Physiological Roles of Bradykinin in Lower Urinary Tract Symptoms and Bradykinin B2 Receptor as a Potential Novel Therapeutic Target

January 2017

Kenichiro FUJIMOTO

Physiological Roles of Bradykinin in Lower Urinary Tract Symptoms and Bradykinin B2 Receptor as a Potential Novel Therapeutic Target

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Bioindustrial Sciences)

Kenichiro FUJIMOTO

Table of Contents.....i

Abbreviations.....ii

Introduction.....1

Chapter 1 Physiological Roles of Bradykinin and Involvement of Bradykinin B2 Receptor in Urethral Function in Humans and Animals.....18

1.1 Background.....19

1.2 Materials and Methods.....21

1.3 Results.....25

1.4 Discussion.....27

Chapter 2 Intratesticular Bradykinin Involvement in Rat Testicular Pain Models.....36

2.1 Background.....37

2.2 Materials and Methods.....39

2.3 Results.....42

2.4 Discussion.....44

Conclusions.....53

References.....61

Acknowledgements.....70

List of Published Articles.....71

Abbreviations

ACE	angiotensin converting enzyme
AUC	area under the curve
BOO	benign outlet obstruction
BPE	benign prostatic enlargement
BPH	benign prostatic hyperplasia
CP	chronic prostatitis
CPP	chronic pelvic pain
CPPS	chronic pelvic pain syndrome
CPSI	chronic prostatitis symptom index
DW	distilled water
EPIC	European Prospective Investigation into Cancer and Nutrition
EPS	expressed prostatic secretion
ICS	International Continence Society
i.p.	intraperitoneal
IUP	intraurethral pressure
i.v.	intravenous
LUTS	lower urinary tract symptoms
MTOPS	Medical Therapy of Prostatic Symptoms
NIH	National Institutes of Health
PDE	phosphodiesterase
p.o.	per os
PSA	prostate specific antigen
QOL	quality of life

REDUCE	Reduction by Dutasteride of Prostate Cancer Events
TURP	transurethral resection of the prostate

Introduction

Along with further advancement of life science and medical technology, it is expected that further cross-border medical care as well as changes in the demographics, globalization of society and economy will proceed in the future. Therefore, in the next 10 years, expectations for superior drugs are supposed to increase on a global scale along with further increase in awareness of health and life. Pharmaceutical industry has to continuously launch innovative medicines so as to contribute to the health and welfare of people around the world, which is its mission.

Although the average life expectancy continues to increase in the world, the future of Japanese society which runs the top among them is being focused. It is a society that people can live a higher quality life, not just live longer, through proactive social participation or selection of a convincing medical and nursing care etc.

Due to illness, aging, operations or anti-cancer agents, it is possible that people are constrained to their life, suffer from pain or can’t live by their own values. Now it is considered important, a medical care to respect patients’ outlook on life or sense of values and maintain their quality of life (QOL) as much as possible. Urogenital disorders such as lower urinary tract symptoms (LUTS) are one of the diseases relating to QOL.

“LUTS” is a term that broadly means the symptoms associated with the storage and discharge of urine. LUTS is subjectively recognized by individuals who are usually patients but sometimes caretakers or partners. Symptoms are reported by patients or through interview. Normally it is qualitative, cannot be used for definitive diagnosis. LUTS is suggestive of a lower urinary tract dysfunction in the background but is also caused by urinary tract infection or the other etiology.

In the terminology of the International Continence Society (ICS) in 2002, the terms related to lower urinary tract function were revised and the part for LUTS was significantly revised (Abrams et al., 2002). LUTS includes major three symptoms, storage symptoms, voiding symptoms and post micturition symptoms, but also urogenital pain, genito-urinary pain syndromes or symptom syndromes (Table 1).

There is no basic difference between the nature of LUTS in men and women. However, voiding symptoms such as slow stream are frequently found in men after middle age and urinary incontinence is apparently common in women. Storage symptoms other than urinary incontinence are somewhat frequently found in women but no difference, especially in the elderly has been reported in the most cases (Irwin et al., 2006, Schatzl et al., 2001, Kakizaki et al., 2002, Araki et al., 2003, Terai et al., 2004, Homma et al., 2006). Prostate disease in men and stress urinary incontinence in women would have influence on the gender difference of the symptom frequency.

To clarify the medical and social positioning of certain diseases and then establish a medical regime, information such as frequency, age distribution or medical care demand, that is, the epidemiological investigation is essential. European Prospective Investigation into Cancer and Nutrition (EPIC) study, which was conducted in Canada, Germany, Italy, Sweden and the United Kingdom in 2005 is one of the examination of LUTS in compliance with the definition of the International Continence Society in 2002 (Irwin et al., 2006). Subjects of this study were 58,139 people of 18-70 years of age (analysis 19,165 people). Percentages having any LUTS were 62.5% in men and 66.6% in women, the storage symptoms 59.2% in women and 51.3% in men, the voiding symptoms 25.7% in men and

19.5% in women, and post micturition symptoms 16.9% in men and 14.2% in women. In men, the frequency of all LUTS increased along with age and was quite significant in the 60 years of age or older. Also in women, the frequency of urgency, nocturia, urinary incontinence, slow stream, intermittent stream and post micturition dribble increased with age.

Benign prostatic hyperplasia (BPH) is a disease in the QOL of elderly men and patients increases as become older and it is said that BPH is pathologically detected in 60-70% of men over 70 years of age (Berry et al., 1984). In BPH, hypertrophy nodule consisting of proliferative prostate epithelial cells and stromal cells is seen around the urethra and LUTS were caused by obstruction at lower urinary tract due to benign prostatic enlargement. That is, clinical BPH is composed of three elements of LUTS, benign prostatic enlargement (BPE) and benign outlet obstruction (BOO) (Figure 1). The concept of the classic BPH was the center the overlapping portions of these three circles, however, actual features of BPH is by no means simple. There are many cases without enlargement or obstruction in LUTS patients.

Recently, an involvement of inflammation in prostate has been focused in BPH. In the clinical studies for 5α-reductase inhibitors, patients in the biopsy specimen of the prostate with inflammation findings showed large prostate volume, high symptom score (Nickel et al., 2008) and also high risk of urinary retention was reported in patients in the prostatectomy tissue with inflammation (Mishra et al., 2007). It was speculated that changes in the hormonal environment associated with aging, chronic inflammation resulting from infection or enhancement of the immune response caused the remodeling of prostate structure and then prostate enlargement (Kramer et al., 2007). Some other recent studies have also suggested

that inflammatory infiltrate would lead to tissue damage and prolonged time to wound healing, which might subsequently induce prostatic enlargement (De Nunzio et al., 2012, De Nunzio et al., 2011, Alcaraz et al., 2009).

For drug therapy of BPH (Table 2), currently, α1 blocker is firstly selected as the standard of care but in case the therapeutic effect of α1 blocker is insufficient and the volume of prostate is large, 5α-reductase inhibitors are used in combination with α1 blocker. Very recently, phosphodiesterase-5 (PED5) inhibitors became available and its blood flow improving effect, anti-inflammatory effect, afferent inhibitory effect other than main smooth muscle relaxant effects have been focused (Andersson et al., 2011).

Prostatitis is seen in men of most of the generation unlike BPH but frequently in the elderly like BPH (Pontari, 2003). The classification of prostatitis by US National Institutes of Health (NIH) indicates that prostatitis is classified in category I-IV (Krieger et al., 1999) (Table 3). Category I is acute bacterial prostatitis and category II chronic bacterial prostatitis in both of which bacteria is detected. Category III is prostatitis in which bacteria is not proven and known as chronic prostatitis/ chronic pelvic pain syndrome (CP/CPPS), accounts for more than 90% of prostatitis. The Category III is further subdivided into IIIA (with inflammation) and IIIB (no inflammation). “Inflammatory” here means the fact that white blood cells are detected in the expressed prostatic secretion (EPS) after prostate massage or in the urine after massage. In addition, prostatitis which does not have symptoms but detects inflammation findings in prostate biopsy tissue specimens and expressed prostatic secretion, semen is classified as category IV, asymptomatic inflammatory prostatitis.

Storage symptoms such as urinary frequency, feeling of incomplete

emptying and urgency are main symptoms of prostatitis but also voiding symptoms can be seen. Pain or discomfort in the various parts of perineum and pelvis is also main symptoms in Category III. In patients with Category III, there is a report that petechiae was detected by the bladder pressure expansion under anesthesia in its 60% (Miller et al., 1995) or a report that its 84% of cases were positive in the potassium test used for examining the permeability of the urothelium (Parsons and Albo, 2002) so that some relation or overlap with symptoms and observations of interstitial cystitis is assumed. NIH-Chronic Prostatitis Symptom Index (NIH-CPSI) has been created as an index to objectively quantify the symptoms of chronic prostatitis (Litwin et al., 1999). There is a possibility that chronic prostatitis is easily diagnosed for the cases of the young less than 50 years of age with storage symptoms, without neurological disease. In the cases of relatively young men with LUTS, interstitial cystitis or overactive bladder in addition to prostatitis needs to be carefully diagnosed. Cernitine pollen extract has been reported to have some efficacy in CP/CPPS but is not recommended drug therapy because of its weak evidence (Elist, 2006).

Kallikrein-kinin system plays an important physiological role as a circulation regulation through modulation of cardiovascular and renal function, and is also involved in inflammation such as asthma, rhinitis and arthritis, allergic diseases, pain, sepsis and tissue injury (Bhoola et al., 1992, Katori and Majima, 1998, Wirth et al., 1995, Calixto et al., 2000). Bradykinin and kallidin (Lys-bradykinin) are main kinins in mammals (Table 4, Figure 2) and produced through serine protease, specifically kallikrein, acting on kininogen. Normal life of bradykinin and kallidin in plasma is short and inactivated rapidly through degradation by enzyme mainly kininase II or angiotensin converting enzyme (ACE). When the arginine residues at the C-terminal side of bradykinin and kallidin are cut by kininase I (arginine decarboxypeptidase), des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin are respectively generated. These peptides act on bradykinin B1 receptor as an agonist, whereas bradykinin acts through B2 receptor and kallidin acts on both B1 and B2 receptors comparably. Bradykinin B1 receptor and B2 receptor have low homology, only 36% in humans (Menke et al., 1994, Marceau et al., 1998).

Protease activation at the site of inflammation/tissue damage cleaves tissue/plasma kininogen precursors to release the nonapeptide bradykinin (Calixto et al., 2000, Dray and Perkins, 1993). Bradykinin is involved in a range of diverse biological functions including smooth muscle contraction, inflammation, pain, and mitogenicity, all of which are mediated by activation of either or both the B1 or B2 receptors (Campbell, 2001, Howl and Payne, 2003). Bradykinin B2 receptors are expressed in a wide range of tissues, whereas B1 receptors are expressed at low levels under normal conditions and up-regulated following tissue damage/inflammation (Wirth et al., 1991, Ahluwalia and Perretti, 1999).

All components of the kallikrein-kinin system exist in human male genital

secretions (Campbell, 2001), suggesting that these molecules participate in physiological and pathophysiological genitourinary function. Indeed, bradykinin has been detected in human prostate tissue as well as in the conditioned medium of human prostate-derived stromal cells in culture (Walden et al., 1999). Bradykinin has been shown to elicit contractile responses in isolated tissue obtained from the bladder, prostate, and urethra in many animal species (Schill and Miska, 1992, Srinivasan et al., 2004, Abdel-Hakim et al., 1983, Chopra et al., 2005, Watts and Cohen, 1991) (Figure 3).

In addition, bradykinin involvement in the regulation of bladder function has been proven in studies demonstrating that both B1 and B2 receptor antagonists can reduce bladder overactivity in a rat model of cyclophosphamide-induced cystitis (Chopra et al., 2005) and in a rat model of spinal cord injury (Forner et al., 2012). These previous findings indicate that bradykinin can evoke an inflammatory response and changes in the urinary bladder reflex either or both by directly activating B2 receptors on afferent fibers or indirectly by releasing ATP and other neurotransmitters of the urothelium (Chopra et al., 2005).

In order to examine bradykinin involvement *via* bradykinin B2 receptor in this study, I used FK3657, also known as FR173657 ((*E)*-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl]acrylamide), a potent, selective, orally active and non-peptide bradykinin B2 receptor antagonist which was obtained by optimization of a lead compound discovered by random screening of Fujisawa pharmaceutical's chemical library (Asano et al., 1997, Asano et al., 1999) (Figure 4). I firstly examined the physiological role of bradykinin in urethral function focusing on the voiding symptoms which are the main complaints of LUTS associated with BPH, and next examined the involvement of bradykinin in rat testicular pain model focusing on the testicular pain which is the main complaints of CP/CPPS. Finally, I discussed and concluded the physiological roles of bradykinin in LUTS and bradykinin B2 receptor as a potential novel therapeutic target.

Table 1. Classification of LUTS defined by ICS in 2002 (Abrams et al., 2002)

1. Storage symptoms		
	Increased daytime frequency	
	Nocturia	
	Urgency	
	Urinary incontinence	
		Stress urinary incontinence, Urge urinary incontinence, Mixed urinary incontinence, Enuresis, Nocturnal enuresis, Continuous urinary incontinence, Other type of urinary incontinence
	Bladder sensation	
		Normal, Increased, Reduced, Absent, Non-specific
2. Voiding symptoms		
	Slow stream, Splitting or spraying, Intermittent stream (Intermittency), Hesitancy, Straining, Terminal dribble	
3. Post micturition symptoms		
	Feeling of incomplete emptying, Post micturition dribble	
4. Symptoms associated with sexual intercourse		
5. Symptoms associated with pelvic organ prolapse		
6. Genital and lower urinary tract pain		
	Bladder pain, Urethral pain, Vulval pain, Vaginal pain, Scrotal pain, Perineal pain, Pelvic pain	
7. Genito-urinary pain syndromes and symptoms syndromes suggestive of lower urinary tract dysfunction		
	Genito-urinary pain syndromes	
		Painful bladder syndrome, Urethral pain syndrome, Vulval pain syndrome, Scrotal pain syndrome, Perineal pain syndrome, Pelvic pain syndrome
	Symptom syndromes suggestive of lower urinary tract dysfunction	
		Overactive bladder syndrome, Urge syndrome, Urgency-frequency syndrome, Lower urinary tract symptoms suggestive of bladder outlet obstruction

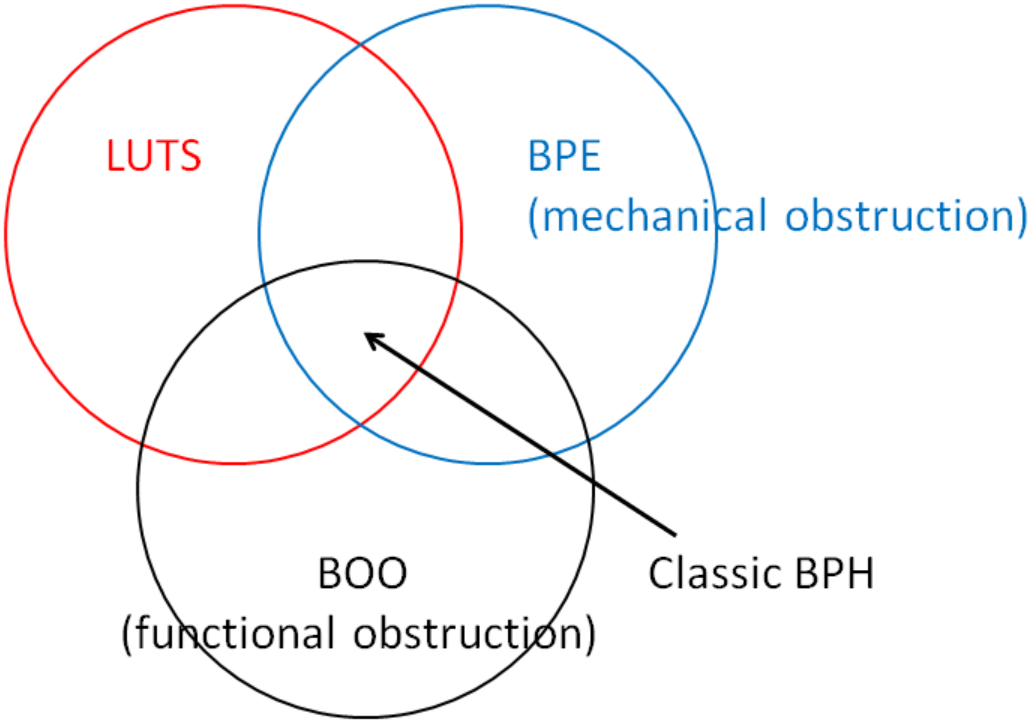


Figure 1. Three elements of clinical BPH (Hald, 1989)

Table 2. Recommended drug therapy for BPH

α1 blocker	
Mechanism of action	Inhibit the α1 adrenergic receptor that is involved in smooth muscle tone in the prostate and bladder neck, reducing the functional obstruction of the urethra by the prostate
Name of drugs	tamsulosin, naftopidil, silodosin etc.
Note	Standard of care (1st choice)
5α-reductase inhibitor	
Mechanism of action	Suppress the production of dihydrotestosterone by inhibiting the 5α-reductase, reducing the mechanical obstruction of the urethra associated with BPH
Name of drugs	dutasteride, finasteride (not approved in Japan)
Notes	In case the volume of prostate is large
PED5 inhibitor	
Mechanism of action	Inhibit the degradation of the local of cGMP by inhibiting the PDE5 in the smooth muscle cells of the urethra and prostate, relaxing smooth muscle
Name of drugs	tadalafil, sildenafil (not approved in Japan)
Notes	New option, recently approved

Table 3. Classification of prostatitis by NIH (Krieger et al., 1999)

I.	Acute bacterial prostatitis
II.	Chronic bacterial prostatitis
III.	Chronic prostatitis / chronic pelvic pain syndrome
	IIIA. Inflammatory
	IIIB. Non-inflammatory
IV.	Asymptomatic inflammatory prostatitis

Table 4. Mammalian kinins

Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Lys-bradykinin (kallidin)	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
des-Arg^{9}-bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
des-Arg^{10}-kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe

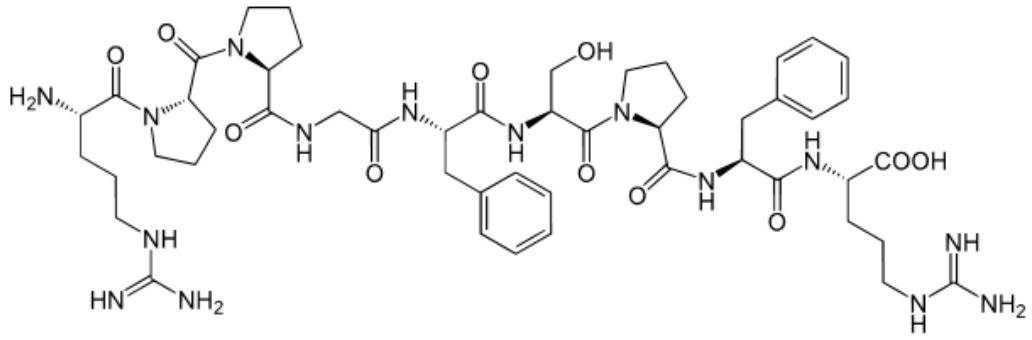


Figure 2. Chemical structure of bradykinin

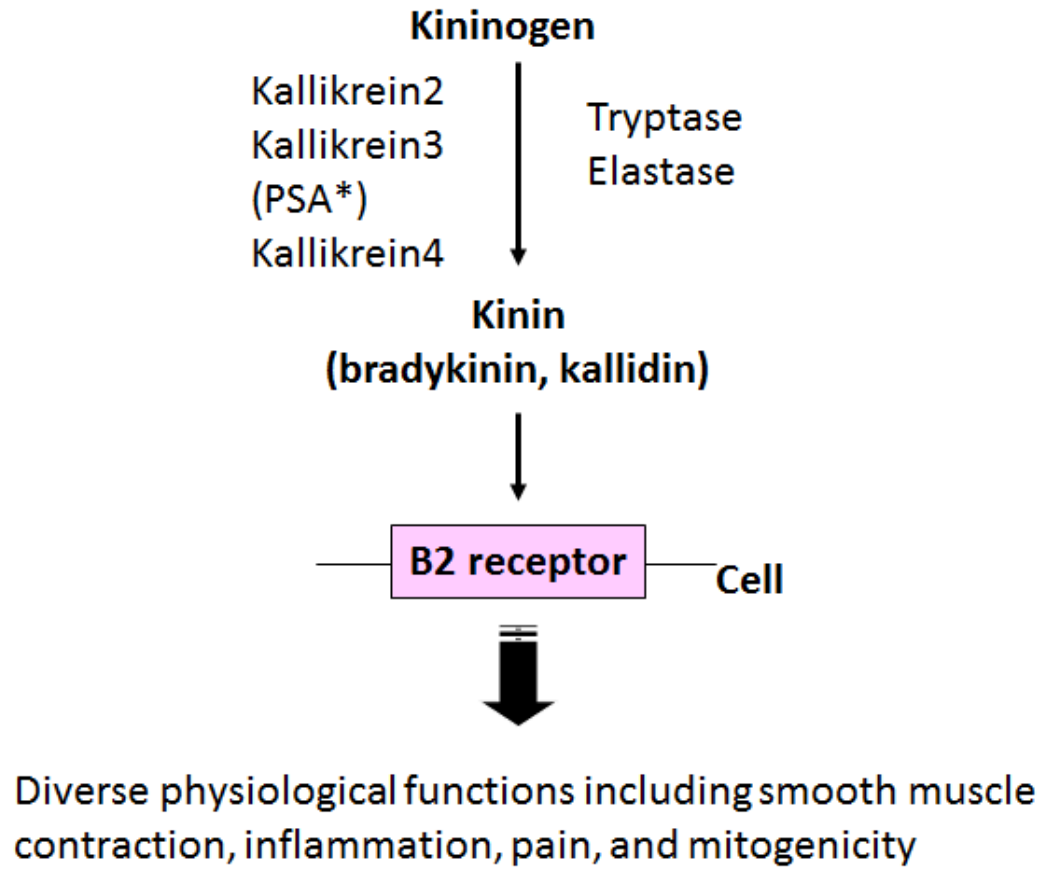


Figure 3. Kallikrein-kinin system and physiological functions *via* B2 receptor

(*PSA: prostate specific antigen)

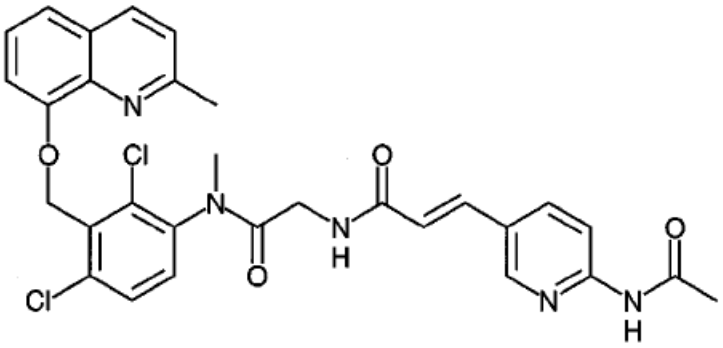


Figure 4. Chemical structure of FK3657

Chapter 1 Physiological Roles of Bradykinin and Involvement of Bradykinin B2 Receptor in Urethral Function in Humans and Animals

1.1 Background

BPH is a common disorder among middle-aged and elderly men which produces BOO. In these patients, LUTS characterized by poor stream, hesitancy in initiation of micturition, urinary frequency, nocturia and urgency were observed. α1-adrenoceptor antagonists are currently the first-line treatment for voiding dysfunction associated with BPH. Although BPH aetiology has not yet been fully elucidated, several mechanisms have been proposed as involved in the pathogenesis and progression of BPH (Gandaglia et al., 2013), and several recent studies have suggested a major role of prostatic inflammation in BPH pathogenesis and progression. Specifically, inflammatory infiltrate has been hypothesized to lead to tissue damage and prolonged time to wound healing, which might subsequently induce prostatic enlargement (De Nunzio et al., 2012, De Nunzio et al., 2011, Alcaraz et al., 2009).

Protease activation at the site of inflammation/tissue damage cleaves tissue/plasma kininogen precursors to release the nonapeptide bradykinin (Calixto et al., 2000, Dray and Perkins, 1993). All components of the kallikrein-kinin system exist in human male genital secretions (Campbell, 2001) suggesting that these molecules participate in physiological and pathophysiological genitourinary function. Indeed, bradykinin has been detected in human prostate tissue as well as in the conditioned medium of human prostate-derived stromal cells in culture (Walden et al., 1999). In addition, bradykinin has been shown to elicit contractile responses in isolated tissue obtained from the bladder, prostate, and urethra in many animal species (Schill and Miska, 1992, Srinivasan et al., 2004, Abdel-Hakim et al., 1983, Chopra et al., 2005, Watts and Cohen, 1991). However, few studies have

examined the pathophysiological roles of bradykinin in the lower urinary tract in humans or *in vivo* urethral function in animals.

Here, to clarify the physiological roles of bradykinin and the involvement of bradykinin B2 receptor in urethral function in humans and animals, I investigated the effects of bradykinin in the urethral function in humans and animals using the bradykinin B2 receptor antagonist FK3657.

1.2 Materials and Methods

1) Materials

Bradykinin was purchased from PEPTIDE INSTITUTE, INC. (Osaka, Japan). FK3657 ((*E)*-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl]acrylamide) synthesized at Astellas Pharma, Inc. (Tokyo, Japan), was dissolved in 0.1 N HCl and then diluted with distilled water. Phenylephrine hydrochloride and epinephrine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2) Preparation of human urethral smooth muscle strips

All human experiments were approved by the Human Ethical Committee of Astellas Pharma, Inc., and performed at the Astellas Research Institute of America. Urinary bladders examined were from three dead male donors who had consented to organ transplant donation or whose next of kin consented to donation of organs for research, and were purchased by a third party organ procurement agency (The International Institute for the Advancement of Medicine, Edison, NJ, USA). After the top, trigon, and mucosa of human urinary bladder were removed, a segment of proximal urethra was excised from a region 1-2 cm from the bladder neck. Connective tissue and the mucosa were removed by dissection. For isometric tension recording, strips approximately 10 mm in length were longitudinally or circumferentially prepared. The urethral smooth muscle strips were suspended in organ baths containing 25 mL of oxygenated Krebs-Henseleit solution (pH 7.4, 118.4 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 25.0 mmol/L $NaHCO_3$, and 11.1 mmol/L glucose) at 37°C. The tension of the strips was measured isometrically with a force displacement

transducer coupled to a carrier amplifier and digitally recorded using a PowerLab (ADInstruments, Dunedin, New Zealand).

3) Contractile response evaluation for human urethral smooth muscle strips

Urethral tissue under a loading tension of 0.5 g was allowed to equilibrate for approximately 1 h, after which KCl was added to an organ bath at 100 mmol/L to confirm contractility. After washing the tissue several times with Krebs-Henseleit solution and equilibrating for a further 30 min, a pre-contraction response for bradykinin (10 μmol/L) was obtained. Cumulative concentration-response curves for bradykinin (0.01-10 μmol/L) were obtained approximately 5 min after pretreatment with FK3657 (0.01-1 μmol/L) or its vehicle. The contractile responses were calculated as the percentage of bradykinin (10 μmol/L)-induced pre-contraction.

4) Measurement of intraurethral pressure in rats

All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma, Inc. Male Wistar rats (Charles River, Kanagawa, Japan; n=4) were anesthetized with urethane [1.2 g/kg, intraperitoneal (i.p.)]. A midline incision was made in the abdominal wall, and the urinary bladder was exposed. A 3.5-Fr sensor-tip transducer catheter (SPR-524; Millar Instruments. Inc., Houston, TX, USA) was inserted approximately 2 cm into the urethra through the superior aspect of the bladder so that the sensor-tip was located in the urethra of bladder neck (Figure 5), and the intraurethral pressure (IUP) was measured with a pressure amplifier (AP-601 G; Nihon Kohden, Tokyo, Japan) and digitally recorded using a PowerLab. For intravenous (i.v.) bolus injection, a polyethylene

catheter (PE-50) was inserted into a femoral vein. Following a stabilization period after surgery of at least 30 min, phenylephrine (30 μg/kg, i.v.) was injected to confirm an increase in IUP, and dose-response curves for bradykinin (0.003-0.1 μg/kg, i.v.) were confirmed. FK3657 (0.03-1 mg/kg, i.v.) was then injected at increasing doses with approximately 30-min intervals, and the dose-response curves for bradykinin were obtained again 5 min after FK3657 injection. All agents were intravenously administered at 1 mL/kg. Area under the curve (AUC) of IUP for 60 sec after administration of phenylephrine or bradykinin was measured with a PowerLab.

5) Surgical procedure and measurement of intraurethral pressure in dogs

Three male beagle dogs weighing 11.7-16.0 kg were anesthetized with pentobarbital sodium (30 mg/kg, i.v.), which was continuously infused (4-5 mg/kg/h) to maintain an anesthetic condition. After endotracheal intubation, animals were artificially ventilated using room air with a tidal volume of 20 mL/kg at a respiration rate of 20 breaths/min (respirator: SN-480-3; Shinano Co., Ltd., Tokyo, Japan). A midline abdominal incision was then made, and the urinary bladder was emptied using a catheter inserted into the bladder through its superior aspect to eliminate any potential effects of residual urine on IUP. A modified thermodilution balloon catheter (5-Fr; Nihon Kohden) was introduced into the urethra *via* the external urethral meatus. The balloon was then inflated with distilled water and placed in the prostatic urethra. The catheter was connected to pressure transducer (TP-400T; Nihon Kohden), and the IUP was measured using pressure amplifier (AP-601G; Nihon Kohden) and recorded on a paper chart (Figure 6). Another catheter was placed in a femoral vein for epinephrine, bradykinin, and

FK3657 administration. Measurements were taken following a stabilization period of at least 60 min after surgery. Epinephrine (3 μg/kg, i.v.) was injected to confirm an increase in IUP, and then the dose-dependent IUP response for bradykinin (0.3-10 μg/kg, i.v.) and inhibitory effects of FK3657 (10-300 μg/kg, i.v.) on IUP change induced by 3 μg/kg bradykinin were evaluated. All agents were intravenously administered at 1 mL/kg.

6) Data analysis

Results are presented as mean ± standard error of mean (S.E.M). Data were statistically analyzed using SAS version 8.2 (SAS Institute, Tokyo, Japan). Differences between the FK3657 and vehicle groups were analyzed using Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

1.3 Results

1) Bradykinin produced a contractile response in smooth muscle strips obtained from human urethra

Bradykinin (0.01-10 μmol/L) induced contraction of urethra isolated from human in a concentration-dependent manner (Figure 7). The maximal response in human urethra was approximately 37% of the KCl (100 mmol/L) response (1.2±0.3 g, n=15). The bradykinin B2 receptor antagonist FK3657 (0.01-1 μmol/L) inhibited bradykinin-induced contraction concentration-dependently.

2) Bradykinin induced an IUP elevation in rats which was reduced by FK3657

In anesthetized rats, intravenously administered bradykinin (0.003-1 μg/kg) dose-dependently increased IUP (Figure 8). The maximal response was close to that induced by the α1-adrenoceptor agonist phenylephrine (30 μg/kg, i.v., data not shown). FK3657 (0.03-1 mg/kg, i.v.) produced a rightward shift of the dose-response curve for bradykinin in rats.

3) Bradykinin induced an IUP elevation in dogs which was reduced by FK3657

In anesthetized dogs, intravenously administered bradykinin (0.3-10 μg/kg) dose-dependently increased IUP (Figure 9). Figure 9a shows a typical trace of epinephrine- and bradykinin-induced IUP elevation in dogs. The maximal response was close to that induced by epinephrine (3 μg/kg, i.v.), although the response induced by 3 μg/kg bradykinin was considered submaximal (Figure 9b). As such, the dose of 3 μg/kg bradykinin was adopted to examine the effects of FK3657 on IUP in dogs. FK3657 (0.3-300 μg/kg, i.v.) inhibited bradykinin-induced (3 μg/kg, i.v.) IUP elevation in a dose-proportional manner, with a significant

reduction observed at 30 and 300 μg/kg (Figure 10).

1.4 Discussion

In the present study, bradykinin concentration-dependently produced contractile responses in human urethral smooth muscle strips. The effect was subsequently inhibited by the bradykinin B2 receptor antagonist FK3657, suggesting that stimulation of bradykinin B2 receptors might contribute to the bradykinin-induced contractile response in human urethral smooth muscle. In addition, intravenous injections of bradykinin induced dose-dependent increases in IUP in both rats and dogs, with maximal responses closely resembling those induced by α1-adrenoceptor stimuli. The increases in IUP induced by bradykinin were also inhibited by FK3657 in a dose-dependent manner, which indicates that the contractile responses induced by bradykinin *via* bradykinin B2 receptor lead to increases in IUP in animals. These results are the first evidence demonstrating bradykinin and bradykinin B2 receptor involvement in urethral function in humans and animals, suggesting that bradykinin may induce an increase in IUP in humans *via* stimulation of bradykinin B2 receptors and potentially contributing to regulation of urethral resistance in humans.

Bradykinin and bradykinin B2 receptor has been reported in the lower urinary tract in both neural and nonneural components and also reported to play an important role in normal and pathological conditions of urinary bladder function (Lecci et al., 1995, Maggi et al., 1993), as activation of the bradykinin B2 receptor stimulates detrusor muscle contractility and evokes bladder hyperreflexia (Lecci et al., 1995, Meini et al., 2000). These effects of bradykinin on bladder contractility may also occur *via* direct activation of pelvic afferent fibers (Lecci et al., 1995). Indeed, bradykinin B2 receptor antagonists have been reported to be effective in bladder overactivity in a model of chemically-induced cystitis (Chopra et al., 2005,

Maggi et al., 1993). However, little information is available on the pathophysiological roles of bradykinin in the lower urinary tract in humans or regarding *in vivo* urethral function in animals. While the present study demonstrated the contracting effect of bradykinin *via* its B2 receptors in human and animal urethral tissue, further studies will be required to clarify the functional roles of bradykinin in regulation of inflammatory and pain systems, including afferent C fiber activity, in the lower urinary tract.

Recent *in vitro*, *in vivo*, and clinical studies have uncovered the crucial role of prostatic inflammation in BPH pathogenesis and progression, suggesting that infiltrating inflammatory cells might act as a link between hormonal changes and the remodeling process promoted by growth factors (Chughtai et al., 2011). Specifically, hormonal changes may induce an increase in inflammatory infiltrates in the prostate responsible for tissue damage at both the epithelial and stromal cellular levels, initiating a chronic process of wound healing which, in turn, might lead to prostatic enlargement (Sampson et al., 2007). Further, the prevalence of chronic prostatic inflammation in patients with LUTS associated with BPH as estimated in the REDUCE trial was 77.6% of 8,224 patients (Nickel et al., 2008). As mentioned above, bradykinin release is triggered by protease activation at the site of inflammation/tissue damage, and bradykinin has been detected in human prostate tissue as well as in conditioned medium of human prostate-derived stromal cells in culture (Walden et al., 1999). The present study showed the role of bradykinin in urethral function. Taken together, these previous findings and this study suggest that continuous release of bradykinin under conditions of chronic prostatic inflammation may be involved in voiding dysfunction in BPH patients who have prostatic inflammation. Further studies will be required to determine

levels of bradykinin in the prostate and the direct involvement of bradykinin in voiding dysfunction in patients with BPH.

Here, I determined that extrinsic bradykinin induced contractile responses in human urethra and increases in IUP in rats and dogs but didn't uncover the role of intrinsic bradykinin in urethral function in patients with voiding dysfunction. Also this study showed the role of bradykinin in urethral function only in male, and therefore further studies will be required for its role in urethral function in female. Although the experimental conditions used in this study may differ from those of clinical settings involving patients, our present findings suggest that inhibition of bradykinin-bradykinin B2 receptor signaling may be a novel target for treating voiding dysfunction associated with BPH in which an increase in intraprostatic bradykinin level is indicated.

The present study provides evidence that bradykinin elicits urethral smooth muscle contraction via bradykinin B2 receptor, suggesting that bradykinin B2 receptor antagonists may be useful in treating voiding dysfunction.

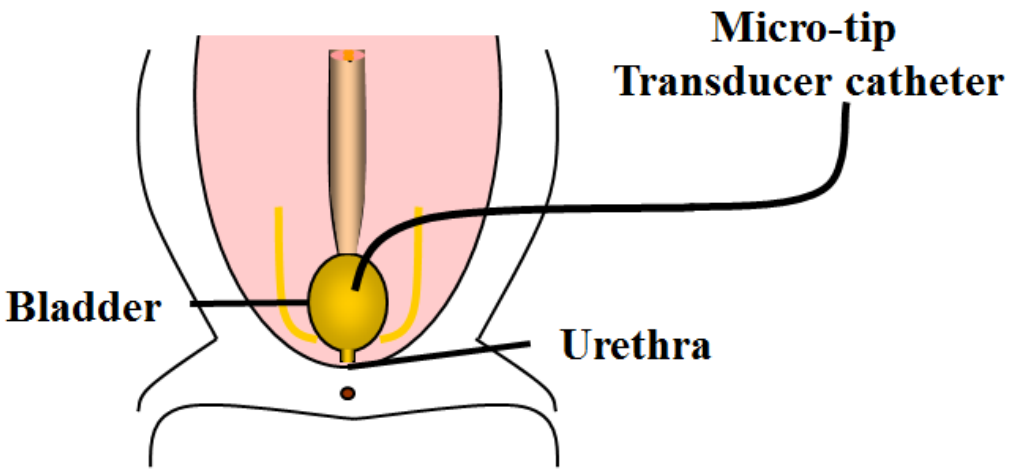


Figure 5. The way to insert the micro-tip transducer catheter from bladder to urethra

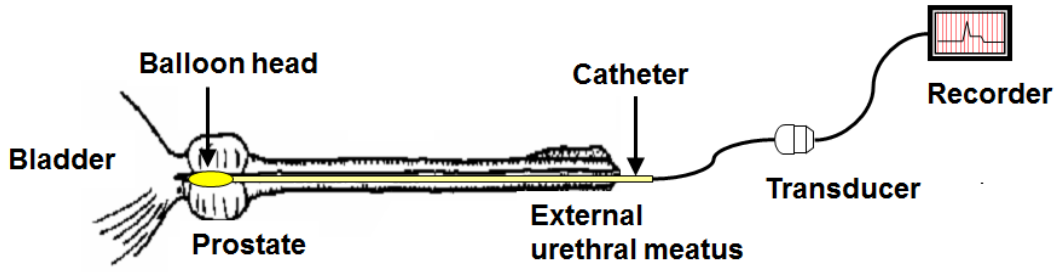


Figure 6. The way to measure IUP in anesthetized dogs

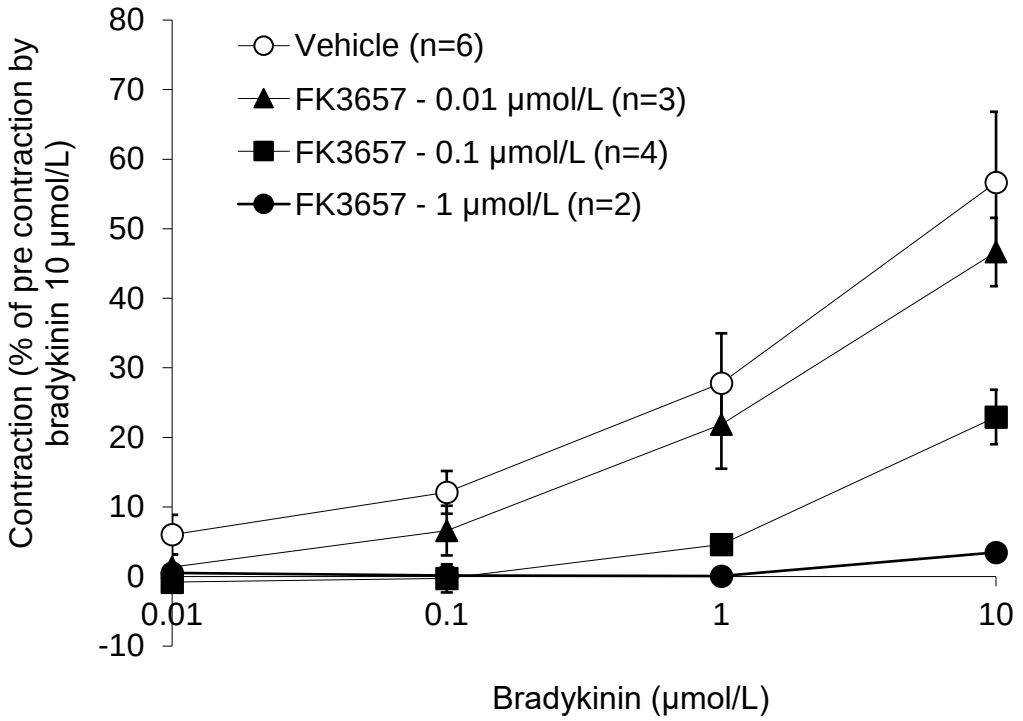


Figure 7. Concentration-dependent contraction induced by bradykinin (0.01-10 µmol/L) in isolated human urethra and its inhibition by FK3657 (0.01-1 µmol/L) (mean ± S.E.M., n=2-6). Results are expressed as the percentage of bradykinin-induced pre-contraction (10 µmol/L).

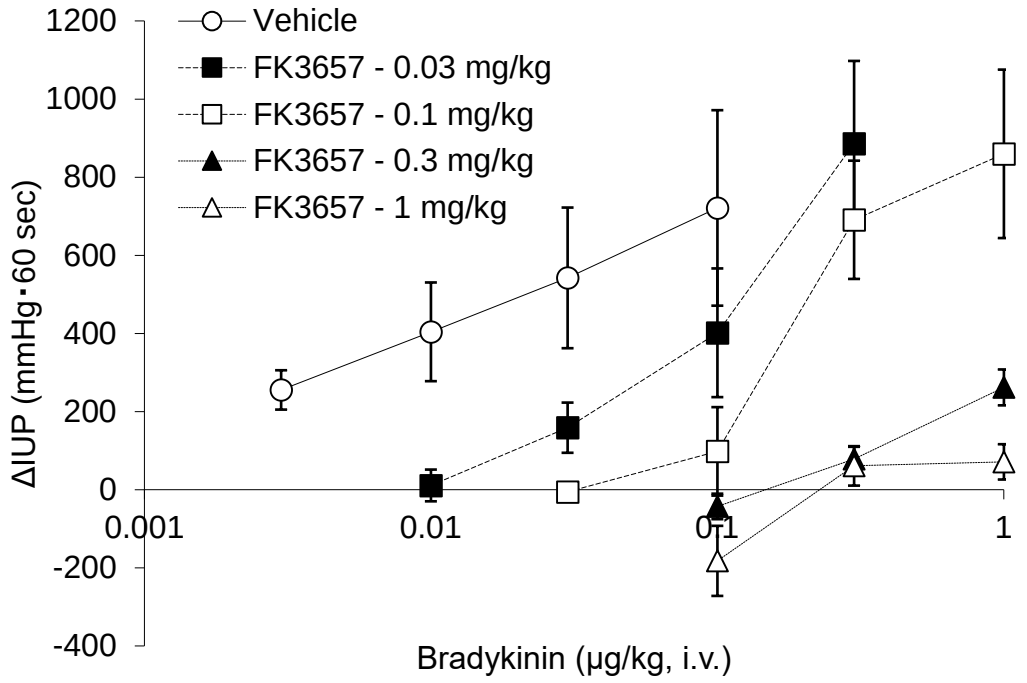
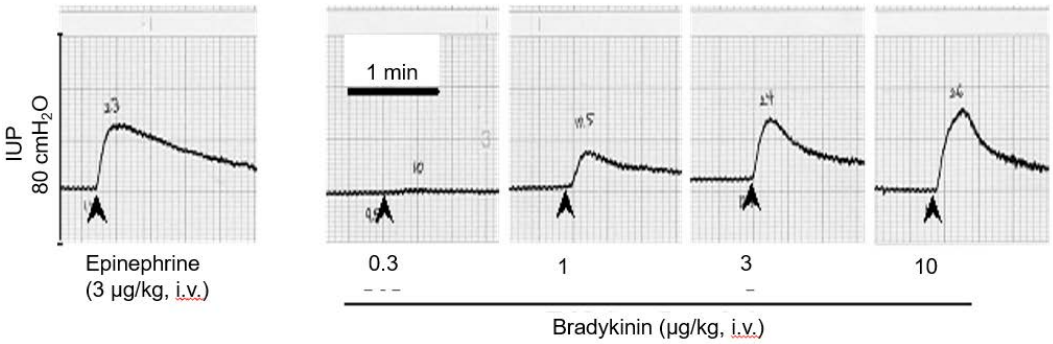


Figure 8. Dose-dependent increases in rat IUP induced by bradykinin (0.003-1 μg/kg, i.v.) and the inhibitory effect of FK3657 (0.03-1 mg/kg, i.v.) on bradykinin-induced IUP increase in rats (mean ± S.E.M., n=4). Results are expressed as the per-minute change in IUP before and after FK3657 administration.

(a)



(b)

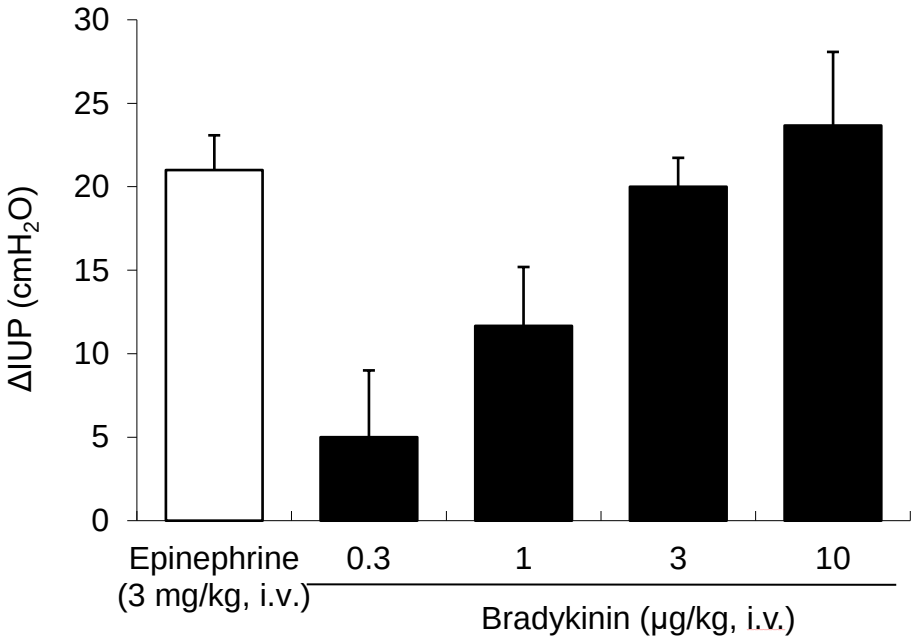


Figure 9. Dose-dependent increases in dog IUP induced by bradykinin (0.3-10 μg/kg, i.v.) compared with that induced by epinephrine (3 μg/kg, i.v.); (a) typical traces of epinephrine- and bradykinin-induced IUP elevation in dogs, (b) results are expressed as the change in IUP before and after administration (mean ± S.E.M., n=3).

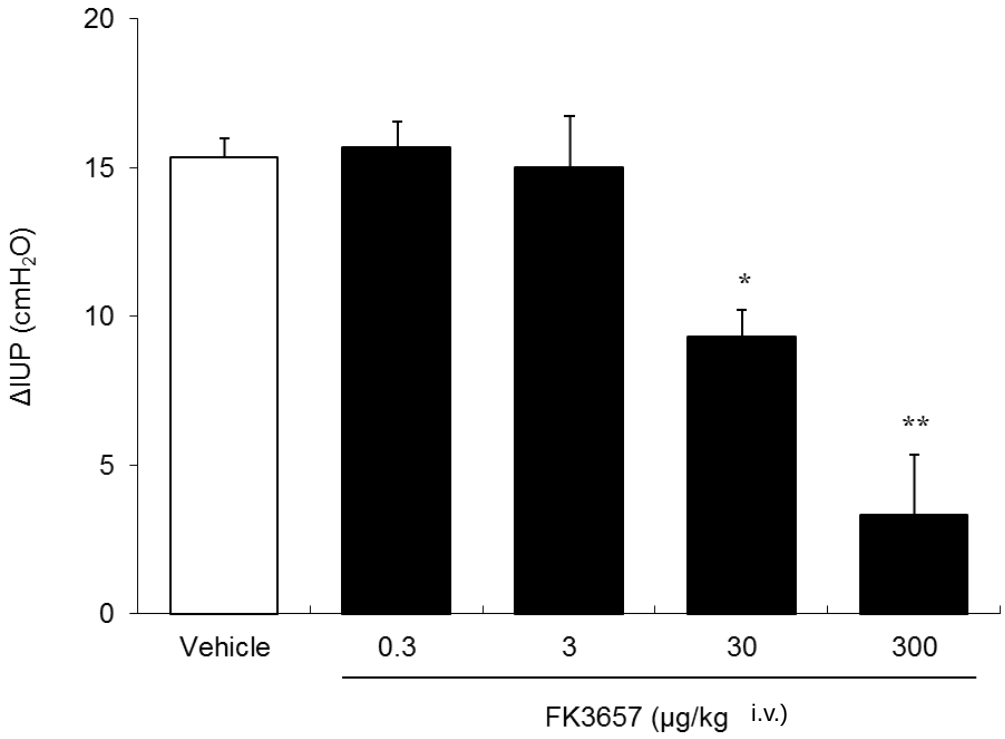


Figure 10. Inhibitory effect of FK3657 (0.3-300 μg/kg, i.v.) on bradykinin (3 μg/kg, i.v.)-induced IUP increase in dogs. Results are expressed as the change in IUP before and after FK3657 administration (mean ± S.E.M., n=3). * $P < 0.05$, ** $P < 0.01$ *vs*. vehicle group by Dunnet's multiple comparison test.

Chapter 2 Intratesticular Bradykinin Involvement in Rat Testicular Pain Models

2.1 Background

Chronic urogenital pain is a severe problem in urology, and chronic pelvic pain (CPP) disorders have been observed in a number of pelvic organs, possibly representing viscerovisceral referred pain. Symptoms of CPP disorders vary; for example, interstitial cystitis is characterized by suprapubic pain and increased urinary frequency and urgency (Teichman and Parsons, 2007), while CP/CPPS is characterized by pain across several organs, including perineum, penis, and lower abdomen, with discomfort during voiding or ejaculation. Some 40-50% of patients with CP/CPPS have reported pain or discomfort in the testes (Litwin et al., 1999, Schaeffer et al., 2002). CP/CPPS is also often accompanied by increased urinary frequency (Krieger et al., 1996). Several potential causes of these symptoms have been suggested, including abnormal activation of afferent nerves in the pelvis, neurogenic inflammation of pelvic nerves, pelvic floor spasticity, pelvic organ ischemia, and autoimmune reactions. However, the etiology remains uncertain, and no effective treatment has yet been developed.

In addition to the genitourinary function of bradykinin described before, bradykinin involvement in the regulation of bladder function has been proven in studies demonstrating that both B1 and B2 receptor antagonists can reduce bladder overactivity in a rat model of cyclophosphamide-induced cystitis (Chopra et al., 2005) and in a rat model of spinal cord injury (Forner et al., 2012). These previous findings indicate that bradykinin can evoke an inflammatory response and changes in the urinary bladder reflex either or both by directly activating B2 receptors on afferent fibers or indirectly by releasing ATP and other neurotransmitters of the urothelium (Chopra et al., 2005).

While bradykinin has been recognized as a modulator of bladder smooth

muscle tone and as a prototypical mediator of increased pain sensation in both inflammatory and non-inflammatory bladder dysfunction, to our knowledge, relatively few studies have evaluated the role of bradykinin in urogenital pain. A rat model of testicular pain induced by injection of diluted acetic acid into the testes was recently established (Yoshioka et al., 2010). However, bradykinin involvement in testicular pain as well as this rat model has not yet been examined.

Here, in order to evaluate bradykinin involvement in rat models of testicular pain, I examined whether or not an injection of bradykinin into the testes induces pain behaviors. I then assessed whether or not those pain behaviors, if present, might be inhibited by the bradykinin B2 receptor antagonist FK3657. In addition, I also examined the analgesic effect of FK3657 in a rat model of acetic acid-induced testicular pain and measured intratesticular concentration of bradykinin at several time points after acetic acid injection.

2.2 Materials and Methods

1) Animals

Male Wistar rats (Charles River, Kanagawa, Japan) were used. For behavioral tests and intratesticular bradykinin measurement, rats weighing 95-180 g were used. All rats for behavioral tests were fasted the evening before experiments. All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma, Inc., and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2) Materials

Bradykinin was purchased from PEPTIDE INSTITUTE, INC. (Osaka, Japan) and diluted with distilled water (DW). Acetic acid was purchased from Kanto Chemical (Tokyo, Japan) and diluted with DW. FK3657 ((*E)*-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl]acrylamide) synthesized at Astellas Pharma, Inc. (Tokyo, Japan), was dissolved in 0.1 N HCl and then diluted with DW.

3) Testicular pain model

The behavioral test was performed in accordance with the method reported by Yoshioka et al. (Yoshioka et al., 2010). Briefly, a 27-gauge needle and 1-mL syringe were used to administer aqueous solutions of 0.1, 0.3, 1, 3, or 10 mmol/L bradykinin, aqueous solution of 1% acetic acid, or DW into the center of both the left and right testes (1 mL/kg each) of rats that were under conscious, but restrained, conditions. Rats were placed individually into a clear, round, plastic container

(diameter: 24.5 cm, height: 30 cm) for observation immediately after the injection. The following behavior was scored as a pain response: contraction of oblique musculature with inward flexion of the hind limb, stretching of the body, and flattening of the lower abdomen against the floor (Figure 11). In the time-course experiment, the number of pain responses 0-10 and 10-20 min after injection of bradykinin (0.1, 0.3, 1, 3, or 10 mmol/L) or DW were counted (n=4-6). In the evaluation of FK3657 pretreatment effect, only the number of responses from 0-10 min after 1 mmol/L bradykinin injection or from 5-20 min after 1% acetic acid injection was counted (n=6-8 for bradykinin injection experiment, n=7-15 for acetic acid). FK3657 (1, 3, 10, or 30 mg/kg) or its vehicle (0.1 N HCl) was orally administered at 5 mL/kg 15 min before the injection of bradykinin or 10 min before acetic acid (Asano et al., 1997, Asano et al., 1999).

4) Intratesticular bradykinin measurement

To measure intratesticular bradykinin level, rats were sacrificed by exsanguination 5, 20, 40, or 60 min after the testicular injection of 1% acetic acid or DW (n=4). Immediately after laparotomy, testes were harvested, weighed, and frozen with dry ice. Frozen testes were then prepared in liquid nitrogen and crushed into powder by applying pulsed pressure using a Cryo-Press (Microtec Co., Ltd., Chiba, Japan). Crushed testes were deproteinized with 200 mg/mL trichloroacetic acid and then homogenized in the T-PER® Tissue Protein Extraction Reagent (Thermo Fisher Scientific KK, Kanagawa, Japan). After centrifugation, collected supernatant was neutralized, and the bradykinin concentration of each sample was measured using a MARKIT®-M Bradykinin ELISA assay kit (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Results were presented as the amount of

bradykinin per wet weight of the testis.

5) Data analysis

Results are presented as mean ± standard error of mean (S.E.M). Data were statistically analyzed using SAS version 8.2 (SAS Institute, Tokyo, Japan) or GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Differences in pain responses between the FK3657 and vehicle groups were analyzed using Dunnett's multiple comparison test, and differences in bradykinin concentrations in testes between the 1% acetic acid and DW groups at 20 min after injection were analyzed using Student's t-test. $P \leq 0.05$ was considered statistically significant.

2.3 Results

1) Intratesticular bradykinin injection induced pain behaviors

Rats injected with aqueous solutions of 0.1, 0.3, 1, 3, or 10 mmol/L bradykinin in testes showed characteristic pain behaviors (Yoshioka et al., 2010), such as contraction of oblique musculature with inward flexion of the hind limbs, stretching of the body, and flattening of the lower abdomen against the floor. These behaviors appeared soon after injection, gradually diminishing over time, and were proportional to the concentration of bradykinin administered (0.1 mmol/L: 6.8±3.1, 0.3 mmol/L: 5.6±1.4, 1 mmol/L: 9.5±1.5, 3 mmol/L: 10.0±0.7, 10 mmol/L: 6.2±2.9, Figure 12a). Injections of DW produced no responses (data not shown). Based on these findings, evaluation of the inhibitory effect by FK3657 was performed from 0 to 10 minutes after injection of aqueous solution of 1 mmol/L bradykinin.

2) FK3657 reduced intratesticular bradykinin injection-induced pain behaviors

Oral administration of FK3657 dose-proportionally inhibited the pain behaviors induced by intratesticular injection of aqueous solution of 1 mmol/L bradykinin, with significant reduction observed at doses of 3, 10, and 30 mg/kg, respectively (3 mg/kg: 6.3±0.9 ($P < 0.05$), 10 mg/kg: 4.2±1.6 ($P < 0.01$), 30 mg/kg: 2.0±0.7 ($P < 0.01$), Figure 13).

3) FK3657 reduced intratesticular acetic acid injection-induced pain behaviors

Oral administration of FK3657 dose-proportionally inhibited the pain behaviors induced by intratesticular injection of 1% acetic acid, with significant reduction observed at doses of 10 and 30 mg/kg (10 mg/kg: 4.0±1.7 ($P < 0.01$), 30 mg/kg: 1.0±0.7 ($P < 0.01$), Figure 14).

4) Intratesticular acetic acid injection induced increases in intratesticular bradykinin

Increases in intratesticular bradykinin concentration were detected soon after acetic acid injection, and further elevation was observed at 20 min after injection. Elevated bradykinin levels diminished to the level observed with DW injection within 40 min (at 20 min, 1% acetic acid: 2.64-3.64 ng/g-tissue, DW: 1.08-1.37 ng/g-tissue, Figure 15).

2.4 Discussion

In the present study, injection of aqueous solution of bradykinin into the testes of conscious rats produced characteristic pain behaviors (Yoshioka et al., 2010), namely contraction of oblique musculature with inward flexion of the hind limb, stretching of the body, and flattening of the lower abdomen against the floor. Similar behaviors have been reported in other pain models, such as the artificial ureteral calculosis model, the visceral pain model induced by intracolonic administration of mustard oil or capsaicin, writhing tests, and the testicular pain model induced by intratesticular injection of acetic acid (Yoshioka et al., 2010, Giamberardino et al., 1995, Laird et al., 2001, Ikeda et al., 2001). The frequency of these bradykinin-induced behaviors was reduced dose-proportionally by FK3657, indicating that the pain behaviors were caused by bradykinin *via* its B2 receptor. In addition, FK3657 dose-dependently inhibited the pain responses induced by intratesticular injection of 1% acetic acid, and the fact that intratesticular bradykinin was released after its injection supported the exertion of its inhibitory effect via bradykinin B2 receptor. I preliminarily confirmed that the doses of FK3657 tested in the present study were well-tolerated in rats even after multiple administrations for 13 weeks (data not shown). The dose and timing of FK3657 administration were determined based on the previous studies, its pharmacokinetics (Tmax shorter than 30 min, data not shown) and the observation period of pain behavior in these models. Indeed, pretreatment is not realistic in clinical setting and further studies will be required to investigate a therapeutic effect of FK3657.

Writhing tests with intraperitoneal injection of acetic acid in rodents have been widely used to evaluate efficacy of analgesics against visceral pain. The pain behaviors induced by intratesticular injection of 1% acetic acid were recently

confirmed and found to be reduced by pretreatment with indomethacin and capsaicin, which indicates that the behaviors may reflect inflammatory pain associated with prostaglandins and are mediated by primary afferent C fibers (Yoshioka et al., 2010). In addition, the testes had obvious tissue damage and increased weight after 1% acetic acid injection, suggesting that inflammation occurred during the observation period. Protease activation at the site of inflammation/tissue damage cleaves tissue/plasma kininogen precursors to release bradykinin (Calixto et al., 2000, Dray and Perkins, 1993), which has been recognized as potent stimulator of afferent nerve fibers and probably the most potent endogenous algesic substance (Juan and Lembeck, 1974). I demonstrated a significant inhibitory effect of FK3657 in our model of acute inflammatory pain associated with activation of afferent C fibers, which was supported by the fact that most of the acute inflammatory effects of bradykinin, including pain, were considered to be induced via activation of bradykinin B2 receptors (Bathon and Proud, 1991, Hall, 1992). In addition, inhibitory effects of bradykinin B2 receptor antagonists against bladder overactivity as well as pain response were reported in a rat model of cystitis previously (Chopra et al., 2005, Maggi et al., 1993). Indeed, intratesticular injection of 1% acetic acid induced bladder overactivity as well (Yoshioka et al., 2010) but I didn't examine the effect of bradykinin B2 receptor antagonist on it nor check whether intratesticular injection of bradykinin would affect the bladder function. Further studies will be required to determine the levels of bradykinin not only in the testes but also in the prostate and the involvement of bradykinin in regulation of pain or bladder overactivity in patients with CP/CPPS.

CP/CPPS, Category III prostatitis (NIH classification) constitutes the vast majority (more than 90%) of cases and is further divided into IIIA and IIIB

subcategories, based on respective presence or absence of evidence of inflammation (white blood cell counts in urine specimen after prostate massage, seminal plasma, or expressed prostatic secretions) (Krieger et al., 1999). Prostatic inflammation has been reported in 33% of patients with CP/CPPS who underwent transperineal prostate biopsy (True et al., 1999), and abnormal levels of inflammatory cytokines have also been reported (Pontari and Ruggieri, 2004). Such chronic inflammation is considered to enhance the activity of afferent C fibers, including pain which is the symptom that distinguishes CP/CPPS from other voiding dysfunctions (Krieger et al., 1996). Given that bradykinin has been detected in human prostate tissue as well as in the conditioned medium of human prostate-derived stromal cells in culture (Walden et al., 1999), elevated bradykinin levels in the prostate due to chronic inflammation likely contribute to pain in patients with CP/CPPS. The pain behavior induced by intratesticular injection of 1 mmol/L bradykinin disappeared at 20 min and that by 1% acetic acid at 1 h, from which these models were considered acute urogenital pain models. I didn't examine the effect of bradykinin B2 receptor antagonist in chronic inflammatory urogenital pain model which is considered closer to the state of the patients with CP/CPPS. Further studies will be required to determine whether bradykinin B2 receptor antagonist is effective for pain in patients with CP/CPPS.

I described the acute analgesic responses of bradykinin B2 receptor antagonist against testicular damage and inflammation in rats but didn't uncover its effect on chronic inflammatory pain nor on bladder overactivity associated with testicular pain. Although the experimental conditions used in this study may differ from those of clinical settings involving patients, my present findings suggest that the inhibition of bradykinin-bradykinin B2 receptor signaling may be a novel target

for treating urogenital pain in CP/CPPS in which an increase in intraprostatic bradykinin level is indicated.

Here, I found that intratesticular bradykinin evokes pain behavior *via* stimulation of bradykinin B2 receptors and that intratesticular acetic acid injection induces intratesticular bradykinin synthesis, consequently leading to pain behavior. These findings suggest that the potential utility of bradykinin B2 receptor antagonists as a novel target for treating urogenital pain.

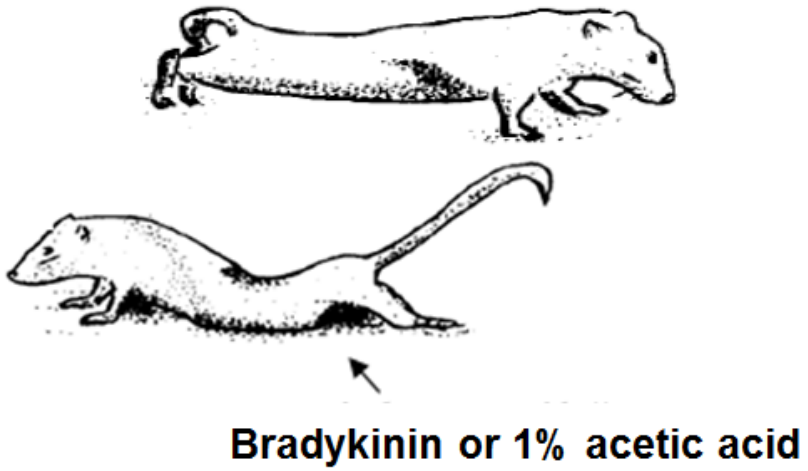


Figure 11. Typical pain behaviors observed after intratesticular injection of bradykinin or 1% acetic acid

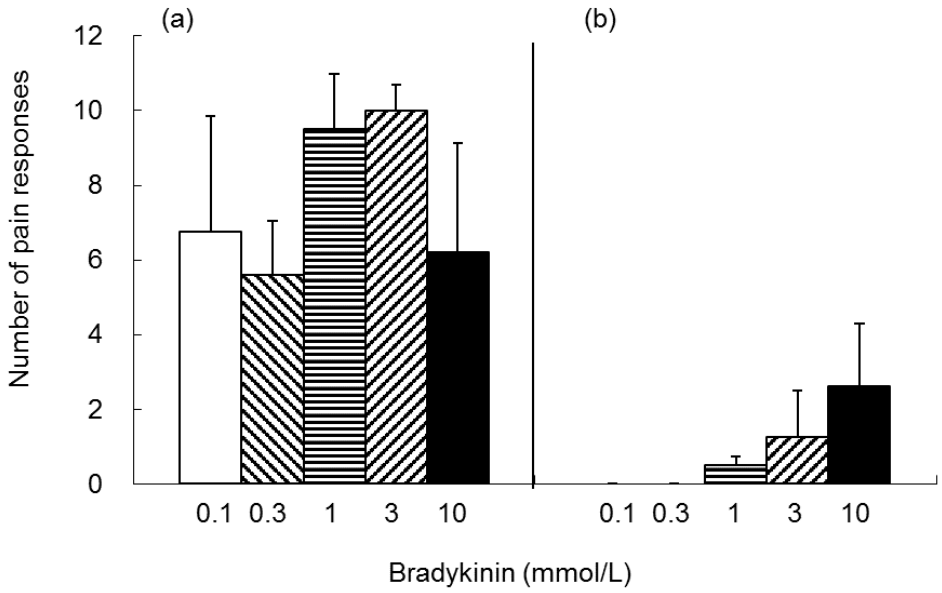


Figure 12. Number of pain behaviors (mean ± S.E.M., n=4-6); (a) 0-10 min and (b) 10-20 min after testicular injection of aqueous solution of 0.1, 0.3, 1, 3, or 10 mmol/L bradykinin.

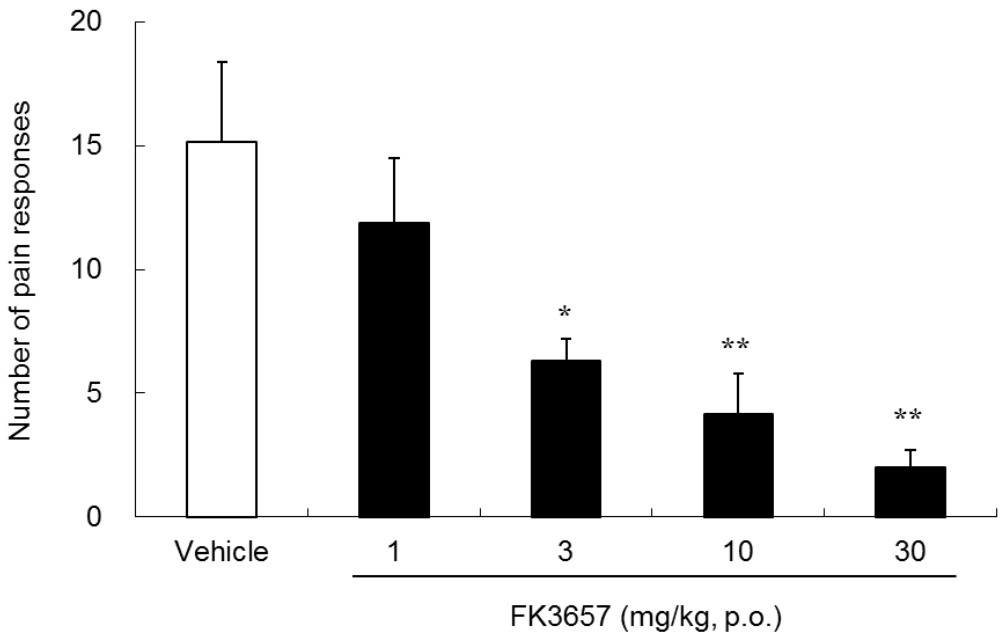


Figure 13. Number of pain behaviors (mean ± S.E.M., n=6-8) 0-10 min after intratesticular injection of aqueous solution of 1 mmol/L bradykinin under pretreatment with either FK3657 [1, 3, 10, or 30 mg/kg per os (p.o.)] or its vehicle 15 min before bradykinin injection. * $P < 0.05$, ** $P < 0.01$ *vs.* vehicle group by Dunnet's multiple comparison test.

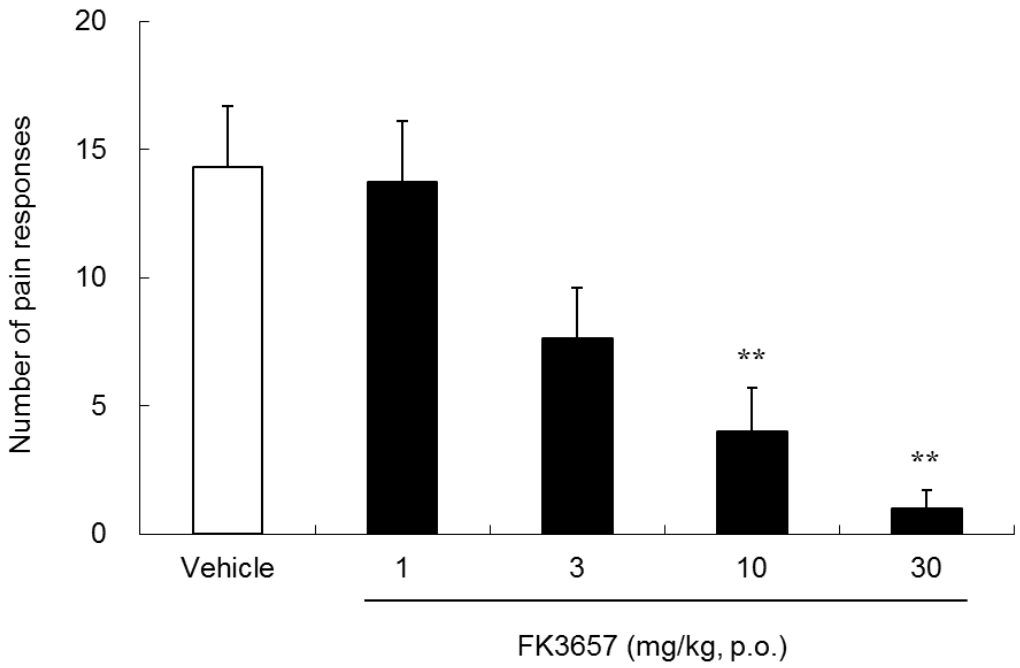


Figure 14. Number of pain behaviors (mean ± S.E.M., n=7-15) 5-20 min after intratesticular injection of 1% acetic acid under pretreatment with either FK3657 (1, 3, 10, or 30 mg/kg p.o.) or its vehicle 10 min before acetic acid injection.

** $P < 0.01$ *vs*. vehicle group by Dunnet's multiple comparison test.

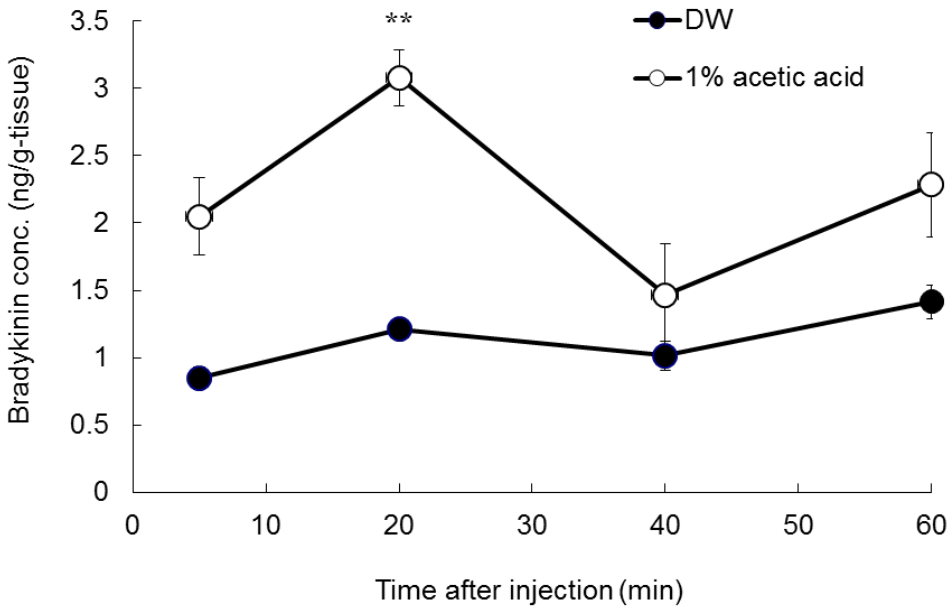


Figure 15 Concentration of intratesticular bradykinin (ng/g-tissue, mean ± S.E.M., n=4) at 5, 20, 40, and 60 min after injection of 1% acetic acid or DW. Statistical analysis was only performed at 20 min after injection. ** $P < 0.01$ *vs* DW group by Student's t-test.

Conclusions

In the present study, bradykinin elicited human urethral smooth muscle contraction and an increase in urethral pressure in anesthetized rats and dogs through the bradykinin B2 receptor. Furthermore, bradykinin, which was produced in the testes by testicular injection of bradykinin or acetic acid, elicited pain behaviors in rats through the bradykinin B2 receptor. Therefore, these findings suggest that bradykinin is involved in LUTS such as voiding symptoms and urogenital pain and potential utility of bradykinin B2 receptor antagonists as a novel target for treating LUTS.

Generation or formation of BPH has not been fully elucidated. In addition, prostate weight and the degree of voiding dysfunction are not necessarily proportional in the actual clinical settings and it is considered that its generation or the progress of symptoms is affected by a number of factors. Especially from the fact that infiltration of inflammatory cells is frequently found in the prostate of symptomatic BPH patients, chronic inflammation as a factor other than the androgen is also believed to play an important role in the induction of BPH or symptoms. BPH patients have been reported to have 7.7-folds history of prostatitis (Collins et al., 2002). It has been reported that about 20% of BPH patients showed ejaculation pain or discomfort and patients with symptoms have a strong degree of LUTS (Nickel et al., 2005). Also, it is known that prostatitis as well as BPH are common in the elderly (Pontari, 2003). Therefore, it has become clearer from some relevant reports that there is some relation between prostatitis and LUTS or BPH.

Prostatitis conventionally been considered syndrome consist of a number of etiologies rather than a single disease. In particular, category III is classified as CP/CPPS and category IV as asymptomatic inflammatory prostatitis (Krieger et al., 1999). Conventionally this category IV was not considered a clinical problem nor

treatment target. However, it has been found that a presence of inflammation is associated with the progression and urinary symptoms of BPH in some large-scale clinical trials such as “Reduction by Dutasteride of Prostate Cancer Events (REDUCE)” trial and “Medical Therapy of Prostatic Symptoms (MTOPS)” trial. For example, in the REDUCE trial, histological chronic inflammation has been reported in approximately 78% of BPH patients regardless of the symptoms associated with chronic prostatitis (Nickel et al., 2007, Nickel et al., 2008). In addition, in the investigation over the average 4.5-years follow-up in MTOPS trial, the rate at which BPH patients lead to acute urinary retention was significantly higher in the group with inflammation. In other words, the group with histological inflammation tends to progress symptoms and the presence of inflammation is considered to be a predictor of BPH progression (Crawford et al., 2006). On the other hand, the pathological analysis of the excised tissue of symptomatic BPH patients without inflammation who underwent trans-urethral resection (TURP) of the prostate showed infiltration of inflammatory cells in almost all cases (Nickel et al., 1999). From these reports, it would be more or less true that the category IV prostatitis would be involved in manifestations of BPH.

Changes in molecular background associated with chronic prostatitis are considered to play an important role in the mechanism of manifestation caused by BPH. Etiology of asymptomatic prostatitis is considered possibility of infection of bacteria or viruses, allergic reaction mechanisms caused by stimulation of semen or urine and induction of inflammatory cells by estrogen. In recent years, BPH is also considered as localized autoimmune diseases, that is, weakening of the immune system due to aging or changes in the hormonal environment causes a reduced function of suppressor cells, infiltration of inflammatory cells and then chronic

inflammation. Chronic inflammation would repeat tissue damages, healing and regeneration and lead to an increase in prostate nodule and at the same time, transformation of stroma into some form of tissue may induce symptomatic BPH (Kramer et al., 2007, Untergasser et al., 2005) (Figure 16).

It is possible in the future that new therapeutic strategies for LUTS associated with BPH are established by further elucidating the meaning of inflammation in the prostate from molecular biology perspective. In the present study, I focused on bradykinin which is known to be produced upon the inflammation or tissue damage and examined its involvement in LUTS such as voiding symptoms or urogenital pain. There is also a report that bradykinin is involved in proliferation of prostate stromal cells (Walden et al., 1999), and thus bradykinin produced by inflammation in the prostate may be involved in the manifestation and progression of BPH. These speculations suggest that bradykinin B2 receptor antagonists would be useful for an inhibition of the progression of BPH as well as for treatment of LUTS such as voiding symptoms or urogenital pain (Figures 17, 18).

As the bradykinin B2 receptor antagonists, Icatibant (Hoe-140), peptide formulation (injection), has been launched for an indication of hereditary angioedema, a rare disease, but oral drugs of low molecule (non-peptide) have not yet been available for any indications including urology or genital diseases. Currently, α1-blockers are used for drug therapy of BPH as standard of care but 5α-reductase inhibitors are added on top of α1-blockers when the therapeutic effect of the blocker is insufficient and when prostate volume is greater than usual. Recently, PED5 inhibitors were also launched as a new alternative choice but a single therapeutic agent useful for suppression of pathology progression as well as LUTS

has not yet been obtained. As for the treatment for CP/CPPS, any therapeutic agent has not been available and further research or development is expected in the future considering its high unmet medical needs. In addition to testicular pain models examined in my study, there are many reports that bradykinin B2 receptor antagonists are effective in variety of pain models in animals. Based on such a background, the present study is expected to contribute to providing a new therapeutic option or possibility for the future of urology and genital disease treatment or even broader therapeutic areas.

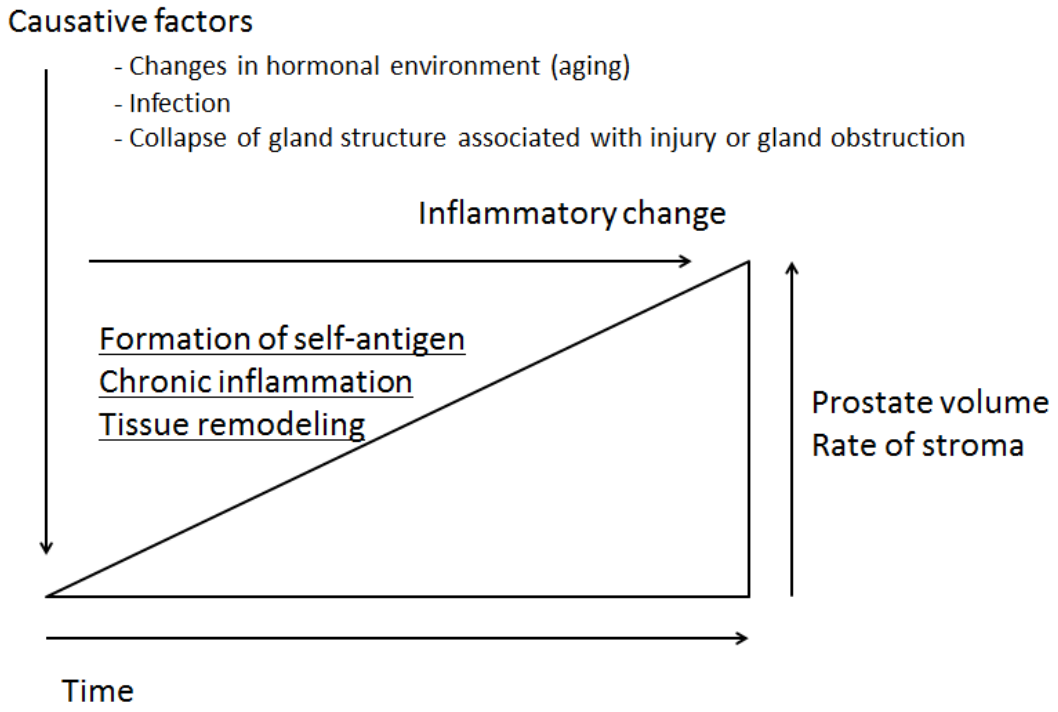


Figure 16. Hypothesis about relation between inflammation (immune response) and prostatic proliferation (Kramer et al., 2007)

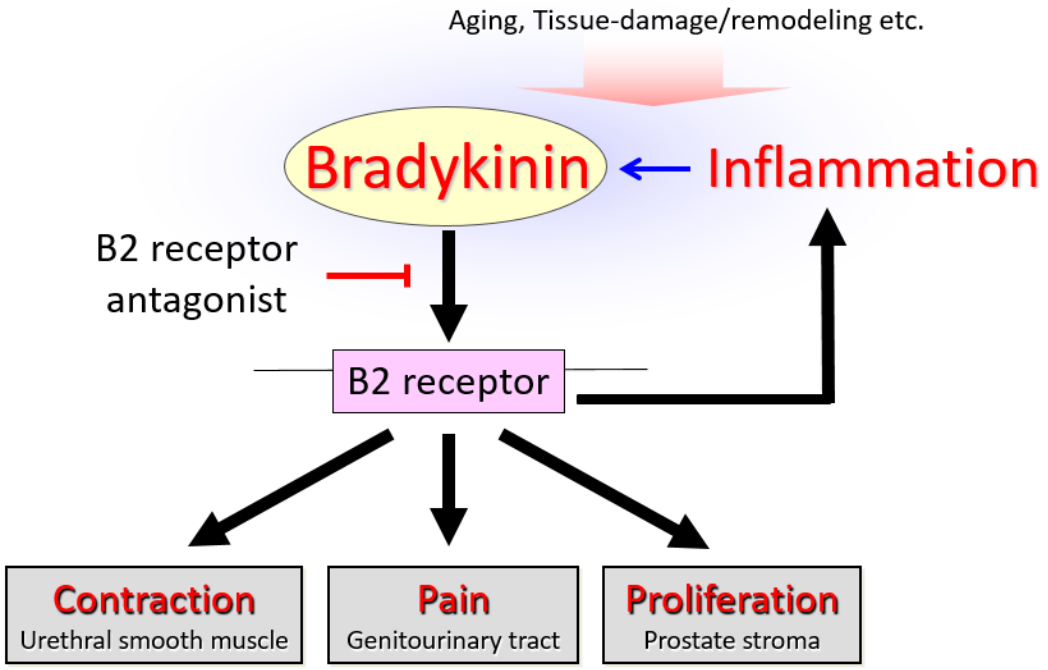


Figure 17. Bradykinin induces urethral smooth muscle contraction, pain in genitourinary tract, proliferation in prostate stroma and inflammation *via* bradykinin B2 receptors

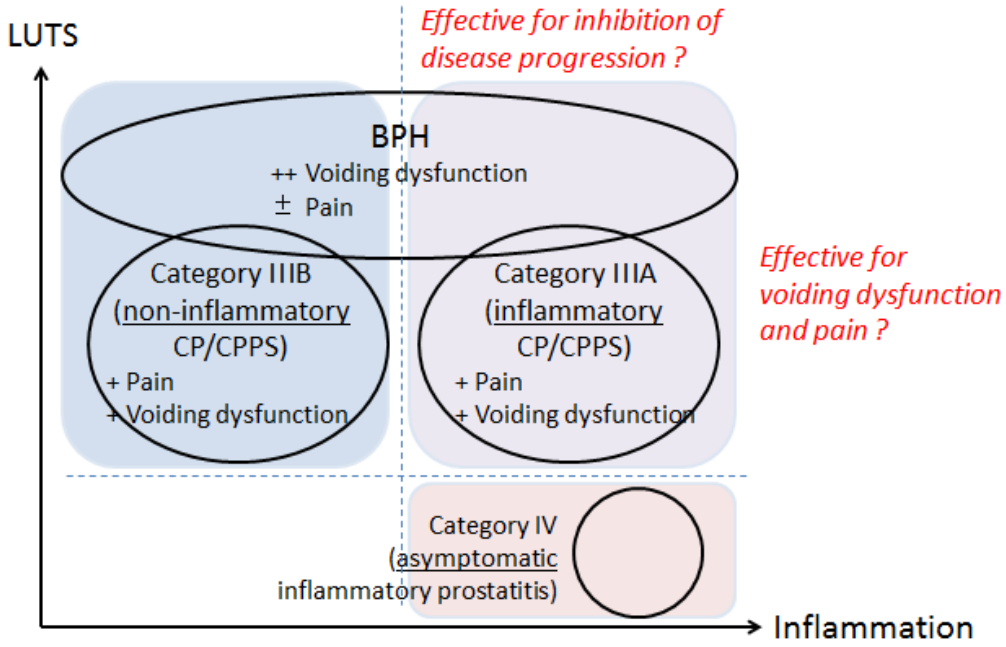


Figure 18. Potential utility of bradykinin B2 receptor antagonists

References

ABDEL-HAKIM, A., HASSOUNA, M., RIOUX, F., ST-PIERRE, S., ABDEL-RAHMAN, M., GALEANO, C. & ELHILALI, M. 1983. Response of urethral smooth muscles to pharmacological agents. II. Noncholinergic, nonadrenergic agonists and antagonists. *J Urol,* 130**,** 988-91.

ABRAMS, P., CARDOZO, L., FALL, M., GRIFFITHS, D., ROSIER, P., ULMSTEN, U., VAN KERREBROECK, P., VICTOR, A. & WEIN, A. 2002. The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Neurourol Urodyn,* 21**,** 167-78.

AHLUWALIA, A. & PERRETTI, M. 1999. B1 receptors as a new inflammatory target. Could this B the 1? *Trends Pharmacol Sci,* 20**,** 100-4.

ALCARAZ, A., HAMMERER, P., TUBARO, A., SCHRODER, F. H. & CASTRO, R. 2009. Is there evidence of a relationship between benign prostatic hyperplasia and prostate cancer? Findings of a literature review. *Eur Urol*, 55**,** 864-73.

ANDERSSON, K. E., DE GROAT, W. C., MCVARY, K. T., LUE, T. F., MAGGI, M., ROEHRBORN, C. G., WYNDAELE, J. J., MELBY, T. & VIKTRUP, L. 2011. Tadalafil for the treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia: pathophysiology and mechanism(s) of action. *Neurourol Urodyn,* 30**,** 292-301.

ARAKI, I., ZAKOJI, H., KOMURO, M., FURUYA, Y., FUKASAWA, M., TAKIHANA, Y. & TAKEDA, M. 2003. Lower urinary tract symptoms in men and women without underlying disease causing micturition disorder: a

cross-sectional study assessing the natural history of bladder function. *J Urol,* 170**,** 1901-4.

ASANO, M., INAMURA, N., HATORI, C., SAWAI, H., FUJIWARA, T., ABE, Y., KAYAKIRI, H., SATOH, S., OKU, T. & NAKAHARA, K. 1999. Discovery of orally active nonpeptide bradykinin B2 receptor antagonists. *Immunopharmacology,* 43**,** 163-8.

ASANO, M., INAMURA, N., HATORI, C., SAWAI, H., FUJIWARA, T., KATAYAMA, A., KAYAKIRI, H., SATOH, S., ABE, Y., INOUE, T., SAWADA, Y., NAKAHARA, K., OKU, T. & OKUHARA, M. 1997. The identification of an orally active, nonpeptide bradykinin B2 receptor antagonist, FR173657. *Br J Pharmacol,* 120**,** 617-24.

BATHON, J. M. & PROUD, D. 1991. Bradykinin antagonists. *Annu Rev Pharmacol Toxicol,* 31**,** 129-62.

BERRY, S. J., COFFEY, D. S., WALSH, P. C. & EWING, L. L. 1984. The development of human benign prostatic hyperplasia with age. *J Urol,* 132**,** 474-9.

BHOOLA, K. D., FIGUEROA, C. D. & WORTHY, K. 1992. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev,* 44**,** 1-80.

CALIXTO, J. B., CABRINI, D. A., FERREIRA, J. & CAMPOS, M. M. 2000. Kinins in pain and inflammation. *Pain,* 87**,** 1-5.

CAMPBELL, D. J. 2001. The kallikrein-kinin system in humans. *Clin Exp Pharmacol Physiol,* 28**,** 1060-5.

CHOPRA, B., BARRICK, S. R., MEYERS, S., BECKEL, J. M., ZEIDEL, M. L., FORD, A. P., DE GROAT, W. C. & BIRDER, L. A. 2005. Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat

urinary bladder urothelium. *J Physiol,* 562**,** 859-71.

CHUGHTAI, B., LEE, R., TE, A. & KAPLAN, S. 2011. Inflammation and benign prostatic hyperplasia: clinical implications. *Curr Urol Rep,* 12**,** 274-7.

COLLINS, M. M., MEIGS, J. B., BARRY, M. J., WALKER CORKERY, E., GIOVANNUCCI, E. & KAWACHI, I. 2002. Prevalence and correlates of prostatitis in the health professionals follow-up study cohort. *J Urol,* 167**,** 1363-6.

CRAWFORD, E. D., WILSON, S. S., MCCONNELL, J. D., SLAWIN, K. M., LIEBER, M. C., SMITH, J. A., MEEHAN, A. G., BAUTISTA, O. M., NOBLE, W. R., KUSEK, J. W., NYBERG, L. M. & ROEHRBORN, C. G. 2006. Baseline factors as predictors of clinical progression of benign prostatic hyperplasia in men treated with placebo. *J Urol,* 175**,** 1422-6; discussion 1426-7.

DE NUNZIO, C., ARONSON, W., FREEDLAND, S. J., GIOVANNUCCI, E. & PARSONS, J. K. 2012. The correlation between metabolic syndrome and prostatic diseases. *Eur Urol,* 61**,** 560-70.

DE NUNZIO, C., KRAMER, G., MARBERGER, M., MONTIRONI, R., NELSON, W., SCHRODER, F., SCIARRA, A. & TUBARO, A. 2011. The controversial relationship between benign prostatic hyperplasia and prostate cancer: the role of inflammation. *Eur Urol,* 60**,** 106-17.

DRAY, A. & PERKINS, M. 1993. Bradykinin and inflammatory pain. *Trends Neurosci,* 16**,** 99-104.

ELIST, J. 2006. Effects of pollen extract preparation Prostat/Poltit on lower urinary tract symptoms in patients with chronic nonbacterial prostatitis/chronic pelvic pain syndrome: a randomized, double-blind, placebo-controlled

study. *Urology,* 67**,** 60-3.

FORNER, S., ANDRADE, E. L., MARTINI, A. C., BENTO, A. F., MEDEIROS, R., KOEPP, J. & CALIXTO, J. B. 2012. Effects of kinin B(1) and B(2) receptor antagonists on overactive urinary bladder syndrome induced by spinal cord injury in rats. *Br J Pharmacol,* 167**,** 1737-52.

GANDAGLIA, G., BRIGANTI, A., GONTERO, P., MONDAINI, N., NOVARA, G., SALONIA, A., SCIARRA, A. & MONTORSI, F. 2013. The role of chronic prostatic inflammation in the pathogenesis and progression of benign prostatic hyperplasia (BPH). *BJU Int,* 112**,** 432-41.

GIAMBERARDINO, M. A., VALENTE, R., DE BIGONTINA, P. & VECCHIET, L. 1995. Artificial ureteral calculosis in rats: behavioural characterization of visceral pain episodes and their relationship with referred lumbar muscle hyperalgesia. *Pain,* 61**,** 459-69.

HALD, T. 1989. Urodynamics in benign prostatic hyperplasia: a survey. *Prostate Suppl,* 2**,** 69-77.

HALL, J. M. 1992. Bradykinin receptors: pharmacological properties and biological roles. *Pharmacol Ther,* 56**,** 131-90.

HOMMA, Y., YAMAGUCHI, O. & HAYASHI, K. 2006. Epidemiologic survey of lower urinary tract symptoms in Japan. *Urology,* 68**,** 560-4.

HOWL, J. & PAYNE, S. J. 2003. Bradykinin receptors as a therapeutic target. *Expert Opin Ther Targets,* 7**,** 277-85.

IKEDA, Y., UENO, A., NARABA, H. & OH-ISHI, S. 2001. Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. *Life Sci,* 69**,** 2911-9.

IRWIN, D. E., MILSOM, I., HUNSKAAR, S., REILLY, K., KOPP, Z.,

HERSCHORN, S., COYNE, K., KELLEHER, C., HAMPEL, C., ARTIBANI, W. & ABRAMS, P. 2006. Population-based survey of urinary incontinence, overactive bladder, and other lower urinary tract symptoms in five countries: results of the EPIC study. *Eur Urol,* 50**,** 1306-14; discussion 1314-5.

JUAN, H. & LEMBECK, F. 1974. Action of peptides and other algesic agents on paravascular pain receptors of the isolated perfused rabbit ear. *Naunyn Schmiedebergs Arch Pharmacol,* 283**,** 151-64.

KAKIZAKI, H., MATSUURA, S., MITSUI, T., AMEDA, K., TANAKA, H. & KOYANAGI, T. 2002. Questionnaire analysis on sex difference in lower urinary tract symptoms. *Urology,* 59**,** 58-62.

KATORI, M. & MAJIMA, M. 1998. Preventive role of renal kallikrein-kinin system in the early phase of hypertension and development of new antihypertensive drugs. *Adv Pharmacol,* 44**,** 147-224.

KRAMER, G., MITTEREGGER, D. & MARBERGER, M. 2007. Is benign prostatic hyperplasia (BPH) an immune inflammatory disease? *Eur Urol,* 51**,** 1202-16.

KRIEGER, J. N., EGAN, K. J., ROSS, S. O., JACOBS, R. & BERGER, R. E. 1996. Chronic pelvic pains represent the most prominent urogenital symptoms of "chronic prostatitis". *Urology,* 48**,** 715-21; discussion 721-2.

KRIEGER, J. N., NYBERG, L., JR. & NICKEL, J. C. 1999. NIH consensus definition and classification of prostatitis. *JAMA,* 282**,** 236-7.

LAIRD, J. M., MARTINEZ-CARO, L., GARCIA-NICAS, E. & CERVERO, F. 2001. A new model of visceral pain and referred hyperalgesia in the mouse. *Pain,* 92**,** 335-42.

LECCI, A., GIULIANI, S., MEINI, S. & MAGGI, C. A. 1995. Pharmacological analysis of the local and reflex responses to bradykinin on rat urinary bladder motility in vivo. *Br J Pharmacol,* 114**,** 708-14.

LITWIN, M. S., MCNAUGHTON-COLLINS, M., FOWLER, F. J., JR., NICKEL, J. C., CALHOUN, E. A., PONTARI, M. A., ALEXANDER, R. B., FARRAR, J. T. & O'LEARY, M. P. 1999. The National Institutes of Health chronic prostatitis symptom index: development and validation of a new outcome measure. Chronic Prostatitis Collaborative Research Network. *J Urol,* 162**,** 369-75.

MAGGI, C. A., SANTICIOLI, P., DEL BIANCO, E., LECCI, A. & GULIANI, S. 1993. Evidence for the involvement of bradykinin in chemically-evoked cystitis in anaesthetized rats. *Naunyn Schmiedebergs Arch Pharmacol,* 347**,** 432-7.

MARCEAU, F., HESS, J. F. & BACHVAROV, D. R. 1998. The B1 receptors for kinins. *Pharmacol Rev,* 50**,** 357-86.

MEINI, S., PATACCHINI, R., GIULIANI, S., LAZZERI, M., TURINI, D., MAGGI, C. A. & LECCI, A. 2000. Characterization of bradykinin B(2) receptor antagonists in human and rat urinary bladder. *Eur J Pharmacol,* 388**,** 177-82.

MENKE, J. G., BORKOWSKI, J. A., BIERILO, K. K., MACNEIL, T., DERRICK, A. W., SCHNECK, K. A., RANSOM, R. W., STRADER, C. D., LINEMEYER, D. L. & HESS, J. F. 1994. Expression cloning of a human B1 bradykinin receptor. *J Biol Chem,* 269**,** 21583-6.

MILLER, J. L., ROTHMAN, I., BAVENDAM, T. G. & BERGER, R. E. 1995. Prostatodynia and interstitial cystitis: one and the same? *Urology,* 45**,** 587-

90.

MISHRA, V. C., ALLEN, D. J., NICOLAOU, C., SHARIF, H., HUDD, C., KARIM, O. M., MOTIWALA, H. G. & LANIADO, M. E. 2007. Does intraprostatic inflammation have a role in the pathogenesis and progression of benign prostatic hyperplasia? *BJU Int,* 100**,** 327-31.

NICKEL, J. C., DOWNEY, J., YOUNG, I. & BOAG, S. 1999. Asymptomatic inflammation and/or infection in benign prostatic hyperplasia. *BJU Int,* 84**,** 976-81.

NICKEL, J. C., ELHILALI, M. & VALLANCIEN, G. 2005. Benign prostatic hyperplasia (BPH) and prostatitis: prevalence of painful ejaculation in men with clinical BPH. *BJU Int,* 95**,** 571-4.

NICKEL, J. C., ROEHRBORN, C. G., O'LEARY M, P., BOSTWICK, D. G., SOMERVILLE, M. C. & RITTMASTER, R. S. 2007. Examination of the relationship between symptoms of prostatitis and histological inflammation: baseline data from the REDUCE chemoprevention trial. *J Urol,* 178**,** 896-900; discussion 900-1.

NICKEL, J. C., ROEHRBORN, C. G., O'LEARY, M. P., BOSTWICK, D. G., SOMERVILLE, M. C. & RITTMASTER, R. S. 2008. The relationship between prostate inflammation and lower urinary tract symptoms: examination of baseline data from the REDUCE trial. *Eur Urol,* 54**,** 1379-84.

PARSONS, C. L. & ALBO, M. 2002. Intravesical potassium sensitivity in patients with prostatitis. *J Urol,* 168**,** 1054-7.

PONTARI, M. A. 2003. Chronic prostatitis/chronic pelvic pain syndrome in elderly men: toward better understanding and treatment. *Drugs Aging,* 20**,** 1111-25.

PONTARI, M. A. & RUGGIERI, M. R. 2004. Mechanisms in prostatitis/chronic pelvic pain syndrome. *J Urol,* 172**,** 839-45.

SAMPSON, N., UNTERGASSER, G., PLAS, E. & BERGER, P. 2007. The ageing male reproductive tract. *J Pathol,* 211**,** 206-18.

SCHAEFFER, A. J., LANDIS, J. R., KNAUSS, J. S., PROPERT, K. J., ALEXANDER, R. B., LITWIN, M. S., NICKEL, J. C., O'LEARY, M. P., NADLER, R. B., PONTARI, M. A., SHOSKES, D. A., ZEITLIN, S. I., FOWLER, J. E., JR., MAZURICK, C. A., KISHEL, L., KUSEK, J. W. & NYBERG, L. M. 2002. Demographic and clinical characteristics of men with chronic prostatitis: the national institutes of health chronic prostatitis cohort study. *J Urol,* 168**,** 593-8.

SCHATZL, G., TEMML, C., WALDMULLER, J., THURRIDL, T., HAIDINGER, G. & MADERSBACHER, S. 2001. A comparative cross-sectional study of lower urinary tract symptoms in both sexes. *Eur Urol,* 40**,** 213-9.

SCHILL, W. B. & MISKA, W. 1992. Possible effects of the kallikrein-kinin system on male reproductive functions. *Andrologia,* 24**,** 69-75.

SRINIVASAN, D., BURBACH, L. R., DANIELS, D. V., FORD, A. P. & BHATTACHARYA, A. 2004. Pharmacological characterization of canine bradykinin receptors in prostatic culture and in isolated prostate. *Br J Pharmacol,* 142**,** 297-304.

TEICHMAN, J. M. & PARSONS, C. L. 2007. Contemporary clinical presentation of interstitial cystitis. *Urology,* 69**,** 41-7.

TERAI, A., MATSUI, Y., ICHIOKA, K., OHARA, H., TERADA, N. & YOSHIMURA, K. 2004. Comparative analysis of lower urinary tract symptoms and bother in both sexes. *Urology,* 63**,** 487-91.

TRUE, L. D., BERGER, R. E., ROTHMAN, I., ROSS, S. O. & KRIEGER, J. N. 1999. Prostate histopathology and the chronic prostatitis/chronic pelvic pain syndrome: a prospective biopsy study. *J Urol,* 162**,** 2014-8.

UNTERGASSER, G., MADERSBACHER, S. & BERGER, P. 2005. Benign prostatic hyperplasia: age-related tissue-remodeling. *Exp Gerontol,* 40**,** 121-8.

WALDEN, P. D., LEFKOWITZ, G. K., ITTMANN, M., LEPOR, H. & MONACO, M. E. 1999. Mitogenic activation of human prostate-derived fibromuscular stromal cells by bradykinin. *Br J Pharmacol,* 127**,** 220-6.

WATTS, S. W. & COHEN, M. L. 1991. Effect of bombesin, bradykinin, substance P and CGRP in prostate, bladder body and neck. *Peptides,* 12**,** 1057-62.

WIRTH, K., HOCK, F. J., ALBUS, U., LINZ, W., ALPERMANN, H. G., ANAGNOSTOPOULOS, H., HENK, S., BREIPOHL, G., KONIG, W., KNOLLE, J. & ET AL. 1991. Hoe 140 a new potent and long acting bradykinin-antagonist: in vivo studies. *Br J Pharmacol,* 102**,** 774-7.

WIRTH, K. J., HEITSCH, H. & SCHOLKENS, B. A. 1995. Kinin receptor antagonists: unique probes in basic and clinical research. *Can J Physiol Pharmacol,* 73**,** 797-804.

YOSHIOKA, K., TANAHASHI, M. & UCHIDA, W. 2010. Behavioral and Urological Evaluation of a Testicular Pain Model. *Urology,* 75**,** 943-948.

Acknowledgements

I would like to thank Prof. Shigemori, Prof. Wang, Associate Prof. Usui, and Associate Prof. Yamada at the Faculty of Life and Environmental Sciences, the University of Tsukuba, for their discussion, advices and guidance on my preparation for this doctoral dissertation.

I would like to thank Mr. Watanabe, Vice President at Japan-Asia Planning & Administration, Astellas Pharma Inc. for giving me an opportunity to prepare this doctoral dissertation and accommodation.

I would like to thank Dr. Takeda, Research Therapeutic Area Head for Urology & Nephrology at Research Portfolio & Sciences, Astellas Pharma Inc., Dr. Masuda, Vice President at Research Planning & Administration, Dr. Yoshino at Medical Affairs and Dr. Yuyama at Research Portfolio & Science for Urology, for their discussion, advices and guidance on my study.

I would like to thank many colleagues at Pharmacology Research Laboratory for Urology (Research Portfolio & Science for Urology, at present), Astellas Pharma Inc. for supporting my study.

At the end, I would also like to thank my wife, son and daughter for always supporting me.

List of Published Articles

Kenichiro Fujimoto, Taiji Yoshino, Satoko Nakajima, Hironori Yuyama, Noriyuki Masuda, Masahiro Takeda. Physiological roles of bradykinin and involvement of bradykinin B2 receptor in urethral function in humans and animals. Low Urin Tract Symptoms, 2016 May, Epub ahead of print.

Kenichiro Fujimoto, Taiji Yoshino, Katsuro Yoshioka, Hironori Yuyama, Noriyuki Masuda, Masahiro Takeda. Intratesticular bradykinin involvement in rat testicular pain models. Low Urin Tract Symptoms, 2016 May, Epub ahead of print.