Functional studies of purinergic and cholinergic interactions in the rat urinary bladder

Characterization of ATP-evoked urothelial release of acetylcholine

Johanna Stenqvist

Department of Pharmacology
Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2020

Cover illustration: Artwork illustration of typical whole bladder *in vitro* recordings of methacholine and ATP responses

Functional studies of purinergic and cholinergic interactions in the rat urinary bladder

johanna.stenqvist@pharm.gu.se

ISBN: 978-91-7833-856-6 (PRINT)
ISBN 978-91-7833-857-3 (PDF)
http://hdl.handle.net/2077/63235

Printed in Bohus, Sweden 2020
Ale Tryckteam

Till Viktor och Akke

Functional studies of purinergic and cholinergic interactions in the rat urinary bladder

Characterization of ATP-evoked urothelial release of acetylcholine

Johanna Stenqvist

Department of Pharmacology, Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Functional interplays between different transmitter systems are known to affect the signalling of the micturition reflex arc. The functional implications of many of these interactions, such as between ATP and acetylcholine, have yet to be unravelled. This thesis aimed to identify and characterize the functional importance of a proposed atropine-sensitive component in purinergic responses. Furthermore, its impact on afferent signalling and changes during experimental (cyclophosphamide-induced) cystitis were to be studied *in vitro* and *in vivo*.

The existence of an atropine-sensitive part of purinergic responses important for direct detrusor contractions was proven. Furthermore, this purinergic-cholinergic link was shown to be dependent on an intact urothelium as well as caveolae. This suggests a pronounced interaction between these structures, tentatively indicating the caveolae in the urothelium to be of importance for the ATP-evoked release of acetylcholine. Blockade of neuronal transmission by tetrodotoxin did not affect this release, suggesting it to be non-neuronal. Cystitis altered the urothelial signalling, diminishing the purinergic atropine-sensitivity, showing this link to be important for healthy bladder signalling and to be affected during cystitis. Also, atropine significantly decreased contractions induced by the stable ATP-analogue α,β-MeATP, demonstrating

the P2X-purinoceptors (likely P2X1 and/or P2X3) to be involved in purinergic release of acetylcholine in the healthy rat urinary bladder. Notably, *in vivo* the purinergic release of acetylcholine was able to activate the micturition reflex arc and trigger afferent signalling. The cholinergic afferent modulation depends on the activation of muscarinic receptors of the M_2/M_4- (inhibitory) and M_3-subtypes (facilitatory).

Thus, a cholinergic part of purinergic signalling exists in the rat urinary bladder, with important function both *in vitro* (*i.e.* direct contractile responses) and *in vivo* (*i.e.* modulation of afferent signalling). Cystitis hinders the purinergic-cholinergic signalling in the urothelium, presumably mediated via caveolae, showing changes in urothelial signalling to be of outmost importance in inflammatory lower urinary tract disorders. These results increase the knowledge of healthy bladder signalling and may provide new insights for future pharmacotherapies.

Keywords: ATP, acetylcholine, urothelium, caveolae, afferents, cystitis

ISBN: 978-91-7833-856-6 (PRINT)
ISBN: 978-91-7833-857-3 (PDF)

Populärvetenskaplig sammanfattning

Interstitiell cystit och överaktiv blåsa är båda exempel på sjukdomar som påverkar de nedre urinvägarna. De karaktäriseras av symtom såsom trängningar och ökat behov av att urinera. Patienter med interstitiell cystit lider också av smärta från urinblåsan eller kringliggande områden. Dessutom är urinblåsan ofta inflammerad vid interstitiell cystit. Inflammation kan påverka funktionen av urinblåsans muskulatur, vilket i sin tur kan bidra till ovannämnda symptom.

Urinblåsans muskulatur kan vara avslappnad (relaxerad) som när den lagrar urin, eller ihopdragen (kontraherad) som vid tömning av urin. Många olika signalsubstanser har visats vara involverade i dessa processer. Särskilt viktig vid kontraktion är signalsubstansen acetylkolin som utövar sin effekt genom att påverka kolinerga receptorer. Signalsubstansen adenosintrifosfat (ATP) är också viktig, särskilt vid vissa sjukdomar (såsom inflammation) och i normalfallet i de nerver som skickar signaler till hjärnan då blåsan fylls (afferenta nerver). Även andra strukturer i urinblåsan är viktiga för signalering. Ett exempel är det innersta cellagret i urinblåsan, urotelet, som både uttrycker många olika receptorer och kan frisätta signalsubstanser. Vidare har caveoler (inbuktningar på cellers membran) visats vara betydelsefulla. Signaleringen i urinblåsan kompliceras ytterligare av att de olika signalsubstanserna kan påverka varandra. Detta verkar vara av stor betydelse för normal funktion av urinblåsan. Ett sådant samspel där ATP verkar öka frisättningen av acetylkolin har identifierats i tidigare studier.

Huvudmålet med denna avhandling var att undersöka och beskriva möjliga funktionella effekter medierade av denna koppling mellan ATP och acetylkolin, samt att ta reda på om (och i så fall hur) den förändras vid inflammation. I denna avhandling har studier gjorts både på isolerad vävnad från urinblåsa (*in vitro*) och på sövd råtta (*in vivo*) för att besvara de vetenskapliga frågeställningarna. Urinblåsans normala funktion har jämförts med den hos råttor som blivit behandlade för att frambringa en lokal blåsinflammation.

Resultaten i avhandlingen visar att ATP kan frisätta acetylkolin i urinblåsan genom att stimulera purinerga receptorer, troligen av typen P2X1 och/eller P2X3, på urotelet. Denna signalering är beroende av såväl fungerande urotel

som caveoler, och visades vara viktig för ATP's sammandragande effekt på blåsmuskulaturen samt dess effekter på afferent nervsignalering. Inflammation visades påverka den urotel- och caveol-beroende signaleringen till den grad att acetylkolin inte längre frisätts vid stimulering (med ATP) av de purinerga receptorerna. Detta visar på betydelsen av ett intakt och välfungerande urotel för urinblåsans funktion, vilket förändras vid sjukdom (inflammation).

Avhandlingen presenterar också en ny roll för acetylkolin i urinblåsans signalering då signalsubstansen visades kunna modulera (öka eller minska) afferent nervsignalering genom att påverka kolinerga receptorer av typen M_3 respektive M_2/M_4. Detta är av intresse då många sjukdomar i urinblåsan, inklusive interstitiell cystit och överaktiv blåsa, tros bero på en ökad signalering från urinblåsan till det centrala nervsystemet. Resultaten visar att inte bara ATP, utan också acetylkolin, är viktigt för afferent signalering.

Vidare studier som undersöker betydelsen av ATP-inducerad acetylkolin-frisättning i andra sjukdomsmodeller behövs. Det måste även undersökas hur väl dessa resultat stämmer med situationen i människa. Avhandlingen presenterar ny kunskap om vikten av samspelet mellan dessa olika signalsubstanser för urinblåsans funktion i hälsa och hur de förändras vid inflammation. Detta kan leda till nya möjliga måltavlor för forskning kring läkemedelsbehandling av sjukdomar i de nedre urinvägarna.

List of Papers

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

I. **Stenqvist J**, Winder M, Carlsson T, Aronsson P & Tobin G. Urothelial acetylcholine involvement in ATP-induced contractile responses of the rat urinary bladder. *Eur J Pharmacol. 2017, 809:253-260; doi:10.1016/j.ejphar. 2017.05.023*

II. **Stenqvist J**, Carlsson T, Winder M & Aronsson P. Effects of caveolae depletion and urothelial denudation on purinergic and cholinergic signaling in healthy and cyclophosphamide-induced cystitis in the rat bladder. *Auton Neurosci. 2018, 213:60-70*; *doi:10.1016/j.autneu. 2018.06.001*

III. **Stenqvist J**, Carlsson T, Winder M & Aronsson P. Functional atropine sensitive purinergic responses in the healthy rat bladder. *Submitted*

IV. **Stenqvist J**, Aronsson P, Carlsson T, Winder M & Tobin G *In vivo* paracrine effects of ATP-induced urothelial release of acetylcholine in the rat urinary bladder. *Submitted*

Reprints were made with kind permission from respective publisher.

Table of Contents

Abbreviations iv

1 Introduction 1

1.1 Anatomy of the urinary bladder 1

1.1.1 The urothelium 2

1.1.2 Caveolae 2

1.2 Physiology of the urinary bladder 3

1.3 Signal transduction 4

1.3.1 Cholinergic signalling 5

1.3.2 Purinergic signalling 7

1.3.3 Relaxatory pathways 9

1.4 Purinergic interactions and the concept of atropine resistance 11

1.5 Overactive bladder syndrome 12

1.6 Bladder pain syndrome/interstitial cystitis 13

1.7 Experimental cystitis 15

1.8 Aims 16

1.8.1 Specific aims 16

2 Materials and methods 18

2.1 Cyclophosphamide-induced cystitis 18

2.2 Removal of the urothelium 18

2.3 *In vitro* organ bath functional studies 18

2.4 Immunohistochemistry 19

2.5 Western blot 19

2.6 Nissl staining 20

2.7 *In vivo* whole bladder setup (cystometry) 20

2.8 *In vivo* split-bladder setup 21

2.9 Statistical analysis 22

2.10 Materials 22
2.10.1 Cholinergic and purinergic substances 22
2.10.2 Other substances 23
2.10.3 Reagents and solutions for Immunohistochemistry and western blot 23
3 Results and Discussion 24
3.1 Atropine-sensitive purinergic functional responses 24
3.1.1 Urothelial involvement 25
3.1.2 Neuronal involvement 27
3.1.3 Characterization of involved receptors 28
3.1.4 Changes in experimental cystitis 30
3.2 Functional roles of caveolae in urinary bladder signalling 31
3.2.1 Changes in response to caveolae depletion 31
3.2.2 Changes in experimental cystitis 32
3.2.3 A link between the caveolae and urothelium important to atropine-sensitive purinergic responses 33
3.3 Afferent and efferent modulation *in situ* 34
3.3.1 Purinergic and cholinergic interplay on afferent signalling 35
3.3.2 Acetylcholine and afferent modulation 36
4 Concluding remarks 37
4.1 Conclusion 38
5 Acknowledgement 39
6 References 41

Abbreviations

α,β-MeATP	α,β-methylene adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ANS	autonomic nervous system
ATP	adenosine 5'-triphosphate
BPS	bladder pain syndrome
CarAT	carnitine-acetyltransferase
ChAT	choline-acetyltransferase
CYP	cyclophosphamide
EFS	electrical field stimulation
IC	interstitial cystitis
i.v.	intravenous
LUTS	lower urinary tract symptoms
Me-β-CD	methyl-β-cyclodextrine
mN	millinewton
MRS2578	N,N"-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)]thio urea
NANC	non-adrenergic, non-cholinergic
NE	norepinephrine
NECA	5'-(N-ethylcarboxamido)adenosine
NO	nitric oxide
OAB	overactive bladder

OCT	organic cation transporter
PAG	periaqueductal grey
PLC	phospholipase C
PMC	pontine micturition center
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid
TTX	tetrodotoxin
VAChT	vesicular acetylcholine transporter
VNUT	vesicular nucleotide transporters
UDP	uridine 5'-diphospate
UTP	uridine 5'-triphospate

1 Introduction

The parasympathetic innervation transmits signals for emptying the bladder and acetylcholine is the principal transmitter in this nervous system. However, more than a 100 years ago, it was observed that a large part of these parasympathetic responses are resistant to the muscarinic antagonist atropine and thus dependent on other transmitters as well. Over the years to come efforts were made in order to identify the compounds responsible for these non-adrenergic, non-cholinergic (NANC) responses. Eventually, in the 1970's, ATP was established to be a co-transmitter in the parasympathetic nervous system. During the following decades, the existence of any NANC-transmission in the human urinary bladder was much debated. However, the knowledge regarding the roles of ATP in the micturition reflex arc has been broadened by more recent findings, namely that ATP has important functions in the initiation of the micturition reflex arc. The importance of ATP is also made evident by its altered functions during pathological conditions in man.

In this thesis, the origin and functional importance of NANC-transmission in the rat urinary bladder is studied. The meaning of NANC-signalling is also examined in a new perspective, where the interactions between acetylcholine and ATP may occur at other sites as well, rather than in the parasympathetic junction.

1.1 Anatomy of the urinary bladder

The urinary bladder is an expandable organ situated in the lower abdomen with the main purpose of storing and eliminating urine. Urine enters the bladder via the two ureters, situated dorsally, which constitute the base of a triangular area called the trigone. The top of the inverted triangle is formed by the inner urethral orifice of the urethra, located at the inferior part of the bladder base. At the end of the urethra an outer urethral sphincter is located which, unlike the rest of the bladder, consists of striated skeletal muscle and is voluntarily controlled. The bladder wall consists of an inner uroepithelial coating (the urothelium), a suburothelial layer, often called the lamina propria, a smooth muscle layer (detrusor muscle) and an outer layer of fibrous connective tissue known as the adventitia or serosal layer. The detrusor smooth muscle in both humans and rats consists of an inner and outer longitudinal muscle layer and a circular layer between. When empty the bladder wall is folded into rugae, wrinkles that allow the bladder to stretch in order to accommodate larger

amounts of urine. At a certain threshold, stretch-induced afferent signals are triggered relaying the need for voiding (see section: 1.2). A median excreted volume in humans has been estimated to be around 330 ml (Fitzgerald et al., 2002).

1.1.1 The urothelium

The innermost cell layers of the bladder wall constitute the transitional epithelium of the bladder called the urothelium. The urothelium consists of three different cell types, the umbrella cells, the intermediate cells and the basal cells. (Jost et al., 1989). The superficial umbrella cells form an important barrier between the urine and the rest of the body through tight junctions, apical uroplakins and the protective mucus-like glucose-amino-glycan layer (GAG-layer) (Hu et al., 2000; Varley et al., 2006).

The urothelium is today known not only to function as a passive barrier against harmful substances but also to be a sensory organ, expressing various receptor subtypes (Merrill et al., 2016). Furthermore, transmitter substances such as adenosine 5'-triphosphate (ATP), acetylcholine and nitric oxide (NO) may be released from the urothelium (Birder et al., 1998; Ferguson et al., 1997). This may stimulate afferent or efferent nerve fibres, trigger smooth muscle contraction and have paracrine effects on the urothelium and/or suburothelium. During inflammation the urothelial release of NANC-substances, primarily ATP and NO, is increased which might sensitize the afferent nerve fibres with urinary frequency and sensations of pain as a result (Andersson et al., 2008; Mansfield & Hughes, 2014; Vesela et al., 2012).

1.1.2 Caveolae

Caveolae are cholesterol rich membrane invaginations that are expressed in most mammalian cells. Since their discovery in the 1950s, the caveolae have been assigned various abilities including exocytosis, endocytosis and signal transduction (Cohen et al., 2004; Palade, 1955; Parton & Simons, 2007). Research has shown that a large variety of receptors can be expressed in, or translocated to, these structures; thus the caveolae mediated signal transduction may be important for bladder contraction (Li et al., 1995; Lisanti et al., 1994; Shaul & Anderson, 1998). Furthermore, the caveolae have been shown to be involved in urinary bladder disorders such as overactive bladder, bladder hypertrophy and bladder cancer (Cohen et al., 2004; Cristofaro et al., 2012; Fu

et al., 2017). A study from 2012 showed a decrease of the caveolae specific protein caveolin-1 in cyclophosphamide-induced (CYP-induced) cystitis (Kim et al., 2012). However, functional studies are missing.

1.2 Physiology of the urinary bladder

The micturition cycle in man can be divided into two separate phases; the filling of the bladder/urine storage and the excretion of urine/micturition respectively. During filling, the sympathetic branch of the autonomic nervous system (ANS) is dominant. These nerves exit the spinal cord at the thoraco-lumbar level (T10-L2). The sympathetic hypogastric nerve reaches the bladder via the inferior mesenteric ganglion, some sympathetic nerves also reach the bladder via the paravertebral sympathetic chain ganglia and the pelvic nerves (De Groat & Ryall, 1969; de Groat et al., 2015; Fehér et al., 1980; Langley & Anderson, 1895). The sympathetic neurotransmitter norepinephrine (NE) stimulates β-adrenergic receptors (mainly of the β_3-subtype) on the detrusor muscle resulting in relaxation of the bladder wall promoting bladder compliance (Abdel-Rahman et al., 1983; Elmér, 1974). Furthermore, NE stimulates contraction of the internal urethral sphincter and bladder neck via α_1-adrenoceptors, counteracting leakage of urine during the storage phase (Caine et al., 1975; Chang et al., 2000).

During bladder filling the release of acetylcholine from the pudendal nerve endings (arising from motor neurons in the S2-S4 segments of the spinal cord) promotes contraction of the external urethral sphincter through the stimulation of cholinergic nicotinic receptors. As the bladder fills, a certain threshold level is eventually reached where stretch-sensitive receptors in the urothelium have been suggested to activate, triggering the release of neurotransmitters such as ATP (Birder et al., 2005; Ferguson et al., 1997; Ishihama et al., 2006). Urothelially derived neurotransmitters may alter afferent firing, however there are also mechanoreceptors expressed directly on afferent nerve endings (Häbler et al., 1993a; Häbler et al., 1993b).

Two types of afferents are present in the bladder, namely myelinated Aδ- and unmyelinated C-fibres (Fowler et al., 2008; Häbler et al., 1990; Satchell & Vaughan, 1994). In the rat the micturition reflex is mainly triggered by Aδ-fibres (Mallory et al., 1989). C-fibres may have a more prominent role during various pathological conditions in the bladder as they generally respond to noxious and nociceptive stimuli for instance during inflammation (Fowler et

al., 2008). Afferent signals are transmitted through the spinal cord via neurons that project to the periaqueductal grey (PAG) (Gable & Kingsley, 1996). The PAG transmit signals to the pontine micturition center (PMC) which in turn sends efferent signals through the spinal cord back to the bladder, inhibiting sympathetic control and promoting the parasympathetic activity (Gable & Kingsley, 1996; Noto et al., 1989). Higher brain centres suggested to be involved in the micturition reflex arc, affecting the PAG activity and thereby the PMC excitation, include for example the prefrontal cortex, the hypothalamus and the bed nucleus of the stria terminalis (Fowler et al., 2008).

During the micturition phase, the parasympathetic branch of the ANS is dominant. These nerves exit the spinal cord at the sacral region (S2-S4). The parasympathetic pelvic nerve reaches the bladder through the pelvic plexus and ganglia. In female rats the ganglion is situated between the uterus and cervix, whereas the larger ganglion in male rats is situated close to the prostate (Gabella, 2004). The parasympathetic transmitter acetylcholine stimulates contraction of the bladder wall mainly via the activation of muscarinic M_3-receptors, but stimulation of M_2-receptors has also been suggested to indirectly enable bladder contraction (see section: 1.3). At the same time, ATP and NO contribute to contraction of the bladder wall, and relaxation of the bladder neck/urethra respectively, enabling voiding when suitable (Andersson et al., 1991; Aronsson et al., 2010; Persson & Andersson, 1994).

1.3 Signal transduction

The autonomic regulation of the urinary bladder, leading to contraction or relaxation of the detrusor smooth muscle, is controlled by the efferent release of transmitter substances from the pelvic and the hypogastric nerve respectively. Contraction is mainly mediated via the release of acetylcholine, acting on muscarinic receptors, and ATP acting on purinoceptors. To which extent the different transmitters are contributing to contraction seems to be species specific with a minor purinergic involvement in the human bladder (under non-pathological conditions) whereas the two transmitters are, under certain circumstances, equally responsible for the contractile response of the rat bladder (Burnstock, 1976; Burnstock, 2009; Kinder & Mundy, 1985; Yoshida et al., 2001). However, during pathological conditions, such as bladder pain syndrome/interstitial cystitis (BPS/IC), ATP seems to have a larger role in bladder signalling (Palea et al., 1993). For instance, urothelial P2X3-purinoceptors are upregulated during this condition (Sun & Chai, 2004).

Relaxation of the bladder wall is largely controlled by the release of NE, acting on adrenergic receptors.

1.3.1 Cholinergic signalling

Acetylcholine was discovered during the early 1900s and was the first acknowledged neurotransmitter. In 1936, the Nobel Prize in physiology or medicine was awarded to Sir Henry Hallett Dale and Otto Loewi for their respective discoveries leading to the identification of acetylcholine as a neurotransmitter. Dale was, in 1914, able to extract acetylcholine from *Claviceps purpurea* ergot, and examined its effect as a pharmacological substance (Dale, 1914; Ewins, 1914). In 1921, Loewi discovered a substance released from the vagal nerve in frogs, which he named "Vagusstoff" (Loewi, 1921). The substance mediated a decrease in heart rate and later turned out to be acetylcholine.

Two acetylcholine synthesizing enzymes exists, called choline-acetyltransferase (ChAT) and carnitine-acetyltransferase (CarAT), that convert choline and acetyl coenzyme A to acetylcholine in some cell types. ChAT is mainly responsible for the synthesis of acetylcholine in the neurons whereas the mitochondrial CarAT seems to be responsible for the non-neuronal synthesis of acetylcholine (Ekstrom & Elmer, 1977; Hanna-Mitchell et al., 2007; Yoshida et al., 2008). In the neurons, acetylcholine is stored in vesicles and the vesicular acetylcholine transporter (VAChT) is responsible for the uptake of the transmitter into vesicles, the subsequent release of acetylcholine is mediated by calcium-dependent exocytosis (Carroll & Goldberg, 1975; Weihe et al., 1996).

The release of acetylcholine from urothelial cells (non-neuronal cells) does not appear to involve vesicular uptake and exocytosis and VAChT is not expressed in these cells (Hanna-Mitchell et al., 2007). Furthermore, studies using Brefeldin (an inhibitor of vesicle formation) did not show any significant effect on the release of acetylcholine (Hanna-Mitchell et al., 2007; Lips et al., 2005). However, the polyspecific organic cation transporter (OCT), more specifically of the OCT3-subtype, has been found in urothelial cells and appears to be involved in the urothelial release of acetylcholine (Hanna-Mitchell et al., 2007). The breakdown of acetylcholine is mediated by the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) that converts acetylcholine to choline and acetate (Colović et al., 2013; Darvesh et al., 2003;

Silman & Sussman, 2008). Reuptake of the choline is mediated by the choline transporter (Kuhar & Murrin, 1978).

1.3.1.1 Muscarinic receptors

Acetylcholine activates two kind of receptors, muscarinic and nicotinic receptors; however, the muscarinic receptors are the most important subtype for contraction of the bladder wall. There are five different muscarinic receptors (M_1-M_5), all of which are guanine nucleotide-binding protein (G-protein) coupled receptors (Eglen et al., 1996). The G-proteins can be divided into subgroups called Gs, Gq, G12 and $G_{i/o}$ (T Gudermann et al., 1996). The M_1-, M_3- and M_5-receptors are excitatory $G_{q/11}$-coupled receptors and lead to the activation of phospholipase C (PLC) and a subsequent increase in calcium via the activation of inositol 1,4,5-triphosphate and diacylglycerol (Wu et al., 2000). The stimulation of the M_2- and M_4-receptors, considered to be inhibitory $G_{i/o}$-coupled receptors, inactivates adenylyl cyclase and prolongs the opening of various ion channels (*e.g.* potassium) (van Koppen & Kaiser, 2003; Zholos et al., 2004).

All five subtypes have been identified in both the human and rat urinary bladder (Tyagi et al., 2006). However, studies have shown the numerically predominating muscarinic receptor to be of the M_2 subtype, followed by the M_3 subtype in a ratio 3:1 (Goepel et al., 1998). This seems to be the case for other species as well (Hegde & Eglen, 1999; Wang et al., 1995; Yamanishi et al., 2000). The muscarinic receptor subtype consistently reported, and widely accepted, as the one most important for generating contraction of the detrusor smooth muscle is the M_3-receptor (Hegde et al., 1997; Tobin, 1995; Tobin & Sjogren, 1995). However, the M_2-receptor may be indirectly involved in the contractile response of the bladder wall through the inhibition of adenylyl cyclase (mentioned above) which in turn hinders relaxations of the detrusor muscle using this pathway (*e.g.* β-adrenergic receptors and P1-purinoceptors) (Giglio et al., 2001; Ma et al., 2002).

Muscarinic autoreceptors are also expressed on efferent neurons in form of inhibitory M_2- or M_4-receptors and facilitatory M_1-receptors in both rats and humans (D'Agostino et al., 2000; Somogyi & de Groat, 1999; Somogyi et al., 1994; Tobin & Sjogren, 1995). Studies in mice have revealed the expression of the M_2-, M_3- and M_4-receptors on afferent nerve fibres in the urinary bladder

(Nandigama et al., 2010). Furthermore, stimulation of muscarinic receptors appears to affect the C-fibre afferents (Kullmann et al., 2008).

All five receptor subtypes are also expressed on the bladder urothelium (Bschleipfer et al., 2007; Tyagi et al., 2006). Some of these receptors may also be expressed in the lamina propria on the interstitial cells of Cajal (Grol et al., 2009).

1.3.2 Purinergic signalling

Although the phenomenon of atropine-resistant bladder signalling has been described as early as 1895 (Langley & Anderson, 1895), the identification of the non-adrenergic-, non-cholinergic-, (NANC) transmitters mainly involved was not clarified until much later. In the beginning of the 70's Burnstock *et al.* introduced the concepts of purinergic neurotransmission as well as purinergic co-transmission (Burnstock, 1972; Burnstock, 1976). Many candidate substances responsible for the atropine-resistant bladder responses were tested and finally, in 1978, ATP was identified as the main NANC-transmitter involved in the parasympathetic neurotransmission in the guinea pig urinary bladder (Burnstock et al., 1978). This challenged the previous definition of neurotransmission, "Dale's Principle", which for a long time was interpreted in a way suggesting that only one type of transmitter could be released from a specific nerve cell (Dale, 1935; Eccles et al., 1954). Today, ATP is an established co-transmitter in almost all nerve types and is co-released with a large variety of neurotransmitters including acetylcholine, NE and dopamine (Banks et al., 2006; Burnstock, 2009; Silinsky, 1975).

ATP can be synthesized in three different ways; during glycolysis, the Krebs cycle and by mitochondrial oxidative phosphorylation. The formation of neuronal ATP is mainly through mitochondrial oxidative phosphorylation and is dependent on the availability of adenosine 5'-diphosphate (ADP) and/or adenosine 5'-monophosphate (AMP). Purines are produced by *de novo* biosynthesis in the cells but also through the re-uptake of purines (*e.g.* adenosine) from the extracellular matrix which can, after conversion to ADP, be used in oxidative phosphorylation. Vesicular uptake, by vesicular nucleotide transporters (VNUT), and vesicular storage of ATP have been shown previously (Gualix et al., 1999; Moriyama et al., 2017; Novak, 2003). The release of acetylcholine is calcium-dependent and subsequent exocytosis has been suggested (Pankratov et al., 2007; Pintor et al., 1992). Similarly,

urothelial release of ATP (*e.g.* stretch-induced release) partly consists of VNUT dependent exocytosis (Nakagomi et al., 2016). However, other proteins have also been shown to be involved in the release of ATP, for example pannexin hemi channels (or connexion), P2X7-purinoceptors and maxi-ion channels (Dahl, 2015; Lazarowski et al., 2011).

After dissociation from the receptor protein, the metabolism of ATP may involve a variety of different proteins. Ecto-nucleoside triphosphate diphosphohydrolases and ecto-nucleotide pyrophosphatase/ phosphodiesterases are able to transform ATP to ADP and/or ADP to AMP. AMP may be further metabolized by ecto-5´-nucleotidases (CD73) to adenosine (Yegutkin, 2008; Zimmermann, 1996; Zimmermann, 2000; Zimmermann et al., 2012). Finally, adenosine may be metabolized to inosine by a range of proteins, for instance adenosine deaminase (Cristalli et al., 2001). There are also alkaline phosphatases which may metabolize both ATP, ADP and AMP (Zimmermann, 2000).

1.3.2.1 Purinoceptors

The purinoceptors are divided into two sub-families, the receptors activated by adenosine belong to the P1-purinoceptors whereas the receptors activated by ATP/ADP belong to the P2-purinoceptors (Burnstock, 1978). The P2-purinoceptros are further separated into two different groups called P2X- and P2Y-purinoceptors (Burnstock & Kennedy, 1985). There are four different P1-purinoceptors (A_1, A_{2A}, A_{2B} and A_3) all of which are G-protein-coupled and seem to mainly affect adenylyl cyclase and hence the levels of cAMP (Abbracchio & Burnstock, 1994). Seven subtypes of P2X-purinoceptors exist (P2X1-P2X7), that are ligand gated ion channels permeable to cations such as calcium and sodium (Valera et al., 1994). Furthermore, they are expressed as hetero- or homomers (Coddou et al., 2011). The P2Y-purinoceptors are, like the P1-purinoceptors, G-protein-coupled and affect either PLC or adenylyl cyclase via their G_s-, $G_{i/0}$- or $G_{q/11}$-protein (Erb & Weisman, 2012). Currently eight different P2Y-purinoceptors have been identified ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$-$P2Y_{14}$). Some of the P2Y-purinoceptors are not only activated by ATP/ADP but can also be activated by uridine 5'-triphospate (UTP) or uridine 5'-diphospate (UDP) (Nicholas et al., 1996).

All seven P2X-purinoceptors have been identified by immunohistochemistry or RT-PCR in the rat and human urinary bladder (Creed et al., 2010; Moore et

al., 2001). However, conflicting evidence exist. For instance Creed *et al*. reported the P2X3- and P2X6-purinoceptors to be exclusively expressed in the male rat bladder and a study from O'Reilly *et al.* reported no RNA expression of these purinoceptors in the human bladder (Creed et al., 2010; O'Reilly et al., 2001). P2X1 is consistently reported as the most abundant purinoceptor in the detrusor smooth muscle in these studies and is considered to be the subtype mainly responsible for detrusor contraction (Aronsson et al., 2010). Involvement of the P2X3-purinoceptor in the contractile response has also been suggested (King et al., 2004).

P2X-purinoceptors have also been detected in the urothelium of many species including man, however some species differences appears to exist (Birder & Andersson, 2013; Elneil et al., 2001; Shabir et al., 2013). For instance, all seven P2X-purinoceptors have been found in the cat urothelium, while a recent study of primary mouse urothelial cells shows the expression of the P2X1-P2X6-purinoceptors but not P2X7 (Birder et al., 2004; Chess-Williams et al., 2019). However, P2X7 has been shown in the urothelium of rats (Lee et al., 2000). Various P2Y-purinoceptors have been detected in the urothelium as well, including the $P2Y_1$-, $P2Y_2$-, $P2Y_4$- and $P2Y_{11}$-subtypes (Birder et al., 2004; Chopra et al., 2008; Shabir et al., 2013). Studies also suggest occurrence of $P2Y_6$-purinoceptors in the urothelium (Timóteo et al., 2014).

The expression of all seven P2X-purinoceptors and $P2Y_1$, $P2Y_2$ and $P2Y_4$ has been shown on bladder afferent nerves (Chen & Gebhart, 2010; Ruan et al., 2005; Xiang et al., 1998), with the P2X2- (and/or P2X2/3-) and P2X3-purinoceptors having a demonstrated functional importance (Cockayne et al., 2005; Cockayne et al., 2000). However, contradictive results regarding these findings exist as well (Takezawa et al., 2016). Among the P2Y-purinoceptors, the $P2Y_2$-subtype has been proposed as a functionally important subtype in afferent signalling (Chen et al., 2010). All P1-purinoceptor subtypes have been detected in the rat and human urinary bladder (Dixon et al., 1996; Yu et al., 2006), with the $P1A_1$-purinoceptor seemingly the one mainly responsible for purinergic efferent modulation (Acevedo et al., 1992; Ikeda et al., 2012).

1.3.3 Relaxatory pathways

Norepinephrine (NE), discovered in the early 20th century, is the main transmitter of the sympathetic nervous system (Ahlquist, 1948; Dale, 1913). There are two groups of adrenoceptors, the α- and β-adrenergic receptors. The

α-adrenergic receptors are further divided into α_1 and α_2 subtypes, where there are three different α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) and three different α_2-adrenoceptors (α_{2A}, α_{2B} and α_{2C}). The β-adrenoceptors are subdivided into β_1, β_2 and β_3. All adrenergic receptors are G-protein coupled receptors blocking or activating adenylyl cyclase (α_2 and β_1-β_3 respectively) or activating PLC (α_1) (Molinoff, 1984). All adrenoceptor subtypes have been identified in the human and rat bladder (Levin et al., 1988; Nomiya & Yamaguchi, 2003). Some α- and all three β-adrenergic receptors have also been detected in the urothelium (Ishihama et al., 2006; Otsuka et al., 2008; Walden et al., 1997).

In the urinary bladder, the sympathetic nervous system is important during the filling phase where NE mediates relaxation of the bladder wall mainly via β-adrenoceptors (Abdel-Rahman et al., 1983; Elmér, 1974). Both β_2 and β_3 are important for bladder relaxation in the rat (Oshita et al., 1997), whereas in humans the β_3-subtype is, to a larger degree, responsible for the relaxation (Igawa et al., 1998). Sympathetic efferent transmission stimulates the contraction of the urethra and bladder neck via α_1-adrenoceptors (Caine et al., 1975; Chang et al., 2000).

Another important neurotransmitter usually considered to be involved in relaxatory responses is the non-adrenergic non-cholinergic substance NO. In 1980, Furchgott et al. identified an endothelium-derived relaxatory factor (EDRF) in arterial smooth muscle, which were later determined to be NO (Furchgott & Zawadzki, 1980; Palmer et al., 1987). As its main mechanism of action, NO is proposed to activate guanylyl cyclase, resulting in increased levels of cGMP. The elevated levels of cGMP in turn activate protein-kinases involved in smooth muscle relaxation (Bredt & Snyder, 1992).

In the urinary bladder, NO released from parasympathetic nerves mediates relaxation of the bladder neck and urethra and may thus be an important player in disorders such as bladder outlet obstruction (BOO) (Andersson et al., 1991; Persson & Andersson, 1994). However, NO also affects both the detrusor smooth muscle and afferent nerve fibres (Ozawa et al., 1999; Yoshimura et al., 2001) and may play a role in the development of cystitis (Aronsson et al., 2014b). Another source of NO in the bladder is the urothelium, mainly released upon activation of adrenergic receptors (Birder et al., 1998; Winder et al., 2017). Furthermore, stimulation of urothelial muscarinic receptors has been

shown to mediate urothelial release of NO, primarily in a state of inflammation (Andersson et al., 2008).

1.4 Purinergic interactions and the concept of atropine resistance

Interactions, or crosstalk, between various transmitter systems are important features in the micturition reflex arc. As an example, NE may affect the efferent release of acetylcholine via prejunctional modulation and vice versa (Somogyi et al., 1995). Purinergic interactions also exist where recent findings suggest adenosine able to act as an inhibitory modulator on neuronal acetylcholine release (Searl et al., 2016; Silva-Ramos et al., 2015). Furthermore, acetylcholine may trigger the release of urothelial ATP which seems to be increased during IC (Birder et al., 2004).

ATP is commonly described as one of the NANC-transmitters responsible for atropine resistant excitatory responses in the urinary bladder of most species (Husted et al., 1980; Levin et al., 1986; Tong et al., 1997). Although other neurotransmitters such as tachykinins, vasoactive intestinal peptide (VIP) and substance P have been identified in functional NANC-signalling in the urinary bladder, the majority of the response is usually accredited to ATP (Levin et al., 1986; Meini & Maggi, 1994; Moro & Chess-Williams, 2012). In favour of this assumption, studies in the rabbit bladder revealed the ATP-induced urinary expulsion to be of similar magnitude as that of electrical stimulation in the presence of atropine (Levin et al., 1986). However, it is possible that ATP is not solely responsible for the NANC contractile responses (Meini & Maggi, 1994; Moro & Chess-Williams, 2012), a theory put forward by Beznak stating that the observed muscle contractions to ATP may be involving acetylcholine (Beznak, 1951). This theory was however, more or less, disregarded as the evidences for direct ATP-induced contractions were presented, although some later findings have been made in support of this idea (Sjogren & Andersson, 1979).

The urothelium is an important structure for neurotransmitter interactions as it expresses a large variety of receptors and may upon stimulation release various transmitters (Winder et al., 2014). Hanna-Mitchell *et al.* demonstrated ATP to be able to stimulate the release of acetylcholine from urothelial cells (Hanna-Mitchell et al., 2007). However, the plausible functional roles of this ATP-

induced release of urothelial acetylcholine in the micturition reflex arc or directly on the detrusor muscle have not been examined.

1.5 Overactive bladder syndrome

Overactive bladder syndrome (OAB) is a chronic bladder disorder characterized by urgency, often in combination with other lower urinary tract symptoms (LUTS) such as nocturia and increased frequency (Haylen et al., 2010). Some patients have a combination of urgency and urge incontinence, referred to as OAB_{WET} (Haylen et al., 2010). In order to diagnose OAB other medical conditions affecting the lower urinary tract such as urinary tract infection and urinary stress incontinence need to be ruled out.

The prevalence of OAB has been estimated to 16.5 % in the USA (the NOBLE-study) and to 11.8 % in the EPIC-study, conducted in Canada, Germany, Italy, Sweden and United Kingdom (Irwin et al., 2006; Stewart et al., 2003). Furthermore, these studies reported an equal prevalence between men and women as well as an increased prevalence with age. The pathophysiology of OAB is not yet unravelled and may vary between individuals. However, theories regarding the pathophysiology of the observed overactivity in these patients have emerged and include for instance sensitization of bladder afferents and/or increased spontaneous contractions (Brading, 2006; de Groat, 1997). Recent research also highlights the plausible importance of the bladder microbiota and chronic urinary tract infection in the development of OAB symptoms (Contreras-Sanz et al., 2016; Drake et al., 2017). Furthermore, an altered urothelial signalling has been observed in OAB patients, for instance patients suffering from OAB as well as BPS/IC show an increase in the stretch-induced release of ATP (Contreras-Sanz et al., 2016; Sun et al., 2001).

Because of the unknown pathophysiology, the pharmacological treatment of OAB today is symptomatic in nature and anticholinergics, such as oxybutynin and tolterodine, are most commonly prescribed (Robinson & Cardozo, 2012). However, due to common side effects, such as dry mouth and constipation, or insufficient effect the discontinuation of anticholinergic treatment for OAB patients is relatively high (Peeker et al., 2010; Sexton et al., 2011). If anticholinergics do not result in clinical improvement or cannot be tolerated due to side effects, other available treatment options are *e.g.* botulinum toxin and the β_3-adrenoceptor agonist mirabegron (Gormley et al., 2012; Lawrence et al., 2010; Leron et al., 2018).

1.6 Bladder pain syndrome/interstitial cystitis

BPS/IC is a chronic bladder disorder often associated with a lowered quality of life and sometimes depression (Hanno et al., 2015). BPS/IC is typically characterized by pelvic pain in combination with various LUTS including frequency, decreased storage capacity and nocturia (Bogart et al., 2007). The diagnosis is obtained after ruling out differential diagnoses such as urinary tract infection and OAB (Doggweiler et al., 2017; Hanno et al., 2015).

The reported prevalence of BPS/IC is somewhat inconsistent, with studies reporting results ranging between 0.45 – 11.20 % in various cohorts (Leppilahti et al., 2002; Lifford & Curhan, 2009). However, BPS/IC appears to be more common among the female population and the prevalence increases with age (Clemens et al., 2005).

Despite much scientific effort, the aetiology of BPS/IC is not yet fully unravelled but a hypersensitivity of afferents or an upregulation of the mechanoreceptor function appear to play a major role. For instance, preclinical studies using an *in situ* half bladder model, enabling investigations of the efferent- and afferent-induced responses of the detrusor muscle, showed that the reflex-evoked responses are increased during CYP-induced cystitis (Aronsson et al., 2014a; Aronsson et al., 2015). This may involve sensitization of the afferent neurons and an upregulation of the mechanoreceptor function.

Furthermore, alterations of the afferent nerve signalling have been widely discussed in the development of cyclophosphamide induced cystitis, for instance the expression of purinergic P2X-purinoceptors on afferent neurons is increased (Dang et al., 2008; Nazif et al., 2007). Likewise, in BPS/IC-patients, the afferent pathway seems to be sensitized likely resulting in symptoms such as frequency and decreased volume voided (Contreras-Sanz et al., 2016; Kumar et al., 2010; Sun et al., 2001). Possible explanations include infection, autoimmune reactions and alterations in neuronal function (Rourke et al., 2014). However, the underlying mechanisms for this disorder remain unknown.

Among the most validated theories for the pathophysiology behind BPS/IC is the adherence theory which suggests disturbances in the glucose-amino-glucan layer (GAG-layer) of the urothelial epithelium and the subsequent increased

permeability for substances such as potassium to be a major cause of LUTS associated with this disease (Lilly & Parsons, 1990; Parsons, 2007). Furthermore, studies of patients suffering from BPS/IC show changes in urothelial integrity and an altered expression of tight junction proteins (Hurst et al., 2015; Liu et al., 2012). The increased influx of various substances observed may lead to inflammation, which has been shown to affect neurotransmitters released from the urothelium as well as sensitizing the afferent nerve endings (Kumar et al., 2010; Vesela et al., 2012). However, it is also possible that the urothelial permeability is a result of inflammation and not the other way around (Chiang et al., 2000).

The only oral treatment for BPS/IC approved by the American food and drug administration (FDA) is called Elmiron® and consists of pentosane polysulphates thought to imitate the GAG-layer and thus may protect the urothelium to some extent (Hanno, 1997; Waters et al., 2000). Other treatments involve amitriptyline, cyclosporine A, transurethral resection of ulcers and bladder distension (Hanno et al., 2015). However, there is no cure for BPS/IC and further scientific effort is needed to fully understand the aetiology of the disorder with the aim to optimize the treatment.

Although ATP-mediated stimulation directly on the detrusor muscle is considered a minor contributor for healthy bladder contraction in humans, the initiation of the micturition reflex via afferent nerve endings may be mediated by stretch-induced release of ATP from the transitional epithelium (Kanai, 2011). Furthermore, the role of ATP during pathological bladder disorders is markedly increased (Palea et al., 1993; Sjogren et al., 1982). For instance, the stretch-induced release of urothelial ATP is elevated in patients suffering from BPS/IC and OAB alike, which may affect the sensory nerves, previously observed in rat models (Contreras-Sanz et al., 2016; Smith et al., 2005; Sun et al., 2001).

An altered expression of purinergic receptors is observed during both disorders. For instance the P2X3-subtype is upregulated in the urothelium of patients suffering from BPS/IC or OAB (Sun & Chai, 2004). Preclinically, immunohistological stainings of bladders from cats with feline interstitial cystitis showed the expression of the P2X1-purinoceptor to be reduced (Birder et al., 2004). An altered expression of muscarinic receptors as well as an

augmented release of acetylcholine have also been identified (Giglio et al., 2005; McDermott et al., 2013). For instance in rat models of experimentally induced cystitis the expression of muscarinic M_5-receptors was shown to be upregulated (Giglio et al., 2005). Furthermore, during inflammation of the bladder the cholinergic contractile responses are generally decreased (Giglio et al., 2005; Mok et al., 2000). The purinergic transmitter system appears to be involved also in the development of bladder inflammation and pain (Aronsson et al., 2012; Cockayne et al., 2000).

1.7 Experimental cystitis

Treatment with the alkylating antineoplastic agent cyclophosphamide (CYP) has adverse effects in patients including a local haemorrhagic cystitis. This knowledge has led to CYP-induced cystitis being a commonly used experimental model for studying BPS/IC in rodents. CYP induced cystitis shares many characteristics with BPS/IC making it a valid model for studying bladder disorders with an inflammatory pathophysiology (Auge et al., 2013; Juszczak et al., 2007). It is not yet fully unravelled how CYP induces bladder inflammation but research has shown the toxic metabolite acrolein to be involved (Cox, 1979). Acrolein has toxic effects on the urothelium eventually leading to a local inflammatory response; therefore, patients treated with CYP are often prescribed the antioxidant Mesna, which incapacitates acrolein and thus alleviates the adverse effects (Haselberger & Schwinghammer, 1995; Luce & Simons, 1988).

Besides CYP-induced cystitis, other models for experimental cystitis exist, all of which have their own advantages and disadvantages. These models include spontaneous feline interstitial cystitis, neurogenic inflammation (induced by *e.g.* electrical nerve stimulation or antigens such as ovalbumin) and bacterial cystitis such as lipopolysaccharide-induced cystitis (LPS-induced cystitis) (Bjorling et al., 2011; Jerde et al., 2000; Kowalski et al., 1990; Saban et al., 1991).

1.8 Aims

The general aims of this thesis were to examine purinergic and cholinergic interactions within the micturition reflex and to identify reflex levels and structures of importance for the interplay. Furthermore, the possible functional implications that this interplay may have on the different levels of the micturition reflex arc were characterized.

1.8.1 Specific aims

In the in *vivo* and *in vitro* experiments the following research issues were addressed:

- *How much of the ATP-induced bladder contraction depends on cholinergic effects?*
- *Does the interplay take place within the urothelium or at the neuronal level involving afferents and/or efferents?*
- *Is the interplay affected by inflammation and/or by disruption of caveolae?*
- *Which purinoceptors are involved?*
- *Do the interactions occur in vivo or only in isolated tissue?*

An *in vitro* whole bladder setup, enabling the administration of substances to the luminal or adventitial side of the bladder wall, was employed in order to determine if the ATP-evoked release of acetylcholine was site-specific (paper I). The origin of the ATP-induced release of acetylcholine was further elucidated by the denudation of the urothelium (paper I, II and IV). Furthermore, the functional interplay between ATP and acetylcholine was examined in CYP-pre-treated animals and compared to healthy controls in order to establish possible impacts of inflammation on this signalling interaction (paper I and II).

The caveolae are important signalling platforms and their involvement in healthy bladder signalling as well as possible alterations during CYP-induced cystitis were unravelled in paper II. Furthermore, the importance of caveolae for the ATP-evoked release of acetylcholine and possible interactions with the urothelium was examined.

The functional interplay between ATP and acetylcholine was further characterized in paper III, which aimed to elucidate the receptors involved in

this interaction by the application of various purinergic agonists and antagonists *in vitro*. In this paper, the possible involvement of the urothelium and/or the efferent neurons in the ATP-evoked release of acetylcholine was further examined.

In paper IV, the functional implications of the atropine-sensitive part of the purinergically-evoked response was examined in an *in vivo* whole bladder setup as well as an *in vivo* half bladder model. The half bladder setup enabled studies of afferent signalling and therefore the effects of the ATP-evoked non-neuronal release acetylcholine on afferent nerve endings could be examined. The possible afferent modulatory effects of acetylcholine were further elucidated.

2 Materials and methods

The experiments conducted in this doctoral thesis were approved by the Animal Ethics Committee in Gothenburg (approval numbers: 196-13 and 1794/18). Male rats of the Sprague Dawley strain were used throughout the studies.

All methods conducted in this thesis are described in detail in each paper.

2.1 Cyclophosphamide-induced cystitis

Experimental cystitis was induced by a single intraperitoneal injection of CYP (100 mg/kg rat) and the rats were euthanized for experiment at the peak of inflammation (60 hours after the injection) (Giglio et al., 2005).

2.2 Removal of the urothelium

Denudation of the urothelium was conducted by filling the bladders with collagenase I (0.1% in saline, 30 min). Urothelial cells were removed by gently massaging the bladder and then rinsing with saline. In addition, for bladder strip preparations, a cotton swab was used to detach urothelial cells.

2.3 *In vitro* organ bath functional studies

The rats were anesthetised and euthanized using an overdose of pentobarbital sodium (>60 mg/kg rat) and a subsequent incision through the heart. The urinary bladder was excised and kept in Krebs solution ($CaCl_2$ 1.25 mM, glucose 5.5 mM, KCl 4.6 mM, KH_2PO_4 1.15 mM, $MgSO_4$ 1.15 mM, NaCl 118 mM and $NaHCO_3$ 25 mM) at all times. Subsequently, two full-thickness bladder strips (6 x 2 mm) excised proximal to the ureteral orifices and above the trigone were prepared. Alternatively, a whole bladder preparation was made in which two catheters were inserted via the urethral opening, for the administration of substances to the luminal side of the bladder and outflow respectively, and secured with threads.

The bladder preparation was mounted in a 25 ml organ bath setup, between a fixed hook and an adjustable steel rod coupled to an isometric force transducer (Linton Instrumentation, Norfolk, UK). The organ baths were filled with Krebs solution kept at a temperature of 38 °C by a warm water circuit and at a constant pH of 7.4 by continuous gassing with 5% carbon dioxide in oxygen. The preparations were pre-stretched to 10-15 mN and left to equilibrate for 45 min, resulting in a basal tension of approximately 5 mN. High potassium Krebs

solution (124 mM, sodium exchanged for an equimolar amount of potassium) was used as a reference value for maximal contraction as well as a measurement of tissue viability throughout the experiments. Responses were recorded by AcqKnowledge software and a data acquisition system (MP100WSW; Biopac, Goleta, USA).

The agonists and antagonists employed were either administered cumulatively into the organ bath in a volume of 125 µL (200 times the final concentration in the baths) or via the catheters (whole bladder setup) in a volume of 0.5 ml (at final concentration). All antagonists used were administered and let to equilibrate for 20 min before stimulation. The same was done for the neurotoxin tetrodotoxin (TTX).

Electrical field stimulation (EFS; square wave pulses with a duration of 0.8 ms, delivered at supramaximal voltage) was applied at increasing frequencies (2, 5, 10 20 and 40 Hz).

When studying relaxatory responses, the tissue was pre-contracted using medium potassium Krebs solution (50 mM, sodium exchanged for an equimolar amount of potassium) for 20 min (until a stable pre-contraction) before the administration of pharmacological substances.

2.4 Immunohistochemistry

Urinary bladders were collected and fixed in 4% paraformaldehyde (in phosphate buffered solution). After paraffin embedment, the tissue was sectioned into 8 µm thick segments (Histolab products AB, Gothenburg, Sweden). The tissue sections were deparaffinised, rehydrated and immersed in blocking solution (1 hour, 0.25% triton X, 25% normal goat serum in phosphate buffered saline) in order to prevent unspecific binding. After overnight incubation (room temperature) with primary antibodies, the sections were incubated with a secondary antibody for 2 hours. The sections were coverslipped with prolong gold antifade reagent (with DAPI, Life technologies, Eugene, OR) and viewed by microscopy (Eclipse 90i, Nikon, Tokyo, Japan).

2.5 Western blot

The excised bladder was placed in homogenization-buffer (protease inhibitor cocktail, phosphatase inhibitor cocktail 2 in Hank's buffered salt solution,

HBSS) and homogenized mechanically with a polytron (Ultra-Torax T8, IKA©-werke, Staufen in Breisgau, Germany) or by ultrasound in a Branson-450-Sonifier (Branson ultrasonics, Danbury, USA). After centrifugation, protein quantification of the supernatant was performed using a Bio-Rad protein assay. 20 µg protein per well was loaded in a 10% w/v SDS gel for electrophoresis. After blotting to a nitrocellulose membrane (Thermo Scientific Rockford, IL, USA), the membrane was incubated in blocking solution (1 hour, using Blotting-Grade Blocker, Bio-Rad or non-fat dry milk, Semper) in order to prevent unspecific binding. Subsequently, the membrane was incubated with primary antibodies overnight (room temperature), washed in tris-buffered saline (0.1% Tween-20) and immersed in solution containing horseradish peroxidase-conjugated secondary antibodies for 2 hours. Finally, the membrane was incubated with ECL (a chemiluminescence system, Clarity™ Western ECL Substrate, Bio-Rad) for 5 min. The bands were visualized and quantified using a luminescence image analyzer (LAS-1000 plus software, Image Reader LAS-1000 Pro, version 2.6, FUJIFILM, Tokyo, Japan). β-actin was used as reference for loading and normalization (for quantification) on stripped membranes (Restore™ Western Blot Stripping Buffer, Thermo Scientific, Rockford, USA).

2.6 Nissl staining

Excised urinary bladders were fixed in 4% paraformaldehyde (in phosphate buffered solution). The tissue was frozen and sectioned into 50-60 µm thick segments (Cryostat; Leica, CM1950; Leica Microsystems, Nussloch, Germany). Free floating segments were stained with cresyl violet acetate (0.2%), mounted and coverslipped with prolong gold antifade reagent (with DAPI, Life technologies, Eugene, OR) and viewed by microscopy (Eclipse 90i, Nikon, Tokyo, Japan).

2.7 *In vivo* whole bladder setup (cystometry)

After induction of anaesthesia with isoflurane (1.5-2%), the rodent was placed on a heating pad connected to a rectal thermistor in order to monitor and keep the body temperature at a constant level of 38 °C. Adequate depth of anaesthesia was controlled by strong paw pinches which did not alter the heart rate or blood pressure. The femoral artery and vein were exposed via a small incision in the inguinal area followed by blunt dissection. After catheterization, the blood pressure was monitored via the femoral artery, whereas the venous

catheter was used for drug administration. After laparotomy, two catheters were inserted through the apex of the exposed bladder, one for intravesical administration of drugs and the other for internal pressure measurements (fig 1; basal pressure between 10-15 mmHg). A ligature around the catheters and the bladder apex prevented leakage, which was validated through an intravesical administration of saline and subsequent voiding. The pelvic nerves were isolated in order to study responses in the intact, as well as denervated, bladder.

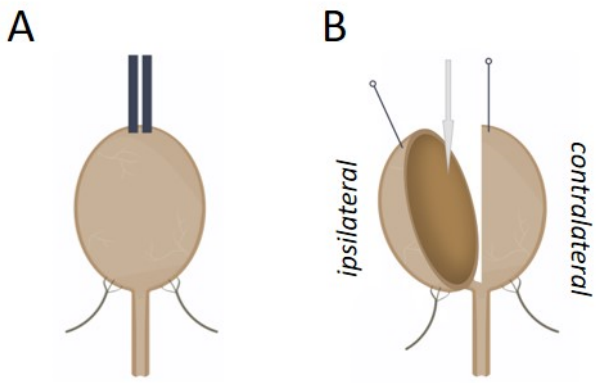

Figure 1: Schematic representations of the whole bladder (A) and half bladder (B) *in vivo* models. In the whole bladder setup two catheters were inserted through the apex of the bladder, for the measurement of intravesical pressure and administration of drugs, respectively. In the half bladder setup, the ipsilateral bladder half (left in B) was used for modulating afferent signalling via stretch-induced responses and topical administration of drugs (white arrow). The contralateral bladder half was connected to a force transducer in order to register contractile responses. The pelvic nerves (at the base of the bladder in the illustration) were used for electrical nerve stimulation (B) or denervation (A, B).

2.8 *In vivo* split-bladder setup

The rat was prepared in the same manner as described for the cystometry setup. However, the bladder was prepared with two ligations at each side of the apex and at each side of the bladder, as well as at each side of the urethra below the pelvic nerves. The bladder was then completely separated into two halves (fig 1). The ligatures at the sides of the urethra and the bladder were fixed to the

underlying tissue. The contralateral bladder half apex was then connected to an isometric force transducer (Linton Instrumentation, Norfolk, UK) coupled to a computer system (MP100WSW data acquisition system and AcqKnowledge software). The other half (ipsilateral) was used for initiating afferent signalling via topical administration of substances (to the luminal side of the bladder) or stretch-induced responses, using weights of 8 g. Topical administration to the contralateral bladder half enabled the study of responses without activation of the micturition reflex arc. The pelvic nerve on the ipsilateral side of the bladder base was placed on a bipolar platinum electrode, enabling afferent stimulation (20 Hz, at supramaximal voltage, square wave pulses with a duration of 0.8 ms), and contractile responses were measured from the contralateral bladder half. An afferent stimulation was confirmed by the absence of contractile responses during deep anaesthesia when reflexive responses are silent. Reflexive responses were usually observed at an isoflurane level of 1-1.2%. Adequate depth of anaesthesia was controlled by strong paw pinches which did not alter the heart rate or blood pressure. A detailed description of this novel *in situ* half bladder preparation enabling afferent stimulation has been published previously (Aronsson et al., 2014a).

2.9 Statistical analysis

The *in vitro* and *in vivo* functional studies were analysed using repeated measurement ANOVA (for multiple comparisons of the same variable) followed by the Bonferroni post-hoc test or Student's t-test when comparing two groups. For the western blots, the densities of the bands were analysed by Mann Whitney's test or Wilcoxon sign test. The result was described as mean ± S.E.M, p-values less than 0.05 were regarded as statistically significant. The GraphPad Prism software was used to generate the graphs and parameters presented (GraphPad Software Inc., San Diego, USA).

2.10 Materials

All substances were purchased from Sigma-Aldrich, St Louis, MO, unless otherwise stated.

2.10.1 Cholinergic and purinergic substances

5'-(N-ethylcarboxamido)adenosine (NECA, Tocris Bioscience, Bristol, United Kingdom), α,β-methylene adenosine 5'-triphosphate (α,β-MeATP, Tocris Bioscience, Bristol, United Kingdom), acetyl-β-methylcholine (methacholine), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate

(ATP), atropine, methoctramine, N,N"-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)]thio urea (MRS 2578, Tocris Bioscience, Bristol, United Kingdom), pirenzepine, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), suramin.

2.10.2 Other substances

$CaCl_2$, citric acid, collagenase type 1 (Invitrogen, Paisley, United Kingdom), cyclophosphamide monohydrate (CYP), dimethyl sulfoxide (DMSO), ethanol (Kemetyl, Stockholm, Sweden) glucose, HCl, heparin (Leo Pharma, Ballerup, Denmark), KCl, KH_2PO_4, methanol, methyl-β-cyclodextrine (Me-β-CD), $MgSO_4$, NaCl, $NaHCO_3$, NaOH, pentobarbitone sodium (Pentobarbitalnatrium vet.; APL, Stockholm, Sweden), sucrose, tetrodotoxin (TTX, Tocris Bioscience, Bristol, United Kingdom), triton X, tris, tween-20, xylene (Kemetyl, Stockholm, Sweden).

2.10.3 Reagents and solutions for Immunohistochemistry and western blot

Alexa Fluor 488 goat anti-rabbit IgG (Thermo Scientific, Rockford, IL, USA), anti-caveolin-1 (ab2910, Abcam, Cambridge, United Kingdom), anti-choline-acetyltranseferase (ChAT, ab178850, Abcam, Cambridge, United Kingdom), anti-carnitine-acetyltransferase (CarAT, PAC400Ra01, Clone Corporation, Houston, TX, USA), β-actin (SC-47778, Santa Cruz Biotechnology, Dallas, TX, USA), blotting-Grade Blocker (Bio-Rad), cresyl violet acetate, horseradish peroxidise-conjugated goat anti-mouse (62-6520, Thermo Scientific, Rockford, IL, USA), horseradish peroxidise-conjugated goat anti-rabbit (65-6120, Thermo Scientific, Rockford, IL, USA), normal goat serum (NGS; Vector Laboratories, Burlingame, Canada), ECL (Clarity ™ Western ECL Substrate, Bio-Rad), phosphate buffered saline tablets (PBS; Thermo Scientific, Rockford, IL, USA), Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), paraformaldehyde, phosphatase inhibitor cocktail 2, prolong gold antifade reagent with DAPI (Thermo Scientific, Rockford, IL, USA), protease inhibitor cocktail, stripping buffer (Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL, USA).

3 Results and Discussion

Crosstalk between different transmitter systems are common in the lower urinary tract and may occur at various levels of the micturition reflex arc. In this thesis, one such interaction between the two important parasympathetic transmitter substances ATP and acetylcholine has been identified and its functional implications have been characterized using different *in vitro* and *in vivo* methods. Furthermore, the role of functional structures such as the urothelium and the caveolae in this link has been elucidated. Finally, a role for acetylcholine in activating the micturition reflex arc on its own has been identified *in vivo*.

3.1 Atropine-sensitive purinergic functional responses

The present results support the hypothesis of an ATP-induced release of acetylcholine with functional implications to exist in the rat urinary bladder, both *in vitro* and *in vivo*. This was made evident by a significant reduction in purinergic contractile responses in the presence of the non-selective muscarinic antagonist atropine. The reduction in contractile response was similar throughout the different setups used (*i.e. in vitro* organ bath and the *in vivo* whole bladder or split-bladder model; table 1), indicating approximately half of the ATP-evoked responses to depend on a subsequent release of acetylcholine (papers I and IV; fig 2).

Table 1: Reduction (%) of ATP-evoked contractile responses in the presence of atropine in different *in vitro* and *in vivo* models. In experiments where more than one concentration of ATP was administered the values from the highest concentration of ATP have been included.

Experimental setup	**Reduction in purinergic contraction in the presence of atropine**
In vitro (*strip preparation; paper II*)	65%
In vitro (*whole bladder; paper I*)	50%
In vivo (*denervated whole bladder; paper IV*)	43%
In vivo (*split bladder, topical administration to the contralateral bladder half; paper IV*)	39%
In vivo (*denervated split bladder, i.v. administration of ATP, paper IV*)	47%

Signs of this functional interaction between ATP and acetylcholine, where ATP induces a release of acetylcholine directly affecting the contractile response of the detrusor muscle, have previously been presented both *in vitro* and *in vivo* (Aronsson et al., 2014a; Sjogren & Andersson, 1979). However, neither the origin of the cholinergic part of ATP-evoked responses in the urinary bladder, the receptors involved in functional responses, nor functional implications beyond direct smooth muscle contraction have been addressed.

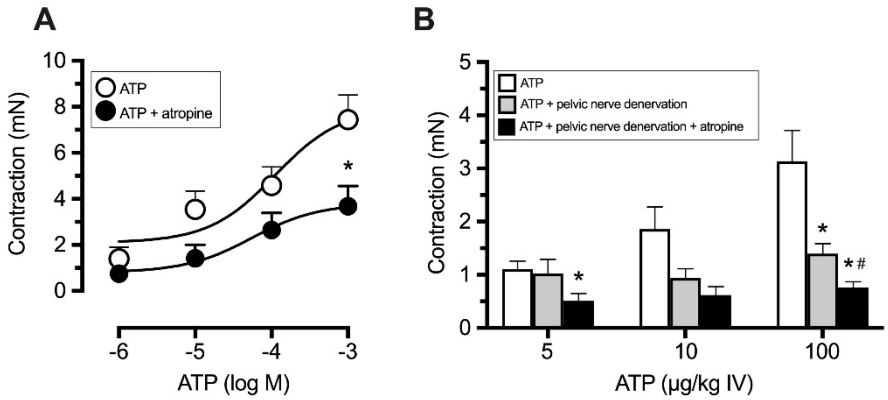

Figure 2: Mean contractile responses to ATP in whole bladder preparations *in vitro* (A; adapted from paper I) and *in vivo* (B; adapted from paper IV), before and after the administration of atropine. *In vivo* (B), the effects of atropine on purinergic contraction were examined after pelvic nerve denervation. $p<0.05$ is denoted by * when compared to ATP, and # when compared to denervated bladder preparations, respectively. n=13 *in vitro* and n=6 *in vivo*. The vertical bars represent S.E.M.

3.1.1 Urothelial involvement

The urothelium with its sensory properties is a likely source for the ATP-induced release of acetylcholine and it is involved in a variety of other such interactions. For instance the stimulation of muscarinic urothelial receptors may induce the release of NO from the urothelium (Andersson et al., 2008). In this thesis the expressions of the acetylcholine synthesising enzymes ChAT and CarAT were investigated, and both enzymes were found to be present in rat urinary bladder tissue. CarAT has previously been suggested to be the main enzyme responsible for non-neuronal synthesis of acetylcholine (Hanna-Mitchell et al., 2007; Yoshida et al., 2008).

Notably, it has previously been observed in (non-functional) transmitter release studies, that an ATP-induced release of acetylcholine from the urothelium may exist (Hanna-Mitchell et al., 2007; Silva et al., 2015). This non-neuronal release of acetylcholine could potentially act either directly on the muscarinic receptors on the smooth muscle, or have paracrine actions stimulating the release of other neurotransmitters from the urothelium or suburothelium.

3.1.1.1 In vitro

An *in vitro* whole bladder setup (see Methods and Material, section: 2.7), enabling site-specific administration to the luminal or adventitial side of the bladder respectively, was used in order to determine whether or not the presumable link between ATP and acetylcholine could be activated from both sides (paper I). The results showed an atropine-sensitive component to be up to half of the functional contraction to intravesically administrated ATP; *i.e.* from tissue that was not accessible from the adventitial side of the bladder wall. Atropine did not affect ATP-induced responses when administered to the external side of the bladder, which favoured the idea of a urothelial origin for the cholinergic part of ATP-evoked responses. Urothelial removal (denudation) using collagenase I was used in order to confirm this (papers I-II and IV). Indeed, urothelial denudation eliminated the atropine-sensitive part of the ATP-evoked responses *in vitro*, strongly indicating the cholinergic part of these responses to emanate from the transitional epithelium (fig 3a).

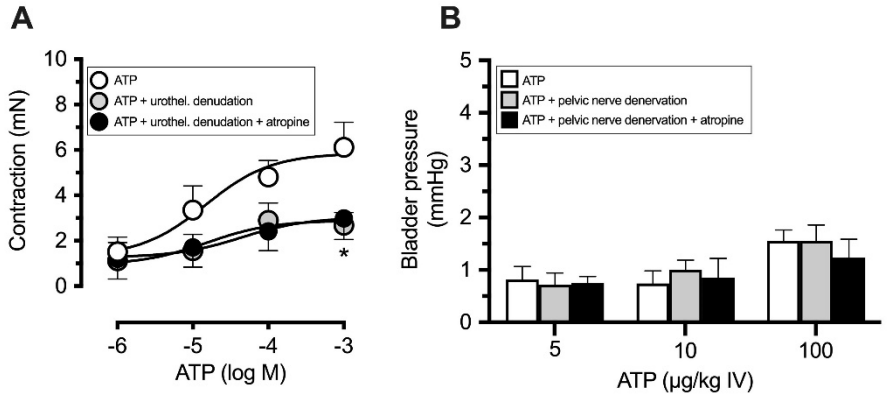

Figure 3: Mean contractile responses to ATP in urothelium-denuded whole bladder preparations *in vitro* (A; adapted from paper I) and *in vivo* (B; adapted from paper IV), before and after the administration of atropine. *In vivo* (B) the effects of atropine were examined after pelvic nerve denervation. *In vitro* experiments (A) include a control administration of ATP before urothelial denudation (open symbol). * indicates $p<0.05$, n=8 *in vitro* and n=5 *in vivo*. The vertical bars represent S.E.M.

3.1.1.2 In vivo

In accordance with the findings *in vitro*, denudation of the urothelium in the *in vivo* whole bladder setup more or less removed the ATP-evoked release of acetylcholine (fig 3b). Furthermore, in intact bladders, denervation of the bladder significantly decreased the purinergic response, suggesting an effect on afferent signalling in addition to any direct detrusor stimulation. This observation was absent in urothelium-denuded preparations, highlighting the importance of an intact urothelium for healthy bladder signalling as both the sensory signalling and direct muscle contraction seem to be impaired in denuded preparations.

3.1.2 Neuronal involvement

Despite the clear evidence for a urothelial origin of the ATP-evoked release of acetylcholine presently observed (paper I, II and IV; see section: 3.1.1) a plausible neuronal origin ought to be examined. Hence, the effects of the potent neurotoxin TTX on this functional interaction was examined *in vitro*. Methacholine-induced responses were used as control and the ability of TTX to abolish efferent nerve signalling was confirmed by EFS. Similar to previous studies ATP- and acetylcholine-evoked responses remained unchanged in the presence of TTX, suggesting their main site of action *in vitro* to be directly on

the smooth muscle and/or on the urothelium/suburothelium (Aronsson et al., 2010; McMurray et al., 1998). Furthermore, the ATP-evoked release of acetylcholine was shown to be TTX-insensitive as atropine still caused a significant decrease of 60% in purinergic response when subjected to the neurotoxin. As expected, the effects of atropine on cholinergic stimulation were TTX-insensitive as well. These results favour the idea of a urothelial, rather than neuronal, origin of the cholinergic part of the ATP-evoked responses presently investigated.

3.1.3 Characterization of involved receptors

3.1.3.1 Functional contractile responses

In order to characterize the purinoceptor subtypes mediating the purinergic-cholinergic interaction described above, a range of purinergic agonists and antagonists with different receptor profiles were applied *in vitro* (paper III). To characterize any involvement of the P2X-purinoceptors in the non-neuronal release of acetylcholine α,β-MeATP was employed. This substance is a stable ATP-analogue, generally regarded to mainly stimulate the P2X1- and P2X3-purinoceptors when administered in single concentration-response experiments (Ralevic & Burnstock, 1998). When administered repeatedly in high concentrations the response is, however, markedly lowered and the substance instead acts as a desensitizer at the same receptors. The plausible sensitivity of α,β-MeATP towards atropine was examined (paper III). Interestingly, similar to the ATP-evoked contractions, the purinergic contractile response to α,β-MeATP (administered as an agonist) was significantly reduced by a third in the presence of atropine. These observations give support to the supposition that it is ATP, rather than any of its metabolites, which mediates the cholinergic interaction. It is suggested that the P2X-purinoceptors, most likely of the P2X1- or P2X3-subtype, are responsible for stimulating the release of non-neuronal acetylcholine *in vitro* in the rat bladder. (Control experiment with repeated administrations of α,β-MeATP were performed in order to exclude these effects to depend on desensitization of the purinoceptors, which could be ruled out.)

Another receptor subtype candidate recently suggested to facilitate release of urothelial non-neuronal acetylcholine is the $P2Y_6$-purinoceptor (Silva et al., 2015). It has, however, not been evaluated whether or not this proposed mechanism constitutes a functionally important one in the rat urinary bladder, *i.e.* if the $P2Y_6$-purinoceptor is able to release acetylcholine to such an extent

that it triggers smooth muscle contraction or if other purinoceptor-subtypes are dominating. Direct purinergic responses can further depend either on ATP stimulating (mainly) P2X-purinoceptors, or metabolites of ATP, such as ADP and adenosine, acting on P2Y- or P1-purinoceptors, respectively. Hence, the effects of MRS2578, a $P2Y_6$-antagonist, on the ATP-evoked release of acetylcholine was examined. However, as the present results indicate the ATP-evoked contractile responses still to be sensitive towards atropine regardless of the presence of MRS2578 (a significant reduction by 48%), there is currently no support for this purinoceptor to be involved in the ATP-induced release of acetylcholine; at least not to such an extent that the subsequent release alone evokes smooth muscle contraction.

3.1.3.2 Functional relaxatory responses

The plausible involvement of acetylcholine in relaxatory purinergic responses was examined in an *in vitro* organ bath setup (paper III). Desensitization of the P2X-purinoceptors, using repeated additions of α,β-MeATP, did not affect the ATP-induced relaxatory responses in pre-contracted tissue, indicating the ATP-evoked relaxatory responses of the detrusor muscle to be dependent on P2Y-purinoceptors. Neither the subsequent administration of atropine, nor the addition of PPADS followed by atropine altered the relaxatory responses to ATP. These results suggest the ATP-induced relaxatory component to be independent of the P2X-purinoceptors as well as any ATP-evoked release of acetylcholine *in vitro*. However, in the bladder also the metabolites to ATP may elicit relaxation of the detrusor smooth muscle. Hence, the sensitivity of relaxations to ADP or NECA (an adenosine analogue) towards atropine was examined, however no significant effects of atropine were observed for these substances.

3.1.4 Changes in experimental cystitis

Bladder inflammation, often present during disorders such as BPS/IC and sometimes OAB, has been shown to affect cholinergic as well as purinergic signalling (McDermott et al., 2013; Sun et al., 2001). For instance, both acetylcholine and ATP are released during stretch of the urothelium, and the released amount is elevated during inflammation (McDermott et al., 2013; Sun et al., 2001). Furthermore, the cholinergic contractile response is reduced during experimentally induced cystitis (Giglio et al., 2005; Mok et al., 2000). Therefore, the functional interaction between ATP and acetylcholine was examined in rats with CYP-induced cystitis (paper I-II). Generally, ATP and methacholine evoked concentration dependent contractile responses that were reduced in CYP-pre-treated animals (by up to approximately two thirds for ATP in paper I). Interestingly, while atropine still caused a significant decrease in cholinergic responses in cystitic bladders, the ATP-evoked contractile response was, unlike the findings in healthy controls, not significantly affected by atropine (fig 4). Similarly, removal of the urothelium did not significantly alter the purinergic responses in bladders from CYP-pre-treated rats (fig 4). The same phenomenon was observed regardless of administration order; *i.e.* atropine being added before denudation or vice versa. These results indicate an alteration in the urothelium dependent non-neuronal cholinergic component of the purinergic response in cystitis.

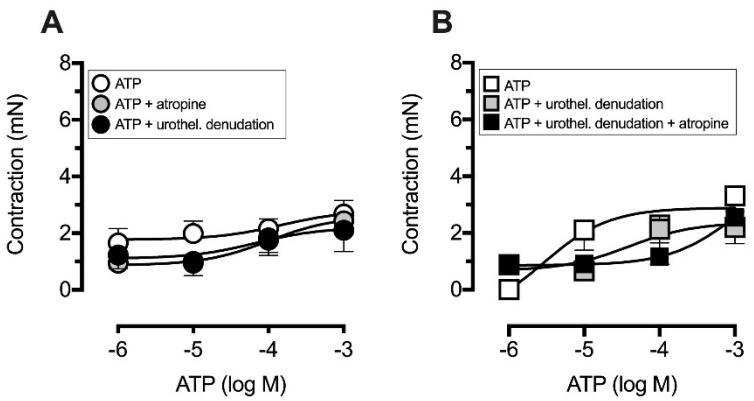

Figure 4: Mean contractile responses to ATP in cystitic intact and urothelium-denuded whole bladder preparations (adapted from paper I) in the absence and presence of atropine *in vitro*. Re-administration of ATP in the presence of atropine was either performed before (A; light grey symbol) or after (B; black symbol) the removal of the urothelium. n=7-8 for each group. The vertical bars represent S.E.M.

In order to unravel the mechanism responsible for the changes in non-neuronal acetylcholine signalling, the expressions of ChAT and CarAT in cystitis were semi-quantified using western blot (paper I). Alterations in the activity or quantity of these enzymes during cystitis could have explained the changes in contractile response currently observed. However, no significant alterations in the expression of the acetylcholine-synthesising enzymes could be found in the cystitic bladders when compared to healthy controls, although a slight indication towards a reduced quantity of CarAT could be seen. Nonetheless, complicating factors such as degradation products and various isoforms need to be taken into consideration when interpreting these results. Regardless, other possible explanations for the reduced release of non-neuronal acetylcholine exists, such as alterations in VAChT or OCT activity. Nonetheless, the present results highlight the important and complex properties of the urothelium for healthy bladder function.

3.2 Functional roles of caveolae in urinary bladder signalling

Another structure often considered important in bladder signalling are the caveolae. These caveolin-containing lipid-rafts have been shown to be vital for healthy bladder signalling due to their ability to function as signalling platforms (Lisanti et al., 1994; Shaul & Anderson, 1998). Consequently, caveolin-1 knockout mice express an impaired ability to elicit normal smooth muscle contraction (Sadegh et al., 2011). Caveolae have also been accredited other functional roles such as endocytosis (Parton & Simons, 2007). During pathological bladder conditions such as detrusor overactivity, the caveolae-mediated signalling appears to be altered due to a change in caveolae density (Cristofaro et al., 2012). In this thesis, the plausible involvement of caveolae-dependent signalling for the ATP-evoked release of acetylcholine was examined. Furthermore, changes in caveolae-mediated signalling during experimentally induced cystitis was characterized *in vitro* (paper II).

3.2.1 Changes in response to caveolae depletion

Presently, any caveolae-mediated signalling was identified by experimental removal of the caveolae lipid rafts by incubation of the tissue with Me-β-CD, interrupting the cholesterol structures in the caveolae membranes (Dreja et al., 2002; Shakirova et al., 2010). Previous studies using this technique have reported a disruption of caveolae without altering other morphological

structures of the smooth muscle (Cristofaro et al., 2007; Shakirova et al., 2010). This is supported by the lack of effect of Me-β-CD-treatment on the general contractile ability of the tissue elicited by high potassium Krebs solution (124 mM), as well as the intact responses to muscarinic stimulation currently observed. However, one should bear in mind that it seems feasible that Me-β-CD could target also other lipid rafts.

In control bladders, incubation with Me-β-CD significantly decreased the purinergic, but not cholinergic contractions to exogenously administered agonists *in vitro* by 61%. Thus, the purinergic signalling is likely, at least to some extent, to be depending on intact caveolae. It was further shown that a part (69%) of the EFS-evoked contractile response was also affected by depletion of the caveolae, suggesting neuronally released transmitters to be dependent on caveolar structures. EFS-evoked responses can be described as a transient phasic component followed by a slower tonic contraction (Brading & Williams, 1990). The phasic component has a shorter duration (0.5-1.5 sec. in this thesis, paper II) and is usually accredited to the stimulation of purinoceptors. The tonic component on the other hand corresponds mainly to cholinergic responses. Hence, in order to examine the effects of caveolae-depletion on neuronally released ATP and acetylcholine the phasic and tonic components of the EFS-evoked responses were analysed (paper II). The results indicated both the phasic and tonic components to be partly caveolae-dependent, where reductions to caveolae depletion by 59% and 87% were seen in the phasic and tonic components, respectively. This suggests a difference in response to exogenously administered methacholine and neuronally released acetylcholine. This may seem contradictive at first, however the difference might be explained by other substances than ATP and acetylcholine being released during EFS.

3.2.2 Changes in experimental cystitis

In bladders from CYP-pre-treated animals, incubation with Me-β-CD did not significantly alter the contractile responses to exogenously administered ATP or methacholine. Likewise, the EFS-induced responses were insensitive to caveolae-depletion in cystitic bladders. This could possibly be explained by an altered expression of caveolae. Hence, expression of caveolin-1, the protein required for caveolae formation, was analysed by western blotting as well as immunohistochemistry. The results indicated a lowered expression of caveolin-1 in cystitic bladders, which in turn is an indirect indication of a

decreased amount of caveolae in the tissue sample. Similar reductions in caveolin-1 expression during CYP-induced cystitis have been presented previously, supporting the current result (Kim et al., 2012).

3.2.3 A link between the caveolae and urothelium important to atropine-sensitive purinergic responses

As mentioned above, caveolae-depletion significantly decreased the ATP-evoked responses in healthy controls. Notably, the atropine-sensitive part of ATP-evoked responses was of a similar magnitude and incubation with Me-β-CD removed this cholinergic part of the ATP-evoked responses (fig 5), which indicates the ATP-induced release of acetylcholine to be caveolae-dependent. However, based on our previous findings this interaction is also dependent on the urothelium (paper I). Hence, a possible interplay between the caveolae and the urothelium was investigated by removal of either structure. A profound interaction was identified where purinergic contractile responses in caveolae depleted bladders were not significantly affected by the subsequent urothelial denudation. Similarly, in denuded preparations no further reduction in purinergic response could be observed after the administration of Me-β-CD.

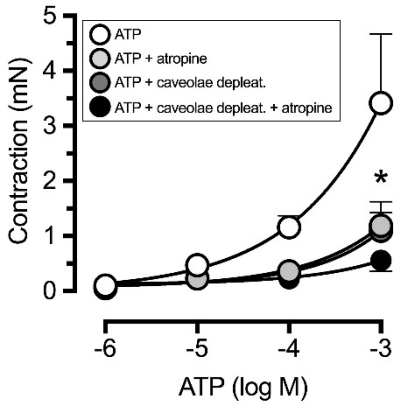

Figure 5: Impact of caveolae depletion on *in vitro* purinergic contractions (adapted from paper II) in control bladder strip preparations. Mean contractile responses to ATP in the absence and presence of atropine, before and after caveolae depletion. * denotes $p<0.05$, n=5. The vertical bars represent S.E.M.

This interaction between the caveolae-mediated signalling and the signalling mediated by the urothelium is probably of fundamental importance for the ATP-evoked release of acetylcholine based on the observation that the disruption of either structure removes the atropine sensitivity of purinoceptor-mediated responses. The present findings might even suggest these caveolae to be located on the urothelium.

To summarize, the caveolae seem to be important for purinergic signalling in the healthy rat urinary bladder. Furthermore, as indicated by the effect of Me-β-CD on the phasic component of the EFS-evoked responses and the effects on the cholinergic part of the ATP-induced responses, two caveolae-dependent pathways for ATP-appear to exist, affecting the neuronal ATP signalling and the ATP-evoked release of acetylcholine respectively.

A functional interplay appear to exist between the urothelium and the caveolae, important for the atropine-sensitive part of the ATP-evoked contractile responses. Furthermore, cystitis seems to alter caveolae-mediated signalling, including the functional link between ATP and the urothelial release of acetylcholine.

3.3 Afferent and efferent modulation *in situ*

In this thesis so far, the ATP-evoked release of acetylcholine has been identified and functional implications such as direct detrusor smooth muscle contraction without any involvement of the micturition reflex arc have been established. However, this urothelial release of acetylcholine may stimulate afferent and/or efferent nerve endings as well. Cholinergic modulation of bladder c-fibres have previously been suggested, even though direct evidence has yet to be presented (Kullmann et al., 2008).

ATP has many roles in the micturition reflex arc and even though the purinergic contribution to direct detrusor contraction appears to be minor in healthy humans, ATP may have paracrine effects and is regarded to be an important mediator for afferent signalling (Cockayne et al., 2005; Cockayne et al., 2000; Silva et al., 2015). Part of the afferent signalling has been shown to be mediated through the stretch-induced release of ATP acting on afferent P2X2-, P2X3- and/or P2X2/3-purinoceptors (Cockayne et al., 2005). In light of the many purinoceptors being expressed in the urothelium and the range of substances being released, ATP may also exhibit paracrine effects on the

urothelium inducing the release of other substances that may influence signalling (Birder & Andersson, 2013; Birder et al., 2004; Elneil et al., 2001; Shabir et al., 2013).

In order to examine afferent and efferent contributions, an *in vivo* whole bladder setup as well as a half bladder model, requiring signals to travel via the CNS in order to elicit any response, were employed (paper IV).

3.3.1 Purinergic and cholinergic interplay on afferent signalling

Similar to the observations in the whole bladder model, the ATP-evoked responses (intravenous, i.v. administration) in the half bladder setup was significantly reduced by about a third in the presence of atropine. Furthermore, topical administration of ATP (ipsilaterally; *i.e.* on the opposite side of contractile measurement) triggered the afferents of the micturition reflex arc resulting in dose-dependent contractile responses of the contralateral bladder half. This is not a novel phenomenon as ATP is known to be important for the initiation of the micturition reflex arc via the stimulation of purinoceptors on the afferent neurons (Cockayne et al., 2005). However, after the topical administration of atropine (ipsilaterally), a significant decrease by 27% in the ATP-induced activation of the reflex arc was observed. This indicates the cholinergic part of ATP-evoked responses to be able to activate afferent nerve fibres.

A role for non-neuronal acetylcholine in the micturition reflex arc has previously been suggested, for instance based on findings in OAB-patients where anticholinergic treatment appears to elicit its effects also during the storage phase of micturition, where no parasympathetic efferent release is thought to occur (Andersson, 2011; Yokoyama et al., 2005). The present finding that a significant part of the ATP-induced activation of the micturition reflex arc is dependent on a non-neuronal release of acetylcholine suggests acetylcholine to be able to initiate the micturition reflex arc on its own. This was further investigated by topical administration of methacholine on the ipsilateral bladder half, which resulted in a contraction of the contralateral side, confirming the hypothesis that acetylcholine may activate reflex responses in itself. However, acetylcholine is a potent paracrine modulator and is known to stimulate the release of other neurotransmitters from the urothelium. One such substance is ATP - known to be important for the initiation of the micturition reflex arc. Therefore, the effects of PPADS on methacholine-induced reflex

responses was examined. Indeed, a significant part of the functional response to methacholine-evoked activation of bladder afferents was lowered (by 26%) by the administration of PPADS, and was thus considered to be dependent on the release of ATP. However, a substantial part of the methacholine-induced afferent signalling remained intact, suggesting a direct effect of acetylcholine on afferent nerve endings to exist. Lastly, stretch-induced responses were significantly reduced in the presence of atropine and PPADS, where PPADS gave rise to twice the reduction compared to atropine in response to ipsilateral stretch, suggesting both transmitters to be involved in afferent signalling.

3.3.2 Acetylcholine and afferent modulation

As shown in the previous section, not only purinergic receptors are expressed on afferent neurons, other receptors including muscarinic-receptors are expressed as well (Nandigama et al., 2010; Yoshimura et al., 2001). Specifically, the M_2, M_4 and M_3-receptors have been identified on afferent neurons in the mouse bladder (Nandigama et al., 2010). Furthermore, the M_1- and M_2-receptors are known to affect neuronal acetylcholine release (Somogyi et al., 1994; Tobin, 1998). In order to characterize any modulatory role for muscarinic receptors in the micturition reflex arc of the rat urinary bladder, the effects of methoctramine (a selective antagonist affecting the M_2/M_4-subtypes) and pirenzepine (a selective antagonist affecting the M_1-subtype) on stretch-induced, as well as ATP-induced, reflex responses were examined in the half bladder model. No effect of the administration of the M_1-antagonist was observed, instead indicating the muscarinic M_3-subtype to be responsible for the stimulation of afferent nerve fibres in this model. However, methoctramine significantly increased the contralateral contractions induced by ipsilateral stretch as well as local ATP administration to the ipsilateral bladder half, where the increase in response to ATP was half of the increase in response to stretch. These observations indicate a muscarinic modulation of afferent signalling to occur either directly on the bladder afferents, or indirectly via the urothelium. Examinations of the effects of methoctramine on ATP-induced contractile responses in the *in vitro* organ bath setup revealed no interaction between these substances, suggesting the cholinergic inhibitory modulation to occur via M_2/M_4-receptors situated directly on the sensory neurons in the rat urinary bladder.

4 Concluding remarks

A substantial part of ATP-evoked contractile responses in the rat urinary bladder depends on the urothelial release of acetylcholine, as demonstrated by the present results. This thesis aimed to characterize the plausible functional implications of this link on the various levels of the micturition reflex arc. By using several *in vitro* and *in vivo* techniques, strong evidence for a cholinergic part, supporting the purinergic functional contractions, has been presented. Furthermore, receptor characterization using various agonists and antagonists indicated the release of acetylcholine to, most likely, be mediated via urothelial P2X1- and/or P2X3-purinoceptors. Regarding functional implications, observations *in vitro* as well as in the denervated bladder *in vivo* showed the ATP-evoked release of acetylcholine to be able to stimulate direct smooth muscle contraction also independent of the micturition reflex arc.

The hypothesis that the urothelium is the source for ATP-evoked release of acetylcholine was investigated. This was confirmed to be the case by urothelial removal, which eradicated the functional ATP-evoked release of acetylcholine both *in vitro* and *in vivo*. Notably, when disrupting the pelvic innervation *in vivo* (whole bladder setup) a significant decrease in the purinergic contractile response was observed, suggesting the removal of afferent reflex responses. This activation of afferent signalling was not present in urothelial denuded animals, highlighting the importance of an undamaged urothelium for healthy bladder signalling. Furthermore, based on the current findings, using the neurotoxin TTX, no direct efferent neuronal involvement could be identified, further supporting the theory of a urothelial origin.

In healthy animals, both the phasic component of the EFS-induced responses (tentatively indicating the neuronal release of ATP) and the ATP-induced release of acetylcholine were shown to be caveolae-dependent. The current results also identified a functional interaction between the urothelium and the caveolae. This may be interpreted as urothelial caveolae being mainly responsible for the purinergic-cholinergic link presently investigated. This, however, remains to be fully proven.

This thesis further elucidated the effects of experimentally induced cystitis on the ATP-evoked release of acetylcholine *in vitro*. Interestingly, this functional interaction was hindered by inflammation, indicating that cystitis affects the

urothelium in such a way that the non-neuronal synthesis of acetylcholine is significantly altered.

Finally, the ATP-induced release of acetylcholine, as well as direct cholinergic activation, was shown to be able to stimulate afferent nerve fibres, triggering the activation of the micturition reflex arc. The cholinergic modulation was shown to depend on the stimulation of facilitatory M_3- and inhibitory M_2/M_4-receptors. Many of the LUTS present in pathological bladder disorders, including OAB and BPS/IC, are thought to occur due to an alteration in sensory signalling. ATP is known to be able to initiate afferent signalling and afferent purinoceptors are often discussed as potential targets in the development of new drugs for LUTS. The novel findings in this thesis suggest that the ATP-evoked non-neuronal release of acetylcholine can modulate bladder afferent activity. This may in turn provide new insights for drug development as well as an increased knowledge of healthy bladder signalling.

4.1 Conclusion

This thesis identifies an ATP-evoked release of urothelial acetylcholine, most likely via the stimulation of the P2X1- and/or P2X3-subtype, which largely contributes to ATP-induced effects in the rat urinary bladder. The release of non-neuronal acetylcholine directly affects smooth muscle contraction as well as afferent nerve signalling. Cystitis seems to alter the urothelial non-neuronal signalling in such a way that the ATP-evoked release of acetylcholine diminishes, highlighting the importance of an intact urothelium and caveolae for normal bladder function.

Furthermore, ATP-induced release of acetylcholine was proven able to modulate reflex responses on its own via the stimulation of M_2/M_4 and M_3-receptors, providing new insights for bladder afferent signalling which might be relevant for the altered sensory signalling observed in OAB- and BPS/IC-patients.

5 Acknowledgement

I would like to express my sincere gratitude to everyone who have provided support and guidance during the years of my doctoral studies as well as during the writing of my thesis. My special thanks to:

My main supervisor **Patrik Aronsson**; during the years of my doctorial studies you have been an inspiring role model both as a person and as a scientist. I would like to thank you for always taking your time and lending me a hand whenever I have needed it. Your future PhD students are lucky to have you as a supervisor! Finally I would like to thank you for always brightening my day with one of your many "word jokes".

My co-supervisor **Thomas Carlsson** for your continuous support and help. Thank you for all the inspiring discussions, for teaching me immunohistological methods and your invaluable input on my articles and papers.

My co-supervisor **Michael Winder** for always taking the time to listen to my thoughts and questions. Thank you for teaching me about nitric oxide and its role in the urinary bladder. I would also like to thank you for the many laughs we have shared, especially during our many conference trips!

Professor **Gunnar Tobin**; you have been a great inspiration and one of the major factors as to why I chose to start a career within research. I would like to thank you for all the help and for teaching me about microsurgery, especially during last year when my back has been ill. Thank you for teaching me the most important lesson, that the "peang" is the best invention in the world.

My colleagues and fellow PhD-students in the Autonomic Neuroscience unit **Maria Del Pilar Murillo Angarita**, **Martin "Markin" Johnsson** and **Özgü Aydogdu** for all your support and help. Thank you for all the adventures during our travels and for the great times we have shared at the office. You are amazing!

Jolie Danial for being a friend and colleague. Thank you for all the positive energy during my doctoral studies. Thank you for the friendship and support!

Yohanna Eriksson for being one of my best friends and colleagues. Thank you for always being there for me and for supporting me in both the good times

and the bad. You are one of the strongest persons I know as well as an amazing friend! I am thankful for all the coffee breaks at “Farmen”, being my partner in crime at dissertation parties and the long walks we have shared with our dogs.

All my fellow PhD-students and other coworkers at the department of Pharmacology for contributing to a great work environment. Special thanks to **Britt-Marie Benbow**, **Maria Björkevik** and **Annalena Carlred** for the administrative help during these years.

My former middle-school teacher **Christina Nilsson** for teaching me to believe in myself and my ability to learn new things, even when I did not. Your guidance led to a fundamental change in my self-esteem. I would never have reached the position where I am today without the guidance you provided.

My **mother** and **father** for encouraging and supporting the choices I have made. Thank you for always being there for me no matter what. My sisters **Cecilia** and **Sofia** for all the love and support always. I love you all!

My **friends** and **extended family**! A special thanks to all the members of the gaming community **“SoD”** for always making me laugh and putting my mind on other stuff than work.

Lastly, I would like to thank my beloved fiancé **Viktor** and our dog **Akke** for all the love and support you both provide**.** You are my everything and without you finishing this thesis would not have been possible. I love you both more than Pringles (and sleeping in on Saturday mornings)!

6 References

Abbracchio MP, & Burnstock G (1994). Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacology & therapeutics 64**:** 445-475.

Abdel-Rahman M, Galeano C, & Elhilali M (1983). New approach to study of voiding cycle in cat. Preliminary report on pharmacologic studies. Urology 22**:** 91-97.

Acevedo CG, Contreras E, Escalona J, Lewin J, & Huidobro-Toro JP (1992). Pharmacological characterization of adenosine A1 and A2 receptors in the bladder: evidence for a modulatory adenosine tone regulating non-adrenergic non-cholinergic neurotransmission. British journal of pharmacology 107**:** 120-126.

Ahlquist RP (1948). A study of the adrenotropic receptors. The American journal of physiology 153**:** 586-600.

Andersson K-E (2011). Antimuscarinic mechanisms and the overactive detrusor: an update. European urology 59**:** 377-386.

Andersson KE, Garcia Pascual A, Forman A, & Tottrup A (1991). Non-adrenergic, non-cholinergic nerve-mediated relaxation of rabbit urethra is caused by nitric oxide. Acta physiologica Scandinavica 141**:** 133-134.

Andersson MC, Tobin G, & Giglio D (2008). Cholinergic nitric oxide release from the urinary bladder mucosa in cyclophosphamide-induced cystitis of the anaesthetized rat. British journal of pharmacology 153**:** 1438-1444.

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

Aronsson P, Carlsson T, Winder M, & Tobin G (2014a). A novel in situ urinary bladder model for studying afferent and efferent mechanisms in the micturition reflex in the rat. Neurourology and urodynamics 33**:** 550-557.

Aronsson P, Carlsson T, Winder M, & Tobin G (2015). Cyclophosphamide-induced alterations of the micturition reflex in a novel in situ urinary bladder model in the anesthetized rat. Neurourology and urodynamics 34**:** 375-380.

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

Aronsson P, Vesela R, Johnsson M, Tayem Y, Wsol V, Winder M*, et al.* (2014b). Inhibition of nitric oxide synthase prevents muscarinic and purinergic functional changes and development of cyclophosphamide-induced cystitis in the rat. BioMed research international 2014**:** 359179-359179.

Auge C, Chene G, Dubourdeau M, Desoubzdanne D, Corman B, Palea S*, et al.* (2013). Relevance of the cyclophosphamide-induced cystitis model for pharmacological studies targeting inflammation and pain of the bladder. European journal of pharmacology 707**:** 32-40.

Banks FCL, Knight GE, Calvert RC, Thompson CS, Morgan RJ, & Burnstock G (2006). The purinergic component of human vas deferens contraction. Fertil Steril 85**:** 932-939.

Beznak ABL (1951). Experiments on the adenosinetriphosphate contraction of the frog's rectus muscle. The Journal of physiology 115**:** 237-248.

Birder L, & Andersson KE (2013). Urothelial signaling. Physiological reviews 93**:** 653-680.

Birder L, Wolf-Johnston A, Buffington C, Roppolo J, De Groat W, & Kanai AJ (2005). Altered inducible nitric oxide synthase expression and nitric oxide production in the bladder of cats with feline interstitial cystitis. The Journal of urology 173**:** 625-629.

Birder LA, Apodaca G, De Groat WC, & Kanai AJ (1998). Adrenergic- and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. The American journal of physiology 275**:** F226-229.

Birder LA, Ruan HZ, Chopra B, Xiang Z, Barrick S, Buffington CA*, et al.* (2004). Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. American journal of physiology Renal physiology 287**:** F1084-F1091.

Bjorling DE, Wang Z-Y, & Bushman W (2011). Models of inflammation of the lower urinary tract. Neurourology and urodynamics 30**:** 673-682.

Bogart LM, Berry SH, & Clemens JQ (2007). Symptoms of interstitial cystitis, painful bladder syndrome and similar diseases in women: a systematic review. The Journal of urology 177**:** 450-456.

Brading AF (2006). Spontaneous activity of lower urinary tract smooth muscles: correlation between ion channels and tissue function. The Journal of physiology 570**:** 13-22.

Brading AF, & Williams JH (1990). Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha,beta-methylene ATP. British journal of pharmacology 99**:** 493-498.

Bredt DS, & Snyder SH (1992). Nitric oxide, a novel neuronal messenger. Neuron 8**:** 3-11.

Bschleipfer T, Schukowski K, Weidner W, Grando SA, Schwantes U, Kummer W*, et al.* (2007). Expression and distribution of cholinergic receptors in the human urothelium. Life sciences 80**:** 2303-2307.

Burnstock G (1972). Purinergic nerves. Pharmacological reviews 24**:** 509-581.

Burnstock G (1976). Do some nerve cells release more than one transmitter? Neuroscience 1**:** 239-248.

Burnstock G (1978). A basis for distinguishing two types of purinergic receptor. Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach**:** 107-118.

Burnstock G (2009). Purinergic cotransmission. Experimental physiology 94**:** 20-24.

Burnstock G, Cocks T, Kasakov L, & Wong HK (1978). Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. European journal of pharmacology 49**:** 145-149.

Burnstock G, & Kennedy C (1985). Is there a basis for distinguishing two types of P2-purinoceptor? General pharmacology 16**:** 433-440.

Caine M, Raz S, & Zeigler M (1975). Adrenergic and cholinergic receptors in the human prostate, prostatic capsule and bladder neck. Br J Urol 47**:** 193-202.

Carroll PT, & Goldberg AM (1975). Relative importance of choline transport to spontaneous and potassium depolarized release of ACh. Journal of neurochemistry 25**:** 523-527.

Chang RS, Chen TB, O'Malley SS, Pettibone DJ, DiSalvo J, Francis B*, et al.* (2000). In vitro studies on L-771,688 (SNAP 6383), a new potent and selective alpha1A-adrenoceptor antagonist. European journal of pharmacology 409**:** 301-312.

Chen X, & Gebhart GF (2010). Differential purinergic signaling in bladder sensory neurons of naive and bladder-inflamed mice. Pain 148**:** 462-472.

Chen X, Molliver DC, & Gebhart GF (2010). The P2Y2 receptor sensitizes mouse bladder sensory neurons and facilitates purinergic currents. The Journal of neuroscience : the official journal of the Society for Neuroscience 30**:** 2365-2372.

Chess-Williams R, Sellers DJ, Brierley SM, Grundy D, & Grundy L (2019). Purinergic receptor mediated calcium signalling in urothelial cells. Scientific Reports 9**:** 16101.

Chiang G, Patra P, Letourneau R, Jeudy S, Boucher W, Green M*, et al.* (2000). Pentosanpolysulfate inhibits mast cell histamine secretion and intracellular calcium ion levels: An alternative explanation of its beneficial effect in interstitial cystitis. The Journal of urology 164**:** 2119-2125.

Chopra B, Gever J, Barrick SR, Hanna-Mitchell AT, Beckel JM, Ford AP*, et al.* (2008). Expression and function of rat urothelial P2Y receptors. American journal of physiology Renal physiology 294**:** F821-829.

Clemens JQ, Meenan RT, Rosetti MC, Gao SY, & Calhoun EA (2005). Prevalence and incidence of interstitial cystitis in a managed care population. The Journal of urology 173**:** 98-102.

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE*, et al.* (2005). P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. The Journal of physiology 567**:** 621-639.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S*, et al.* (2000). Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature 407**:** 1011-1015.

Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, & Stojilkovic SS (2011). Activation and regulation of purinergic P2X receptor channels. Pharmacological reviews 63**:** 641-683.

Cohen AW, Hnasko R, Schubert W, & Lisanti MP (2004). Role of caveolae and caveolins in health and disease. Physiological reviews 84**:** 1341-1379.

Colović MB, Krstić DZ, Lazarević-Pašti TD, Bondžić AM, & Vasić VM (2013). Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol 11**:** 315-335.

Contreras-Sanz A, Krska L, Balachandran AA, Curtiss NL, Khasriya R, Kelley S*, et al.* (2016). Altered urothelial ATP signaling in a major subset of human overactive bladder patients with pyuria. American journal of physiology Renal physiology 311**:** f805-f816.

Cox PJ (1979). Cyclophosphamide cystitis--identification of acrolein as the causative agent. Biochemical pharmacology 28**:** 2045-2049.

Creed KE, Loxley RA, & Phillips JK (2010). Functional expression of muscarinic and purinoceptors in the urinary bladder of male and female rats and guinea pigs. Journal of Smooth Muscle Research 46**:** 201-215.

Cristalli G, Costanzi S, Lambertucci C, Lupidi G, Vittori S, Volpini R*, et al.* (2001). Adenosine deaminase: Functional implications and different classes of inhibitors. Med Res Rev 21**:** 105-128.

Cristofaro V, Peters CA, Yalla SV, & Sullivan MP (2007). Smooth muscle caveolae differentially regulate specific agonist induced bladder contractions. Neurourology and urodynamics 26**:** 71-80.

Cristofaro V, Yalla SV, & Sullivan MP (2012). Altered caveolar mediated purinergic signaling in spontaneously hypertensive rats with detrusor overactivity. The Journal of urology 188**:** 1017-1026.

D'Agostino G, Bolognesi ML, Lucchelli A, Vicini D, Balestra B, Spelta V*, et al.* (2000). Prejunctional muscarinic inhibitory control of acetylcholine release in the human isolated detrusor: involvement of the M4 receptor subtype. British journal of pharmacology 129**:** 493-500.

Dahl G (2015). ATP release through pannexon channels. Philos Trans R Soc Lond B Biol Sci 370**:** 20140191.

Dale H (1935). Pharmacology and Nerve-endings (Walter Ernest Dixon Memorial Lecture): (Section of Therapeutics and Pharmacology). Proc R Soc Med 28**:** 319-332.

Dale HH (1913). On the action of ergotoxine; with special reference to the existence of sympathetic vasodilators. The Journal of physiology 46**:** 291-300.

Dale HH (1914). The action of certain esters and ethers of choline, and their relation to muscarine. Journal of Pharmacology and Experimental Therapeutics 6**:** 147-190.

Dang K, Lamb K, Cohen M, Bielefeldt K, & Gebhart GF (2008). Cyclophosphamide-induced bladder inflammation sensitizes and enhances P2X receptor function in rat bladder sensory neurons. Journal of neurophysiology 99**:** 49-59.

Darvesh S, Hopkins DA, & Geula C (2003). Neurobiology of butyrylcholinesterase. Nature Reviews Neuroscience 4**:** 131-138.

De Groat W, & Ryall R (1969). Reflexes to sacral parasympathetic neurones concerned with micturition in the cat. The Journal of physiology 200**:** 87-108.

de Groat WC (1997). A neurologic basis for the overactive bladder. Urology 50**:** 36-56.

de Groat WC, Griffiths D, & Yoshimura N (2015). Neural control of the lower urinary tract. Compr Physiol 5**:** 327-396.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, & Freeman TC (1996). Tissue distribution of adenosine receptor mRNAs in the rat. British journal of pharmacology 118**:** 1461-1468.

Doggweiler R, Whitmore KE, Meijlink JM, Drake MJ, Frawley H, Nordling J*, et al.* (2017). A standard for terminology in chronic pelvic pain syndromes: A report from the chronic pelvic pain working group of the international continence society. Neurourology and urodynamics 36**:** 984-1008.

Drake MJ, Morris N, Apostolidis A, Rahnama'i MS, & Marchesi JR (2017). The urinary microbiome and its contribution to lower urinary tract symptoms; ICI-RS 2015. Neurourology and urodynamics 36**:** 850-853.

Dreja K, Voldstedlund M, Vinten J, Tranum-Jensen J, Hellstrand P, & Sward K (2002). Cholesterol depletion disrupts caveolae and differentially impairs agonist-induced arterial contraction. Arteriosclerosis, thrombosis, and vascular biology 22**:** 1267-1272.

Eccles JC, Fatt P, & Koketsu K (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. The Journal of physiology 126**:** 524-562.

Eglen RM, Hegde SS, & Watson N (1996). Muscarinic receptor subtypes and smooth muscle function. Pharmacological reviews 48**:** 531-565.

Ekstrom J, & Elmer M (1977). Choline acetyltransferase activity in the denervated urinary bladder of the rat. Acta physiologica Scandinavica 101**:** 58-62.

Elmér M (1974). Inhibitory β-adrenoceptors in the urinary bladder of the rat. Life sciences 15**:** 273-280.

Elneil S, Skepper JN, Kidd EJ, Williamson JG, & Ferguson DR (2001). Distribution of P2X1and P2X3Receptors in the Rat and Human Urinary Bladder. Pharmacology 63**:** 120-128.

Erb L, & Weisman GA (2012). Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdiscip Rev Membr Transp Signal 1**:** 789-803.

Ewins AJ (1914). Acetylcholine, a New Active Principle of Ergot. The Biochemical journal 8**:** 44-49.

Fehér E, Csanyi K, & Vajda JJ (1980). Intrinsic innervation of the urinary bladder. Acta Anat (Basel) 106**:** 335-344.

Ferguson DR, Kennedy I, & Burton TJ (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory mechanism? The Journal of physiology 505 (Pt 2)**:** 503-511.

Fitzgerald MP, Stablein U, & Brubaker L (2002). Urinary habits among asymptomatic women. American journal of obstetrics and gynecology 187**:** 1384-1388.

Fowler CJ, Griffiths D, & de Groat WC (2008). The neural control of micturition. Nature reviews Neuroscience 9**:** 453-466.

Fu P, Chen F, Pan Q, Zhao X, Zhao C, Cho WC*, et al.* (2017). The different functions and clinical significances of caveolin-1 in human adenocarcinoma and squamous cell carcinoma. OncoTargets and therapy 10**:** 819-835.

Furchgott RF, & Zawadzki JV (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288**:** 373-376.

Gabella G (2004). CHAPTER 3 - Autonomic Nervous System. In The Rat Nervous System (Third Edition). ed Paxinos G. Academic Press: Burlington, pp 77-109.

Gable SR, & Kingsley T (1996) *Concise text of neuroscience*. Williams & Wilkins.

Giglio D, Delbro DS, & Tobin G (2001). On the functional role of muscarinic M2 receptors in cholinergic and purinergic responses in the rat urinary bladder. European journal of pharmacology 428**:** 357-364.

Giglio D, Ryberg AT, To K, Delbro DS, & Tobin G (2005). Altered muscarinic receptor subtype expression and functional responses in cyclophosphamide induced cystitis in rats. Autonomic neuroscience : basic & clinical 122**:** 9-20.

Goepel M, Gronewald A, Krege S, & Michel MC (1998). Muscarinic receptor subtypes in porcine detrusor: comparison with humans and regulation by bladder augmentation. Urological Research 26**:** 149-154.

Gormley EA, Lightner DJ, Burgio KL, Chai TC, Clemens JQ, Culkin DJ*, et al.* (2012). Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline. The Journal of urology 188**:** 2455-2463.

Grol S, Essers PBM, Van Koeveringe GA, Martinez-Martinez P, De Vente J, & Gillespie JI (2009). M3 muscarinic receptor expression on suburothelial interstitial cells. BJU international 104**:** 398-405.

Gualix J, Pintor J, & Miras-Portugal MT (1999). Characterization of Nucleotide Transport into Rat Brain Synaptic Vesicles. Journal of neurochemistry 73**:** 1098-1104.

Häbler H, Jänig W, & Koltzenburg M (1990). Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. J Physiol 425**:** 545-562.

Häbler H, Jänig W, & Koltzenburg M (1993a). Myelinated primary afferents of the sacral spinal cord responding to slow filling and distension of the cat urinary bladder. J Physiol 463**:** 449-460.

Häbler H, Jänig W, & Koltzenburg M (1993b). Receptive properties of myelinated primary afferents innervating the inflamed urinary bladder of the cat. Journal of neurophysiology 69**:** 395-405.

Hanna-Mitchell AT, Beckel JM, Barbadora S, Kanai AJ, de Groat WC, & Birder LA (2007). Non-neuronal acetylcholine and urinary bladder urothelium. Life sciences 80**:** 2298-2302.

Hanno PM (1997). Analysis of long-term Elmiron therapy for interstitial cystitis. Urology 49**:** 93-99.

Hanno PM, Erickson D, Moldwin R, & Faraday MM (2015). Diagnosis and treatment of interstitial cystitis/bladder pain syndrome: AUA guideline amendment. The Journal of urology 193**:** 1545-1553.

Haselberger MB, & Schwinghammer TL (1995). Efficacy of mesna for prevention of hemorrhagic cystitis after high-dose cyclophosphamide therapy. The Annals of pharmacotherapy 29**:** 918-921.

Haylen BT, de Ridder D, Freeman RM, Swift SE, Berghmans B, Lee J*, et al.* (2010). An International Urogynecological Association (IUGA)/International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. Neurourology and urodynamics 29**:** 4-20.

Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM*, et al.* (1997). Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. British journal of pharmacology 120**:** 1409-1418.

Hegde SS, & Eglen RM (1999). Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. Life sciences 64**:** 419-428.

Hu P, Deng F-M, Liang F-X, Hu C-M, Auerbach AB, Shapiro E*, et al.* (2000). Ablation of Uroplakin III Gene Results in Small Urothelial Plaques, Urothelial Leakage, and Vesicoureteral Reflux. Journal of Cell Biology 151**:** 961-972.

Hurst RE, Greenwood-Van Meerveld B, Wisniewski AB, VanGordon S, Lin H, Kropp BP*, et al.* (2015). Increased bladder permeability in interstitial cystitis/painful bladder syndrome. Translational andrology urology 4**:** 563.

Husted S, Sjögren C, & Andersson KE (1980). Mechanisms of the responses to non-cholinergic, non-adrenergic nerve stimulation and to ATP in isolated rabbit urinary bladder: evidence for ADP evoked prostaglandin release. Acta pharmacologica et toxicologica 47**:** 84-92.

Igawa Y, Yamazaki Y, Takeda H, Akahane M, Ajisawa Y, Yoneyama T*, et al.* (1998). Possible beta 3-adrenoceptor-mediated relaxation of the human detrusor. Acta physiologica Scandinavica 164**:** 117-118.

Ikeda Y, Zabbarova IV, Birder LA, de Groat WC, McCarthy CJ, Hanna-Mitchell AT*, et al.* (2012). Botulinum neurotoxin serotype A suppresses neurotransmitter release from afferent as well as efferent nerves in the urinary bladder. European urology 62**:** 1157-1164.

Irwin DE, Milsom I, Hunskaar S, Reilly K, Kopp Z, Herschorn S*, et al.* (2006). Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. European urology 50**:** 1306-1314.

Ishihama H, Momota Y, Yanase H, Wang X, de Groat WC, & Kawatani M (2006). Activation of alpha1D adrenergic receptors in the rat urothelium facilitates the micturition reflex. The Journal of urology 175**:** 358-364.

Jerde TJ, Bjorling DE, Steinberg H, Warner T, & Saban R (2000). Determination of mouse bladder inflammatory response to E. coli lipopolysaccharide. Urological research 28**:** 269-273.

Jost SP, Gosling JA, & Dixon JS (1989). The morphology of normal human bladder urothelium. Journal of anatomy 167**:** 103-115.

Juszczak K, Krolczyk G, Filipek M, Dobrowolski ZF, & Thor PJ (2007). Animal models of overactive bladder: cyclophosphamide (CYP)-induced cystitis in rats. Folia medica Cracoviensia 48**:** 113-123.

Kanai AJ (2011). Afferent mechanism in the urinary tract. Handbook of experimental pharmacology**:** 171-205.

Kim SO, Song SH, Lee SC, Cho KA, Yu HS, Hwang IS*, et al.* (2012). Expression of caveolin-1 in rat urinary bladder with cyclophosphamide-induced cystitis. International neurourology journal 16**:** 169-174.

Kinder RB, & Mundy AR (1985). Atropine blockade of nerve-mediated stimulation of the human detrusor. Br J Urol 57**:** 418-421.

King BF, Knowles ID, Burnstock G, & Ramage AG (2004). Investigation of the effects of P2 purinoceptor ligands on the micturition reflex in female urethane-anaesthetized rats. British journal of pharmacology 142**:** 519-530.

Kowalski ML, Sliwinska-Kowalska M, & Kaliner MA (1990). Neurogenic inflammation, vascular permeability, and mast cells. II. Additional evidence indicating that mast cells are not involved in neurogenic inflammation. J Immunol 145**:** 1214-1221.

Kuhar MJ, & Murrin LC (1978). Sodium-dependent, high affinity choline uptake. Journal of neurochemistry 30**:** 15-21.

Kullmann FA, Artim DE, Birder LA, & de Groat WC (2008). Activation of muscarinic receptors in rat bladder sensory pathways alters reflex bladder activity. J Neurosci 28**:** 1977-1987.

Kumar V, Chapple CR, Rosario D, Tophill PR, & Chess-Williams R (2010). In vitro release of adenosine triphosphate from the urothelium of human bladders with detrusor overactivity, both neurogenic and idiopathic. European urology 57**:** 1087-1092.

Langley JN, & Anderson HK (1895). The Innervation of the Pelvic and adjoining Viscera: Part II. The Bladder. Part III. The External Generative Organs. Part IV. The Internal Generative Organs. Part V. Position of the Nerve Cells on the Course of the Efferent Nerve Fibres. The Journal of physiology 19**:** 71-139.

Lawrence GW, Aoki KR, & Dolly JO (2010). Excitatory cholinergic and purinergic signaling in bladder are equally susceptible to botulinum neurotoxin a consistent with co-release of transmitters from efferent fibers. Journal of Pharmacology and Experimental Therapeutics 334**:** 1080-1086.

Lazarowski ER, Sesma JI, Seminario-Vidal L, & Kreda SM (2011). Molecular mechanisms of purine and pyrimidine nucleotide release. Advances in pharmacology (San Diego, Calif) 61**:** 221-261.

Lee HY, Bardini M, & Burnstock G (2000). Distribution of P2X receptors in the urinary bladder and the ureter of the rat. The Journal of urology 163**:** 2002-2007.

Leppilahti M, Tammela TL, Huhtala H, & Auvinen A (2002). Prevalence of symptoms related to interstitial cystitis in women: a population based study in Finland. The Journal of urology 168**:** 139-143.

Leron E, Weintraub AY, Mastrolia SA, & Schwarzman P (2018). Overactive Bladder Syndrome: Evaluation and Management. Curr Urol 11**:** 117-125.

Levin RM, Ruggieri MR, & Wein A (1986). Functional effects of the purinergic innervation of the rabbit urinary bladder. Journal of Pharmacology Experimental Therapeutics 236**:** 452-457.

Levin RM, Ruggieri MR, & Wein AJ (1988). Identification of Receptor Subtypes in the Rabbit and Human Urinary Bladder by Selective Radio-Ligand Binding. The Journal of urology 139**:** 844-848.

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH*, et al.* (1995). Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. The Journal of biological chemistry 270**:** 15693-15701.

Lifford KL, & Curhan GC (2009). Prevalence of painful bladder syndrome in older women. Urology 73**:** 494-498.

Lilly JD, & Parsons CL (1990). Bladder surface glycosaminoglycans is a human epithelial permeability barrier. Surgery, gynecology & obstetrics 171**:** 493-496.

Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D*, et al.* (2005). Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. American journal of respiratory cell and molecular biology 33**:** 79-88.

Lisanti MP, Scherer PE, Tang Z, & Sargiacomo M (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. Trends in cell biology 4**:** 231-235.

Liu H-T, Shie J-H, Chen S-H, Wang Y-S, & Kuo H-C (2012). Differences in mast cell infiltration, E-cadherin, and zonula occludens-1 expression between patients with overactive bladder and interstitial cystitis/bladder pain syndrome. Urology 80**:** 225. e213-225. e218.

Loewi O (1921). Über humorale übertragbarkeit der Herznervenwirkung. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere 189**:** 239-242.

Luce JK, & Simons JA (1988). Efficacy of mesna in preventing further cyclophosphamide-induced hemorrhagic cystitis. Medical and pediatric oncology 16**:** 372-374.

Ma FH, Higashira-Hoshi H, & Itoh Y (2002). Functional muscarinic M2 and M3 receptors and beta-adrenoceptor in cultured rat bladder smooth muscle. Life sciences 70**:** 1159-1172.

Mallory B, Steers WD, & De Groat WC (1989). Electrophysiological study of micturition reflexes in rats. American Journal of Physiology-Regulatory, Integrative Comparative Physiology 257**:** R410-R421.

Mansfield KJ, & Hughes JR (2014). Effect of inflammatory mediators on ATP release of human urothelial RT4 cells. BioMed research international 2014**:** 182862.

McDermott C, Chess-Williams R, Mills K, Kang S, Farr S, Grant G*, et al.* (2013). Alterations in acetylcholine, PGE2 and IL6 release from urothelial cells following treatment with pyocyanin and lipopolysaccharide. Toxicology in Vitro 27**:** 1693-1698.

McMurray G, Dass N, & Brading AF (1998). Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. British journal of pharmacology 123**:** 1579-1586.

Meini S, & Maggi CA (1994). Evidence for a capsaicin-sensitive, tachykinin-mediated, component in the NANC contraction of the rat urinary bladder to nerve stimulation. British journal of pharmacology 112**:** 1123-1131.

Merrill L, Gonzalez EJ, Girard BM, & Vizzard MA (2016). Receptors, channels, and signalling in the urothelial sensory system in the bladder. Nature reviews Urology 13**:** 193-204.

Mok MS, Knight GE, Andrews PL, Hoyle CH, & Burnstock G (2000). The effects of cyclophosphamide on neurotransmission in the urinary bladder of Suncus murinus, the house musk shrew. Journal of the autonomic nervous system 80**:** 130-136.

Molinoff PB (1984). α- and β-Adrenergic Receptor Subtypes. Drugs 28**:** 1-15.

Moore KH, Ray FR, & Barden JA (2001). Loss of Purinergic P2X3 and P2X5 Receptor Innervation in Human Detrusor from Adults with Urge Incontinence. Journal of Neuroscience 21**:** RC166-RC166.

Moriyama Y, Hiasa M, Sakamoto S, Omote H, & Nomura M (2017). Vesicular nucleotide transporter (VNUT): appearance of an actress on the stage of purinergic signaling. Purinergic signalling 13**:** 387-404.

Moro C, & Chess-Williams R (2012). Non-adrenergic, non-cholinergic, non-purinergic contractions of the urothelium/lamina propria of the pig bladder. Autonomic & autacoid pharmacology 32**:** 53-59.

Nakagomi H, Yoshiyama M, Mochizuki T, Miyamoto T, Komatsu R, Imura Y*, et al.* (2016). Urothelial ATP exocytosis: regulation of bladder compliance in the urine storage phase. Scientific Reports 6**:** 29761.

Nandigama R, Bonitz M, Papadakis T, Schwantes U, Bschleipfer T, & Kummer W (2010). Muscarinic acetylcholine receptor subtypes expressed by mouse bladder afferent neurons. Neuroscience 168**:** 842-850.

Nazif O, Teichman JM, & Gebhart GF (2007). Neural upregulation in interstitial cystitis. Urology 69**:** 24-33.

Nicholas RA, Watt WC, Lazarowski ER, Li Q, & Harden K (1996). Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. Molecular pharmacology 50**:** 224-229.

Nomiya M, & Yamaguchi O (2003). A quantitative analysis of mRNA expression of alpha 1 and beta-adrenoceptor subtypes and their functional roles in human normal and obstructed bladders. The Journal of urology 170**:** 649-653.

Noto H, Roppolo J, Steers W, & De Groat W (1989). Excitatory and inhibitory influences on bladder activity elicited by electrical stimulation in the pontine micturition center in the rat. Brain research 492**:** 99-115.

Novak I (2003). ATP as a Signaling Molecule: the Exocrine Focus. Physiology 18**:** 12-17.

O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, Rymer JM*, et al.* (2001). A quantitative analysis of purinoceptor expression in human fetal and adult bladders. The Journal of urology 165**:** 1730-1734.

Oshita M, Hiraoka Y, & Watanabe Y (1997). Characterization of beta-adrenoceptors in urinary bladder: comparison between rat and rabbit. British journal of pharmacology 122**:** 1720-1724.

Otsuka A, Shinbo H, Matsumoto R, Kurita Y, & Ozono S (2008). Expression and functional role of beta-adrenoceptors in the human urinary bladder urothelium. Naunyn-Schmiedeberg's archives of pharmacology 377**:** 473-481.

Ozawa H, Chancellor MB, Jung SY, Yokoyama T, Fraser MO, Yu Y*, et al.* (1999). Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis. The Journal of urology 162**:** 2211-2216.

Palade GE (1955). A small particulate component of the cytoplasm. The Journal of biophysical and biochemical cytology 1**:** 59-68.

Palea S, Artibani W, Ostardo E, Trist DG, & Pietra C (1993). Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. The Journal of urology 150**:** 2007-2012.

Palmer RM, Ferrige AG, & Moncada S (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327**:** 524-526.

Pankratov Y, Lalo U, Verkhratsky A, & North RA (2007). Quantal release of ATP in mouse cortex. The Journal of general physiology 129**:** 257-265.

Parsons CL (2007). The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis. Urology 69**:** 9-16.

Parton RG, & Simons K (2007). The multiple faces of caveolae. Nat Rev Mol Cell Biol 8**:** 185-194.

Peeker R, Samsioe G, Kowalski J, Andersson A-S, & Bergqvist A (2010). A prospective observational study of the effects of treatment with extended-release tolterodine on health-related quality of life of patients suffering overactive bladder syndrome in Sweden. Scand J Urol Nephrol 44**:** 138-146.

Persson K, & Andersson KE (1994). Non-adrenergic, non-cholinergic relaxation and levels of cyclic nucleotides in rabbit lower urinary tract. European journal of pharmacology 268**:** 159-167.

Pintor J, Diaz-Rey MA, Torres M, & Miras-Portugal MT (1992). Presence of diadenosine polyphosphates--Ap4A and Ap5A--in rat brain synaptic terminals. Ca2+ dependent release evoked by 4-aminopyridine and veratridine. Neuroscience letters 136**:** 141-144.

Ralevic V, & Burnstock G (1998). Receptors for purines and pyrimidines. Pharmacological reviews 50**:** 413-492.

Robinson D, & Cardozo L (2012). Antimuscarinic drugs to treat overactive bladder. BMJ 344**:** e2130.

Rourke W, Khan SA, Ahmed K, Masood S, Dasgupta P, & Khan MS (2014). Painful bladder syndrome/interstitial cystitis: aetiology, evaluation and management. Archivio italiano di urologia, andrologia : organo ufficiale [di] Societa italiana di ecografia urologica e nefrologica / Associazione ricerche in urologia 86**:** 126-131.

Ruan HZ, Birder LA, de Groat WC, Tai C, Roppolo J, Buffington CA*, et al.* (2005). Localization of P2X and P2Y receptors in dorsal root ganglia of the cat. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 53**:** 1273-1282.

Saban R, Christensen M, Keith I, Graziano FM, Undem BJ, Aagaard J*, et al.* (1991). Experimental model for the study of bladder mast cell degranulation and smooth muscle contraction. Semin Urol 9**:** 88-101.

Sadegh MK, Ekman M, Rippe C, Sundler F, Wierup N, Mori M*, et al.* (2011). Biomechanical properties and innervation of the female caveolin-1-deficient detrusor. British journal of pharmacology 162**:** 1156-1170.

Satchell P, & Vaughan C (1994). Bladder wall tension and mechanoreceptor discharge. Pflügers Archiv 426**:** 304-309.

Searl TJ, Dynda DI, Alanee SR, El-Zawahry AM, McVary KT, & Silinsky EM (2016). A1 adenosine receptor–mediated inhibition of parasympathetic neuromuscular transmission in human and murine urinary bladder. Journal of Pharmacology Experimental Therapeutics 356**:** 116-122.

Sexton CC, Notte SM, Maroulis C, Dmochowski RR, Cardozo L, Subramanian D*, et al.* (2011). Persistence and adherence in the treatment of overactive bladder syndrome with anticholinergic therapy: a systematic review of the literature. Int J Clin Pract 65**:** 567-585.

Shabir S, Cross W, Kirkwood LA, Pearson JF, Appleby PA, Walker D*, et al.* (2013). Functional expression of purinergic P2 receptors and transient receptor potential channels by the human urothelium. Am J Physiol Renal Physiol 305**:** F396-F406.

Shakirova Y, Mori M, Ekman M, Erjefalt J, Uvelius B, & Sward K (2010). Human urinary bladder smooth muscle is dependent on membrane cholesterol for cholinergic activation. European journal of pharmacology 634**:** 142-148.

Shaul PW, & Anderson RG (1998). Role of plasmalemmal caveolae in signal transduction. The American journal of physiology 275**:** L843-851.

Silinsky EM (1975). On the association between transmitter secretion and the release of adenine nucleotides from mammalian motor nerve terminals. The Journal of physiology 247**:** 145-162.

Silman I, & Sussman JL (2008). Acetylcholinesterase: How is structure related to function? Chemico-Biological Interactions 175**:** 3-10.

Silva-Ramos M, Silva I, Faria M, Magalhães-Cardoso M, Correia J, Ferreirinha F*, et al.* (2015). Impairment of ATP hydrolysis decreases adenosine A 1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder. Purinergic signalling 11**:** 595-606.

Silva I, Ferreirinha F, Magalhaes-Cardoso MT, Silva-Ramos M, & Correia-de-Sa P (2015). Activation of P2Y6 Receptors Facilitates Nonneuronal Adenosine Triphosphate and Acetylcholine Release from Urothelium with the Lamina Propria of Men with Bladder Outlet Obstruction. The Journal of urology 194**:** 1146-1154.

Sjogren C, & Andersson KE (1979). Inhibition of ATP-induced contraction in the guinea-pig urinary bladder in vitro and in vivo. Acta pharmacologica et toxicologica 44**:** 221-227.

Sjogren C, Andersson KE, Husted S, Mattiasson A, & Moller-Madsen B (1982). Atropine resistance of transmurally stimulated isolated human bladder muscle. The Journal of urology 128**:** 1368-1371.

Smith CP, Vemulakonda VM, Kiss S, Boone TB, & Somogyi GT (2005). Enhanced ATP release from rat bladder urothelium during chronic bladder inflammation: effect of botulinum toxin A. Neurochemistry international 47**:** 291-297.

Somogyi GT, & de Groat WC (1999). Function, signal transduction mechanisms and plasticity of presynaptic muscarinic receptors in the urinary bladder. Life sciences 64**:** 411-418.

Somogyi GT, Tanowitz M, & de Groat WC (1994). M1 muscarinic receptor-mediated facilitation of acetylcholine release in the rat urinary bladder. The Journal of physiology 480 (Pt 1)**:** 81-89.

Somogyi GT, Tanowitz M, & de Groat WC (1995). Prejunctional facilitatory alpha 1-adrenoceptors in the rat urinary bladder. British journal of pharmacology 114**:** 1710-1716.

Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R*, et al.* (2003). Prevalence and burden of overactive bladder in the United States. World journal of urology 20**:** 327-336.

Sun Y, & Chai TC (2004). Up-regulation of P2X3 receptor during stretch of bladder urothelial cells from patients with interstitial cystitis. The Journal of urology 171**:** 448-452.

Sun Y, Keay S, De Deyne PG, & Chai TC (2001). Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis. The Journal of urology 166**:** 1951-1956.

T Gudermann, F Kalkbrenner a, & Schultz G (1996). Diversity and Selectivity of Receptor-G Protein Interaction. Annual review of pharmacology and toxicology 36**:** 429-459.

Takezawa K, Kondo M, Kiuchi H, Ueda N, Soda T, Fukuhara S*, et al.* (2016). Authentic role of ATP signaling in micturition reflex. Scientific Reports 6**:** 19585.

Timóteo MA, Carneiro I, Silva I, Noronha-Matos JB, Ferreirinha F, Silva-Ramos M*, et al.* (2014). ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors. Biochemical pharmacology 87**:** 371-379.

Tobin G (1995). Muscarinic receptor subtypes in the submandibular gland and the urinary bladder of the rabbit: in vivo and in vitro functional comparisons of receptor antagonists. Journal of autonomic pharmacology 15**:** 451-463.

Tobin G (1998). Presynaptic muscarinic M1 and M2 receptor modulation of auriculotemporal nerve transmission in the rat. Journal of the autonomic nervous system 72**:** 61-71.

Tobin G, & Sjogren C (1995). In vivo and in vitro effects of muscarinic receptor antagonists on contractions and release of [3H]acetylcholine in the rabbit urinary bladder. European journal of pharmacology 281**:** 1-8.

Tong YC, Hung YC, Shinozuka K, Kunitomo M, & Cheng JT (1997). Evidence of adenosine 5'-triphosphate release from nerve and P2x-purinoceptor mediated contraction during electrical stimulation of rat urinary bladder smooth muscle. The Journal of urology 158**:** 1973-1977.

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor MB, & Miguel Fd (2006). Qualitative and Quantitative Expression Profile of Muscarinic Receptors in Human Urothelium and Detrusor. The Journal of urology 176**:** 1673-1678.

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A*, et al.* (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. Nature 371**:** 516-519.

van Koppen CJ, & Kaiser B (2003). Regulation of muscarinic acetylcholine receptor signaling. Pharmacology & therapeutics 98**:** 197-220.

Varley CL, Garthwaite MAE, Cross W, Hinley J, Trejdosiewicz LK, & Southgate J (2006). PPARgamma-regulated tight junction development during human urothelial cytodifferentiation. J Cell Physiol 208**:** 407-417.

Vesela R, Asklund H, Aronsson P, Johnsson M, Wsol V, Andersson M*, et al.* (2012). Coupled nitric oxide and autonomic receptor functional responses in the normal and inflamed urinary bladder of the rat. Physiological research / Academia Scientiarum Bohemoslovaca 61**:** 371-380.

Walden PD, Durkin MM, Lepor H, Wetzel JM, Gluchowski C, & Gustafson EL (1997). Localization of mRNA and receptor binding sites for the alpha 1a-adrenoceptor subtype in the rat, monkey and human urinary bladder and prostate. The Journal of urology 157**:** 1032-1038.

Wang P, Luthin GR, & Ruggieri MR (1995). Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. The Journal of pharmacology and experimental therapeutics 273**:** 959-966.

Waters MG, Suleskey JF, Finkelstein LJ, Van Overbeke ME, Zizza VJ, & Stommel M (2000). Interstitial cystitis: a retrospective analysis of treatment with pentosan polysulfate and follow-up patient survey. The Journal of the American Osteopathic Association 100**:** S13-18.

Weihe E, Tao-Cheng JH, Schäfer MK, Erickson JD, & Eiden LE (1996). Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 93**:** 3547-3552.

Winder M, Tobin G, Zupančič D, & Romih R (2014). Signalling molecules in the urothelium. BioMed research international 2014**:** 297295-297295.

Winder M, Vesela R, Aronsson P, Patel B, & Carlsson T (2017). Autonomic Receptor-mediated Regulation of Production and Release of Nitric Oxide in Normal and Malignant Human Urothelial Cells. Basic & clinical pharmacology & toxicology 121**:** 257-265.

Wu G, Bogatkevich GS, Mukhin YV, Benovic JL, Hildebrandt JD, & Lanier SM (2000). Identification of Gbetagamma binding sites in the third intracellular loop of the M(3)-muscarinic receptor and their role in receptor regulation. The Journal of biological chemistry 275**:** 9026-9034.

Xiang Z, Bo X, & Burnstock G (1998). Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. Neuroscience letters 256**:** 105-108.

Yamanishi T, Chapple CR, Yasuda K, & Chess-Williams R (2000). The role of M2-muscarinic receptors in mediating contraction of the pig urinary bladder in vitro. The Journal of urology 131**:** 1482-1488.

Yegutkin GG (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta 1783**:** 673-694.

Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, & Akino H (2005). Effects of tolterodine on an overactive bladder depend on suppression of C-fiber bladder afferent activity in rats. The Journal of urology 174**:** 2032-2036.

Yoshida M, Homma Y, Inadome A, Yono M, Seshita H, Miyamoto Y*, et al.* (2001). Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. Exp Gerontol 36**:** 99-109.

Yoshida M, Masunaga K, Satoji Y, Maeda Y, Nagata T, & Inadome A (2008). Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in urothelium and its clinical significance. Journal of pharmacological sciences 106**:** 193-198.

Yoshimura N, Seki S, & de Groat WC (2001). Nitric oxide modulates Ca(2+) channels in dorsal root ganglion neurons innervating rat urinary bladder. Journal of neurophysiology 86**:** 304-311.

Yu W, Zacharia LC, Jackson EK, & Apodaca G (2006). Adenosine receptor expression and function in bladder uroepithelium. Am J Physiol Cell Physiol 291**:** C254-C265.

Zholos AV, Zholos AA, & Bolton TB (2004). G-protein–gated TRP-like Cationic Channel Activated by Muscarinic Receptors : Effect of Potential on Single-channel Gating. Journal of General Physiology 123**:** 581-598.

Zimmermann H (1996). Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog Neurobiol 49**:** 589-618.

Zimmermann H (2000). Extracellular metabolism of ATP and other nucleotides. Naunyn-Schmiedeberg's archives of pharmacology 362**:** 299-309.

Zimmermann H, Zebisch M, & Sträter N (2012). Cellular function and molecular structure of ecto-nucleotidases. Purinergic signalling 8**:** 437-502.