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Studies on Inhibitory Modulation of Urinary Tract Motility

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You inspired me to start and provided me with everything at the outset.

ABSTRACT

Overactive Bladder (OAB) is a symptom syndrome characterized by urgency and frequency, with or without urge incontinence. Research concerning the mechanisms underlying OAB and finding new inhibitory factor(s) is intensive.

By using a cascade superfusion system, we confirmed the existence of urothelium-derived inhibitory activity in the guinea pig urinary bladder. The unknown inhibitory activity was transmissible over a significant distance, thus allowing attempts at isolation. The unknown factor(s) was found unlikely to be nitric oxide, an adenosine receptor agonist or cyclo-oxygenase products.

During isolation of the unknown transmissible inhibitory factor(s) using HPLC and bioassay technologies, we observed considerable amounts of PGE_2 and PGD_2 being released from guinea pig urinary bladder urothelium, in the resting state. PGE_2 and PGD_2 were not only released from the bladder but also exerted modulatory effects regulating the lower urinary tract motility.

Organ bath experiments using pharmacological tools in guinea pig urothelium-denuded bladder strips showed that PGE_2 increased basal tone and spontaneous contractions. Exogenous PGE_2 potentiated contractile responses to EFS, ACh and ATP, while PGD_2 caused inhibition of EFS, ACh and ATP induced contractions. The inhibitory effect of PGD_2 was exerted via DP_1 receptors as judged from agonist and antagonist experiments. PGD_2 also had a low affinity excitatory effect via TP receptors.

The bladder trigone and urethra have different innervations compared with the bladder dome. Both trigone and proximal urethra are important in continence. The effects of PGE_2 and PGD_2 were investigated also in these regions in the guinea pig. PGE_2 and PGD_2 in a dose-dependent manner inhibited trigone contractions induced by EFS and spontaneous contractions of the proximal urethra.

Immunohistochemical studies of DP_1 and DP_2 receptor proteins indicated that DP_1 and DP_2 receptors were localized in the guinea pig bladder dome, trigone and urethra. Both urothelium/sub-urothelium and smooth muscle cells were immunolabelled with DP_1 and DP_2 receptor antibodies. Hematopoietic prostaglandin D synthase was abundant in the immediate sub-urothelium.

These findings will be of importance in understanding normal function and pathophysiology of the lower urinary tract, hopefully opening up new future treatment modalities.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which in the text will be referred to by their Roman numerals:

I. **Guan NN**, Thor A, Hallén K, Wiklund NP, Gustafsson LE (2014). Cascade bioassay evidence for the existence of urothelium-derived inhibitory factor in Guinea pig urinary bladder. *PLoS One. 2014 Aug 1;9(8):e103932.*

II. **Guan NN**, Nilsson KF, Wiklund NP, Gustafsson LE (2014). Release and inhibitory effects of prostaglandin D2 in guinea pig urinary bladder and the role of urothelium. *Biochim Biophys Acta. 2014 Sep 16;1840(12):3443-3451.*

III. **Guan NN**, Svennersten K, de Verdier PJ, Wiklund NP, Gustafsson LE. Receptors involved in Prostaglandin D_2 modulation of guinea pig urinary bladder motility. *Submitted Manuscript.*

IV. **Guan NN**, Svennersten K, de Verdier PJ, Wiklund NP, Gustafsson LE. Prostaglandin D_2 effects and DP_1/DP_2 receptor distribution in guinea pig urinary bladder outflow region. *Manuscript.*

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
ATP	adenosine 5'-triphosphate
CGRP	calcitonin gene-related peptide
CRTH2	chemoattractant homologous receptor expressed on Th2 cells
DP_1	prostaglandin D_2 receptor type 1
DP_2	prostaglandin D_2 receptor type 2
EFS	electrical field stimulation
HPLC	high performance liquid chromatography
L-NAME	N^G-nitro-L-arginine methyl ester
LUT	lower urinary tract
NANC	non-adrenergic non-cholinergic
NO	nitric oxide
NO_2^-	nitrite
NPY	neuropeptide Y
OAB	overactive bladder
PACAP	pituitary-adenylate cyclase- activating polypeptide
PGDS	prostaglandin D synthase
TP	thromboxane receptor
TTX	tetrodotoxin
UD	urothelium-denuded
UDIF	urothelium-derived inhibitory factor
UI	urothelium-intact
VIP	vasoactive intestinal polypeptide
8-PST	8-(*p*-sulfophenyl)theophylline

1 INTRODUCTION

1.1 LOWER URINARY TRACT SYMPTOMS

In 2003, the standardization sub-committee of the International Continence Society (ICS) issued the latest report of the standardization of terminology in lower urinary tract function (Abrams *et al.*, 2003). This updated version included all the new and changed definitions of previous ICS reports. The terminology covered the definitions of lower urinary tract symptoms, signs suggestive of lower urinary tract dysfunction, urodynamic observations, conditions and treatment. Symptoms are defined as the subjective indicators of a disease or change in condition witnessed by the patient, caregiver or persons around and may result in seeking help from professionals. There are three groups of lower urinary tract symptoms: storage symptoms, voiding symptoms and post micturition symptoms.

1.1.1 Storage Symptoms

The bladder relaxes and stores urine for more than 99% of the lifetime. Storage symptoms are experienced during the storage phase of the bladder and include increased daytime frequency, nocturia (frequency during sleep), urgency and urinary incontinence (not applicable to infants and small children).

1.1.2 Voiding and Post Micturition Symptoms

Voiding symptoms are experienced during the voiding phase which include slow stream, splitting or spraying of urine stream, intermittent stream, hesitancy, straining to void and terminal dribble.
Post micturition symptoms are experienced immediately after micturition including feeling of incomplete emptying and post micturition dribble.

Lower urinary tract symptoms also include the symptoms associated with sexual intercourse, with pelvic organ prolapse, genital and lower urinary tract pain etcetera.

1.1.3 OAB

The ICS committee recommended the use of overactive bladder syndrome and detrusor overactivity after 2002 ICS standardization of lower urinary tract function/dysfunction terminology (Abrams *et al.*, 2002; Abrams, 2003). Detrusor overactivity is an urodynamic diagnosis which defines the signs and symptoms during urodynamic observation. The report described two types of detrusor overactivity: phasic and terminal. In order to simply describe the condition, the term “Overactive Bladder (OAB) Symptom Complex” has been introduced. OAB is a symptomatic diagnosis with the definition as urgency, with or without urge incontinence, usually with increased daytime frequency and nocturia (Abrams *et al.*, 2002). However, OAB is not synonymous with detrusor overactivity. A study among 1457 patients showed that 82% of men with OAB had detrusor overactivity (sensitivity) and 82% with detrusor overactivity had OAB (positive predictive value), while the rate for women were 58%

and 85% respectively (Hashim *et al.*, 2006). The bladder seems to be a more reliable witness in men than in women (*op.cit*.).

OAB affects nearly 34 million in the US and 66 million people in the European Union One epidemiological study concerning the prevalence of overactive bladder syndrome, before the ICS 2002 definition and among 16776 interviews, in six European counties found the overall prevalence of overactive bladder symptoms in individuals above age 40 was 16.6% (15.6% of men and 17.4% of women) (Milsom *et al.*, 2001). Later it was believed that this number was too high (Hashim *et al.*, 2007). Many other studies regarding the overactive bladder syndrome prevalence showed similar results. The National Overactive Bladder Evaluation (NOBLE) Program using a sample of 5204 adults over age 18 revealed an OAB prevalence of 16.0% in men and 16.9% in women in the United States (Stewart *et al.*, 2003). A population-based study of OAB symptoms among 1000 adults of age over 18 reported 13.9% (13.1% in men and 14.7% in women) prevalence in Canada (Herschorn *et al.*, 2008). Among 1827 community-dwelling adults in Taiwan, the age-adjusted prevalence of OAB was 16.9% (Yu *et al.*, 2006). The largest multinational population-based study was the EPIC study using the current ICS definition included Canada, Germany, Italy, Sweden and the United Kingdom. The overall prevalence of OAB was 11.8% within the 19165 participating individuals. Rates were similar in men and women and increased with age (Irwin *et al.*, 2006a). A recently study limited to individuals aged 40 and older from US, UK and Sweden showed that the prevalence of OAB ranged from 26-33% across races for men and from 27-46% for women (Coyne *et al.*, 2012).(Figure 1)

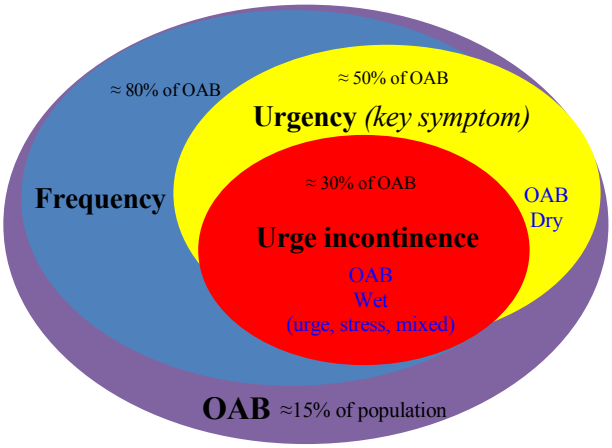

Figure 1. *Ring diagram showing that the average prevalence of OAB from cited studies was about 15% of the entire population. 80% of the OAB patients expressed frequency especially nocturia, 50% of the OAB patients reported urgency and 30% had urge incontinence symptom. OAB dry, the term from the NOBLE study defining the individuals who had OAB symptoms but no urge incontinence, was more prevalent in men. OAB wet was more prevalent in women which might be due to weak bladder neck and sphincter mechanism.*

Although OAB symptoms conditions are not life threatening, they seriously affect quality of life of the individual, including employment, social interactions and

emotional well-being (Irwin *et al.*, 2006b). Frequency and urge incontinence also increase the risk of urinary tract infection and falls (Brown *et al.*, 2000). OAB patients with nocturia suffering from poor sleeping quality had lower level of vitality (Kobelt *et al.*, 2003). These lower urinary tract symptoms are more prevalent in the older population, where 40% of individuals over the age of 70 are affected (Milsom *et al.*, 2001). As a consequence, OAB produces major problems for individuals, health professions and society. Importantly, as people are generally living longer, the numbers of affected people are increasing. It is obvious that the combined problems give a heavy economic burden to the society. The main costs include treatment costs, routine care costs, consequences costs and indirect costs (Hu *et al.*, 2004). The mainly non-pharmacotherapeutic costs can thus lead to an under-estimation of the financial magnitude of the problem. It has been indicated that the estimated total cost of urinary incontinence and OAB was $19.5 billion and $12.6 billion respectively in the United States in year 2000 using data from the NOBLE program (Hu *et al.*, 2004). In 2007, OAB resulted in total costs of $65.9 billion in the US with projected total national costs of $76.2 billion and $82.6 billion respectively in year 2015 and 2020 (Ganz *et al.*, 2010). In a report covering Germany alone, 6.48 million adults over 40 years of age were affected by OAB, and 2.18 million of these individuals experience incontinence, with annual financial implications in the range of €3.98 billion, costs comparable to other chronic diseases such as dementia or diabetes mellitus (Klotz *et al.*, 2007). Estimation of the economic costs of OAB in six countries (Canada, Germany, Italy, Spain, Sweden and the UK) by using the prevalence data from the EPIC study and healthcare resource-use data showed the total annual direct cost plus nursing care and work absenteeism cost was €9.7 billion (Irwin *et al.*, 2009). The direct cost in Sweden was estimated to be €333 million per year.

The primary clinical problem and major impact to the patient is urgency to void (Abrams *et al*. 2002). The ICS definition of urgency is the complaint of a sudden compelling desire to pass urine which is difficult to defer. Urgency is the key symptom and must be a symptom for OAB diagnosis and is also the driver for other lower urinary tract symptoms (Abrams *et al.*, 2012). Remarkably, despite the prevalence and costs involved, the mechanisms underlying urgency are currently unknown. There are three main pathophysiology theories about OAB and detrusor overactivity: myogenic theory (Brading, 1997), neurogenic theory (de Groat, 1997) and autonomous bladder theory (Drake *et al.*, 2001). These theories all involve the unbalanced release of inhibitory and excitatory neurotransmitters or modulating mediators. Little understanding of the mechanism leads to the lack of effective pharmacological treatment. Antimuscarinic drugs are the most commonly used agents to treat OAB, but their clinical utility is limited due to side effects and low efficacy. Undoubtedly, there are needs for exploring the mechanisms underlying this process and finding new therapeutic targets to treat OAB effectively.

OAB debate

Debate on the definition of OAB as a symptom syndrome has been continuing since it was first issued in the report by the standardization committee of International

Continence Society in 2002. Among all the OAB debates, the main arguments are whether the concept of OAB simplifies the clinical conditions, with their different causes, underlying 'overactive bladder' patients (Zinner, 2011; Tikkinen *et al.*, 2012) and if the concept of OAB was created by the pharmaceutical industry as marketing method to sell drugs (Tikkinen *et al.*, 2012).
In my opinion, the driving force of issuing the descriptive term OAB is not the above debates but medical needs behind. Before the concept of OAB was introduced, similar medical conditions were referred to as *unstable bladder* which is a urodynamic diagnosis. In the clinic, many patients are hesitant to undergoing the urodynamic test before receiving any drug treatments. Though OAB describes a set of symptoms which does not provide any pathophysiological explanation, there is a need for finding a term that patients, nurses, and doctors can easily understand and use it to begin empirical management. By using the term OAB, more attention will be brought into the scientific fields and more research concerning the mechanisms behind OAB will be done. This will lead to modifications of and more accurate description of overactive bladder conditions. Already in the seventies, emepronium bromide and later terodiline as anticholinergic drugs were introduced in urology to treat smooth muscle overactive conditions. Due to severe side effects, these drugs were abandoned in many countries. This led to the need of new anticholinergic drugs with less side effects such as tolterodine and to find new drugs that target on other signaling pathways. Despite having some utility in OAB, many patients are not treated effectively even with the most recent agents (Ko *et al.*, 2006), despite supporting reports (Van Kerrebroeck *et al.*, 2001; Nabi *et al.*, 2006).

1.2 LOWER URINARY TRACT ANATOMY AND PHYSIOLOGY

The lower urinary tract consists of the bladder and urethra. The bladder is a hollow muscular organ to collect and store urine generated by the kidneys. The structure of the bladder is similar between male and female. The urethra is directly connected to the bladder and is a somewhat muscular passage for micturition. The structure and function of urethra is different between the male and female. In the male, genital glands are connected and have openings towards the urethra which thus carries semen and other fluids ejaculated from genital ducts, as well as urine. In the female, the urethra is shorter and only responsible for urination. Besides gender differences, there are also species variation between the human and other mammals.

1.2.1 Gross Anatomy of Bladder and Urethra

Bladder
The bladder is a distensible organ in the pelvis. The size and shape of the bladder change when it fills with different volumes of urine. In healthy adults, the bladder can hold 300-500 ml of urine. The bladder wall consists of several layers. The internal surface is lined with transitional epithelium (urothelium), which becomes thinner and flatter during bladder distension and folds when the bladder is empty. The urothelium has three layers with different cell types, the apical umbrella cells, the intermediate cells and basal cells (Richter *et al.*, 1963). The lamina propria underlying the

urothelium is a collagenous fibroelastic connective tissue rich in blood vessels, nerves, interstitial cells and some muscle bundles. The ensuing smooth muscle layer (detrusor) which accounts for the most of the bladder wall, mixed with connective tissue, forms parallel muscle bundles. These smooth muscle bundles are irregularly oriented, loosely arranged into inner longitudinal, middle circular, and outer longitudinal layers. The inner and outer layers are not clearly separated; muscle bundle can cross layers and branch into a longitudinal or circle direction.

The bladder trigone is defined as the region within the three openings: two ureters and urethra. This region is relatively smooth and constant in thickness compared with the rest of the bladder. The trigone is composed of three muscle layers: a superficial layer, derived from the longitudinal muscle of the ureter extending into the urethra, a deep layer and a detrusor layer originally coming from the bladder outer longitudinal and middle circular smooth muscle layers.

Bladder neck

Smooth muscle cells in the bladder neck become smaller in size and the muscle bundles are more uniformly organized. The smooth muscle around the bladder neck is morphologically and pharmacologically distinct from the remainder of the bladder. The anterior of the bladder neck is more closely akin to the bladder detrusor muscle, while the posterior fibers are more similar to the trigone (Gosling *et al.*, 1987; Delancey *et al.*, 2002). The structure of the bladder neck seems to differ between men and women. In male, three distinct layers of smooth muscle are present: inner layer of longitudinal smooth muscle bundles continuous with the inner longitudinal layer of the urethra, the middle circular layer that becomes the proximal sphincter at the bladder neck level, and the outer longitudinal layers. At the female bladder neck, the majority of muscle bundles extend obliquely or longitudinally into the urethral wall and the existence of sphincter like circular muscle layer is debatable (Chung *et al.*, 2012)

Urethra

The length of the human male urethra is approximately 20 cm and is usually described as three parts: prostatic, membranous and spongy. The female urethra is a fibromuscular tube only about 3-4 cm long. The urethra is lined with transitional epithelium which changes to a nonkeratinized stratified squamous epithelium in the very distal end of the female urethra. The lamina propria separates the urothelium and muscle layer and has many fibroblasts, small glands, blood vessels and nerves. Compared with bladder detrusor, the muscle bundle cells are relatively small and interspersed with large amounts of connective tissue. A continuation of bladder inner longitudinal smooth muscle forms the inner longitudinal smooth layer of urethra and runs the entire course of the urethra. The bladder outer longitudinal muscle cells travel obliquely around bladder neck and proximal urethra. As a result, the longitudinal arranged muscle cells become more and more circular from the neck to urethra. This urethra outer circular smooth muscle layer is only present in the proximal urethra, which ends at where it meets the striated muscle that forms the intrinsic rhabdosphincter (Hutch *et al.*, 1968). In the male, the outer circular smooth muscle

fuses with the ventral face of the prostate in the prostatic urethra region. The outer circular muscle only covers the anterior part of the proximal urethra, while in female the proximal urethra is surrounded by outer circular smooth muscle. Due to the anatomical position of the male prostate and the female vaginal wall, the superior part of striated muscle only covers the anterior part of the urethra. The inferior part of the striated muscle becomes horseshoe-shaped and covers the anterior and posterior of the urethra. In the female, the distal part of the striated muscle extends and covers the urethra and vagina (Yucel *et al.*, 2004; Wallner *et al.*, 2009).

Urethral sphincter

The muscular sphincters in the male prostatic + membranous urethra and the proximal 60% of the female urethra are especially implicated in urinary continence. Two major sphincter mechanisms are described in this region: the internal urethral sphincter and the external urethral sphincter (intrinsic rhabdosphincter for the striated component). The internal sphincter is located at the junction of the urethra with the bladder. The internal sphincter is a continuation of the bladder smooth muscle. The external sphincter is located at the 15 to 60% of the proximal urethra in females and at the level of the membranous urethra in the male. The external sphincter consists of an outer striated muscle and an inner longitudinal thin layer of smooth muscle. (Figure 2)

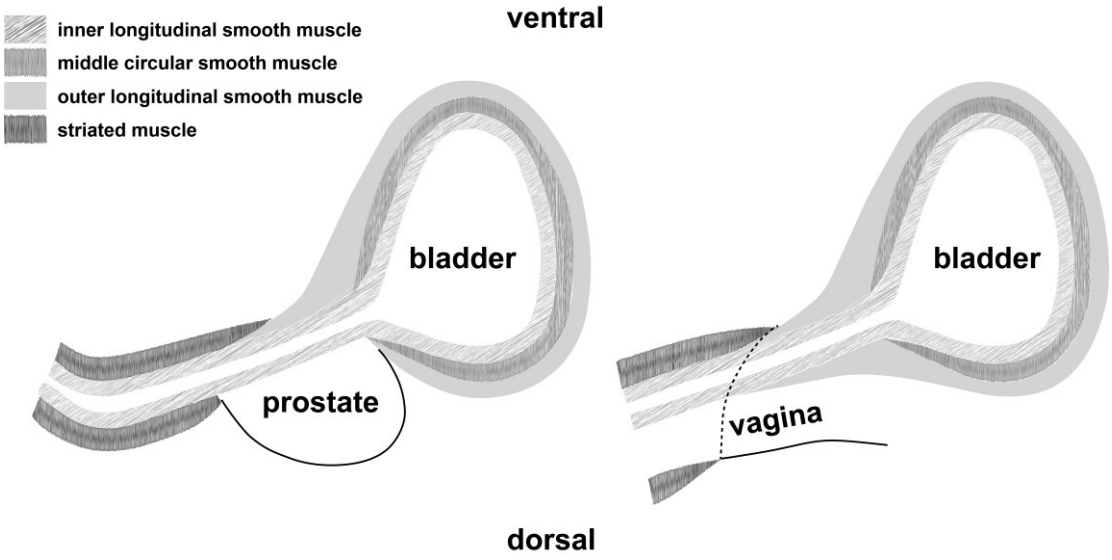

Figure 2. *Schematic drawing of different muscle layers arranged in the male (left) and female (right) bladder and urethra. The external urethra sphincter is composed of urethral inner longitudinal smooth muscle, which extends from bladder inner longitudinal detrusor, and of outer striated muscle. The outer longitudinal detrusor muscle bundles extend obliquely into the urethral wall. As a result, muscle bundles travel across to the other side of the bladder to become circularly oriented. This sphincter-like circular muscle layer is referred to as the internal urethra sphincter.*

Male guinea pig lower urinary tract

Many different kinds of mammals are used to study the function of the lower urinary tract. However, the anatomy, pathophysiology and pharmacology might not be the same at all. When performing animal experiments, it is important to be aware of the differences. Guinea pigs are widely used for both *in vivo* and *in vitro* studies on the biological function of the urinary tract. Studies have shown the basic structure of the guinea pig LUT to be comparable to the human. However, the accessory glands and gland locations are different and it does not seem to be the right model to study the glands related diseases (Figure 3). The sphincter mechanisms to maintain continence are comparable to human, and it might be suitable to use guinea pig isolated muscle strips or cell cultures in this context (Neuhaus *et al.*, 2001).

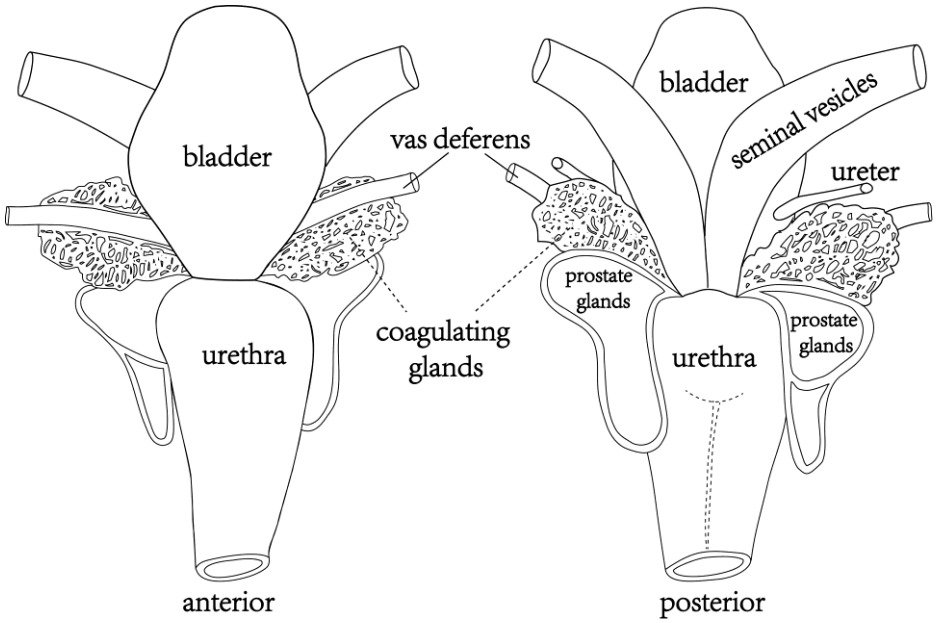

Figure 3. *Cartoon showing the locations and structural relations of elements in male guinea pig lower urinary tract and accessory glands. The bladder in the figure is empty. The prostate glands have several lobes as in the human, but they do not surround the proximal urethra lumen. The coagulating glands are unique for rodents and do not exist in the human. The prostate glands, coagulating glands, seminal vesicles and vas deferens all discharge through the dorsal wall of the proximal urethra.*

1.2.2 Innervation

The efferent innervation

A complex network of efferent (motor) and afferent (sensory) signaling in the autonomic and somatic nervous system is involved in regulating the lower urinary tract function. The cerebral cortex, brain stem and spinal cord are all involved in the central nervous system control. In both male and female, the bladder wall is mainly supplied with parasympathetic cholinergic nerve endings derived from the S2-S4 spinal cord segments. A nonadrenergic, noncholinergic (NANC) component of the autonomic nervous system has been proposed in activating the detrusor. Sparse sympathetic

neurons derived from Th10-L2 segments may also participate in mediating detrusor relaxation during urine storage. Down along the tract, the male bladder neck receives rich sympathetic innervation. In contrast, the female bladder neck has little adrenergic innervation. The trigone and male preprostatic sphincter (prevents retrograde ejaculation) are the only regions to show a distinct innervation by adrenergic neurons. The bladder neck and trigone also receive innervation from nitric oxide synthase containing nerves. They both relax during micturition.

Being regulated by both excitatory and inhibitory nerves, the innervation of the urethral smooth muscle is in this aspect more complicated than the detrusor. The female urethral smooth muscle has relatively few noradrenergic sympathetic nerves but receives an extensive presumably cholinergic parasympathetic nerve supply from the spinal cord S2-S4 segments similar as in the detrusor. In the pig urethra, it was suggested that two independent components were involved in the NANC-nerve derived urethral relaxation, one of which being nitrergic nerves, the other is still unknown (Werkström *et al.*, 1995). Urethral smooth muscle is activated in a stimulatory and inhibitory fashion to become shortened and widened during micturition. Similar preganglionic parasympathetic innervation to the smooth muscle components of the membranous urethra is likely to be present in the male. The striated muscle of the external urethral sphincter receives dual somatic innervations from the pudendal and pelvic somatic nerves from S2-S4 nerve roots in both the male and female (Delancey *et al.*, 2002; Chung *et al.*, 2012).

The afferent innervation

Afferent nerves sense stimuli and provide information to the central nervous system. Compared with efferent innervation in the lower urinary tract, the afferent innervation is less well understood. Many sensations and nervous pathways have been proposed arising from bladder and urethra: the sensation of normal filling; the sensation of voiding; the sensation of urgency incontinence; the sensation that urine is passing (Nathan, 1956). The afferent innervation in human lower urinary tract arises from neurons located in the dorsal root ganglia at the level of S2-S4 and Th11-L2 spinal segments. Two kinds of afferent fibers are found in the bladder: myelinated A-δ fibers and unmyelinated C-fibers. Sensation of bladder filling is conducted by A-δ fibers. C-fibers synthesize and release various neuropeptides, including calcitonin-gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), pituitary-adenylate cyclase-activating polypeptide (PACAP) and tachykinins (substance P and neurokinin A). C-fibers also express many neuropeptide receptors and other receptors, including acetylcholine, ATP, prostaglandins and transient receptor potential (TRP) receptors (de Groat *et al.*, 2009; de Groat *et al.*, 2012).

In the human, by using immunohistochemistry, peptidergic afferent axons were found distributed throughout the bladder and co-located within the subepithelial region, encircling ganglia cells and around blood vessels (Smet et al., 1997). In the urethra, the afferent nerves were distributed between the muscle fibers, in the urothelium, and especially dense in the suburothelial layer (Crowe et al., 1986; Tainio, 1993). The striated sphincter muscle seem to have very little afferent innervation. As indicated

before, the morphology of striated muscle in human urethra reveals relatively small cells compared with skeletal muscle. Specialized tension receptors which are prominent in most skeletal muscles were either absent or present in low density in human rhabdosphincter (Gosling et al., 1981; Lassmann, 1984).

1.2.3 Bladder Filling and Voiding

Bladder storage
During bladder filling, the intravesical pressure remains relatively constant until the volume reaches the threshold of voiding although there is increasing afferent activity during filling. The ability of keeping the pressure stable is a passive phenomenon of smooth muscle called compliance together with spinal modulation of the incoming afferent signals. Smooth muscle has the intrinsic ability to change length without changing tension within a certain range. de Groat proposed a "gating" mechanism that might be involved in bladder storage. In short, an inhibitory interneuron mechanism in the spinal cord helps to keep afferent impulses, until they reach a threshold, from being transmitted into efferent signals. When reaching the threshold, intensive impulses are then transmitted down the postganglionic efferent neuron to the bladder resulting in smooth muscle contraction (deGroat *et al.*, 1980). In addition, there are inhibitory effects of the sympathetic neurons that have postganglionic branches ending on parasympathetic ganglion cells in the bladder. This promotes urethral outlet closure and inhibits contractions of the bladder (de Groat *et al.*, 1976). During filling, a local spinal reflex with an increased efferent firing in the pudendal nerves results in an increased outlet resistance. Contractions mediated by pudendal motorneurons in the urethral sphincter are activated by bladder afferent input (Park *et al.*, 1997a), and urethral and pelvic floor afferent inputs (Fedirchuk *et al.*, 1992). Contraction of the urethral sphincter also sends afferent input to activate the central inhibitory mechanisms, further suppressing the micturition reflex (McGuire *et al.*, 1983).

Bladder emptying
When the bladder volume reaches a threshold, a voiding process will be triggered. As mentioned above, increased intensive afferent signaling transmits onward to the pons to activate the parasympathetic efferent activity and inhibits the sympathetic and somatic pathway. This reflex results in sufficient amplitude and duration of contraction to empty the bladder and in relaxation of the intrinsic rhabdosphincter to allow voiding. The drop of the urethral pressure is not the consequence of bladder contraction as it happens before the bladder contraction (Tanagho *et al.*, 1970). An important inhibitory transmitter, NO, is released to relax the bladder neck and urethral smooth muscle during micturition, a response mediated by activation of a parasympathetic pathway (Andersson *et al.*, 1994; Bennett *et al.*, 1995). The activation of the parasympathetic pathway to the urethra also counteracts the excitatory inputs to the urethra (Mundy, 2010; Yoshimura *et al.*, 2010).

1.2.4 Peripheral Control of Smooth Muscle

Mechanisms of smooth muscle contraction
Detrusor smooth muscle cells are spindle shaped single nucleated cells with plasticity and diversity. The phasic contraction of the smooth muscle cell is triggered by myosin light chain phosphorylation. Inactivation of myosin (dephosphorylation) results in the relaxation. The phosphorylation requires Ca^{2+}-calmodulin complex activated myosin light chain kinase (MLCK) while dephosphorylation needs myosin light chain phosphatase (MLCP). Protein kinase C (PKC) activates the phosphorylation of both structural and regulatory components of actin which regulate the tonic contraction (Rasmussen *et al.*, 1987). The intracellular free calcium level is the major determinant during smooth muscle contractility. Other modulators that may regulate the activity of key enzymes can also affect the contraction and relaxation of smooth muscle, including cAMP-dependent protein kinase A (PKA), mitogen-activated protein kinase, CaM kinase II (Yamaguchi, 2004) and *rho*-associated kinase (ROK) (Kimura *et al.*, 1996).

Neurotransmitters and modulators
In healthy human bladder, acetylcholine (ACh) is the contractile neurotransmitter, while in many pathologies including OAB, hypertrophy, interstitial cystitis, neurogenic damage (Palea *et al.*, 1993; Bayliss *et al.*, 1999), and in physiological signaling of many animals (Burnstock *et al.*, 1978), ATP is regarded as an additional activator of muscle contraction. The source of ACh is not only parasympathetic and somatic motor nerves but also urothelium (Lips *et al.*, 2007). There are five subtypes of muscarinic receptors and they are expressed throughout the LUT. In human, rat, rabbit and guinea pig detrusor, immunoprecipitation analyses have shown that m2 and m3 subtypes are expressed (Wang et al., 1995). Although more m2 receptors are expressed, the contractile activity is via m3 subtype. In most mammalian species, ATP is co-released with ACh from parasympathetic nerves, which activates P2 (P2X and P2Y) receptors to initiate detrusor contraction (Burnstock, 2011). Unlike parasympathetic activation, direct sympathetic innervation in detrusor is less important. In pig, few evenly distributed adrenergic nerve terminals were found in detrusor, while a greater number of terminals were observed in the trigone, bladder neck and urethra (Larsen *et al.*, 1978). All three beta-receptor subtypes are expressed in detrusor of which β3 is the most abundant subtype (Yamaguchi, 2002). Activation of β3 receptors elevates smooth muscle cAMP levels and causes detrusor relaxation, making it a potential interesting target for OAB treatment (Andersson *et al.*, 2013).

In most species, nitric oxide does not have a role as neurotransmitter to cause direct relaxation of the detrusor. The density of nitric oxide synthase (NOS) immunoreactivity was higher in trigone and urethra than in the detrusor (Persson *et al.*, 1993). In human bladder neck and urethra, the areas of marked relaxations activity to nerve stimulation and the areas of high NOS abundance were in good agreement, suggesting nitric oxide as a mediator for the neurogenic dilation of the bladder neck and urethra during the micturition reflex (Ehrén *et al.*, 1994). The synthesis, storage and release of neuropeptides including vasoactive intestinal polypeptide (VIP), calcitonin gene–

related peptide (CGRP), substance P, neurokinin A, and pituitary adenylate cyclase-activating peptide (PACAP) from efferent and afferent nerves and urothelial cells have been demonstrated in the lower urinary tract. VIP was shown to inhibit spontaneous contractions in isolated detrusor and urethral muscle from human and animals (Sjögren *et al.*, 1985). CGRP is contained within capsaicin-sensitive sensory nerves in the urinary bladder. CGRP could relax rat bladder neck, and inhibited neuromuscular transmission in the guinea pig detrusor. In vivo, high concentrations of CGRP inhibited the rat micturition reflex (Hoyle, 1994). Tachykinins (substance P and neurokinin A) had contractile effects in the isolated bladder smooth muscle (Negri *et al.*, 1973; Maggi, 1991). Tachykinin NK1 receptor seems more commonly found in blood vessels and the urothelium than in muscle cells (Candenas *et al.*, 2005). NK2 receptors are present in detrusor muscle (Templeman *et al.*, 2003). PACAP- immunoreactivity is present primarily in afferent neurons. Exogenous PACAP has both relaxant and excitant effects on bladder and urethral smooth muscle depending on species (Yoshiyama *et al.*, 2008). Prostaglandins as one of the modulator groups are produced locally in the bladder urothelium and muscle layers. Exogenous PG alters bladder motor activity in vitro and in vivo and it can also influence the micturition reflex in humans and many different species as discussed before.

Spontaneous contraction

Lower urinary tract tissues especially bladder, are capable of generating a TTX resistant spontaneous activity (Sibley, 1984). Moreover, some reports found that spontaneous contractions were observed to an increased extent in OAB patients (Mills *et al.*, 2000; Sui *et al.*, 2009). The mechanisms behind spontaneous activity is not clear yet, although some have been proposed. 1) Neurogenic hypothesis: de Groat reported that reduced neurogenic inhibition could increase the activation of micturition reflex and contractions associated with smooth muscle overactivity (de Groat, 1997). 2) Myogenic hypothesis: changes to the smooth muscle excitability and intercellular coupling to the other muscle cells would modify the contractile activity (Brading, 1997). In addition, pacemaker cells such as interstitial cells could generate spontaneous contractions (Hashitani, 2006). Oscillators located in the interstitial cells generate repetitive Ca^{2+} transients that activate inward currents. The currents spread through the gap junctions providing a depolarizing signal which opens L-type voltage-operated channels (VOCs). The result of increased Ca^{2+} thus triggers smooth muscle contraction (Berridge, 2008). 3) Urotheliogenic hypothesis: changes in the sensitivity and coupling of the mucosa (urothelium/suburothelium) network results in the changes of spontaneous activity (Ikeda *et al.*, 2008).

1.2.5 Mediator Release

The bladder urothelium has long been thought to be a protective barrier between detrusor and urine. In the late 1980's it was noticed that contraction responses to the sensory nerve mediator substance P in the guinea pig urinary bladder were smaller when the urothelium was intact (Maggi *et al.*, 1987a). Direct evidence for the release of a relaxant mediator from bladder was obtained by Andersson's group, when they

co-incubated urothelium containing urinary bladder with an endothelium-denuded rat aorta strip (Fovaeus *et al.*, 1998; Fovaeus *et al.*, 1999). Similar bladder-derived relaxant factor was also shown in the rat, using bladder as assay tissue by coaxial bioassay system (Bozkurt *et al.*, 2004). Unlike the evidence from the previous studies that the relaxant factor(s) was non-urothelium dependent, Chess-Williams group showed the existence of a diffusible urothelium-derived inhibitory factor (UDIF) in pig urinary bladder by using sandwich-type bioassay experiments (Hawthorn *et al.*, 2000; Templeman *et al.*, 2002). UDIF related effects were also observed in mouse urinary bladder by using electrophysiology (Meng *et al.*, 2008) and by quantitative analysis of contractile responses in human urinary bladder (Chaiyaprasithi *et al.*, 2003). The nature of the bladder-derived non-urothelium dependent relaxant factor(s) and UDIF have not been elucidated. One substance group to be considered is arachidonic acid derivatives from the cyclo-oxygenase (COX) system. Experiments in urothelium-intact and -denuded preparations had shown that COX products had a role in regulation of ureteral motility (Mastrangelo *et al.*, 2007). The data suggested that prostacyclin was released from the urothelium, but they furthermore suggested that this to a great extent acted indirectly, via stimulation of the release of an unknown inhibitory factor.

1.3 PROSTANOIDS AND PROSTANOID RECEPTORS

Fatty acids as sources of energy and as precursors of mediators are usually derived from triglycerides or phospholipids. Eicosanoids are a group of important signaling molecules made by oxidation of the 20-carbon fatty acid arachidonic acid (20:4 ω 6). One important subclass of eicosanoids is prostanoids, consisting of prostaglandins, thromboxanes, and prostacyclins. Prostanoids exert their bioactivities locally via specific receptors. The modulatory roles of prostanoids in the lower urinary tract had come into focus for many years, but the mechanisms of prostanoids effects are still not clear due to their multitude and to lack of pharmacological tools. In recent years, the molecular and genetic information as well as specific agonists and antagonists of prostanoids receptors have become available, making it possible to evaluate the effects of prostanoids from a more detailed perspective. In this study, we focused on biosynthesis and bioactivities of prostaglandin D_2 and E_2 in the lower urinary tract.

1.3.1 Prostanoids Biosynthesis

Eicosanoids can be metabolized from arachidonic acid (AA) through enzymatic or non-enzymatic processes. There are three major well known enzymatic pathways catalyzed by cyclo-oxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (epoxygenase, CYP) respectively (Buczynski *et al.*, 2009). In addition, prostaglandin-like compounds called isoprostanes are formed via non-enzymatic process where free radicals act on AA. Prostanoids that are synthesized via cyclooxygenase pathways are a group of hydroxyl fatty acids containing an oxygenated cyclopentane unit. PGH_2 as the precursor for prostanoids is formed from free AA by prostaglandin endoperoxide synthases known as constitutively expressed cyclo-oxygenase-1 (COX-1) or inducible cyclo-oxygenase-2 (COX-2) via an unstable intermediate product PGG_2. PGG_2 is then reduced to PGH_2 in the peroxidase active site. PGH_2 is rapidly

converted to prostaglandin D, E, F, and I series and to thromboxane A_2 via prostaglandin D, E, F, I synthases and thromboxane synthase (Smith *et al.*, 2000). (Figure 4)

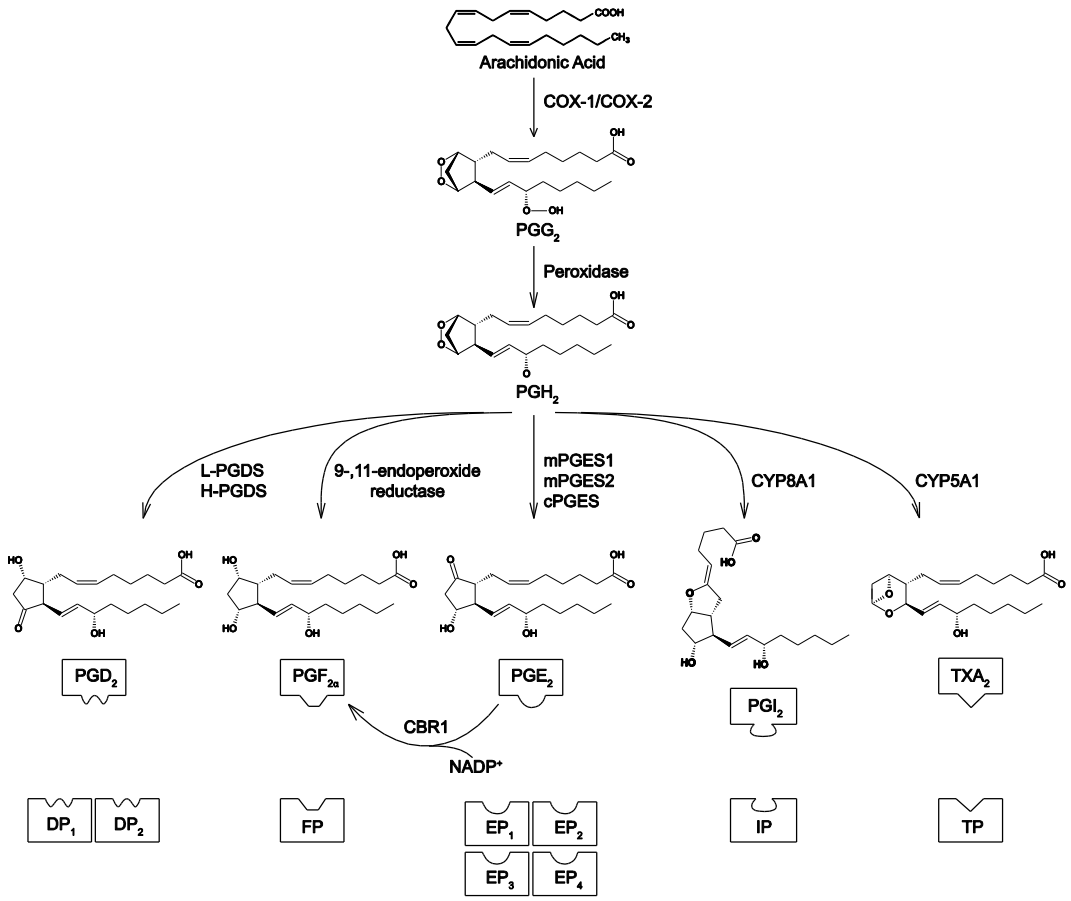

Figure 4. *Schematic overview of prostanoids synthesis in the cyclooxygenase pathway and the corresponding prostanoid receptors. Prostanoid synthases nomenclature is from the IUPHAR database (http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=243).*

1.3.2 Prostanoid Receptors

Based on the IUPHAR prostanoids receptors classification updated in year 2009, there are nine prostanoids receptors classified as DP_1, DP_2 (CRTH2), EP_1, EP_2, EP_3, EP_4, FP, IP, and TP preferentially activated by the major prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and TxA_2 (Woodward *et al.*, 2011) (Figure 4). Some receptors such as EP_3 have several splice variants. Moreover, ligand binding specificity studies have shown that prostaglandins have different binding affinities not only to the corresponding receptors but also to the other prostanoid receptor subtypes (Kiriyama *et al.*, 1997). Primary G protein coupling studies classified DP_1, EP_2, EP_4 and IP receptors as Gs-coupled receptors. EP_1, FP and TP receptors were shown to be Gq-coupled, while EP_3 receptors seemed capable of coupling both Gq and Gi proteins. In smooth muscle, activation of Gs-coupled receptors leads to the increase of intracellular cAMP levels, thereby activating protein kinase A (PKA) which could phosphorylate myosin light chain kinase resulting in smooth muscle relaxation. Activation of Gq-coupled EP_1, FP and TP

receptors could increase the level of second messenger intracellular Ca^{2+} concentration, leading to contractile responses. EP_3 receptors can couple Gi protein to decrease the cAMP level and couple Gq protein to increase the intracellular Ca^{2+} concentration. Activation of EP_3 receptors in the smooth muscle would thus induce a contractile response. Studies on DP_2 receptor signaling pathways are limited. DP_2 receptors are Gi-coupled which may decrease the intracellular cAMP level and cause Ca^{2+}mobilization (Woodward *et al.*, 2011).

1.3.3 Prostanoids and LUT

1.3.3.1 Production of prostanoids in the LUT

It is well established that prostanoids are produced locally within the bladder smooth muscle and mucosa in human and other species (Abrams *et al.*, 1979; Jeremy *et al.*, 1987; Andersson, 2009). Their production increases by various stimuli under pathological conditions such as bladder outlet obstruction (Park *et al.*, 1997b; Masick *et al.*, 2001), spinal cord injury induced bladder overactivity (Masunaga *et al.*, 2006) and inflammation (Saban *et al.*, 1994; Wheeler *et al.*, 2001; Wheeler *et al.*, 2002). The production of prostanoids also responds to physical stretch (Downie *et al.*, 1984; Jeremy *et al.*, 1984) and EFS of urinary bladders strips *in vitro* (Alkondon *et al.*, 1980). Although the prostanoid effects differ slightly between different species, prostaglandin E, F series and thromboxane usually contract bladder strips and increase the spontaneous activities during *in vitro* experiments (Abrams *et al.*, 1975; Andersson *et al.*, 1977; Ueda *et al.*, 1985; Palea *et al.*, 1998). In *in vivo* experiments, urodynamic tests showed an increased detrusor pressure and reduced bladder capacity after intravesical administration of PGE_2 (Schüssler, 1990; Ishizuka *et al.*, 1995; Schröder *et al.*, 2004). The mechanisms that, apart from inflammation, increase prostanoids production are unclear. They could be neuronal, hormonal or via a local reflex. .

In addition to release of the prostaglandins E and F series, some release studies also showed significant release of PGD_2 from pieces of normal rat urinary bladder (Kasakov *et al.*, 1985; Whigham *et al.*, 2002) and from guinea pig urinary bladder after ovalbumin sensitization or dietary fat supplementation (Saban *et al.*, 1994; Whigham *et al.*, 2002). Whether PGD_2 also plays a role in regulating bladder muscle contractility has not been reported. In this study, we not only measured the release and recovery of PGD_2 from the whole urinary bladder but also for the first time investigated PGD_2 effects in modulating guinea pig lower urinary tract motility (Paper II, III and IV).

1.3.3.2 Prostanoids bioactivity and receptors in the LUT

Expression of all EP_1 EP_2 EP_3 and EP_4 receptor subtypes have been shown in guinea pig urinary bladder (Ponglowhapan *et al.*, 2010; Rahnama'i *et al.*, 2012a). No data suggests expression of DP, FP and IP prostaglandins receptors (Rahnama'i *et al.*, 2012b). Other than expression investigations, there are some mouse knockout studies and pharmacological studies dealing with the function of different receptor subtypes in urinary bladders. One study using EP_1 knockout mice showed that, compared with wild type, EP_1 knockout mice did not respond to intravesical PGE_2 instillation (Schröder et

al., 2004). In normal rats, an EP_1 antagonist significantly increased the bladder capacity, voiding volume and micturition interval (Lee et al., 2007). Urodynamic tests of EP_3 knockout mice revealed an enhanced bladder capacity in the basal state and reduced hyperactivity response to application of PGE_2 (McCafferty et al., 2008). In rats, intravesical instillation of the EP_4 antagonist MF191 suppressed the bladder overactivity induced by PGE_2 but had no significant effects on control responses (Chuang et al., 2012). No data was found concerning the expression of DP receptors or the effect of PGD_2 and its receptors involved in the urinary bladder except one human data set that suggested PGD_2 to have a low potency to contract bladder strips (Palea et al., 1998). In the respiratory system, PGD_2 was found to be a potent mediator, exerting biological excitatory and inhibitory effects through DP_1, DP_2 receptors, and was also able to activate TP receptors (Larsson et al., 2011). Expression of DP receptors in guinea pig esophageal nodose ganglia was reported by Zhang and colleagues by use of immunohistochemistry, Western blotting and RT-PCR (Zhang *et al.*, 2013).

Effects of PGE_2 on bladder trigone and proximal urethra have been studied for decades. Andersson and colleagues showed that PGE:s relaxed pre-contracted human circularly cut urethral rings (Andersson *et al.*, 1977). PGE_2 was shown to relax the pre-contracted trigone and longitudinally cut urethra in the human and pig (Klarskov *et al.*, 1983a). Similar results were also shown in other species, that PGE_2 relaxed the pre-contraction of circularly cut hamster and longitudinally cut dog urethra (Mutoh *et al.*, 1983; Pinna *et al.*, 1998). In one study of cat urethra, PGE_2 contracted the longitudinal urethra strips but relaxed the circular urethral muscle (Abdel-Hakim *et al.*, 1983). In animal experiments using rabbit and dog trigone, PGE_2 contracted the tissue and increased spontaneous activities (Mutoh *et al.*, 1983; Gotoh *et al.*, 1986). So far, there is no data available on the effect of PGD_2 in trigone and proximal urethra. Whether PGD_2 is involved in the regulation of trigone and urethra motility and if there is expression of DP_1 and DP_2 receptors in these regions have until now been open questions.

1.3.3.3 PGD_2 in other systems

PGD_2 has a variety of biological roles via CRTH2 (DP_2), DP_1 and TP receptors. Effects of Gi-coupled CRTH2 receptor activation include recruitment of Th2 cells (Honda *et al.*, 2003; Xue *et al.*, 2005) and eosinophils (Woodward *et al.*, 1990; Hirai *et al.*, 2001; Monneret *et al.*, 2001) resulting in production of cytokines . Gs-coupled DP_1 receptor activation induces inhibition of platelet aggregation (Whittle *et al.*, 1983; Trist *et al.*, 1989), bronchodilatation (Matsuoka *et al.*, 2000) , vasodilatation (vary according to species) (Giles *et al.*, 1989), tocolysis (Senior *et al.*, 1992; Fernandes *et al.*, 1995), and is implicated in central regulation of sleep (Urade *et al.*, 1999) and in pain (Eguchi *et al.*, 1999). Suppression of cytokine production, via DP_1 activation in inflammation and immune responses, occurs in certain leukocytes and in dendritic cells (Hammad *et al.*, 2003). The effects of DP_1 activation in inflammation and immune responses are usually accompanied by DP_2 activation in an opposing fashion. This could switch PGD_2 from a pro- to an anti-inflammatory mediator. PGD_2 can also bind to the Gq-coupled TP receptor. Activation of TP receptors results in platelet aggregation, bronchoconstriction

and vasoconstriction (Pettipher *et al.*, 2007), and PGD_2 can either by itself or via its metabolites exert TP-mediated airway constriction (Coleman *et al.*, 1989).

PGD_2 is biosynthesized in brain, platelets, mast cells and some neoplastic cells, and is rapidly metabolized by 15-hydroxy prostaglandin dehydrogenase (15-hydroxy PGDH) and 11-ketoreductase (AKR1C3) to 13,14-dihydro-15-keto PGD_2 (DK-PGD_2) and 9α, 11β-PGF_2. Alternatively, through non-enzymatic means, PGD_2 is converted to J-ring products PGJ_2, Δ12-PGJ_2, 15-deoxy-Δ12,14-PGJ_2 (15d-PGJ_2) or the D-ring products Δ12-PGD_2, 15-deoxy-Δ12,14-PGD_2 (15d-PGD_2). Conversion of Δ12-PGD_2 and Δ12-PGJ_2 from PGD_2 and PGJ_2 are albumin-catalyzed isomerization processes (Fitzpatrick *et al.*, 1983; Shibata *et al.*, 2002). (Figure 5) Although a micromolar range of ligand concentrations was used to generate intracellular effects, some of these metabolites might be endogenous agonists on CRTH2 and DP_1 receptors (Pettipher *et al.*, 2007). The influence of PGD_2 metabolites in the LUT under physiological and pathophysiological conditions is unknown.

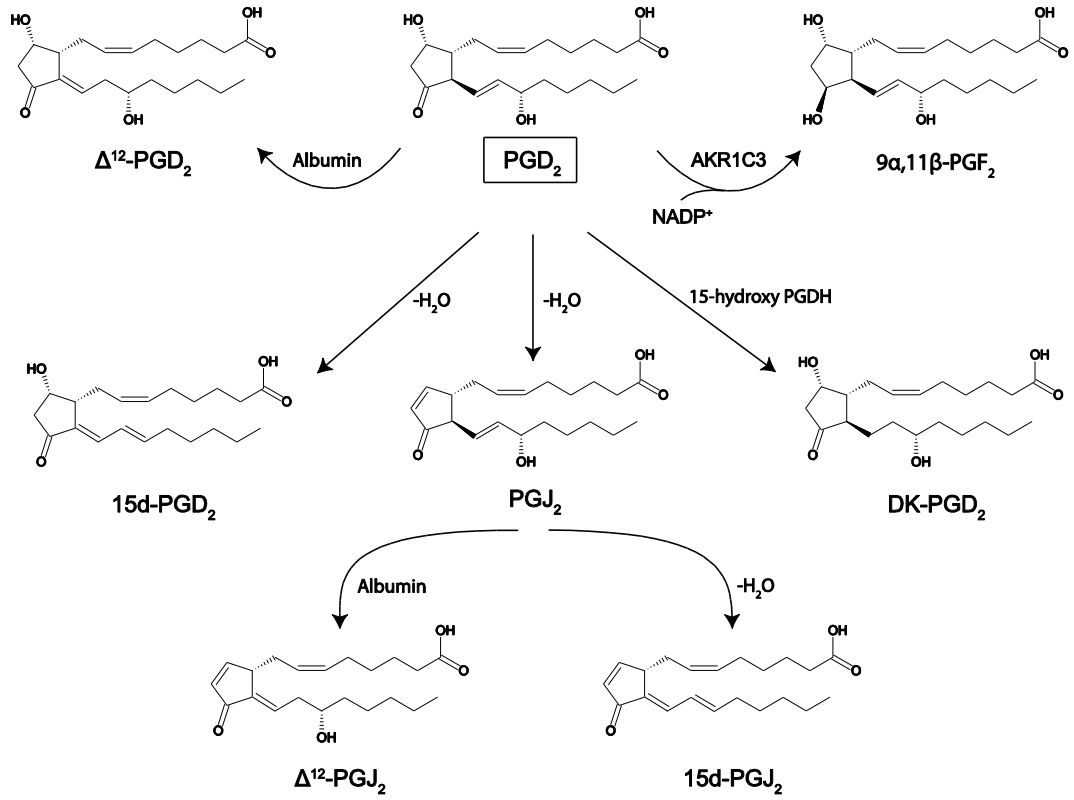

Figure 5. *Schematic overview of PGD_2 metabolism. PGD_2 spontaneous by dehydrates to PGJ_2, which can undergo a second dehydration to generate 15d-PGJ_2. 15d-PGD_2, 15d-PGJ_2 and Δ12-PGD_2 and Δ12-PGJ_2 are agonists for CRTH2 receptor (Monneret et al., 2002; Heinemann et al., 2003; Gazi et al., 2005). 15d-PGJ_2 is the most studied PGD_2 metabolite, and can bind to both DP_1 and DP_2 receptors. The main target of 15d-PGJ_2 is peroxisome proliferator-activated receptor-γ (PPAR-γ)(Bell-Parikh et al., 2003). Activation of PPAR-γ in turn mediates anti-inflammatory effects. 9α,11β-PGF_2 might be an agonist of the Gq-coupled TP receptor, activation of which leads to airway contraction (Beasley et al., 1987). 9α,11β-PGF_2 can also activate CRTH2 receptors (Sandig et al., 2006).*

2 HYPOTHESIS AND AIMS

The general project aims at gaining a further understanding of the Overactive Bladder Syndrome. The main hypothesis was that, if proven to exist, the urothelium-derived inhibitory factor (UDIF) would be of importance in normal bladder function and if chemically identified could be shown to be disturbed in overactive bladder. The main aims were:

1. To investigate whether guinea pig urothelium-derived inhibitory factor could be demonstrated by in vitro serial bioassay and to attempt its chemical characterization.

2. To analyze whether adenosine/purinergic P1 receptor agonists, nitric oxide or prostaglandins might explain any observed activity.

During chemical analysis of the observed UDIF activity we found considerable amounts of prostanoids released from guinea pig urinary bladder, with a PGD_2-like component. As a consequence of obtained results the following aims were formulated:

3. To identify and quantify the release of prostaglandins under physiological conditions and to investigate the activity of PGD_2 in modulation of bladder motility.

4. To study receptors involved in PGD_2 action on the bladder and their distribution.

5. To investigate whether PGD_2 might affect the bladder outflow region.

3 METHODS

3.1 TISSUES PREPARATION

Albino guinea pigs of either sex weighing 300 to 800 g were anaesthetized with midazolam 1 mg kg^{-1} + sodium pentobarbital 120 mg kg^{-1} and exsanguinated.

PAPER I: The kidneys, ureters and urinary bladders of guinea pigs were removed en bloc. The proximal ureter about 2 cm long with part of the renal pelvis was removed. The pelvic-ureteral preparations were cut open longitudinally, and in some preparations the urothelium was removed by scraping with a blunt instrument. Guinea pig bladders were kept intact and everted. In some experiments the bladder urothelium/suburothelium, which accounts for approximately one third of the thickness of the bladder wall, was removed as far as possible by cutting with scissors. Removal of the urothelium was after experiments confirmed by NADPH-diaphorase staining.

PAPER II: The abdominal aorta was perfused with warm saline in order to achieve blood-free tissue preparations. Then the urinary bladder was removed and cleaned from fat and connective tissue. Two kinds of preparations were made. For the release experiments, the whole bladder was spirally cut into a 2-3 cm long strip. For bioassay and pharmacological studies, one single bladder dome was cut into several 8 X 2 mm strips. In some experiments, the bladder urothelium was removed as much as possible by fine dissection with iris scissors and forceps under the microscope. Differential assay was performed also on guinea pig colon longitudinal strips prepared as previously described (Olgart *et al.*, 1998).

PAPER III: The urinary bladder was separated from the urethra and ureters. Detrusor from bladder dome was cut into several strips. Urothelium was either intact or removed as described before.

PAPER IV: Male guinea pig urinary bladder and proximal urethra were taken en bloc. The bladder trigone was dissected by locating the urethra and ureter openings. A trigone strip about 7 X 2 mm was made from each guinea pig with the urothelium intact. The proximal urethra was cut into several rings with urothelium intact. Urethral rings were then cut open into strips for organ bath experiments.

After dissection, tissues were placed in a storage bath for equilibration for 30-60 min in Tyrode′s solution (136.9 mM NaCl, 4.8 mM KCl, 23.8 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 5.5 mM glucose) and aerated with 5% CO_2 in O_2 at 37℃.

3.2 CASCADE SUPERFUSION

PAPER I. Three water-jacketed and thermostatted chambers were mounted in series. The top chamber was preceded by a warming coil through which was pumped aerated

(5% CO_2 in O_2) Tyrode′s solution at 1.5 mL per min by means of a Gilson peristaltic pump. The fluid was led onto the tissues via the suspending cotton ligature. In the upper chamber the donor tissue, a whole guinea pig bladder, was connected to an isometric transducer (FT03, Grass Instruments, Quincy, MA). The initial tension of the bladder was adjusted to 20 mN. An assay ureter with one end connected to Harvard isotonic transducers (Harvard Apparatus, Holliston, MA) was mounted in each of the two lower chambers. The distance between the donor tissue and the assay tissue in two chambers was 20 cm. The time required for flow to travel between donor and assay tissues was approximately 3 s (Figure 6).
Carbachol was introduced either by injection onto tissues directly or infusion into the supplying flow just before the donor chamber. By infusion at the bottom of the donor chamber, compounds such as scopolamine could be directly infused onto assay tissues, thus bypassing the donor tissue. Blocking agents (L-NAME, 8-PST or diclofenac) were added into the superfusion reservoir separately and exposed to the tissues for at least 30 min before carbachol application.

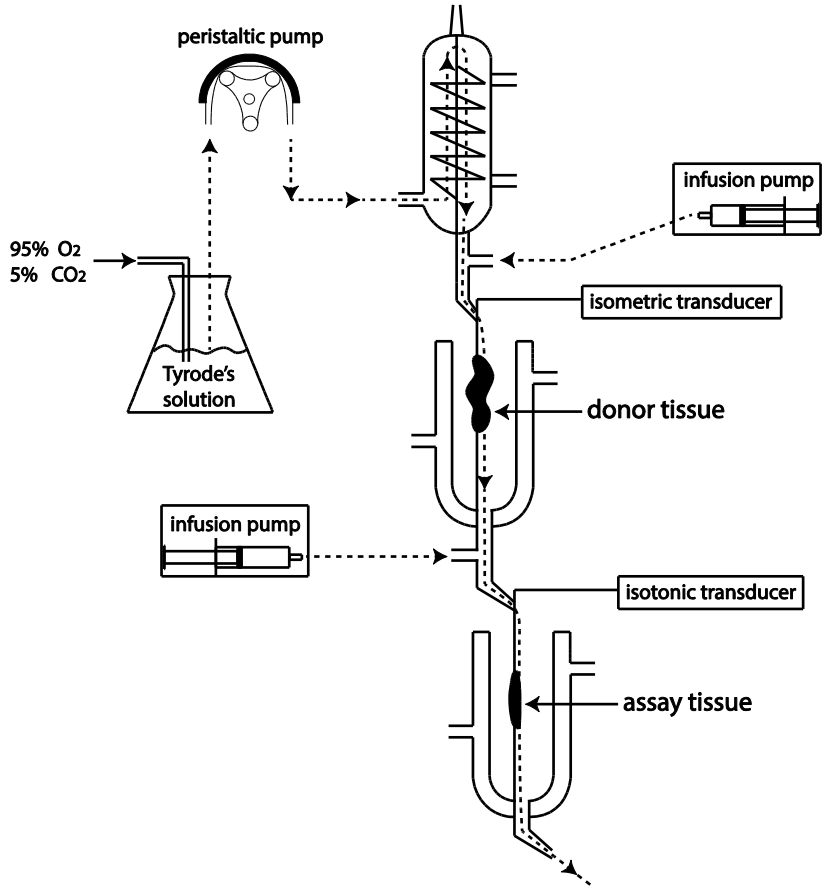

Figure 6. *Schematic drawing of the employed cascade serial superfusion, based on the Vane technique.(Supporting Figure S1, Paper I; modified from(Gryglewski et al., 1986).*

3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

PAPER II. Superfusate of the urinary bladder was for 66 min collected after the donor chamber onto 35cc Sep-Pak tC18 cartridges (Waters) or directly collected as 15 mL

fractions in ice-cold test tubes with acetic acid (2% final concentration). Superfusate samples were analyzed by two steps of reversed phase HPLC, first using a C18 column (150 x 8 mm, particle size 5 μm; ReproSil-Pur C18-AQ, Dr Maisch GmbH, Ammerbuch-Entringen, Germany). The gradient profile was from water to 50% methanol in 10 min, 50 to 80% methanol in 60 min and 80 to 100% methanol in 10 min with 1% acetic acid throughout. The flow rate was 1.6 mL min^{-1}. Column eluate was monitored at 265 nm and 287 nm by two detectors and a scanning diode array UV absorbance detector (Waters 991, scanning the range 190-500 nm at 5 s intervals) in series. Eluate from the HPLC was collected as 1.5 min fractions in polyethylene test tubes. Fractions were dried by overnight by vacuum centrifugation and then analysed further in a second step of HPLC. The second purification step was by isocratic elution in reversed phase HPLC. The stationary phase was a C18 column (150 X 2 mm, particle size 5 μm; Kromasil 100 C18, Dr Maisch GmbH, Ammerbuch-Entringen, Germany). The mobile phase was 36% acetonitrile and 1% acetic acid at a flow rate of 0.45 mL min^{-1}. UV absorbance was monitored at 290 nm by a fixed-wavelength detector and at a 190-500 nm by the scanning UV absorbance detector (Waters 991). Eluate from this HPLC was collected in 0.5 min fractions in polyethylene test tubes. The fractions were freeze-dried overnight and then immediately bioassayed on bladder detrusor smooth muscle preparations. Standard compounds were analyzed under the same conditions in the HPLC system for comparison of retention times and calculations of recovery.

3.4 MEASUREMENTS OF NO/NO_2^- BY CHEMILUMINESCENCE

PAPER I. The superfusate of the guinea pig urinary bladder tissue was collected during periods of 1 min before and during ACh stimulation. The NO-synthase substrate L-arginine (10^{-5} M) was added to the Tyrode′s solution, in order to maintain the synthesis of NO during collection. Aliquots of 1 mL were injected into a reaction glass chamber, containing 100 mL to 150 mL of 1% sodium iodide in deoxygenated and concentrated hot (89°C) acetic acid. In the acidic and reducing milieu, NO_2^- is reduced to NO (Walters *et al.*, 1987) which is by a stream of N_2 carried into a reaction chamber. In the reaction chamber NO together with ozone reacts under vacuum giving rise to photons, the amount of which is counted by a photo- multiplier (Palmer *et al.*, 1987; Walters *et al.*, 1987). The detection limit was 0.5-2 pmol $NaNO_2$ per mL. The system was calibrated by injecting freshly made aliquots of $NaNO_2$ solution and using peak heights for construction of standard curves for calculation of unknown samples (Wiklund *et al.*, 1993; Hallén *et al.*, 2007).

3.5 ORGAN BATH EXPERIMENTS

PAPER II. Bladder strips were mounted vertically in 3.5 mL organ baths with one end fixed to a hook at the bottom of the bath and the top end connected to an isometric transducer. The initial tension was adjusted to 10 mN. Electrical field stimulation (EFS) was applied to the preparations by means of two platinum electrodes on the walls of the organ baths (50 V, single pulse of 0.2 ms every 30 s). The evoked contractile responses were recorded with a computerised acquisition system (MP100, Biopac). When stable

contractile responses developed, diclofenac 10^{-6} M was given to the tissue. After 1 h equilibration, PGE_2, PGD_2 and the freeze-dried fractions from the HPLC were applied and assayed onto the bladder strips in organ baths.

PAPER III. Urothelium denuded bladder strips of either gender were transferred to 2.5 to 6.5 mL organ baths to pharmacologically study the effects of PGD_2 and its receptors, using agonists and antagonists on isolated bladder strips. Tissues were stimulated electrically and pretreated with diclofenac 10^{-6} M as in Paper II. When stable contractile amplitudes developed, PGD_2 and the selective DP_1 agonist BW-245C were applied to the tissue. When investigating the effects of prostaglandin receptor DP_1 and TP antagonists, tissues were exposed to these antagonists alone or combined for at least one hour due to high lipophilicity/slow onset properties of prostaglandin receptor blockers used in this study (Jones *et al.*, 2011). Agonist dose-response curves were then carried out in the presence of different concentrations of receptor antagonists.

PAPER IV. Male urothelium intact trigone and proximal urethra rings, prepared into strips, were mounted in the organ baths. The initial tension of the trigone and urethra strips was adjusted to 5 mN. When stable tension developed, EFS (50 V, 15 pulses of 0.2 ms every 60 s) was applied to trigone strips. Proximal urethra ring strips were after initial tests left unstimulated to record the spontaneous contractions. Upon stabilization, PGE_2 and PGD_2 were applied cumulatively to the tissues in half-log increments.

The corresponding solvent used to dissolve agonists and antagonists was given to tissues in the same way without compounds dissolved in it. The final concentration of solvent used in each organ bath was less than 0.1% ethanol.

3.6 WESTERN BLOT

PAPER III. Guinea pigs were anesthetized and the descending aorta was perfused with 30-40 mL warm saline after which the bladder dome was isolated as described above. Separate tissue samples of either intact wall, urothelium with suburothelium, or muscle were prepared. For protein extraction, each mg wet tissue was subjected to 20μL of lysis buffer with protease inhibitors followed by homogenization. 50 μg of proteins was loaded into each well of 8-16% SDS gels followed by electrophoresis. Proteins were transferred onto PVDF membranes subsequently probed 1 h at room temperature with a anti-human hematopoetic prostaglandin D synthase antibody, a anti-human DP_1 receptor antibody or a mouse IgG1 anti-human β-actin antibody. HRP-conjugated secondary antibodies and a chemiluminescent substrate were used to detect protein signal on autoradiographs (Kodak X-Omat).

PAPER IV. Male guinea pigs were anesthetized as above and the bladder trigone, neck and proximal urethra were dissected and isolated apart. Protein extraction was prepared as above. 7 μg of protein was loaded onto 8-16% SDS gels and separated by electrophoresis. Membranes were probed overnight with rabbit anti-human DP_1 receptor antibody or rabbit anti-human DP_2 (CRTH2) receptor antibody. For protein signal detection, HRP-conjugated secondary antibodies were used as above.

3.7 MORPHOLOGICAL STUDY

PAPER I. NADPH-diaphorase staining.
To confirm successful removal of urothelium from ureters and bladders, the whole preparation of urothelium-intact and -denuded ureters and bladders were incubated at pH 8 in Tris-HCl buffer solution containing 1 mM β-NADPH, 0.5 mM nitroblue tetrazolium and 0.2% Triton X-100 at 37°C for 10 min (Persson *et al.*, 1999). Tissues were then washed in saline and directly placed onto slides for microscopic observation.

PAPER III. Guinea pigs were anesthetized and blood-free tissues obtained as above. The bladder dome tissues were fixed for 4 h at 4°C in ice-cold 4% paraformaldehyde 0.1 M phosphate buffer. After fixation, tissues were cryoprotected with sucrose solution for 16 to 20 h and then quickly frozen in cooled isopentane and stored at -80°C. 10 µm transverse bladder dome sections were mounted on coated slides and labeled with an anti-human hematopoetic prostaglandin D synthase antibody or a polyclonal antibody raised against human DP_1 receptor C-terminal. To visualize the basal membrane, sections were incubated with an anti-laminin antibody. The sequential control sections were treated with blocking buffer without primary antibody. Sections were washed followed by application of secondary antibody labeled with ALEXA Fluor 568. Fluorescein isothiocyanate-labelled phalloidin or a Cy3 conjugated anti-α-smooth muscle actin antibody were applied followed by secondary antibody and nuclei counterstaining. The sections were then mounted with mounting medium and covered with cover slips.

PAPER IV. Blood-free male guinea pig urinary bladder trigone and proximal urethra were prepared, fixed and sectioned as in Paper III. Sections were either labelled with antibody raised against human DP_1 receptor C-terminal or labelled with antibody raised against human DP_2 (CRTH2) receptor. Counterstaining of smooth muscle actin, F-actin and nuclei was by anti-α-smooth muscle actin antibody, phalloidin and Hoechst33258 respectively. Secondary antibodies were applied, followed by mounting and fluorescence immunohistochemistry.

All immunolabeled sections in Paper III and IV were observed under an Axioplan 2 imaging fluorescence microscope (Carl Zeiss) and photographed with a Nikon D3000 digital camera.

3.8 STATISTICAL ANALYSIS

All the data were presented as means ± standard error of the mean (SEM). Statistical significance was analyzed by Student's paired or unpaired *t*-test, or by one-way analyses of variances (ANOVA). A p-value less than 0.05 was considered significant. Agonist and antagonist characteristics such as EC50, IC50, maximal effect values and pA_2 values were calculated by Prism software (GraphPad Software Inc).

4 RESULTS AND DISCUSSION

4.1 UROTHELIUM DERIVED INHIBITORY ACTIVITY (PAPER I, II).

Urothelium being one of the key elements throughout the whole project, its presence or absence matters. To confirm the removal of urothelium from guinea pig ureters and bladders, NADPH-diaphorase staining followed by imaging in reflective light was carried out. Several staining techniques were investigated but yielded poor or no staining of the urothelium whereas the NADPH diaphorase reaction exhibited prominent staining of the urothelium (Figure 7). The difference between urothelium intact and urothelium denuded areas was clearly visible, allowing confirmation of successful urothelium removal in urothelium-denuded bladders and ureters.

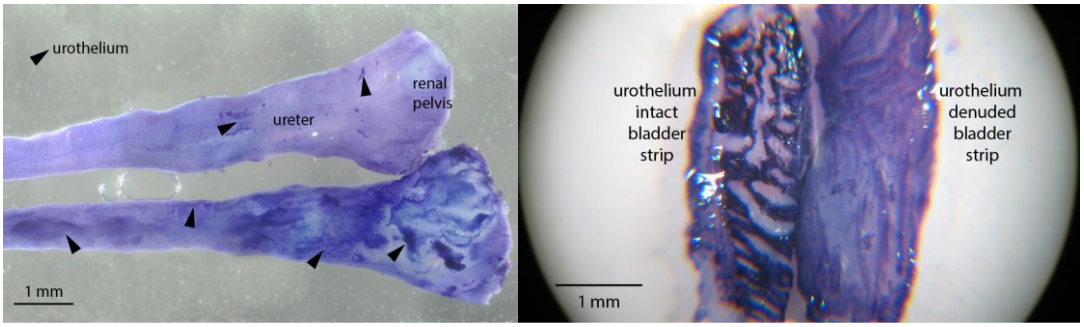

Figure 7. *NADPH-diaphorase staining of guinea pig ureters after a cascade superfusion experiment and of strips from intact and denuded bladder. Left image: ureters were opened longitudinally with their originally internal side facing towards the viewer. Top tissue was denuded from urothelium as much as possible. Urothelium was stained dark blue by the diaphorase reaction (bottom tissue, and some small specks in top tissue). Non-urothelium tissue exhibited light purple staining (top tissue). Some urothelium fell off from urothelium-intact tissue in the renal pelvis part (lower tissue), but the majority of urothelium was still present on the surface. Right image: two strips dissected from the same bladder with the internal side facing towards the viewer, one strip with urothelium intact, one removed of urothelium. Strips were exposed to the same diaphorase reaction as ureters and a clear difference was seen between the strips. When immersing the urothelium-intact bladder in the dye, the urothelium was folding, like when the bladder is empty, causing poor penetration of the dye into foldings (white streaks between dark blue). (Figure 6, Paper I)*

Urothelium-derived inhibitory factor (PAPER I)

Guinea pig ureters exhibited regular spontaneous contractions when superfused by Tyrode´s solution at 1.5 mL min^{-1} (Figure 8, lower panel). The spontaneous activities were phasic contractions at a rate of 0.5-1.5 beats min^{-1} and were relatively stable over 1 to 2 h and then declined in both frequency and amplitude, which is a phenomenon more prominent in urothelium-intact ureters. Urothelium-derived inhibition of ureter spontaneous contractions was observed by using the cascade serial superfusion system (Figure 6). Guinea pig whole urinary bladder was mounted in the donor tissue bath, urothelium-denuded ureters were the assay tissues. After

applications of carbachol $5x10^{-6}$ M 0.5 mL directly to scopolamine-treated urothelium-denuded ureters, either no effects or only excitatory effect was seen, whereas the same amount of carbachol injected over the urothelium intact bladder, subsequently reaching the ureter, showed significant inhibition of assay ureter contractions, sometimes preceded by an initial excitation (Figure 8). The inhibitory effect was reproducible by repeated injections of carbachol. The time course of inhibitory effects was somewhat slow.

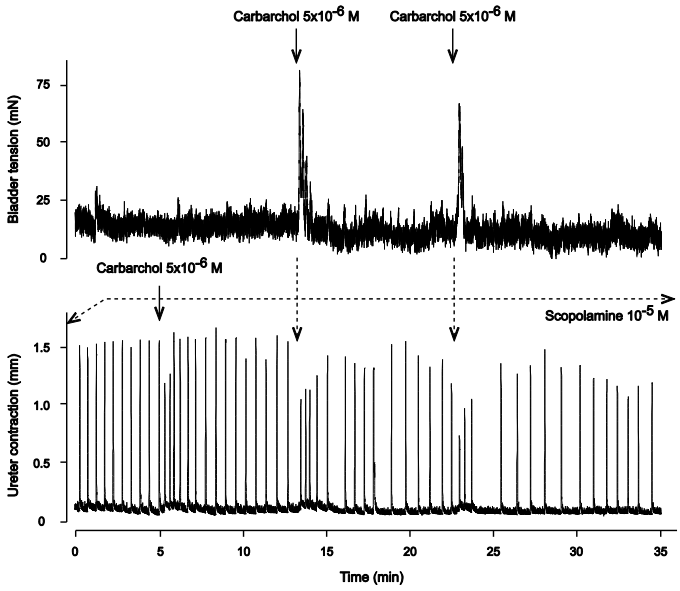

Figure 8.*Experimental recording of an everted urothelium-intact bladder (upper tracing) and an urothelium-denuded ureter (lower tracing) in the cascade superfusion mode. (Figure 1, Paper I)*

In order to investigate whether the observed transmissible inhibitory activity was emanating from the urothelium, the same experiment was performed but using an urothelium-denuded bladder as donor tissue. No inhibition of assay ureter contractions was seen indicating the source of inhibitory activity to be the urothelium. Adenosine and nitric oxide exert inhibitory actions on smooth muscle and are well known inhibitors in the urinary tract (Canda *et al.*, 2007). Also prostaglandins can inhibit the peristalsis of ureters and may also be very important in maintaining spontaneous activity of the ureter (Davidson *et al.*, 2000). We investigated if the transmissible inhibitory activity was due to one of these well-known compounds. Nitric oxide synthase inhibitor (L-NAME), adenosine receptor antagonist (8-PST) or cyclo-oxygenase inhibitor (diclofenac) were added separately into the superfusion reservoir. In the presence of blocker, the same superfusion experiments were repeated as described before. The treatments had a tendency of slightly lowering the spontaneous contractions of the ureters, but no modification of the transmissible inhibitory activity was observed (Figure 9).

Based on study II, we know that the bladder was able to synthesize PGE_2 and PGD_2, and that PGD_2 could inhibit the bladder. However, diclofenac removed the release of prostaglandins from the bladder (Paper II), showing that diclofenac was effective in this respect. Thus, it is unlikely that the unknown inhibitory factor derived from urothelium is PGD_2 or PGE_2.

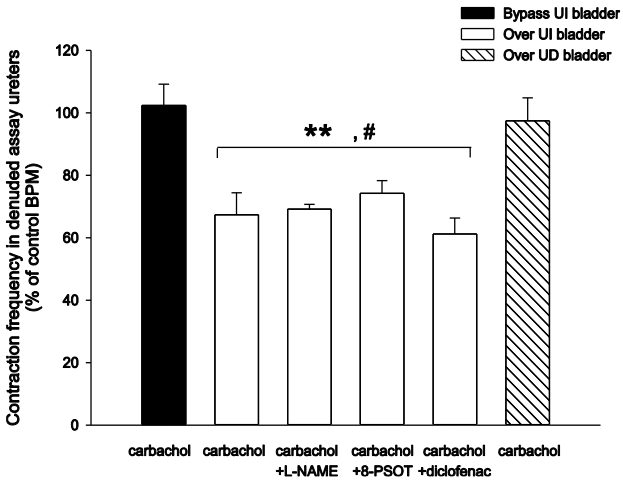

Figure 9. *Effect of transmissible urothelium derived activity on ureter contractions in different treatment groups (n=8). ** denotes p<0.01 after unpaired t-test for the comparison between bypass urothelium intact (UI) bladder group with over UI bladder groups. # denotes no significant difference between antagonist/inhibitor treatments. Ureters in carbachol bypass UI bladder group exhibited similar effect as carbachol perfusing over urothelium denuded (UD) bladder group (P>0.62). (Figure 4, Paper I)*

Prostaglandin actions were blunted when urothelium was intact (PAPER II)
In the organ bath, exogenous PGE_2 and PGD_2 effects on the nerve stimulated guinea pig bladder strip contractions were compared under different conditions. In the presence of urothelium, PGE_2 and PGD_2 were less effective in modulating bladder motility compared with in the absence of urothelium. Removal of urothelium and application of cyclo-oxygenase inhibitor to a similar degree modified PGE_2 and PGD_2 effects (Figure 10), these being larger when the endogenous prostanoids production was abolished. This is in similarity with exogenous adenosine which is more efficacious when endogenous adenosine has been blocked (Nilsson *et al.*, 2010).

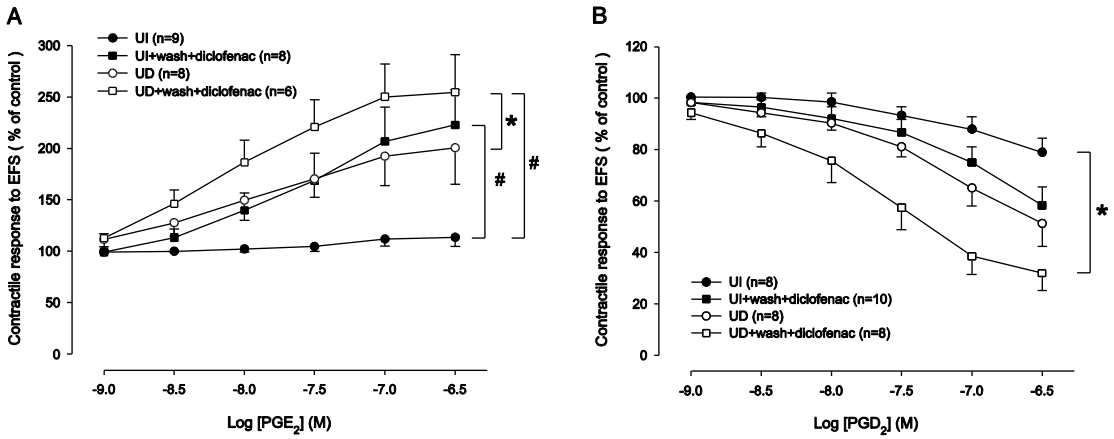

Figure 10. *Contractile responses to EFS in guinea pig urinary bladder detrusor strips. Dose-response curves of (A) PGE_2 enhancing effect and (B) PGD_2 inhibitory effect on the contractile response. Data from 6 to 10 strips from at least 4 animals, and presented as mean±standard error mean (SEM). # denotes p<0.005 and * denotes p<0.05 for comparison of maximal response. (Figure 6A and C, Paper II)*

4.2 QUALIFICATION OF MEDIATOR RELEASE (PAPER I, II).

Bladders are able to generate and release many bioactive compounds (Birder *et al.*, 1998; Knight *et al.*, 2002; Tanaka *et al.*, 2011). The cascade superfusion results in Paper I showed that there was an inhibitory factor(s) released from guinea pig

urothelium which was unlikely to be one of the known compounds, such as NO. In order to confirm that L-NAME was sufficient to block the NO generation, measurements of NO/nitrite release before and after L-NAME were carried out. The results showed that $3x10^{-4}$ M L-NAME was able to reduce the NO production by around 80%, rendering NO unlikely to be the released inhibitory factor. (Figure 5, Paper II)

During our analysis of the urothelium-derived inhibitory factor, we found significant amounts of bioactivity being released from bladder urothelium, and when the HPLC fractions initially were bioassayed an excitatory activity was observed. We therefore subjected excitatory fractions from the gradient elutions to isocratic separation (Kunz *et al.*, 2002) as shown in figure 11B. Subsequent bioassay on isolated urothelium-denuded bladder tissues revealed an excitatory activity corresponding to the retention time of authentic PGE_2 and an inhibitory activity corresponding to the retention time of PGD_2.

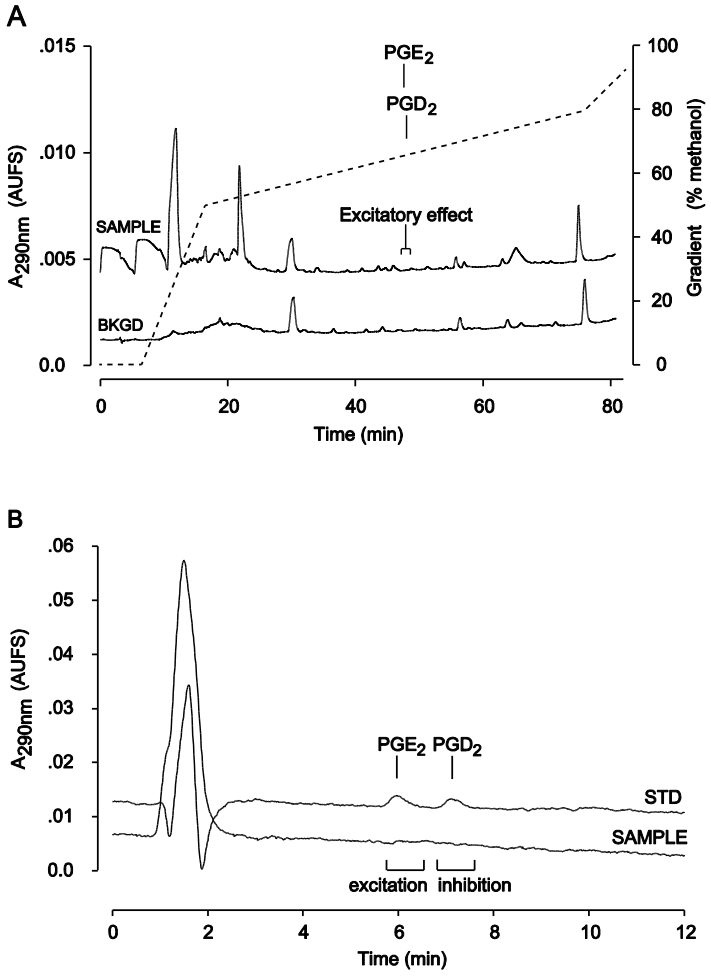

Figure 11. *Isolation and purification of excitatory and inhibitory prostaglandin-like bioactivity from guinea pig urinary bladder. (Figure 1, Paper II)*

(A) Gradient HPLC chromatograms of guinea pig urinary bladder superfusate collected from an urothelium intact urinary bladder (SAMPLE) and elution profile of a run with a sham tissue (BKGD).

(B) Purification and isolation by isocratic reversed phase chromatography of excitatory activity fractions obtained at 48 min from gradient HPLC as in panel A (SAMPLE) and compared with standard PGE_2 and PGD_2 (STD).

Removal of the urothelium abolished more than 90% of PGE_2 release, and essentially all of PGD_2 in the sense that it now was undetectable with our method. The data indicated that the urothelium was the main source of PGE_2 and PGD_2. One hour of diclofenac 10^{-7} M pretreatment abolished more than 68% of PGE_2 and 94% of PGD_2 release. This validates that the released bioactivities are likely to be PGE_2 and PGD_2,

and also that the lack of effect by diclofenac on released inhibitory activity in Paper I is a true negative result.

4.3 EFFECTS OF PGD_2 AND PGE_2 IN THE LUT (PAPER II, IV).

Based on the HPLC analysis, PGE_2 and PGD_2 were constantly produced in the urinary bladder. The effects of PGE_2 and PGD_2 in modulating the lower urinary tract (the bladder dome, trigone and proximal urethra) motilities were therefore investigated. Results are summarized in figure 12, panel A. Dose-response curves are shown in figure 12, panel B to D.

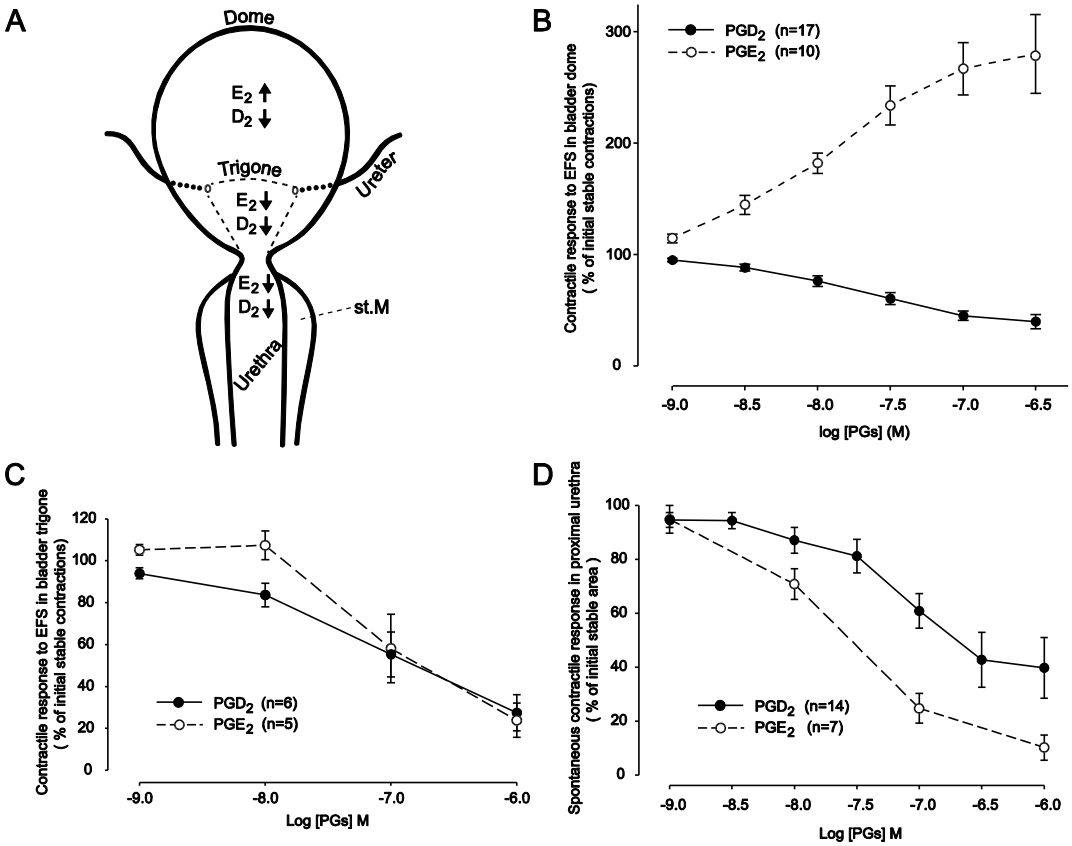

Figure 12. *Summary (panel A) and dose-response curves of PGD_2 and PGE_2 effects (panel B to D) in guinea pig LUT. Panel B: bladder dome, panel C: bladder trigone, panel D: proximal urethra. Bladder dome and trigone tissues were electrically stimulated whereas spontaneous contractions of proximal urethra were recorded. PGD_2 and PGE_2 were applied cumulatively in log or half-log increments. Data were presented as mean±standard error mean (SEM), n denotes number of tissue strips in panel B and number of animals in panel C and D. st.M denotes striated muscle in the urethra.(parts of Figure 6, Paper II; Figure 2 and 3, Paper IV)*

Effects of PGD_2 and PGE_2 on acetylcholine and ATP induced contractions were also investigated (Paper II). In diclofenac pretreated (10^{-6} M) urothelium-denuded bladder strips with EFS turned off, acetylcholine ($3x10^{-6}$ M) and ATP (10^{-5} M) caused contractions which were reproducible when repeated. Application of PGE_2 (10^{-8} M) increased the tone and also enhanced the contractile response to exogenous ACh and

ATP (Figure 13 panel A and C). When PGD_2 (10^{-8} M) was similarly applied, responses to exogenous ACh and ATP were significantly inhibited (Figure 13 panel B and D).

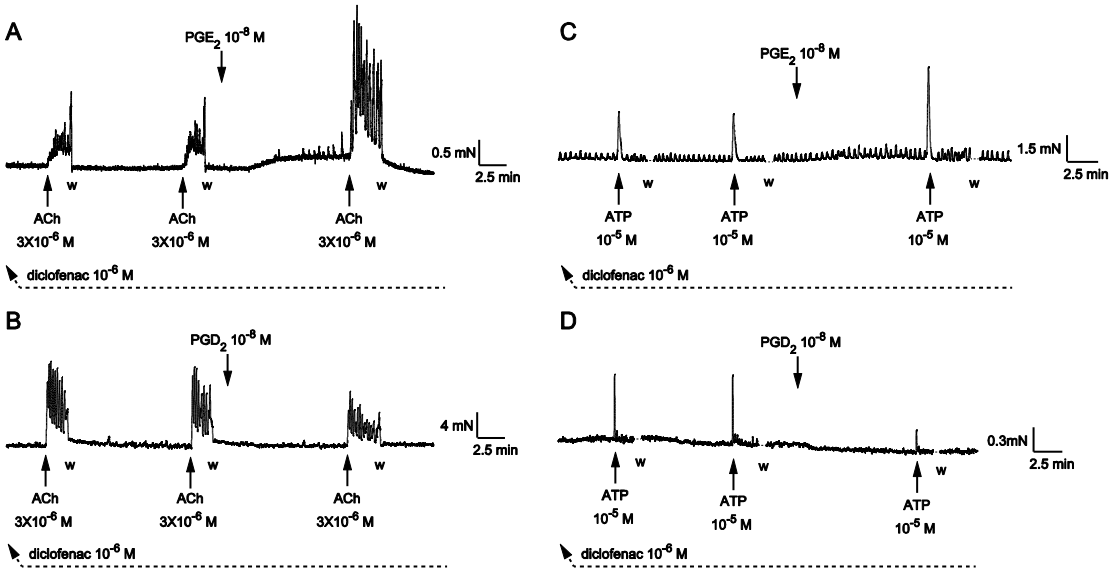

Figure 13. *Urothelium denuded guinea pig urinary bladder detrusor strips monitored by isometric recording of muscle responses in the absence of EFS. Original experimental recordings of enhancing PGE_2 (panel A and C) and inhibitory PGD_2 (panel B and D) modulation of contractile responses induced by repeated applications of exogenous acetylcholine (ACh) or ATP, with washings indicated by w. Tissues were pretreated with diclofenac 10^{-6} M, repeated after each wash. (Figure 7, Paper II)*

Presently, PGD_2 in addition to its inhibitory effect on contractile responses to nerve stimulation also inhibited the responses to the neurotransmitters acetylcholine and ATP. It is reasonable to conclude that PGD_2 mainly acts by a postjunctional inhibitory effect. Whether this involves changes in membrane potential or changes in second messengers or both remains to be studied and additional prejunctional effects by PGE_2 and PGD_2 can not be excluded from the present results.

4.4 RECEPTORS INVOLVED IN PGD_2 MODULATION (PAPER III).

In guinea pig urinary bladder detrusor, the DP_1 receptor antagonist BW-A868C caused a progressive dose-dependent reversal of the PGD_2 inhibitory effect on the contractile responses to EFS, where first the higher concentrations of PGD_2 (during BW-A868C 10^{-8} M) and then all concentrations of PGD_2 (during BW-A868C 10^{-6} M) now evoked an enhancing effect on the contractile responses. The enhancement by PGD_2, after blocking DP_1 receptors with BW-A868C, was antagonized by the TP receptor antagonist SQ-29548 10^{-7} M which alone did not modify the effect of PGD_2 (Figure 14).

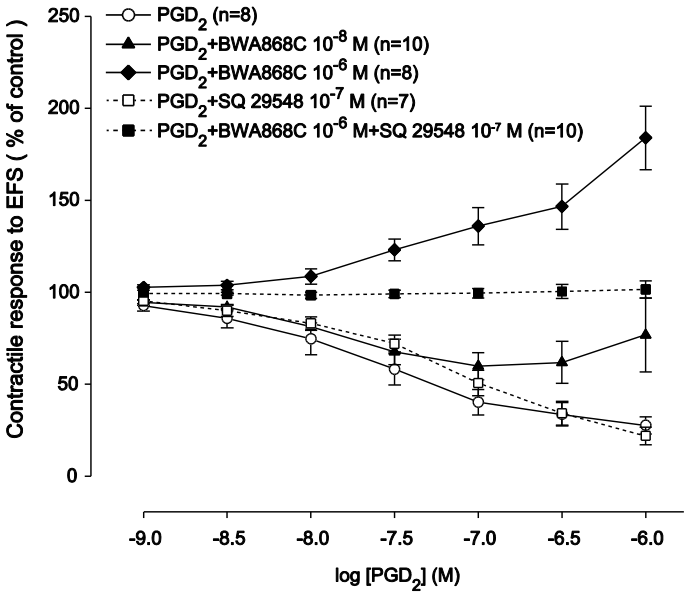

Figure 14. *Cumulative dose-response curves to PGD_2, in the absence and presence of the DP_1 antagonist BW-A868C and/or the TP antagonist SQ-29548, on contractile responses in urothelium-denuded guinea pig urinary bladder detrusor strips evoked by electrical field stimulation (50 V, single pulses of 0.2 ms every 30 s). Data presented as mean±standard error of the mean (SEM), n denotes number of tissues. (Figure 2, Paper III)*

In summary, PGD_2 and PGE_2 are not only released from guinea pig urinary bladder but are also involved in the regulation of the lower urinary tract motilities. The action and potency of PGD_2 and PGE_2 were different depending on the tissue type (Figure 12 panel A and Table 1). PGD_2 inhibition of bladder detrusor strip EFS-induced contractions was mediated via DP_1 receptor. PGD_2 also activates TP receptors, where this effect can only be seen after blocking of DP_1 receptors

Table 1. *The presently estimated potency of prostanoids receptor agonists and antagonists in different parts of guinea pig lower urinary tract. Data from Paper II, III and IV.*

	Bladder	Trigone	Proximal Urethra	Antagonism in Bladder
PGE_2	pEC_{50} 8.0±0.38 (n=6)	pIC_{50} 7.05±0.27 (n=5)	pIC_{50} 7.65±0.17 (n=7)	
PGD_2	pIC_{50} 7.6±0.23 (n=8)	pIC_{50}7.08±0.27 (n=6)	pIC_{50} 7.09±0.22 (n=14)	
BW 245C	pIC_{50} 8.11±0.07 (n=11)			
BW A868C*	pIC_{50} 7.68±0.28 (n=9)			pA_2 8.68±0.06 (n=6)

**Partial agonist (Liu et al., 1996).*

4.5 EXPRESSION AND DISTRIBUTION OF PGDS AND DP_1/DP_2 RECEPTORS IN LUT (PAPER III, IV).

PGDS and DP_1 receptor in guinea pig bladder dome. The expression of hematopoetic prostaglandin D synthase (PGDS) and DP_1 receptor protein in the guinea pig bladder dome were examined by Western blot. The whole bladder dome extract as well as dome detrusor muscle and dome urothelium extracts were exposed to anti-PGDS and anti-DP_1 antibodies, which were the same antibodies used in the immunofluorescence histochemistry. Western blot showed a band slightly above 24 kD in agreement with the predicted of size 24 kD for the hematopoetic prostaglandin D synthase protein. In the guinea pig bladder dome it was mainly from the urothelium/suburothelium as judged from the bands in Figure 4 of Paper III. This result agrees with a strong

urothelium-dependency in the release of PGD_2 from the guinea pig urinary bladder as in the Paper II. The guinea pig urinary bladder dome expressed significant levels of DP_1 protein. Two groups of protein bands were seen with one slightly above 40 kD as predicted from the size of DP_1 receptor protein and additional bands at around 95 kD.

In the immunohistochemistry performed in the bladder dome, the smooth muscle was relatively faintly labeled with anti-PGDS antibody compared with urothelium/suburothelium (Figure 15A and B). Staining for actin was prominent in the detrusor muscle and also in vessels of the lamina propria, with only minor overlap with the PGDS stain (Figure 15B). The urothelium basal membrane was identified first by laminin staining subsequently allowing its localization from distribution of nuclei. It is marked by white dashed line in Figure 15A and C according to the anti-laminin in combination with nuclear stain results. In Figure 6C it can be seen that a basal row of cell nuclei line up in the urothelium just above the basal membrane laminin stain. In comparison, the similar lining up of nuclei allows the major PGDS stain to be determined as localized to the suburothelium (Figure 4F). DP_1 receptors were seen in several cell types throughout the guinea pig urinary bladder, as visualized by the red stain in figure 15C and D. There was only moderate staining for DP_1 receptors in the urothelium, and suburothelial interstitial cells-like profiles stained slightly more for DP_1 receptors (Figure 15C under dashed line). The most prominent staining for DP_1 receptors was in the detrusor muscle (Figure 15D). In the urothelial cells (Figure 15C) and especially in the smooth muscle (Figure 15D) the DP_1 receptor localization was mainly to the cell membrane, although intracellular staining could also be seen.

DP_1 and DP_2 receptors in male guinea pig trigone and proximal urethra. Western blot results found that in the male guinea pig trigone, neck and urethra, significant levels of DP_1 and DP_2 receptor proteins were expressed in whole tissue extracts. Fluorescence immunohistochemistry results, in sequential vertical sections of the trigone with transitional urothelium (u) and part of the smooth muscle (sm) layers, are shown in Figure 15E and F. Immunoreactivity for DP_1 and DP_2 receptors was seen throughout the trigone urothelium and smooth muscle. DP_1 receptors were more prominent in the smooth muscle layer (Figure 15E) while DP_2 receptors were more evenly distributed in the urothelium and smooth muscle layers but not as strong and localized as DP_1 receptors in the muscle cells (Figure 15F). The distribution of DP_1 receptor in the proximal urethra was similar as in the trigone where fluorescence of DP_1 receptor antibody was seen more heavily stained in the smooth muscle than in the urothelium. DP_2 receptor immunoreactivity was also seen distributed in the proximal urethra urothelium and smooth muscle. The urothelium exhibited much stronger fluorescence for DP_2 receptor compared with the smooth muscle. The border between smooth muscle (sm) and striated muscle (st) in proximal urethra is shown in Figure 15G and H. Longitudinal and circular smooth muscle layers can be identified by red label in the anti-α-smooth muscle actin antibody. DP_1 and DP_2 receptors were found both in smooth muscle and striated muscle components, but DP_2 receptor was stained at a much lower degree. Merged image of muscle anti-DP_1 receptor (green) and anti-α-smooth muscle actin (red) shows strong yellow fluorescence indicating co-localization of DP_1 receptor on the smooth muscle bundles (Figure 15G).

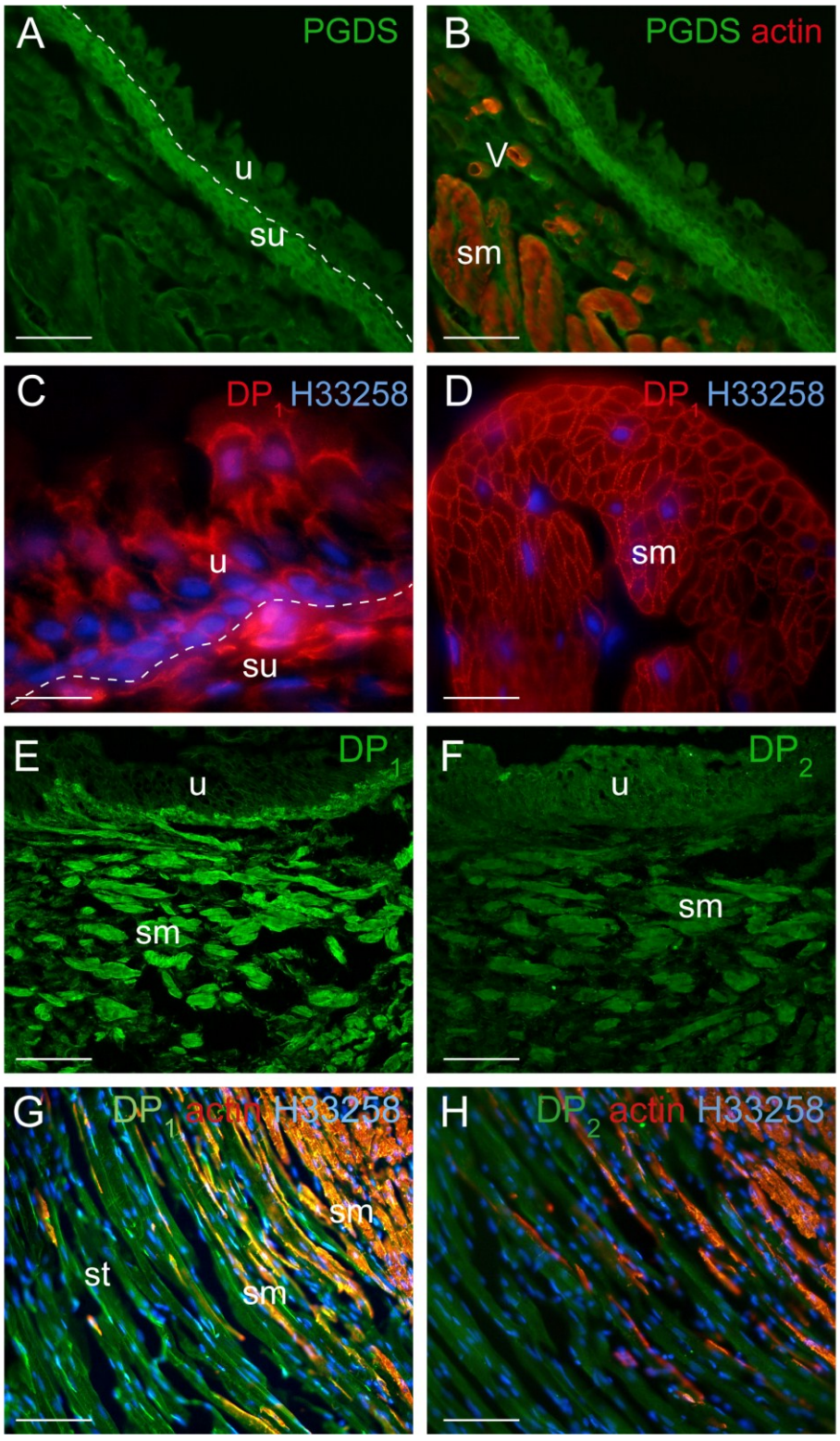

Figure 15. *Transverse section showing PGDS immunofluorescence (green) in guinea pig bladder dome (A, B); DP_1 receptor fluorescence (red) in dome urothelium (C) and smooth muscle (D); DP_1 and DP_2 receptors fluorescence (green) in trigone urothelium and smooth muscle (E, F); DP_1 and DP_2 receptors fluorescence (green) in proximal urethra smooth muscle and striated muscle (G, H). Nuclei were counterstained with Hoechst33258 in blue (C, D, G, H).Smooth muscle cells were stained with anti-α-smooth muscle actin antibody in red (B, G, H). Symbols legend: u denotes urothelium, su denotes the immediate suburothelium of lamina propria, v denotes blood vessels, sm denotes smooth muscle, st denotes striated muscle. White dashed line indicates the location of the urothelium basal membrane. Scale bars indicate 100 µm in A, B, E, F, G and H, 20 µm in C and D.*

5 GENERAL DISCUSSION

5.1 INHIBITORY MEDIATORS IN LUT

The current studies have been focused on the peripheral control of the LUT motility. As mentioned in the introduction, studies had shown an unidentified smooth muscle relaxing factor(s) being released from the bladder smooth muscle (Fovaeus *et al.*, 1998; Fovaeus *et al.*, 1999; Bozkurt *et al.*, 2004). Subsequently a urothelium-derived inhibitory factor(s) had been suggested (Hawthorn *et al.*, 2000; Templeman *et al.*, 2002; Chaiyaprasithi *et al.*, 2003; Meng *et al.*, 2008).The results from Paper I confirmed the existence of the urothelium-derived inhibitory activity. We could ascertain that the inhibitory effect seen on assay ureters was coming from bladder urothelium. That merely the mechanical contraction was a cause for the release of inhibitory bioactivity seems unlikely since, in a previous study, stimulating the bladder with α-adrenoceptor agonist failed to release inhibitory factor although it induced significant contraction of the bladder tissue (Templeman *et al.*, 2002). High concentration of KCl and neurokinin A (NKA) evoked contractile responses on human detrusor which were not affected by urothelium removal (Chaiyaprasithi *et al.*, 2003). We therefore believe that released inhibitory activity is not simply a reflection of direct bladder detrusor muscle contraction, but seems to be a more complicated process involving muscarinic receptor activation and where urothelium is a key component in this process.

Some studies suggested that the relaxation of trigone, bladder neck and urethra was not only due to the inhibition of excitatory neurotransmitter release but might also involve the release of non-adrenergic non-cholinergic (NANC) mediator(s) (Klarskov *et al.*, 1983b; Hills *et al.*, 1984; Speakman *et al.*, 1988). Nitric oxide was suggested to be responsible for the NANC factor mediated relaxation (Thornbury *et al.*, 1992; Werkström *et al.*, 1995). The existence of other relaxant mediator(s) can't be ruled out. Some NANC mediator candidates apart from NO and ATP will be discussed below.

Neuropeptides

Vasoactive intestinal polypeptide (VIP) had been shown to inhibit the isolated bladder spontaneous activities in many species including human (Larsen *et al.*, 1981; Levin *et al.*, 1981; Finkbeiner, 1983; Klarskov *et al.*, 1984; Sjögren *et al.*, 1985) but seemed to have no effect on the EFS or ACh induced contractions (Sjögren *et al.*, 1985). Intravenous injection of VIP induced relaxation of dog bladder (Andersson *et al.*, 1990) but had no effect on human bladder with the dose that was able to increase the heart rate (Klarskov *et al.*, 1987). In the guinea pig urinary bladder, the effects of VIP were contradictory. Burnstock and his colleagues found that VIP produced contractions of isolated guinea pig bladder strips (Mackenzie *et al.*, 1984). Other studies showed that VIP either had a very weak effect (Johns, 1979) or no effect (Finkbeiner, 1983; Pessina *et al.*, 2001) on baseline tension, or on magnitude or frequency of contractions of guinea pig bladder muscle strips. VIP was also found inhibiting the noradrenaline contracted urethra in the rabbit, cat, pig and human (Larsen *et al.*, 1981; Klarskov *et al.*,

1984). No data suggest a VIP effect in modulating the guinea pig ureter spontaneous contractions, but it can't exclude the possibility that the urothelium-derived inhibitory activity bioassayed on the ureter is VIP. However, it is unlikely that VIP is the unknown mediator since VIP has little inhibitory effect on the isolated guinea pig bladder.

Neuropeptide Y (NPY) rich nerves were found abundant in both human (Gu *et al.*, 1984; Crowe *et al.*, 1995; Dixon *et al.*, 1997) and rat bladders (Mattiasson *et al.*, 1985; Keast *et al.*, 1989). Moderate numbers of NPY containing nerve fibers were observed in the pig trigone and urethra, while very few were found in the detrusor (Persson et al., 1995). In the rat, NPY had an excitatory influence on spontaneous contractions and resting tone of the detrusor, and it also potentiated EFS-induced contractions but did not affect ACh-evoked contractions (Iravani *et al.*, 1994). Different results were observed when NPY from other species was studied. Porcine and human NPY inhibited the EFS-induced contractions of rat detrusor, and in the rat urethra adrenergic transmission was primarily inhibited. The degree of inhibitory effects in the rat detrusor differed with human and porcine NPY (Zoubek *et al.*, 1993). In guinea pig bladder, NPY had no effect on noradrenaline, ATP or α,β-methylene ATP induced contractions, but inhibited the NANC components of the EFS induced contractions (Lundberg *et al.*, 1984). The conflicting results of NPY in modulating the LUT activities could be due to the different amino acid sequences in the different species and these NPY-like peptides might have different affinity to the receptors. Since NPY has inhibitory effect in guinea pig bladder, the possibility of NPY being the unknown inhibitory factor in Paper I can't be excluded. There is a need for investigation of the NPY-like peptides effects in human LUT. However, NPY seems less plausible as the urothelium-derived inhibitory factor since it has no effect on tone or spontaneous contractions and enhances induced contractions in the equine ureter (Prieto *et al.*, 1997).

Calcitonin gene-related peptide (CGRP) is a sensory neuropeptide from capsaicin-sensitive nerves and found distributed in all bladder layers of human and many other species (Nimmo *et al.*, 1988; Smet *et al.*, 1996). CGRP effects differ according to the different intensity in distribution between regions of LUT even in the same species. CGRP was more potent and effective as inhibitor of the EFS induced contraction in the guinea pig bladder neck than in the dome (Maggi *et al.*, 1988). In hamster bladder, CGRP also exerted an inhibitory effect in the bladder smooth muscle (Giuliani *et al.*, 2001), while it had no effect on modulating the rat and human bladder contractility (Maggi *et al.*, 1987b; Maggi *et al.*, 1989). Immunoreactivity studies showed that CGRP colocalized with tachykinin in primary sensory neurons in bladders (Ghatei *et al.*, 1985; Su *et al.*, 1986). Tachykinins exert excitatory effects in the urinary bladder in many species (Anderson, 1993). Stimulation of capsaicin-sensitive nerves will result in the release of both inhibitory peptide CGRP and excitatory tachykinin such as substance P. Thus, effects of combined mediator release from sensory nerves in the bladder onto the assay ureter might exhibit as contraction, relaxation or no effect at all. CGRP can inhibit induced contractile responses in the guinea pig ureter (Hua *et al.*, 1986; Maggi *et al.*, 1996) and induced spontaneous contractions in rat ureter (Maggi *et al.*, 1987b).

Further investigations are needed on whether CGRP might be a urothelium-derived inhibitor.

Prostanoids

Prostanoids are known to be synthesized by the cyclo-oxygenases localized in the urinary tract. Our results using the cyclo-oxygenase inhibitor diclofenac in the superfusion fluid were that it did not abolish the carbachol induced transmissible inhibitory activity. However, prostanoids seem to play important roles in modulation of urinary tract motility. It was proposed that the spontaneous motility of urinary tract depends on local release and balance of both excitatory and inhibitory prostanoids (Mastrangelo *et al.*, 2007). Meanwhile, studies also showed difficulties to fully inhibit the release of prostanoids from urothelium-containing bladder tissue by application of a cyclooxygenase inhibitor (Abrams *et al.*, 1979). In Paper II, we observed and quantified the biological release of PGE_2 and PGD_2 from guinea pig bladder without any stimuli. The concentrations of endogenous PGE_2 and PGD_2 from donor bladder to assay ureter were about 1 to 2 nanomolor and were profoundly decreased by cyclo-oxygenase inhibitor (Paper II, Figure 4). Furthermore, the investigation of exogenous PGE_2 and PGD_2 effects on ureter spontaneous contraction indicated that with concentration of biologically released PGE_2 and PGD_2, only excitation of ureters were seen. So, prostanoids being the unknown inhibitor(s) seems quite unlikely, at least by generation from cyclo-oxygenase.

Isoprostane

Isoprostanes are prostaglandin-analoguos compounds formed in vivo through free radical catalyzed peroxidation of arachidonic acid. In 1990, the PGF_2 like compounds were found from fresh plasma of normal volunteers. Formation of these compounds was found to occur by a non-enzymatic oxidative process (Morrow *et al.*, 1990). Since then, several other classes of isoprostanes were discovered as D_2-isoprostane (D_2-isoP), E_2-isoP, J_2-isoP, and A_2-isoP (Milne *et al.*, 2008). The isoprostanes were able to bind to the normal prostanoids receptors (Kinsella *et al.*, 1997; Audoly *et al.*, 2000) and exerted similar activities as cyclooxygenase products. As a biomarker of oxidative stress, 8-isoprostane was shown elevated in exhaled breath condensate, plasma and urine in asthmatic adults and children compared to healthy controls (Montuschi *et al.*, 1999; Zanconato *et al.*, 2004). Isoprostanes were also found to be involved in regulation of vascular smooth muscle (Fukunaga *et al.*, 1993) and platelet function (Ting *et al.*, 2010). The isoprostane 8-epi $PGF_{2\alpha}$ was also shown to be synthesized in rabbit bladders and to cause detrusor smooth muscle contraction (Tarcan *et al.*, 2000). Based on Paper II and III, PGD_2 can inhibit the EFS induced or spontaneous contractions in guinea pig LUT. D_2-isoP and PGD_2 metabolites (Figure 5) are able to activate the DP receptors and induce similar effects as PGD_2. The possibility of the unknown inhibitor(s) being isoprostanes can't be excluded.

5.2 PGD_2 IN LUT

The roles of PGD_2 in the allergic diseases and inflammation have been widely discussed and are now better understood (Pettipher *et al.*, 2007), while knowledge

about the PGD_2 effects in the LUT seems to have been minimal. One *in vitro* study by using human bladder strips showed no effect of PGD_2 application up to $3x10^{-7}$ M at resting tension (Palea *et al.*, 1998). In Paper II, III and IV, the release and effects of PGD_2 in the guinea pig bladder dome, trigone and urethra have for the first time been discribed. The receptors involved in the functional effects together with DP_1/DP_2 distribution in LUT was also investigated for the first time. PGD_2 was shown to be released mainly from guinea pig urothelium/suburothelium. Immunofluorescent detection with anti-DP_1 and anti-DP_2 showed that DP_1/DP_2 receptors were distributed both in the urothelium and suburothelium and DP_1 prominently in the smooth muscle cells. Although presently not studied, in the smooth muscle cells it seems likely that PGD_2 directly activates G_s-coupled DP_1 receptors leading to the increase of intercellular cAMP levels, hence activating PKA which could phosphorylate myosin light chain kinase and result in smooth muscle relaxation. Presently, PGD_2 in guinea pig urinary bladder was also able to inhibit the contractions induced by nerve stimulation, ACh and ATP, favouring the idea of a direct smooth muscle relaxing action. In the urethra, PGD_2 inhibited the spontaneous contractions which in the LUT are generally thought to be generated or modulated by interstitial cells (Brading *et al.*, 2005; Brading, 2006). Some remaining questions are: apart from direct effect on the smooth muscle, is PGD_2 indirectly involved in the modulation of the bladder and urethra motilities through action on the interstitial cells? If DP_1/DP_2 receptors are expressed in the urothelium, what is the function of activating DP_1/DP_2 receptors located on the urothelial cells? (Figure 16)

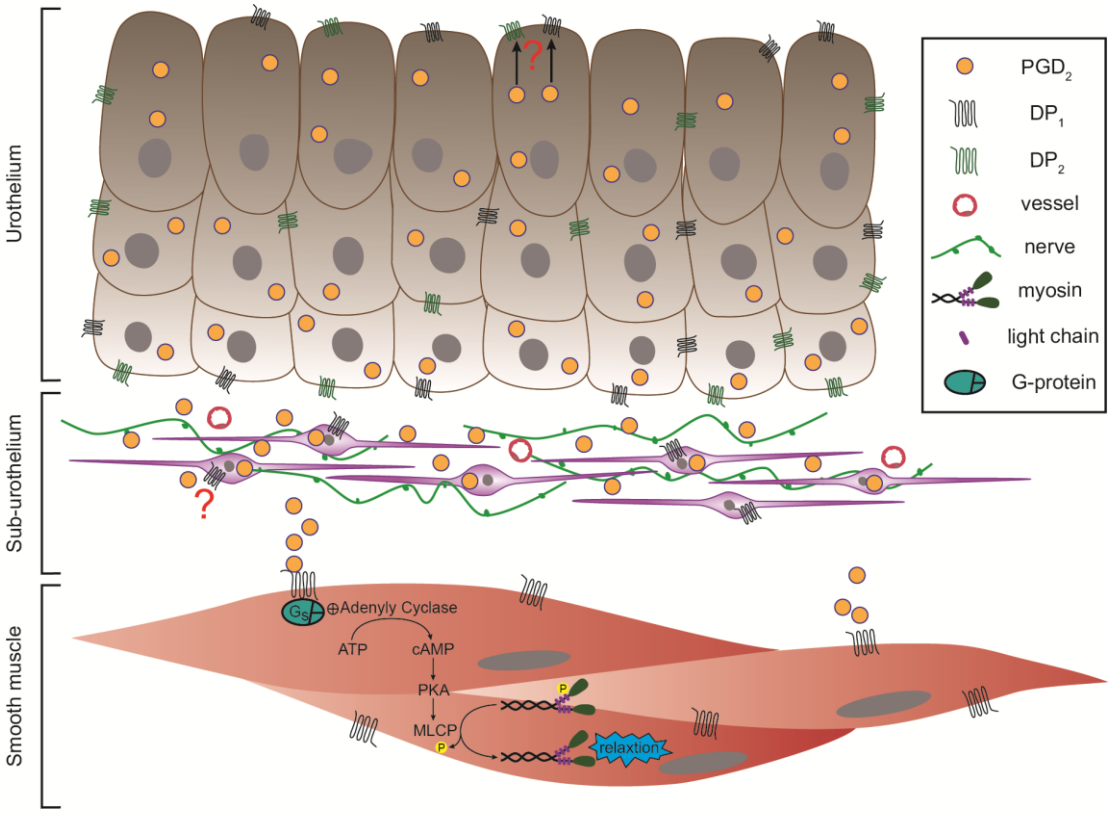

Figure 16. *Schematic drawing summarizing the bladder structure, PGD_2 released from urothelium and suburothelium, localization of DP_1 and DP_2 receptors and the effect of PGD_2 relaxing the smooth muscle in guinea pig urinary bladder.*

The inhibitory effect of PGD_2 in modulating guinea pig LUT motility in vitro is valuable information to understand the pathophysiology of bladder and urethral sphincter. Depending on the expression and distribution of DP receptors in the LUT, the functions of PGD_2 might be different in other animal species and the human. DP_1−/− and CRTH2−/− mice were generated and used in many allergy and inflammation studies (Mohri *et al.*, 2006; Satoh *et al.*, 2006; Murata *et al.*, 2013). These knock-out animals provide us interesting tools to further study the effects of PGD_2 in the LUT. Moreover, there is also a need for in vivo urodynamic studies to understand the systematic role of PGD_2 in LUT.

5.3 ENDOGENOUS PROSTANOIDS AND RECEPTOR ANTAGONISTS

Diclofenac was applied to the bladder tissue in our pharmacological experiments to inhibit the production of endogenous prostanoids. After applying diclofenac to a naive guinea pig bladder strips, less than 5% inhibition of EFS induced contractions was observed. After washing the diclofenac treated bladder tissues, more than 20% inhibition was shown which was not seen when washing naive bladder strips. This indicates that similar to endogenous adenosine (Nilsson *et al.*, 2010), considerable amounts of endogenous prostanoids or prostanoid-like compounds are present in the tissues. The concentrations of endogenous prostanoids will be decisive for the effects of further application of exogenous prostanoids, which could be one of the reasons that some studies reported negative results of prostanoids application to certain tissues. I suggest that when working with prostanoids, removal of the endogenous prostanoids that have already been formed is as important as inhibition of their production.

Prostanoid agonists and antagonists are a group of compounds with high affinity and high lipophilicity. These properties could explain the slow onsets of some prostanoid receptor analogs and blockers in multilayer tissues (Jones *et al.*, 2011), which is one of the likely reasons why reports on estimated pA_2 values of prostanoid receptor antagonists vary and may be distorted in the same species and same tissue. There is a need for caution in using highly lipophilic prostanoid receptor antagonists as research tools. When performing pharmacological experiments in the present studies, the minimal incubation time of antagonists was at least one hour, aiming for the blockers to exert an optimal antagonism with each given dose.

5.4 PHARMACOLOGICAL IMPLICATIONS

The framework of prostanoids and their receptors is very complicated. Ligands do not exclusively bind to their corresponding receptors. If follows that the receptors are not entirely specific for the correspondent prostanoids. Moreover, clinical studies by using non-steroidal anti-inflammatory drugs to treat bladder instabilities have shown contradictory results (Cardozo *et al.*, 1980; Delaere *et al.*, 1981; Chaudhuri *et al.*, 1993; Park *et al.*, 2000). It seems that globally inhibiting the prostanoids production might take away the excitatory factors but also inhibitory factors as well. The treatments should specifically aim at certain prostanoids or target at prostanoids receptors. Urodynamic studies showed that intravesical administration of PGE_2 and PGE_2-derivative to women not only increased bladder pressure but also reduced bladder capacity and resulted in measurable bladder instability (Andersson *et al.*, 1978;

Schüssler, 1990). Treatment targeting EP_1 receptors has been attempted. A recent clinical phase II study using the EP_1 receptor antagonist ONO-8539 to treat non-neurogenic OAB suggested a minimal role for EP_1 receptor antagonism (Chapple *et al.*, 2014).However, the work leading up to this trial provided concepts of targeting the prostanoid receptors to treat OAB. Our work revealed roles of PGD_2 in regulating the LUT motility, which opens up new possibilities to target PGD_2 signaling pathways when in the future designing treatments for OAB and other bladder instability situations.

6 FUTURE PERSPECTIVES

Prostaglandin D_2 as an inhibitory modulator in guinea pig lower urinary tract (bladder and proximal urethra), its release, synthase expression, receptor activation, receptor expression and pharmacological properties, has been studied and thoroughly discussed in the present work. The roles of PGD_2 in influencing human bladder motility and the outflow region will be valuable future information for us to understand the physiology of the human lower urinary tract. To compare the effects of PGD_2 in the healthy human lower urinary tract tissues with that in OAB patient tissues will help us to understand the roles of PGD_2 under pathological conditions, which might lead to new treatment targets for overactive bladder situations. The expression and effects of inhibitory or relaxing prostanoid receptors are also of interest in the human tissue under both physiological and pathological conditions.

During chemical analysis of the prostanoids released from guinea pig urinary bladder in Paper II, we have developed a method to minimize the abundant background of impurities from buffer salts and other system components. The described technique is generally applicable for isolation attempts of endogenous compounds. Introducing the use of the ureter as a bioassay tissue might prove useful in future studies on urinary tract signalling compounds, including UDIF, especially if combined with mass spectrometry methodology.

7 GENERAL CONCLUSIONS

1. Urothelium-denuded guinea pig ureters can be used in serial superfusion to bioassay released inhibitory activities from the guinea pig urinary bladder.

2. An inhibitory activity originates from the urothelium and is transmissible over a significant distance. This opens up the possibility of attempting isolation of the elusive urothelium derived inhibitory factor(s).

3. The unknown urothelium-derived inhibitory activity is unlikely to be induced by or mediated through nitric oxide, adenosine receptor agonists or cyclooxygenase products.

4. The urothelium/immediate suburothelium is a major source of PGD_2 production and PGD_2 can inhibit the bladder motility mainly through a postjunctional action.

5. PGD_2 exerts a dual action on contractile responses, with an inhibitory effect at relatively low concentrations through DP_1 receptors localized in the smooth muscle, masking an excitatory effect exerted via TP receptors.

6. In the guinea pig outflow region, PGD_2 has an inhibitory influence on the trigone and proximal urethra which is consistent with the expression and distribution of DP_1 and DP_2 receptors in these regions.

7. The role of PGD_2 in regulation of the human bladder, and especially the outflow region, should be considered in future investigations, not least in the context of overactive bladder.

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