	Spleen, brain, lymph nodes, bone marrow	ADP = 2MeSADP >> ATP and 2MeSATP	
P2 Y_{14}	Placenta, adipose tissue, stomach, intestine, discrete brain region	UDP glucose = UDP-galactose	

Tab. 2.3: PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; BzATP, 2′,3′-O-(4-benzoyl-benzoyl) ATP; 2meSATP, 2-methylthioATP; RB2, Reactive blue 2;

2.7.2 Purinergic receptors and pain

The involvement of ATP in the initiation of pain was recognized in the early 60's. It was Burnstock who put forward a unifying purinergic hypothesis for the initiation of pain by ATP acting *via* $P2X_3$ and $P2X_{2/3}$ receptors associated with causalgia, reflex sympathetic dystrophy, angina, migraine, pelvic and cancer pain. This has been followed by an increasing number of papers expanding this concept for acute, inflammatory, neuropathic and visceral pain. A hypothesis was proposed that purinergic mechanosensory transduction occurs in visceral tubes and sacs, including the ureter, bladder and gut, where ATP released from epithelial cells during distension acts on $P2X_3$ homomultimeric and $P2X_{2/3}$ heteromultimeric receptors on subepithelial sensory nerves to initiate impulses in sensory pathways to pain centres in the CNS (Burnstock, 2006b).

2.7.3 Purinergic receptors and inflammation

$P2X_7$ and $P2Y_1$ and $P2Y_2$ receptors located on inflammatory and immune cells play a pivotal role in inflammation and immunomodulation. ATP and its breakdown product adenosine are released at sites of inflammation. ATP is involved in the development of inflammation through a combination of actions: release of histamine from mast cells, provoking production of prostaglandins, and the production and release of cytokines from immune cells. In contrast, adenosine exerts anti-inflammatory actions (Burnstock, 2006b).

Inflammatory urinary bladder

It was shown that mice with blocked $P2X_3$ receptors exhibited reduced inflammatory pain and marked urinary bladder hyporeflexia with reduced voiding frequency and increased voiding volume, suggesting that $P2X_3$ receptors are involved in mechanosensory transduction underlying both inflammatory pain and physiological reflexes (Cockayne *et al.*, 2000). Recent studies have shown significant increase in the release of ATP from the urothelium in different animal models of interstitial cystitis as well as in interstitial cystitis in humans; in response to stretch in the cat model, in the cyclophosphamide mouse model as well as from urothelial cells from patients with interstitial cystitis. Release of ATP from urothelial cells with hypo-osmotic mechanical stimulation was increased by over 600% in inflamed bladder from cyclophosphamide-treated animals, which was inhibited by botulinum toxin (Burnstock, 2006a). Furthermore, an increase in stretch-evoked urothelial release of ATP has been reported from porcine and human bladders with sensory disorder (urgency) compared with normal bladders (Kumar *et al.*, 2004).

3 Aims

The aim of the thesis is to investigate the presence and function of selected purinergic receptor subtypes, namely $P1A_1$ and $P2X_1$, in the urinary bladder of the rat. Three questions were raised:

1. Do these receptors exist in the urinary bladder?

2. If they do exist, where are they located?

3. Do their expression, as well as location change during the inflammatory process of the urinary bladder?

The aim for the functional studies is to describe if the bladder reacts differently according to this receptor change during the inflammation. This investigation should also show us what is the role of these receptors.

4 Methods and Materials

4.1 Immunohistochemistry

4.1.1 Introduction of the method

Immunohistochemistry is used for the localization of antigens or proteins in tissue sections by employing labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold. Since immunohistochemistry involves specific antigen-antibody reaction, it has an apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Therefore, immunohistochemistry has become a crucial technique and widely used in many medical research laboratories as well as clinical diagnostics. There are numerous immunohistochemistry methods that may be used to localize antigens. The selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity required.

An important part of immunohistochemistry is the tissue preparation, such as fixation and sectioning. It is essential to ensure the preservation of tissue architecture and cell morphology, prompt and adequate fixation. The most common fixatives used for immunohistochemistry are 4% paraformaldehyde in 0.1M phosphate buffer, 2% paraformaldehyde with 0.2% picric acid in 0.1M phosphate buffer, 4% paraformaldehyde with 0.05% glutaraldehyde (TEM immunohistochemistry), 4% paraformaldehyde, 0.2% periodate and 1.2% lysine in 0.1M phosphate buffer (PLP fixative). Sectioning is provided by vibratome.

The demonstration of many antigens can be significantly improved by the pretreatment with the antigen retrieval reagent that break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. Microwave Oven, Pressure Cooker and Steamer are the most commonly used heating devices. Citrate buffer of pH6.0 is the most popularly used retrieval solution and is suitable for most of antibody applications. Next step in immunohistochemistry is a non-specific background blocking. The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and

electrostatic forces to certain sites within tissue sections. This form of background staining is usually uniform and can be reduced by blocking those sites with normal serum. The solution for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody.

In the immunohistochemistry, as well as in other experiments through the whole scientific field, controls are needed for comparisons. A positive control serves to test a protocol or procedure and make sure it works. A negative control serves to test the specificity of an antibody involved. First, no staining must be shown when omitting primary antibody or replacing an specific primary antibody with normal serum (must be the same species as primary antibody). This control is easy to achieve and can be used routinely in immunohistochemical staining.

There are two principal methods in immunohistochemistry, direct and indirect. The direct method is an one step staining method, and involves a labelled antibody (i.e. FITC conjugated antiserum) reacting directly with the antigen in tissue sections. The indirect method involves an unlabelled primary antibody (first layer),which reacts with tissue antigen, and a labelled secondary antibody (second layer) reacting with primary antibody (Note: The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised).

Many indirect methods are used for the localization of proteins. For example PAP (peroxidise anti- peroxidise method), ABC (Avidin-Biotin Complex) method, LSAB (Labeled StreptAvidin Biotin) method or various polymeric methods.

Avidin-Biotin Complex (ABC) Method:

ABC method is standard IHC (use the abbreviation all the time and explain it the first time or avoid it constantly) method and one of widely used technique for immunhistochemical staining (Fig. 4.1). Avidin, a large glycoprotein, can be labelled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules such as antibodies.

The technique involves three layers. The first layer is unlabelled primary antibody. The second layer is biotinylated secondary antibody (secondary antibody,

Santa Cruz ABC- kit). The third layer is a complex of avidin-biotin peroxidise (AB enzyme reagent, Santa Cruz ABC- kit). The peroxidase is then developed by the DAB (peroxidise substrate, Santa Cruz ABC- kit) or other substrate to produce different colorimetric end products (World IHC, 2007).

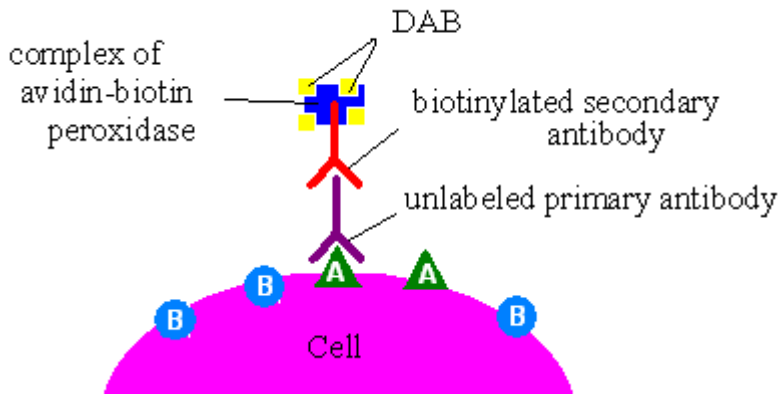


Fig. 4.1: ABC- method

4.1.2 Materials

The following solutions and compounds were used in the current study:

Xylene; ethanol- 99.5%, 95%, 85%, 85%, 70%; deionised water; TBS (4 l; pH= 7.5; 52.9 g Trizma- HCl; 7.8g Trizma-Base; 36g NaCl); citrate buffer (1 l; pH= 6.0; 10mM; 2.1 g citric acid of MW= 210.14 g/mol; 0.97 g NaOH or 6 ml 2M NaOH); H_2O_2 (0.03%); BSA(bovine serum albumin); primary antibody diluted in 1% BSA in TBS (125 µl of the antibody diluted 1:10, 1125 µl TBS, 1.25 mg BSA); 1% BSA in TBS (1125 µl TBS, 1.25 mg BSA); Santa Cruz ABC- kit, hematoxylin; glue

4.1.3 Immunohistochemistry procedure

If we have got the glasses with two thin slices of urinary bladder ready on each glass we can start to run the immunohistochemistry procedure.

This experiment takes two days. First day the paraffin that the bladder slices are fixed in is removed and the membranes are ruptured for enabling binding of the primary antibody. This is followed by overnight incubation and on the second day the incubating with antibody and staining is proceeded. There are two slices on each glass. One is marked as a positive and the other one as a negative control. To have a

negative control is necessary because it shows selectivity of the primary antibody. The more accurate, the more correct result will we get at the end by comparing the negatives with the positives.

Protocol

DAY ONE

1. The sections were incubated at 60°C for 60 minutes which made the paraffin soft.

2. Second step is the deparaffination itself. The glasses need to be rinsed twice for 30 minutes in xylene (twice means after first 30 minutes the glasses are emerged from the pot filled by the liquid and merged back for another 30 minutes), then twice for 5 minutes in 99.5% ethanol, twice for 5 minutes in 95% ethanol, for 5 minutes in 85% ethanol and finally for 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 were rinsed under flowing deionised water for 10 minutes so residues of ethanol and paraffin are washed out. The glasses are rinsed twice for 5 minutes in TBS. The TBS is used almost after every step. It is supposed to wash out solutions used in previous step, inactivate the reactions which had been going on and get the sample to the "normal state".

4. Then the glasses need to be rinsed in citrate buffer for 5 minutes. Boiling the samples in citrate buffer in a microwave oven four times for 6 minutes follows this. 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. Then the glasses are supposed to cool in citrate buffer for 30 minutes.

5. The glasses are washed in TBS twice for 5 minutes.

6. Next step is blocking the endogen peroxidise by adding 0.03% H_2O_2 for 30 minutes.

7. The glasses are washed again in TBS twice for 5 minutes.

8. The area around each section was demarcated with a hydrophobic pen. The glasses have to be let to get dry a bit. Then the blocking of non-specific background using 5% BSA in TBS for 30 minutes has to be done.

9. Only this step varies the positives and negatives samples from each other. That is why it needs to be done very carefully. First the positive and negative sample on each of the glasses has to be marked. The positives are then incubated over night with primary antibody diluted in 1% BSA in TBS. The negative controls on the other hand are incubated just in 1% BSA in TBS (1125 µl TBS + 1.25 mg BSA). The box used for incubation has to be closed and filled by water so the water can not touch the glasses but it can prevent the sections from becoming dry.

DAY TWO

1. The Santa Cruz ABC-kit was used for binding the secondary antibody and staining the samples. The ABC-kit has to suit to the primary antibody, which was used the previous day. According to the manual the secondary antibody, AB enzyme reagent and peroxidise substrate were mixed.

2. The glasses were washed in TBS twice for 5 minutes. The best way how to prevent mixing two drops on the glass together is by removing the drop using the automatic pipette. It does not really matter if they mix during other steps of the whole procedure but "the step 9 of the day one" and this step are only two points where is needed to be precise and treat positive and negative control just by what they are supposed to be.

3. The glasses are ready now to be incubated with secondary antibody for 30 minutes in the same box under the same conditions they were incubated during the night.

4. They were washed twice in TBS for 5 minutes.

5. Then the incubation with AB enzyme reagent for 30 minutes was proceed.

6. The glasses were washed again in TBS twice for 5 minutes.

7. The sections were incubated in peroxidise substrate until they got brown. Recommended time is 5 minutes.

8. The samples were then carefully rinsed in flowing deionised water for 10 minutes.

9. Next step was staining with hematoxylin until stained enough, usually between 2-4 minutes.

10. The glasses were rinsed in 37ºC water until they became blue.

11. The samples now need to be merged to the ethanol in increasing concentrations and in xylene in the end for 10 minutes in each.

12. When almost dry, the sections had to be glued and covered by the cover glass.

4.2 In vitro functional studies

4.2.1 Rats

Male rats of the Sprague-Dawley strain with the weight of 250-300 g were used in the current thesis. The study was approved by to local ethical committee of Göteborg University.

4.2.2 Urinary bladder strip preparation

The rats were anaesthetized with carbon dioxide and then killed with overdose of the same gas. The urinary bladder was removed. After cutting and opening the bladder two or three strips 6x2 mm big were excised from the middle of the organ. In order to keep the bladder wet, it was constantly kept in Krebs bicarbonate solution.

For the contraction experiments, organ bathes were used. The strips were fastened between two steel rods. One of these two rods was adjustable. The organ bath containing the strip and the two steel rods device was filled by Krebs bicarbonate solution of the following composition: deionised water, NaCl 6.9g/l, KCl 0.34g/l, KH_2PO_4 0.16g/l, $MgSO_4$ 0.14g/l, $NaHCO_3$ 2.10g/l, glucose 0.99g/l, the solution was bubbled by gas mixture of 95% O_2 and 5% CO_2 for at least 45 minutes before $CaCl_2$ 0.18g/l was added and then it was bubbled by the same gas all the time to keep a stable and neutral pH. The temperature was kept at 37 ºC by a thermostat. The strips were pre-stretched to a tension of 10 mN and left to equilibrate for 45 minutes. It resulted to fluent relaxation and the strips got stabilised about 3- 5 mN.

After 45 minutes all strips were treated by “high potassium krebs” (124 mM; 4.41g KCl in 0.5 l krebs bicarbonate solution), which was used as a reference solution employed in each experiment. When the maximum contraction was reached, the high potassium krebs was washed out and the bathes were refilled by the “normal physiologic” bicarbonate krebs. Then the tension has to be stabilised on 3- 5 mN again if needed and after at least 20 minutes the experiment started.

4.2.3 Adenosine effect

For functional studies of $P1A_1$ receptors, a concentration of adenosine (its agonist) was used since it gives the most suitable response. This response shows a significant, rather sharp and relatively fast relaxation. To find the appropriate concentration of adenosine giving relaxation, a contractile response has to be present. Therefore, when the relaxatory experiment is performed, the tissue strips were exposed to "low potassium krebs". The concentration of this solution is "50mM in bath" (1.86g KCl in 10ml krebs bicarbonate solution). It means the real concentration of the solution in a tube is actually 50 times higher. Thus, after adding 0.5 ml of it in to the organ bath of 25 ml, the solution is diluted to 50mM. By pre-contracting the tissue we wanted to get a tension around 8 and 10 mN, so when relaxatory agent is added the tissue would have a space to relax. In order to get the right precontractile value, low potassium krebs was sometimes needed to be added in somewhat higher concentrations. Right after the administration of the low potassium krebs, a concentration of phentolamine and propranolol (both in concentration of 10^{-5}mol/l) were added to block possible adrenergic responses.

The tissue was let to be pre-contracted for 30 minutes, then the adenosine in increasing concentrations was added within 1-2 minutes (after minimum response was reached). The used concentrations of adenosine were 10^{-7}, $5x10^{-7}$, 10^{-6}, $5x10^{-6}$, 10^{-5}, $5x10^{-5}$, 10^{-4} mol/l. The procedure was repeated once again after washing the baths.

4.2.4 Determination of response to adenosine caused by $P1A_1$ receptors in healthy and cystitis rats

When the effects of antagonists were examined, the protocol described above was repeated four times, every time a new antagonist was added. After phentolamine and propranolol it was a reversible antagonist of the muscarinic receptors 4-DAMP of concentration 10^{-5} mol/l. The next round was the relaxation performed with all previous antagonist plus reversible selective P2 purinoceptors antagonist PPADS of 10^{-4} mol/l. In the final round five antagonists were used including DPCPX, an irreversible specific $P1A_1$ antagonist of concentration 10^{-5} mol/l. All drugs were

administrated in volume 125µl. The incubation time for all antagonists was 20 minutes. The very same procedure was performed with the bladder strips from control and cystitis rats.

4.2.4.1 Cystitis rats

Inflammation of the urinary bladder was induced by the pre-treatment with cyclophosphamide. The rats were given cyclophosphamide in concentration 100 mg/kg i.p. in combination with the anaesthetic buprenorphinum 10 µg/kg i.m. The urinary bladder was removed 60 hours later (Giglio *et al.*, 2005b).

The compound N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide *(Cyclophosphamide, $C_7H_{15}Cl_2N_2O_2P$, 261.085)* is a drug used for treatment of various types of cancer or autoimmune disorders. One of its many side effects is that it actually cause a cystitis and for this "property" it is used for pre- treatment for inducing inflammation in the "cystitis rats" group in the experiments showed in this project.

4.2.5 Determination of relaxatory response caused by $P1A_1$ using electric field stimulation (EFS) in healthy and cystitis rats

A contraction experiment using electric field stimulation (EFS) was run in the similar way as the previous ones. The procedure of the strip preparation is the same. No pre-contraction was performed in these experiments, but "high potassium krebs" for the reference response was used. After this step the baths were washed, refilled with physiological krebs and the strips had to equilibrate for 20 minutes before the first EFS was performed.

First the tissue was stimulated three times within 20 minutes with no antagonist present at a frequency of 40 Hz (this frequency elicits the maximal response as shown in previous preparatory experiments). Then the antagonists were added all in the volume of 125 µl as in the "agonist experiments".

Propranolol and phentolamine were first added to the organ baths and after 20 minutes the strips were stimulated by the electric field. Without any rewashing 4-DAMP was put on and again incubated for 20 minutes in presence of the three

antagonists, before another measuring the tone. Then PPADS was added and lastly DPCPX.

The baths were then washed. When the experiments were repeated within the same strips, the antagonists had to be added in another way of the rounds, starting with DPCPX since it is only irreversible antagonist of the used ones. It means this kind of experiment can be performed just twice with the same strips while using irreversible antagonist.

4.2.6 Materials

For immunohistochemistry the following materials and substances were used: ABC Staining system sc-2018 for use with rabbit primary antibody *(Santa Cruz Biotechnology, USA)*, Anti-P2X1 Purinergic Receptor- developed in rabbit *(Sigma, Sweden)*, Anti-P1A1 Purinergic Receptor- developed in rabbit *(Sigma, Sweden)*, Mayer´s hematoxyline *(Histolab, Göteborg, Sweden)*.

Substances used in contraction experiments were used as follows: 9-β-D-Ribofuranosyladenine, Cell Culture Tested *(Adenosine, $C_{10}H_{13}N_5O_4$, 267.24,Sigma, Sweden)*,2-[N-(3-Hydroxyphenyl)-p-toluidinomethyl]-2-imidazolidine hydrochloride *(Phentolamine, $C_{17}H_{19}N_3O \cdot HCl$, 317.81, Sigma, Sweden)*, DL-Propranolol-[4-^{3}H] hydrochloride *(Propranolol, $C_{16}H_{21}NO_2 \cdot HCl$, 295.80, Sigma)*, 4-Diphenylacetoxy-N-methylpiperidine methiodide, powder *(4-DAMP, $C_{21}H_{26}INO_2$ 451.34, Sigma)*, Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate, solid, ≥98% (HPLC) *(PPADS, $C_{14}H_{10}N_3Na_4O_{12}PS_2 \cdot xH_2O$, 599.31 (anhydrous basis), Sigma)*, 1,3-Dipropyl-8-cyclopentylxanthine, solid *(DPCPX, $C_{16}H_{24}N_4O_2$, 304.39, Sigma)*. Chemical structure of used antagonists shows Fig. 4.2.

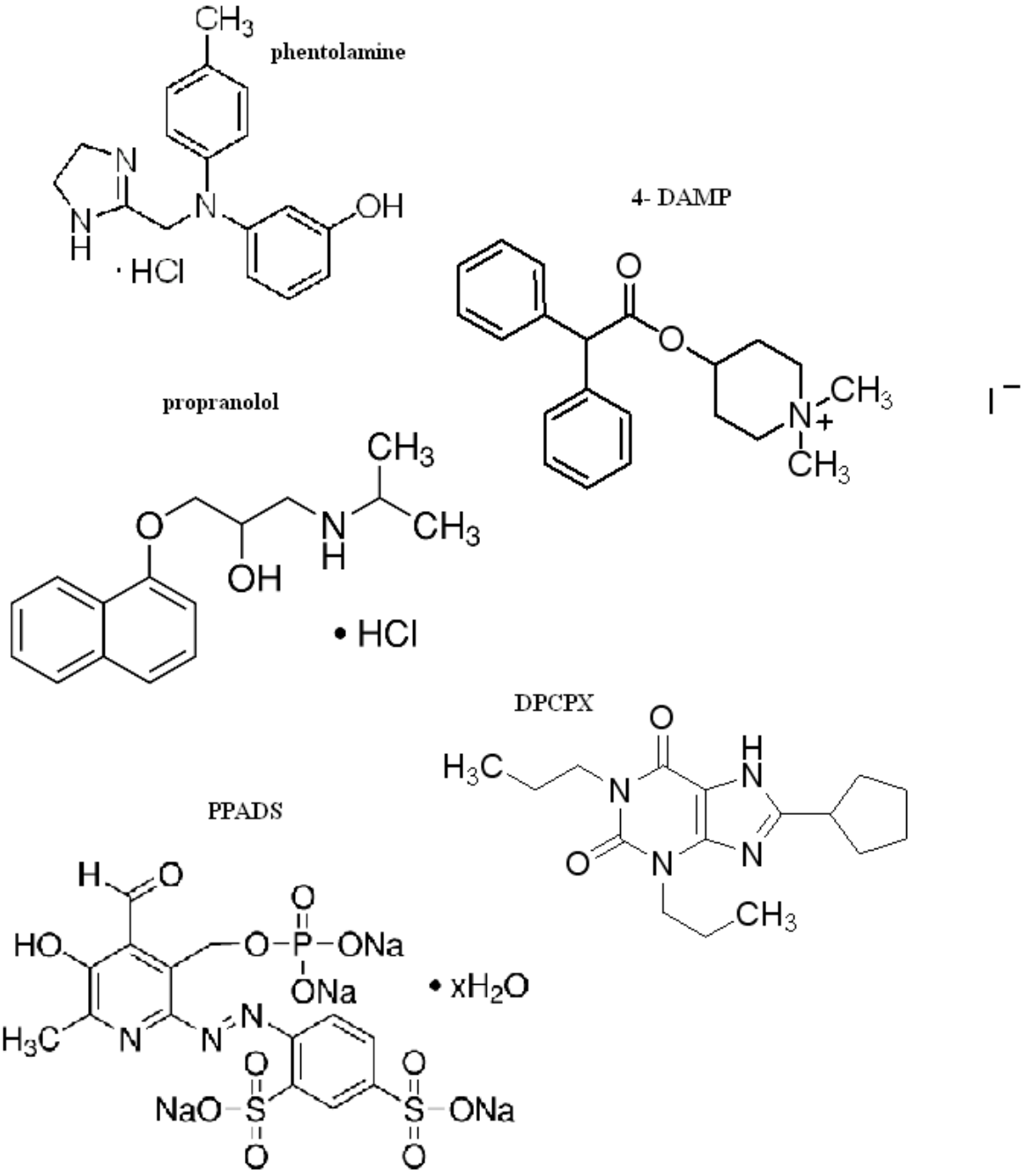


Fig. 4.2: Chemical structure of used antagonists

5 Results

5.1 Immunohistochemistry

5.1.1 $P1A_1$ receptors

The staining colour showing presence of $P1A_1$ receptors is brown. In the negative samples it is clear the primary antibody was highly selective to $P1A_1$ receptors since there was a lack of brown colour at all (pictures A, C, E, G).

Brown areas in the positive saline samples (Fig. 5.1) show where the $P1A_1$ receptors are located in the healthy urinary bladder (pictures B, D). We can see that all parts of urinary bladder (urothelium, sub- urothelium and muscle) is brown and with about the same colour intensity. Thus, $P1A_1$ receptors seem to be present in the urinary bladder just everywhere, and the intensity shows density or frequency of the receptors in different areas. In the case of $P1A_1$ receptors the frequency of appearance is thus the same in urothelium, sub- urothelium and in the muscle.

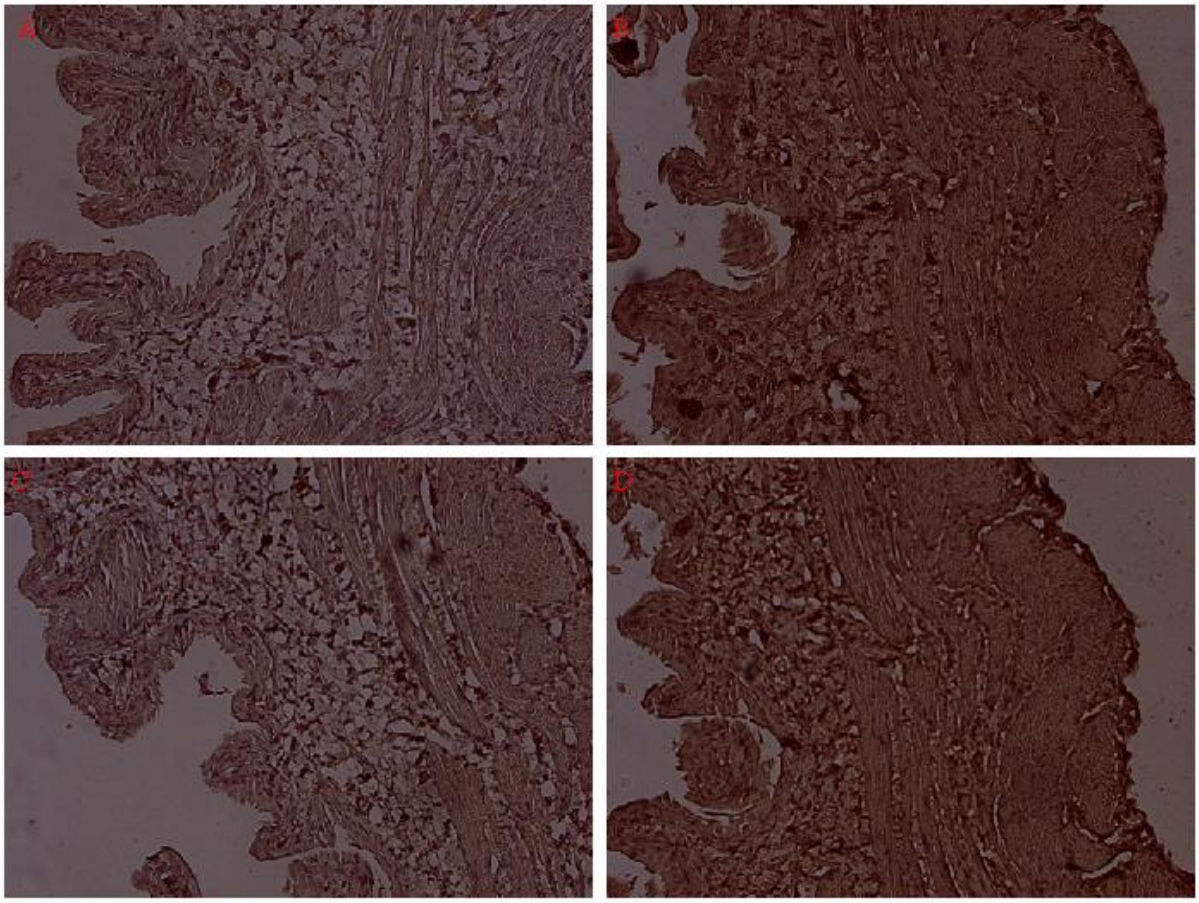


Fig. 5.1: Immunohistochemical labeling of bladders negative controls (*A, C*) and positive saline samples (*B, D*). All sections are counterstained with hematoxyline. Right column (*B, D*) shows the staining for $P1A_1$ receptors. The pictures all show a cut through whole urinary bladder with urothelium on the left, sub- urothelium in the middle and muscle on the right side.

The appearance of the receptor seemed to be different in inflamed urinary bladders as showed in figure 5.2. Even here it is obvious staining was specific to $P1A_1$ receptors since there was no brown colour in the negative samples (picture E, G). Also the cystitis bladder (pictures F, H) was coloured brown compare to the negatives. The frequency of the brown areas was the same in urothelium, sub-urothelium and in the muscle, but intensity of brown colour differs comparing to saline positives (pictures B, D). This led us to the conclusion that there exist $P1A_1$ receptors and that they are present in all parts of the bladder but their amount decreases during inflammatory process.

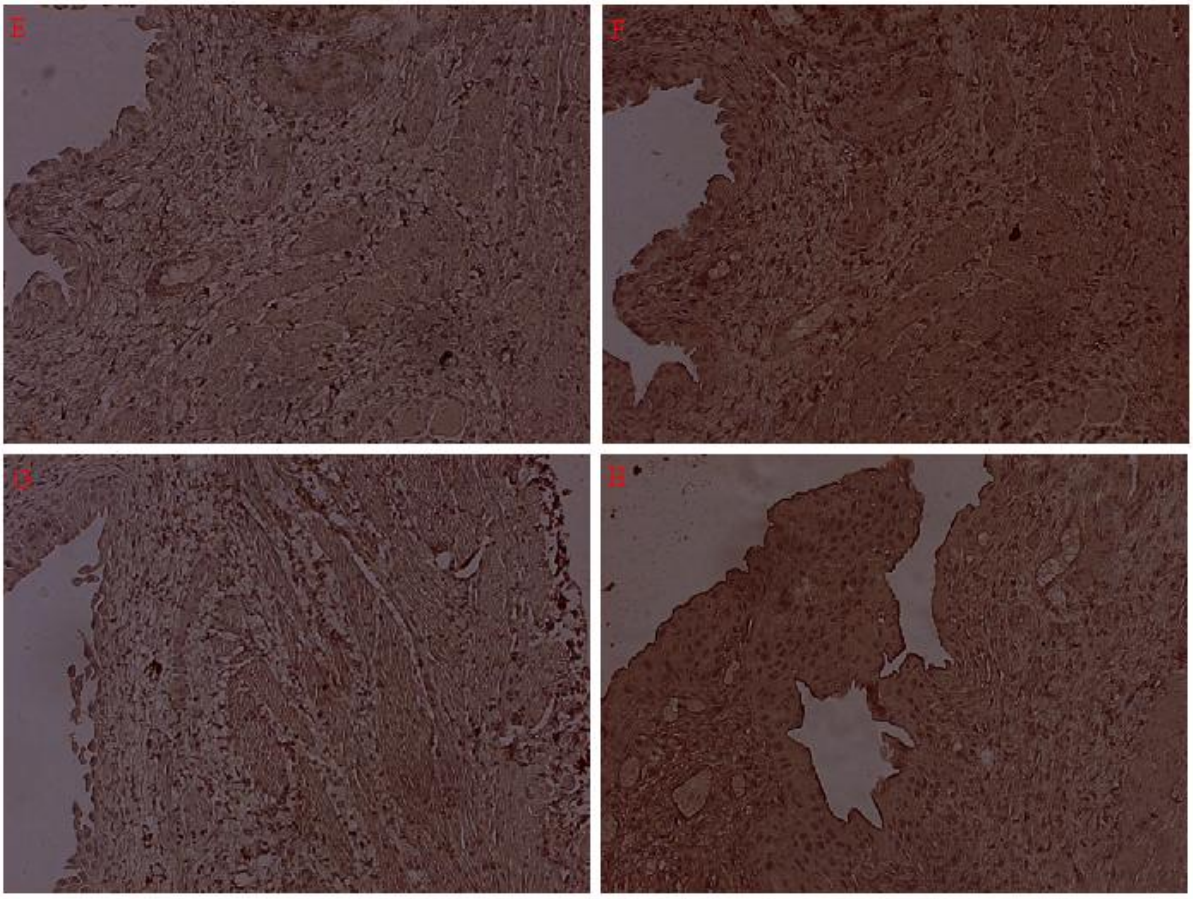


Fig. 5.2: Immunohistochemical labeling of bladders negative controls (*E, G*) and positive cystitis samples (*F, H*). All sections are counterstained with hematoxyline. Right column (*F, H*) shows the staining for $P1A_1$ receptors. The pictures are situated all the same direction so urothelium would be on the left side and muscle tissue on the right. Also picture *H* shows typical view of cystitis in urinary bladder: incrassate urothelium- consisted from more than one layer of urothelium cells, increased connective tissue and vacuols in sub- urothelium.

5.1.2 $P2X_1$ receptors

According to the specific staining for $P2X_1$ receptors (Fig.5.3 and 5.4), these are mainly localized to the muscle tissue but also to the urothelium of the healthy rat urinary bladder (Fig. 5.3).

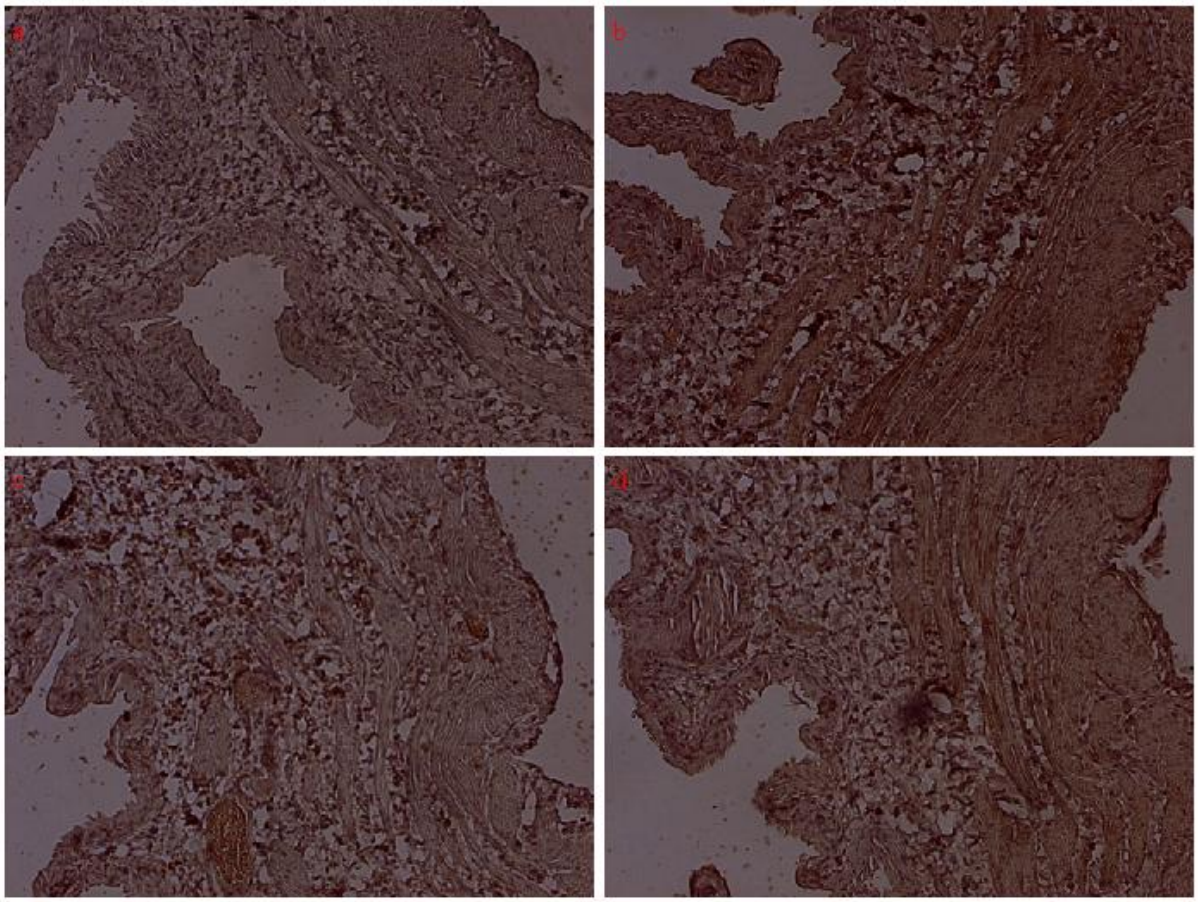


Fig. 5.3: Immunohistochemical labeling of bladders negative controls (*a, c*) and positive saline samples (*b, d*). All sections are counterstained with hematoxyline. Right column (*b, d*) shows the staining for $P2X_1$ receptors. The pictures show the cut through whole bladder, on the left it is an urothelium, sub-urothelium and connective tissue and muscle on the right side of the each picture.

However, the same observation was made even in the cystitis urinary bladder sections (Fig. 4). The brown staining shows where the $P2X_1$ receptors are present and the intensity of the colour the density of the receptors. The brown colour intensity (comparison of right columns of Fig. 5.3 (b, d) and Fig. 5.4 (f, h, j)) does not seem to show any significant differences in normal and inflamed bladders. For this reason we concluded there is no change in amount of $P2X_1$ receptors between healthy and cystitis urinary bladders.

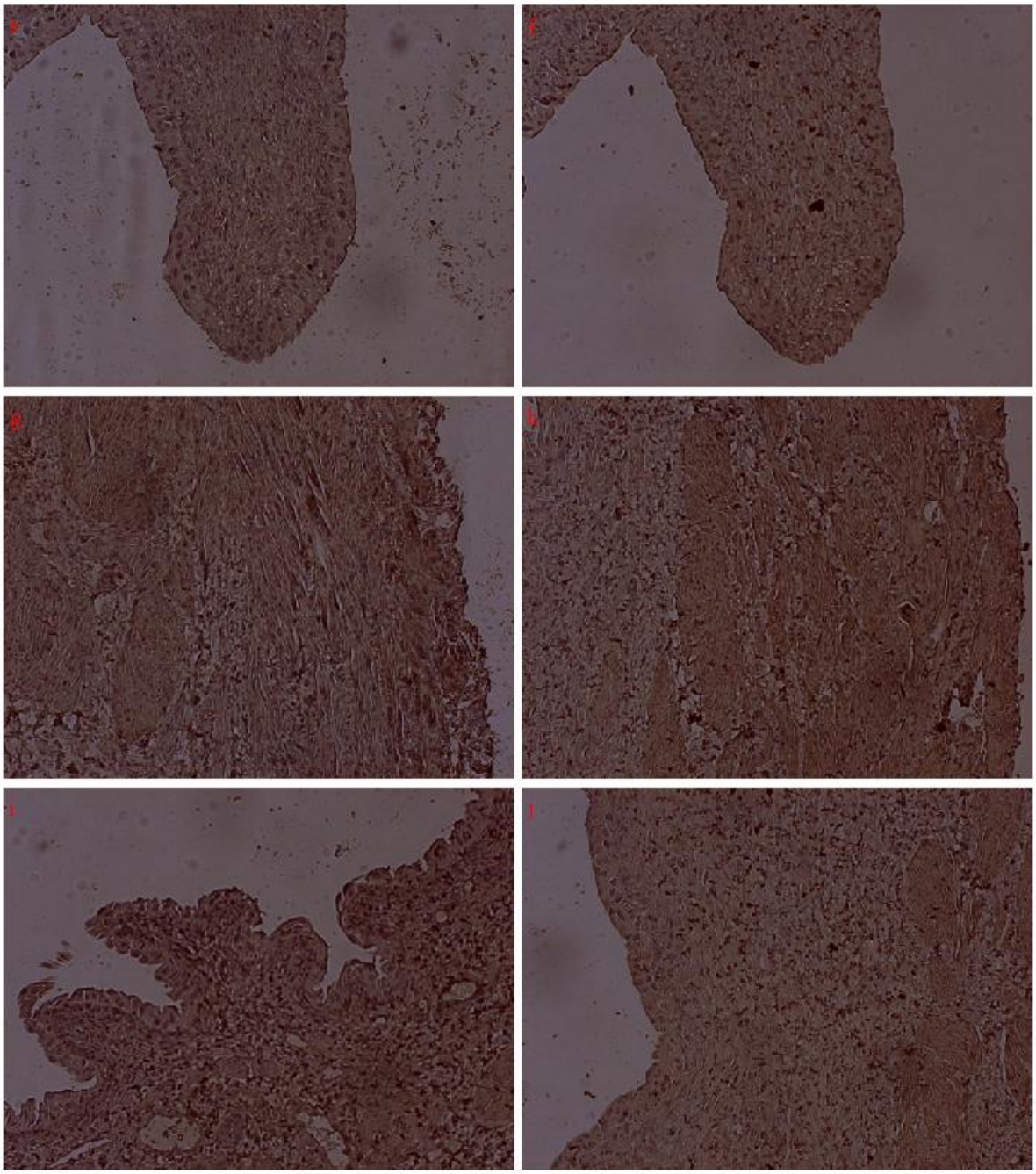


Fig. 5.4: Immunohistochemical labeling of bladders negative controls (*e, g, i*) and positive cystitis samples (*f, h, j*). All sections are counterstained with hematoxyline. Right column (*f, h, j*) shows the staining for $P2X_1$ receptors. Pictures *e, f* show the urothelium and suburothelium, *g,h* muscle, *i, j* urothelium and sub-urothelium.

5.2 Contraction experiments

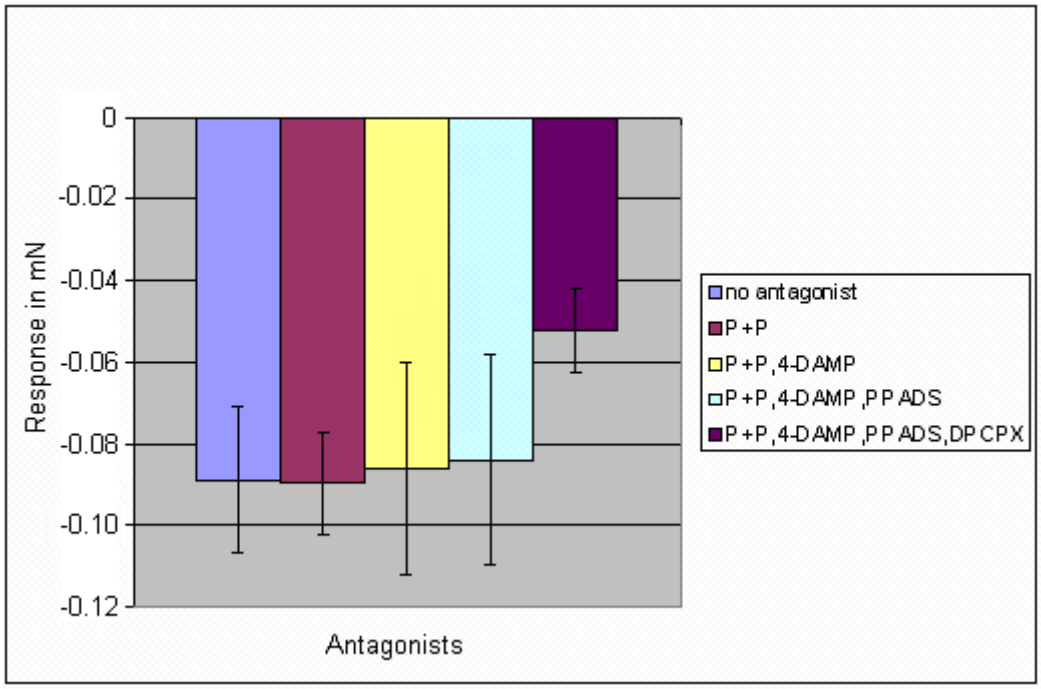
5.2.1 Stimulation by agonist

The adenosine in concentration of $5x10^{-5}$ M was used as an agonist. Five types of antagonists were added. Propranolol and phentolamine to block unspecific

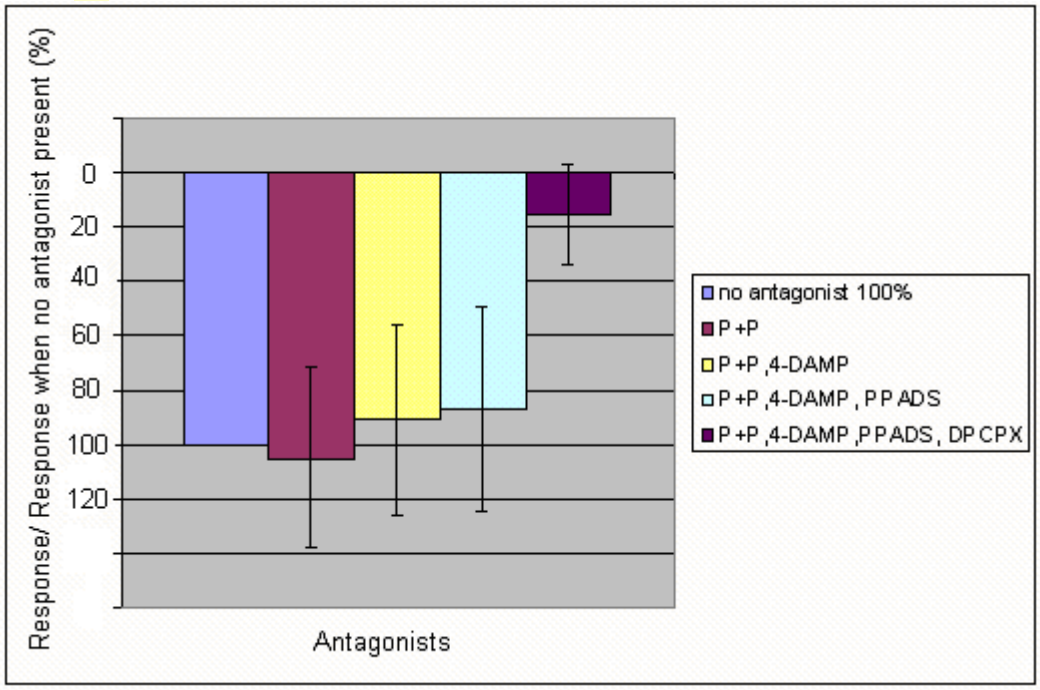
andrenergic responses, 4- DAMP as a blocker of muscarinic receptors, PPADS which blocks the P2 receptors generally and DPCPX- a specific irreversible antagonist of $P1A_1$ receptors.

The healthy rats

The relaxation of the bladder detrusor was observed and measured within one minute after the agonist was added. These graphs show the average values of five strips of the detrusor. Results for each of the graphs were obtained at the same day so the conditions for the measuring would be as similar as possible and the errors would be minimised.



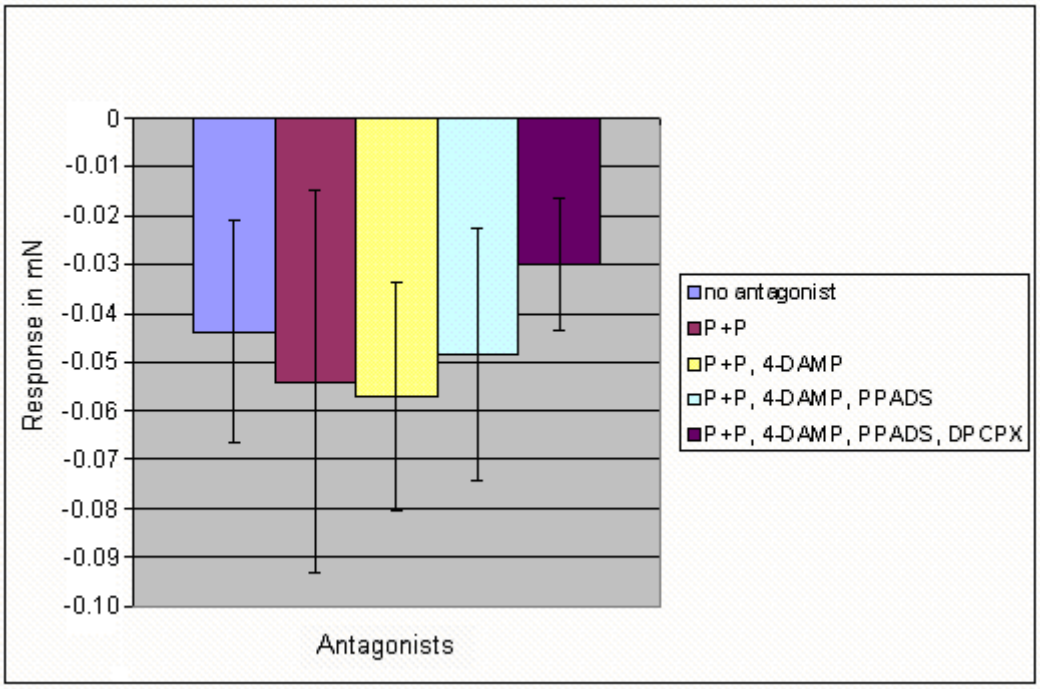
Graph 5.1: Healthy rats, stimulation by agonist. After agonist added, we obtained five values (five strips). Each of these values was divided by maximal response to high potassium krebs. Then the average was noted to the graph. There is a significant difference between the states before and after DPCPX was added. Vertical bars represent standard error deviation.



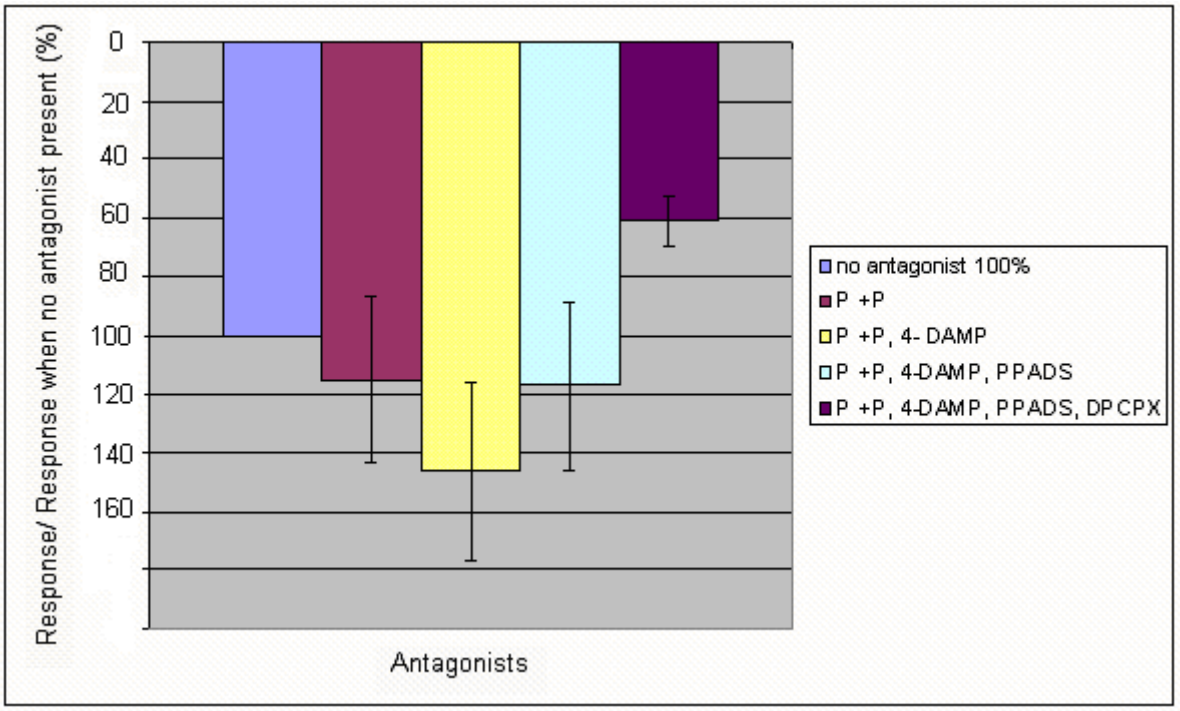
Graph 5.2: Healthy rats, stimulation by agonist. For this type of graph the same values like for the previous one were used. The procedure of getting the numbers for this graph was very similar to the graph 1. The difference makes the last step, when after dividing the values by response to high potassium krebs, the average value is divided by response of tissue to no antagonist. Using this way we get percentage expression of results. Vertical bars represent standard error deviation.

The cystitis rats

The exactly same experiments were performed with the cystitis rats. Results were processed in the same way. In comparison with the normal rats, adenosine evoked a larger relaxatory response in the presence of 4-DAMP and the relaxation was to a lesser degree inhibited by the specific irreversible antagonist of $P1A_1$ receptors DPCPX.



Graph 5.3: Cystitis rats, stimulation by agonist. Again there is a significant difference in response of the tissue when DPCPX added. A significant differences in responses were noted after adding 4- DAMP and DPCPX. Vertical bars represent standard error deviation.



Graph 5.4: Cystitis rats, stimulation by agonist. This graph expresses the experiment in percentages. Vertical bars represent standard error deviation.

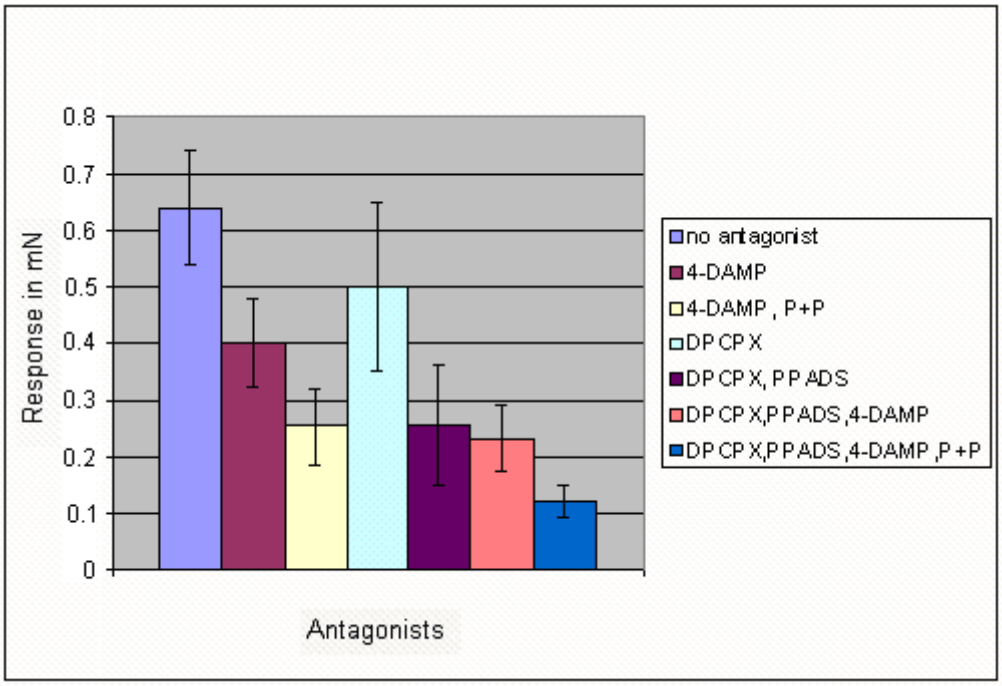
5.2.2 Electric field stimulation (EFS)

Electric field stimulation is another possible way to stimulate a tissue. The electric field stimulation at a frequency of 40 Hz was used in the experiments. For about 20 seconds the detrusor strips were exposed to an EFS and the maximum response was used for the calculation of the responses. All ion channels are open during EFS, which means both systems, i.e., the one for relaxation and also the one for contraction, are activated at the same time. The measured value is then a sum of maximums and minimums, i.e., a composite response is actually recorded.

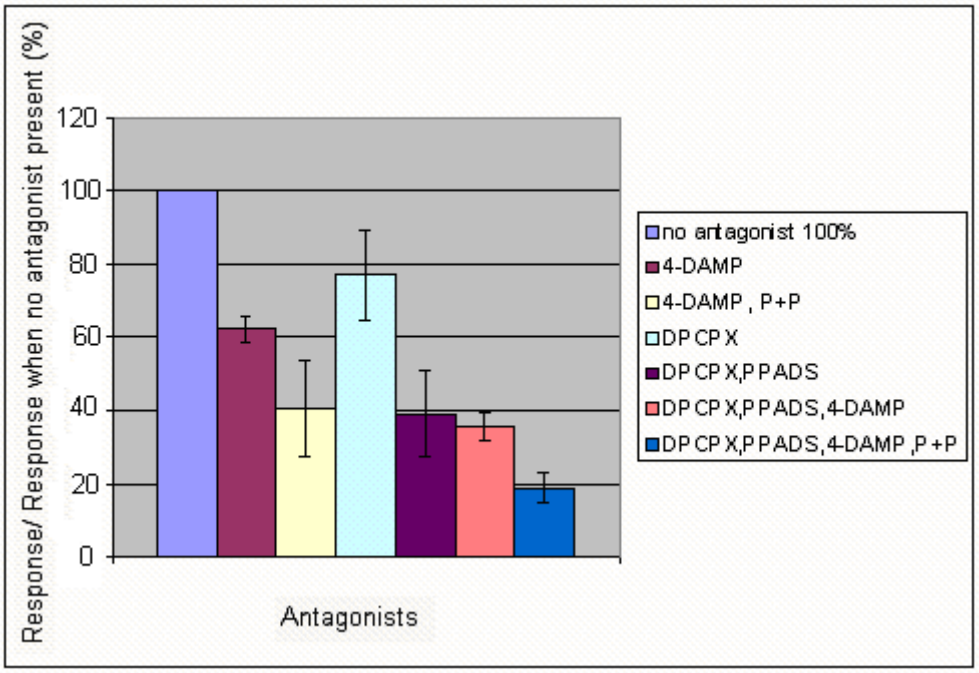
The experiment with EFS was run with small differences compared to the agonist stimulation. The main difference is the order of the added antagonists. First of all the 4-DAMP was added to show what happens when muscarinic receptors are blocked. Then phentolamine and propranolol blocked the adrenergic responses. After washing these three antagonists away, just DPCPX, which should block just $P1A_1$ receptors was added to see how the response will be changed. Since DPCPX is an irreversible antagonist, it is impossible to wash it out, and that is why the experiment continued by adding all of the other antagonist.

Healthy and cystitis rats

The graph shows the results in the EFS experiments and also the differences in the results between agonist and electric field stimulation. The same procedure was done with the bladder strips of cystitis rat. In the normal rats, muscarinic blockade reduced the contraction by 35% and the adrenergic blockade by a further 40%. In the presence of these antagonists DPCPX increased the response. Thus, it antagonized a adenosine relaxatory effect. In cystitis, this effect was close to absent.

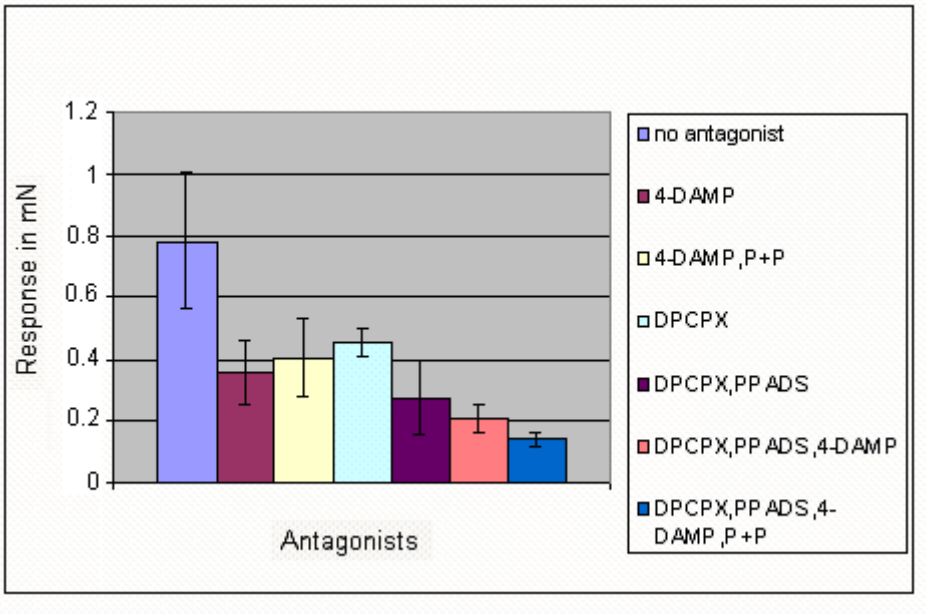


Graph 5.5: Healthy rats, electric field stimulation. Noted average of values divided by maximal response to high potassium krebs. Contrary to agonist stimulation, the EFS one show us more significant differences in response according to added antagonist. It is significant when 4- DAMP is added, significant chamge in response is also after DPCPX (according to "no antagonist" column), following PPADS adding affects the response very obviously, in the end phentolamine and propranolol change the response remarkably. Vertical bars represent standard error deviation.

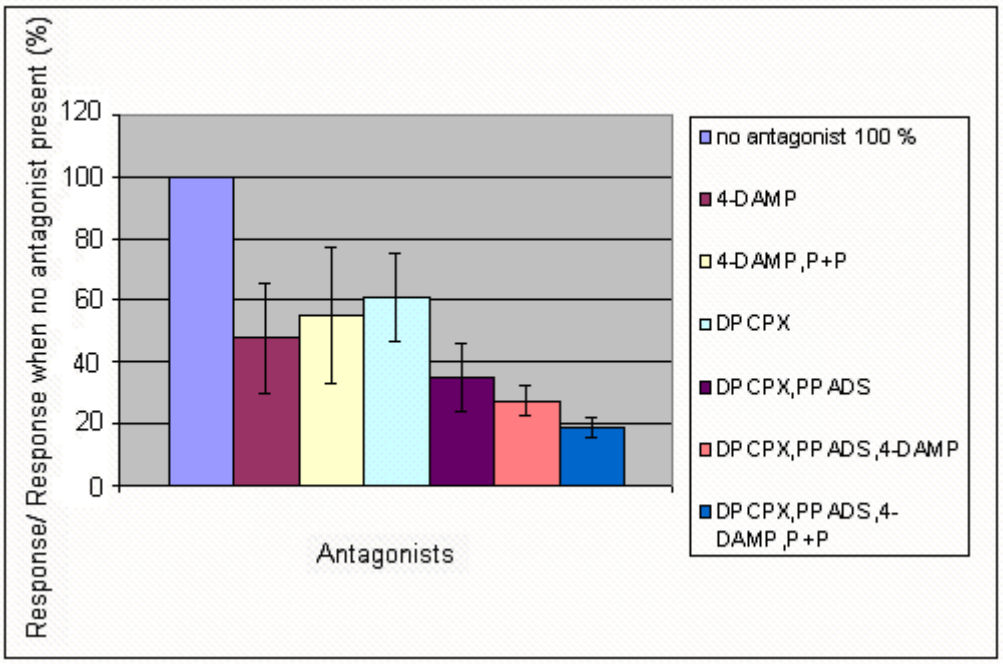


Graph 5.6: Healthy rats, electric field stimulation. This graph expresses the experiment in percentages. Vertical bars represent standard error deviation.

Cystitis rats



Graph 5.7: Cystitis rats, electric field stimulation. In this case we can observe a significant difference after adding 4- DAMP, propranolol and phentolamine kept the significant change. When washed just DPCPX was added and also this antagonist showed significant effect. PPADS, 4- DAMP and phentolamine and propranolol added in a row then lowered the response even more. Vertical bars represent standard error deviation.



Graph 5.8: Cystitis rats, electric field stimulation. This graph express the experiment in percentages. Vertical bars represent standard error deviation.

6 Discussion

There are five subtypes of muscarinic receptor present in urinary bladder and the importance of muscarinic M_3 receptors for the micturition is very well known (Lecci, 2002). The functional significance of all the five subtypes has not been established yet. But it has been proved that the subtypes of muscarinic receptors interact on neuronal as well as on non- neuronal cells in regulation of autonomic responses (Tobin *et al.*, 2009). Lately it has been suggested that the muscarinic receptors subtypes are connected also with inflammation and proliferation (Ventura *et al.*, 2002).

The contraction of the urinary bladder is primarily dependent on the activation of muscarinic receptors. It has been showed that muscarinic M_3 receptors are mainly responsible for the bladder contraction, more than any other of the subtypes, even though M_2 receptors may have contractile effects also. However, the M_2 receptors contributes most significantly by facilitating contractions by opposing relaxations induced by adenylate cyclase- coupled receptors such as the β- adrenoceptors and P1 purinoceptors (Hedge *et al.*, 1997).

It has been suggested that exogenous ATP and adenosine relax the smooth muscle of the pig urethra (Werkström, 2005). The highest levels of A_{2B} receptors are present in cecum, colon and bladder, followed by blood vessels, lung, eye and mast cells (Volpini, 2003). The immune system cells also express P1 receptors, and the cells are then responsive to the modulatory effects of adenosine in an inflammatory environment. In animal models, the adenosine receptors have been shown to be involved in inflammatory processes such as asthma, arthritis, sepsis and inflammatory bowel (Haskó, 2008).

According to already published experimental results, this project was focused on $P1A_1$ receptors. The aim of this project was to give the answer to three questions: Exist these receptors in the urinary bladder, and if they do, where are they located and do their location and expression change during the inflammatory process of the urinary bladder. The functional studies were run to find out how the change of the number of receptors would change the bladder function.

Immunohistochemistry results indicated clearly that $P1A_1$ receptors exist in the urinary bladder of the male rat. Very specific staining of a brown colour showed us that $P1A_1$ receptors are localized in all parts of the urinary bladder- in urothelium, suburothelium and even in the muscle.

The change of amount of the receptors during an inflammatory process was another question that I addressed. Immunohistochemistry run on the samples of the urinary bladder of rats pre- treated with cyclophosphamide, showed a different pattern compared to that in the healthy rats. The specific brown staining of the cystitis rat bladder was much less intensive than the glasses with healthy bladder. These findings support the suggestion that during the inflammation of the rat urinary bladder the expression of $P1A_1$ receptors is decreased.

As a control the same immunohistochemistry experiment was run with the $P2X_1$ receptors. The specific staining for $P2X_1$ receptors was used and showed, as been shown previously, that the receptors occur in the bladder, and that the intensity of the brown colour does not change significantly in inflammation. Tentatively, there is no change in expression of $P2X_1$ in the cystitis urinary bladder of rat.

The difference between staining for $P1A_1$ and $P2X_1$ receptors is considerable, which indicates that the immunohistochemistry results for $P1A_1$ are true reflections the an objective situation. This is of course strongly supported by the functional findings as well.

To find out how does this change affect the bladder function, the set of functional experiments was run. The functional studies were based on relaxation and contraction experiments respectively. In the first part of the functional experiment adenosine was used in concentration $5x10^{-5}$ mol/l as an agonist for $P1A_1$. To discover the best concentration of the agonist, we designed adenosine contraction curve. The adenosine concentration of $5x10^{-5}$ mol/l gave us a significant, sharp and relatively fast relaxation, and that was why this concentration was chosen for the next experiments.

By adding adenosine we only affected postsynaptic $P1A_1$ receptors. This means that the relaxation of tissue caused by adenosine is just due to receptors localized postsynaptically. As a selective $P1A_1$ antagonist we used DPCPX, which has been used for this purpose in many other experiments.

To blockade as many unspecific influences as possible, the group of specific antagonists for all receptors defined in urinary bladder of rat were used. Phentolamine and propranolol for blocking any unspecific reactions of adrenergic receptors, 4- DAMP for inactivation of muscarinic receptors and nonselective purinergic receptor PPADS to blocking all P2 receptors (Gulbransen, 2009).

At the beginning of each experiment the relaxation caused by the pure agonist was measured. Then the antagonists were added to the baths consecutively, and the relaxation caused by adenosine was measured every time after a new antagonist was added. According to these gained values, the graph was compiled. This experimental protocol was run twice, with healthy rat urinary bladders and with cystitis rat urinary bladders respectively. At the end of each experiment the bladder strips were weighed.

Several different ways to approach the questions raised in the thesis work were applied. First we tried to divide the individual responses of the strip in mN by its individual weight. But this option showed to be very inexact, since size and weight of the strips seem to have little correlation with the strength of contraction or relaxation of the strip. Mostly because the strips are prepared manually, which gives a variation of the preparations. More accurate information about the strip potency was obtained when the responses in mN wre normalized by maximal response of the strip to the high potassium krebs. These results are showed in graphs with odd numbers (Graph 5.1, 5.3, 5.5 and 5.7). Also a very clear impression was received when we divided these numbers by the response in the absence of antagonist. Then the data are of course given in percentage, as are shown in graphs with even number (Graph 5.2, 5.4, 5.6 and 5.8).

In healthy rat urinary bladders no significant change of the response to adenosine occurred when all the non-specific antagonists were added. The only significant difference in the responses occurred when DPCPX (selective antagonist of $P1A_1$ receptors) was added. This change shows how big the importance of $P1A_1$ receptors is for the relaxation of the urinary bladder tissue. The relaxation was reduced by over 80% by DPCPX . Therefore I conclude that $P1A_1$ receptors play a fundamental role in urinary bladder relaxation.

A significant reduction of the relaxation of the urinary bladder strips was recorded also in inflammatory urinary bladder. In this case the reduction caused by DPCPX amounted to 40% (reduction from 100% to 60% of relaxation). This change in the amount of the reduction could be caused by decreased quantity of the $P1A_1$ receptors, which would support the immunohistochemistry suggestion.

Electric field stimulation showed a different pattern. EFS opens all ion channels and EFS affect both presynaptic and postsynaptic receptors, since the intratissual neurons are activated also. Consequently, significant reductions in contraction occurred in the presence of 4- DAMP (muscarinic receptor blocker) and phentolamine and propranolol (adrenergic receptor blocker). However, after adding DPCPX the contractions to EFS once again almost reached the originally values before adding any blocker. That means that there has been a relaxation occurring that is counteracted by DPCPX. In the cystitis rats, this increase was absent. Also when the second round of administration of the antagonists, a similar pattern emerges. It is clear, there is remarkable difference between healthy and cystitis urinary bladder. There are few suggestions why this is happening which will be the objectives of next experiments. It is possible that cystitis would affect some other receptors, which are also sensitive to DPCPX. It is necessary run some more experiments to ensure that DPCPX is really just $P1A_1$ antagonist and that the solution is clean and does not affect anything else, especially some kind of contractile receptors. The next step should be to find out if there is any influence of DPCPX on M_5 receptors since we know their expression goes up during cystitis (Giglio, 2005).

There is a remarkable difference in behaviour of the specific antagonist of $P1A_1$ receptors DPCPX in urinary bladder strips stimulated by agonist of $P1A_1$ receptors adenosine and urinary bladder strips stimulated by electric field. The difference may be caused by location of the stimulation. While adenosine stimulate just the postsynaptic receptors, EFS acts on both, presynaptic and postsynaptic sides. Also it is important to find out more about properties of DPCPX. To answer these new raised questions will be object of following laboratory work.

7 Conclusion

The present study shows:

1. Both $P2X_1$ and $P1A_1$ purinoreceptors are present in the rat urinary bladder. This suggestion was proved by a very specific staining of a brown colour.

2. The staining also showed us that $P1A_1$ receptors are localized in all parts of the urinary bladder- in urothelium, suburothelium and even in the muscle. For this experiment the Avidin-Biotin Complex (ABC) method was used.

3. The expression of $P1A_1$ receptors during inflammatory process of the bladder is decreased. Purinergic response induced by adenosine (agonist of $P1A_1$ receptors) can be blocked by 80% (in healthy rat) or 40% percent respectively (in cystitis rat) by adding specific antagonist of $P1A_1$ purinoceptors DPCPX. Electric field stimulation showed differences in effect of DPCPX comparing to adenosine stimulation.

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