
# Regulation of autonomic activation and inhibition in the urinary bladder

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# *1.* Introduction

## *1.1. General introduction*

The optimal function of the urinary bladder is a complex of regulatory mechanisms that is mediated by the interaction between the autonomic nervous system and the voluntary nervous control. In normal individuals, there's no awareness of the bladder until it is full and the need to void is necessary. The bladder pathologies interfere dramatically with normal daily life influencing the quality of life because of its delicate nature. According to the ICS (International continence society), women are three times more frequently affected by regular urinary incontinence than men.

## *1.2. The anatomy and physiology of the bladder and urethra*

The urinary bladder is situated in the lower abdomen and it consists of several muscular layers. In its empty state, it forms a tetrahedral shape and in its filled state, it forms an ovoid shape. In the empty bladder, there is a superior surface, two inferolateral surfaces and a posteroinferior surface or base, where the bladder neck forms the lowest point. The urachus, which is connected at the apex of the bladder, anchors the bladder to the anterior abdominal wall. There are several anatomical differences between male and female bladder setout, but the most important thing is, that the body and fundus of the bladder is highly mobile and distensible and has a large capability of expanding into the abdomen during the filling phase *(Walsh et al., 2002)*. Histological, histochemical and pharmacological differences were found between the bladder neck and the bladder body. The bladder neck is a separated functional unit apart from the bladder body and it is also different in males and females. The bladder outlet is formed by the bladder base, urethra and external urethral sphincter. Whilst the male urethra is composed of the preprostatic portion, the prostatic urethra, the membranous portion and the penile urethra *(see Figure 1 for outline)*, the female urethra is only 4 cm long, protruding from the anterior wall to the external meatus *(Walsh et al., 2002).*

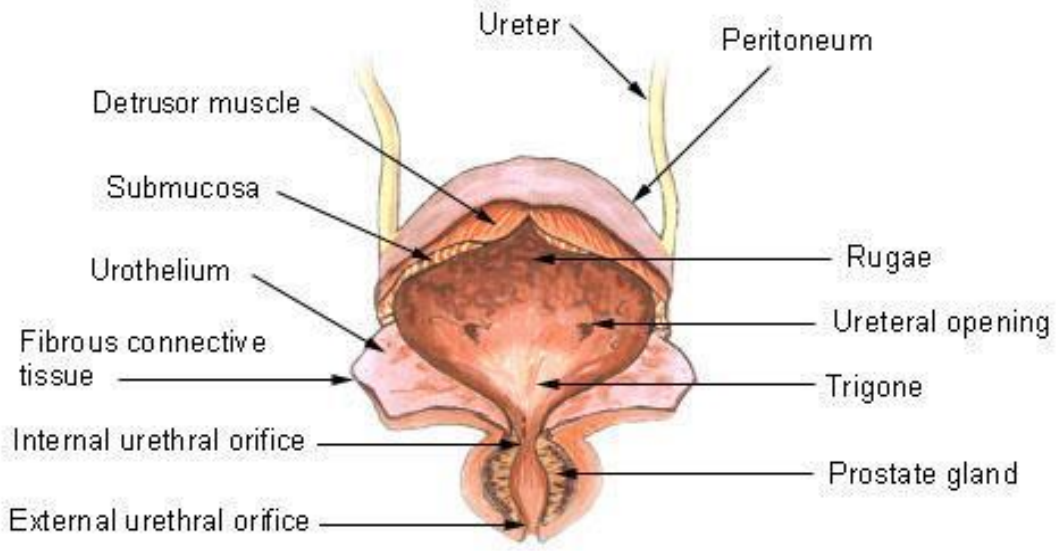


**Figure 1:**

Outline of the male urinary bladder. Adapted from Wikipedia.

(Wikipedia; 06-10-03, http://en.wikipedia.org/wiki/Image:Illu_bladder.jpg)

The main functions of the bladder are to store urine and also, when the bladder is full, to perform controlled voiding in controlled situations and time. The correct function is a result of cooperation of the somatic, parasympathetic and sympathetic nervous system. The main function of the bladder is divided into two phases, the storage phase and the micturition phase. During the storage phase the bladder distends, while the rise in intravesical pressure is notably small when the bladder volume is under the threshold volume for inducing voiding *(Torrens et al., 1987; Walsh et al., 2002)*. It has been discovered, that the hypogastric nerve inhibits bladder contractions thereby optimizing bladder filling *(de Groat, 1976)*. Furthermore, when the bladder is distended in response to bladder filling, the vessels need to stretch, whilst still maintaining sufficient blood supply to the smooth muscle *(Brading et al., 1999)*. Unfortunately, there is only partial knowledge about the relaxatory mechanism during the storage phase *(Andersson, 1999)*. After the storage phase comes the voiding (micturition) phase. The physiological capacity of the

human bladder is approximately 500 ml but the urge to urinate is already generated through the stretch receptors in the bladder wall at the level of about 200 ml of urine. Impulses from the bladder wall are transduced to the spinal cord via the pelvic nerves, initiating the micturition reflex *(Andersson, 2002; Morrison, 1999).* Furthermore, the hypogastric and pudendal nerve are also involved in the storage and micturition process initiating the micturition reflex *(Andersson, 2002).* The awareness of the bladder fullness is further generated through transmission via projection fibres to the cerebral cortex *(Walsh et al., 2002)*. During the bladder filling and of course increase of urine, the sensation of the bladder fullness becomes more and more acute. The center that controls the micturition is known as the pontine micturition centre (PMC), or “Barrington’s nucleus” *(Barrington, 1921)*. So, the impulse to expel urine from the bladder seems to be a result of pelvic nerve and muscarinic receptor activation together with an increase of the intraabdominal pressure *(Hemat et al., 2003).* During this process a domination of parasympathetic input could be seen while the sympathetic stimulation leads to relaxation of the internal urethral sphincter. The external sphincter, which is innervated by the pudendal nerve, relaxes voluntarily during micturition and after that the bladder is voided.

### *1.3. The urothelium*

The urothelium in vivo is a cell multiayer, that has an endodermal origin. It is composed of 3-6 cell layers and each of them has a different cell type. The basal cell layer is germinal in nature, having cells with diameter of 5-10 μm, the intermediate layer cells have a diameter of 20 μm and the superficial cells, also known as umbrella cells, have a changing diameter, depending on the degree of bladder stretch (50-120 μm across). The cell replacement happens by fusion of the basal cell layer to form intermediate cells and fusion of the intermediate cells to form umbrella cells *(Martin, 1972)*.

There are no special morphological aspects of basal and intermediate cells, whilst the superficial umbrella cells have two unique morphological

features. First the apical membrane is covered with scalloped-shaped plaques *(see Figure 2)* that are separated by plasma membrane domains called the “hinge”. Because of these plaques, the outer leaflet of the apical membrane appears thicker than the inner leaflet *(Porter et al., 1967)*. The second feature is the high density of cytoplasmatic vesicles of the umbrella cells, which are composed of two apposing plaques joined together by hinge membrane, with an associated cytoskeletal network of fine fibrils *(Hicks, 1975)*. The polygonal-shaped plaques occupy 70 to 90% of the apical surface area, while the hinge membrane, surrounding the individual plaques, occupy the remaining 10-30%. The plaques are composed of subunits, and each plaque contains approximately 1,000 subunits, which have a sixfold symmetry and are composed of an inner and outer ring, each consisting of six particles. There are also two 4 transmembrane domain proteins, UPIa and UPIb and two type 1 proteins, UPII and UPIII. These proteins form an inner and outer ring. The superficial umbrella cells are connected by interconnecting strands, which form tight junctions *(Peter, 1978)*. Resulting in that the combination of umbrella cells and tight junctions offer a physical barrier to the movement of substances between the urine and the blood.

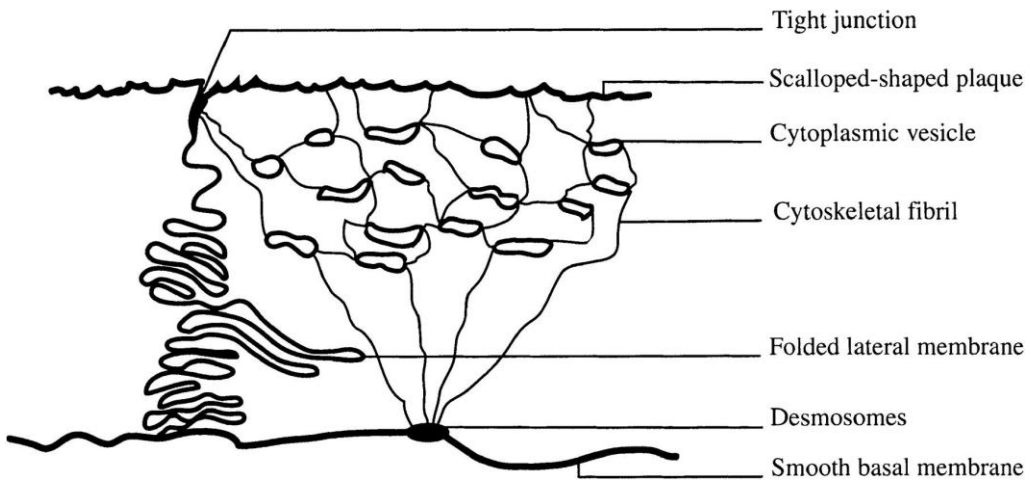


**Figure 2:**

Schematic of stretched umbrella cell. Adapted from Lewis, Everything you wanted to know about the bladder epithelium but were afraid to ask *(Lewis, 2000)*

### *1.4. Cholinergic regulation of the urinary bladder*

The parasympathetic part of the autonomic nervous system is widely expressed in the urinary bladder. The evidence for mRNA of all five muscarinic receptor subtypes has been found in the human urinary bladder *(Sigala et al., 2002)*. Muscarinic $M_1$, $M_3$ and $M_5$ receptors are coupled to $G_{q/11}$ proteins and mobilize phosphoinositides, to generate inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol, via activation of phosphoinositide-specific phospholipase Cβ, thereby increasing intracellular calcium. However, muscarinic $M_2$ and $M_4$ receptors inhibit elevated adenylate cyclase activity, as well as prolonging potassium channel, non-selective cation channel and transient receptor potential (TRP) channels opening *(see Figure 3 for outline; Zholos et al., 2004).*

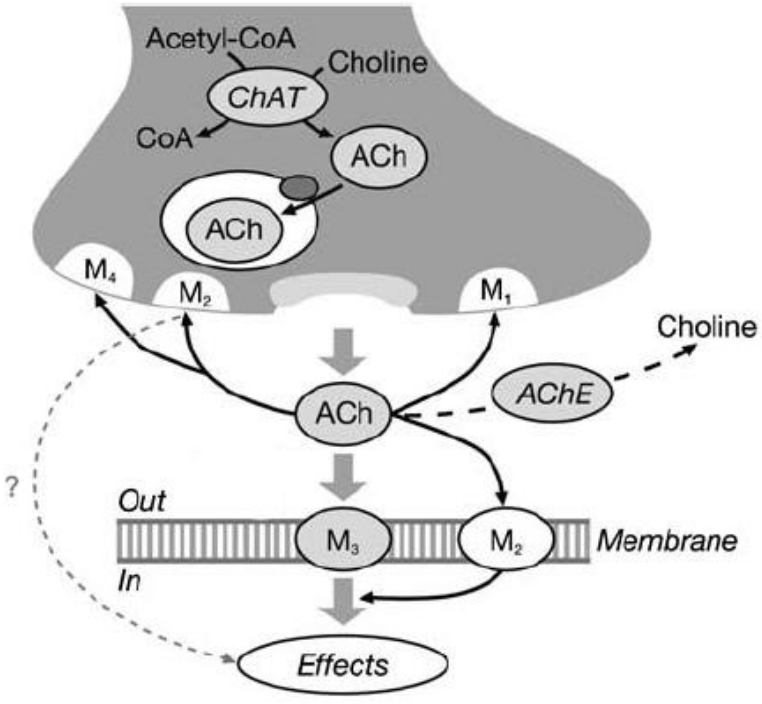


**Figure 3:**

The outline of the parasympathetic transmission in the detrusor. Adapted from Abrams, Muscarinic receptors: their distribution and function in body systems, and the implications for treating urge incontinence connected with overactive bladder *(Abrams et al., 2006)*

### *1.5. Functional role of muscarinic receptors in the urinary bladder*

The contraction of the urinary bladder is primarily dependent on the activation of muscarinic receptors. The mRNA of muscarinic $M_2$ and $M_3$ was identified in the smooth muscle of the bladder *(Kamai et al., 1994; Lambrecht et al., 1989)*. The immunoprecipitation analysis has shown that muscarinic $M_2$ population of receptors dominate quantitatively over $M_3$ in ratio 3:1. This was revealed in rats and humans, respectively *(Wang et al., 1995)*. Although in minority, the muscarinic $M_3$ receptors are responsible for almost entire cholinergic contractile response of the bladder, as was observed in functional and knockout studies *(Chess-Williams et al., 2001; Hegde et al., 1997; Matsui et al., 2002)*. The function of predominant $M_2$ remains only partly understood. However, in vivo studies, a contractile effect, mediated by $M_2$ receptors, has been observed on guinea-pig urinary bladder *(Sundquist, 1998)*. Furthermore, in vitro studies on rat bladder showed the indirect contractile effect of $M_2$ receptors, caused by the inhibition of the relaxatory mechanism of β-adrenoceptors, leading to optimization of voiding *(Hegde et al., 1997; Hegde et al., 1999)*. Besides the receptor population on the detrusor itself, muscarinic receptors are also located prejunctionally (excitatory $M_1$ likewise inhibitory $M_2$ and $M_4$ receptors) modifying the release of acetylcholine into the synaptic clefts *(Braverman et al., 1998; D'Agostino et al., 1986; Tobin et al., 1995)*. It was discovered that muscarinic $M_2$ and $M_4$ receptors are also located prejunctionally on adrenergic nerve terminals, inhibiting the release of noradrenaline in the bladder and urethra *(Trendelenburg et al., 2005)*. In connection with presence of muscarinic receptors in urothelium, a relaxatory factor, affecting the detrusor contractile response, has been found *(Hawthorn et al., 2000)*. The knowledge of role of the muscarinic $M_5$ receptor in the urinary bladder is limited at present, but it has been clarified that this subtype is closely related to the muscarinic $M_3$ receptor *(Bonner et al., 1988)*. It has not yet been shown whether the urinary bladder expresses functional muscarinic $M_5$ receptors but the evidence for mRNA of this subtype has been discovered. The reason why it is so difficult to characterize the presence and function of the $M_5$ receptor is that there is currently no specific muscarinic $M_5$ antagonist available and therefore it cannot be discriminated from the $M_3$ subtype *(Eglen et al., 2000).*

### *1.6. Adrenergic regulation of the urinary bladder*

Both subtypes of adrenoceptors are present in the urinary bladder. Whilst α-adrenoceptors populate the trigonum and the urethra, β-adrenoceptors are situated in the bladder body *(Levin et al., 1988)*.

It has been revealed that the $\alpha_1$ receptor subtype induces contraction in the detrusor of many species *(Andersson, 2007)*. There is an unclear opinion which subtype of α-adrenoceptor dominates in the urinary bladder. Some studies discovered that $\alpha_{1A}$- adrenoceptor is the predominant receptor subtype, while the expression of $\alpha_{1B}$- adrenoceptor and $\alpha_{1D}$- adrenoceptor on the mRNA and protein level is not detectable *(Walden et al., 1997)*. This is in contrast with the statement that the expression of $\alpha_{1D}$-adrenoceptor dominates over the $\alpha_{1A}$-adrenoceptor subtype in the urinary bladder, observed in the study from Hampel *(Hampel et al., 2002)* and Malloy *(Malloy et al., 1998)*. It has been revealed that $\alpha_2$-adrenoceptor population mediates the inhibition of both acetylcholine and noradrenaline release in the bladder of several species including rat, rabbit and man *(Mattiasson et al., 1987; Tobin et al., 1998)*, likewise in the urethra of rabbit and human *(Mattiasson et al., 1984)*.

Activation of β-adrenoceptors leads to relaxation of the urinary bladder smooth muscle *(Longhurst et al., 1999)*. The specific β-adrenoceptor subtypes differ between species. In the rabbit bladder, the relaxation seems to be mediated solely by $\beta_2$-adrenoceptors, while in the human bladder primarly by the $\beta_3$-adrenoceptors *(Igawa et al., 2001)*.

### *1.7. Purinergic mediated processes in the urinary bladder*

First knowledge about presently named Non-Adrenergic Non-Cholinergic (NANC) nerve transmission comes from the beginning of the 2Oth century. Furthermore, there was later discovered that the cholinergic nerve terminals contain two types of vesicles: clear vesicles and small dense-cored vesicles, suggesting the release of another transmitter together with acetylcholine *(Hoyes et al., 1975)*. In the urinary bladder, adenosine 5´-triphosphate (ATP) appears as the most important among the NANC transmitters (*Burnstock, 1980)*. ATP can be released by vesicular exocytosis

in response to cholinergic stimulation as well as in response to pelvic nerve stimulation as was shown in the cat. The release of ATP evokes bladder contractions, also clarified with HPLC technique *(Theobald, 1996)*. The mechanism of the bladder contraction mediated by ATP is the transient activation of ion-ligated $P_{2X}$ purinoceptors, mobilising calcium and sodium *(Benham et al., 1987)*. Moreover the role of ATP in the urinary bladder is to induce the biosynthesis of prostaglandins leading to another mechanism of contraction *(Husted et al., 1983)*. The predominant subtype of $P_{2X}$ purinoceptors in the human urinary bladder is the $P_{2X1}$ subtype *(O'Reilly et al., 2001)*. However, the relaxation of the bladder is probably dependent on the metabolite of ATP, adenosine, stimulating relaxatory $P_1$ purinoceptors *(Burnstock et al., 1972)*.

### *1.8. Urinary bladder pathology*

Lower urinary tract symptoms (LUTS) are common in the population and increase with age. Nowadays the urinary tract symptoms are subdivided into two main categories, obstructive and storage symptoms *(Roehrborn, 2001)*. The categories are related to the two main functions of the bladder – storage and voiding.

When the bladder becomes full in healthy individual, signals are conveyed to the brain that leads to awareness of the filled bladder and urgency to void appears. In certain time period the urgency to void can be restrain until the time and place to urinate are adequate. When the bladder is filled and the situation is suitable, micturition occurs rapidly with constant urinary flow until the bladder is entirely emptied. However, the urgency is normally suppressed during the night.

The most common pathology among the lower urinary tract symptoms is the urinary incontinence. According to the ICS definition, it's the "involuntary loss of urine that is objectively demonstrable and a social or hygienic problem" *(Abrams et al., 1988)*.

The urinary incontinence is furthermore divided into three different subgroups. The acute incontinence, chronic incontinence and mixed incontinence *(see Figure 4)*.

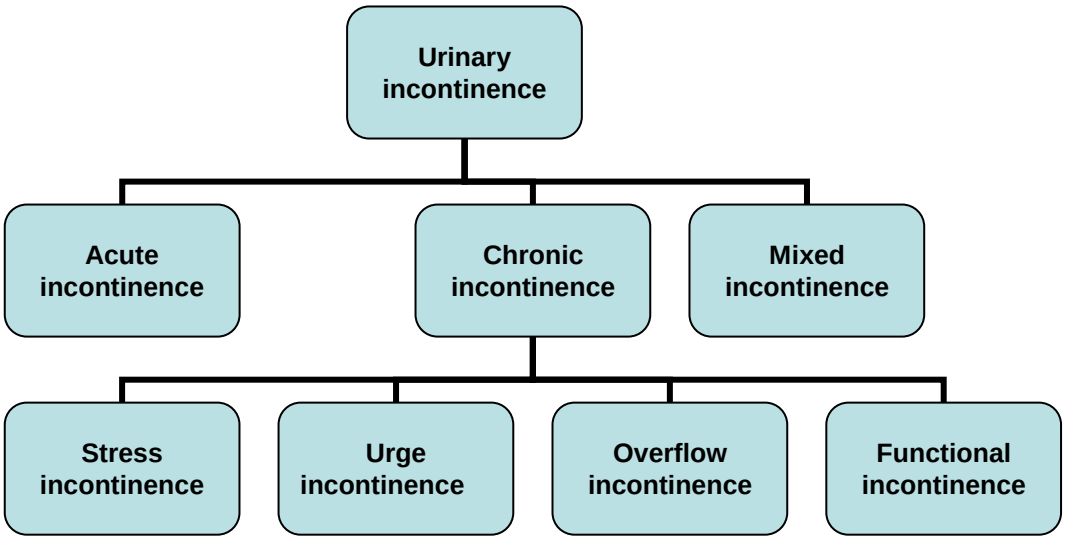


**Figure 4:**
Basic subdivision of the urinary incontinence problems

The etiologies of the acute incontinence are multiple and are often caused by medications such as antihypertensives that may affect the bladder negatively likewise as various mental conditions.

The chronic incontinence is subdivided into stress incontinence, urge incontinence, overflow incontinence and functional incontinence.

The stress incontinence is caused by an increase of the intra-abdominal pressure (e.g. coughing, sneezing, laughing, physical activities). The nature of the stress incontinence is damage of the innervation of the bladder neck and sphincters that could be likewise caused by neurological conditions such as myelodysplasia or by a structural damage after pelvic

surgery or trauma. Furthermore, birth, age and lack of oestrogen can cause breakdown in the bladder supporting structures and lead to leakage *(Walsh et al., 2002)*. Nowadays, the treatment of the stress incontinence is rather conservative such as weight loss, pelvic floor exercise or surgical by reinforcing the sphincter function *(Underwood, 2003)*.

The urge incontinence is defined as a sudden urge to urinate without any previous sensations *(Walsh et al., 2002)*. The terms connected with the urge incontinence is the “Overactive bladder” syndrome (OAB or unstable bladder) and the “Detrusor overactivity” (detrusor hyperreflexia). The overactive bladder syndrome occures during bladder filling, when the patient tries to inhibit micturition *(Underwood, 2003)*. The symptom that provides this disorder is frequent and urge expelling of small volumes of urine often during the night-time. Further assumptions may also be dementia or stroke influencing the cerebral cortex that normally suppresses the micturition reflex *(Underwood, 2003)*.

The overflow incontinence is defined as expelling of small amounts of urine without any previous urges, caused by a hindrance in the bladder outlet such as enlarged prostate gland due to benign prostate hyperplasia or cancer. Detrusor insufficiency can also occur due to diabetes or overdistension of the bladder.

The functional incontinence is a collective name for incontinence caused by immobility, orthopaedic limitations, depression or delirium.

The mixed incontinence is defined as a combination of two or more contributing factors leading to urine leakage.

The evidence revealed that women are more often affected by stress incontinence than by the other forms of incontinence whilst this condition is very uncommon among men. The urge incontinence occurs more among men but also older women are often affected. Nowadays, an under-diagnoses and undertreatment among this disorder is observed. This happens because incontinence is a hidden disorder and affected individuals do not often seek help *(Malmsten et al., 1997).*

### *1.9. Pharmacotherapeutical treatment of urinary incontinence*

Nowadays, first choice for women with stress incontinence is the hormonal treatment that balances back the lack of oestrogen (*Fantl et al., 1994)*, as already mentioned in the Urinary bladder pathology part. The urge incontinence is mostly treated with anticholinergics *(see Figure 5)*, but tricyclic antidepressants are also used for their beneficial relaxant effect on the bladder. However, long-term studies with antimuscarinic drugs revealed only poor effect on the condition and showed that placebo effect contributes markedly (by 30-50%) on the clinical improvement of patients with OAB. Also bad compliance among the anticholinergic medication was reported, because of poor treatment efficacy and various side-effects *(Kelleher at al., 1997)*.

| Agent | Mechanisms of action | Evidence | Reference |
|---|---|---|---|
| **Oxybutinin** | Muscarinic M1/M3 receptor antagonist, calcium antagonist and local anesthetic actions | In vitro smooth muscle relaxant effect (500 times weaker than antimuscarinic activity) Efficacy in OAB shown in clinical studies. Effective on intravesical administration | Reviewed by Andersson & Chapple (2001) |
| **Dicyclomine** | Nonselective muscarinic receptor antagonist, calcium antagonist action | Efficacy in OAB shown in clinical studies | Reviewed by Andersson et al. (1999) |
| **Propiverine** | Nonselective muscarinic receptor antagonist, calcium antagonist action | Efficacy in OAB shown in clinical studies | Reviewed by Andersson et al. (1999) |
| **Temiverine** | Selective muscarinic M3 receptor antagonist, calcium antagonist action | In-vitro inhibition of carbachol- and Ca-induced contractions in human detrusor muscle No published clinical data | Yono et al. (2000) |
| **Terodiline** | Nonselective muscarinic receptor antagonist, calcium antagonistic action | Efficacy in OAB shown in clinical studies Induced ventricular arrhythmias (Torsades de Pointes) | Reviewed by Andersson (1984; 1988) |

**Figure 5:**

Outline of muscarinic receptor antagonists with secondary mechanism of action.

Adapted from Abrams, Muscarinic receptors: their distribution and function in body systems, and the implications for treating urge incontinence connected with overactive bladder *(Abrams et al., 2006)*

## *2.* Aims of thesis

The aim of the thesis was to investigate the regulation mechanisms in the urinary bladder. The interaction between muscarinic $M_2$ receptors and $P_1$ purinoceptors as well as ATP-induced relaxation was in the center of interest. Furthermore, different drugs and substances were used in combinations with each other to reveal functional mechanisms in vitro conditions.

## *3.* Materials and methods

### *3.1. Experimental part*

All experiments were performed at Göteborg University (Sweden) and were approved by the regional ethical committee at Sahlgrenska hospital.

In the study, 58 male rats (300-350g) of the Sprague-Dawley strain were used. The urinary bladder was removed from the rats after anaesthetization with pentobarbital. The rats were killed with an overdose of pentobarbital after each experiment. Full thickness strips (6x2 mm), with the average weight of 5.1 mg, were excised from the detrusor smooth muscle. For the contraction experiments, the detrusor strip was mounted between two steel rods, of which one was fixed and the other adjustable. The strips were immersed in 20-ml organ baths *(see Figure 6)* containing Krebs bicarbonate solution (pH=7.25) of the following composition (mM): NaCl 118; KCl 4.6; CaCl 1.25; $KH_2PO_4$ 1.15; $MgSO_4$ 1.15; $NaHCO_3$ 25, and glucose 5.5. The solution was gassed with 5% $CO_2$ in $O_2$. The temperature of the organ baths was controlled and kept at 37°C by thermostat. The detrusor preparations were pre-stretched, which resulted in gradual tension relaxation. The preparations were repeatedly stretched resulting in a stable tension of about 3 mN, obtained after 30–45 min. The initial dose of carbachol (reference concentration, $10^{-5}$ M) was administered first before and then after the renewal of the Krebs buffer in each experiment. The contractile responses were expressed in percentage compared to the last reference response.

The volume of drugs, administered to the organ baths, was in all experiments 100 µl. The antagonists (pirenzepine, methoctramine, 4-DAMP, 8-sulfophenyltheophylline) were administered 20 minutes before the addition of agonists (carbachol, ATP, 2-chloro-adenosine). When the effect of ATP was observed in combination with carbachol, the nucleotide was added to the bath just prior to the administration of carbachol. In the testing of 2-chloro-adenosine, the administration of the nucleoside was performed when a relatively stable plateau of the response to the single concentration of carbachol was reached, which occurred within 2 min. after the peak of the response. The peak of the response to carbachol was reached within 30 s upon carbachol administration, and after 2 min. had declined to a level of 85–75% of the peak response. The concentration of carbachol was $10^{-5}$ M when a single carbachol concentration was used, since this concentration gives a response at the log phase in the concentration–response curve.

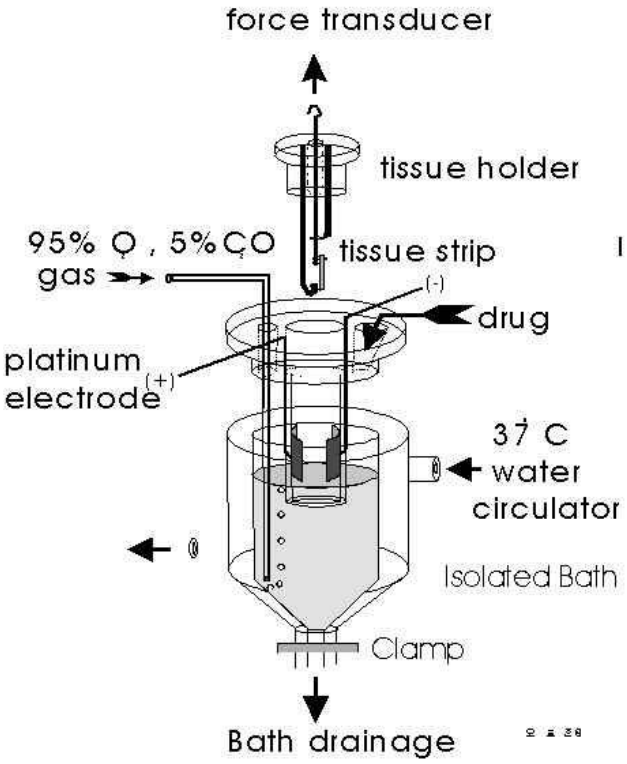


**Figure 6:**

Outline of the organ bath with functional description. Adapted from web.snuh.org. (08-03-26, web.snuh.org/~urology/html/organ_bath_str.jpg)

### *3.2. Substances and drugs*

All substances, used in this study were purchased from Sigma-Aldrich (*St. Louis, MO, USA*). Following drugs were used during the experiment: adenosine 5´-triphosphate (ATP), carbamylcholine chloride (carbachol), 2-chloro-adenosine, 4-diphenylacetoxy-*N*-methylpiperidine methobromide (4-DAMP), methoctramine hydrochloride, pirenzepine dihydrochloride and 8-*p*-sulfophenyltheophylline.

### *3.3. Results counting and statistical analyse*

Schild plots for competitive antagonism were used to estimate the $pA_2$ values. Statistical significance was obtained by Student's t-test for unpaired data or for paired data when relevant. For multiple comparisons with the same variable, a t-test of Bonferroni method was used. The level of significance was set to $p<0.05$ which represented the probability of non-random results higher than 95%. Microsoft Excel *(Microsoft Corp., Redmond, WA, USA)* and Prism *(GraphPad Software Inc., CA, USA)* software was used for creating tables, graphs and necessary calculations.

# *4.* Results

## *4.1. Carbachol-evoked responses*

An immediate response to carbachol in the urinary bladder strip preparations was observed when administered above a threshold of $5x10^{-8}$ to $5x10^{-7}$ M. The maximal contraction of the isolated muscle strips was 4.6 mN/mg tissue wet weight and was observed at a concentration of $5x10^{-6}$ to $5x10^{-5}$ M. 4-DAMP, methoctramine and pirenzepine all gave a rise to right shifts of the carbachol-evoked concentration-response curves *(see Figure 7)*.

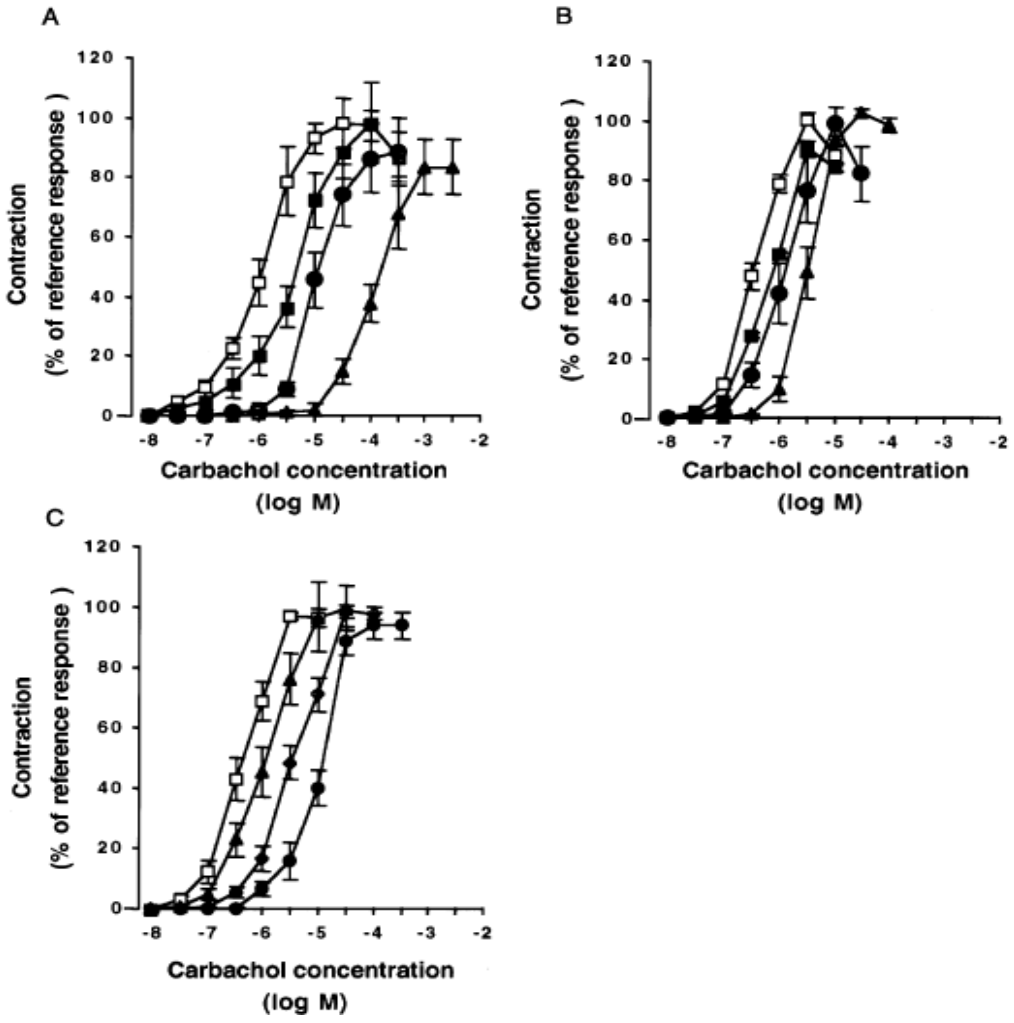


**Figure 7:**

Contractile responses in the absence of antagonists ( □ ) and in presence of ( A ) pirenzepine (■ $10^{-7}$ M; ● $10^{-6}$ M; ▲ $10^{-5}$ M), ( B ) methoctramine (■ $10^{-7}$ M; ● $10^{-6}$ M; ▲ $10^{-5}$ M) and ( C ) 4-DAMP (▲ $10^{-10}$ M; ◆ $10^{-9}$ M; ● $10^{-8}$ M). Contractile responses are expressed as percentages of an initially evoked reference carbachol response ( $10^{-5}$ M ). Vertical bars represent S.E.M.

4-DAMP shifted the curve to the right most noticeably from all the antimuscarinic drugs. The $pA_2$ value, according to the Schild analysis, for 4-DAMP was 9.8, while the $pA_2$ values for less potent pirenzepine were 7.0 and 6.5 respectively. A significance for methoctramine ($P<0.05$) was found. Administration of 4-DAMP ($10^{-9}$ and $10^{-8}$ M) in combination with low doses of methoctramine ($5x10^{-9}$, $5x10^{-8}$ and $10^{-7}$ M) showed no additional inhibition of the contractile carbachol-evoked contractions. However, when 4-DAMP ($10^{-8}$ M) was given in combination with methoctramine ($10^{-6}$ M) the contractions were additionally inhibited *(see Figure 8)*.

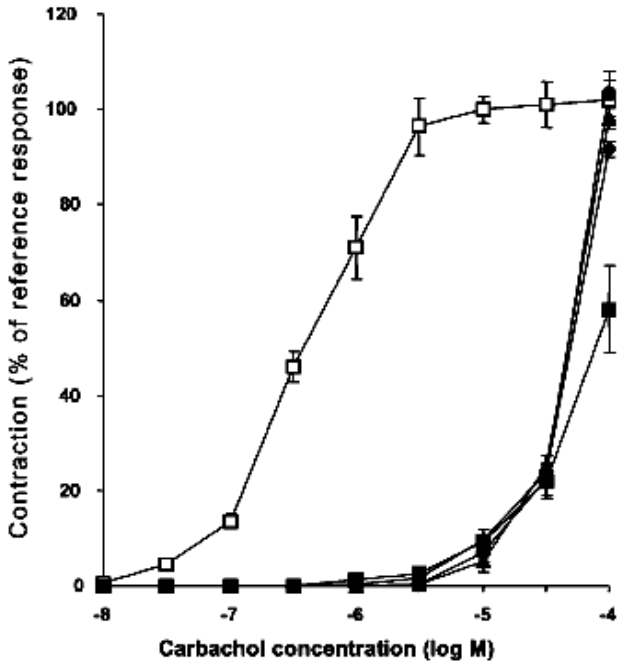


**Figure 8:**

Contractile responses to carbachol of isolated detrusor strips in the absence of antagonists ( □ ) and in the presence of methoctramine (◆ $5x10^{-9}$ M; ▲ $5x10^{-8}$ M; ● $10^{-7}$ M; ■ $10^{-6}$ M;) and 4-DAMP ($10^{-8}$ M). at different concentrations. Contractile responses are expressed as percentages of an initially evoked reference carbachol response ( $10^{-5}$ M ). Vertical bars represent S.E.M.

### *4.2. ATP-evoked responses*

ATP provoked concentration-dependent contractions, with a threshold concentration of $10^{-7}$ to $10^{-6}$ M. The maximal effect was observed at $5x10^{-3}$ M. A right shift tendencies of the concentration-response curve were observed when a low concentration of ATP ($10^{-5}$ M) was administered in combination with carbachol. Significantly smaller carbachol-evoked contractile responses occurred with concentrations of carbachol higher than $10^{-6}$ M, suggesting that ATP also evokes relaxation. This may result from the breakdown of ATP producing adenosine and was tested by pre-incubating the preparations with the adenosine receptor antagonist 8-p-sulfophenyltheophylline ($10^{-6}$ M). In the presence of this agent, the relaxatory effect of ATP on the carbachol-evoked responses was attenuated.

On the other hand, methoctramine ($5x10^{-8}$ M) quantitatively inreased the relaxatory response to ATP *(see Figure 9)*.

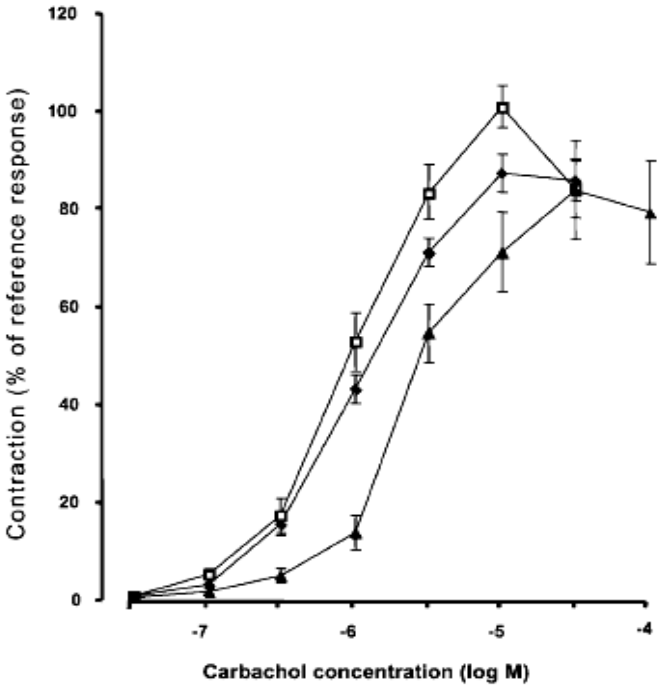


**Figure 9:**

Mean contractile responses to carbachol alone ( □ ), to carbachol after administration of ATP (◆ $10^{-5}$ M) and to carbachol after administration of ATP ($10^{-5}$ M) in the presence of methoctramine (▲ $5x10^{-8}$ M) of isolated detrusor strips at different carbachol concentrations. Contractile responses are expressed as percentages of an initially evoked reference carbachol response ($10^{-5}$ M). Vertical bars represent S.E.M.

### *4.3. Adenosine-evoked responses*

The agonist 2-chloro-adenosine, administered at concentrations of $10^{-8}$, $5x10^{-8}$ and $10^{-7}$ M, to pre-contracted urinary bladder strips (carbachol; $10^{-6}$ M), showed concentration-dependent relaxations, but no statistical significance was proved in comparison with carbachol-induced tension curves in the absence of 2-chloro-adenosine.

For further examination of 2-chloro-adenosine effects in the presence and absence of methoctramine, the concentration of $5x10^{-8}$ M of 2-chloro-adenosine was selected and the preparations were challenged by this concentration initially as well as at the end of the testing protocol. The administrations resulted in the same tension reductions (-28.4 ± 3.4% compared to -27.5 ± 3.1% respectively). The presence of methoctramine at $5x10^{-8}$ M and $10^{-7}$ M significantly enhanced the 2-chloro-adenosine-evoked relaxations *(*$p<0.05$; *see Figure 10)*. However, methoctramine at lower concentrations ($5x10^{-9}$ M; $10^{-8}$ M) did not affect the 2-chloro-adenosine-evoked relaxations.

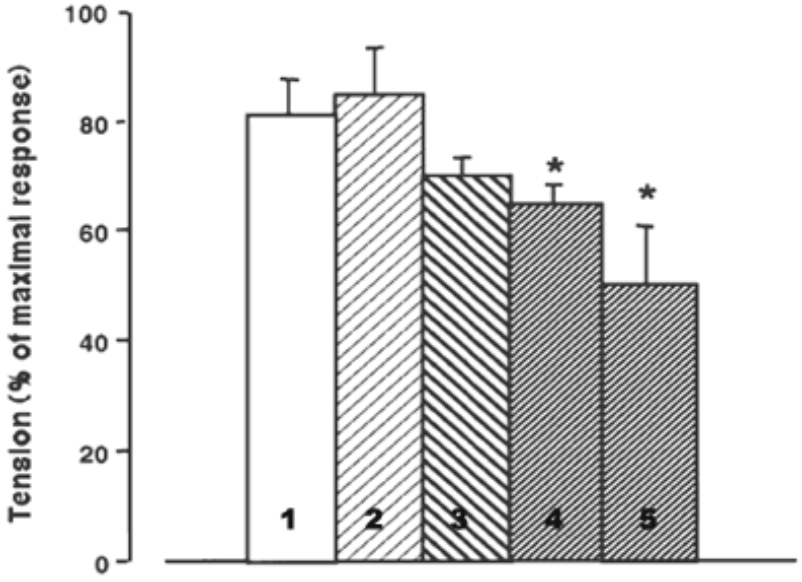


**Figure 10:**

Mean contractile tensions of isolated detrusor strips 2 min. after the peak of the response to carbachol ($5x10^{-6}$ M) in the absence (column no.1) and presence of methoctramine ($10^{-7}$ M; column no. 2), 2-chloro-adenosine ($5x10^{-8}$ M; column no. 3) and 2-chloro-adenosine+methoctramine ($5x10^{-8}$ M; column no. 4 and $10^{-7}$ M; column no. 5, respectively). Tensions are expressed as percentages of the peak contraction. Vertical bars represent S.E.M. ∗ $p<0.05$, in comparison with the decline in the absence of 2-chloro-adenosine and methoctramine.

## *5.* Discussion

The present study confirmed that muscarinic receptors play an important role in mediation of contractile responses in the urinary bladder. Furthermore, the evidence leads to conclusion that the subtype responsible for contraction in the rat urinary bladder is the $M_3$ receptor subtype. However, there was no evidence found for a direct muscarinic $M_2$ receptor mediation of cholinergic bladder contractions. The further study of the muscarinic $M_2$ receptors showed a modulating and inhibitory role of purinoceptor-mediated relaxation of the detrusor. In contrast, the muscarinic $M_2$ receptors did not affect purinergic contractile responses. Furthermore, the relaxatory part of the ATP induced biphasic response seemed to be due to effects of products from the breakdown of ATP acting on $P_1$ purinoceptors.

Furthermore, the present study showed the inhibitory potencies of the muscarinic antagonists on carbachol-evoked contractile responses of the urinary bladder strips which correspondents with the present knowledge about the antimuscarinic agents in the treatment of the urinary bladder pathologies *(Wang et al., 1995; Tobin, 1995)*. The analysis revealed 2000 times larger potencies of 4-DAMP on inhibition of carbachol-evoked contraction than methoctramine and 600 times larger than pirenzepine. The differences between the $pA_2$ values for the antagonists were consistent with a tissue expressing only functional $M_3$ receptors. Noteworthy, competitive antagonists have been reported to behave non-competitively at high concentrations *(Melchiorre, 1988)*.

Studies on contractile mechanisms in the gastrointestinal tract of the guinea pig revealed a direct contractile effect of the muscarinic $M_2$ receptor subtype after blockade of the muscarinic $M_3$ receptor subtype *(Ehlert et al., 1999)*. Therefore, it was presently wondered whether the administration of carbachol during muscarinic $M_3$ receptor blockade, in the presence of different concentrations of 4-DAMP, would cause reduction of contractions by muscarinic $M_2$ receptor antagonism. Because of this, methoctramine was administered at concentrations lower than $10^{-6}$ M, at which, a selective blockade of the muscarinic $M_2$ receptors occurs and this concentration doesn't

lead to further inhibition. At larger concentrations of 4-DAMP, a decrease in inhibition was observed, probably caused by a contemporary non-selective receptor blockade, leading to a contraction evoked by cholinergic stimulation via activation of a homogenous muscarinic receptor population. This was previously observed in study with mice lacking the muscarinic receptor gene for $M_3$ receptor subtype. However, the authors were not able to pharmacologically characterize the response, similarly to the present study *(Matsui et al., 2000)*.

Some studies describe that the contractile capacity of the muscarinic $M_2$ receptor subtype is smaller in vitro, than in vivo. This could probably be because of the effect of carbachol which is a nicotinic, as well as muscarinic receptor agonist and may activate postganglionic nerve fibres in vivo, which, at least in part, could explain the changes observed. The finding leads to speculation whether the $M_2$ receptors on the detrusor may have a similar opposing effect on parasympathetically evoked relaxatory responses, as on β-adrenoceptor-mediated relaxations *(Hegde et al., 1997; Hegde and Eglen, 1999)*. However, in this context, ATP seems to be particularly interesting, since this purine nucleotide was demonstrated to co-exist with acetylcholine in the parasympathetic neurons of the urinary bladder *(Theobald, 1996)*.

In the present study, ATP showed a dual response. The relaxatory effect was most evident on carbachol pre-contracted bladder strips, whilst the adenosine receptor antagonist 8-*p*-sulfophenyltheophylline attenuated the ATP-evoked relaxation. However, this relaxation seems at least partly to be due to the breakdown of ATP to adenosine that acts through the $P_1$ purinoceptors. The evidence revealed that a certain production of adenosine by ATP degradation also occurs in the rat urinary bladder.

An inhibitory effect on direct or indirect relaxatory effects by other parasympathetic transmitters than acetylcholine could be an explanation for an antagonistic effect of muscarinic $M_2$ receptor activation, evident in vivo, but hardly in vitro. For the examination of possible muscarinic influence on purinergic relaxatory responses, 2-chloro-adenosine was administered to the carbachol pre-contracted strips, but no significant responses were observed. Although tendencies to dose-dependent relaxatory responses occured. On

the other hand, administration of 2-chloro-adenosine in the presence of methoctramine induced significant relaxations of the carbachol pre-contracted urinary bladder strips, corresponding with suggestion that ATP generated products which activated adenosine receptors ($P_1$ purinoceptors). Apart from this, a certain direct muscarinic $M_3$ receptor antagonism could contributed to the reduction of the tension.

Notably, methoctramine did not affect ATP evoked relaxations of potassium pre-contracted strip preparations, indicating that muscarinic effects are necessary for the methoctramine-induced enhancement of ATP relaxation.

### *5.1. Conclusions*

The current study demonstrates a modulatory role of the muscarinic $M_2$ receptor population on effects connected with adenosine receptors that may be interpreted as an antagonism of contractions caused by stimulation of parasympathetic nerves. The evidence for this is that, first, the adenosine-evoked relaxation of carbachol pre-contracted strips was significantly enhanced by muscarinic $M_2$ receptor blockade. Second, this muscarinic $M_2$ receptor blockade showed no effect in the absence of purine agonists, and third, the muscarinic $M_2$ receptor blockade also enhanced ATP-induced relaxation. Furthermore, other facilitatory and inhibitory mechanisms, such as a direct muscarinic $M_2$ receptor contractile effect, could play important role on parasympathetic nerve terminals and may complicate the interpretation of results from the experiments *(Somogyi and De Groat, 1992; Tobin and Sjögren, 1998)*.

### *5.2. Závěr*

Tato studie prokazuje vliv muskarinových $M_2$ receptorů na adenosinové receptory, který by mohl být interpretován jako antagonismus kontrakcí, vyvolaných stimulací parasympatických nervů. Důkazy pro to jsou, za prvé, adenosinem vyvolaná relaxace karbacholem pre-kontrahovaných preparátů byla signifikantně zesílena blokádou muskarinových $M_2$ receptorů. Za druhé, tato blokáda neměla žádný efekt bez přítomnosti purinových agonistů, a za třetí, blokáda těchto receptorů také posílila relaxaci způsobenou ATP. Na těchto efektech se ale můžou podílet i další aktivační a inhibiční mechanismy, např. přímý kontrakční efekt $M_2$ receptorů může hrát významnou roli na parasympatických nervových zakončeních a komplikovat tak interpretaci výsledků.

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