# Inhibition of cholinergic neurotransmission by $\beta_3$-adrenoceptors in the rat urinary bladder: Role of adenosine release and $A_1$ receptors activation

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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# Abbreviations

A - Amplitude

AR – Adenosine Receptors

ABC - ATP-binding cassetetransporters

ACh - Acetylcholine

ADO – Adenosine

ADP – Adenosine diphosphate

AMP – Adenosine 5'- monophosphate

ATP - Adenosine triphosphate

ANOVA – Analysis of Variance

$\beta_n$-AR - $\beta_n$- adrenoceptors

BPM - Beats per minute

cAMP - Cyclic adenosine 5'-monophosphate

CNS - central nervous system

CHO – Chinese hamster ovary

DAPR – Decerebrate artificially-perfused rat

DPCPX - 1,3-dipropyl-8-cyclopentylxanthine

$\Delta$t - Contraction duraction

Ecto - NTPDase - Ectonucleoside triphosphate diphosphohydrolase

ENaC - Epithelial sodium channel

ENT- Equilibrative nucleoside transporters

GAG - glycosaminoglycan

HPLC - High-performance liquid chromatography

HX – Hypoxanthine

ICI- Intercontraction interval

INO – Inosine

iNos - Inducible nitric oxide synthase

LUTS - Lower urinary tract symptoms

OAB - Overactive bladder

PTh - Pressure threshold

$M_n$ - Muscarinic receptors

NaCl- Sodium chloride

NADPH - Nicotinamide adenine dinucleotide phosphate

NBTI - S-(4-nitrobenzyl)-6-thioinosine

NO - nitric oxide

NVC - Non-voiding contractions

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Another work was presented in IJUP' 16 (9º Encontro de Jovens Investigadores da Universidade do Porto), in a form of a poster: Timóteo, M. A., **Costa, A.F.**, Vieira, C., Silva, I., Correia-de-Sá, P. Complex roles of muscarinic receptor subtypes in the bladder of urethane-anaesthetized male wistar rats. (Abstract in supplement 1)

# Abstract

The overactive bladder syndrome (OAB) is diagnosed in patients presenting urgency, with or without urge incontinence, usually accompanied by frequency and nocturia. The clinical success of $\beta_3$-adrenoceptor agonists, like mirabegron, for the management of the OAB syndrome has generated a great interest regarding their mechanisms of action. *In vivo* urodynamic studies demonstrated the inhibitory effect of these drugs in the storage phase of the urinary cycle without affecting the voiding phase; in contrast to other β-adrenoceptor agonists, selective $\beta_3$ agonists do not affect heart rate. Studies performed in isolated bladder preparations indicate that activation of $\beta_3$-adrenoceptors improve overactive bladder symptoms by directly relaxing detrusor smooth muscle contractions as a consequence of intracellular cyclic AMP accumulation. Activation of $\beta_3$-adrenoceptors may also inhibit the release of ACh from parasympathetic nerves innervating the detrusor, but there is no evidence for $\beta_3$ receptors being present on bladder cholinergic nerve terminals. In this study, we hypothesized that adenosine formed from the extracellular catabolism of cyclic AMP in detrusor smooth muscle fibers could be the retrograde messenger responsible for the indirect inhibitory control of cholinergic activity by $\beta_3$-adrenoceptors in the rat urinary bladder, via the activation of prejunctional $A_1$ receptors. Cystometry recordings in urethane-anaesthetized rats demonstrated that intravesical application of isoprenaline, a non-selective β-adrenoceptor agonist, and the selective $\beta_3$-adrenoceptor agonist, CL316,243 (0.001-1 μM), increased the inter-contraction interval (ICI), i.e. decreased the voiding frequency, in a concentration dependent-manner. The inhibitory effect of isoprenaline was prevented by selectively blocking $\beta_3$-adrenoceptors with SR59230A (100 nM) and $A_1$-receptors with DPCPX (100 nM). Incubation of detrusor strips of the rat urinary bladder with isoprenaline (1 μM) increased significantly the concentration of adenosine in the incubation fluid. However, endogenous adenosine does not seem to result from the extracellular catabolism of release cyclic AMP in the rat urinary bladder. This was concluded because we detected only small amounts of the nucleoside in preparations incubated with cyclic AMP (30 μM) for 45 min. Interestingly, inhibition of equilibrative nucleoside transporters (ENT) with dipyridamole (0.5 μM) or S-(4-nitrobenzyl)-6-thioinosine (NBTI) (30 μM) also prevented the inhibitory action of isoprenaline on the voiding frequency. In addition to the inhibitory effect of isoprenaline (1 μM) in urodynamic studies *in vivo*, we show here that this compound decreases by 38±1% the release of [$^3$H]-ACh from urothelium-denuded bladder strips *in vitro* stimulated electrically (10 Hz, 200 pulses). Again, isoprenaline (1 μM) inhibitory action on transmitter release was

prevented by SR59230A (100 nM) and by DPCPX (100 nM), as well as after blocking ENTs with dipyridamole (0.5 μM) and NBTI (30 μM). The selective $\beta_3$-adrenoceptor agonist, CL316,243 (1 μM), and the adenylyl cyclase activator, forskolin (3 μM), mimicked the inhibitory effect of isoprenaline (1 μM). Immunolocalization studies using confocal microscopy confirmed that $\beta_3$-adrenoceptors are widely distributed on detrusor smooth muscle membranes, but no co-localization was observed with the $A_1$ receptor immunoreactivity on VAChT-positive cholinergic nerves. We also found that ENT1 immunoreactivity is located predominantly on the plasma membrane of smooth muscle fibers, whereas the ENT2 mainly appears in interstitial spaces of detrusor muscle bundles. Data presented here suggest that inhibition of cholinergic neurotransmission by $\beta_3$-adrenoceptors activation results from adenosine release via equilibrative nucleoside transporters (probably of the ENT1 subtype) leading to retrograde activation of prejunctional $A_1$ receptors in the rat urinary bladder.

**Keywords:** Urinary bladder; Overactive bladder; $\beta_3$-adrenoceptors; Adenosine $A_1$ receptors; Adenosine

# Resumo

A síndrome da bexiga hiperativa (OAB) é diagnosticada em doentes com urgência miccional, com ou sem incontinência, geralmente caracterizada por polaquiúria e noctúria. O sucesso clínico dos agonistas dos recetores adrenérgicos $\beta_3$, como o mirabegron, no tratamento da OAB alertou para a necessidade de descobrir com exatidão o seu real mecanismo de ação. Ensaios urodinâmicos *in vivo* demonstraram o efeito inibitório destes fármacos sobre a fase de armazenamento da urina durante o ciclo miccional sem afetar de forma considerável a fase de esvaziamento nem o ritmo cardíaco, ao contrário de outros agonistas dos recetores β-adrenérgicos. Por outro lado, experiências *in vitro* em preparações de bexiga isolada sugeriram que a estimulação dos adrenocetores $\beta_3$ melhora os sintomas de hiperatividade da bexiga devido ao relaxamento das contrações do detrusor resultantes da acumulação intracelular de AMP cíclico. Sabe-se, ainda, que a ativação dos adrenocetores $\beta_3$ pode inibir a libertação de ACh dos nervos parassimpáticos da bexiga, mas não existe nenhuma evidência de que os adrenocetores $\beta_3$ estejam presentes nos terminais nervosos colinérgicos. Este trabalho foi delineado para explicar a ação indireta dos recetores adrenérgicos $\beta_3$ sobre a transmissão colinérgica na bexiga de ratazana, partindo do princípio que esta pode ser mediada pela formação de adenosina a partir do catabolismo extracelular do AMP cíclico libertado pelas fibras musculares lisas e, subsequente, ação retrógrada sobre recetores inibitórios do subtipo $A_1$ nos terminais nervosos colinérgicos. Os estudos urodinâmicos *in vivo* em ratazanas Wistar Han anestesiadas com uretano mostraram que a isoprenalina, um agonista não-seletivo dos adrenorecptors β, e o agonista seletivo dos receptores $\beta_3$, CL316,243 (0,001-1 μM), aumentam o intervalo entre contrações esvaziantes da bexiga (ICI), i.e. reduzem a frequência miccional, de forma dependente da concentração. O efeito inibitório da isoprenalina foi prevenido após o bloqueio seletivo dos adenocetores $\beta_3$ com SR59230A (100 nM) e dos recetores $A_1$ da adenosina com DPCPX (100 nM). A incubação de tiras isoladas de bexiga de ratazana sem a camada urotelial com isoprenalina (1 μM) promoveu a libertação de adenosina para o meio de incubação. No entanto, a adenosina libertada não parece provir do catabolismo extracelular do AMP cíclico, já que a incubação das preparações com AMP cíclico (30 μM) não aumentou significativamente os níveis de adenosina no meio de incubação. A inibição dos transportadores equilibrativos de nucleósidos (ENT) com dipiridamole (0,5 μM) ou NBTI (30 μM) também foi capaz de prevenir o efeito inibitório da isoprenalina (1 μM) sobre a frequência miccional. Os resultados deste trabalho mostraram, ainda, que a isoprenalina é capaz de reduzir em 38±1% a libertação de [$^3$H]-ACh estimulada

electricamente (10 Hz, 200 pulsos) no detrusor de ratazana. O efeito inibitório da isoprenalina (1 μM) sobre a libertação de [$^3$H]-ACh foi prevenido pelo SR59230A (100 nM) e pela DPCPX (100 nM), bem como após o bloqueio dos ENT pelo dipiridamole (0,5 μM) e NBTI (30 μM). Tanto o agonista seletivo dos recetores $\beta_3$, CL316,243 (1 μM), como o ativador da adenilciclase, forskolin (FSK, 3 μM), mimetizaram o efeito inibitório da isoprenalina (1 μM) sobre a libertação de [$^3$H]-ACh. Ensaios de imunolocalização por microscopia confocal mostraram que os recetores $\beta_3$-adrenérgicos estão difusamente distribuídos nas membranas das fibras musculares lisas do detrusor da ratazana, mas não colocalizam com o recetor $A_1$ da adenosina nos nervos colinérgicos marcados contra o transportador vesicular da ACh (VAChT). Já o ENT1 foi encontrado predominantemente na membrana plasmática das fibras musculares lisas, enquanto o ENT2 existe em maior quantidade nos espaços intersticiais dos feixes musculares do detrusor. Estes resultados sugerem que a inibição da neurotransmissão colinérgica causada pela ativação dos recetores adrenérgicos $\beta_3$ parece resultar da libertação retrógrada de adenosina por intermédio de transportadores equilibrativos nucleósidos (muito provavelmente do tipo ENT1), cuja atividade se manifesta através de recetores inibitórios do subtipo $A_1$ localizados nos terminais nervosos colinérgicos da bexiga de ratazana.

**Palavras-chaves:** Bexiga; Bexiga hiperativa; adrenocetores $\beta_3$, Recetores da adenosina $A_1$; Adenosina

# 1. Introduction

## 1.1 Lower urinary tract symptoms: Overactive bladder

Lower urinary tract symptoms (LUTS) represent one of the most common clinical complaints in men and women due to structural or functional abnormalities in one or more parts of the lower urinary tract, which involves the bladder, bladder neck, prostate (in men), distal sphincter mechanism and urethra (Abrams et al., 2002, 2013).

LUTS can be divided into three groups: storage symptoms (increased daytime urinary frequency, nocturia, urgency and incontinence), voiding symptoms (slow stream, splitting or spraying, intermittent stream, hesitancy, straining and terminal dribble) and post-micturition symptoms (feeling of incomplete emptying and post-micturition dribble). Overactive bladder syndrome (OAB) is a subset of storage LUTS and a common condition that is associated with a negative impact on quality of life. OAB is defined as urgency, with or without urge incontinence, usually accompanied by frequency and nocturia (Abrams et al., 2002).

Population-based estimates suggest that OAB affects 12–17% of adults in Europe and the United States (Irwin et al., 2006). Despite not being a terminal disease, it is normally a chronic condition and has a devastating impact on quality of life. The patient has fear of wetting accidents leading to social isolation and depression (McGhan, 2001). Frequent nocturnal voids, resulting in sleep disturbances, can prejudice daytime function, especially in older individuals (MacDiarmid, 2008). Recurrent urinary tract infections and urinary dermatitis are more prevalent among these patients, especially in ones that have urge incontinence (Brown et al., 2000).

The treatment of OAB, normally, consists of a combination of non-pharmacologic and pharmacologic approaches. Non-pharmacologic treatment options include dietary and fluid modifications, behavioral therapy, and pelvic floor muscle rehabilitation (MacDiarmid, 2008). The pharmacologic treatment of choice for OAB has been muscarinic receptor antagonists. However, these drugs often cause unwanted side effect resulting in treatment discontinuation (Andersson, 2013). So, it is urgent to find new pharmacologic targets, with less adverse effects in order to improve the quality of life of these patients. Recent studies have explored purinergic signaling pathways and β-adrenoceptors as new therapeutic targets for OAB.

## 1.2. The urinary bladder anatomy

The lower urinary tract comprises the urinary bladder and an outlet consisting of the bladder neck, the urethra and the urethral sphincter (Aldousari and Corcos, 2016). The urinary bladder is a reservoir. Its size, shape, position and relations vary according to its content and to the physiological state of neighboring viscera. The base of the bladder is triangular and located posteroinferiorly. In females it is closely related to the anterior vaginal wall. In males it is related to the rectum although it is separated from it above by the rectovesical pouch, and below by the seminal vesicle and vas deferens on each side (Gray's Atlas of Anatomy, pag 1245).

Anatomically, the bladder is divided into the bladder body, which consists of smooth muscle, and the base, which includes the trigone, urethrovesical junction and bladder neck that are intimately connected to the pelvic floor. The bladder outlet has two urethral sphincters, the internal (smooth muscle) sphincter in the bladder neck and proximal urethra and the external (striated muscle) sphincter of the membranous urethra (Figure 1) (Dorsher and McIntosh, 2012).

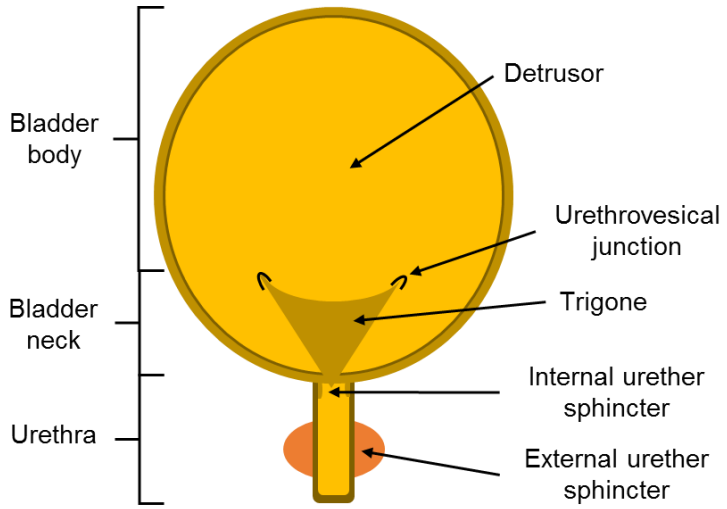


**Figure 1**: Schematic drawing of the bladder and the urethra. The bladder can be divided into two main components: the bladder body and the bladder base.

The bladder's principal function is to store urine and, then, when it reaches a certain volume, contract to be able to expel the urine. For that, it needs to have several specifications. First, it must be highly compliant, allowing wide variations in volume, without significant changes in pressure. Second, both the smooth muscle fibers and the nerves must be protected from urine's contact through the urothelium. Third, emptying requires a synchronous activation of all the smooth muscle cells of the bladder body (Yoshimura, 2012).

The bladder wall has three well-defined layers: the mucosa (the innermost portion), the *muscularis propria* (or detrusor), and the external adventitia/serosa (Figure 2). The *muscularis propria* is also called detrusor smooth muscle. It can be described as a sphere of smooth muscle bundles without well-defined orientation, consisting of three indistinct layers: an inner and outer longitudinal and an intermediate circular layer. The individual smooth muscle cells in the detrusor are typical, similar to those in other hollow muscular organs. They are long, spindle-shaped cells with a central nucleus (Andersson and Arner, 2004). Near the bladder neck, these three layers are well defined. The muscle fibers of the inner longitudinal layer extend down into the urethra in a funnel-shaped structure allowing continence and emptying of the bladder (Aldousari and Corcos, 2016). The orientation and interaction between the smooth muscle cells in the bladder are important, since this will determine how the bladder wall behaves and what effect will have on its shape and intravesical pressure (Andersson and Arner, 2004). The serosa partially covers the bladder. It is lined externally by mesothelium, beneath which there is a variable amount of vascularized adipose tissue that frequently extends into the *muscularis propria* and occasionally into the lamina propria (Gray's Atlas of Anatomy, pag 1250).

The mucosa includes the urothelium, basement membrane and the lamina propria (Birder and Andersson, 2013). The urothelium forms the interface between the urinary lumen and the underlying vasculature, connective, nervous, and muscular tissues. The urothelium is a pseudostratified epithelium presenting three layers: a basal cell layer, attached to a basement membrane, an intermediate layer, and a superficial or apical layer composed of large hexagonal cells known as "umbrella cells" (Birder and Andersson, 2013). The basal layer consists of small cuboidal cells from which the upper layers arise. The intermediate layers are polygonal and possess the capacity to stretch and flatten. The superficial layer forms a protective, almost impermeable surface for the bladder mucosa and consists of large, and sometimes multinucleated, cells displaying degenerative changes in their cytoplasm. Usually, these cells end up to be pulled out into the urine. An urothelial glycosaminoglycan (GAG) layer covers the umbrella cells and has been suggested to contribute to urothelial barrier function and may have a number of roles including a defense mechanism against microorganisms, carcinogens and toxic substances in the urine (Apodaca et al., 2004). The lamina propria lies between the basement membrane of the mucosa and the *muscularis propria*, forming a relatively thick layer of fibroelastic connective tissue that supports the urothelium. It is composed of an extracellular matrix containing several types of cells, including fibroblasts, adipocytes, interstitial cells, and sensory nerve endings it also contains a rich vascular

network, lymphatic channels, elastic fibers, and smooth muscle fascicles (muscularis mucosae) (Birder and Andersson, 2013).

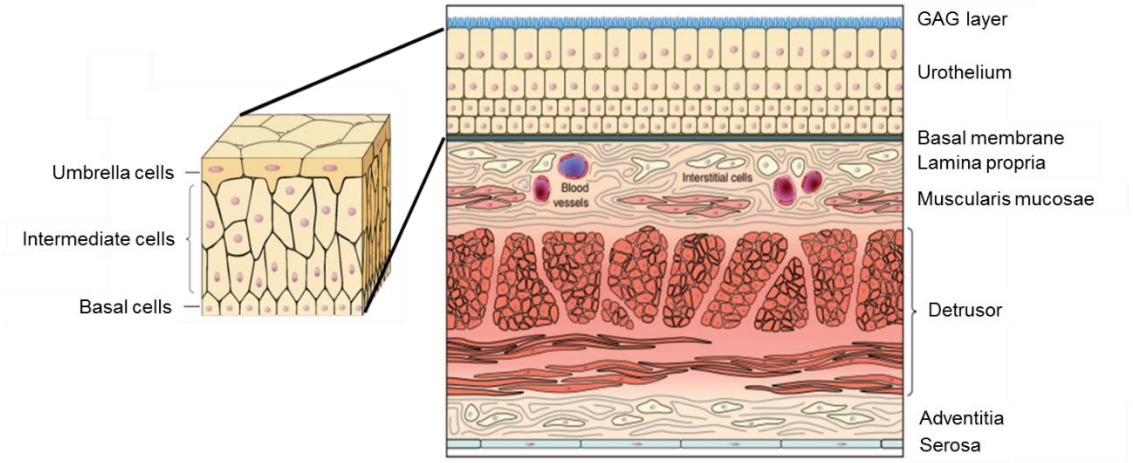


**Figure 2:** The several components of the bladder wall view from a transverse section. Magnification on the urothelium was made in order to depict the multiple epithelial cell layers (Adapted from Birder and Andersson, 2013).

## 1.3. Micturition

Micturition is a process in which neural circuits in the brain and spinal cord coordinate the activity of smooth muscle in the lower urinary tract. These circuits act as on-off switches to alternate the lower urinary tract between two modes of operation: storage and elimination. Injuries or diseases of the nervous system in adults can disrupt the voluntary control of micturition and cause the reemergence of reflex micturition, resulting in bladder hyperactivity and urge incontinence (Yoshimura and Chancellor, 2003).

The requirement for voluntary control over the lower urinary tract needs complex interactions between the autonomic nervous system, mediated by sympathetic and parasympathetic nerves, and the somatic nervous system, mediated by pudendal nerves, efferent pathways. The sympathetic innervation arises in the thoracolumbar outflow of the spinal cord ($Th_{11}$-$L_2$), whereas the parasympathetic and somatic innervations originate in the sacral segments of the spinal cord ($S_1$-$S_4$) (Fowler et al., 2008). Briefly, during the storage phase sympathetic postganglionic nerves release noradrenaline, which produce contractions of bladder base and urethral smooth muscle, via α-adrenoceptors and relaxation of the bladder body, via β-adrenoceptors. Somatic cholinergic motor nerves that supply the striated muscles of the external urethral sphincter cause the release of ACh, which acts on nicotinic receptors to induce a muscle

contraction. When the storage threshold is reached, the parasympathetic postganglionic nerves release ACh. These results in detrusor contraction and consequent urinary flow and is mediated principally by the $M_3$ muscarinic receptor and less by $M_2$ (Yoshimura and Chancellor, 2003; Fowler et al., 2008) (Figure 3).

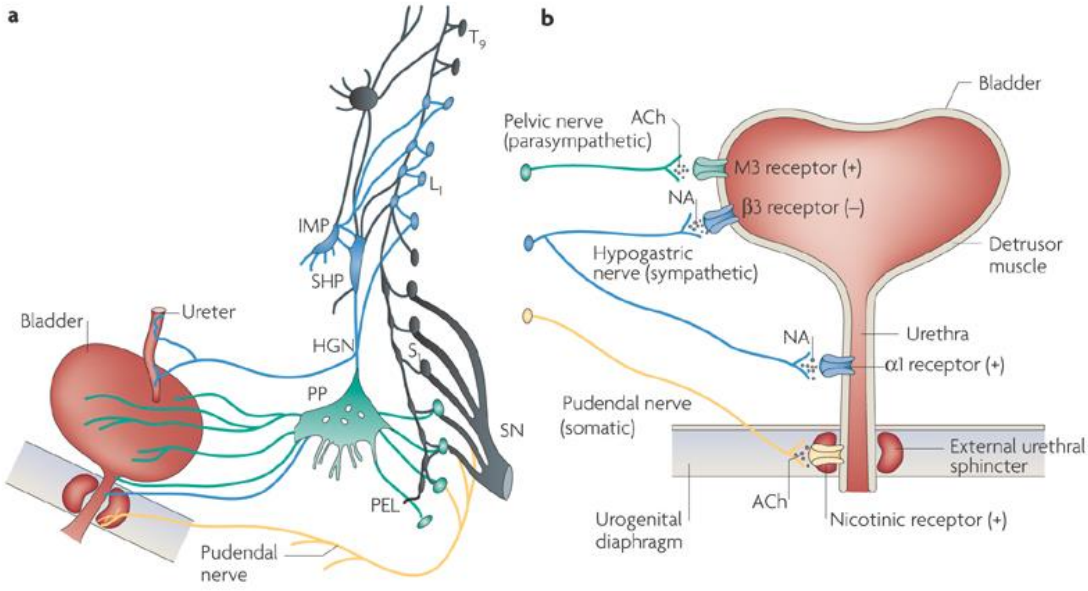


**Figure 3:** Innervation, efferent pathways and neurotransmitter mechanisms that regulate the lower urinary tract. **a)** Sympathetic fibers (shown in blue) originate in the T11–L2 segments in the spinal cord. Parasympathetic preganglionic fibers (shown in green) arise from the S2–S4 spinal segments. Somatic motor nerves (shown in yellow) that supply the striated muscles of the external urethral sphincter arise from S2–S4 motor neurons and pass through the pudendal nerves. **b)** Parasympathetic postganglionic axons in the pelvic nerve release acetylcholine (ACh), which produces a bladder contraction by stimulating $M_3$ muscarinic receptors in the bladder smooth muscle. Sympathetic postganglionic neurons release noradrenaline (NA), which activates $\beta_3$ adrenergic receptors to relax bladder smooth muscle and activates $\alpha_1$ adrenergic receptors to contract urethral smooth muscle. Somatic axons in the pudendal nerve also release ACh, which produces a contraction of the external sphincter striated muscle by activating nicotinic cholinergic receptors (Adapted from Fowler et al., 2008).

Sensations of bladder fullness are conveyed to the spinal cord by the pelvic and hypogastric nerves, whereas sensory input from the bladder neck and the urethra is carried out in the pudendal and hypogastric nerves. They transmit information from the lower urinary tract to the lumbosacral spinal cord. Afferent components of these nerves of small myelinated Aδ-fibers and unmyelinated C-fibers monitor the volume of the bladder and the amplitude of bladder contractions (Yoshimura and Chancellor, 2003; Fowler et al., 2008). Aδ-fibers are located primarily in the detrusor smooth muscle layer, whereas the unmyelinated sensory C-fibers are more widespread and reside in the detrusor muscle, close to the urothelium in the lamina propria and directly adjacent to urothelial cells (Kanai and Andersson, 2010). The Aδ-fibres respond to passive

distension and active contraction and thus convey information about bladder filling. The C-fibers are insensitive to bladder filling under physiological conditions and they're called 'silent' C-fibers and respond primarily to noxious stimuli such as chemical irritation or cooling. However, there is also evidence that C-fibers can carry information on bladder volume changes (Kanai and Andersson, 2010).

## 1.4. Muscarinic receptors

The treatment of choice for OAB has been the use of muscarinic receptor antagonists or antimuscarinic drugs. Although they have been generally recognized as safe and effective, these drugs have some drawbacks, like poor tolerability and frequent adverse events (Andersson, 2013). Dry mouth, cognitive impairment, tachycardia, blurred vision and constipation frequently lead to treatment discontinuation (Benner et al., 2010).

Muscarinic receptors are stimulated by ACh released from efferent parasympathetic nerves leading to detrusor contraction during the voiding phase. Five muscarinic receptor subtypes have been cloned and defined pharmacologically: $M_1$, $M_2$, $M_3$, $M_4$ and $M_5$. The $M_1$, $M_3$ and $M_5$ receptor subtypes couple to $G_{q/11}$ and activate phospholipase C to induce inositol phosphate turnover, while the $M_2$ and $M_4$ receptor subtypes inhibit adenylate cyclase via $G_i$ proteins (Cauldfield and Birdsall, 1998). Muscarinic receptors are widely distributed throughout the human body and mediate distinct physiological functions according to location and receptor subtype. In the bladder, all the muscarinic receptors can be found (1) on the urothelium where they cause the release of a yet unknown factor that inhibits detrusor contraction, (2) on parasympathetic and sympathetic nerve endings where they influence transmitter release, and (3) on the detrusor smooth muscle where they cause contraction (Chess-Williams, 2002). Inhibitory and facilitatory muscarinic mechanisms have been identified which influence transmitter release. The facilitatory mechanism appears to involve the activation of prejunctional $M_1$ and $M_3$ receptors, which enhance ACh release by a pathway involving protein kinase C and L-type calcium channels. The inhibitory mechanism, on the other hand, reduces ACh release and is mediated via $M_2$ or $M_4$ receptors, however, there is some confusion as to whether the inhibitory receptor is due to the $M_2$ or $M_4$ receptor subtype or both (Chess-Williams, 2002; Abrams et al., 2006). $M_2$ and $M_3$ receptors are predominant in the detrusor, with the $M_2$ subtype outnumbering the $M_3$ receptor subtype. However, it is the minority population of $M_3$ receptors that

directly mediate detrusor contraction (Chess-Williams, 2002), this was observed *in vivo* (Hegde et al., 1997) and *in vitro (*Hegde et al., 1997; Chess-Williams et al., 2001). Muscarinic $M_2$ activation, on the other hand, seems to contract the bladder indirectly by inhibit the adenylyl cyclase activity, i.e. by counteracting the β-adrenoceptor mediated relaxation (Hegde et al., 1997) (Figure 4). This dual mechanism may allow the parasympathetic nervous system, which is activated during voiding, to cause more efficient and complete emptying of the bladder.

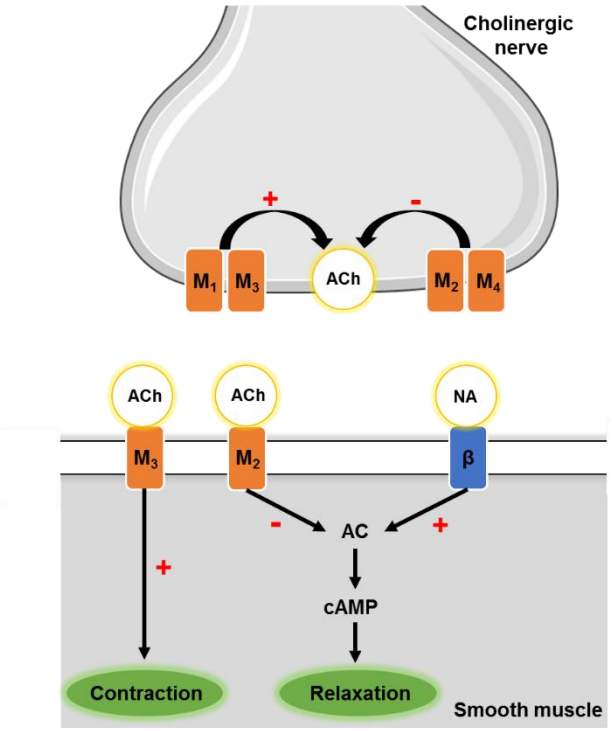


**Figure 4**: Schematic localization and role of muscarinic receptors at the neuromuscular junction of the urinary bladder. Actions of muscarinic receptors on transmitter release and on detrusor muscle contraction. Inhibitory ($M_2$ and $M_4$) and facilitatory ($M_1$ and $M_3$) muscarinic mechanisms influence transmitter release. In the muscle, $M_3$ have a direct action on the detrusor contraction, whereas $M_2$ lead to an indirect contraction of the muscle by inhibiting the relaxation pathway triggered by β-adrenoceptors activation and cyclic AMP formation.

## 1.5. Noradrenaline and β-adrenoceptors

Because of the common discontinuation of antimuscarinic drugs, it was necessary to find an alternative pharmacologic target for the treatment of OAB. It has been known for a long time that stimulation of β-adrenoceptors (AR) can relax the bladder, so it was a matter of time before studies targeting β-adrenoceptors activation started. A selective $β_3$-adrenoceptor agonist, mirabegron, was developed for the treatment of overactive bladder and was recently launched as the first $β_3$-adrenoceptor

agonist for the treatment of overactive bladder with symptoms of urge urinary incontinence, urgency, and urinary frequency.

Sympathetic stimulation of the lower urinary tract can lead to both excitatory and inhibitory effects. The highest density of adrenergic nerves is in the urethra, bladder neck and bladder body. In the urethra and bladder neck, the smooth muscle express predominantly $\alpha_1$-receptors which are responsible for generating muscle tone and occluding the urethral orifice during the storage phase of the micturition cycle (De Groat and Yoshimura, 2001). In the bladder body, adrenergic fibers are found manly in the smooth muscle bundles. Relaxation of the smooth muscle is mediated by β-adrenergic receptors (Andersson, 1993). Studies show that noradrenaline was an effective inhibitor of bladder muscle strip contractions in men and cats (Åmark et al., 1986). In the guinea-pig, noradrenaline reduce the contractions induced by muscarinic stimulation, being this effect due to $\alpha_1$ and $\beta_3$ receptor subtypes (Gillespie, 2004). However, the noradrenaline effect observed in the bladder body is mainly attributed to the $\beta_3$-adrenoceptor, being the $\alpha_1$-adrenoceptors less important (Chou et al., 2003).

The β-adrenoceptors belong to the superfamily of G protein-coupled receptors that can be activated by endogenous catecholamines, in particular adrenaline and noradrenaline (Wallukat, 2002). In 1948, the existence of two subtypes of adrenoceptors, α-ARs and β-ARs, were first demonstrated. In the late 1960, two subtypes of β-adrenoceptors, $\beta_1$ and $\beta_2$, were identified and characterized. In the isolated human bladder, the non-selective β-adrenoceptor agonist isoprenaline has a pronounced inhibitory effect, and administration of isoprenaline-like drugs can increase bladder capacity in humans (Andersson, 1993). Early functional studies on bladders from animals and humans suggested that such effects may be mediated by an "atypical" β-AR, since the β-AR involved had functional characteristics typical of neither $\beta_1$-, nor $\beta_2$-ARs. The isoprenaline effects could be blocked by propranolol (a non-selective β-antagonist), but not by practolol ($\beta_1$), metoprolol ($\beta_1$) or butoxamine ($\beta_2$) (Larssen, 1979). This prompted to the hypothesis of the existence of a third β adrenoceptor. Similar observations were seen in other organs where atypical β-adrenoceptors have also been suggested to exist, such in the ileum and colon (Bianchetti and Manara, 1990), skeletal muscle (Challis et al., 1988), and heart (Kaumann, 1990) of rodents. $\beta_3$-AR was isolated and cloned in 1989 in the adipose tissue (Emorine et al., 1989).

β-adrenoceptors are present in several organs performing several functions. For instance in the heart their principal role is to control heart rate and contractility in response to norepinephrine and epinephrine. $\beta_1$-AR is the predominant subtype in the

normal myocardium, representing 75% to 80% of the total β-AR density, followed by $\beta_2$-AR, which comprises 15% to 18% of total cardiomyocyte β-ARs, and the remaining 2% to 3% is $\beta_3$-ARs (Brodde, 1993; Lymperopoulos et al., 2013). In the brain, the three β-adrenoceptors are present in all the main brain regions. The amounts of $\beta_3$ found in the brain are very low compared to those of the $\beta_1$ and $\beta_2$-AR mRNAs, being hardly detectable in the adult brain. In contrast, the brain of very young infants contained about 100-fold more $\beta_3$ mRNA than the adult brain, whereas the amounts of $\beta_1$ and $\beta_2$-AR transcripts were essentially the same (Rodriguez et al., 1995). β-adrenoceptors are also present in the lung, where $\beta_2$ agonists are effectively use as bronchodilators due primarily to their ability to relax the airway smooth muscle (Cazzola et al., 2013).

Imunolocalization studies demonstrate the β-adrenoceptors are present throughout the bladder. The $\beta_1$-adrenoceptor is present in the urothelium and, in lesser amounts in interstitial cells and detrusor smooth muscle fibers. The $\beta_2$-adrenoceptor localizes in the urothelium, interstitial cells and detrusor muscle, but its distribution is not uniform, being the urothelium the most enriched zone and the detrusor muscle the less labeled region. $\beta_3$- adrenoceptors are expressed in higher amounts in interstitial cells and lesser in the urothelium and detrusor smooth muscle fibers (Otsuka et al., 2013).

Some authors tried to measure the expression of β-AR in the bladder. The first approach was to study the protein level based upon binding studies with various radioligands. Limited attempts have been made to identify the β-AR subtypes in the bladder by radioligand binding, however the radioligand used in these studies had much lower affinity for $\beta_3$- than for $\beta_1$- and $\beta_2$-ARs (Hoffmann et al., 2004). The problem is that the currently available radioligand binding techniques are probably inadequate to detect the presence of $\beta_3$-ARs and may lead to wrong interpretations because it can underestimate the density of $\beta_3$-ARs in the bladder (Niclau et al., 2006; Yamaguchi and Chapple, 2007).

Another option was to study the receptor expression at the mRNA level. Using Northern blots, *in situ* hybridization and RT-PCR it was possible to detect the presence of β-adrenoceptors in the rat and human bladder. Quantitative PCR experiments demonstrate that of all subtypes of β-AR mRNA were present in the human bladder. 97% was represented by the $\beta_3$-AR subtype and only 1.5% and 1.4% by $\beta_1$- and $\beta_2$-AR subtypes, respectively (Nomiya and Yamaguchi, 2003; Michel and Vrydag, 2006).

Igawa and collaborators, in 1999, studied the effect of β-adrenoceptors in the human detrusor. The non-selective β-adrenoceptors agonist, isoprenaline, relaxed the

detrusor smooth muscle in a concentration-dependent manner, but this effect was not reproduced by dobutamine or by procaterol. Only $\beta_3$ receptor agonists managed to relax the tissue. In agreement with this, only $\beta_3$ antagonists caused a rightward shift in the isoprenaline concentration-relaxation curve. This prompted to the conclusion that relaxation of the human detrusor by adrenergic stimulation is mediated mainly by $\beta_3$-ARs *in vitro*. Similar experiments to test the relaxing effect of isoprenaline in bladder strips pre-contracted with carbachol (Takeda et al., 1999; Takatsu et al., 2007) and KCl (Wuest et al., 2009) reached the same conclusions. The importance of the $\beta_3$-AR to cause relaxation of the urinary bladder has been reproduced *in vitro* in other animal species, like porcine (Yamanishi et al., 2002), ferret (Takeda et al., 2000a), dog (Hicks et al., 2007), mouse (Deba et al., 2009) and rat (Takeda et al., 2000b; Takatsu et al., 2007).

*In vivo* effects of β-ARs on bladder function have been studied in various animal models. In urethane-anesthetized rats, isoprenaline and Cl316,243 (a selective $\beta_3$ agonist) reduced intravesical pressure, with the effect of CL316,243 being verified for longer periods of time. On the other hand, while isoprenaline was able to reduce blood pressure and increase heart rate denoting an effect on other β-adrenoceptor subtypes, the selective $\beta_3$ receptor agonits,CL316,243, was devoid of cardiovascular effects (Takeda et al., 2000b).

Intravesical administration of acid acetic is frequently used to mimic bladder overactivity (Zhang et al., 2003). The result is a shortening of the voiding interval and the appearance of spontaneous contractions during the filing phase - non-voiding contractions. Intravenous application of CL316,243 increases the voiding interval, the bladder compliance and capacity, and decreases the voiding threshold pressure (Woods et al., 2001). Detrusor instability can be seen in hypertrophied bladders due to outlet obstruction, which can be used as a good model to study bladder overactivity (Malmgren et al., 1987). Cystometry recordings using this model are characterized by an increase in the number of small non-voiding contractions during the filing phase, which can be reduced by the administration of a $\beta_3$ receptor agonist (Woods et al., 2001). Likewise, ovariectomy leads to hormonal changes causing an increase in the voiding frequency and reduced voided volume in conscious and anaesthetized female rats. These parameters can also be improved by several $\beta_3$ agonists and these authors showed that prevention of spontaneous *in vitro* bladder contractions were significantly decreased using the same $\beta_3$ agonists, indicating that $\beta_3$-mediated actions are operated at the bladder level independently of central reflexes. The involvement of $\beta_3$ receptors was confirmed because the selective $\beta_3$ antagonist, SR59230A, but not $\beta_1$ and $\beta_2$ antagonists, could revert the relaxing effects of $\beta_3$ receptor agonists. Moreover, these authors

concluded that hormonal changes did not affect $\beta_3$-AR agonist activity, which is a good premise for the treatment of postmenopausal females with OAB (Kullmann et al., 2009). Isoprenaline and mirabegron favored storage, increase bladder capacity and prolonged the intervoid interval in decerebrate artificially-perfused rats (DAPR) (Sadananda et al., 2011). These rats exhibit a loss of the autonomic control, thus increasing non-voiding contractions (NVC) amplitude and the micturition frequency. These changes were attenuated by selective $\beta_3$ antagonists leading to the conclusion that blockade of the basal sympathetic tone operated by $\beta_3$ receptors activation is sufficient to control LUTS (Sadananda et al., 2013).

The presence of $\beta_3$ receptors in the urothelium raise questions related to their role in bladder relaxation. The presence of urothelium in human bladder strips causes a parallel rightward shift of the concentration-response curve to isoprenaline compared to urothelium-denuded preparations (Murakami et al., 2007). Apparently, activation of urothelial $\beta_3$-adrenoceptors triggers the release of an urothelium-derived factor that is responsible for attenuation of $\beta_3$-induced relaxation of the detrusor smooth muscle. Although activation of urothelial β-adrenoceptors can release NO, the inhibitor effect of isoprenaline was not prevent by the inhibition of NO synthase, indicating that NO is not involved in $\beta_3$-operated detrusor relaxation. So, the signaling messenger released from the urothelium in response to $\beta_3$-adrenoceptors activation that is responsible for downsizing the relaxant effect of isoprenaline, both *in vitro* and *in vivo*, remains to be elucidated. Conversely, other authors using isolated strips of the rat bladder *in vitro* showed that $\beta_3$ receptor agonists relax the detrusor smooth muscle independently of the presence of urothelium (Kullmann et al., 2011). Discrepancies between these two studies may be related to species differences (rat *vs.* human) and to experimental conditions, according to the authors. Urothelial-derived factors may act on smooth muscle fibers, but, even more importantly, they may modulate the activity of afferent nerves. This was concluded because intravesical application of $\beta_3$-adrenoceptor agonists reduced the amplitude of voiding contractions and increased the intervoid interval in the anaesthetized animal, whereas removal of the urothelium and lamina propria from isolated bladder strips did not alter $\beta_3$-induced smooth muscle relaxation (Kullmann et al., 2011).

In 2015, D'Agostino and colleagues showed that isoprenaline and selective $\beta_3$-AR agonists (BRL37344 and mirabegron) inhibited electrical field stimulation (EFS)-evoked contractions and the release of [$^3$H]-ACh from isolated human bladder strips *in vitro* and the inhibitory effects of these drugs were significantly attenuated by selective $\beta_3$-AR antagonists (L-748,337 and SR59230A). This was the first study demonstrating

that $\beta_3$-AR may reduce cholinergic neurotransmission in the human bladder besides its more reported relaxing action directly on the detrusor. Yet, the mechanism(s) underlying the inhibitory effect of $\beta_3$ agonists on nerve-evoked transmitter release requires further elucidation.

It is known that β-adrenoceptors couple to $G_s$ protein in order to activate adenylyl cyclase and increase intracellular cyclic AMP (cAMP) levels. This was demonstrated in Chinese hamster ovary cells (CHO) expressing rat and human $\beta_3$-adrenoceptors in response to isoprenaline and mirabegron (Hatanaka et al., 2013). In keeping with this, forskolin, a direct activator of adenylyl cyclase, mimicked the effect of isoprenaline on bladder tone (Frazer et al., 2005). Intracellular cAMP is a key molecule in cell signaling. Its levels are tightly controlled by intracellular phosphodiesterases, which degraded cAMP to AMP and adenosine (Chiavegatti et al., 2008). When generated in higher amounts surpassing phosphodiesterase inactivation capacity, intracellular cAMP can be pumped out from cells via ATP-binding cassete (ABC) transporters (Jedlitschky et al., 2000). Once outside the cell, cAMP may be degraded by an enzymatic cascade consisting of ecto-phosphodiesterase and ecto-5'-nucleotidase/CD73 to generate AMP and, ultimately, adenosine, respectively. Alternatively, adenosine generated from the intracellular action of phosphodiesterases degrading cAMP can be translocated to the extracellular milieu via equilibrative nucleoside transporters (ENT), namely ENT1 and ENT2 (King et al., 2006). In view of this, we hypothesized that $\beta_3$-adrenoceptors-induced intracellular cAMP accumulation may indirectly trigger the activation of adenosine P1 receptors in neighboring cells (e.g. muscle fibers, nerve terminals and blood vessels) via an autocrine/paracrine signaling pathway (Chiavegatti et al., 2008) (Figure 5).

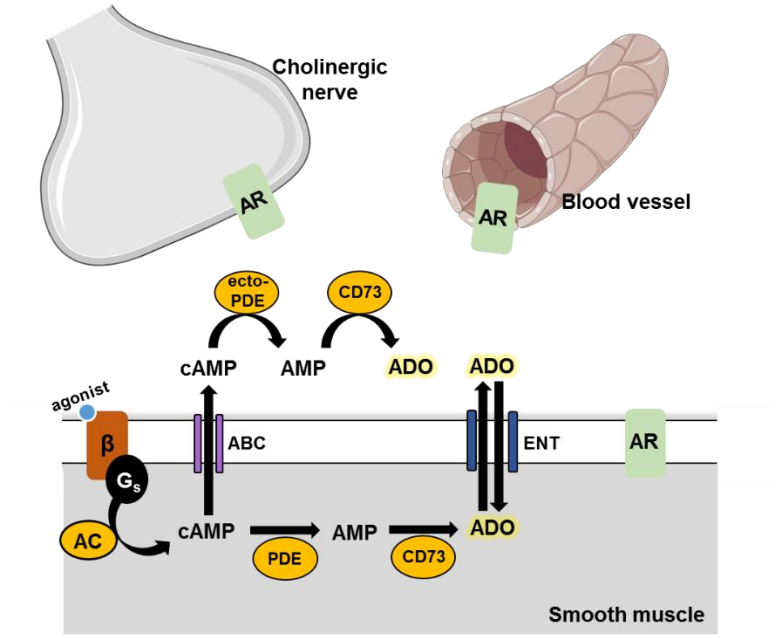


**Figure 5**: Schematic model of cAMP signalling pathway. cAMP generated by adenylyl cyclase (AC) can be degraded by intracellular phosphodiesterases (PDEs) and CD73 or transported out from muscle fibers by ABC transporters. Outside the cell, cAMP is degraded into AMP and adenosine by ecto-phosphodiesterase and ecto-5'-nucleotidase /CD73, respectively. Adenosine (ADO) can then activate specific receptors (AR) at the muscle fiber itself or at neighboring cells, like cholinergic nerves or blood vessels.

## 1.6. Purinergic receptors

For many years, ATP has been considered almost exclusively in terms of its role as an intracellular energy source. Nowadays, mounting evidences show that ATP and related purine nucleotides and nucleosides have potent extracellular actions on excitable membranes and that these may be involved in physiological regulatory mechanisms (Burnstock, 1976). Actually, extracellular purines (adenosine, ADP, and ATP) and pyrimidines (UDP and UTP) are important signaling molecules that mediate diverse biological effects via cell-surface receptors termed purine receptors (Ralevic and Burnstock, 1998). Two types of purinoceptor were first identified: the P1, for adenosine (explored in 1.7), and the P2, for adenine nucleotides (Burnstock, 1978a). Later on, pharmacology studies allowed us to distinguish the P2 into two receptor subtypes, P2X (ligand-gated ion channels) and P2Y (G-protein coupled) receptor families (Burnstock and Kennedy, 1985). P2X receptors are ATP-gated ion channels which mediate rapid (within 10 ms) and selective permeability to cations ($Na^+$, $K^+$ and $Ca^{2+}$). They are distributed essentially on excitable cells (smooth muscle cells, neurons, and glial cells) and their role is to mediate fast excitatory neurotransmission in response to ATP in both

the central and peripheral nervous systems. This contrasts with the slower onset of response (less than 100 ms) to ATP acting at metabotropic P2Y receptors. To date seven mammalian P2X receptors (P2X1–7) have been cloned, characterized, and accepted as valid members of the P2 receptor family (Ralevic and Burnstock, 1998). P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins (Burnstock, 2007). There are eight receptors and they can be further divided into two subfamilies based on sequence homology and coupling to second messengers: five $G_q$-coupled $P2Y_1$-like ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and three $G_i$-coupled $P2Y_{12}$-like ($P2Y_{12}$–$P2Y_{14}$) receptors. The first native agonists of P2Y receptors (P2YRs) with recognized biologic effects were ATP and ADP, and later on, UTP, UDP, and UDP glucose (UDPG) were found to activate certain P2YRs (Jacobson et al., 2015).

In the bladder, ATP exerts its effects predominantly through the activation of P2 purinoceptors located in the detrusor smooth muscle (P2X1) and in sub-urothelial pelvic nerve afferents (P2X3). ATP released from urothelial cells during bladder distention is a major mediator to initiate the voiding reflex via the activation of P2X3 receptors on suburothelial sensory nerve fibers (Cockayne et al., 2000; Ito et al., 2008). ATP exerts mutually exclusive effects through the activation of excitatory P2X1 receptors on the detrusor smooth muscle and of inhibitory $P2Y_1$ receptors controlling acetylcholine release from cholinergic nerve endings, after its catabolism into ADP by ENTPDases (Silva et al., 2011). In response to mechanical stretch, inflammatory mediators and chemical irritation, large amounts of ATP are released from urothelial cells (Ferguson et al., 1997). Studies from our group showed that urinary ATP may be a dynamic biomarker of detrusor activity in women with overactive bladder syndrome (Silva-Ramos et al., 2013). Botulinum toxin is currently being used against bladder overactivity. It has two different sites of action: it reduces the release of both ACh and ATP from parasympathetic nerves (Lawrence et al., 2010). It also reduces the release of ACh and ATP from urothelial cells, so reducing the stimulatory drive of the voiding reflex (Smith et al., 2005, Chancellor et al., 2008).

Uracil nucleotides only recently began to be explored. Previous studies showed that rat urothelial cells express $P2Y_2$ receptors recognizing UTP as the most potent agonist, which activate the receptor and lead to the release of ATP (Chopra et al., 2008). UDP-sensitive $P2Y_6$ receptors have been involved in the generation of large spontaneous contractions and propagating waves of intracellular $Ca^{2+}$ and membrane depolarization originating in suburothelial myofibroblasts and spreading to the detrusor smooth muscle, in rats submitted to spinal cord transection (Fry et al., 2012). Our group recently reported that activation of highly expressed UDP-sensitive $P2Y_6$ receptors

increased the voiding frequency in the anaesthetized rat, indirectly by releasing ATP from the urothelium via pannexin-1 hemichannels leading to a subsequent activation of P2X3 receptors on sub-urothelial sensory nerve fibers (Timóteo et al., 2013; Carneiro et al., 2014). On the other hand, it was also observed that activation of $P2Y_6$ receptor amplifies mucosal ATP release underlying bladder overactivity in patients with benign prostatic hyperplasia. These results suggest that selective $P2Y_6$ blockade may be a novel therapeutic strategy to control persistent symptoms in obstructed patients (Silva et al., 2015).

## 1.7. Adenosine and adenosine receptors

More than thirty years ago it was shown that the breakdown product of ATP, adenosine, decreased the tone and the spontaneous activity of the urinary bladder in guinea-pig (Burnstock et al., 1978). In the rat, adenosine can inhibit carbachol-induced bladder contractions (Nicholls et al., 1992), an effect that was also shown in acetylcholine pre-contracted bladder detrusor strips in humans (Rubinstein et al., 1998).

Adenosine is produced primarily from the catabolism of ATP and the nucleoside exerts multiple functions throughout the body. In the central nervous system (CNS), adenosine plays important functions such as modulation of neurotransmitter release, synaptic plasticity, and neuroprotection in ischemic, hypoxic and oxidative stress events (Fredholm et al., 2011; Sheth et al., 2014). In addition, adenosine plays different roles in a large variety of tissues, participating in the normal function of the cardiovascular, respiratory, renal, gastrointestinal, musculoskeletal and immune systems (Silva-Ramos et al., 2015b).

Intracellularly, adenosine is generated from the hydrolysis of S-adenosyl-L-homocysteine and from catabolism of adenine nucleotides, ATP, ADP, AMP or cAMP, which can also occur extracellularly (Fredholm et al., 2001). Adenosine's immediate precursor, AMP, is converted by the action of ecto-5'-nucleotidase/CD73. AMP is produced by the catabolism of ATP or ADP, by ecto-NTPDases (e.g. ecto-NTPDase1 / CD39) (Figure 6) or of cyclic AMP, by ecto-phosphodiesterases (Figure 5). After adenosine production, it can be metabolized into inosine, by adenosine deaminase and, then to hypoxanthine by purine nucleoside phosphorylase. Adenosine can also be converted back to AMP by the action of adenosine kinase and, subsequently, to ADP and ATP (Figure 6). Adenosine can also be transported out of the cell to the extracellular

space by specific bi-directional nucleoside transporters, e.g. ENT (Latini and Pedata, 2001; Sheth et al., 2014).

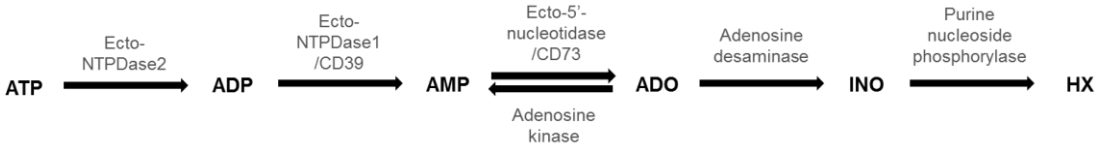


**Figure 6**: Schematic of adenosine synthesis from ATP or ADP.

In human detrusor strips, the ectoNTPDase1/CD39 enzyme exerts a dominant role converting ATP directly into AMP, which is then sequentially hydrolyzed into adenosine and inosine by ecto-5′-nucleotidase/CD73 and ecto-adenosine deaminase, respectively (Silva-Ramos et al., 2015a). Ecto-5′-nucleotidase/CD73 is present exclusively in the detrusor smooth muscle together with ecto-NTPDase1/CD39 (Yu et al., 2011). So, in the bladder, the biosynthesis of adenosine from released ATP seems to happen in the suburothelial and detrusor muscle layers, being the adenosine formation insignificant in the urothelium where ecto-5′-nucleotidase/CD73 is almost absent (Mohlin et al., 2009).

**Table 1:** Expression of adenosine receptors based in RT-PCR studies in the rat (Adapted from Dixon et al., 1996).

| | **$A_1$ receptors** | **$A_{2A}$ receptors** | **$A_{2B}$ receptors** | **$A_3$ receptors** |
|---|---|---|---|---|
| **High level** | Brain, heart, aorta, spleen, kidney, liver, testis, eye and bladder | Brain, eye and skeletal muscle | CNS, proximal colon, eye, lung, uterus and bladder | Brain, spleen, lung, uterus and testis |
| **Low levels** | Lung, uterus, stomach | Heart, lung, bladder, uterus, aorta, spleen, stomach, testis, skin, kidney and liver | Aorta, stomach, testis, skeletal muscle, jejunum, kidney, heart, spleen and liver. | Liver, bladder, aorta, stomach, jejunum, proximal colon, kidney and eye |

Adenosine can activate four adenosine receptors, previously known as P1 receptors: $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$, to elicit numerous physiological effects in many organs. These adenosine receptors are G protein-coupled receptors, being that the $A_1$ and $A_3$

receptors are coupled to $G_i$ or $G_o$ proteins, which lead to inhibition of adenylate cyclase and activation of hyperpolarizing potassium currents, while the $A_2$ receptors are coupled to the $G_s$ protein leading to activation of adenylate cyclase and cyclic AMP accumulation (Fredholm et al., 2001). Table 1 shows the expression of adenosine receptors throughout the body.

The $A_1$ receptor was the first identified adenosine receptor. The role of $A_1$ receptors in the central nervous system (CNS) and heart have been especially well characterized. The $A_1$ receptor is abundantly expressed in the brain where it plays a major neuroprotective role by decreasing the release of excitatory neurotransmitters, such as glutamate. This receptor acting via $G_i$-proteins, decreases cAMP levels, opens $K^+$ channels and reduces calcium influx by blocking $Ca^{2+}$ channels leading to inhibition of neurotransmitter release (Stone et al., 2009). In the heart, the dramatic increase of adenosine during myocardial ischemia was first demonstrated in 1970 (Olsson, 1970). Studies on $A_1AR^{-/-}$ mice have shown that the receptor is involved in aortic and coronary vasoconstriction and protects the heart against injury caused by myocardial infarction and ischemia by inhibiting the production of cAMP and activating ATP-dependent potassium channels (Tawfik et al., 2006). In addition, cardiac-specific $A_1$ overexpression studies showed that the receptors were involved in reduction of diastolic dysfunction during ischemia/reperfusion and prolonged cardioprotection (Matherne et al., 1997). Despite the beneficial roles of $A_1$ receptors, the activity of this receptor under certain pathological conditions could lead to harmful effects. Numerous reports have been made where activation of $A_1$ during inflammation or pathological conditions has adverse effects. For instance, the expression of $A_1$ receptors is upregulated in asthma patients and activation of these receptors lead to bronchoconstriction, leukocyte activation and inflammation, bronchial hyperresponsiveness and mucous secretion (Brown et al., 2008). Because of this, one anti-$A_1$ drug to treat asthma is currently in clinical trials (Wilson et al., 2008), to avoid the unwanted side effects of broad adenosine receptor antagonists, such as methylxanthines, which are used for decades in the treatment of asthmatics. In conclusion, $A_1$ receptors have a beneficial role under normal physiological conditions, however during many pathological conditions and inflammatory conditions, its activity further aggravates the pathological state.

$A_2$ receptors play a major role in many disease conditions including asthma, inflammation, wound healing, cancer, reperfusion injury of the heart and neurodegeneration (Haskó et al., 2009). Physiologically, the role of $A_2$ receptors in modulating organ function depends on their ability to limit inflammation and tissue injury.

$A_{2A}$ receptors are highly enriched on inflammatory cells including neutrophils, mast cells, macrophages, eosinophils, platelets, and T cells (Lappas et al., 2005a). The anti-inflammatory properties of $A_{2A}$ receptors include inhibition of T-cell activation and limitation of the production of inflammatory mediators such as IL-12, TNF-α and INF- γ (Lappas et al., 2005b). In human urothelial cells infected with *Escherichia coli*, the expression of $A_1$ and $A_{2B}$ mRNA was decreased and the $A_{2A}$ receptor was up regulated suggesting that adenosine may control the inflammatory responses triggered by infectious agents via $A_{2A}$ receptors (Save et al., 2009). Furthermore, $A_{2A}^{-/-}$ mice have increased oxidative stress in the lungs and airway smooth muscles due to activation of inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) (Nadeem et al., 2007). Regarding $A_{2B}$ receptors activation it aids in the progression of inflammatory diseases such as asthma. $A_{2B}$ can activate $G_q$ proteins and downstream cytokines, including IL-4, IL-8 and IL-13, which can in-turn induce immunoglobulin E (IgE), the immunoglobulin involved in progression of allergic response and asthma (Ryzhov et al., 2004). In conclusion, $A_2$ receptors are beneficial in instances where inflammation is a detrimental component, but possess adverse effects in the treatment of cancer or of neurological diseases where an immune response is required. For instance, up regulation of $A_2$ receptors protects cancer cells from attack by T-cells; as a matter of fact, $A_{2A}^{-/-}$ mice showed a remarkable decrease in tumor size and increased survival rate (Ohta et al., 2006).

The $A_3$ receptor was the last member of the adenosine receptor family to be cloned. These receptors are also highly expressed in inflammatory cells including human eosinophils, neutrophils, monocytes, macrophages, lymphocytes and dendritic cells (Borea et al., 2009). The $A_3$ receptor activation on immune cells has both pro- and anti-inflammatory responses and their role in the progression of inflammatory diseases is controversial. Selective $A_3$ receptor antagonism prevented airway eosinophilia and mucous production in adenosine deaminase knockout ($ADA^{-/-}$) mice. Similar results were found in $ADA^{-/-}/A3^{-/-}$ double knockout mice suggesting that $A_3$ receptors activation plays a damaging role in chronic lung diseases (Young et al., 2004). Yet, the $A_3$ receptor may have controversial roles in other organ systems. For instance, $A_3$ activation plays a protective role in lung injury (Inoue et al., 2008) and has a positive role at the diurnal rhythm and temperature regulation (Yang et al., 2010). However, its damaging role was shown in studies where activation of the receptor intensifies renal dysfunction, recommending the use of $A_3$ antagonists (Lee and Emala, 2000). The expression of the receptors is increased in ocular ischemic diseases and in conditions with oxidative stress (Avila et al., 2002).

All the adenosine receptors are present in the urinary bladder, being the $A_1$ and the $A_{2B}$ the most expressed. Previously, it was reported that $A_{2B}$ protein expression in the detrusor muscle was more significant compared to the others adenosine receptors (Yu et al., 2006). However, immunolocalization studies of our group showed that $A_1$ and $A_{2A}$ are the most expressed receptors in the human detrusor, being the immunoreactivity of $A_{2B}$ and $A_3$ less evident. Co-localization experiments showed that $A_1$ receptors are localized on cholinergic nerves and that $A_{2A}$ receptors are diffusely expressed on smooth muscle fibers (Silva-Ramos et al., 2015a). Both receptors are also present in the urothelium. Localization studies in the rat revealed that $A_1$ receptors are expressed predominantly in the umbrella cells and in the connective tissue underlying the urothelium. $A_{2A}$ receptors were identified in the urothelium and also in the connective tissue beneath the urothelium. Both $A_{2B}$ and $A_3$ receptors are expressed along the basolateral surface of umbrella cells, but the latter receptor is also present in the subepithelial connective tissue although to a lesser extent (Yu et al., 2006).

Only few studies have been focusing on the role of subtype-specific adenosine receptors in the control of micturition under normal and pathological conditions. Fry and collaborators showed that adenosine reduces the strength of nerve-induced contractions by acting predominantly at presynaptic inhibitory $A_1$ receptors and that adenosine analogues, such NECA and CCPA, had the same effect (Fry et al., 2004). The selective adenosine $A_1$ receptor antagonist, DPCPX, completely prevented the inhibitory effect of CCPA. Moreover, it has also been shown that adenosine relaxes the isolated urinary bladder through the activation of $A_1$ receptors, whereas no relaxation was produced by activation of $A_{2A}$ receptors; the same authors observed an increase in the magnitude of adenosine-induced relaxation in the presence of the $A_3$ receptor antagonist, MRS 1523 (Vesela et al., 2011).

Intrathecal administration of the adenosine analogues, NECA and R-PIA, delayed the voiding reflex, but the same was not observed when these compounds were applied intravenously or intravesically *in vivo* (Sosnowski and Yaksh, 1990). Moreover, other authors corroborated these results showing that administration of adenosine into the bladder of *in vivo* rats did not cause any change in micturition. Maybe this happened because adenosine cannot pass the barrier made by umbrella cells of the urothelium or it is rapidly inactivated in the lumen of the bladder. In contrast to adenosine, its analogue acting selectively on $A_1$ receptors, CCPA, decreased the pressure threshold for voiding and this effect was attenuated by DPCPX; surprisingly, CCPA did not affect the intervoid interval or other parameter of the micturition cycle. Paradoxically, intravesical

administration of CCPA increased bladder overactivity in rats with cyclophosphamide-induced cystitis (Prakasam et al., 2012), but this finding was not confirmed in rats whose bladder was irritated with acetic acid. Administration of the $A_1$ agonist, CCPA, or the $A_{2A}$ antagonist, ZM241385, had an inhibitory effect on the micturition reflex in normal and acetic acid-treated rats, because both compounds increased the intervoid interval. Despite certain controversies, the majority of the studies agree that the micturition reflex is modulated by adenosine through dual activation of inhibitory $A_1$ and excitatory $A_{2A}$ receptors (Kitta et al., 2013).

More recently, studies from our group demonstrated that adenosine-induced relaxation of detrusor contractions requires higher (milimolar) concentrations than those (in the micromolar range) required to inhibit [$^3$H]-ACh released from stimulated cholinergic nerves of the human detrusor. The inhibitory adenosine effect on evoked transmitter release was mimicked by two adenosine analogues, including the selective $A_1$ receptor agonist, R-PIA, and it was specifically blocked by DPCPX (Silva-Ramos et al., 2015a). These results clearly indicate that adenosine exerts a predominant inhibitory effect on cholinergic neurotransmission in the human detrusor via the activation of prejunctional $A_1$ inhibitory receptors. Loss of the $A_1$ receptor-mediated inhibitory tone may contribute to promote bladder overactivity in men with outflow obstruction due to benign prostatic hyperplasia, which may be caused in part by deficits in adenosine formation from the extracellular hydrolysis of released ATP (Silva-Ramos et al., 2015a). Interestingly, our group showed that cholinergic nerve hyperactivity in these patients could be partially reversed by blocking the uptake of adenosine via ENT (with dipyridamole) or the extracellular adenosine deamination by ADA (with EHNA).

# 2. Objectives

Mounting evidences suggest that stimulation of $\beta_3$-adrenoceptors improve overactive bladder symptoms by directly relaxing detrusor smooth muscle contractions as a consequence of intracellular cyclic AMP formation. Activation of $\beta_3$-adrenoceptors may also down-modulate nerve-evoked acetylcholine (ACh) release, but there is no evidence for $\beta_3$-adrenoceptors being present on bladder cholinergic nerve terminals. In this study, we hypothesized that adenosine formed from the catabolism of cyclic AMP in the detrusor might act retrogradely via prejunctional $A_1$ receptors to explain the inhibitory control of cholinergic activity by $\beta_3$-adrenoceptors in human and rat urinary bladder.

We addressed this question by doing *in vitro* experiments using isolated urinary bladder strips form rats to measure the release of [$^3$H]-ACh and adenosine, to study the kinetics of the extracellular cyclic AMP catabolism by HPLC, and to evaluate the tissue distribution of $\beta_3$ and $A_1$ receptors in the urinary bladder by immunofluorescence confocal microscopy. In addition, we tested whether a similar phenomenon occurs to control the sensory bladder drive of the micturition cycle by $\beta_3$-adrenoceptors. To this end, we evaluated the urodynamic effects of selective $\beta_3$- and $A_1$-receptor antagonists on isoprenaline-induced inhibition of bladder activity caused by instillation of this drug into the bladder lumen in urethane-anaesthetized rats.

# 3. Materials and Methods

## 3.1. Animals

Animal care and experimental procedures were in accordance with the guidelines prepared by the Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Male rats (Wistar Han, 300–450 g; Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06:30–19:30 h)–dark (19:30–06:30 h) cycle, with food and water provided *ad libitum*.

## 3.2. *In vivo* cystometry recordings

The experiments were carried out in spontaneously breathing and anaesthetized rats with urethane (1.0−1.2 $g \cdot kg^{-1}$). The body temperature of the animal was kept around 38°C with the help of a heating pad controlled by a thermosensor.

The surgical procedure begins with a tracheostomy where a cannula is inserted into the trachea to facilitate breathing. A catheter, connected to an injection pump, was inserted into the left jugular vein to permit saline infusion (4 $mL \cdot h^{-1} \cdot kg^{-1}$) and intravenous drugs application. Then, the urinary bladder was exposed through a medial abdominal incision and a three-barrel catheter was inserted through its dome. One barrel was connected to an automated perfusion pump for saline and/or drugs infusion (2.4 $ml \cdot h^{-1}$); a second barrel was attached to a pressure transducer for continuous monitoring of intravesical pressure; a third barrel was used either to drain or to close the bladder circuit, in order to initiate the micturition reflex. The bladder pressure was continuously monitored on a computer screen with a PowerLab data acquisition system (Chart 5, version 4.2 software; AD Instruments, Colorado Springs, CO, USA), which was also used to record the electrocardiogram in the anaesthetized rat (Figure 7).

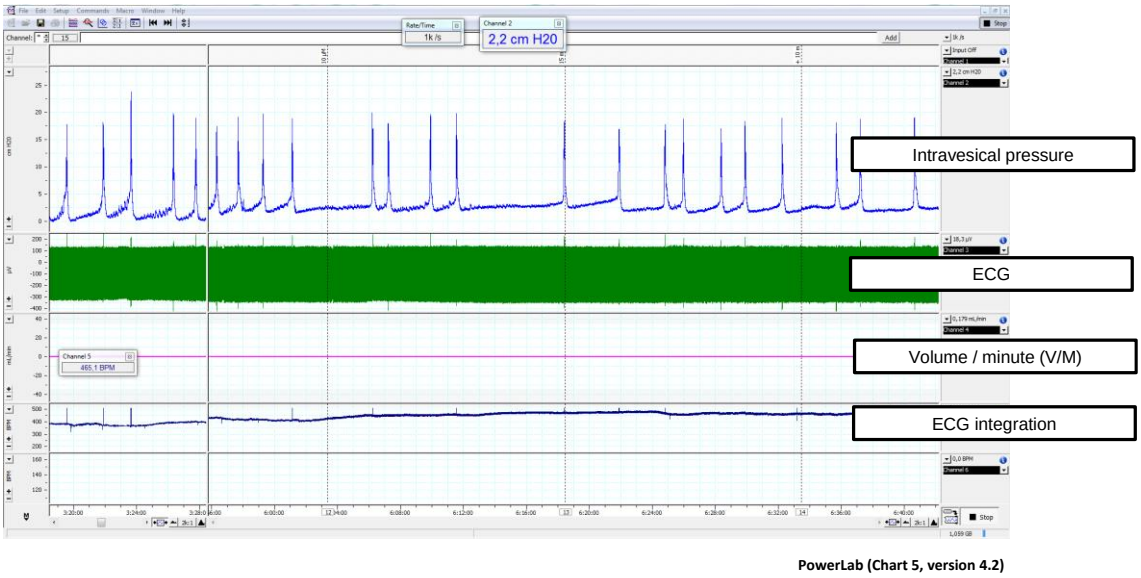


**Figure 7:** Example of typical recording traces obtained with the PowerLab data acquisition system (Chart 5, version 4.2 software; AD Instruments, Colorado Springs, CO, USA). Intravesical pressure of the bladder and the electrocardiogram (ECG) were monitoring continuously during all the experimental procedure.

After surgical preparation, a 60 min equilibration period was undertaken during which saline was infused into the urinary bladder and allowed to freely drain out of the bladder (open circuit). The micturition reflex was initiated by closing the draining barrel while keeping intravesical infusion of saline at a constant flow rate. Voiding contractions were assumed as large-amplitude rhythmic bladder contractions accompanied by urine draining through the urethra, when bladder pressure reached a certain threshold. Test drugs were applied either directly into the bladder lumen, by changing the syringe connected to the automate perfusion pump (2.4 ml·h$^{-1}$) or intravenously through the catheter inserted into the left jugular vein.

The four cystometric parameters represented in figure 8 were evaluated.

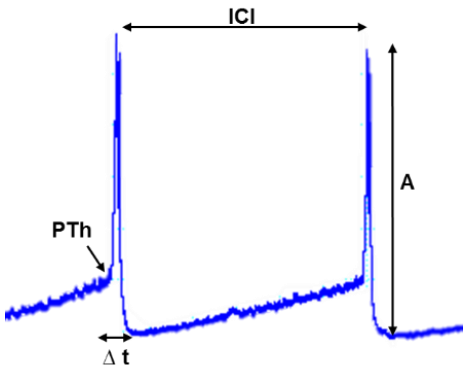


**Figure 8:** Representation of the cystometric parameters evaluated. The parameters evaluated were pressure threshold (PTh), intercontraction interval (ICI), contraction duration (Δt) and amplitude (A).

The intercontraction interval (ICI, min) and the pressure threshold (PTh, cm of $H_2O$) that is required to initiate the voiding reflex are normally associated with the sensitive component of the micturition reflex (filling phase); on the other hand, the amplitude (A, cm of $H_2O$) and the duration (Δt, s) of the voiding contractions are mostly associated with the motor component of the micturition reflex (emptying phase).

## 3.3. Kinetics of cyclic AMP extracellular catabolism and adenosine formation by HPLC

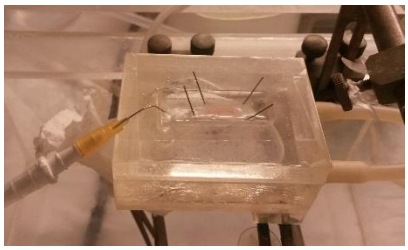

**Figure 9:** Rat bladder mounted in a horizontal organ bath to perform enzymatic kinetic studies.

For the kinetic experiments of the extracellular catabolism of AMPc isolated rat bladders without the urothelium were mounted in a 2-mL organ bath, as shown in figure 9. All experiments were performed at 37 °C. Preparations were superfused with gassed (95 % $O_2$ and 5 % $CO_2$) Tyrode's solution (pH=7.4, containing (mM) 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.4 $NaH_2PO_4$, 11.9 $NaHCO_3$, 11.2 glucose). After a 30 min equilibration period, the preparations were incubated for 45 min with Tyrode's solution to eliminate cellular debris and endogenous interfering compounds. After a 10 min washout, the preparations were incubated with 30 µM cAMP (zero time). Samples of 75 µL were collected from the incubation fluid at different times up to 45 min (0, 1, 2, 5, 10, 15, 30, 45) for further analysis by HPLC (with UV detection) system (LaChrome Elite; Merck, Germany) of the variation of substrate disappearance and product formation (Figure 10).

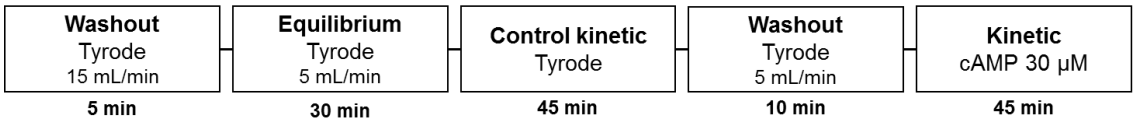


**Figure 10***:* Scheme of experimental protocol to evaluate the kinetics of the extracellular catabolism of cyclic AMP.

## 3.4. Quantification of [$^3$H]-ACh release

The experiments were performed in isolated detrusor muscle strips without the mucosa of the rat urinary bladder. The mucosa was dissected by gently rubbing the

urothelium with a cottonwood swab. Isolated detrusor muscle strips were mounted in 365 µL capacity chambers of a Brandel SF-12 automated superfusion system (Valley Internationnal Corp., Austin, TX, USA) heated at 37°C. Then, the preparations were continuously superfused with gassed (95% $O_2$ and 5% $CO_2$) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, $NaH_2PO_4$ 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period, cholinergic neurons were loaded during 40 min with 1 µM [$^3$H]choline (specific activity 5 µCi nmol$^{-1}$) under electrical field stimulation (EFS, 1 Hz frequency, 0.2 ms pulse width, 75 mA) using two platinum-made grid electrodes placed above and below the muscle strip (transmural EFS stimulation). Following loading, washout superfusion (1 ml/min) of the preparations was performed during 120 min with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 µM). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, and Boston, USA; % counting efficiency: 56±2%) after appropriate background subtraction, using 1-ml bath samples automatically collected every 1-min using the SF-12 suprafusion system. [$^3$H]-ACh release was evoked by two periods of EFS ($S_1$ and $S_2$), each consisting of 200 square wave pulses of 0.2 ms duration delivered at 10-Hz frequency. Test drugs were added 6 min before $S_2$ and were present up to the end of the experiments (Figure 11). The evoked [$^3$H]-ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period. In control conditions, $S_2/S_1$ ratio was 0.96±0.02 (n=4). None of the drugs significantly (P>0.05) changed the basal tritium outflow.

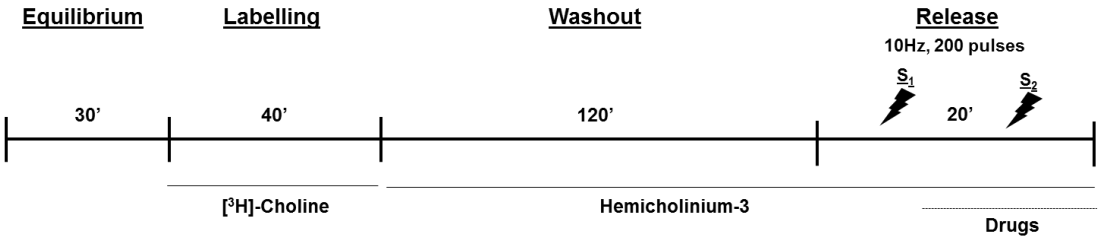


**Figure 11:** Scheme of the experimental protocol used to measure [$^3$H]-ACh release from the rat bladder.

## 3.5. Measurement of adenosine release

Experiments were performed in isolated rat detrusor strips without the mucosa using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection). After a 30-min equilibration period, the preparations were incubated with 2.8 mL gassed Tyrode's

solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. In these experiments, samples retained for analysis were collected 15, 30, 45 and 60 min after starting the experimental protocol (zero time). Isoprenaline (1 μM) was added to the incubation solution after the first collection (Basal, 15 min) and was kept in contract with the preparations at least for 15 min (Isop, 30 min) before starting electrical field stimulation (Isop + EFS, 45 min), which consisted in 3000 square wave pulses of 1 ms duration delivered at a 10-Hz frequency (Figure 12). Bath aliquots (500 μl) were snap-frozen in liquid nitrogen immediately after collection, stored at −20ºC (the enzymes are stable for at least 4 weeks) and analyzed within 1 week from collection by HPLC with diode array detection (Finnigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 μM, 2.1mm × 150 mm) equipped with a guard column (5 μm, 2.1mm × 1 mm) using an elution gradient composed of ammonium acetate (5mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 μl per min and the column temperature was maintained at 20ºC. The autosampler was set at 4ºC and 50 μl of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine.

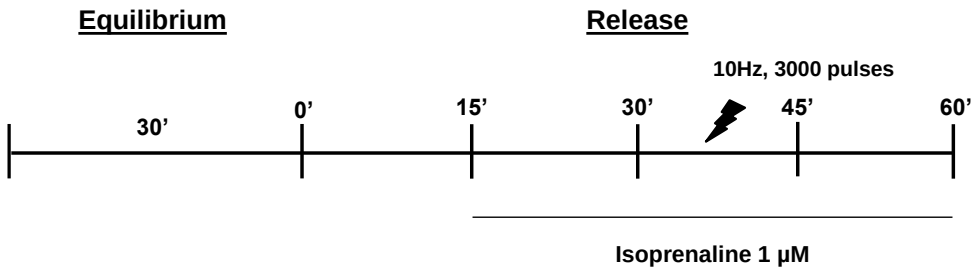


**Figure 12:** Scheme of the experimental procedure for measuring adenosine release by HPLC.

## 3.6. Immunofluorescence staining and confocal microscopy observation

Rat detrusor strips without the mucosa were stretched to all directions and pinned onto a Petri dish coated with Sylgard®. The strips were then fixed in PLP solution

(paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4ºC. Fourteen-micron sections were incubated with selected primary antibodies (Table 2) diluted in an incubation buffer (fetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4ºC, for 16 h. For double immunostaining, antibodies were combined before application to tissue samples. After washing away unbound primary antibody, the sections were incubated with secondary antibodies in the dark for 2 h at room temperature. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity of the secondary antibodies was tested in control experiments in which primary antibodies were omitted. The specificities of primary antibodies were all previously validated in heterologous expression systems according to references provided in manufacturers' websites. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield as antifade mounting media (VectorLabs) and stored in the dark at 4ºC. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

**Table 2:** Primary and secondary antibodies used to stain rat detrusor strips.

| Primary antibodies | Code | Host | Dilution | Source |
|---|---|---|---|---|
| **Anti-A1 receptor** | Ab75177 | Rabbit (rb) | 1:200 | Abcam |
| **Anti-β3 receptor** | AAR-017 | Rabbit (rb) | 1:50 | Alomone |
| **Anti-VAChT** | SAB5200240 | Mouse (ms) | 1:50 | Sigma |
| **Anti-ENT1** | ANT-051 | Rabbit (rb) | 1:50 | Alomone |
| **Anti-ENT2** | ANT-052 | Rabbit (rb) | 1:50 | Alomone |
| **Secondary antibodies** | **Code** | **Host** | **Dilution** | **Source** |
| **Alexa Fluor 488 anti-rb** | A-21206 | Donkey | 1:1000 | Molecular Probe |
| **Alexa Fluor 633 anti-ms** | A-21050 | Goat | 1:1000 | Molecular Probes |

## 3.7. Drugs and Solutions

Acetylcholine, adenosine 3',5'-cyclic monophosphate sodium salt monohydrate (3',5' cyclic AMP), hemicholinium-3, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol,

fetal 235 bovine, 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (Forskolin, FSK), S-(p- 236 nitrobenzyl)-6-thioinosine (NBTI) were obtained from Sigma (St Louis, MO, USA); isoproterenol hydrochloride (Isoprenaline), 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid disodium salt (CL316,243), 1-(2-ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride (SR59230A hydrochloride) were obtained from Tocris Bioscience (Bristol, UK); dipyridamole was obtained from Boehringer Ingelheim (Germany); [methyl-$^{3}$H] choline chloride (ethanol solution, 80.6 Ci mmol-1) was obtained from PerkinElmer (Boston, USA); serum albumin, Triton X-100 were obtained from Merck (Darmstadt, Germany). DPCPX was dissolved in a 5-mM stock solution in 99% dimethylsulfoxide (DMSO) + 1 % NaOH 1 M (v/v). FSK and NBTI were made up in 3- and 50- mM stock solutions in DMSO, respectively. Other drugs were prepared in NaCl (0.9 % (W/V)) or Tyrode's solution. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stocks solutions were made daily and appropriate solvent controls were done. No statistical differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed.

## 3.8. Presentation of data and statistical analysis

Results are expressed as mean ± SD, with n indicating the number of individuals used for a particular set of experiments. Only one experimental procedure (e.g. agonist in the absence and in the presence of the antagonist) was performed per individual. Statistical analysis of data was carried out using Graph Pad Prism 6.04 for Windows software (La Jolla, USA). Paired and unpaired Student's t-test with Welch's correction was used for statistical analysis when parametric data was considered. One-way or Two-Way analysis of variance (ANOVA) followed by the Dunnett's post test was used for multiple comparisons. $P<0.05$ (two-tailed) values were considered statistically significant.

# 4. Results

## 4.1. *In vivo* cystometry recordings in urethane-anaesthetized rats

Figure 13A shows original recordings obtained during *in vivo* cystometry experiments performed in urethane-anaesthetized rats after infusion of isoprenaline (1 μM, a non-selective β-adrenoceptor agonist) into the lumen of the urinary bladder (intravesical) compared with control saline (NaCl 0.9%) infusion. The urodynamic parameters analyzed are shown in figure 13B. Isoprenaline (0.001-1 μM) concentration-dependently increased the intercontraction interval (ICI), meaning that it decreased the voiding frequency, without much affecting both the pressure threshold (PTh), the amplitude (A) and the duration (∆t) of the voiding contractions (Figure 13B). Isoprenaline also decreased the number and amplitude of non-voiding contractions (Figure 13A). It is worth to note that instillation of isoprenaline (1 μM) directly into the bladder lumen did not significantly (P>0.05) affect heart rate (430±49 bpm, n=5) compared to the control situation (405±52 bpm, n=5) where only NaCl 0.9 % (w/v) was present inside the bladder (Figure 14).

The inhibitory effect of isoprenaline on the voiding frequency was mimicked by CL316,243, which is a selective $\beta_3$ receptor agonist in rodents (Evans et al., 1999). Infusion of CL316,243 (0.001-1 μM) into the bladder lumen also increased the ICI in a concentration-dependent manner, without significantly (P>0.05) affecting the other cystometry parameters (PTh, A and ∆t) (Figure 15B). Like the effect observed with isoprenaline, CL316,243 (1 μM) also decreased the number and amplitude of non-voiding contractions (Figure 15A). Intravesical CL316,243 (1 μM) was also without (P>0.05) effect on heart rate (468±50 bpm, n=5) compared to the control situation (460±75 bpm, n=5) where only NaCl 0.9 % (w/v) was present inside the bladder (Figure 16).

## *In vivo* rat bladder cystometry

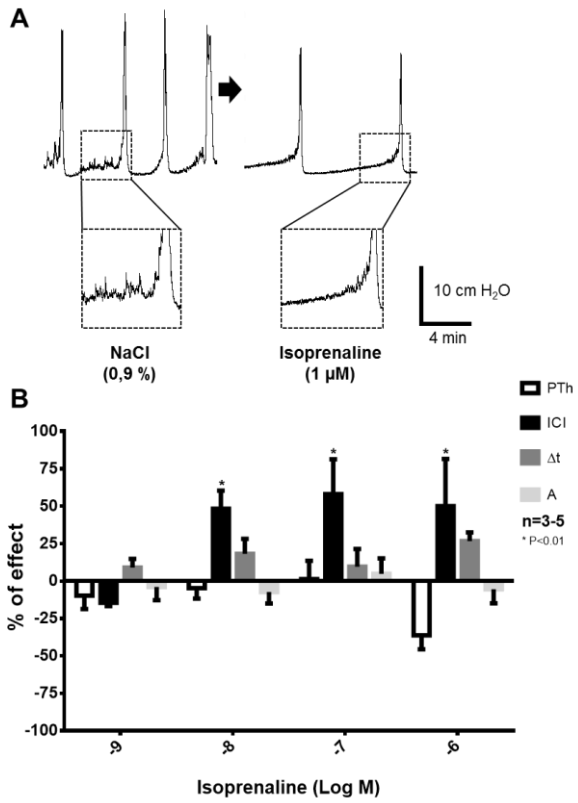


**Figure 13: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and isoprenaline (1 µM) infusion into the urinary bladder of urethane-anaesthetized rats. Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Traces obtained during the filling phase of the micturition reflex (dotted rectangles) were enlarged to show non-voiding spontaneous microcontractions. Stable urodynamic responses to isoprenaline were reached in 10-15 min. **(B)** Show the effect of increasing the concentration of isoprenaline (0.001-1 µM) inside the lumen of the urinary bladder on the cystometry parameters recorded: Pressure threshold (PTh), intercontraction interval (ICI), contraction duration (Δt) and amplitude (A); control values correspond to zero percent variation. The vertical bars represent ± SD of three to five animals. *P<0.01 (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the control situation (zero percent).

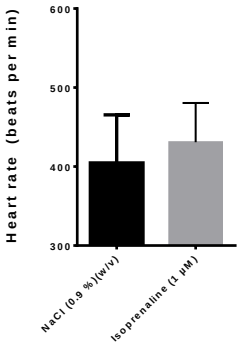


**Figure 14**: Effect of isoprenaline (1 µM) instilled directly into the bladder lumen on the heart rate of anaesthetized rats. Unpaired Student's t-test with Welch's correction was used for statistical analysis. The vertical bars represent ± SD of five animals.

## *In vivo* rat bladder cystometry

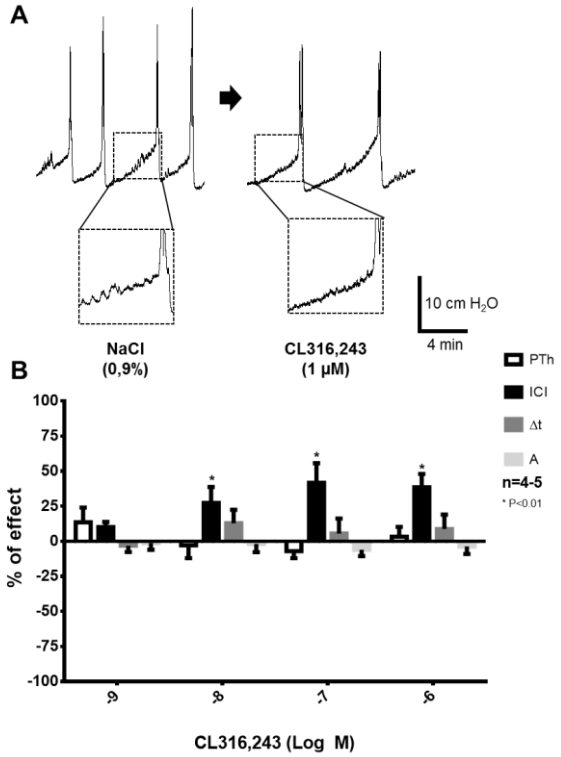


**Figure 15: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and CL316,243 (1 µM, a selective $\beta_3$ receptor agonist) infusion into the urinary bladder of urethane-anaesthetized rats. Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Traces obtained during the filling phase of the micturition reflex (dotted rectangles) were enlarged to show non-voiding spontaneous microcontractions. Stable urodynamic responses to CL316,243 were reached in 10-15 min. **(B)** Show the effect of increasing the concentration of CL316,243 (0.001-1 µM) inside the lumen of the urinary bladder on the cystometry parameters recorded: Pressure threshold (PTh), intercontraction interval (ICI), contraction duration (Δt) and amplitude (A); control values correspond to zero percent variation. The vertical bars represent ± SD of four to five animals. *P<0.01 (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the control situation (zero percent).

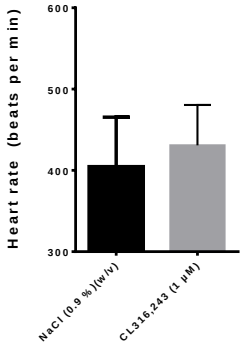


**Figure 16:** Effect of CL316,243 (1 µM) instilled directly into the bladder lumen on the heart rate of anaesthetized rats. Unpaired Student's t-test with Welch's correction was used for statistical analysis. The vertical bars represent ± SD of five animals.

The selective $\beta_3$ receptor antagonist, SR59230A (100 nM), applied either directly into the lumen of the bladder or intravenously via the jugular vein, prevented ($P<0.05$) the inhibitory effect on the voiding frequency of intravesical isoprenaline (0.001-1 μM) (Figure 17). This was evidenced by the reversal of the isoprenaline-induced increase in the ICI by SR59230A (100 nM). A similar result was obtained when the selective $\beta_3$ receptor agonist, CL316,243 (0.001-1 μM), was used instead of isoprenaline (Figure 18).

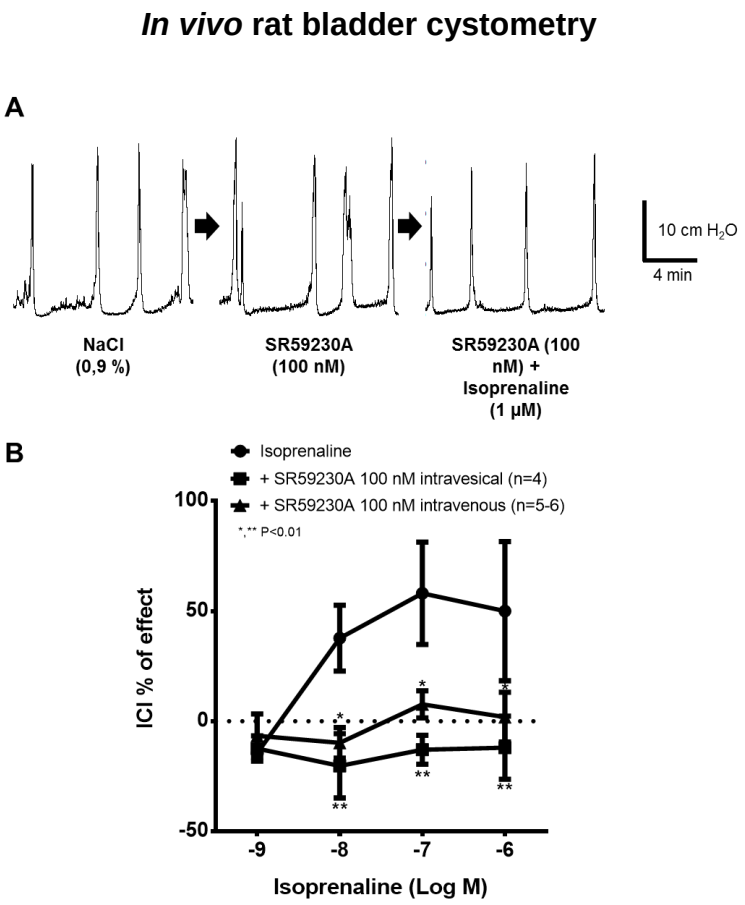


**Figure 17: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and isoprenaline (1 μM) infusion into the urinary bladder of urethane-anaesthetized rats in the absence and in the presence of the selective $\beta_3$-receptor antagonist, SR59230A (100 nM). Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Stable urodynamic responses to isoprenaline were reached in 10-15 min. **(B)** Show concentration-response curves of isoprenaline (0.001-1 μM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of SR59230A (100 nM), applied either directly into the lumen of the bladder (intravesical) or via the jugular vein (intravenous); control values correspond to zero percent variation. The vertical bars represent ± SD of five to six animals. *,**$P<0.01$ (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline applied alone.

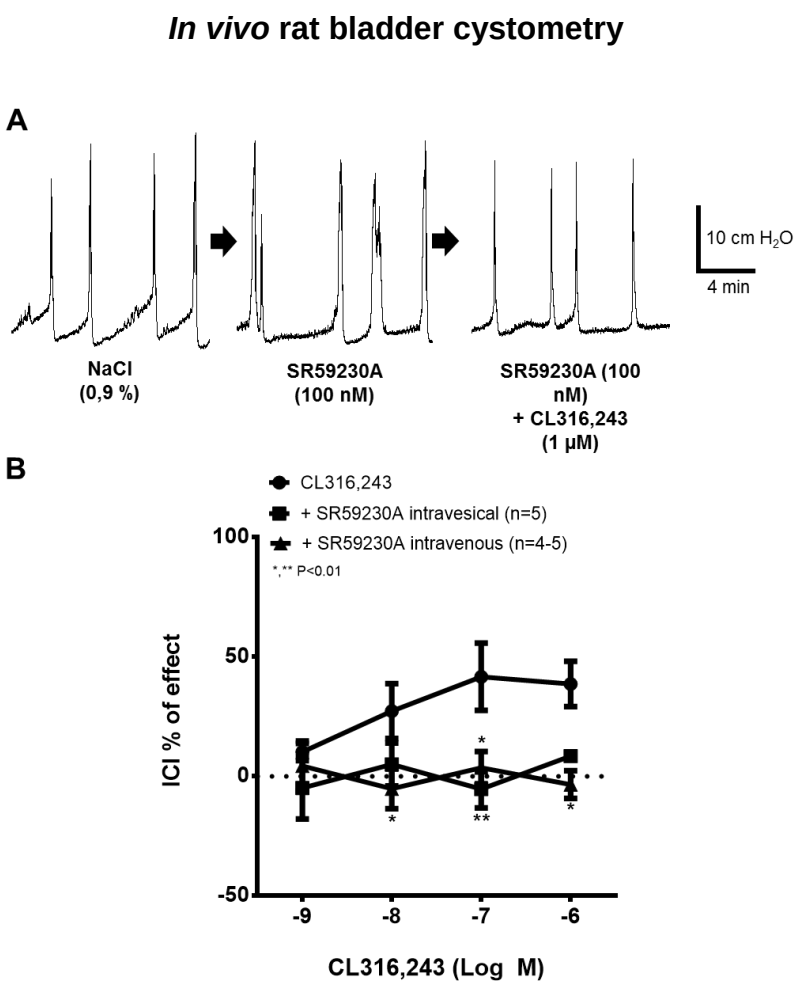


**Figure 18**: **(A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and CL316,243 (1 µM) infusion into the urinary bladder of urethane-anaesthetized rats in the absence and in the presence of the selective $\beta_3$-receptor antagonist, SR59230A (100 nM). Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Stable urodynamic responses to CL316,243 were reached in 10-15 min. **(B)** Show concentration-response curves of CL316,243 (0.001-1 µM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of SR59230A (100 nM), applied either directly into the lumen of the bladder (intravesical) or via the jugular vein (intravenous); control values correspond to zero percent variation. The vertical bars represent ± SD of four to five animals. *,**P<0.01 (Two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of CL316,243 applied alone.

Selective blockade of the adenosine $A_1$ receptor with DPCPX (100 nM), applied either directly into the lumen of the bladder or intravenously via the jugular vein, prevented ($P<0.05$) isoprenaline (0.001-1 µM)-induced reduction of the voiding frequency when the β-adrenoceptor agonist was applied inside the lumen of the bladder (Figure 19). This was revealed by the reversal of isoprenaline-induced increase in the ICI by DPCPX (100 nM). Co-administration of isoprenaline (0.001-1 µM) plus DPCPX (100 nM) did not affect other urodynamic parameters, namely PTh, $\Delta$t and A, as well as

heart rate (data not shown). On its own, DPCPX (100 nM) was unable to modify any of the cystometry paramaters evaluated in this study, independently of the route of administration (data not shown).

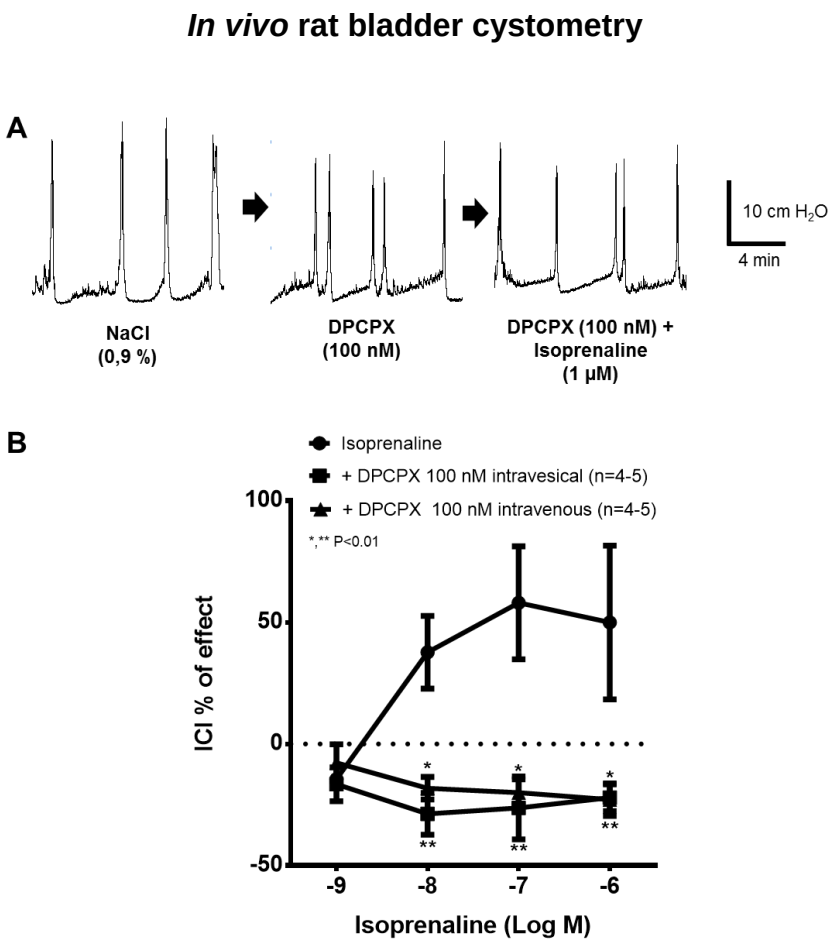


**Figure 19: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and isoprenaline (1 µM) infusion into the urinary bladder of urethane-anaesthetized rats in the absence and in the presence of the selective $A_1$-receptor antagonist, DPCPX (100 nM). Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Stable urodynamic responses to isoprenaline were reached in 10-15 min. **(B)** Show concentration-response curves of isoprenaline (0.001-1 µM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of DPCPX (100 nM), applied either directly into the lumen of the bladder (intravesical) or via the jugular vein (intravenous); control values correspond to zero percent variation. The vertical bars represent ± SD of five to six animals. *,**P<0.01 (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline applied alone.

Intracellular cyclic AMP accumulation resulting from $\beta_3$ adrenoceptors activation by isoprenaline and CL316,243 (Hatanaka et al., 2013) may be linked to enhanced levels of extracellular adenosine and to the activation of $A_1$ receptors in neighboring cells. Adenosine may be originated (1) from the extracellular catabolism of cyclic AMP pumped out from cells via ATP-binding cassete (ABC) transporters (Jedlitschky et al., 2000) and

then subsequently converted into AMP and adenosine via an enzymatic cascade involving ecto-nucleotide pyrophosphatase / phosphodiesterase 1 (ENPP1) and ecto-5'-nucleotidase/CD73 (Chiavegatti et al., 2008; Sassi et al., 2014), or (2) through the release of adenosine, as such, via equilibrative bi-directional nucleoside transporters (ENTs) as a consequence of increased degradation of cyclic AMP into AMP and adenosine inside cells (King et al., 2006).

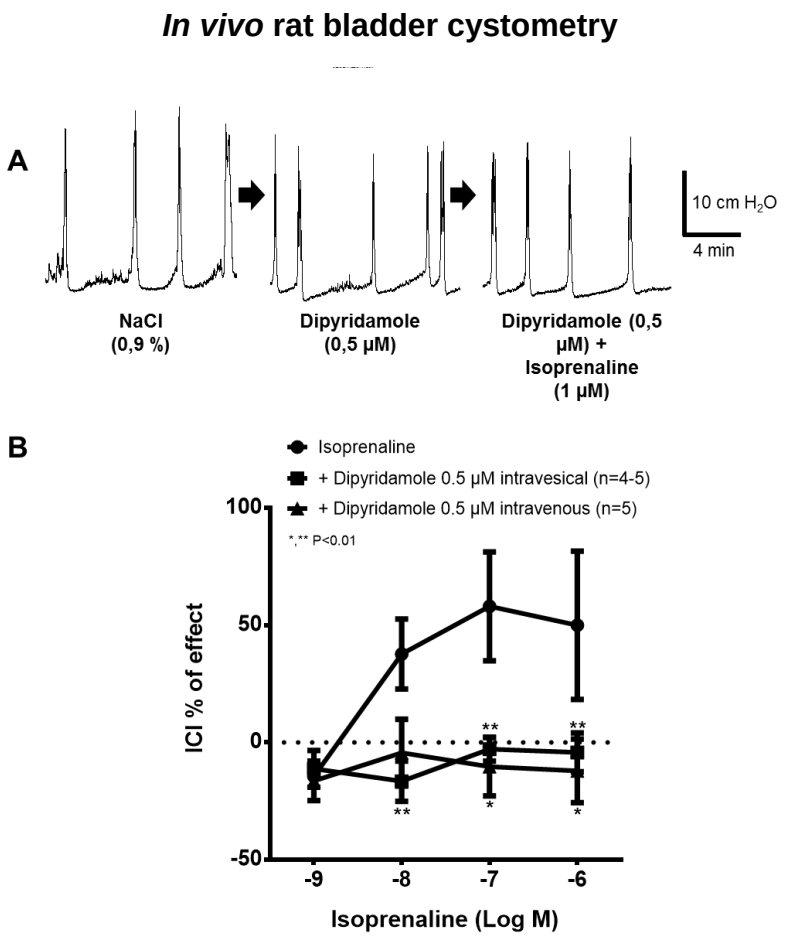


**Figure 20: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and isoprenaline (1 µM) infusion into the urinary bladder of urethane-anaesthetized rats in the absence and in the presence of the equilibrative nucleoside transport (ENT) inhibitor, dipyridamole (0.5 µM). Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Stable urodynamic responses to isoprenaline were reached in 10-15 min. **(B)** Show concentration-response curves of isoprenaline (0.001-1 µM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of dipyridamole (0.5 µM), applied either directly into the lumen of the bladder (intravesical) or via the jugular vein (intravenous); control values correspond to zero percent variation. The vertical bars represent SD of four to five animals. *,**P<0.01 (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline applied alone.

In this study, we evaluated the role of equilibrative nucleoside transporters (ENTs) on isoprenaline-induced decrease in the voiding frequency in urethane-anaesthetized rats using dipyridamole (0.5 μM), a non-selective ENT inhibitor, and NBTI (30 μM), a selective ENT1 inhibitor. On their own, applied either directly into the lumen of the bladder or intravenously via the jugular vein, dipyridamole (0.5 μM) and NBTI (30 μM) were devoid of effects on evaluated urodynamic parameters (Figures 20 and 21, respectively). Like that observed with the $A_1$ receptor antagonist, DPCPX (100 nM), blockade of adenosine outflow from cells via ENTs with dipyridamole (0.5 μM) and NBTI (30 μM) fully prevented ($P<0.05$) the inhibitory effect of isoprenaline (0.001-1 μM) on the voiding frequency when the β-adrenoceptor agonist was instilled into the bladder lumen; this was concluded because prolongation of the ICI caused by isoprenaline (0.001-1 μM) alone was revert to control levels in the presence of dipyridamole (0.5 μM) (Figure 20) and NBTI (30 μM) (Figure 21), independently of the route of administration (intravesical or intravenous) of the two ENT inhibitors. Co-administration of isoprenaline (0.001-1 μM) with dipyridamole (0.5 μM) or NBTI (30 μM) did not affect heart rate and other urodynamic parameters (PTh, ∆t and A) besides the ICI (data not shown).

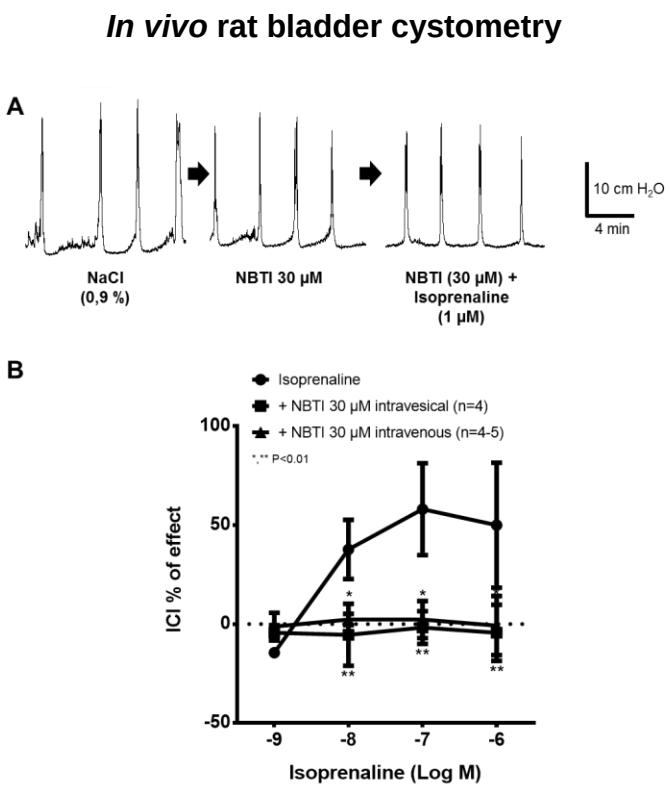


**Figure 21: (A)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and isoprenaline (1 μM) infusion into the urinary bladder of urethane-anaesthetized rats in the absence and in the presence of the equilibrative nucleoside transport (ENT) inhibitor, NBTI (30 μM). Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Stable urodynamic responses to isoprenaline were reached

in 10-15 min. **(B)** Show concentration-response curves of isoprenaline (0.001-1 μM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of NBTI (30 μM), applied either directly into the lumen of the bladder (intravesical) or via the jugular vein (intravenous); control values correspond to zero percent variation. The vertical bars represent ± SD of four to five animals. *,**P<0.01 (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline applied alone.

## 4.2. Measurement of adenosine release

Incubation of detrusor strips of rat urinary bladder with isoprenaline (1 μM) for 15 min increased significantly (to 174±21 nM, n=4, *P<0.0001*) the concentration of adenosine released, while the amount of inosine (40±4 nM, n=4, *P>0.05*) remained constant compared to basal conditions (43±4 nM, n=4). Isoprenaline (1 μM)-induced adenosine release was further increased to 241±13 nM (n=4, *P<0.0001*) after electrical stimulation of the preparations (Figure 22). The extracellular concentration of adenosine measured under these experimental conditions is probably underestimated, because it was obtained in a rather large volume of incubation media (2.8 ml) compared with the reduced interstitial volume of the bladder in vivo.

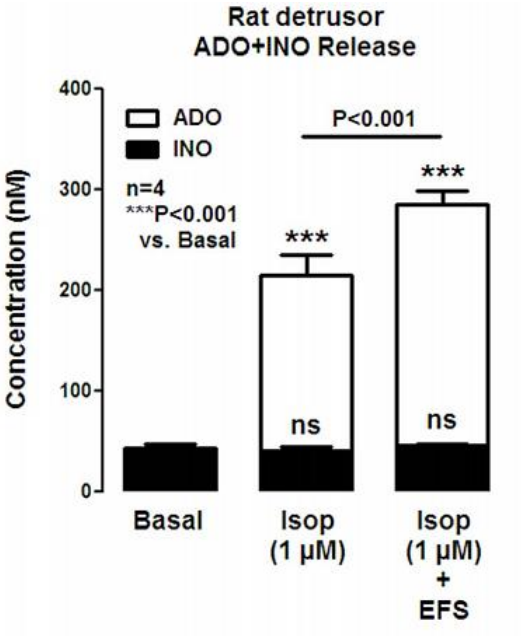


**Figure 22:** Isoprenaline (Isop, 1 μM) promotes the release of adenosine from urothelium- denuded rat detrusor strips, both under resting conditions and during electrical field stimulation (EFS: 10 Hz, 3000 pulses of 1 ms duration) of the preparations. Isoprenaline (1 μM) was added 15 min before EFS and remained in the incubation fluid until the end of the experiment. The ordinates represent the concentration of adenosine (ADO, white bars) and inosine (INO, black bars) detected by HPLC with diode array detection in samples collected from the incubation media at 15 min intervals. The data are means ± SD of four animals; duplicates were performed for each individual experiment. ****P<0.001* (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation (Basal).

## 4.3. Kinetic of the extracellular catabolism of cyclic AMP

As previously mentioned, cyclic AMP pumped out from many cell types, including the rat detrusor (Uchida et al., 2005), can be converted into adenosine extracellularly via an enzymatic cascade involving ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and ecto-5'- nucleotidase/CD73 (Chiavegatti et al., 2008; Sassi et al., 2014). In figure 23 shows the enzymatic kinetic experiment where we studied the time course of the extracellular catabolism of cyclic AMP (30 µM) and formation of its metabolites, including AMP, adenosine, inosine and hypoxanthine, in urothelium-denuded strips of the rat urinary bladder. The activity of ENPP1 calculated 15-min after cyclic AMP (30 µM) application by the ratio of [AMP+ADO+INO+INO]:[cyclic AMP] per min was $10.7 \times 10^{-3}$ (n=3). Under similar experimental conditions dephosphorylation of AMP (30 µM) by ecto-5'-nucleotidase/CD73 calculated by the ratio of [ADO+INO+INO]:[AMP] per min was also low ($7.4 \times 10^{-3}$, n=3) (data not shown). Consequently, the amount of adenosine (plus inosine and hypoxanthine) in the incubation fluid originating from the extracellular catabolism of cyclic AMP (30 µM) and AMP (30 µM) in the rat bladder did not surpass 2.27 µM and 2.14 µM, respectively, during the first 15-min of incubation.

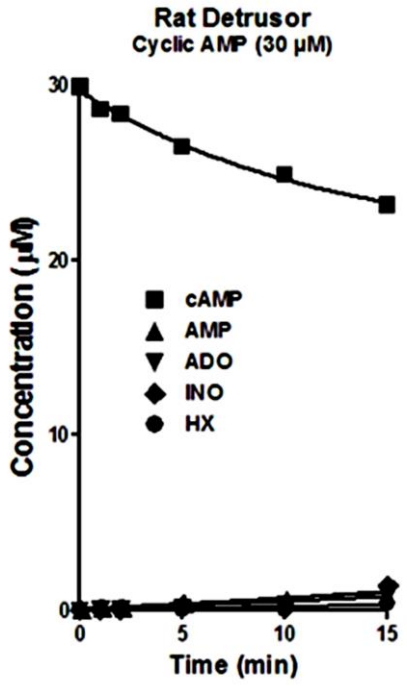


**Figure 23***:* Kinetics of the extracellular catabolism of cyclic AMP in urothelium-denuded strips of the rat urinary bladder. Cyclic AMP (30 µM) was added at zero time to the incubation medium. Samples were collected from the incubation fluid at the indicated times on the abscissa and analyzed by HPLC with UV detection to quantify cyclic AMP (squares), AMP (upward triangles), adenosine (ADO, downward triangles), inosine (INO, lozenges) and hypoxanthine (HX, circles).

Average results obtained from four animals made in triplicate; the vertical bars represent ± SD and are shown when they exceed the symbols in size.

## 4.4. Quantification of [$^3$H]-ACh release

Figure 24A show that isoprenaline (1 μM) decreased ($P<0.05$) by 38±1% (n=4) the evoked [$^3$H]-ACh release (10 Hz, 200 pulses) in rat urinary bladder strips without urothelium. Moreover, the isoprenaline (1 μM) inhibitory action on evoked [$^3$H]-ACh outflow was mimicked by CL316243 (1 μM), which selectively activates the $\beta_3$-adrenoceptor in rodents (Evans et al., 1999) (Figure 24B). Involvement of $\beta_3$-adrenoceptors mediating inhibition of transmitter release caused by isoprenaline (1 μM) and CL316,243 (1 μM) was further confirmed by showing that their effects were significantly ($P<0.05$) attenuated by the $\beta_3$-adrenoceptor antagonist, SR59230A (100 nM) (Figure 24B).

$\beta_3$-adrenoceptors usually couple to adenylyl cyclase via $G_s$ proteins to generate intracellular cyclic AMP. In this context, we show in Figure 24B that the inhibitory effects of isoprenaline (1 μM) and CL316,243 (1 μM) were reproduced by the adenylyl cyclase activator, forskolin (3 μM), which on its own decreased evoked [$^3$H-]ACh release by 61±7% (n=5).

In many tissues, intracellular cyclic AMP accumulation translates into increased extracellular adenosine amounts, which negatively modulates ACh release from cholinergic bladder nerves via $A_1$ receptors activation, as our group observed in the human bladder (Silva-Ramos et al., 2015a). Therefore, we tested whether this mechanism was responsible for the $\beta_3$-adrenoceptor inhibitory control of cholinergic neurotransmission in rat detrusor strips. Figure 24A shows that transmitter release inhibition caused by isoprenaline (1 μM) was significantly attenuated ($P<0.05$) by blocking selectively the adenosine $A_1$ receptor with DPCPX (100 nM). Likewise, inhibition of adenosine release from cells by blocking ENTs with dipyridamole (0.5 μM) and NBTI (30 μM) also prevented the inhibitory effect of isoprenaline (1 μM) on evoked [$^3$H]-ACh release from the rat detrusor strips (Figure 24A).

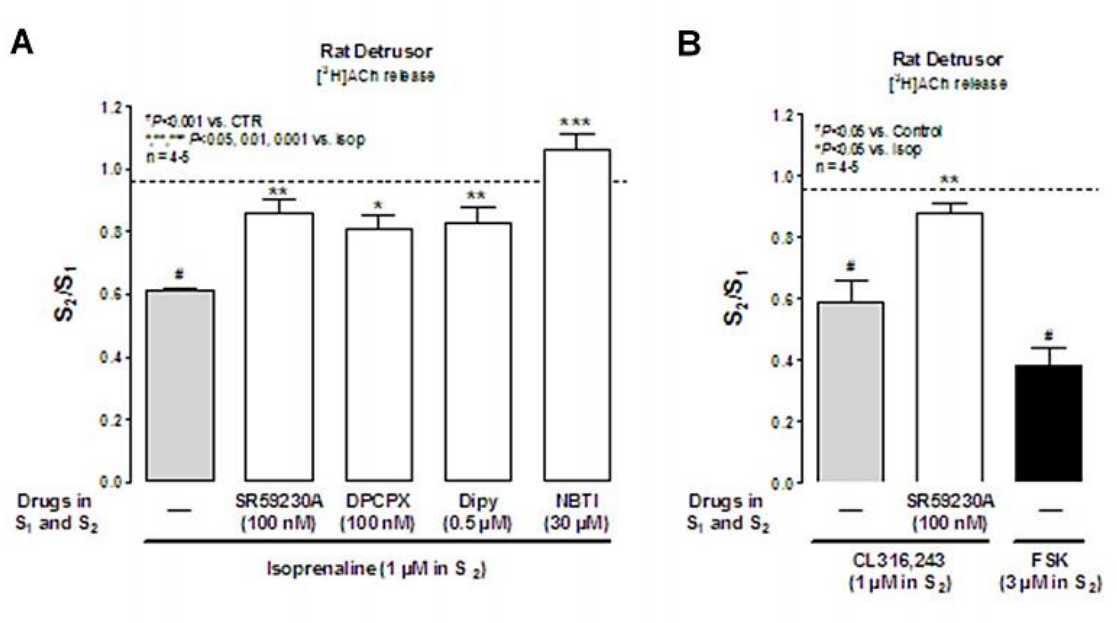


**Figure 24: (A)** Changes in the inhibitory effect of isoprenaline on electrically-evoked [$^3$H]-ACh release from urothelium-denuded rat detrusor strips in the presence of SR59230A (100 nM, $\beta_3$-adrenoceptor antagonist), DPCPX (100 nM, adenosine $A_1$ receptor antagonist), dipyridamole (Dipy, 0.5 µM, ENT1 and ENT2 inhibitor) and NBTI (30 µM, selective ENT1 inhibitor). [$^3$H]-ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4$^{th}$ ($S_1$) and 13$^{th}$ ($S_2$) minutes after the end of washout (zero time). Isoprenaline (1 µM) was added to the incubation media 6 min before $S_2$; SR59230A (100 nM), DPCPX (100 nM), Dipy (0.5 µM) and NBTI (30 µM) were present throughout the assay, including $S_1$ and $S_2$. Ordinates are changes in $S_2/S_1$ ratios compared to the $S_2/S_1$ ratio obtained without addition of any drug (dotted horizontal line). The data are means ± SD of four to five individuals; duplicates were performed for each individual experiment. $^{\#}$P<0.001 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; *P<0.05, **P<0.01 and ***P<0.001 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the inhibitory effect of isoprenaline. **(B)** For comparison purposes we also tested the effect of the selective $\beta_3$-adrenoceptor agonist, CL316,243 (1 µM), and the adenylyl cyclase activator, FSK (3 µM), on evoked [$^3$H]-ACh release from rat detrusor strips under similar experimental conditions. The inhibitory effect of CL316,243 (1 µM) on evoked [$^3$H]-ACh release was also prevented in the presence of SR59230A (100 nM) applied throughout the assay, including $S_1$ and $S_2$. The data are means ± SD of four to five individuals; duplicates were performed for each individual experiment. $^{\#}$P<0.05 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; *P<0.05 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the inhibitory effect of CL316,243.

## 4.5. Immunolocalization of $\beta_3$-adrenoceptors and $A_1$ receptors in rat detrusor layer

Immunofluorescence confocal micrographs shown in Figure 25 indicate that $\beta_3$-adrenoceptors are diffusely expressed on smooth muscle fibers of the human detrusor, whereas the adenosine $A_1$ receptor co-localizes with the vesicular ACh transporter (VAChT) on bladder cholinergic nerve terminals, as already observed in human bladder strips (Silva-Ramos et al., 2015). Using higher magnification images (right hand-side

panels) we failed to co-localize $\beta_3$-adrenoceptors with VAChT immunostaining, thus suggesting that these receptors are not expressed on cholinergic nerve terminals (*cf.* Otsuka et al., 2013).

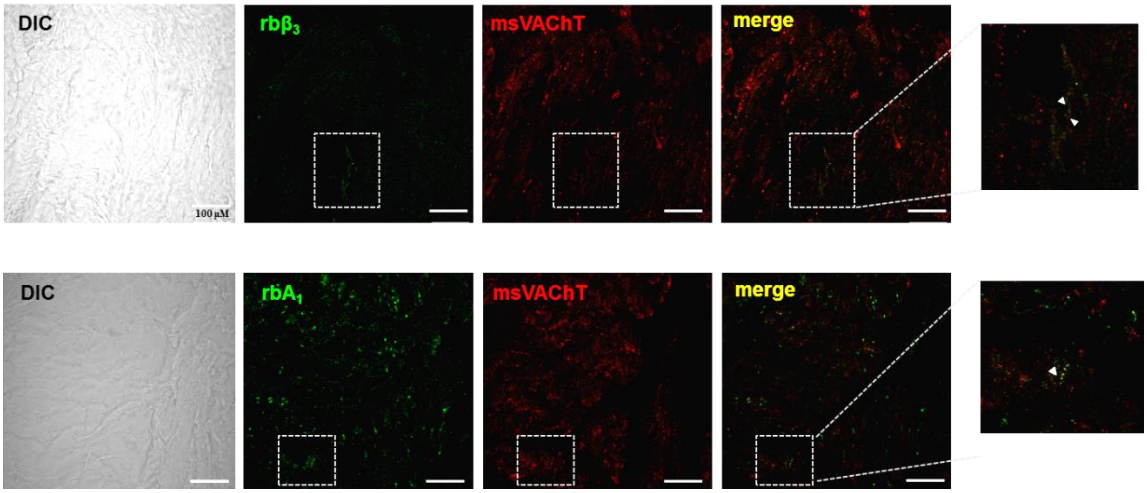


**Figure 25:** Upper panels, represent confocal micrographs of transverse sections of the rat detrusor showing that the $\beta_3$-adrenoceptor immunoreactivity (green) is diffusely distributed among smooth muscle fibers, but is not present on cholinergic nerve fibers stained with the vesicular ACh transporter (VAChT, red); this aspect is indicated by the open arrowheads in the higher magnification image shown on the right hand-side panel. Bottom panels, show that the adenosine $A_1$ receptor (green) is predominantly expressed on VAChT-positive cholinergic nerve terminals (red) of the human detrusor; co-localization of $A_1$ and VAChT immunofluorescence is identified by closed arrowheads in the high magnification image shown on the right hand-side panel. Images are representative of four different individuals. Differential interference contrast (DIC) images are also shown for comparison. Scale bar= 100 µm.

## 4.6. Expression of equilibrative nucleoside transporters (ENTs) in the rat detrusor

In support for the involvement of equilibrative nucleoside transporters mediating adenosine outflow in response to $\beta_3$-adrenoceptors activation in the urinary bladder, we show in Figure 26 that the rat detrusor exhibits significant immunoreactivity against NBTI-sensitive ENT1 (SLC29A1) and NBTI-insensitive ENT2 (SLC29A2) subtypes, which are localized predominantly on the plasma membrane of smooth muscle fibers and at interstitial spaces of detrusor muscle bundles, respectively.

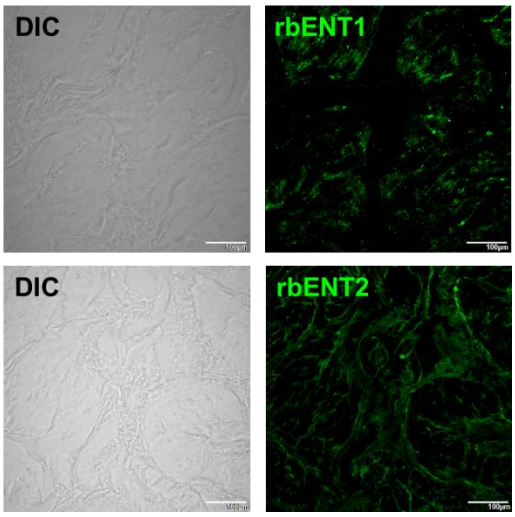


**Figure 26**: Confocal micrographs showing the immunoreactivity against equilibrative nucleoside transporters, ENT1 and ENT2, in transverse sections of rat detrusor strips. ENT1 (upper panel) and ENT2 (bottom panel) immunoreactivity is shown in green; differential interference contrast (DIC) images are also shown. Images are representative of four individuals. Scale bars = 100 µm.

# 5. Discussion

There is an increasing interest concerning the role of $\beta_3$-adrenoceptor agonists for the management of the OAB syndrome as an efficacious and more tolerable alternative to antimuscarinic drugs (Thiagamoorthy et al., 2015). It was first thought that improvement of OAB symptoms by $\beta_3$-adrenoceptor agonists, like mirabegron and solabegron, were mostly due to direct relaxation of the detrusor smooth muscle (Igawa et., 1999; Takeda et al., 1999; Wuest et al., 2009). However, newer theories suggest that $\beta_3$-adrenoceptor agonists decrease OAB symptoms also by reducing bladder afferent nerve activity (Woods et al., 2001) and/or ACh release from parasympathetic postganglionic nerves innervating the detrusor (D'Agostinho et al., 2015). These new paradigms may be clinically relevant because, contrary to the initial belief that normally no parasympathetic nerve drive was observed during bladder storage, recent evidences demonstrate that there is release of ACh from both neuronal and non-neuronal sources, such as the urothelium and lamina propria, during the filling phase of the micturition cycle (Yoshida et al., 2004; Winder et al., 2014). Moreover, this cholinergic trend may be directly or indirectly exaggerated in overactive bladder patients via the release of ATP and other "danger" molecules (Silva et al., 2015).

In our study, we verified by immunofluorescence confocal microscopy that $\beta_3$-adrenoceptors are localized on detrusor smooth muscle fibers (*cf*. Fujimura et al., 1999; Igawa et al., 1998; Igawa et al., 1999) but these receptors are absent from VAChT-positive cholinergic nerves of the rat urinary bladder regardless of their presence have been reported in intramural ganglia and in Schwann cells of intramural nerves (Limberg et al., 2010). These findings challenge the current view about the mechanism and site of action of $\beta_3$-adrenoceptor agonists on cholinergic bladder nerves activity and open the door for novel targets (e.g. adenosine) indirectly mediating inhibition of neurogenic bladder contractions. Another puzzling finding that has been insufficiently explored so far is the higher effectiveness of $\beta_3$-adrenoceptor agonists in decreasing neurogenic-induced [$^3$H]-ACh release than in producing bladder smooth muscle relaxation (D'Agostino et al., 2015; Gillespie et al., 2015; Rouget et al., 2014). This led several authors to suggest that in vivo concentrations of these drugs following clinical application are below the minimum required to affect detrusor myogenic contractions as a mechanism underlying relief of OAB symptoms (Andersson, 2015). Our results strongly suggest that isoprenaline-induced $\beta_3$-adrenoceptors activation significantly increases adenosine release from detrusor smooth muscle fibers, via NBTI-sensitive equilibrative

nucleoside transporters (ENT1), leading to retrograde activation of $A_1$ receptors and, thereby, to inhibition of [$^3$H]-ACh release from cholinergic nerves of the rat urinary bladder. Reduction of the cholinergic tone responsible for non-voiding bladder contractions, acting in coordination with the sensory inhibitory drive caused by $\beta_3$-adrenoceptors agonists and endogenous adenosine (via $A_1$ receptors), may increase bladder compliance during the filling phase of the micturition cycle resulting in improvement of OAB symptoms. This is an interesting feature that might put adenosine signaling pathways, consisting of metabolizing enzymes, nucleoside transporters, and membrane receptors, in the center of research for novel therapeutic targets to manage OAB syndromes.

However, in contrast to the relatively well characterized actions of ATP in the urinary bladder both in physiological and pathological conditions, limited information is available on the role of its breakdown product, adenosine. First reports of the effect of adenosine in the lower urinary tract suggested that it reduces the tone and the spontaneous activity of the urinary bladder in different species (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978b; Nicholls et al., 1992), including humans (Rubinsterin et al., 1998). Although information regarding location of adenosine receptors on the detrusor is still scant there are reports suggesting that low affinity $A_{2B}$ receptors are abundantly expressed in the rat bladder smooth muscle (Stehle et al., 1992; Yu et al., 2006), while our group recently showed that in the human detrusor the $A_{2A}$ receptor seems to be the more abundant receptor subtype (Silva-Ramos et al, 2015a). Conflicting pharmacological evidences for the presence of $A_1$, $A_{2A}$ and $A_3$ receptors in detrusor smooth muscle fibers subsist in the literature depending on the animal species and the experimental settings (Gopalakrishan et al, 2002; Vesela et al.,2011; Yang et al., 2000). Despite this, a remarkable parallelism seems to exist between the potency of adenosine and $\beta_3$-adrenoceptor agonists to inhibit transmitter release and to promote relaxation of myogenic detrusor contractions (see above). Data from our group show that relaxation of human detrusor strips pre-contracted with ACh required 30-fold higher concentrations (milimolar range) of adenosine than the inhibitory effect of the nucleoside on evoked [$^3$H]-ACh release operated by prejunctional $A_1$ receptors (Silva-Ramos et al., 2015a). This difference was even more significant in the bladder of patients with benign prostatic hyperplasia, a pathological condition that may be accompanied by OAB-like symptoms. It, thus, appears that bladder outlet obstruction (BOO) increases the sensitivity of nerve-evoked transmitter release to exogenous adenosine receptor agonists, a situation that occurs in parallel to the up-regulation of inhibitory $A_1$ receptors localized in VAChT-positive cholinergic nerve fibers. It remains to

be investigated whether up-regulation of the $A_1$ inhibitory control of ACh release also occurs in OAB patients, which might contribute to explain the clinical success of $\beta_3$-adrenoceptors agonists in the treatment of this syndrome. The potency of several $\beta_3$-adrenoceptor agonists, including isoprenaline, was very similar in patients with low bladder compliance, hyperreflexic bladders and controls (Igawa et al., 2001), but the relaxing effect of clembuterol was significantly greater in neurogenic-contracted bladder strips from patients with urodynamically confirmed urge incontinence compared to continent patients (Hundman et al., 2001). Amplification of $\beta_3$-adrenoceptor signaling by adenosine $A_1$ receptors may gain further relevance considering that no significant changes in the mRNA expression of $\beta_3$-adrenoceptors were observed in humans and rats with BOO (Barendrecht et al., 2009; Nomiya and Yamaguchi, 2003), yet $\beta_3$-adrenoceptors agonists decreased more efficiently non-voiding contractions in obstructed bladders in vivo (Igawa and Michel, 2013).

Using Western blot analysis and immunofluorescence localization, Yu and col. detected the expression of all four types of adenosine receptors in the bladder urothelium of female Sprague-Dawley rats (Yu et al., 2006). The $A_1$ receptors were predominantly localized to the apical membrane of umbrella cells, whereas $A_{2A}$, $A_{2B}$ and $A_3$ receptors were expressed intracellularly or on the basolateral membrane of umbrella cells and the plasma membrane of underlying cell layers. The $A_1$ receptor immunoreactivity was also observed in the underlying submucosal connective tissue, but suburothelial tissue elements (possibly connective cells, myofibroblasts or blood vessels) were more strongly labeled with the $A_{2A}$ antibody. Based upon quantitative PCR experiments, it appears that the $\beta_3$-adrenoceptor accounts for more than 95% of all β-adrenoceptor mRNA in the bladder (Nomiya and Yamaguchi, 2003; reviewed in Michel and Vrydag, 2016), which is consistent with the $\beta_3$ receptor protein immunoreactivity found by us in the detrusor (Figure 1). This finding reduces tremendously any lack of discriminative power of the antagonist, SR59230A, regarding β-adrenoceptor subtypes in functional studies (Honfmann et al., 2004) and strengthens our hypothesis that the $\beta_3$-adrenoceptor is the main responsible for the adrenergic inhibitory tone in rat bladder.

Besides the presence of $\beta_3$-adrenoceptors on smooth fibers of the rat detrusor (see above), these receptors are equally abundant in vimentin-positive interstitial cells of the suburothelium. A minor density of expression of $\beta_3$-adrenoceptors was found in the urothelium, with the majority of these receptors present in the apical umbrella cells layer (Limberg et al., 2010; Otsuka et al., 2008). The close proximity of adenosine $A_1$ and $\beta_3$ receptors in umbrella cells may explain their crosstalk to decrease sensitivity of the

urinary bladder to isoprenaline-containing saline distension in the anaesthetized rat, which resulted in reduction of the voiding frequency and in enhancement of the bladder storage capacity. Although isoprenaline-induced inhibition of the micturition cycle was prevented by co-application with selective $\beta_3$ and $A_1$ receptor antagonists, SR59230A and DPCPX, respectively, one cannot exclude the participation of suburothelial structures endowed with the two receptors, including afferent and efferent nerves (or both) and interstitial cells that may influence bladder spontaneous activity and detrusor tonus (Limberg et al., 2010). In support of this possibility, our results showed that both blockers were equally effective when instilled into the bladder lumen or when infused intravenously. Moreover, intravenous application of isoprenaline also reduced the intravesical pressure and increased bladder storage capacity in a model of distension-induced bladder activity under isovolumetric conditions in urethane-anaesthetized rats (Lecci et al., 1998).

Interestingly, previous studies demonstrated that $\beta_3$-adrenoceptors inhibit detrusor contractions of the pig urinary bladder via the urothelium (Masunaga et al., 2010), most probably through the release of an urothelium-derived factor of unidentified nature as it has been proposed in porcine and human bladders ( Murakami et al., 2007; Otsuka et al., 2013). Our best hypothesis is in favor of adenosine, which might be released in response to urothelial $\beta_3$-adrenoceptors activation via ENTs existing at the basolateral surface of epithelial cells (Loffler et al., 2007). We showed here for the first time that $\beta_3$-adrenoceptor-induced adenosine release from urothelial cells via ENTs is crucial to decrease bladder activity in the anaesthetized rat in response to infusion of isoprenaline and CL316,243 into the bladder lumen. This was concluded because the increase in the intervoid interval (ICI) promoted by the $\beta_3$-adrenoceptor agonists was reversed to control levels after pretreatment with ENT inhibitors, dipyridamole and NBTI, in a similar manner to that observed with the selective blockade of adenosine $A_1$ receptors with DPCPX.

Differences in the kinetics of the extracellular catabolism of adenine nucleotides by ecto-nucleotidases between luminal and abluminal sides of the urothelium indicate that adenosine biosynthesis predominates in the basal layer of the urothelium where ecto-5'-nucleotidase/CD73 is dominantly expressed, at least in the human bladder (Silva-Ramos et al., 2015b). This feature strengthens our hypothesis that adenosine is predominantly originated via the nucleoside transport system in the more superficial layers of the urothelium. Once in the extracellular milieu adenosine starts an inhibitory signaling cascade at more superficial urothelial layers via the activation of neighboring

cells endowed with $A_1$ receptors. It has been demonstrated that adenosine negatively modulates stretched-induced ATP release over many minutes by modulating exocytosis in the apical membrane of umbrella cells (Yu et al., 2000). A more acute (within seconds) effect to modulate distension-induced ATP release from the urothelium has also been suggested (Dunning-Davies et al., 2013). Distension-induced ATP release was decreased by adenosine (1-10 μM) and enhanced by adenosine deaminase and DPCPX, but not by blocking $A_2$ receptors with 3,7-dimethyl-1-propargyl-xanthine (DMPX). The mechanism by which adenosine reduces urothelial ATP release remains unknown, but it might depend on changes in the transepithelial potential by interference with mechanosensitive $Na^+$ channels (identified as amiloride-sensitive epithelial $Na^+$ channels or ENaC), which may ultimately favor $Ca^{2+}$ outflow via $Na^+/Ca^{2+}$ exchange in urothelial cells (Wu et al., 2011). These findings support a dominant role of adenosine $A_1$ receptors in regulating ATP release from the urothelium and, thus, in the control of the sensory filling phase of the micturition cycle. In fact, prolongation of the intercontraction interval without affecting the amplitude of voiding contractions was observed when $A_1$ receptor agonists were applied into the lumen of the urinary bladder in the rat *in vivo* (Kitta et al., 2014).

Alternatively, the production and release of NO from urothelial cells may follow β-adrenoceptors activation leading to increases in intracellular $Ca^{2+}$ triggered by activation of the cyclic AMP pathway (Birder et al., 2002). However, these authors did not specifically test the role of any selective $\beta_3$-adrenoceptor agonist/antagonist. Because NO has only minimal relaxing effects on bladder smooth muscle (Andersson and Persson, 1995), it was suggested that NO can down-regulate bladder activity by suppressing excitability of sensory nerve afferents (Pandita et al., 2000). Even though we cannot discount the participation of NO in the $\beta_3$-adrenoceptor-induced inhibitory pathway controlling bladder activity in the anaesthetized rat, full prevention of the isoprenaline effect by DPCPX, NBTI and dipyridamole strongly suggests that adenosine released via ENTs and subsequent activation of $A_1$ receptors play a relevant role.

Controversy still exists on whether the prototypical activation of adenylyl cyclase and cyclic AMP generation coupled to $\beta_3$-adrenoceptors activation is the exclusive mechanism triggering the inhibitory effects on urinary bladder tone (reviewed in Michel and Vrydag, 2006; Yamaguchi and Chapple, 2007). Other possibilities include modulation of membrane potential, ion-channels activity and intracellular ion concentrations. Isoprenaline was found to hyperpolarize the cells, prevent action potentials and decrease $Ca^{2+}$ transients. Activation of potassium currents may cause

membrane hyperpolarization and relaxation of the urinary bladder (Uchida et al., 2005), but differences in species and in the stimulation protocol used by distinct research groups impair more positive conclusions. Notwithstanding this, data from the present study indicate that both isoprenaline (via $\beta_3$-adrenoceptors activation) and the receptor-independent adenylyl cyclase activator, forskolin, decreased evoked [$^3$H]-ACh release from rat bladder strips without the urothelium. Furthermore, we showed that the inhibitory effect of isoprenaline was prevented in the presence of two equilibrative nucleoside transport inhibitors, NBTI and dipyridamole. Distribution of the immunoreactivity against NBTI-sensitive equilibrative nucleoside transporter 1 (ENT1) on the plasma membrane of detrusor smooth muscle fibers in close proximity to $\beta_3$-adrenoceptors contrast with the predominance of NBTI-insensitive ENT2 immunostaining surrounding smooth muscle bundles. Thus, imaging and functional studies suggest that $\beta_3$-adrenoceptor-induced adenosine release from detrusor bladder strips result from translocation of the nucleoside across the plasma membrane via the ENT1 subtype. This is possible because increased cyclic AMP levels in stimulated cells are effectively degraded into AMP and adenosine by highly active phosphodiesterases and 5'-nucleotidase, respectively, to control its intracellular effects. Consequently, this raises the transmembrane gradient forcing the nucleoside outflow to the extracellular compartment. Alternatively, cyclic AMP may be actively transported outside cells via several members of the group of ATP-binding cassette (ABC) transporters (Chen et al., 2001; Guo et al., 2003; Jedlitschky et al., 2000; Van Aubel et al., 2002). Even though this mechanism has been described at the urinary bladder (Uchida et al., 2005) and cyclic AMP can originate adenosine in the extracellular space in many cell types through the sequential hydrolysis by two ectoenzymes, ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and ecto-5'-nucleotidase/CD73 (see e.g. Chiavegatti et al., 2008; Sassi et al., 2014), our results demonstrate that ENPP1, converting cyclic AMP into AMP, and ecto-5'-nucleotidase/CD73 that is responsible for AMP dephosphorylation into adenosine, exhibit low productivity in the rat detrusor. Given that low adenosine amounts resulted from the extracellular cyclic AMP-adenosine pathway in rat detrusor strips, it might not account significantly for the extracellular adenosine accumulation detected following $\beta_3$-adrenoceptors activation with isoprenaline.

# 6. Conclusion

In conclusion, data from the present study suggest that inhibition of cholinergic neurotransmission by $\beta_3$-adrenoceptors activation results from adenosine release from stimulated detrusor smooth muscle fibers, via ENT1, leading to retrograde activation of prejunctional $A_1$ receptors in the rat urinary bladder (see Figure 27). $\beta_3$-adrenoceptor agonists may also exert a clinically useful effect by fine-tuning regulating the sensory bladder drive during urine storage through urothelium-derived adenosine release from mechanically-sensitive umbrella cells, which are also endowed with ENT1 nucleoside transporters. The molecular mechanism underlying adenosine release downstream $\beta_3$-adrenoceptor-induced adenylyl cyclase activation and cyclic AMP generation in smooth muscle fibers and urothelial cells, remain to be elucidated. Notwithstanding this, we propose here a novel mechanism involving endogenous adenosine release and $A_1$ receptors activation which contribute to explain, at least partially, the therapeutic success of $\beta_3$-adrenoceptor agonists on OAB symptoms. $\beta_3$-adrenoceptor agonists with the participation of endogenous adenosine may exert inhibitory effects on bladder functions increasing the storage capacity and prolonging the micturition interval, without affecting the voiding pressure or post-void residual volume. These drugs may act both (a) on the sensory bladder drive operated by mechanically-sensitive urothelial cells, sensory nerve afferents and interstitial pace-maker cells, and (b) on the efferent motor component resulting in the reduction of non-voiding cholinergic microcontrations and in the increase in bladder storage capacity by relaxing the detrusor. Apparently these processes are effectively cut-short during the voiding command by yet unknown mechanisms, which may involve the unrestrained parasympathetic boost (reviewed in Yamaguchi and Chapple, 2007); in fact, $\beta_3$-adrenoceptor agonists do not affect voiding urodynamic paramaters (maximum urinary flow and detrusor pressure at maximal urinary flow) in clinical settings (Nitti et al., 2013 ) and we did not observe any changes in the duration and amplitude of voiding urinary contractions in response to isoprenaline in the anaesthetized rat, nor these parameters were affected by blockage of adenosine outflow with dipyridamole and NBTI and, subsequent, $A_1$ receptors activation with DPCPX. Thus, we propose that pharmacological manipulation of endogenous adenosine levels and/or $A_1$ receptor activation may act synergistically with $\beta_3$-adrenoceptors activation to control bladder overactivity (see also Silva-Ramos et al., 2015a).

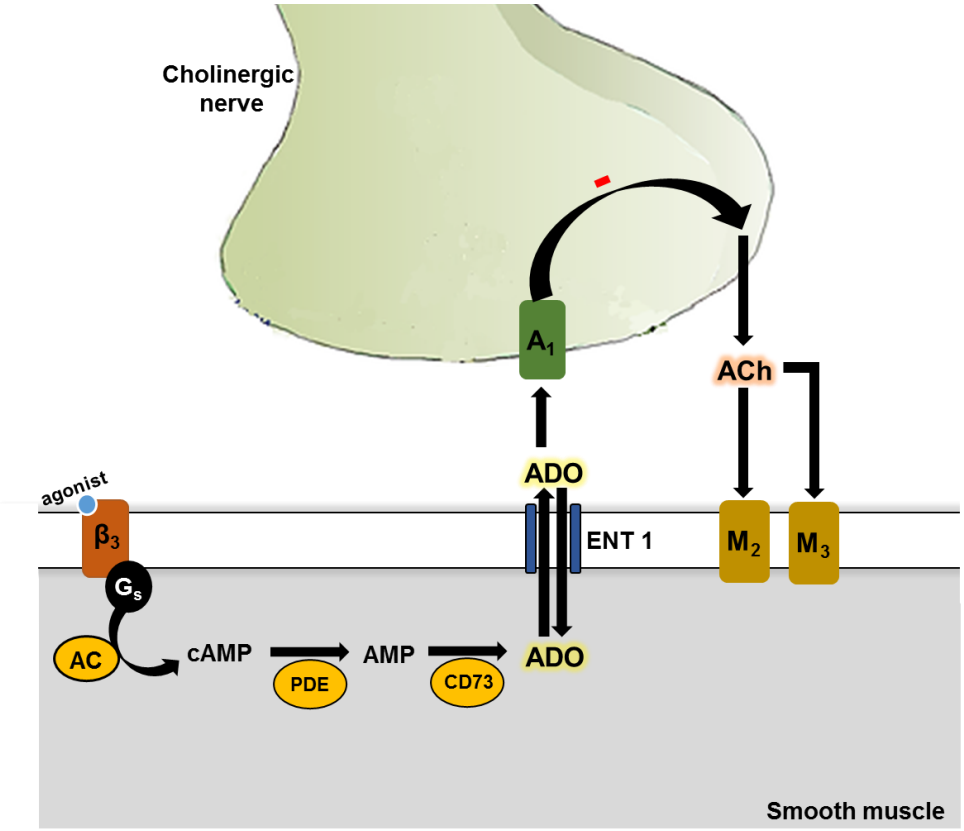


**Figure 27:** Representation of the putative mechanism underlying the action of $\beta_3$-adrenoceptors in the rat bladder. $\beta_3$-adrenoceptors activation results from adenosine release from stimulated detrusor smooth muscle fibers, via ENT1, leading to retrograde activation of inhibitory prejunctional $A_1$ receptors on cholinergic nerve terminals innervating the rat detrusor. Thus inhibition of ACh release result in less muscarinic ($M_3$ and $M_2$) receptors tone and muscle relaxation.

# 7. References

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# Supplement 1

## COMPLEX ROLES OF MUSCARINIC RECEPTOR SUBTYPES IN THE BLADDER OF URETHANE-ANAESTHETIZED MALE *WISTAR* RATS

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Antimuscarinic drugs are frequently used to treat overactive bladder syndromes. The role of muscarinic ($M_1$-$M_5$) receptor subtypes in the control of micturition is a matter of debate. Intravesical administration of the muscarinic agonist, oxotremorine (Oxo), increased the voiding frequency (VF) induced by bladder distension in urethane-anaesthetized male *Wistar* rats. The excitatory effect of Oxo was counteracted by the $M_2$ antagonist, methoctramine. On its own, methoctramine concentration-dependently decreased the VF to a maximum of 60% of control levels; a similar effect was observed with the $M_1$ antagonist, pirenzepine, although the maximal inhibitory effect was smaller (40%). The selective $M_3$ receptor antagonist, J104129, increased (by 30%) the VF when administered into the bladder, but its effect was by far more relevant in decreasing the contractile activity of the detrusor when used IV (continuous perfusion). The $M_4$ antagonist, tropicamide, was virtually devoid of effect. In contrast to the excitatory effect of intravesical Oxo (3 µM), this compound decreased electrically-evoked [$^3$H]-ACh release from detrusor strips *in vitro* through the activation of methoctramine (100 nM) and J104129 (10 nM)-sensitive receptors. Data demonstrate that muscarinic receptor subtypes $M_1$, $M_2$ and $M_3$ may exert opposite actions in different layers of the bladder wall, namely urothelium, suburothelial nerves and detrusor, thus contributing to increase the complexity of antimuscarinic drug actions.