EXPRESSION AND FUNCTION OF UROTHELIAL NICOTINIC ACETYLCHOLINE RECEPTORS

by

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Classically, the epithelial lining of the urinary bladder, also called the urothelium, has been thought of as a passive barrier against toxins present in urine. However, recent studies are beginning to emerge that demonstrate an active role for the urothelium in the sensory functions of the bladder. For example, the urothelium expresses a number of the same receptors as sensory nerves and can respond to and release transmitters. One such transmitter, acetylcholine, has been shown to be released from the urothelium in response to physical stimuli, and is thought to act back on the urothelium in an autocrine/paracrine manner to effect urothelial signaling. This study was undertaken to determine if the urothelium expresses the proper receptors to respond to acetylcholine, specifically nicotinic acetylcholine receptors, and if these receptors play a role in influencing bladder physiology. Our research indicates that the urothelium expresses the proper nicotinic receptor subunits to form two classes of receptor: 1) α3 heteromeric receptors and 2) α7 homomeric receptors. Both of these classes of urothelial receptor are functional and can alter bladder reflexes in the anesthetized rat. Specifically, α7 receptors mediate an inhibitory pathway as measured by a bladder cystometrogram, while α3 receptors mediate an excitatory pathway. Finally, we examined intracellular and extracellular pathways that may mediate these physiological effects *in vivo.* These experiments suggest that nicotinic receptors in the urothelium mediate their effects through intracellular calcium signaling, resulting in the modulation of the release of the excitatory transmitter ATP. Specifically, our research indicates that α3 stimulation can potentiate the release of ATP from urothelial cells, while α7 stimulation

inhibits it. This effect may be due to the fact that each receptor subtype modulates $[Ca^{+2}]_i$ through distinct pathways: α3 receptors through influx of extracellular Ca^{+2} and α7 receptors through release from intracellular stores. Additionally, our research indicates that α7 receptors can inhibit signaling through α3 receptors, indicating another possible mechanism for the inhibitory effects α7 receptors exhibit *in vivo*. This research, which is the first to indicate an interaction between two types of nicotinic receptor, suggests that urothelial nicotinic receptors could play a significant role in bladder physiology and may represent a viable target for treatments into bladder pathology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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"Dear old Pittsburgh, Alma Mater, God preserve thee evermore!"

1.0 INTRODUCTION

The urinary bladder has two physiological functions; the storage and eventual elimination of waste products in the form of urine [1-5]. In order to operate correctly, the bladder must properly perform these two functions at the proper time (i.e. store urine when the bladder empty and release urine only when the subject is consciously attempting to do so). To accomplish this, the bladder and its outlet, the urethra, are carefully coordinated by neural pathways that act in concert to either promote storage or initiate elimination (also known as micturition). These pathways are driven through activity of afferent nerves innervating the bladder, which communicate to the central nervous system information on the fullness of the bladder, which the brainstem can translate into the sensations of urgency felt when the bladder is full.

In the past, it was believed that sensory aspects of micturition were performed solely by the afferent nerves innervating the bladder [1-9]. However, it has been recently hypothesized that the epithelial lining of the bladder, known as the urothelium, can also play a sensory role in the bladder [10-16]. For example, the urothelium has been shown to release a number of neurotransmitters, which are thought to play a role in modulating afferent excitability. These transmitters can be released either by mechanical or chemical stimuli, which suggests a role for the urothelium in transmitting information on conditions in the bladder to the underlying afferent nerves. The research presented here aims to further the hypothesis that the urothelium can

participate in the function of the urinary bladder by demonstrating a role of urothelial nicotinic acetylcholine receptors in modulating micturition reflexes.

1.1 OVERVIEW OF THE BLADDER

1.1.1 Anatomy of the Bladder

The urinary bladder is made up of two functional units: 1) a reservoir for storage of urine (the bladder) and 2) and an outlet that allows for emptying (the bladder neck and the urethra) [2]. The bladder itself is commonly divided into three sections, known as the trigone, the equatorial region and the dome (Figure 1.1). The trigone consists of the base of the bladder, and is where urine enters the bladder from the kidney through the ureters. The dome consists of the top portion of the bladder, where innervation is the greatest and bladder contractions begin. The equatorial section makes up the central part of the bladder.

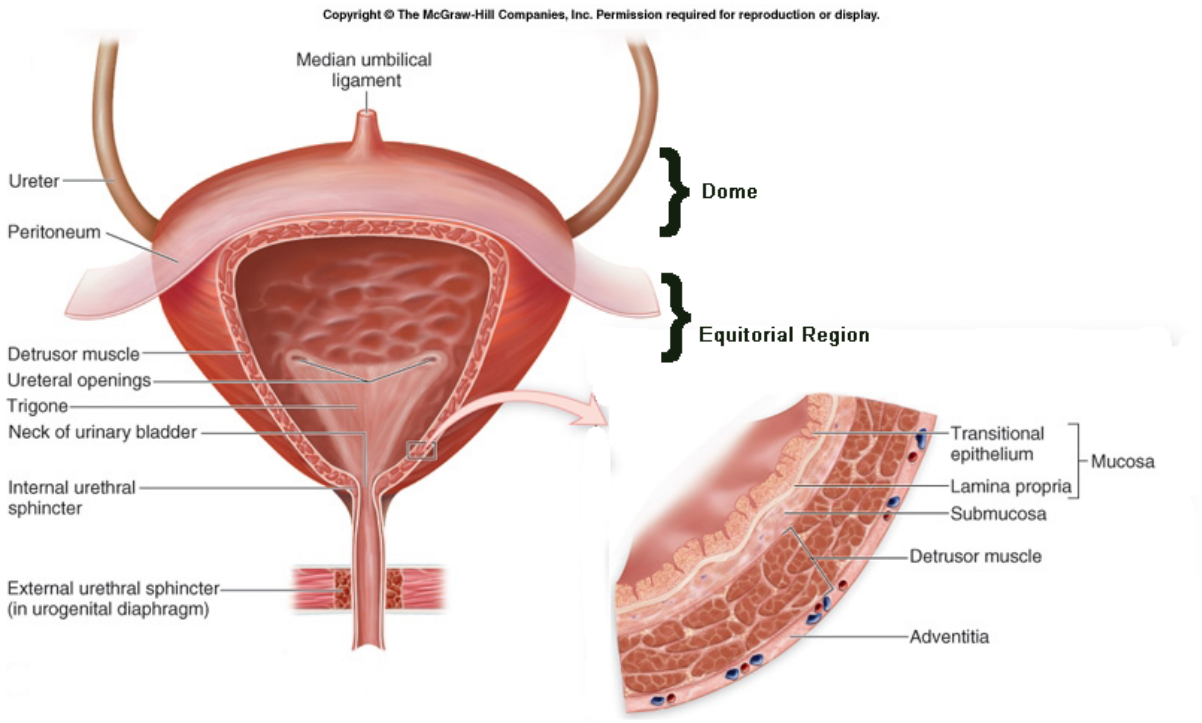

Figure 1.1 - Anatomy of the Bladder

Artist's depiction of the major anatomical features of the urinary bladder. Inset: a cross section of the bladder wall. Reprinted from [17] with permission from The McGraw-Hill Company.

The wall of the urinary bladder is made up of 5 distinct layers of tissue (Figure 1.2) [18-20]. The outside of the bladder wall is composed of three separate layers of smooth muscle, collectively described as the detrusor [20, 21]. These layers of smooth muscle are oriented in 3 separate directions with a layer of circular smooth muscle sandwiched between layers of longitudinal smooth muscle (known as the inner and outer longitudinal smooth muscle layers). These layers of smooth muscle thicken towards the trigone of the bladder and into the neck forming what is referred to as the internal urethral sphincter. The internal urethral sphincter acts as the final barrier in the bladder to urine release into the bladder outlet. During voiding, these muscles relax and the smooth muscle in the rest of the bladder contracts, inducing urine flow.

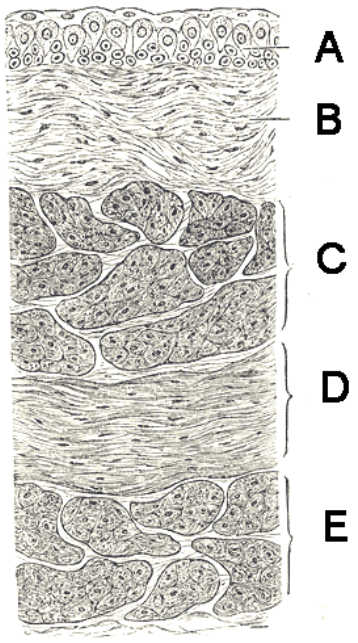

Figure 1.2 - Cross Section of the Bladder

(A) Urothelium. (B) Submucosa or the lamina propria. (C) Inner layer of longitudinal smooth muscle. (D) Middle circular smooth muscle. (E) Outer layer of longitudinal smooth muscle. Figure adapted from Gray (1901) [21].

Surrounding the lumen (or inside) of the bladder sac is a layer of transitional epithelial cells known as the urothelium [12, 19, 22]. The urothelium is also made up of three cells layers: 1) umbrella cells which line the bladder lumen and express the tight junctions that are responsible, in part, for the urothelium's barrier function, 2) intermediate cells and 3) basal cells, which anchor the urothelium to the underlying tissue. The urothelium will be discussed in greater detail Section 1.2.

Between the urothelium and the smooth muscle layers is the lamina propria (sometimes referred to as the submucosa), a layer of connective tissue consisting of fibers of collagen and elastin [23]. The main purpose of the lamina propria is to anchor the urothelium to the

surrounding smooth muscle. The lamina propria is populated by afferent nerve terminals forming the sensory portion of the bladder pathway [24] and is also home to myofibroblasts [25, 26], which may play a role in bladder function by acting as pacemaker cells during bladder contraction.

1.1.2 The Innervation of the Bladder and the Control of Micturition

The bladder receives a dual autonomic innervation that works in concert to maintain normal bladder function; i.e. storage and voiding [1, 20, 21]. When the bladder is empty, sympathetic nerves originating from the thoracolumbar spinal cord and innervating the bladder neck and urethra release norepinephrine, which activate α_1-adrenoceptors in the smooth muscle to maintain tone, keeping the outlet closed. At the same time, sympathetic neurons activate β-adrenoceptors in the detrusor to cause relaxation (Figure 1.3).

Information on conditions in the lower urinary tract is conveyed to the central nervous system by afferent nerves contained in the pelvic nerve, as well as the hypogastric and pudendal nerves [2, 3, 5-8, 27]. These afferents consist of small myelinated fibers (Aδ) and unmyelinated (C) fibers and are responsible for conveying impulses from various parts of the bladder. Aδ afferent nerves that originate near or in the detrusor smooth muscle of the bladder wall convey impulses from tension or volume changes as the bladder stretches to accommodate greater amounts of urine. Afferents that originate near the urothelium can respond to transmitters (e.g. NO, ATP, ACh, prostaglandins) that are released from the urothelium in response to changes in urine composition or in response to mechanical stretch. C-fiber afferents have been shown to function mainly as nociceptive neurons, only responding to noxious stimuli in the bladder, such as overdistention, physical damage or inflammation in response to bacterial infection [28].

Research has indicated that as the bladder fills, increased afferent nerve activity drives increased sympathetic efferent activity, maintaining tone in the urethra and inhibiting the bladder, maintaining continence [1-3, 5, 6, 27]. Increased afferent activity also activates a spinobulbospinal pathway that passes through a center in the rostral brain stem called the pontine micturition center (PMC) [29, 30]. Activation of this pathway results in feelings of bladder fullness and urgency. In an infant (under approximately 4 years of age), activation of this pathway results in a switch in the pathways activated in the bladder. During micturition, descending neurons in the spinal cord inhibit the sympathetic pathways maintaining urethral tone and inhibiting the bladder and activate parasympathetic neurons in the pelvic nerve (Figure 1.3B). These nerves release transmitters that act on purinergic and cholinergic receptors on detrusor smooth muscle to evoke a contraction [1, 31-34]. At the same time, nitrergic nerves innervating the urethra release NO, relaxing the urethra and resulting in voiding [35-37]. This process occurs reflexly in infants, however after the age of 4-6, neural pathways in the cerebral cortex and diencephalon develop that can modulate the PMC-driven spinobulbospinal reflex, allowing for voluntary control of the bladder.

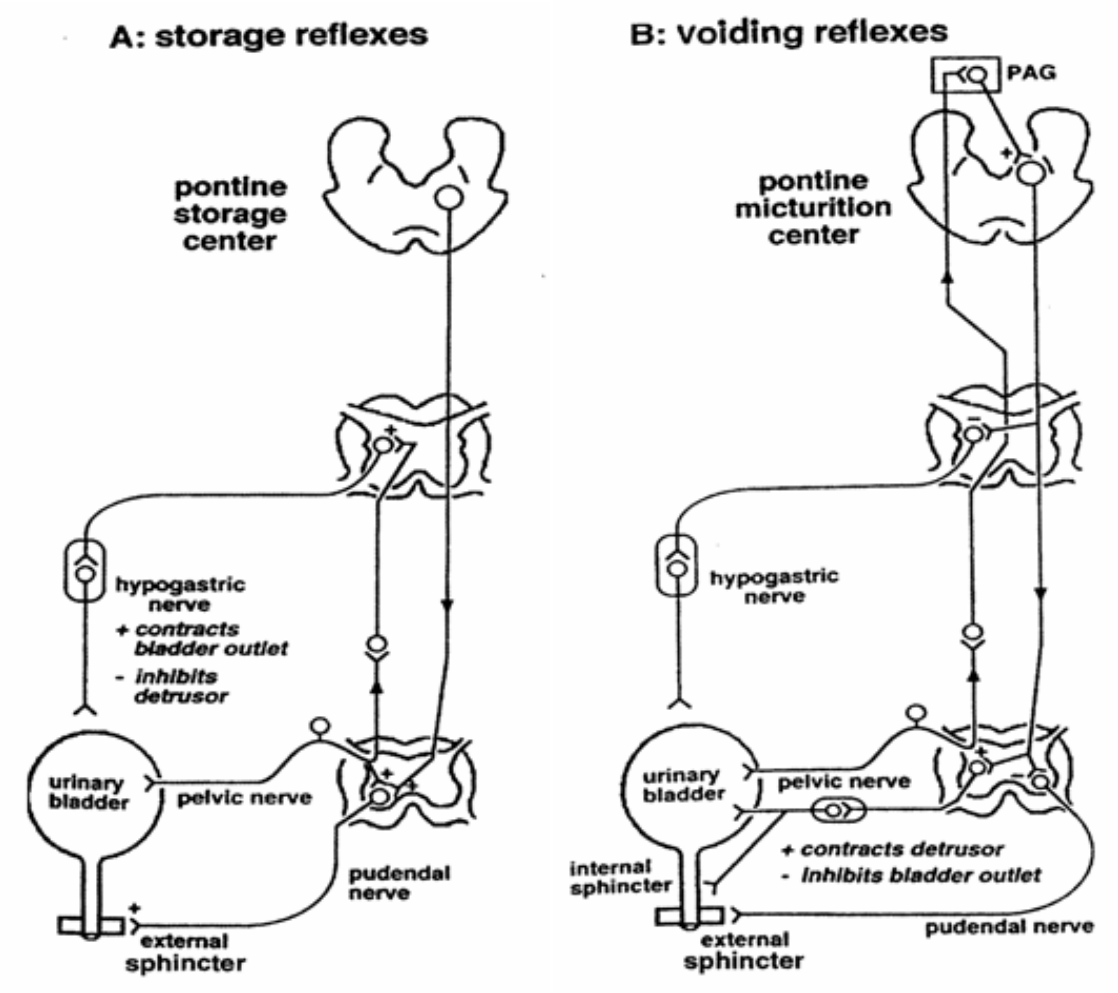

Figure 1.3 - Neural Pathways Involved in Storage and Voiding

(A) During storage, activity in pelvic afferent nerves drives sympathetic nerves that inhibit the bladder (hypogastric nerve) and excite the external urethral sphincter (pudendal nerve). **(B)** During voiding, descending pathways from the pontine micturition center in the brainstem inhibit the sympathetic pathways and activate parasympathetic pelvic efferents that contract the bladder and relax the sphincter. Figures reprinted from [9] with permission of Wiley-Blackwell.

1.2 THE UROTHELIUM

1.2.1 The Urothelium as a Barrier

Classically, the urothelium has been thought of as a simple, yet highly effective barrier, preventing harmful waste being stored in urine from harming the bladder [38-40]. The urothelium performs this function extremely well, as it has been shown to have one of the lowest permeabilities of any epithelial layer in the body, with some studies putting its transepithelial resistance as high as 300,000 $\Omega \cdot cm^{-2}$ (in the frog; in the rabbit the range is 10,000-75,000 $\Omega \cdot cm^{-2}$). This low permeability makes the urothelium the ultimate "liner" for the bladder, holding waste away from where it could damage bladder tissue.

The urothelium's impermeability is a function of its composition. The major players in this impermeability are the umbrella cells; large, flat, hexagonally-shaped cells that line the superficial surface of the bladder. These cells express two distinct morphological features that contribute to the impermeability of the urothelium. The first of these is the expression of scalloped-shaped plaques of proteins called uroplakins that line the luminal surface of the umbrella cells (Figure 1.4) [41-43]. These polygonal shaped plaques are approximately 0.5μm in diameter, 12nm in thickness and occupy almost 90% of the apical surface of the umbrella cells. They are made up of over 1,000 protein subunits, with each subunit composed of 12 proteins arranged in a hexagonal pattern. It is thought that these plaques, in conjunction with specialized apical membrane lipids [44], limit the exposure of the umbrella cell membrane to small

molecules (water, urea, ions) to reduce permeability across the apical membrane of the umbrella cells.

The second morphological feature expressed in umbrella cells that attributes to its high impermeability is the expression of tight junctions (Figure 1.5) [19, 45]. Tight junctions are a dense network of cytoplasmic proteins, cytoskeletal elements and transmembrane proteins that link adjacent cells and form a barrier to prevent movement of solutes and ions between them (also called paracellular transport) [19, 40, 46, 47]. Tight junctions are made up of a number of proteins such as occludin, ZO-1 and various members of a group of transmembrane proteins called the claudins. The claudins comprise a multigene family, of which there are 24 identified members [48]. Many different claudins can exist in the same junction, where they can interact in both heterotypic and homotypic manners. Permutations of these claudin interactions in each type of epithelial tissue are thought to be responsible for the unique paracellular properties of each epithelium. This is evidenced best in the kidney, where claudin expression, as well as paracellular permeability to various ions, varies by segment [49]. The bladder epithelium also expresses a number of claudins, including -4, -8 and 12; subtypes which have been previously shown to increase transepithelial resistance in heterologous expression systems [50].

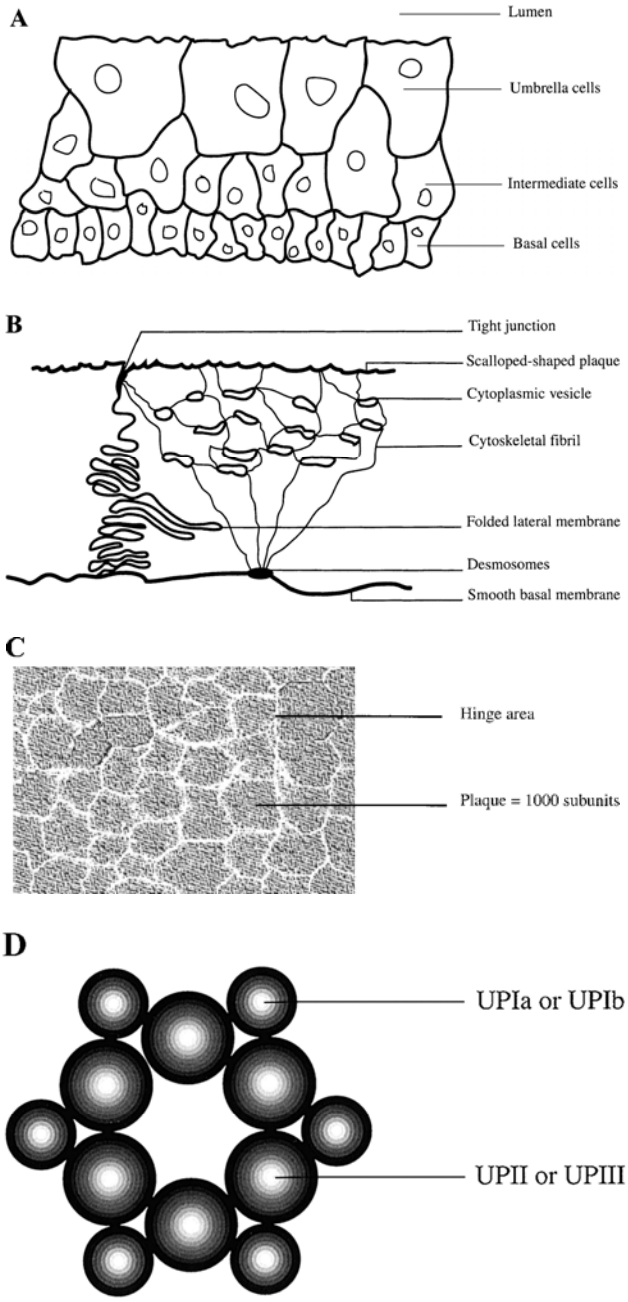

Figure 1.4 - Composition of the Urothelium

(A) Schematic diagram depicting the three layers of transitional epithelium which comprise the urothelium. (B) Cross-sectional diagram depicting the structural elements of the umbrella cell layer. (C) Electron micrograph of the apical surface of the umbrella cells, depicting the uroplakin plaques. (D) A schematic diagram of the composition of a uroplakin plaque unit. Figure reprinted from Lewis [19], with permission from the American Physiological Society.

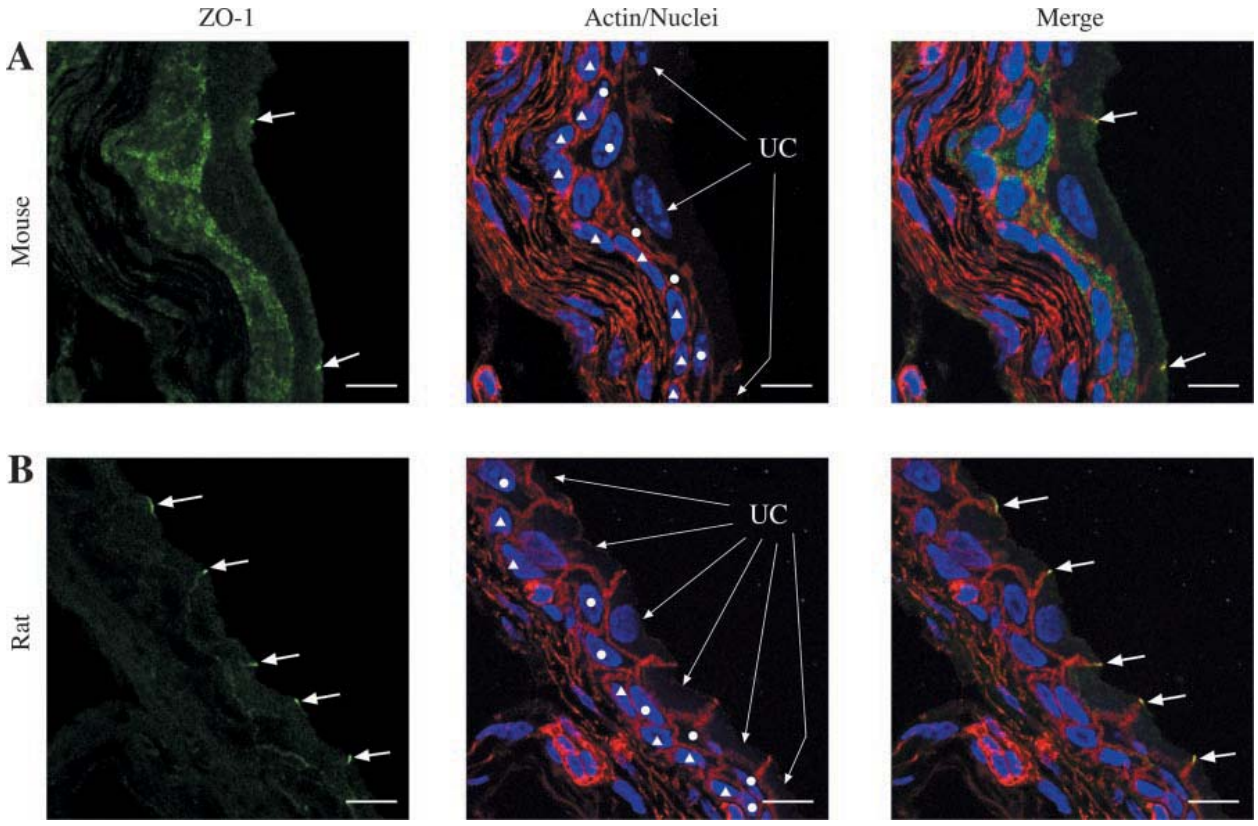

Figure 1.5 - Tight Junction Expression in the Rat/Mouse Urothelium

Distribution of the tight junction marker ZO-1 in mouse and rat uroepithelium. Cryosections of bladder tissue from mice (*A*) or rats (*B*) were labeled with anti-ZO-1 antibodies (*left*) or rhodamine-phalloidin and TO-PRO3 (*middle*). *Right*: merged images. Images were collected as a *z*-series with a confocal microscope and then summed and displayed as a single composite projection. *Right* and *left*: arrows show location of tight junctions. *Middle*: UCs are labeled with arrows, intermediate cells with filled circles, and basal cells with filled triangles. Bar = 50 μm. Figure taken from Acharya, et. al. [50], permission to reprint not required under the American Physiological Society's rules for publication concerning republication of figures by authors of the original manuscript.

1.2.2 The Urothelium as a Sensor/Transducer

While the urothelium has traditionally been thought as only a barrier to contain urine in the bladder, more recently it has been discovered that the urothelium can also play a role in the regulation of bladder activity. The first evidence that the urothelium may be more than a barrier came from Hypolite, et. al, who demonstrated that the urothelium had a much higher metabolic rate than the underlying detrusor smooth muscle of the bladder, suggesting that the urothelium may play an active role in bladder physiology rather than a passive one [51]. This led to further studies of the urothelium to determine what role it might play in bladder function. These studies have determined that the urothelium expresses a large number of "neuronal" receptors (those receptors commonly present in sensory nerves), and can respond to various chemical and physical changes in the bladder to release neurotransmitters [10-12, 47]. It is thought that the urothelium, through this transmitter release, can modulate the excitability of nearby afferent nerves, hence modulating bladder function. In the following sections, we will review the properties of the urothelium that play a role in bladder function.

1.2.2.1 The Sensory Properties of the Urothelium

In that it surrounds the luminal surface of the bladder, the urothelium is positioned in the ideal location to sense physical, chemical or pathological changes in the bladder. Therefore it should be no surprise that recent data from a number of investigators have demonstrated that the urothelium does indeed respond to mechanical stimuli such as stretch when the bladder fills [52-59], chemical mediators present in the urine [13, 60-68], or pathological conditions such as a bacterial infection [69-71]. Each of these responses demonstrates the sensory capability of the

urothelium and supports the hypothesis that the urothelium plays an important role in bladder function.

During bladder filling, the urothelium must accommodate the growing volume of urine by increasing its apical surface area and hence maintain the urine-blood barrier. To accomplish this, the urothelium responds to stretch by movement of a population of cytoplasmic discoid vesicles into the plasma membrane [38, 72]. This cAMP and PKA dependent process results in: 1) increases in apical surface area, 2) increases in uroplakin expression on the cell surface, 3) excretion of secretory proteins apically, and 4) the release of neurotransmitters such as NO, ACh and ATP. It is thought that these neurotransmitters can act on afferent nerve terminals underlying the urothelium to modulate sensory input from the bladder into the spinal cord [6, 73].

In addition to responding to physical stimuli, such as stretch, the urothelium possesses the capability to respond to chemical stimuli as well. Ongoing studies in a number of laboratories have demonstrated that the urothelium expresses a number of "sensory" receptors, i.e. receptors/ion channels common to nociceptor/mechanosensor sensory nerves. Examples of these urothelial "sensors" are depicted in Table 1 and include receptors for bradykinin [63], acetylcholine [65, 74-77], norepinephrine [61, 78], purines (P2X and P2Y) [79-81], neurotrophins (trkA and p75) [82], protease-activated receptors (PARs) [83], amiloride/mechanosensitive Na^+ channels (ENaC) [84, 85], calcium activated potassium channels (SK channels) [86] and transient receptor potential channels (TRPs) [13, 60, 87]. Pharmacological or genetic manipulation of many of these receptors present on the urothelium can result in the alteration of bladder reflexes in the rat and mouse, confirming a role for urothelial receptors in the sensory systems controlling micturition.

Table 1 - Similarities Between Urothelial and Sensory Nerve Receptors

Sensor Function/Stimulus	Urothelial Sensor Molecules	Neuronal Sensor Molecules
ATP	P2X/P2Y	P2X/P2Y
Capsacin/resiniferatoxin	TRPV1	TRPV1
Heat	TRPV1/TRPV2/TRPV4	TRPV1/TRPV2/TRPV3/TRPV4
Cold	TRPM8/TRPA1	TRPM8/TRPA1
H^+	TRPV1	TRPV1/ASIC/DRASIC
Osmolarity	In part TRPV4	In part TRPV4
Bradyknin	B1/B2	B1/B2
Acetylcholine	Nicotinic/Muscarinic	Nicotinic/Muscarinic
Norepinephrine	α,β-subtypes	α,β-subtypes
Nerve Growth Factor	P75/trkA	P75/trkA
Mechanosensitivy	Amiloride-Sensitive Na^+ Channels	Amiloride-Sensitive Na^+ Channels

(adapted from Birder [12], with permission from the American Physiological Society)

The urothelium can also respond to foreign bodies, such as bacteria, that may be present in urine. Studies in the bladder suggest that the urothelium can internalize bacteria, such as *Escherichia coli* or *Mycobacterium bovis,* through interactions between bacterial cell wall glycoproteins and urothelial surface receptors. For example, the hyaluronic acid binding protein CD44, which is expressed on urothelial cells, is the target for *E. coli* binding; mice with CD44 genetically knocked out are highly resistant to *E. coli* induced urinary tract infections [71]. Internalization of bacteria by the urothelium leads to degradation in lysosomes and the eventual presentation of antigens to CD4+ T cells in the suburothelial layer, leading to the activation of the innate immune response to fight off the infection [70].

Taken together, the evidence is clear that the urothelium plays a role in sensing the physical, chemical and pathological conditions inside the bladder. As we will discuss in the next section, the urothelium can then transduce this sensory information to underlying tissues such as nerves in order to modulate bladder physiology.

1.2.2.2 The Transducer Properties of the Urothelium

In addition to possessing the proper receptors to sense mechanical and chemical changes in the bladder, the urothelium can also respond to these changes by releasing various factors [16, 58, 59, 61, 77, 78, 88-91]. It has been hypothesized that this release demonstrates the "transducer" properties of the urothelium, in which the urothelium communicates sensory information with nerves and other cell types in the bladder through the release of neurotransmitters [10, 12]. In this way, the urothelium would play an important role in the function of the urinary bladder.

Many studies have demonstrated the release of transmitters from the urothelium in response to chemical or mechanical stimuli. Acetylcholine is released from bladder tissue in response mechanical stretch [16, 92]. Since this release was significantly diminished following surgical removal of the mucosa, it was hypothesized that this release originated in the urothelium. This hypothesis has been strengthened by studies demonstrating the presence of the proper machinery to synthesize, store, release and metabolize acetylcholine in urothelial cells [89, 93]. In addition to ACh, a number of other chemical mediators have been shown to be released from the urothelium in response to chemical or mechanical stimulation: nitric oxide [61, 65, 78], ATP [58-60, 63, 79, 88, 90, 91], substance P [94], prostaglandins [95], nerve growth factor [96]. This release of transmitters, coupled with the proximity of afferent nerves to the urothelium, has led to the hypothesis that the urothelium can transmit information about conditions in the bladder to sensory nerves through transmitter release.

This hypothesis was furthered through research by Chapple and Chess-Williams, who demonstrated that the urothelium could release a factor that could prevent bladder smooth muscle contractions in response to cholinergic stimulation [14, 15]. These experiments demonstrated that in a tissue bath, bladder smooth muscle contracted in response to muscarinic

receptor activation through carbachol stimulation to a much higher degree when present in the bath alone than when a separate piece of tissue containing the urothelium was present. This led the researchers to hypothesize that the urothelium released a soluble factor in response to cholinergic stimulation that could inhibit bladder smooth muscle contraction, which they named the "urothelial derived inhibitory factor (UDIF)". To date, no one has discovered the identity of the UDIF, however experiments have confirmed that it is not nitric oxide, ATP, a cyclooxengenase product, adenosine, a catecholamine, GABA nor does it mediate its effect through the opening of potassium channels.

Aside from the UDIF and its apparent effects on smooth muscle, other transmitters have been shown to be released from the urothelium and may act on various other sites in the bladder. For example, cholinergic or adrenergic stimulation of urothelial cells can cause the release of nitric oxide [61, 75, 78]. NO has been shown decrease afferent firing, and may represent an inhibitory factor in the bladder [97]. ATP is also released by the urothelium in response to mechanical or hypotonic stretch [58, 59, 88, 90] and chemical stimulation by ACh [77], which may then act on a subpopulation of bladder afferent nerves that express the $P2X_3$ receptor. Additionally, hyperreflexia induced by intravesical administration of bradykinin or muscarinic agonists can be blocked by the administration of the purinergic antagonist PPADS, suggesting that the effects of these agents are mediated through the release of ATP [63, 65].

In addition to the urothelium's possible interaction with afferent nerve fibers innervating the bladder, a number of other cell types present near the urothelium may also participate. In addition to afferent nerve terminals ending in close proximity to the urothelium, it also appears that efferent nerve project to the basal layer of the urothelium. In some cases, these efferents run in close proximity to afferent nerves, suggesting the possible communication between

urothelium, afferents and efferents (Figure 1.6). The sub-urothelium also contains a layer of cells that possess the cytological characteristics of both smooth muscle cells and fibroblasts, which are termed myofibroblasts (or occasionally interstitial cells) [26, 98, 99]. These myofibroblasts form an uninterrupted layer in the lamina propria and are linked to each other and to surrounding nerves and smooth muscle through gap junctions. This connectivity may allow bladder myofibroblasts to act as pacemaker cells by integrating signals in the bladder wall, much like the interstitial cells of Cajal in the gut. These cells express a number of purinergic receptors (P2X and P2Y), and respond to ATP by generating an intracellular Ca^{+2} transient, raising the possibility that urothelial derived ATP may activate myofibroblasts in the lamina propria (Figure 1.6) [100-102]. Given its proximity to these other cell types in the bladder, the possibility exists that the urothelium could participate in a number of cell-cell interactions important in bladder physiology.

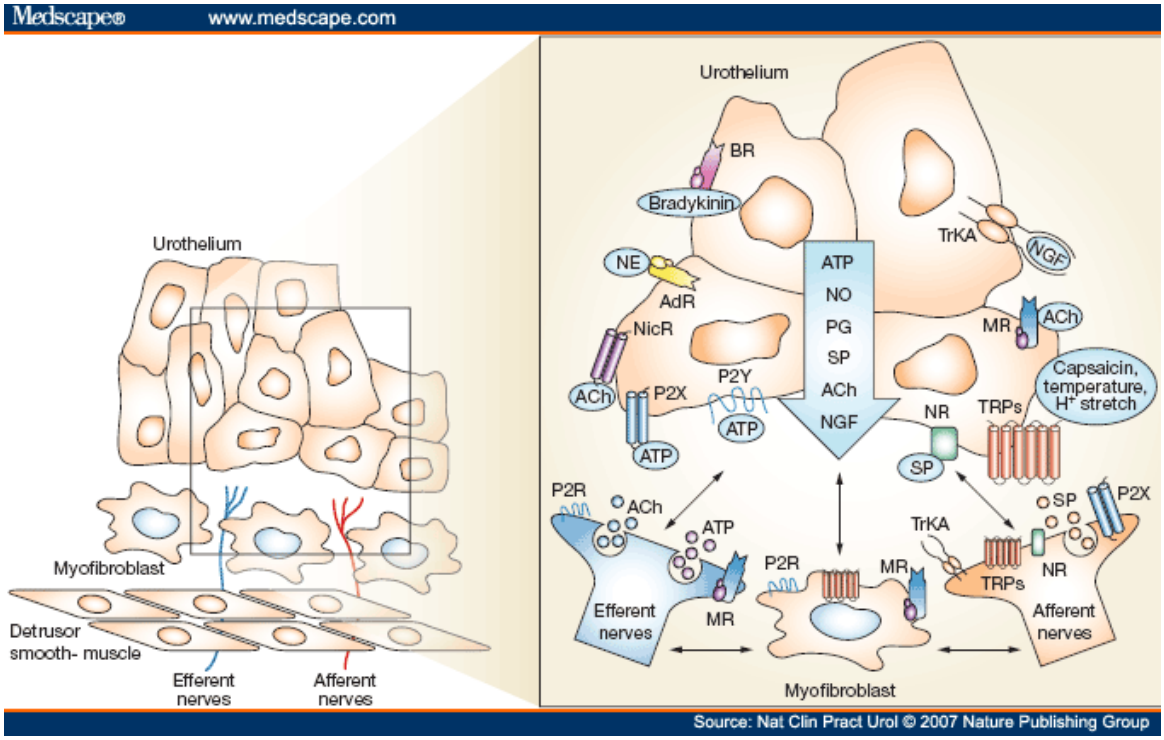

Figure 1.6 - Hypothetical Model of "Crosstalk" between Cell Types in Bladder Signaling

Schematic depicting possible interactions between cell types in the urothelium. Chemical or physical stimulation can cause the release of a number of transmitters from the urothelium, which may then act on myofibroblasts, afferent or efferent nerves residing in close proximity to the urothelial layer. It is also possible that factors released from nerves or myofibroblasts can act on urothelial cells to modulate urothelial signaling. Reprinted with permission from the Nature Publishing Group / Macmillan Publishers Ltd.: Nature Clinical Practice – Urology [103], © 2007.

1.2.2.3 TRPV1 as an Example of the Sensor/Transducer Role of the Urothelium

A prime example of the sensor/transducer role of the urothelium concerns the TRPV1 receptor. The TRP (transient receptor potential) superfamily of receptors is a diverse family of proteins that are expressed throughout the body in tissues such as neurons, smooth muscle and epithelial cell layers [104]. TRPV1 (originally named VR1) was the earliest TRP channel cloned and has been studied extensively due to its prominent role in nociception [105]. It was discovered to be the receptor responsible for the painful sensations induced by capsaicin, the pungent vanilloid present in hot peppers. It was further discovered that TRPV1 could be activated by heat, protons, a toxin present in a cactus-like plant (resiniferatoxin) [106] and

certain lipid metabolites such as anandamide [107]. In addition to being expressed in the unmyelinated C-fiber nociceptors that respond to noxious bladder stimuli, TRPV1 is also expressed in urothelial cells, where it is thought to modulate responses to non-noxious stimuli in the bladder [13]. For example, bladders from TRPV1 knockout mice exhibit decreases in stretch-evoked ATP release and membrane capacitance [60]. Cultured urothelial cells from TRPV1 KOs also showed less ATP release in response to hypotonic stretch than cells cultured from wild-type counterparts. Because ATP can excite afferent nerves in the bladder through activation of P2X receptors, it has been hypothesized that this lack of ATP release is responsible for the higher bladder volumes exhibited by TRPV1 KO mice [6, 73].

1.2.3 The Role of the Urothelium in Bladder Pathology

As we have discussed, control of the urinary bladder can be a complicated process, involving a number of different cell types in the bladder, peripheral and central nervous pathways, and a host of different transmitters. Therefore it should be no surprise that a number of pathological conditions exist as a result of defects in normal bladder physiology. Symptoms of pathological bladder conditions, such as a urinary tract infection, bladder outlet obstruction (as a result of enlargement of the prostate), interstitial cystitis or overactive bladder (OAB) often include increased bladder sensations, such as urgency or pain, leading investigators to focus on changes or defects in the sensory (afferent) nerve pathways in the bladder [7, 8, 12, 32, 74, 108]. Given its newly discovered sensory role, however, many investigators are beginning to believe that the urothelium may also play a role in bladder pathology.

The most common form of bladder pathology is the urinary tract infection (UTI). As stated above, the urothelium can internalize bacteria through interactions with the bacterial wall,

leading to digestion of the bacteria in lysosomes [109]. It appears that the urothelium may then be responsible for presenting bacterial antigens to CD4+ T-cells in the suburothelium to initiate the immune response [110-112]. Cytokines, released either from activated T-cells or by damaged urothelial cells, can activate mast cells in the suburothelium, initiating the release of vasoactive, inflammatory and nociceptive mediators. These mediators, such as nerve growth factor [113] and histamine [114] have been shown to act on C-fiber nociceptive neurons to induce pain [115, 116]. Additionally, activated mast cells can release factors that disrupt the urothelial barrier, increasing urothelial permeability [117]. This could allow substances in the urine, such as ATP, acetylcholine or H^+ or K^+ ions access to afferent nerves, sensitizing them to normally non-noxious stimuli. The ultimate result is the pain and urgency to void felt by the patient with a UTI.

While the urothelium's response to a bladder infections and the subsequent inflammatory response to a UTI is acute; other, more chronic bladder pathologies exist. Generally, these conditions present themselves as a result of aging, through mechanisms that are not yet fully understood, but can also be caused by a physical trauma, such as surgery or stress on muscles in women during a pregnancy [118]. These bladder disorders, which include overactive bladder (OAB), stress incontinence, bladder outlet obstruction or painful bladder syndrome/interstitial cystitis (PBS/IC), are thought to be quite common among adults; for example, it is estimated that 1 in every 6 men and women in the U.S. and Europe are affected by overactive bladder [74]. However, given the embarrassing nature of these disorders, their prevalence are thought to be under-reported, indicating that the number of people affected by bladder pathology may be much higher.

Not much is currently known about the etiologies of most of the bladder disorders, specifically PBS/IC or OAB, two chronic bladder disorders that are characterized by frequent urgency to void. One possibility may be an increase in the permeability of the urothelial barrier, much like that described above for UTIs [119]. A number of IC animal models show increased permeability of the urothelial barrier which may allow toxic substances in the urine to seep into the bladder wall [108]. It has also been shown that urine taken from human patients diagnosed with IC contains a protein known as anti-proliferative factor (APF), which has been demonstrated to prevent proliferation of urothelial cells and hence hinder the bladder's ability to maintain the urothelial barrier [120, 121]. In addition to these changes in urothelial permeability, however, other factors may play a role in the emergence of bladder pathology.

A common symptom of bladder pathologies is detrusor overactivity (DO), or the involuntary contractions of the bladder smooth muscle during the filling phase [74, 122]. Because these contractions often lead to leakage and increased frequency of urination, treatments were designed to attempt to block the activation of bladder smooth muscle and hence decrease these symptoms. Since previous research had identified ACh as the major transmitter responsible for smooth muscle contractions in the bladder through actions on muscarinic receptors, antimuscarinic agents were developed as treatments for DO, as well as for OAB [7, 8, 31, 32, 74, 123, 124]. These agents are very efficacious in treating the frequency of urination in patients with OAB, however it became clear that the results were due to actions elsewhere than bladder smooth muscle. To begin, antimuscarinic agents are active during the filling phase of the micturition cycle, when parasympathetic nerve activity is generally non-existent [1]. This suggested that antimuscarinic agents were working at a different site. Interest was piqued in the urothelium as one possible target following the discovery of two things: 1) the urothelium can

release ACh in response to stretch [16] and 2) the urothelium expresses muscarinic receptors, in some cases at a much higher density than in the detrusor [31]. Thus, researchers hypothesized that urothelial muscarinic receptors, possibly activated in an autocrine/paracrine manner by urothelially released ACh, could be playing a role in bladder pathology.

This hypothesis has been supported by further research into the role of cholinergic signaling in the urothelium. The instance of bladder pathology increases as a population ages, a trend which is mirrored by an increase in both basal and stretch-evoked release of ACh from the urothelium [92]. Muscarinic receptors can play an excitatory role in the urothelium; intravesical stimulation with the high concentrations of the general agonist oxotremorine M can excite bladder reflexes in the anesthetized rat [65]. This effect is thought to be mediated through the release of an actions on afferent nerves by ATP; muscarinic stimulation of cultured urothelial cells can evoke ATP release [77] and the *in vivo* excitation of bladder reflexes by muscarinic stimulation can be blocked by the purinergic antagonist PPADS [65]. Additionally, anti-muscarinics can decrease bladder afferent activity, however it is not entirely clear whether this is an effect of blocking ATP release from the urothelium or a direct effect of the anti-muscarinics on afferent nerves [125].

Bladder pathology can also change the expression of muscarinic receptors in the urothelium. For example, bladder outlet obstruction causes a marked increase in the expression of both the M2 and M3 muscarinic receptor subtypes in rats [122]. Conversely, induction of cystitis in rats through an injection of cyclophosphamide increased M1 and M5, without affecting the expression of the other muscarinic receptors [126]. While these results conflict in the subtypes that exhibit plasticity in a given animal model of bladder pathology, they both

suggest that increases in muscarinic receptor expression could play a role in increasing cholinergic signaling in the urothelium, leading to excitation of the bladder reflex.

In addition to the cholinergic system, plasticity in other transmitter/receptor systems may play a role in bladder pathology. For example, ATP release from the urothelium is also increased in older patients [92]. ATP release is also increased in interstitial cystitis patients [55], as well as in a feline model of the disease (FIC)[58]. While it is possible that ATP can directly activate bladder afferent nerves, research has demonstrated that ATP, acting through $P2X_3$ receptors, can also sensitize afferent nerves, lowering the threshold for activation by other stimuli [127, 128]. This is especially relevant in C-fiber nociceptors, where ATP has been shown to lower the sensitivity of TRPV1 to capsaicin, protons and heat, suggesting increased urothelial ATP release may play a role in sensitizing normally silent C-fibers in response to innocuous stimuli, causing urgency and pain [129].

A number of pathological bladder models have also demonstrated urothelial receptor plasticity. For example, normal rat bladders express only the bradykinin receptor B2R, however following induction of bladder inflammation using chronic cyclophosphamide treatment; this phenotype switches to the B1R [63]. This change in the prominent receptor phenotype may play a role in the bladder hyperreflexia observed in CYP treated rats, as the number of non-voiding contractions in CYP-treated anesthetized rats were significantly blocked by bradykinin antagonists. This bladder hyperactivity may be mediated through an ATP dependant process, as bradykinin evoked ATP release from cultured urothelial cells and bradykinin-induced bladder hyperactivity can be blocked by the purinergic antagonist PPADS.

1.3 NICOTINIC RECEPTORS

Acetylcholine is an important transmitter in the control of the bladder, being responsible for neurotransmission in the brain stem, spinal cord and autonomic ganglia as well as being the major compound responsible for bladder smooth muscle contractions [1, 130-134]. ACh mediates its effects *in vivo* through actions on the two types of cholinergic receptors: 1) the metabotropic muscarinic receptors and 2) the ionotropic nicotinic receptors [135]. While the muscarinic receptors play an important role in bladder function, our focus for this project is the nicotinic receptors, and we will constrain our focus to those receptors.

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels sensitive to acetylcholine and nicotine [136-141]. To date, sixteen nicotinic subunits have been cloned in the mammal, consisting of α1-10, β1-4, δ, γ, and ε. Nicotinic receptor subtypes are separated into two main categories based on the tissue in which they are normally found; those found in nervous tissue and those found at neuromuscular junction [136, 139]. Generally, it is the subunit composition of the receptor that differs between classes of receptor. nAChRs found at the neuromuscular junction are comprised of α1, β1, and δ subunits together with a γ subunit in the fetus or the ε subunit in the adult. In skeletal muscle, these receptors are activated by ACh released by motor neurons that innervate the muscle to cause a contraction.

Neurons have their own types of receptors, which are further divided into three categories: 1) the high-affinity nicotine binding receptors containing the α4 subunit, 2) α-bungarotoxin sensitive homomeric receptors composed of α7 subunits and 3) the complex heteromeric receptors of the autonomic nervous system composed of variations on a basic α3/β4 receptor [136, 137, 139-141]. These receptors are generally responsible for fast synaptic transmission in the brain (in the case of the α4 receptors) [142] or in the autonomic ganglia (as is

the case with the α3β4 and α7 receptors) [130]. Interestingly though, in addition to their long-discovered role as a fast-excitatory ion channel, nAChRs are also responsible for a number of other cellular processes in nerves, such as modulation of transmitter release from the presynaptic terminal [143], activation of calcium dependant second messenger systems [144-147] and initiation of transcriptional systems [148-150].

The consequence of such a wide array of cellular processes being influenced by the same endogenous transmitter would be the separation of signals, i.e. how does stimulation of a cell with ACh activate one process and not another? While the cellular location of nAChR on a nerve would play a role in determining which cellular process would be activated (e.g. α7 receptors on the presynaptic terminal can modulate transmitter release while those located in the soma can activate transcriptional systems, Figure 1.7) [151], subunit composition also seems to contribute in determining the function of a particular nAChR. Each nAChR subunit combination studied so far has a distinct pharmacological and electrophysiological profile which distinguishes it from the other nAChRs [137, 140, 152-156]. For example, each combination of nicotinic receptor subunits has different gating properties in response to their endogenous ligand, ACh (Figure 1.8). It is thought that these different electrophysiological properties account for the wide variety of functions attributed to nAChR signaling. In the following sections we will discuss the major pharmacological and electrophysiological differences between the three types of neuronal nicotinic receptors, as well as their known physiological roles *in vivo*.

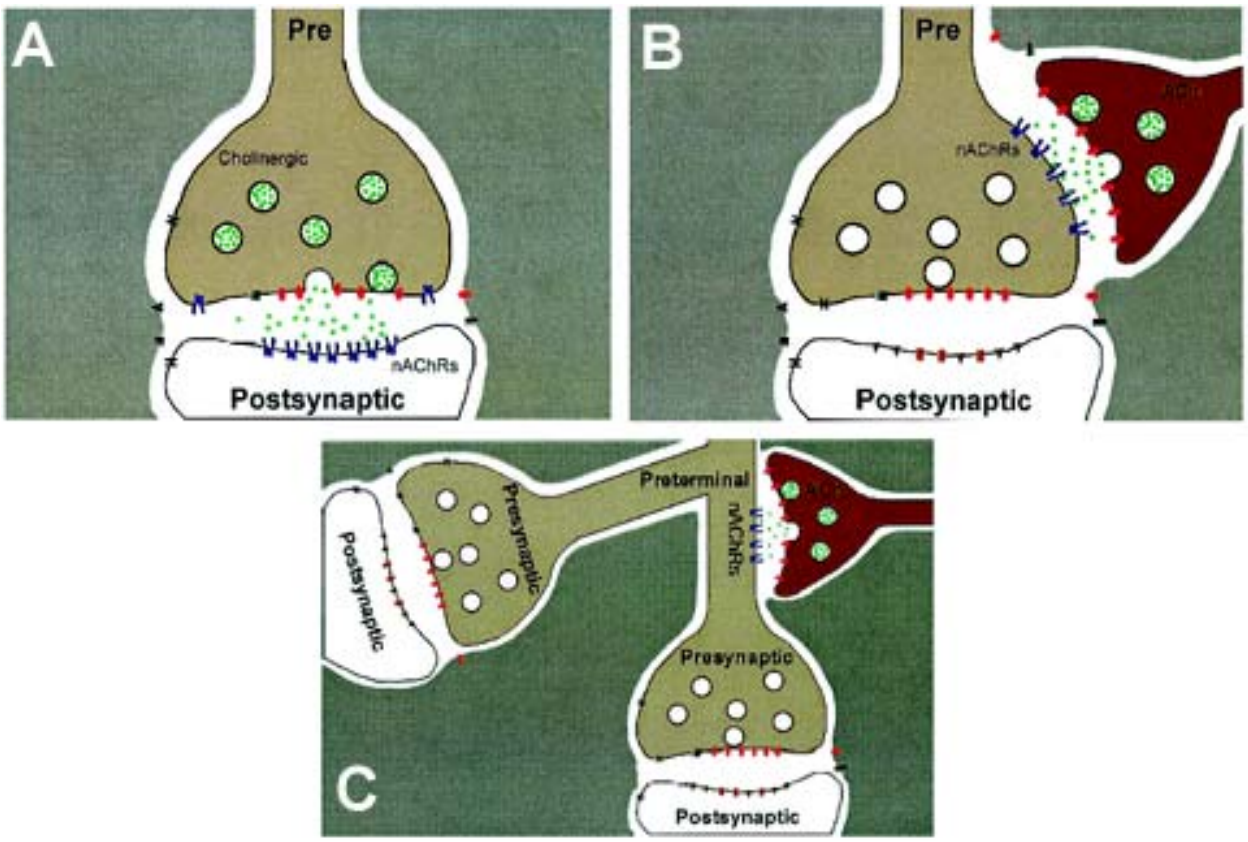

Figure 1.7 - nAChR Function is Influenced by Their Location

Series of figures depicting how cellular location can influence nAChR function. **(A)** The classic depiction of fast synaptic transmission. nAChRs present on the postsynaptic terminal are activated by ACh released by the presynaptic terminal, propagating the action potential across the synaptic cleft. **(B)** Transmitter modulation. nAChRs located on the presynaptic terminal can be activated by ACh from an adjacent terminal to modulate transmitter release from the presynaptic nerve. **(C)** Axonal or preterminal nicotinic modulation. nAChRs situated on the axonal arbor in a position where they may affect propagation of the action potential to one synapse, but not another. Reprinted from [151]: Biological Psychiatry 49(3) by J.A. Dani, "Overview of nicotinic receptors and their role in the central nervous system" © 2001, with permission from Elsevier Ltd.

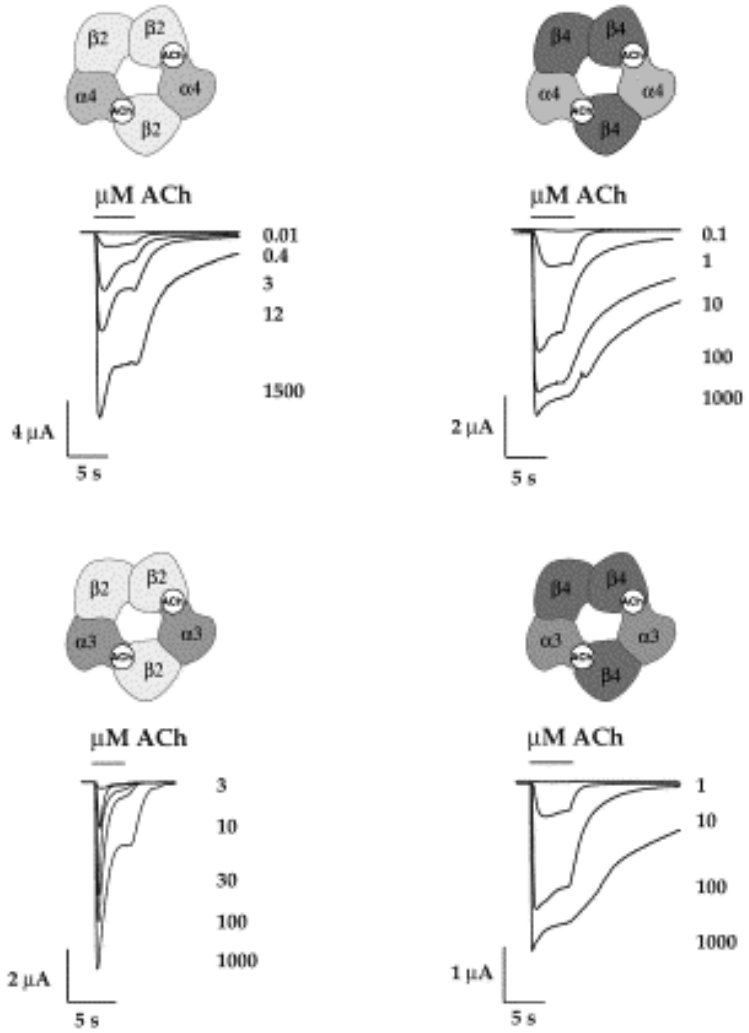

Figure 1.8 - Differences in nAChR Channel Properties Due to Subunit Composition

Inward currents recorded during voltage clamp experiments following stimulation with increasing concentrations of ACh to demonstrate the influence of subunit composition on channel gating properties. All traces were done in oocytes injected with mRNA coding for the subunits depicted in each graph. Cells were held at -100mV during recording. Reprinted from [137]: FEBS Letters 504(3) by V. Itier and D. Bertrand "Neuronal nicotinic receptors: from protein structure to function" © 2001 with permission from Elsevier Ltd.

1.3.1 α4 Containing Receptors

α4 containing receptors were the first neuronal nAChRs discovered in the CNS and are expressed throughout the cortex, thalamus, hippocampus, substantia nigra, striatum and cerebellum [139, 140, 157, 158]. α4 receptors are heteropentameric ion channels which consist of two α4 subunits paired with either three β2 subunits or, more rarely, three β4 subunits. α4 receptors account for the majority of nAChRs in the brain, with α7 receptors making up the rest. These receptors exhibit the highest affinity for nicotine of all the nAChRs yet studied, which has lead most investigators to focus on them when studying the pharmacology of smoking addiction [159]. In addition to their role in smoking cessation, α4 receptors have been found to be important in certain neurological pathologies, such as schizophrenia [160, 161], Parkinson's [162] and Alzheimer's disease [163]. These studies have increased interest in specific α4 agonists in the treatment of cognitive disorders.

While α4 receptors have been found to be very important in nicotinic signaling pathologies of the brain, they are rarely observed outside of the CNS. As our research centers on the periphery, where α4 receptors are not present, we will move on to discuss nAChRs more relevant to our research.

1.3.2 α3 Containing Receptors

α3 containing nAChRs (referred to henceforth as α3* receptors) are another classical example of neuronal nicotinic receptors found predominantly in sensory and autonomic nerves [130, 164]. These receptors are also comprised as heteropentamers, most commonly consisting

of two α3 subunits with three β4 subunits [137, 140]. Unlike other nAChRs though, α3 receptors seem to exhibit much more variability in their composition. α3 receptors have been demonstrated to co-express with α5, β2 and β4 subunits, suggesting that they can form functional receptors with these subunits as well [152, 153, 165, 166]. Studies in heterologous systems such as oocytes confirm that the α3 receptor can at least form functional receptors of the following compositions (more may be possible): α3β4 (stoichiometry 2:3), α3β2 (2:3), α3β2β4 (2:1:2), α3α5β2 (2:1:2) and α3α5β2β4 (2:1:1:1). While these receptors have not yet been shown to be pharmacology distinct, each receptor has measurably different electrophysiological properties that distinguish it from the others. For example, substitution of β4 subunits with β2 subunits in the α3β4 receptors increases open time, increases conductance and decreases the rate of desensitization of the receptor [165]. Additionally, the presence of an α5 subunit in either an α3β2 or α3β4 receptor can alter a receptor's sensitivity to ACh and increase the Ca^{+2} permeability of the channel [152, 165]. It has been hypothesized that these changes in the properties of α3* receptors could help "fine tune" nicotinic receptor signaling to perform specific functions.

This heterogeneity of the α3* receptor, however, makes physiological studies difficult; given that, as yet, no specific pharmacological agents have been found to differentiate between them. α3* receptors demonstrate a high affinity for nicotine or acetylcholine (approximately 10 fold lower than α4 receptors) [137, 139, 140] and are also activated by cytisine, dimethylphenylpiperazinium (DMPP) and epibatidine (see Table 2 for a listing of nicotinic agents). However, none of these compounds or any α3* antagonist (mecamylamine, TMPH, hexamethonium) demonstrates a sufficient selectivity to a particular α3* receptor subtype in order to use as a specific agent. Therefore it becomes near impossible to parse out each

receptor's physiological role in a given system. In an attempt to answer some of these questions, researchers have taken a genetic route, knocking out specific receptor subtypes in the hope to observe differences in autonomic function [130, 156, 167]. Most of these studies, however, resulted in lethality due to multiple organ failure, suggesting that each receptor subunit plays a significant role in autonomic function.

Table 2 - Subtype Specific nAChR Agents*

Agonists

Agent	Receptor Specificity	Potency (EC_{50} in μM)
ACh	α4>α7≈α3	0.5-68 / 28-180 / 35-203
Nicotine	α4>α3>α7	0.35-5 / 8.1-110 / 49-113
Choline	α7>>α3	400-16,000 (for α7)
PNU 282987	α7>>α3	~0.120 (for α7)
Cytisine	α4>α3>α7	0.019 / 5.6 / 72
Epibatidine	α4>α3>>α7	0.0045-0.0085 / 0.024-0.07 / 1.2-1.3
Dimethylphenylpiperazinium (DMPP)	α4>α3>α7	1.9-18 / 14-19 / 26-64

Antagonists

Agent	Receptor Specificity	Potency
Methyllycaconitine Citrate (MLA)	α7	10-200 nM
α-Bungarotoxin	α7	1-100 nM
Mecamylamine	α3*	1-10 μM
TMPH	α3*	0.1-10 μM
Hexamethonium	α3*	1-100 μM
α-Conotoxin AuIB	α3β4	1-10 μM
α-Contoxin MII	α3β2	10-120 nM

* Data taken from the online review by Wonnacott and Barik [168], and the references contained within.

1.3.3 α7 nAChRs

α7 nAChRs are unique in that they are comprised of five copies of the same subunit, as opposed to the general heteromeric structure of the other nicotinic receptors, which consist of at least two separate types of subunits [169]. This homomeric structure gives the α7 receptor some unique

pharmacological and physiological properties. For instance, the α7 receptor is the only common nAChR that is sensitive to choline, the metabolic product of acetylcholine [170, 171]. While choline is a much less potent agonist against α7 receptors as compared to nicotine or ACh (approximately 10-100 fold), it is much more selective [168]. Additionally, α7 receptors are susceptible to the snake venom, α-bungarotoxin, which can block α7 receptors almost irreversibly [138].

α7 receptors exhibit very fast activation and desensitization kinetics, as compared to other nAChRs [137, 138, 140, 169, 172, 173]. In fact, it has been hypothesized that α7 nicotinic receptors can desensitize much more rapidly than modern perfusion systems take to reach maximum concentrations of drug, leading to an underestimation of the desensitization kinetics at higher concentrations [169, 173]. These kinetics are largely concentration and time dependant, with lower concentrations of agonist activating and desensitizing slowly (see Figure 1.9). Higher concentrations of agonist, or longer durations of application can significantly increase the number of fully desensitized receptors. This phenomenon appears to be unique to α7 receptors and is certain to be important for nAChR signaling in the brain, however further studies must be performed, as no specific benefit has yet been ascertained.

In addition to their unique gating properties, α7 receptors also exhibit a higher Ca^{+2} permeability than the other ligand-gated ion channels yet studied [174, 175]. It has been estimated that the ratio of Ca^{+2} ions flowing through an open α7 channel as compared to other cations could be as high as 10:1, much higher than the 1:1 ratio exhibited by other nAChRs. This raises the possibility that α7 receptors may play a role in calcium homeostasis, as well as activate a number of calcium-dependent processes in cells. For example, α7 receptors have been shown to activate the PI3-kianse/Akt pathway in a calcium dependant manner [176]. Calcium

chelators also block nAChR-mediated nitric oxide production in the myenteric plexus, suggesting that α7 receptors also mediate this pathway [177]. Additionally, α7 receptors can activate transcriptional systems [148, 149] as well as modulate some proteolytic processes [178, 179], further confirming the receptors role in calcium-dependant cellular processes.

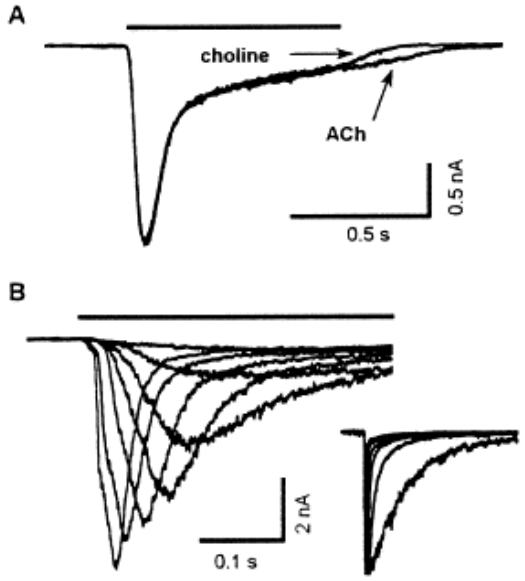

Figure 1.9 - α7 Currents in Response to ACh and Choline

Kinetic characteristics of α7 mediated whole cell currents in rat hippocampal neurons. **(A)** Currents elicited by a 750ms pulse of 300μM ACh or 2mM choline in the same neuron, superimposed over one another to show their near identical trace. **(B)** Concentration dependent kinetics of ACh evoked whole cell currents. Traces show the initial 0.4s of a 4s pulse of ACh at 10 (the smallest amplitude), 20, 40, 80, 160, 320 and 3000μM (the largest amplitude). The inset shows the entire 4s pulse scaled to the same peak amplitude in order to highlight the concentration dependence of the decay. Reprinted from [171]: Brain Research 882(1-2) by A. Mike, et. al. "Choline and acetylcholine have similar kinetic properties of activation and desensitization on the α7 receptors in rat hippocampal neurons" © 2000 with permission from Elsevier Ltd.

1.3.4 The role of nAChRs in the Control of the Urinary Bladder

As mentioned previously, ACh is an important transmitter in the bladder. Not only is ACh, released for parasympathetic nerves, responsible for bladder smooth muscle contraction during micturition [1, 2, 5, 7, 30, 31, 74], it is an important transmitter at many sites along the spinal-bulbo-spinal pathways that control micturition [29, 132, 180, 181]. In the following sections, we will discuss the role nicotinic receptors have in areas of the body important for controlling micturition, such as the brainstem, spinal cord and autonomic ganglia.

1.3.4.1 Brainstem

Nicotinic receptors are prevalent in a number of areas of the brain, including the cortex, thalamus, hippocampus, substantia nigra, striatum and cerebellum [146, 157, 182-185]. Therefore, it is reasonable to expect that nicotinic receptors play a role in synaptic transmission in areas of the brain that control micturition. For example, injections of cholinergic agents into the pontine micturition center of the cat elicited both inhibition and excitation of bladder reflexes in the cat, depending on the site of injection [186]. In both anesthetized and unanaesthetized rats, low doses (0.01-0.01 μg) of the ultrapotent agonist epibatidine, injected intracerebroventricularly (i.c.v.), inhibited bladder reflexes, suggesting that α4 receptors in the brain play a role in micturition [133]. Larger doses (1 μg) of epibatidine excited bladder reflexes, however this dose also increased the peak micturition pressure, therefore it may be that this excitation is due to non-specific effects elsewhere in the body, such as the autonomic ganglia. Additionally, nicotine injected i.c.v. decreased bladder activity; an effect that returned to normal after 40 minutes and which was blocked by the antagonist mecamylamine [180].

1.3.4.2 Spinal Cord

Both the afferent and efferent limbs of the micturition pathway run through spinal interneurons that also rely on nicotinic receptors for synaptic transmission [29]. Intrathecal administration of nicotine increased voiding frequency transiently without significantly changing any other voiding parameters, such as pressure threshold or maximum voiding pressure [180]. This effect of nicotine could be reversed using the nicotinic antagonist mecamylamine. Interestingly, the effect of nicotine was also blocked by the NMDA receptor antagonist MK-801, indicating that the spinal effects of nicotine are mediated though the stimulation of the glutamate-NMDA pathway. This was consistent with other studies that demonstrate hyperalgesia and/or irritation in behavior responses following intrathecal administration of nAChR agonists [187, 188]. These studies found a significant increase in glutamate and aspartate levels in spinal fluid of rats following stimulation with nicotine, cytisine or epibatidine. Capsaicin treatment of the bladder also significantly blocked nicotine's spinal effects, suggesting that C-fiber afferents may play a limited role in nicotine-induced glutamate release [180].

1.3.4.3 Autonomic Ganglia

The urinary tract is innervated by autonomic sympathetic and parasympathetic nerves which form the basis of the two phase of micturition: storage and voiding [130, 131, 135, 181]. These pathways consist of preganglionic nerves, which originate in the lumbar or sacral spinal cord and postganglionic nerves, which innervate the urinary tract. In the rat or mouse, the postganglionic fibers originate in the major pelvic ganglion, while in higher mammals they generally originate in intramural ganglia in the bladder wall.

nAChRs play a major role in bladder function by mediating synaptic transmission for both sympathetic and parasympathetic neurons through the autonomic ganglia. Studies have

shown that autonomic ganglia express a number of nAChR subunits, including α3, α4, α5, α7, β2 and β4, with α3β4 receptors acting as the major functional receptor [131]. This is demonstrated both by perforated whole cell patch clamp, where ACh-induced currents could be blocked by mecamylamine and α-conotoxin AuIIB [181]; as well as genetic knockout studies, where knockout of either the α3 or β4 subunit blocks nicotine induced bladder contractions *in vitro* [131].

1.3.4.4 Myofibroblasts or Interstitial Cells

The bladder contains a population of cells similar to the interstitial cells of Cajal (ICC), found in the gut [25, 100, 101]. In the gut ICC cells are responsible for maintaining peristaltic activity and transmission of signals from nerves to the smooth muscle of the GI tract. They are thought to perform roughly the same function in the bladder; performing "pacemaker" functions to propagate smooth muscle contractions along the length of the bladder and act as an intermediate in neuron-smooth muscle signaling. ICC cells of the bladder exist in two locations, in the detrusor smooth muscle and in the lamina propria. ICC cells in the detrusor, also called detrusor myofibroblasts, have been shown to release ACh and can respond to cholinergic stimulation with increases in intracellular calcium [102]. This is not the case, however, for ICC cells present in the lamina propria. While nicotinic receptors have not yet been located to either type of bladder myofibroblasts, they are important players in fibroblast proliferation and signaling [189] in other tissues, suggesting that they may also play the same role in the bladder.

1.3.4.5 The Role of nAChRs in Afferents

Nicotine has long been known to possess anti-nociceptive properties, as smoking tobacco has been used as a folk remedy for pain since the plant was discovered [190]. While undoubtedly

some of nicotine's anti-nociceptive effects are due to actions in the brain and spinal cord, nicotinic receptors are also present on peripheral afferent nerves. While no study to date has directly located nicotinic receptors to bladder specific afferent nerves, indirect evidence suggests that they could play a significant role in the bladder sensory pathway.

For example, a number of nicotinic receptor subunits (α2, α3, α4, α5, α6, α7, β2, β3 and β4) have been localized to dorsal root ganglion cells, including cells from DRGs known to contain bladder afferent nerves (in the rat, L6-S1) [164]. These nicotinic subunits form functional receptors that the authors separated into 4 categories based on their pharmacological and electrophysiological properties. For example, 32% of small diameter DRG cells (a common measurement to identify C-fiber nociceptors) and 77% of large cells (indicative of A-β and A-δ fibers) express the "Category I" nAChR response, characterized by a rapidly desensitizing current that was sensitive to choline and α-bungarotoxin, suggesting the presence of α7 receptors. A smaller number of cells (9% of small cells, 16% of large cells) exhibit a non-desensitizing current in response to nicotine and cytisine, suggesting the involvement of α3β4 receptors. The other two categories of responses occur in less than 5% of all cells, which did not allow the authors to complete an extensive pharmacological profile, however they believe that these two responses may be attributed to either α4-containing receptors, or more complex forms of the α3β4 receptor (such as α3α5β4). It is unclear what this wide variety in nicotinic signaling in the DRG might mean in terms of sensory function.

The site of nicotine's anti-nociceptive actions is also unclear, however a few hypotheses exist. nAChRs have been localized to afferent nerves terminals entering the spinal cord at the level of lamina I-III [191]. Following noxious stimuli, C-fiber afferent axons in these laminae of the spinal cord release peptides, such as Substance P (SP) or calcitonin gene-related peptide

(CGRP). Because intrathecal injection of SP or CGRP causes nociceptive behavior in rats and mice [192, 193], it is thought that these transmitters modulate the pain response. Conversely, nicotinic agonists such as epibatidine can have anti-nociceptive properties when injected intrathecally [194]. It is thought, then, that this analgesic effect of epibatidine is accomplished in the same manner as that of opioids, i.e. by acting on receptors located presynaptically on afferent nerve axons in the spinal cord. Activation of these presynaptic receptors would deplete the releasable stores of SP or CGRP that are normally released following noxious stimuli in the periphery, resulting in analgesia. Additionally, it is possible that nAChRs present on the peripheral terminal of C-fiber afferents play a role in epibatidine's anti-nociceptive properties. It is believed that epibatidine could desensitize C-fiber nociceptive neurons; much in the same manner as capsaicin does, acting on TRPV1 receptors.

Evidence for these hypotheses can be found both *in vivo* and *in vitro.* nAChR-mediated release of SP or CGRP has been well characterized in C-fiber afferent nerves innervating the rat trachea [195], as well as the guinea pig bronchi and lungs [196-198]. Epibatidine also causes SP release from F11 cells (a DRG cell line with C-fiber properties) [199]. And while release of SP or CGRP from afferent nerves seems counterintuitive to anti-nociception, a second, subsequent activation of afferent nerves or F11 cells with nicotinic agents results in little peptide release, supporting the hypotheses that the epibatidine either desensitizes the nerve or depletes the pool of releasable peptides [199]. Additionally, it has been discovered that stimulation of DRGs with nicotinic agents significantly decreases calcium transients in response to a subsequent stimulation by capsaicin, indicating that nAChRs can desensitize C-fibers to other stimuli. [200]

In relation to the bladder, little is known about the role of nicotinic receptors on afferent nerves. As mentioned previously, intrathecal injection of nicotine in the rat can transiently excite

bladder reflexes [180]. It appears that this effect is modulated at least partly through C-fiber afferents, as pretreatment with capsaicin diminished nicotine's effect. Additionally, intravesical administration of nicotine (in concentrations thought high enough to pass through the urothelial barrier to activate underlying nerves) can cause excitation of the bladder reflex. Given this data, it appears that nicotinic receptors on both the peripheral and central terminals of bladder afferent nerves play a role in the micturition pathway.

1.3.5 The Role of Neuronal nAChR in Non-Neuronal Cells

While we have spent a good deal of time discussing "neuronal" nicotinic receptors and their role in neural physiology, it should be noted that these receptors have recently been gaining notoriety in non-neuronal systems. While the "neuronal" receptors that were first cloned in the brain are found throughout the nervous system, increasing evidence points towards important roles for these receptors in other tissues such as adipose tissue, epithelial and endothelial cells, keratinocytes, and immune cells such as lymphocytes and macrophages (See Table 3 for a summary of nAChR expression in non-neuronal cell types). These receptors have been found to be responsible for a number of cellular processes, such as transcription, cell growth, cell differentiation and secretion. Given that the bladder also contains a number of these non-neuronal tissues, it is conceivable that nAChRs in non-neuronal tissue may also play a role in bladder function.

For example, a growing number of studies have indicated a role for nAChRs in the maintenance of epithelial or endothelial barriers. Human skin keratinocytes have been shown to express the α3, α5, α7, α9, α10, β1, β2 and β4 subunits [144], while vascular endothelial cells in the human aorta express α3, α5, β2 and β4 [201]. Stimulation of these cells with nicotinic agents

have a number of effects that can influence the permeability of the barriers these cells maintain. For example, stimulation of human umbilical vein endothelial cells with nicotine increased the expression of the cellular adhesion molecules VCAM1, ICAM and E-selectin, as well as increased the ability of *E. coli* to penetrate the endothelial barrier [202]. Additionally, nicotine can increase paracellular permeability of the blood-brain barrier, presumably due to decrease in connexin 43 expression [203, 204]. Conversely, mecamylamine treatment of organotypic co-cultures (a experimental model of human skin), diminished epithelial thickness and expression of terminal differentiation markers [205], indicating that tonic nAChR activation may be necessary for maintaining the epithelial barrier in the skin. Finally, nAChRs have been shown to stimulate the proliferation of bronchial epithelial cells, as well as inhibit apoptosis [206, 207]. Therefore, the role of nAChRs in the proliferation and differentiation of epithelial/endothelial cells has been well-established.

In addition to maintenance of epithelial/endothelial barriers, nAChRs have also been shown to play a significant role in the immune system. ACh, acting on α7 receptors, prevents activation of macrophages by inhibiting the nuclear translocation of NF-κB [208] as well as activating the Jak/Stat3 pathway [209], which in turn prevents the macrophages from releasing pro-inflammatory cytokines such as TNF, MIP-2 and IL-6 [210]. This anti-inflammatory pathway can also be activated by nicotine, which has led to its use in the treatment of various chronic inflammatory diseases, such as ulcerative colitis or pancreatitis [211, 212]. In addition to macrophages, nAChRs are also important in down-regulating other inflammatory cell types, such B- and T-lymphocytes, as well as dendritic cells, reducing their ability to release inflammatory mediators and increasing apoptosis [181, 213-216]. When combined, these effects of nAChRs in the immune system results in a significant anti-inflammatory response. Therefore,

clinically, nAChR agents are being examined to combat a host of inflammatory diseases, such as sepsis [208].

These results are only a small sample of the non-neuronal tissues throughout the body where nicotinic receptors are present. However, they illustrate other areas, outside of nervous pathways, where nAChRs may play an important role in bladder physiology or pathophysiology. For example, nAChRs present on vascular endothelial cells in blood vessels in the bladder could modulate vascular permeability and hence influence the ability of immune cells to reach the site of a bladder infection. It is also possible that nicotinic receptor signaling could be responsible for changes in the permeability of the urothelial barrier following infection, allowing macrophages and other inflammatory responders to gain access to the lumen of the bladder in the event of a bladder infection. Some bladder pathologies, such as PBS/IC are thought to have an inflammatory etiology, therefore the anti-inflammatory properties of nicotinic agents may help alleviate the symptoms associated with PBS/IC. A thought that may be more pertinent to our present research however; given the prevalence of nAChRs in epithelial tissues coupled with the knowledge that the bladder epithelium can respond to and release ACh as a part of its "sensor/transducer" properties, is the potential exists for nAChRs to be expressed in the urothelium and play a significant role in urothelial biology.

Table 3 - Expression of nAChRs in Non-Neuronal Tissues

Cell Type	***Tissue***	***Nicotinic Receptors Expressed***
Epithelial Cells	*Airway (Human)*	
	Surface epithelium	α1, α3, α5, α7, α9, β1, β2, β4, δ, ε
	Alveolar type 2 cells	α4, α7, β1, β2 (rhesus monkey)
	Glands	α4 (rhesus monkey)
	Skin (Human)	
	Keratinocytes	α3, α5, α7, α9, α10, β1, β2, β4
	Pilosebaceous unit	α3, α4, α5, α7, α9, α10, β1, β2, β4
	Sweat glands - Myoepithelial	α3, α4, α5, α7
	Sweat glands - Acinar cells	α9, β2
	Melanocytes	α1, α3, α5, α7, β1, β2, γ, δ
	Intestine	
	Surface epithelium	α3
	Colonic epithelial cell line	α4, α5, α7, β1
	Glands (salivary cells, gastric cells, pancreatic acinar cells)	α2, α3, α4, α5, α7, β2 (beta cell line, rat)
Endothelial Cells	*Aorta/pulmonary vessels*	
	Human	α3, α5, α7, β2, β4
	Rat	α2, α3, α4, α5, α6, α7, α10, β2, β4
	Mouse	α3, α5, α7, β2 (cerebral microvasculature)
Immune Cells		
	MNLs (Human)	α2, α4, α5, α6, α7, α9, α10, β2, β4 (variable expression with dominant expression of α2, α5, α7)
	Eosinophiles (Human)	α3, α4, α7
	Macrophages (Human)	α1, α7, α10
	Macrophages (Mouse)	α2, α4, α5, α6, α7, α10, β2, β4
	DC cells (Mouse)	α2, α5, α6, α7, α10, β2, β4
	Mast cells (Human)	α3, α5, α10

1.4 FINAL THOUGHTS AND GOALS FOR THIS DISSERTATION

In summary, we have presented significant evidence in support of the probability of nAChR signaling in the urothelium. As mentioned previously, ACh is a major transmitter in a variety of locations along the micturition pathway, acting on cholinergic receptors in the brain, spinal cord, autonomic ganglia, afferent nerves and smooth muscle. ACh has been shown to be released from the urothelium in response to stretch and chemical stimulation, and can act on muscarinic receptors on the urothelium to modulate bladder reflexes, further suggesting a role for non-neuronal cholinergic signaling in the urothelium. Therefore, given the role of nicotinic signaling in a number of non-neuronal cell types, as well as the proven role of cholinergic modulation of bladder reflexes by the urothelium, the following question arises: does the urothelium express functional nAChRs that can influence bladder function?

To date, only a few cursory studies have examined this question. For example, two recent studies have examined the expression of nAChRs in the urothelium, demonstrating that the human urothelium expresses the α7, α9 and α10 subunits [218], while the mouse expresses the α2, α4, α5, α6, α7, α9 and α10 subunits [219]. However, no study has yet examined if these receptors mediate any type of physiological effects, such as modulation of transmitter release or, in the whole animal, modulation of bladder reflexes. As we have discussed, the urothelium can respond to a number of chemical and physical stimuli, including acetylcholine, therefore the possibility of nAChRs also participating in urothelial signaling is strong.

Given the established role anti-muscarinics play in lessening the urge to urinate in some cases of overactive bladder, the possibility of a second class of cholinergic drugs to treat bladder

pathology could have important clinical ramifications, as well. If urothelial nAChR agents can be shown to modulate bladder reflexes, then this research may have uncovered another potential target in addition to muscarinic receptors for the treatment of bladder pathologies. Additionally, since muscarinic and nicotinic receptors often interact in tissues where they are co-expressed, it is possible that nicotinic receptor agents could be used as an adjunct therapy with anti-muscarinic agents; lessening the doses of anti-muscarinics necessary to effect the desired result, while diminishing the undesirable side effects commonly caused by anti-muscarinic treatment.

The following chapters will describe our research into the potential role of nAChRs in urothelial signaling by first demonstrating the expression of nAChRs in the urothelium. We will then demonstrate the functionality of these urothelial nAChRs by examining their ability to modulate intracellular calcium concentrations and the release of the neurotransmitter ATP. Finally, we will examine if stimulation of urothelial nAChRs can alter bladder reflexes in the anesthetized rat, as measured by a cystometrogram. Our research indicates that nicotinic receptors are present in the urothelium and that they do play a role modulation of bladder reflexes, an effect most likely mediated through intracellular Ca^{+2} signaling and modulation of ATP release from the urothelium. These results not only further our understanding of the urothelium and how it mediates its "sensor/transducer" properties, but could have a significant effect on the treatment of bladder disorders, as they demonstrate a pathway capable of modulating bladder reflexes *in vivo.*

2.0 EXPRESSION AND DISTRIBUTION OF NICOTINIC ACETYLCHOLINE RECEPTORS IN THE URINARY BLADDER EPITHELIUM

Although nicotinic acetylcholine receptors (nAChRs) in both the central and peripheral nervous systems have been shown to play a prominent role in the control of urinary bladder function, little is known regarding expression or function of nicotinic receptors in the bladder epithelium, or urothelium. Nicotinic receptors have been described in epithelial cells lining the upper GI tract, respiratory tract, and the skin where they are responsible for a number of physiological functions such as cell migration, differentiation, homeostasis and intracellular signaling. Because recent studies have demonstrated that the urothelium can participate in the control of micturition, the urothelium may represent another epithelial tissue in which nAChR signaling may play a physiological role. Thus, as a first step into determining if urothelial nicotinic receptors play a role in the bladder, the present study examined the expression and distribution of nicotinic receptors in the urothelium. mRNA for the α3, α5, α7, β3, and β4 nicotinic subunits was identified in rat urothelial cells using RT-PCR. Similar subunits were also found in the cat and the human urothelium. Western blot analysis also supported the presence of nAChR protein in urothelial tissue. We attempted to localize the expression of nAChRs to specific layers of the urothelium using fluorescently tagged antibodies or toxins against nAChR subunits and co-localizing with known urothelial markers. Our experiments indicate that α3 receptors co-localize with cytokeratin 20, a marker for the umbrella cell layer in the urothelium. Conversely, α7

receptors co-localize with both cytokeratin 17 and 20, suggesting that these receptors are expressed in all three layers of the urothelium (umbrella, intermediate and basal). These data, which indicate the presence of nAChRs in the urothelium, are the first step in determining if nicotinic acetylcholine receptors could play a role in urothelial physiology.

Note: Figures 2.1 and 2.3 and the text describing them have been previously published in the American Journal of Physiology [220]© 2005, however permission to reprint is not required under the American Physiological Society's rules for reprinting published material by authors of the original manuscript.

2.1 INTRODUCTION

The afferent nerves in the urinary bladder respond to a variety of stimuli including distension and contraction of the detrusor muscle, as well as noxious chemicals contained in the urine or released during infection [1, 2, 6, 7]. Activity in afferent nerves initiates sensations of bladder fullness or pain and can also trigger voiding and urine storage reflexes. Previous studies of sensory mechanisms in the bladder, therefore, have primarily focused on the properties of the afferent nerves.

Recently, however, it has been shown that urothelial cells may influence afferent nerve activity by expressing "neuronal-like" properties including: 1) release of neurotransmitters (e.g. NO and ATP) [55, 61], 2) expression of receptors commonly found in nerves, such as vanilloid receptors (TRPV1) [13] and calcium-activated K^+ channels (SK channels) [86] and 3) sensitivity to neurotransmitters, such as acetylcholine [58, 77], norepinephrine [61] and ATP [11]. The

urothelium may also release acetylcholine in a fashion similar to that already reported for other non-neural tissues including pulmonary endothelial cells [221], placental epithelial cells [202, 222], and bronchial epithelial cells [223], where it acts in an autocrine/paracrine fashion to influence cellular functions. In this context, recent data suggests that urothelial cells may release acetylcholine during the filling phase of micturition [16, 89, 92, 93]. Given the established role for nicotinic acetylcholine receptors (nAChRs) in the neural control of bladder function [130, 131], the current study was aimed at determining if nAChRs are also expressed in the urothelium.

The nicotinic acetylcholine receptor family is currently known to consist of at least 17 different subunits (α1-10, β1-4, γ, δ, and ε) [140, 141]. These subunits form pentameric channels that can be categorized into 2 different groups; neuronal nicotinic receptors (consisting of α2-10 and β2-4 subunits) and muscle nicotinic receptors (consisting of α1, β1, γ, δ, and ε subunits). Neuronal nAChRs can be further classified into 3 groups: 1) homomeric pentamers (such as α7 or α9), 2) simple heteromeric pentamers consisting of one type of α subunit and one type of β subunit in a 2:3 stoichiometry (e.g. α3β2 receptors) and 3) complex heteromeric pentamers consisting of three or more different subunits (e.g. α3α5β2 receptors) [137, 138]. Each type of receptor has different electrophysiological and pharmacological properties, which have been hypothesized to be the basis for the widely varying effects of acetylcholine throughout the central and peripheral nervous system [152, 164, 165, 223-226]. The present study detected, in the urothelium of the rat, cat and human, mRNA for several nicotinic receptor subunits known to form functional receptors. We also detected protein for α3 and α7 subunits, utilizing western blot and fluorescent staining of bladder tissue. These data are the first step in determining if nicotinic receptors can play a role in urothelial signaling, as well as bladder physiology.

2.2 RESULTS

2.2.1 Nicotinic Subunit mRNA Expression in the Urothelium

As a first step to determining if nAChRs are expressed in the urothelium, we examined the expression of nicotinic receptor subunit mRNA in urothelial tissue of the rat, cat and human. The following sections summarize these experiments in each species.

2.2.1.1 nAChR Expression in the Rat

In order to determine nAChR expression in rat urothelial tissue, the bladder was first removed, cut open and pinned into a dish filled with oxygenated KREBS buffer. The urothelium was then gently teased away from the underlying smooth muscle using fine forceps and scissors and the RNA extracted. RT-PCR experiments using this extracted RNA indicated the presence of message for α3, α5, α7, β3, and β4 nicotinic receptor subunits in urothelial tissue (Figure 2.1A).

In the previous experiment, the possibility exists that contaminating tissue such as nerves, myofibroblasts or even small amounts of smooth muscle may be present in our samples, resulting in false positives. In order to rule out products amplified by contaminating tissue, we also extracted RNA from primary cultures of rat urothelial cells (Please refer to Section A.1.2 for details on our urothelial cell culture technique). Because the media maintaining our cultured cells only supports urothelial cells, we can assume that mRNA extracted from these cells, grown in culture for 48 hours, would lack contamination from other tissue types. RT-PCR in cultured cells also indicated the presence of α3, α5, α7, β3 and β4 mRNA (results not shown).

In both urothelial tissue and cultured cells, the observed bands corresponded to expected product sizes (see Table 5 for expected product sizes) and results were repeatable in samples

from three separate rat bladders or three independent cultures. We did not detect products for α2, α4, and β2 subunits in either urothelial tissue or cultured cells, however these subunits were amplified using RNA from rat brain extract (Clontech) as a positive control (results not shown).

Because extracted mRNA is sometimes contaminated with genomic DNA, which would result in a PCR product being amplified in our experiments, a negative control was also performed. This negative control involved the amplification of a housekeeping gene, GAPDH, from first-strand reactions of urothelial RNA where reverse transcriptase (RT) was omitted. If contaminating genomic DNA was present in our RNA samples, then a PCR reaction on these RT negative samples would have resulted in amplification of GAPDH DNA. As shown in Figure 2.1D, GAPDH is amplified in PCR experiments when RT is included in the first strand synthesis reaction, but not when it is omitted, indicating that contaminating genomic DNA is not present. Finally, in order to rule out the possibility of amplification of an incorrect product through non-specific binding of our PCR primers, each product's identity was confirmed through DNA sequencing and subsequent matching through a BLAST search.

2.2.1.2 nAChR mRNA Expression in the Human

In order to determine if nAChR subunits are expressed in human urothelial tissue, we obtained human bladder tissue from deceased organ donors through the University of Pittsburgh's Center for Organ Recovery and Education (CORE). The urothelium was obtained in the same manner as described above for the rat; the bladder was cut open, pinned in a dish containing oxygenated KREBS buffer and the urothelium teased away using fine forceps and scissors. mRNA was obtained from three such samples and RT-PCR was performed using subunit specific primers. PCR amplified products for nAChR subunits very similar to that found in the rat: α3, α5, α7, β2 and β4 (Figure 2.1B).

To rule out contamination from non-urothelial tissue in our RT-PCR experiments, human urothelial cells were also cultured, as described in Section A.1.2. RT-PCR performed on RNA extracted from three independent cultures of human urothelial cells exhibited identical results to urothelial tissue: products were observed for α3, α5, α7, β2 and β4 (data not shown).

As a negative control to determine if our RNA samples from either urothelial tissue or cultured human cells contained contaminating genomic DNA, PCR was performed on samples where reverse transcriptase was omitted from the first-strand synthesis reaction. These reactions showed no amplification, indicating that no genomic DNA was present (data not shown). Unfortunately, we could not obtain control tissues in the human such as brain or dorsal root ganglia to use as positive controls. The specificity of these primer sets, however, have been demonstrated by another group [227] in human brain and skeletal muscle, therefore we are confident that our negative results are not due to failure of the primers. Positive results were analyzed through DNA sequencing and their identities confirmed through a BLAST search.

2.2.1.3 nAChR mRNA Expression in the Cat

In addition to the rat and human, we also examined nAChR expression in the cat. Cats are often used in bladder physiology experiments due to the natural occurrence of a pathological bladder condition similar that which affects humans known as interstitial cystitis. Therefore, information into the expression of nicotinic receptors in the cat urothelium may help further research into this disease. Unfortunately, few nAChR subunits have been cloned in the cat, therefore a thorough examination of which subunits are expressed by the urothelium is not yet possible. Primer sets do exist for α3, α4, α7 and β2 [228], which we used to determine if these subunits are expressed in the cat urothelium. Urothelial tissue was obtained from three cat bladders in the same manner as described above for the rat; bladders were removed, cut open, pinned into a dish containing

oxygenated KREBS buffer and the urothelium gently teased away using fine forceps and scissors. RT-PCR performed on RNA extracted from these tissues indicated the presence of α3 and α7 mRNA, but not α4 and β2 (Figure 2.1C). Positive controls for α4 and β2 were performed in mRNA extracted from dorsal root ganglia (data not shown). Additionally, controls for GAPDH expression in RT negative reactions were also negative, indicating a lack of non-specific amplification by genomic DNA contamination (data not shown).

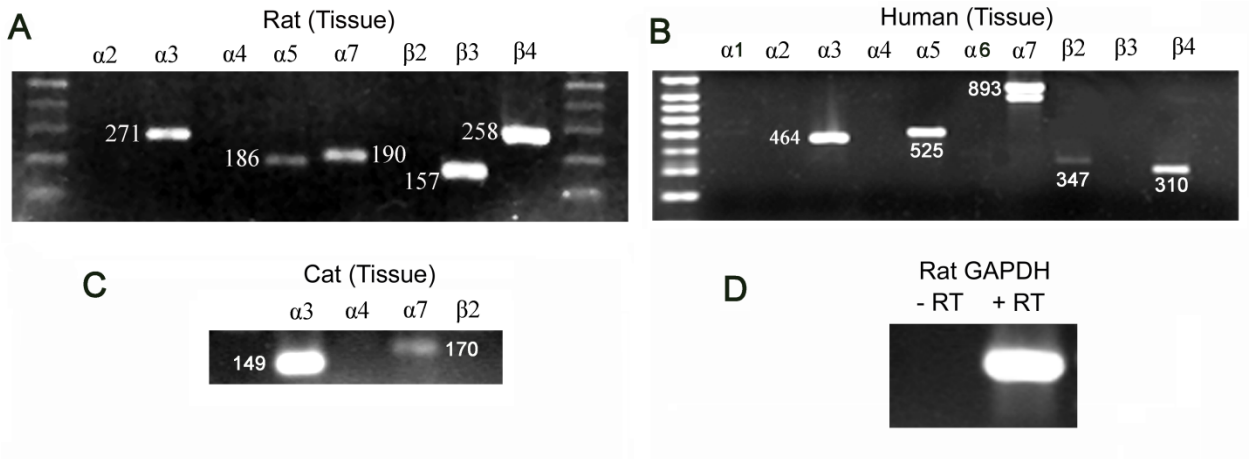

Figure 2.1- Expression of nAChR mRNA in the Urothelium

(A) Nicotinic acetylcholine subunit expression was detected in urothelial tissue taken from rat bladders. Notice positive results in α_3, α_5, α_7, β_3 and β_4 lanes. 1.2% agarose gel in 1X TBE buffer stained with ethidium bromide. Results are identical from tissue taken from three separate rat bladders. Numbers indicate the size, in base pairs, of the product band. **(B)** RT-PCR of human urothelial tissue. Positive results are present for the α3, α5, α7, β2 and β4 subunits. Results are identical from tissue taken from three separate bladders. Numbers indicate the size, in base pairs, of the product band. **(C)** RT-PCR of cat urothelial tissue. Proper bands are present for α3 and α7. Results are identical from tissue taken from three bladders. Numbers indicate the size, in base pairs, of the product band. **(D)** Negative control experiment demonstrating that the housekeeping gene GAPDH fails to amplify when the reverse transcriptase (RT) enzyme is omitted from the cDNA synthesis reaction. This indicates that no genomic DNA contamination exists in our samples.

2.2.1.4 Quantitative PCR of nAChRs in the Rat Urothelium

To determine the relative expression levels of the nAChR subunits found in the urothelium, we performed real-time quantitative PCR (qPCR) in rat urothelial tissue. Urothelial tissue was removed as described above and the RNA extracted for use in cDNA synthesis. This cDNA was then used as a template, along with the subunit specific primers in SYBR Green based qPCR reactions (Bio-Rad, Inc.).

The field of real-time qPCR is relatively new, and as a result, a number of popular methods for analyzing data have emerged, all having specific strengths depending on the experimental setup. Because we are interested in the relative expression of nAChR mRNA as compared to each other, we utilized the $2^{-\Delta\Delta C}{}_T$ method [229] for comparing gene expression through normalization to an endogenous control gene. In our case, β-actin was used as our control gene. This technique will also allow us to express our data relative to the expression of one of our target genes, assuming that the efficiencies of amplification between the subunits are the same. The efficiency of amplification was experimentally tested for each subunit and β-actin by performing qPCR reactions on 4 serial dilutions (1X-1,000X dilutions) of cDNA and plotting CT versus cDNA concentration. The efficiency of the amplification was then calculated as $E= 10^{(-1/slope)}$. All efficiencies (average: 87.4%) were determined to be not significantly different, allowing us to use the $2^{-\Delta\Delta C}{}_T$ method of analyzation.

We chose to express our data relative to the α5 subunit, as that subunit was expressed at the lowest levels. Therefore the equation used to determine the relative expression of our nAChRs was:

$$\text{Relative expression} = 2^{-(\Delta C_{T,q} - \Delta C_{T,\alpha 5})}$$

where $\Delta C_{T,q}$ is the difference in the threshold cycle between the gene of interest and the housekeeping gene (β-actin) and $\Delta C_{T,\alpha5}$ is the difference in the threshold cycle between α5 and the housekeeping gene.

It should be noted, though, that in our experiments the cDNA to be amplified and quantified using qPCR is created using general primers consisting of 15 thymine bases (dT15) instead of primers specifically designed for each mRNA target. These oligo(dT)15 primers bind to the poly(A) tail present on all mRNA, which allows binding of reverse transcriptase and conversion to cDNA. Use of these primers may have the potential to skew our results, as the reverse transcription of each mRNA may not occur with the same efficiency due to such factors as length of the transcript or the presence of a complex secondary structure. This could cause incorrect interpretation of our results, as any differences in the measured C_T between two targets may not be due to differences in their mRNA levels within the cells, but instead be due to differences in the efficiency of the reverse transcriptase reaction between the two targets. This could be controlled for by measuring the efficiency of each reverse transcriptase reaction and correcting for it in the ΔCT equation (for review, see [230]). However, our goal for this part of our project was to use qPCR to make an educated guess about the subunit composition of the nAChRs that may play a significant role in urothelial signaling based on their prevalence in the urothelium. This only requires us to determine a rough estimate of the relative expressions of the nAChRs present in the urothelium, not to determine exact figures on the number of copies of mRNA present for each nAChR subunit. Therefore, for the purposes of our experiments the efficiency of the reverse transcriptase reactions for each target was assumed to be equal.

As shown in Figure 2.2, we estimated that the β4 subunit is expressed at the highest levels, followed by the α7, α3, β3 and α5 subunits. These data are consistent with the α7 and

α3β4 subunits being expressed at the highest levels, with α3* receptors containing the α5 or β3 subunits being much less common.

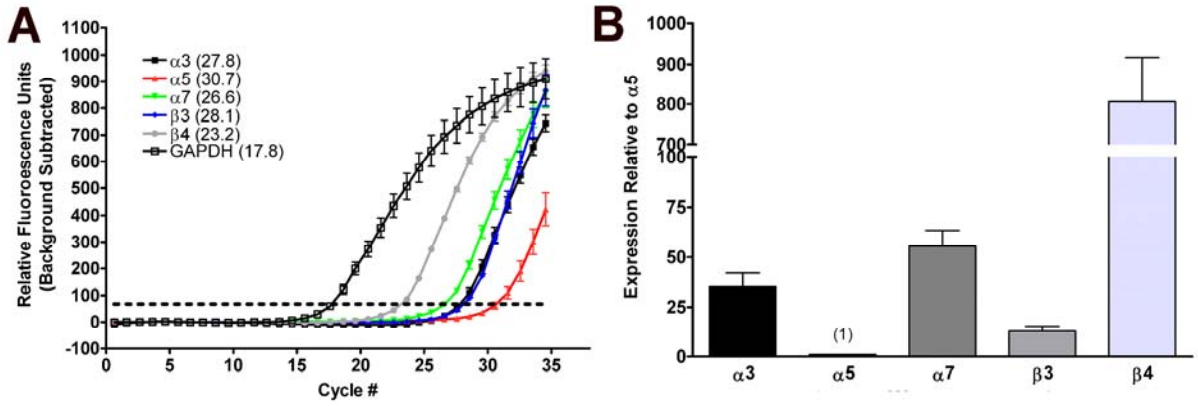

Figure 2.2 - Relative Expression of nAChR Subunit mRNA in the Rat Urothelium

Relative expression of nAChR mRNA in the rat urothelium. **(A)** Real-time PCR data from one experiment, showing the increase in relative fluorescent units (RFUs) as a function of cycle number. Each trace is an average of triplicate samples prepared identically from the same cDNA (see Appendix for methods). The dashed line indicates the threshold level of fluorescence, as determined by the computer (68.2 RFUs). The numbers indicated in the legend are the cycle number where the signal crossed the threshold (C_T). **(B)** Graph summarizing the relative expression of nAChRs. Levels are expressed in relation to the α5 subunit, which was expressed at the lowest level. Results shown are the average of experiments before from four separate rat bladders.

2.2.2 nAChR Protein Expression in the Rat Urothelium

While our experiments have indicated the presence of mRNA for nAChRs in the urothelium, they do not determine if the actual nicotinic proteins are expressed. In order to determine if the urothelium of the rat expresses nAChR proteins, we utilized both immunoblotting techniques and fluorescent labeling of bladder tissue.

2.2.2.1 Western Blots of nAChR Subunits in Rat Urothelium

To determine the distribution of the nAChRs within the urothelium, we first performed immunoblots on protein extracted from urothelial tissue. Tissue was collected as described above for PCR, and the extracted proteins run on a SDS-PAGE gel under denaturing conditions. Following transfer to a nitrocellulose membrane, we probed for nicotinic receptor subunits using commercially available antibodies (Santa Cruz). We only probed for the α3 and α7 receptors, due to the lack of suitable antibodies against the other subunits. However, given what is known about how nAChR subunits combine to form functional receptors, we believe that any functional receptors in the urothelium would most likely include either of these two subunits [139]. Additionally, only rat tissue was examined due to limitations either in antibody selectivity (i.e. antibody does not react with cat receptors) or inability to obtain sufficient amounts of tissue (such as in the human).

As shown in Figure 2.3, immunoblotting revealed a major and minor band for both the α3 and α7 subunits, with no other bands being present in the lane. These bands migrated to a molecular weight of 30 and 35 kDa, respectively. These bands were consistent with those observed with protein extracted from rat dorsal root ganglia, which we used as a positive control (data not shown) [164, 231, 232]. Additionally, bands observed from both DRG and urothelially-derived protein disappeared when the antibodies were pretreated with the appropriate blocking antigen, suggesting that the binding is specific for nAChR subunits.

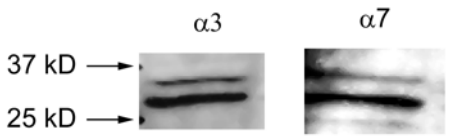

Figure 2.3 - Western Blot of nAChR Subunits in Protein Extracted from Rat Urothelial Tissue

Western blots stained for α3 (left) and α7 (right) nAChR subunits. Both blots revealed a major band at 30 kDa and a minor band at 35 kDa.

2.2.2.2 Co-localization of nAChRs with Urothelial-Specific Markers in the Rat Bladder

Our immunoblots suggest that urothelial tissue expresses nAChR subunit proteins, however they do not give us information on where in the urothelium the receptors are expressed. In order to localize each receptor subunit to a particular layer of the urothelium, we performed co-localization studies in rat bladder tissue with cytokeratins 17 and 20, which are known to be differentially expressed in the urothelium of the rat. Cytokeratin 20 has been shown to be expressed in the umbrella cells of the urothelium [233], while cytokeratin 17 localizes to the intermediate and basal cells [234].

In order to localize the α3 subunit within the urothelium, we stained sections of the rat bladder with a commercially available antibody (Santa Cruz). α3 subunits are also prevalent in small to medium sized neurons of the rat DRG [232], which we used as a positive control (Figure 2.4B). In rat bladder tissue, the α3 subunit stained a layer of tissue consistent with the urothelium, as the staining was prevalent in tissue directly adjacent to the lumen of the bladder (Figure 2.5A-C). This staining disappeared if the antibody was pre-incubated with its antigen or if only the secondary antibody was used (Figure 2.5D-G). Co-localization studies with

cytokeratin 20 suggested that the α3 receptor is expressed mainly in the umbrella cells (Figure 2.6A-C), as the two antibodies co-localize almost exclusively.

To localize the α7 nAChR subunit, a fluorescently tagged (AlexaFluor 488) epitope of the neurotoxin α-bungarotoxin (α-BTX) was used instead of an antibody. This was done because commercially available antibodies against α7 receptors have not yet been fully developed and α-BTX binds α7 receptors with high affinity and high specificity. Figure 2.4A shows α-BTX binding in all small to medium sized DRG cells, confirming earlier research [231] and acting as a positive control for our staining. α-BTX binding in rat bladder sections revealed staining consistent with urothelial localization (Figure 2.7A-C), as staining was prevalent in tissue surrounding the bladder lumen. We also observed α-BTX staining consistent with blood vessels (Figure 2.7, arrow), in line with earlier reports that α7 receptors are expressed in vascular endothelial cells. α-BTX staining in the rat bladder was diminished when pre-incubated with a 100-fold higher concentration of unlabelled α-BTX (Figure 2.7D-F). The intensity of this staining was analyzed using image analysis software (Simple PCI, Hamamatsu, Inc., Sewickley, PA). This was accomplished by analyzing the intensity of staining in select regions of interest (ROIs) in the urothelium and comparing between tissue stained with fluorescent α-BTX and tissue where α-BTX staining was competed off using un-labeled α-BTX. These ROIs, which were carefully selected to be the same size, were corrected by subtracting background fluorescence. Intensities were averaged from three tissue slices for each type of staining and compared using a students' t-test. Using this method, it was determined that pre-incubation with the unlabeled bungarotoxin decreased the intensity of staining 47.3% ($p<0.01$), suggesting that the staining was specific (Figure 2.7 G-I).

In order to determine which layers of the urothelium express α7 receptors, we co-stained rat bladder sections with α-BTX and cytokeratins -17 and -20. As shown in Figure 2.8, α-BTX staining co-localized with both cytokeratin 17 (Figure 2.8A-C), suggesting α7 expression in intermediate and basal cells, and with cytokeratin 20 (Figure 2.8D-F), suggesting α7 expression in umbrella cells.

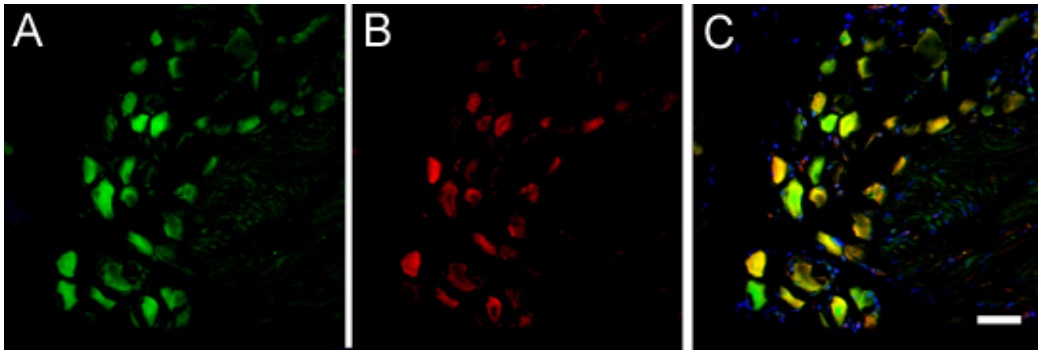

Figure 2.4 - Positive Controls for nAChR Receptor Localization

(A) α7 and (B) α3 nAChR staining in the rat DRG. (C) An overlay of the green and red channels coupled with a DAPI nuclear stain to show co-localization. Note that the majority of small to medium sized cells (cell diameter < 50μm) are positive for both α7 and α3, while no cells larger than 50μm are labeled, supporting earlier research [231, 232]. Pictures are a layered merge of a series of 50 images taken with a confocal microscope with a 40X oil immersion objective at 0.08 μm intervals along the z-axis (4 μm total depth). Calibration bar = 50 μM.

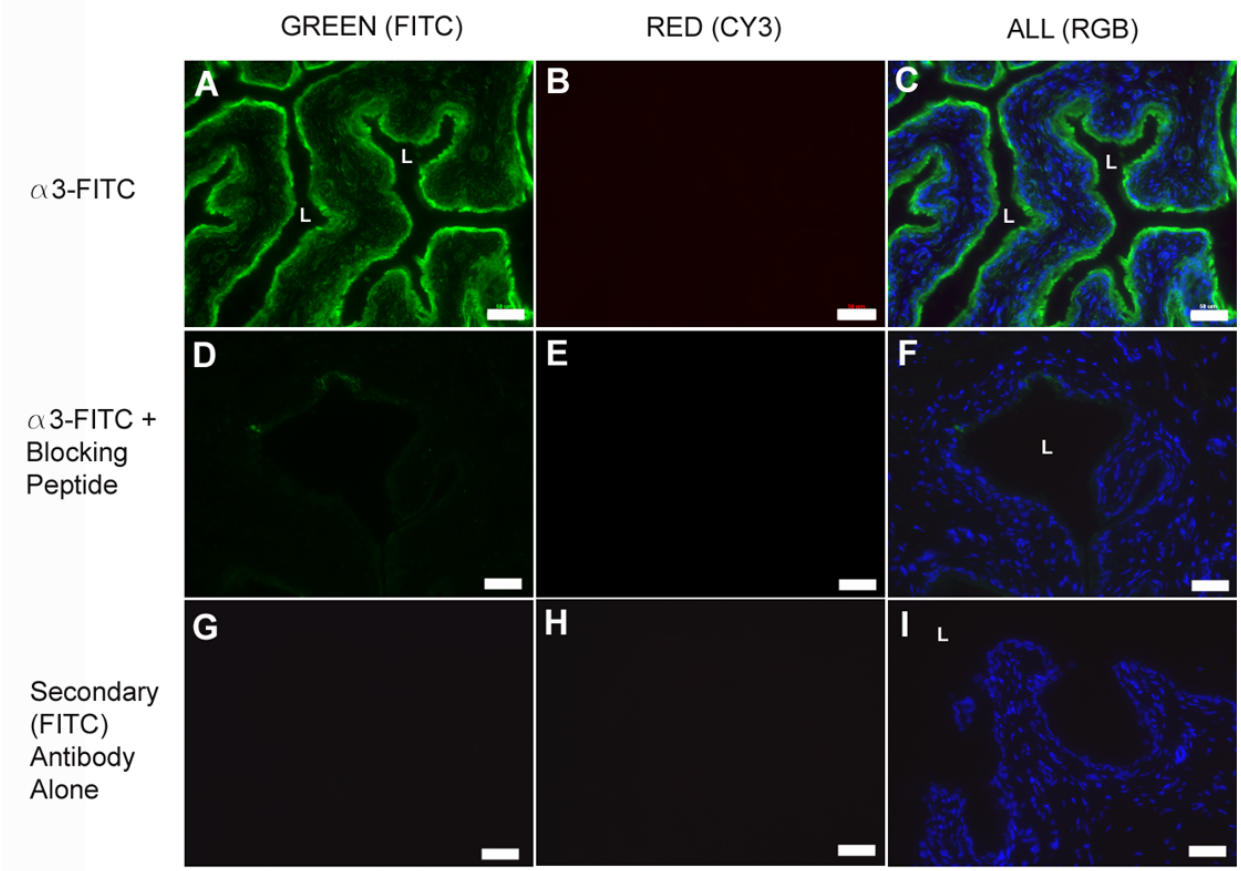

Figure 2.5 - Expression of the α3 nAChR Subunit in the Rat Bladder

Photomicrographs depicting α3 staining in the rat bladder. The left column depicts the FITC labeling of the α3 subunit, the middle column shows the Cy3 channel as a control and the right column merges the first two with a DAPI nuclear stain. The Cy3 channel is shown to demonstrate that no "bleed through" of the FITC signal occurs, which may affect the interpretation of the co-localization experiments shown in the next figure. **(A-C)** Staining for the α3 subunit depicts labeling of tissue directly adjacent to the lumen (L), consistent with the location of the urothelium. **(D-F)** Pre-incubation of the antibody with its blocking peptide antigen abolishes the staining. **(G-I)** secondary antibody (donkey anti-goat FITC) control to demonstrate that the secondary antibody does not bind non-specifically. All photos were taken with a 20X oil immersion objective, calibration bars show 50μm.

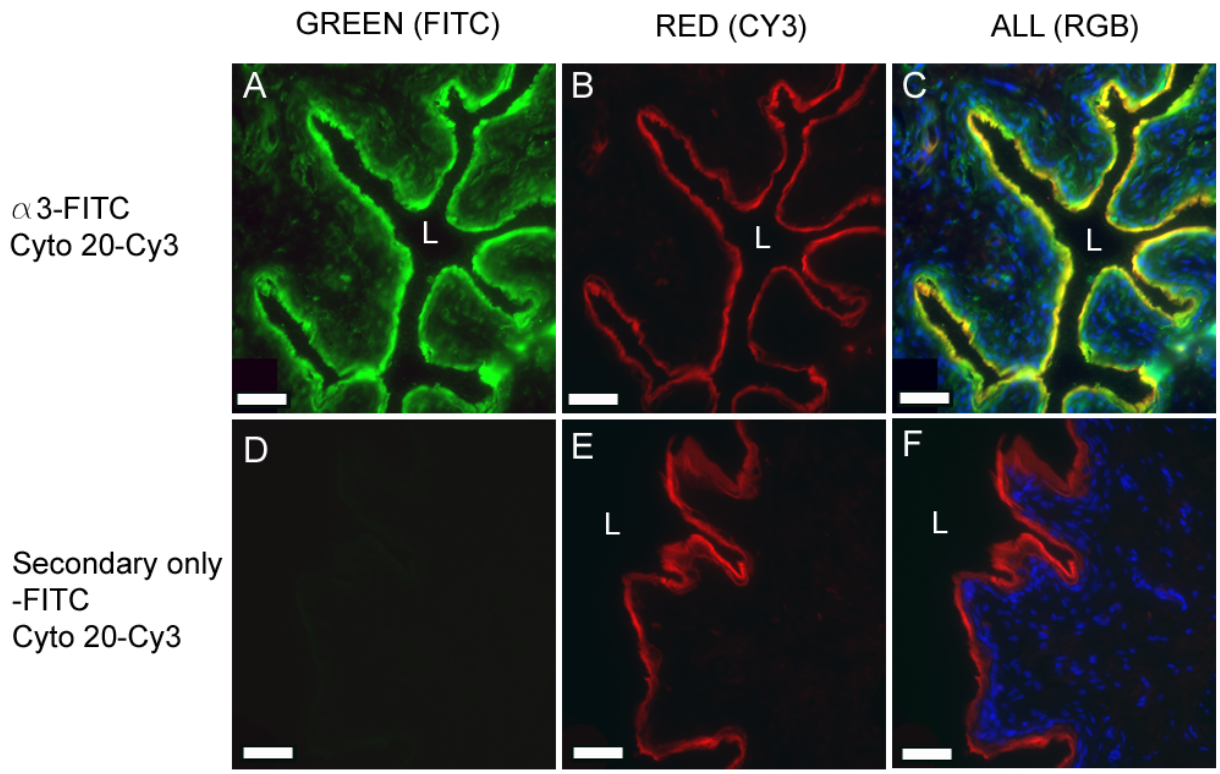

Figure 2.6 – Co-localization of the α3 nAChR with Cytokeratin 20

Staining for the α3 nAChR subunit and the umbrella cell specific marker cytokeratin 20 in the rat urothelium. The left column depicts FITC labeling of the α3 subunit, the middle column shows Cy3 labeling of cytokeratin 20 and the right column overlays the first two with a DAPI nuclear stain. **(A-C)** α3 staining co-localizes with cytokeratin in the umbrella cells of the urothelium. Note the presence of the color yellow directly adjacent to the bladder lumen (L) in the right hand column, which depicts co-localization of α3 staining with cytokeratin staining. **(D-F)** A control experiment to demonstrate that the secondary FITC antibody used to visualize the α3 antibody does not non-specifically bind in the bladder. Note the lack of signal in (D), indicating that no cross reactivity is present. All pictures were taken with a 20X oil immersion objective, calibration bars show 50μm.

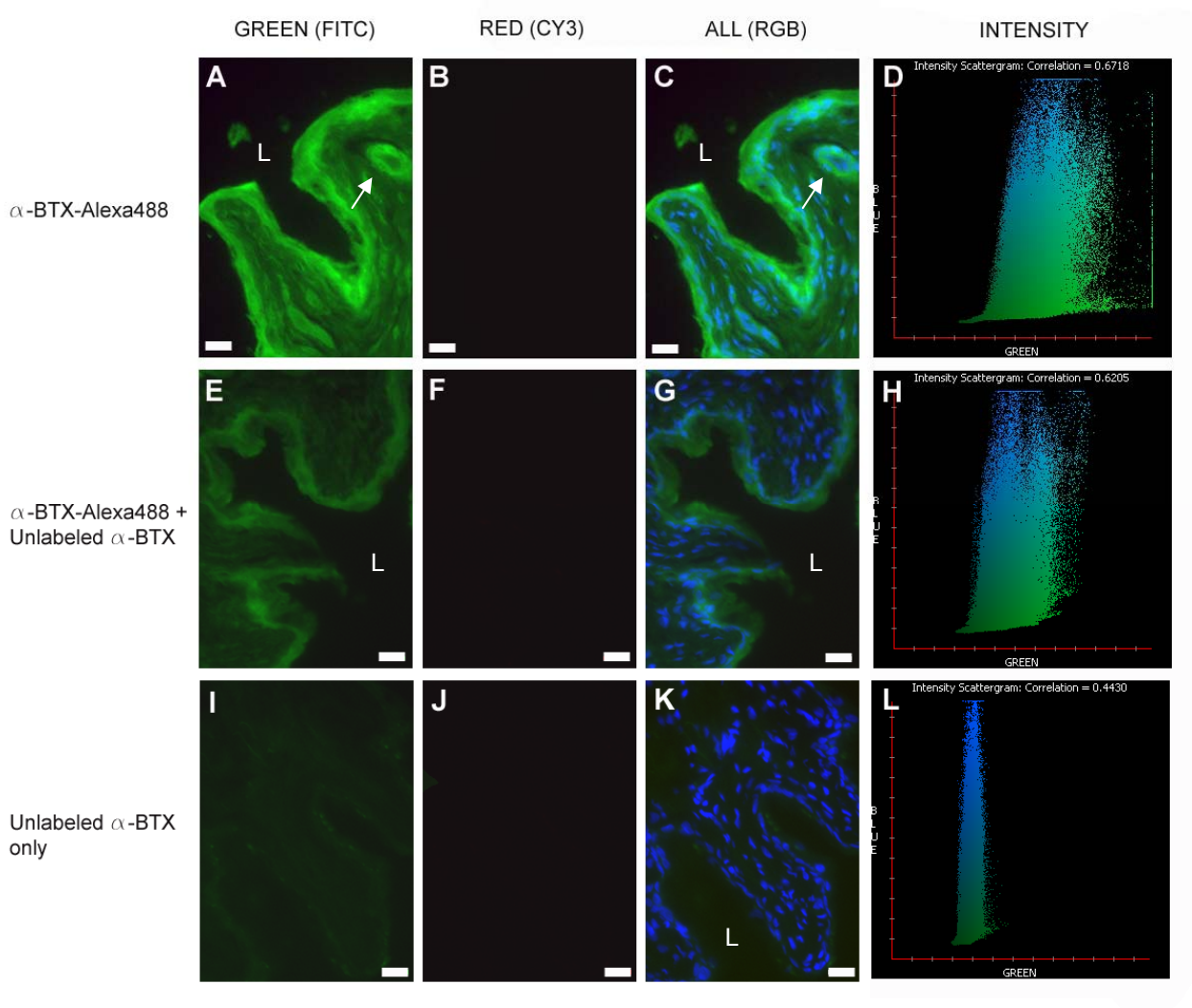

Figure 2.7 - α7 Staining in the Rat Bladder

Photomicrographs showing α-bungarotoxin (α-BTX) binding in the rat bladder. The left column depicts α-BTX binding, the 2nd column shows the Cy3 channel as a control and the 3rd column merges the first two with a DAPI nuclear stain. The Cy3 channel is shown to demonstrate that no "bleed through" of the FITC signal occurs, which may affect the interpretation of the co-localization experiments shown in the next figure. The right column depicts an intensity scatterplot of the green (FITC) and blue (DAPI) signals. **(A-D)** Staining with α-BTX shows labeling in the bladder directly adjacent to the bladder lumen (L), consistent with the location of the urothelium. Staining is also seen in structures consistent with blood vessels (arrow) which have been previously shown to express α7 receptors [201, 235, 236]. Panel D shows an intensity scatterplot of the green and blue channels; notice the full distribution of both channels across the spectrum. **(E-H)** Pre-incubation of the tissue with unlabeled α-BTX diminishes the staining. Panel H shows an intensity scatterplot of the green and blue channels; notice the decrease in green intensity as compared to D. **(I-L)** Control slide where tissue was incubated with unlabeled α-BTX. Notice the lack of signal in I and the decrease in green intensity as measured in L. All pictures were taken with a 20X oil immersion objective at the same shutter speed and light intensity so that differences in staining intensity between slices could be measured. Calibration bars show 50μm.

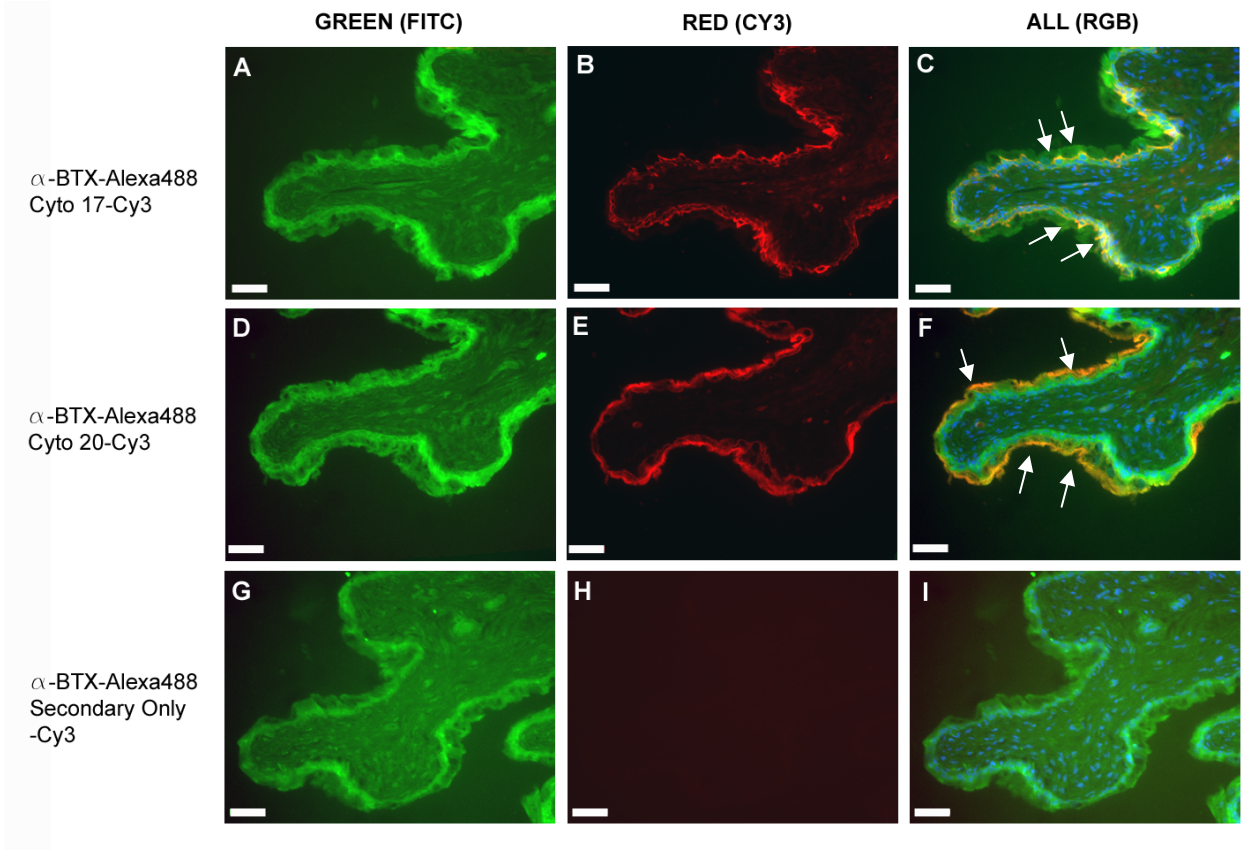

Figure 2.8 - α7 Co-localization with Cytokeratins

Photomicrographs showing α-BTX binding in rat urothelial cells positive for cytokeratins 17 and 20. The left column depicts the FITC labeling of the α7 subunit, the middle column shows the Cy3 staining of cytokeratins 20 or 17 and the right column merges the first two with a DAPI nuclear stain. **(A-C)** Fluorescent α-BTX co-localizes with cytokeratin 17 in the intermediate and basal cells of the urothelium, as demonstrated by the yellow staining seen in C (arrows). **(D-F)** Fluorescent α-BTX co-localizes with cytokeratin 20 in the umbrella cells of the urothelium as demonstrated by the yellow staining seen in F (arrows). **(G-I)** As a control to determine if the secondary antibody used to visualize the cytokeratins (donkey anti-mouse Cy3) exhibits non-specific binding that would cause a misinterpretation in co-localization. As seen in Panel H, staining with the secondary antibody alone results in no labeling, indicating that non-specific binding does not occur. All pictures were taken with a 20X oil immersion objective, calibration bars show 50μm.

2.2.3 nAChR Expression in Cultured Urothelial Cells

We have shown evidence of nAChR expression in rat urothelial tissue, however many of the experiments we will perform in later chapters of this thesis will utilize cultured urothelial cells to study nAChR function and signaling. It is possible that the culturing procedure could alter nAChR expression in rat urothelial cells, however, which could influence our results. Our earlier experiments demonstrated that both tissue and cultured urothelial cells from the rat express message for the same nAChR subunits, however, we have not yet demonstrated that nicotinic proteins are expressed in cultured cells. Therefore, we stained cultured rat cells (48 hours in culture) for the α3 and α7 nAChR subunits, as we did in bladder tissue above. As shown in Figure 2.9, cultured cells expressed both α3 and α7 subunits. This staining was observed throughout all cells in the culture, and was located both cytoplasmically as well as along the cell membrane.

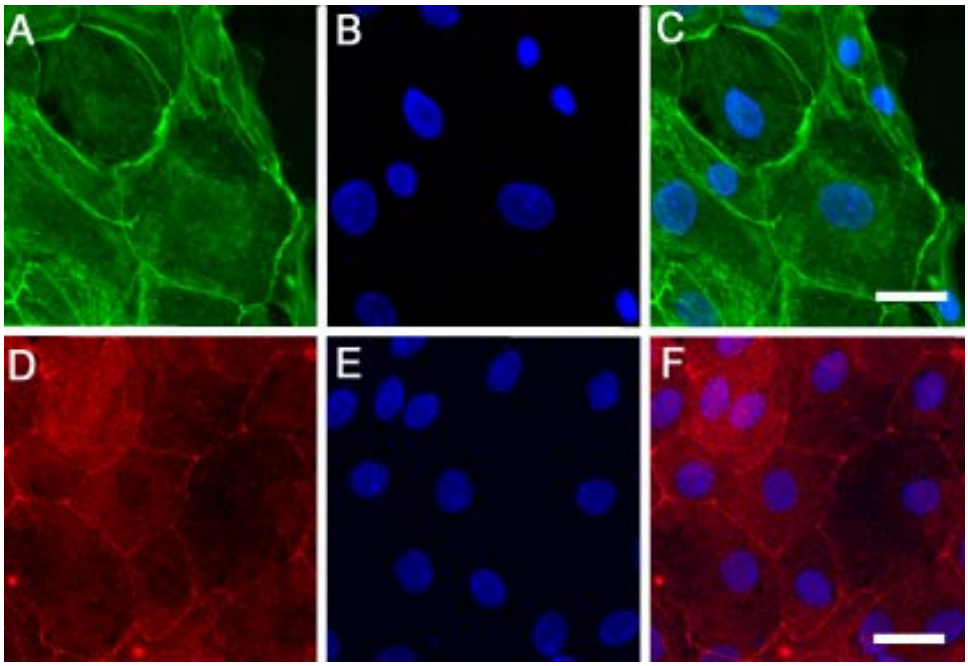

Figure 2.9 - nAChR Expression in Cultured Rat Urothelial Cells

Staining for α3 and α7 nAChRs in cultured rat urothelial cells. **(A-C)** FITC labeling of α7 receptors using α-BTX. **(D-F)** Cy3 labeling of α3 receptors. In both rows, the left column depicts staining for the nAChR subunit in question, the middle column depicts DAPI nuclear staining and the right column shows a merge of the two. Pictures were taken with a 20X oil immersion objective and zoomed 200% to show detail. Calibration bar denotes 50μm.

2.3 DISCUSSION

Acetylcholine is an important transmitter in the neural pathways controlling bladder function; being responsible for neurotransmission in the brain, spinal cord, autonomic ganglia and detrusor smooth muscle [1, 130-134]. The present study raises the possibility of an additional site for cholinergic modulation of bladder function. RT-PCR revealed that urothelium of the rat, cat and human expresses the appropriate nicotinic receptor subunits that can interact to generate commonly expressed receptors, while immunoblotting and tissue staining indicated that nAChR proteins are also expressed in the urothelium of the rat.

RT-PCR analyses indicate that there may be more than one type of nicotinic receptor present in the urothelium. While many combinations of the 16 known subunits are possible, research in heterologous expression systems has indicated that only a few of these combinations actually form functional receptors [136-138, 140]. Possible functional receptors in the rat urothelium include α7 homomeric receptors and α3β4, α3β3β4, α3α5β4, and α3α5β3β4 heteromeric receptors (commonly referred to collectively as α3*) (see Figure 2.10). Additionally, recent evidence suggests that α7 subunits may form functional receptors with β subunits in heterologous expression systems, such as oocytes, in addition to their homomeric form [226]. These atypical receptors, however, have not yet been demonstrated to exist *in vivo,* therefore their contributions to cellular function are not yet known. Each subtype of receptor has specific pharmacological and electrophysiological properties [140, 152, 165, 166, 170, 172, 174] that may be responsible for mediating different effects in the bladder. nAChRs are responsible for a number of physiological effects *in vivo* such as cell motility [237], differentiation [144, 148], apoptosis [238], adhesion [237], calcium homeostasis [144, 147, 239] and intracellular signaling [231, 240]. Therefore it is possible that urothelial nAChRs mediate a number of different physiological effects in the bladder.

We first examined the expression of nicotinic subunit protein through the use of western blot. One concern in our experiments is that the bands we observed migrated consistent with sizes of 30 and 35 kDa, while both the α3 and α7 receptor subunits are 50-55 kDa proteins. This may indicate non-specific binding, however two pieces of data suggest that it is not. First, these bands migrate to the same position in our positive control, DRG protein, where both subunits have been previously reported. Secondly, both bands disappear when the antibody is pre-incubated with its commercially available antigen. While these results do not definitively prove

that the observed bands are indeed nAChR subunits, as the antigen could also block non-specific binding, when considered in conjunction with our PCR and tissue staining, we can conclude that urothelial cells express nAChRs.

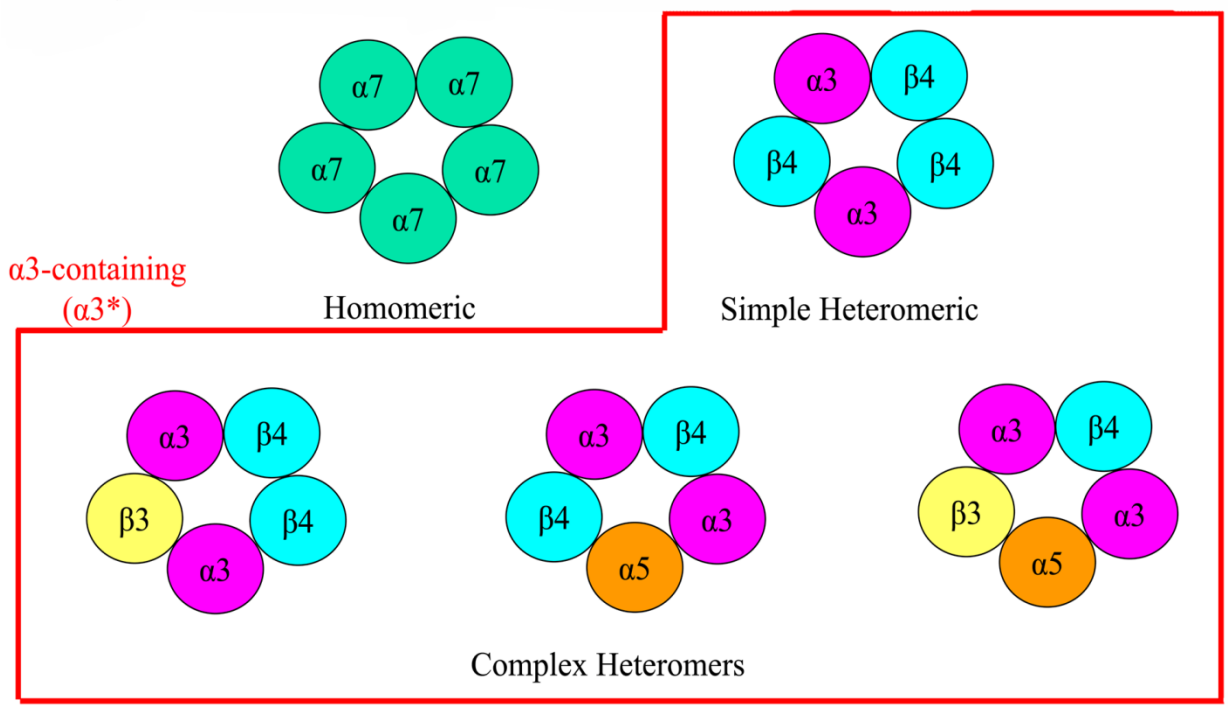

Figure 2.10 - Possible Composition of Urothelial nAChRs

Cartoon representation of the possible nAChR subunit compositions that might form functional receptors in the urothelium. While many more combinations are possible, only these have been found to form functional receptors either *in vivo* or in heterologous expression systems, such as oocytes. Note that each of the possible heteromeric receptors are grouped into the "α3-containing" category, as these receptors may be functionally different but are so far pharmacologically indistinguishable.

Why then would the proteins migrate so far from their widely accepted molecular weight? A number of possibilities exist. First, it is possible that the nAChR subunit protein was subject to proteolytic degradation, reducing the apparent size of the protein. We believe that this possibility is unlikely however. First, a cocktail of protease inhibitors is included when the urothelial tissue is homogenized to extract the protein, which should protect against proteolytic

degradation. Additionally, we would expect that proteolytic degradation would result in more than two bands on the blot.

Additionally, though, a smaller protein may indicate the presence of a slice variant of the nAChR subunit. For example, it has been recently shown that a splice variant of the α7 receptor exists which arises from an alternate splicing of intron 9 and results in a protein that is missing a large intracellular loop and most of transmembrane region four [241]. This results in a protein that migrates to approximately 38-42 kDa in a non-SDS polyacrilamide gel. Given this data, it is possible that the nAChR observed with anti-α7 antibodies in urothelially-derived protein samples may represent an alternatively spliced version of the receptor. To determine if this is indeed the case, we would need to probe the urothelium for the splice variant, either by *in situ* hybridization experiments using a cRNA probe that would bind to the alternate version of intron 9 or through PCR using primers that flank the splice site and would amplify different sized products depending on which splice variant was present. It should be noted however, that these experiments would only detect the specific splice variant described by Saragoza, et. al. [241] and not any other variants that may exist. It would also be possible to collect and sequence the protein(s) that bind to our antibody through affinity chromatography and determine if they have the same sequence as known nAChRs.

Another question regarding our immunoblots could be raised by the presence of two bands for each subunit. The presence of two bands could mean that our antibody is also binding non-specifically. However, once again, both bands are present in our positive control and disappear when the antibodies are pre-incubated with their respective antigen. While these data do not rule out the possibility of non-specific binding, it is also possible that our antibodies are binding two forms of the nAChR subunits, possibly the subunit with and without post-

transcriptional modulation. Additions of adjuncts, such as glycosylation, myristoylation or acetylation could add mass to a finished protein and slow its movement through a gel [242]. Therefore, the two separate bands may not indicate non-specific binding, but post-translational modifications that could alter migration. To determine if post-translational modifications are responsible for this shift, the proteins would need to purified and analyzed through mass spectroscopy.

While some questions remain following immunoblot analysis, our fluorescent binding studies in the rat bladder strengthen the argument that urothelial cells express nAChR protein. We have demonstrated that staining bladder tissue with an antibody against the α3 receptor subunit or a toxin that binds the α7 receptor binds to bladder tissue directly adjacent to the lumen, where the urothelium is located. Both the antibody and the toxin co-localize to tissue that is stained by urothelial-specific markers, suggesting urothelial distribution of these nAChRs. Taken together with the immunoblot data, then, we are confident that the urothelium expresses nAChRs.

Our research localizes the α3 subunit to the umbrella cells of the urothelium. These studies, however, do not determine which of the possible α3* receptors are actually expressed in the urothelium. Ideally, co-localization studies between α3, α5, β3 and β4 subunits should be performed in order to determine the prevalence of each possible receptor combination. However, the lack of proper antibodies prevents us from performing these experiments. Our qPCR experiments suggest that the α3β4 receptor is the most prevalent in the rat urothelium, as mRNA for the β4 subunit is expressed at much higher levels than the β3 subunit. Our studies also demonstrate that the α5 and β3 subunits are expressed at a much lower level than the others, suggesting that receptors containing these subunits may be much less common. These findings

are consistent with the prevalence of nAChR receptors demonstrated in other tissues; α3β4 receptors are much more common than receptors containing α5 or β3 subunits [139, 152, 243]. It should be pointed out though, that we only examined the relative levels of nAChR message, not actual protein, which may differ depending on differences in the control of translation for each subunit. Each of these receptors has distinct pharmacological and electrophysiological properties, therefore even though α3β4 receptors may greatly outnumber β3 or α5-containing receptors in the urothelium; it is possible that each receptor subtype may have important physiological roles in urothelial signaling. We will attempt to determine the physiological effects of α3* receptor stimulation in the following chapter of the dissertation.

Clues to the physiological roles of nAChRs in the urothelium may be deduced by their localization in the urothelium. Given the low permeability of the umbrella cells maintaining the urothelial barrier [19, 38, 39, 119], receptors expressed on the apical surface of the umbrella cells, as our research indicates both types of receptor are, would likely only be stimulated by ACh present in the lumen of the bladder. nAChRs have been shown to be present on the luminal surface of a number of epithelial/endothelial "sacs", such as bronchial alveoli [244], where ACh is released into the lumen as it is in the bladder. In these other tissues, ACh released by epithelial cells acts in an autocrine or paracrine fashion on epithelial nAChRs to promote cell adhesion and inhibit apoptosis, maintaining the epithelial layer [245]. It is possible that ACh, released into the lumen of the bladder and acting on urothelial nAChRs, also maintains the urothelial barrier in a similar fashion. Additionally, it is possible that urothelial nAChRs play a role in the sensor/transducer role of the urothelium. ACh, as well as norepinephrine and bradykinin, cause the release of transmitters such as NO or ATP from cultured urothelial cells [58, 61, 63, 77], which are thought to act on afferent nerves to modulate bladder activity [6, 55,

66, 67, 97]. As mentioned previously, it is our aim to determine if urothelial nAChRs can influence bladder activity in the next chapter of the dissertation.

α7 receptors are also expressed in the intermediate and basal layers of the urothelium, where they could only be stimulated by luminal ACh when the urothelial barrier is disrupted, such as after an injury or infection. Since α7 receptors have been shown to be responsible for motility and terminal cell differentiation in stratified squamous epithelial cells [148], it is possible that urothelial α7 receptors mediate terminal differentiation of intermediate cells into the polarized umbrella cells in order to maintain the urothelial barrier. Additionally, there exist nerves and fibroblasts directly below the urothelium which may also release ACh [10, 25, 98, 99]. It is possible that these sources of ACh may also be responsible for nAChR signaling in the basal urothelium.

Our research indicates a high level of conservation in the subunits expressed in the urothelium throughout the rat, cat and human. One variance would be the expression of the β2 subunit in the human over the expression of the β3 subunit in the rat. It is difficult to say what difference this would make physiologically, if any. In heterologous expression systems such as the frog oocyte, replacing the β2 subunit with the β4 subunit alters the rate of desensitization of the receptor and the efficacy of the agonists cytisine and ACh [165]. It is unclear whether this would alter function of the receptor between the two species, however.

Other investigators have also examined the expression of nicotinic receptors in the urothelium of the human and the rat and found similar results. Bschleipfer, et. al. [218] examined the expression of nAChRs in human urothelial biopsies and found α7, α9 and α10 receptor expression using RT-PCR. Other subunits, such as those examined in our study, were not studied. Little is known about the α9 or α10 receptors, as they are infrequently found *in vivo.*

They can form α-BTX-sensitive homomeric channels, like the α7 receptor, but can also form heteropentamers with each other (α9α10) [246]. In addition to RT-PCR, Bschleipfer also examined α7 expression using immunohistochemistry and found expression throughout the urothelial layers of human urothelium, much the same as we found in the rat.

Additionally, the same group has studied nAChR in the mouse bladder. RT-PCR and immunohistochemical analysis of FVB mice determined that the α2, α4, α5, α6, α7, α9 and α10 subunits are present in the urothelium [219]. α7 staining in the mouse urothelium was comparable to the rat and human (spread throughout the urothelial layers), however the absence of the α3 receptor subunit represents a major difference from the other species. α4 subunits form another major subtype of AChR commonly found in neuronal tissue, however localization of the α4 subunit in the mouse bladder was confined to the intermediate and basal cells, unlike the umbrella localization of the α3 receptor in the rat. The investigators were unable to determine the localization of the α2 or α6 receptor since antibodies for those subunits do not exist as yet, so it is possible that one of these subunits may be expressed in the umbrella cells of the mouse. One last complication we must consider in the mouse is strain differences; it is possible that the nAChR subunits present in the urothelium may change depending on the strain of mouse used. Therefore, the results obtain by the Lips group in FVB mice may not be representative of all mouse strains.

One additional question raised by our research involves what our studies may suggest about the phenotype of the cells in our urothelial cultures. Our research demonstrates that both the α3 and α7 receptors subunits are expressed in the umbrella cells of rat bladder tissue as well as throughout all cells in our primary culture. This would suggest that our cultured cells are of umbrella cell origin, which would contradict the current dogma concerning urothelial cell

culture. Umbrella cells in the urothelium are terminally differentiated and would, most likely, not multiply upon plating. Indeed, cells cultured in the manner described in our studies lack a number of umbrella cell characteristics, such as multi-nucleation (as shown in Figure 2.9) or the expression of the umbrella cell marker cytokeratin 20 [77]. This is generally believed to be a consequence of plating and growing the cells on plastic or glass dishes; only culturing urothelial cells on a porous support would allow them to differentiate into the polarized layer of cells characteristic of umbrella cells [247]. A possible explanation then, for the expression of both types of nicotinic receptors in these cultured cells could lie in the regulation of nicotinic receptor expression following chronic cholinergic stimulation. Exposure of cultured cells to nicotinic agents can cause an up-regulation in the expression of nAChRs [149, 158, 248]. Choline, a specific α7 agonist, has also been shown to be a potent growth factor for keratinocytes [249] and is included in defined keratinocyte media, such as those we use to maintain urothelial cell cultures (at approximately 100μM, personal communication with Invitrogen, Inc.). It is possible, then, that the expression of nAChRs in cultured urothelial cells is driven by chronic stimulation of α7 receptors by choline in the cell culture media. Therefore, while cultured urothelial cells may not be derived wholly from umbrella cells, the expression of both types of nAChRs in cultured cells allows us to utilize them as a model of umbrella cell signaling in these *in vitro* studies. It would be interesting, then, to determine what the effect on α3 receptor expression in these cultured cells would be if choline was removed from the media, or if α7 stimulation was blocked using a subtype specific antagonist. However, while these experiments could lead to interesting advancements in the understanding of urothelial cell proliferation and differentiation, they are out of the scope of our current project and will not be studied.

Our results are the first indication that the rat urothelium expresses nicotinic acetylcholine receptors. These receptors are differentially expressed throughout the urothelium, suggesting that they may play different roles in bladder function. Further experimentation, detailed in the following chapters, will examine the role of urothelial nicotinic receptors in bladder physiology and the signaling pathways involved.

3.0 FUNCTIONALITY OF UROTHELIAL NICOTINIC RECEPTORS: MODULATION OF CALCIUM SIGNALING AND ATP RELEASE

The experiments outlined in the previous chapter demonstrated that the urothelium expresses mRNA and protein for nicotinic receptor subunits consistent with known functional receptors. However, we have not yet demonstrated that the urothelium expresses functional nAChRs. Nicotinic receptors are highly permeable to calcium; therefore we examined the ability of nAChRs to increase intracellular calcium using Fura-2. Our research revealed two types of functional nAChR in the urothelium through the use of subtype-specific agonists and antagonists; α7 and α3-containing receptors (α3*). Additionally, our studies indicated that each of these receptors influenced intracellular calcium through distinct pathways. For example, stimulation of cultured urothelial cells with cytisine, an α3* receptor agonist, resulted in increases in the Fura-2 ratio that were dependent on extracellular calcium, indicating that α3* receptor stimulation increased intracellular calcium through flow of extracellular calcium through the receptor's channel. Stimulation of α7 receptors with choline also resulted in increases in the Fura-2 ratio, however these signals were independent of extracellular calcium, indicating that α7 receptors mediate a release of Ca^{+2} from intracellular stores. In addition to modulating intracellular calcium, nAChRs have also been shown to play a significant role in modulating transmitter release in a number of cell types, including nerves. Because of the similarities in transmitter release previously demonstrated between nerves and urothelial cells,

we also examined the role of nAChRs to modulate the release of ATP from cultured urothelial cells. Stimulation of both types of nAChRs with specific agonists demonstrated that each subtype of nAChRs modulates ATP release from urothelial cells, however in different manners. α7 receptor stimulation with choline inhibited mechanically-induced ATP release from urothelial cells. α3* receptor stimulation with cytisine, however, revealed a biphasic response, where low concentrations of agonist (1-10μM) inhibited mechanically-induced ATP release while larger concentrations (50-100μM) increased release. These effects were all blocked using subtype specific antagonists (α7: methyllycaconitine citrate; α3*: TMPH), confirming that each effect was due to specific actions by agonists on urothelial nAChRs. In addition to demonstrating the functionality of nAChRs present on urothelial cells, the present research also uncovered evidence of cross-modulation between the two types of nAChRs in the urothelium. Stimulation of cultured urothelial cells with α7 agonists such as choline and PNU-282987 prevented both the calcium response and ATP release normally elicited by cytisine stimulation. These effects were prevented in the presence of the α7 antagonist MLA, suggesting that stimulation of α7 receptors can effectively turn off α3* receptors. These data demonstrate that urothelial nicotinic receptors are functional and can participate in intercellular and intracellular signaling.

3.1 INTRODUCTION

The experiments in the preceding chapter demonstrated that the urothelium of the rat expresses mRNA for the α3, α5, α7, β3 and β4 nicotinic subunits, as well as protein for the α3 and α7 subunits. As we have discussed, the subunits revealed by RT-PCR in the urothelium have been shown to form functional receptors when found in other tissues or when expressed in heterologous expression systems such as CHO cells or frog oocytes [139, 140, 250]. However, no research has yet demonstrated that the nicotinic receptors expressed in the urothelium join to form functional receptors. Hence, the experiments outlined in this chapter were designed to determine if functional nAChRs exist in the urothelium.

In order to determine the functionality of urothelial nAChRs, we examined the ability of nAChR stimulation to influence both intercellular and intracellular signaling through the use of two functional assays. The first of these experiments was intracellular calcium imaging, using the calcium sensitive dye, Fura-2AM. This technique was used due to the fact that nAChR receptors are ligand-gated ion channels that are permeable to calcium [174, 175]. nAChRs, especially the α7 receptor, have been shown to play an important role in modulating intracellular calcium concentrations in a number of cell types, such as nerves [251], skeletal muscle [138] or endothelial cells [235]. Indeed, many of the cellular processes modulated by nAChRs, such as transmitter release, proliferation or differentiation are dependent on intracellular calcium [144, 237]. Additionally, calcium plays a significant role in many cellular processes in the urothelium, such as ATP release [58], transcription [252], cell-cell communication and differentiation [253]. Therefore, given the potential for nAChRs to modulate urothelial function through influencing

intracellular calcium, we examined the ability of nicotinic agonists to increase intracellular calcium.

To examine the effect of nicotinic receptor stimulation on intracellular calcium, we utilized the calcium sensitive dye Fura-2AM. Fura-2 is a ratiometric fluorescent dye that, in the presence of calcium, undergoes a blue-shift in its maximum excitable wavelength (363nm to 335nm), while its emission spectra remains relatively unchanged (max: 510nm) [254]. This molecule is made cell-permeable by the addition of an acetoxymethyl ester, which is cleaved off by esterases present inside the cell, sequestering the dye in the cytoplasm, where it can bind with intracellular calcium. During the experiment, cells are excited at 340 and 380nm and the emission at 510nm measured. The ratio of these readings (340/380) is related to the concentration of intracellular calcium; as intracellular calcium increases, the ratio increases. Using this technique, we examined if nAChR stimulation could lead to increases in intracellular calcium in cultured urothelial cells. We found that the two subtypes of nAChR both increased intracellular calcium (indicated by increases in the Fura-2 ratio from control), however through different mechanisms. For example, α3* receptors, when stimulated by the specific agonist cytisine, were responsible for increases in the Fura-2 ratio through influx of extracellular calcium while α7 receptors, when stimulated by choline, mediated their increase through release from intracellular stores. Nicotinic receptors also modulate calcium in neurons, where they are responsible for, among other things, modulation of transmitter release. For example, both α7 and β2-containing receptors (most likely α4β2) are present in neurons of the rat prefrontal cortex, where they play a role the release of aspartate [255]. Each nAChR in prefrontal neurons modulates the release of aspartate through distinct, calcium-dependent cellular mechanisms. α7 receptors modulate aspartate release by increasing intracellular calcium through a ryanodine

sensitive pathway and subsequent activation of the ERK1/2 pathway, while β2-containing receptors mediated transmitter release by increasing calcium through a coupling to voltage-operated calcium channels. Similar signaling has also been shown in hippocampal cells [256-258], PC12 cells [259], and isolated synaptosomes [260, 261]. Like neurons, urothelial cells can also release transmitters, such as ATP [58, 60, 63]. Given the similarities in signaling between neurons and urothelial cells (the sensor/transducer properties of the urothelium, as discussed in Chapter 1.2.2), and evidence that urothelial nicotinic receptors can also influence intracellular calcium through similar cellular mechanisms, we examined if stimulation of nAChRs could influence the release of ATP from cultured urothelial cells.

To study ATP release from urothelial cells, we utilized the luciferin-luciferase assay to directly measure ATP levels in the medium supporting cultured urothelial cells. The luciferin-luciferase assay is a commonly used technique that utilizes an ATP-dependent chemical reaction that naturally occurs in the firefly [262]. Firefly luciferase is an enzyme that catalyzes its substrate, luciferin with the help of the co-substrate ATP. This reaction creates light and is responsible for the luminescent glow of fireflies. Since ATP is used as a co-substrate for this reaction, in the presence of large amounts of luciferin and luciferase, the amount of light is related to the concentration of ATP present in the assay. Therefore, this assay is used to give sensitive measurements of the concentration of ATP in experimental samples. For our experiments, we used this technique to measure the concentrations of ATP in samples of media taken from cultured urothelial cells. We will demonstrate that cultured urothelial cells, maintained in a chamber continuously infused with HBSS buffer releases a consistent baseline concentration of ATP, thought to be caused by mechanical stimulation caused by the continuous flow of buffer. This mechanically-evoked release can be modulated by nicotinic receptor

stimulation; for example α7 stimulation can decrease basal release of ATP from urothelial cells, while ATP release can be either decreased or increased through stimulation of α3* receptors, depending on the concentration of agonist. These data indicate that nAChRs can modulate the release of ATP from urothelial cells and therefore nAChRs may play a role in sensor/transducer properties of the urothelium.

3.2 RESULTS

3.2.1 Intracellular Calcium Increases Following α3* Receptor Stimulation are Due to Extracellular Calcium Influx

To determine the functionality of urothelial nAChRs, we examined their ability to influence intracellular calcium using the calcium sensitive dye Fura-2. To setup our experiments, cultured urothelial cells were grown on glass coverslips. After allowing the cells to grow 2 to 3 days *in vitro*, a coverslip was removed from the culture media and placed into a Hanks Balanced Salt Solution (HBSS) containing 1μM Fura-2AM for 30 minutes in an incubator (37ºC) to load the dye into the cells. The coverslip was then transferred to an inverted microscope where the cells were maintained through a gravity-fed perfusion system containing HBSS (flow rate: ~2.4ml/min). To examine the effects of nicotinic receptor stimulation, drug solutions were perfused onto the coverslip, also using the gravity-fed perfusion system. In order to measure each transient, each cell in the microscope's field of view was carefully traced along the outside of its plasma membrane and denoted as a region of interest (ROI). During the experiment, the

fluorescence at 510nm was recorded for each ROI when illuminated at 340 and 380nm and the ratios (340/380) for each cell were calculated after subtracting the background fluorescence. Data was collected at the rate of 1 sample per second. For each cell, the magnitude of the Fura-2 signal was measured using the following equation:

$$((\text{Peak} - \text{Baseline}) / \text{Baseline}) \times 100 = \% \text{ change from baseline}$$

where "Peak" is the Fura-2 ratio taken at the peak of the calcium transient and "Baseline" is an average of five samples taken just prior to drug stimulation.

To examine the functionality of urothelial nAChRs, we first examined the effect of α3* stimulation. Cytisine (1-100μM in HBSS, EC_{50}: 5.6μM), an α3* receptor agonist, caused a concentration-dependent increase in the Fura-2 ratio (2.83 ± 0.54%, 9.35 ± 0.65% and 24.46 ± 0.80% increase over baseline for 1, 10 and 100μM cytisine, respectively; Figure 3.1). This effect lasted approximately 2 minutes before returning to baseline levels. These cytisine-induced calcium transients could be blocked by a 10 minute pre-incubation with the α3* receptor antagonist TPMH (30μM in HBSS, 3.74 ± 0.36% increase over baseline), suggesting that the previous response was due to specific actions of the agonist on the receptor. Additionally, cytisine-induced calcium transients were blocked when the cells were perfused with calcium-free HBSS with 0.5mM EDTA. These data indicate that the source of the increase in intracellular calcium was due to extracellular calcium entering the cell, as opposed to release of calcium from intracellular stores.

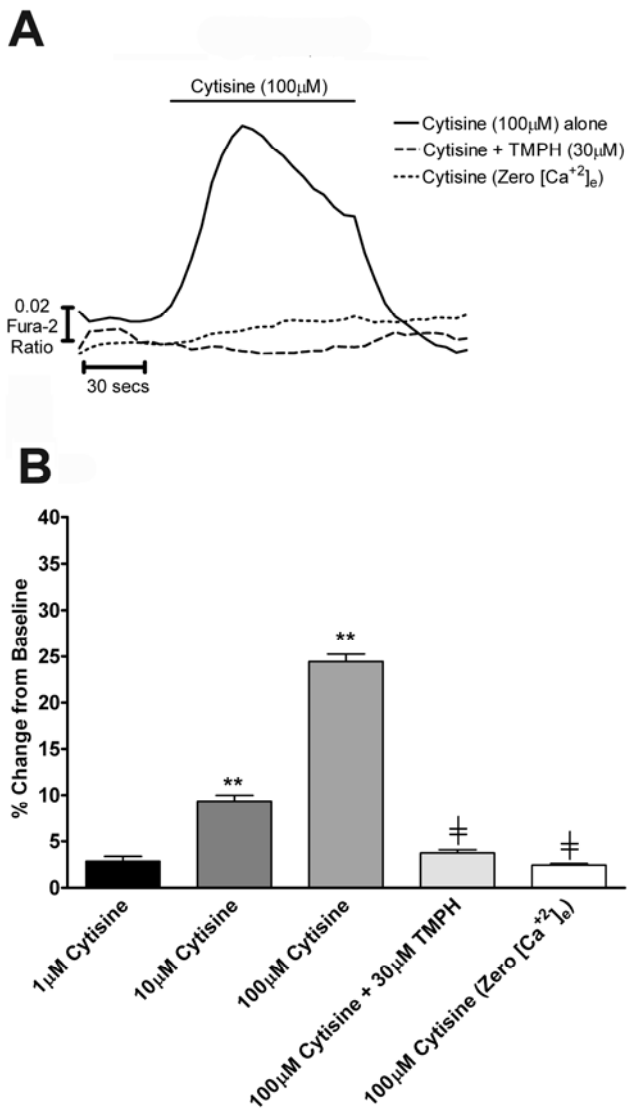

Figure 3.1 - Cytisine Induced Calcium Transients

A: Representative traces of calcium transients following stimulation with 100μM cytisine alone (solid trace), cytisine following pre-incubation with α3* receptor antagonist TMPH (30μM, dashed trace) and cytisine in HBSS containing no extracellular calcium (dotted trace). **B:** Summary graph depicting the changes in intracellular calcium following cytisine stimulation as a percent change in the Fura-2 ratio from baseline (HBSS perfused). Statistical significance was determined using a one-tailed ANOVA with Tukey's multiple comparison post test. **$p<0.005$ compared to HBSS baseline. ╪ $p<0.05$ as compared to 100μM cytisine. n = 96, 73, 201, 142 & 214 cells, respectively.

3.2.2 Activation of α7 Receptors Increases Intracellular Calcium Through a Ryanodine Sensitive Pathway

To determine the contribution of α7 receptors to urothelial calcium signaling, cultured rat urothelial cells were stimulated with the α7 agonist choline (1-100μM, in HBSS). While these concentrations are in the lower range of the range of concentrations that can activate α7 receptors (EC50: ~400μM), these concentrations were chosen in order to eliminate any effect of stimulation of muscarinic receptors, which may also be activated by higher concentrations of choline (concentrations greater than 100μM). Additionally, half of these experiments were carried out in the presence of 10μM atropine, a general muscarinic receptor antagonist, with no significant differences observed (Figure 3.2C). When choline was applied, a concentration-dependent increase in the Fura-2 ratio is observed (4.63 ± 0.19%, 14.25 ± 0.69% and 34.1 ± 3.30% increase over baseline for 1, 10 and 100μM, respectively; Figure 3.2). This increase lasted for 5-7 minutes (Figure 3.2A), and was completely blocked by pre-incubation with the α7-specific antagonist α-bungarotoxin (1μM in HBSS, 5 minute incubation), indicating a specific action on the α7 receptor. Unlike cytisine, however, removal of extracellular calcium and the addition of EDTA to the HBSS did not block the choline-induced signal, suggesting the involvement of calcium release from intracellular stores. Pre-incubation with ryanodine (10μM in HBSS, 15 minutes) blocked this response. This concentration of ryanodine is generally accepted to block ryanodine receptor-mediated release of calcium from the ER [263], thus indicating a role for ryanodine receptors in choline-induced release (Figure 3.2B).

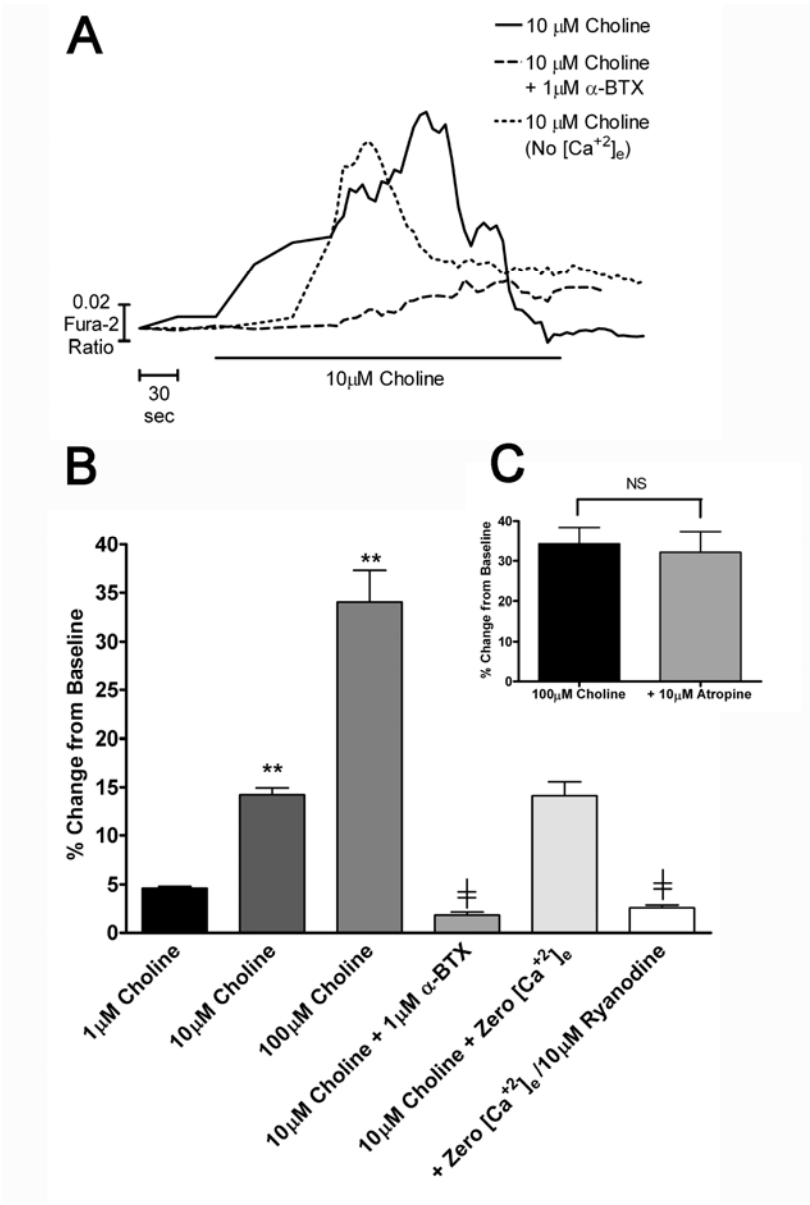

Figure 3.2 - Choline Increases Intracellular Calcium Through a Ryanodine Sensitive Pathway

(A) Representative traces of calcium imaging experiments following stimulation with 10μM choline alone (solid trace), choline following pre-incubation with α7 receptor antagonist α-BTX (1μM, dashed trace) and choline in HBSS containing no calcium (dotted trace). **(B)** Summary graph depicting the changes in intracellular calcium following choline stimulation as a percent change in the Fura-2 ratio from baseline (HBSS perfusion). Statistical significance was determined using a one-tailed ANOVA with Tukey's multiple comparison post test. **$p<0.005$ compared to HBSS baseline. ⧺ $p<0.05$ as compared to 10μM choline. n = 104, 117, 75, 113, 115 & 220 cells, respectively. **(C)** Summary graph demonstrating no difference between Fura-2 signals following stimulation of cells with 100μM choline alone and 100μM choline in the presence of 10μM atropine. NS - not statistically significant as compared by students' t-test. n= 42 and 33, respectively.

3.2.3 Cross-Modulation of nAChRs in the Urothelium

In an attempt to confirm the role of α7 nAChRs in the control of urothelial calcium signaling, we utilized another selective agonist of α7 receptors, PNU 282987. PNU 282987 is highly selective and potent α7 agonist [264], which would eliminate the need to include atropine in the bath to block potential non-selective activation of muscarinic receptors. However, stimulation of cultured urothelial cells with PNU 282987 (10nM - 1μM, in HBSS) elicited no change in the Fura-2 ratio (Figure 3.3A). It was noted that application of PNU 282987, while having no effect itself to elicit calcium transients (100nM, 2 minute pre-incubation), blocked any increase in the Fura-2 ratio observed following subsequent stimulation with cytisine (100μM, Figure 3.3A). This inhibition of the α3*-mediated transient by an α7 receptor agonist was blocked following pre-incubation with the α7 antagonist MLA (100μM, Figure 3.3B), but not if the antagonist was given after PNU 282987, indicating that the result was not due to actions of PNU 282987 on the α3* receptor. It was also possible to recover the cytisine response if PNU 282987 was washed out of the bath for 10 minutes with normal HBSS prior to stimulation with cytisine.

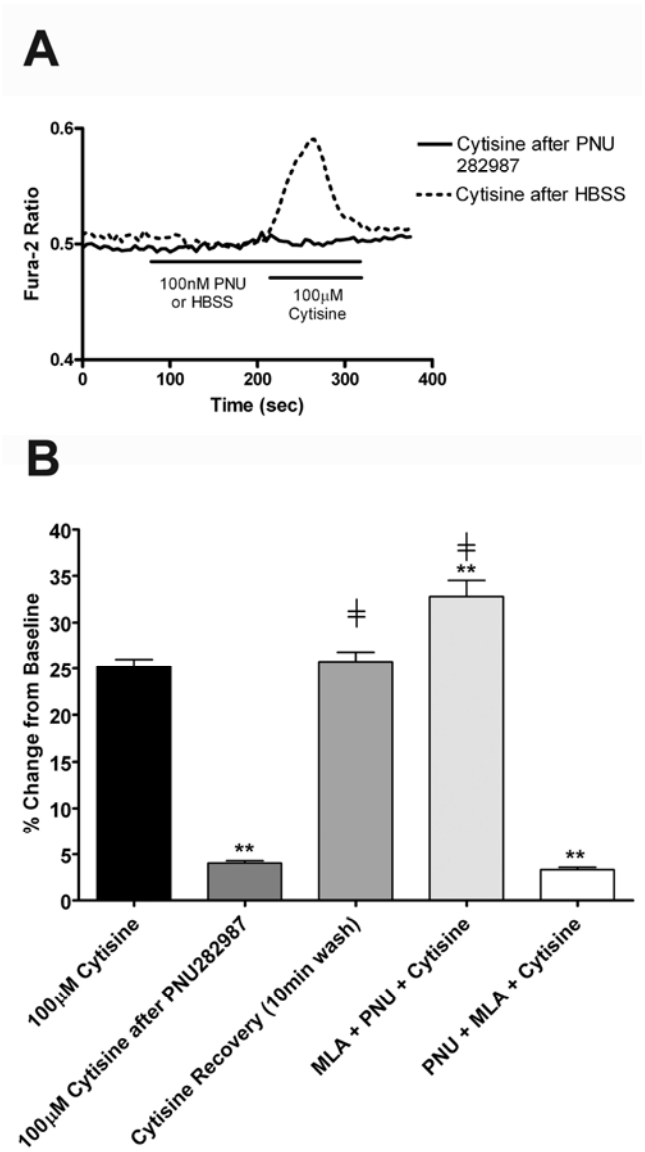

Figure 3.3 - Inhibition of Cytisine-Induced Calcium Signals by the α7 Agonist, PNU 282987

(A) Representative traces of increases in the Fura-2 ratio following either cytisine stimulation alone (100μM, dashed line) or cytisine following PNU 282987 (100 nM, solid line). Drug applications are denoted by the solid lines below the traces. Note that stimulation of cells with PNU 282987 alone elicited no change in the Fura-2 ratio. **(B)** Summary graph showing the percent change in Fura-2 ratio from baseline (HBSS perfusion) following stimulation of cultured urothelial cells with 100μM cytisine alone and following stimulation with various α7 nAChR agents. The cytisine induced signal is blocked following stimulation with the α7 agonist PNU 282987 (100nM, 2nd column). This block is recoverable after 10 minute wash with HBSS (3rd column) and is prevented if the cells are pre-incubated with the α7 antagonist methyllycaconitine citrate (MLA, 100μM, 4th column). The inhibitory effect is not blocked, however, if the antagonist is given after the agonist (5th column). Statistical significance was determined using a one-tailed ANOVA with Tukey's multiple comparison post test. **$p<0.05$ compared to 100μM cytisine. ╪ $p<0.05$ as compared to cytisine after PNU 282987. n = 201, 166, 131, 66 & 29 cells, respectively.

One well-established mechanism to modulate ion channels is through phosphorylation by protein kinases. While phosphorylation of a receptor could lead to either inhibition or potentiation, depending on the ion channel, research has indicated that phosphorylation of nicotinic receptors accelerates the rate of desensitization, leading to receptor inhibition [265]. Therefore, we hypothesized that this inhibition of the α3*-mediated response may be due to phosphorylation of the α3* receptor. Nicotinic receptors are commonly phosphorylated by PKC, a group of kinases that are expressed ubiquitously throughout the body. However, no study to date has determined if any PKC isoforms are expressed in the urothelium. Therefore, to examine the isoforms of PKC that are expressed in the urothelium, we performed RT-PCR on rat urothelial tissue. As demonstrated in Figure 3.4, mRNA for a number of PKC isoforms are expressed in the urothelium, including PKCα, PKCγ, PKCδ, PKCε, PKCλ, and PKCζ. Another kinase known to modulate nAChRs in other tissues is PKA, a cAMP-dependent kinase that may also be expressed in the urothelium [266]. To determine if either of these kinases can influence α3* nAChR signaling, we examined how pre-incubation of urothelial cells with PKA or PKC modulators influenced cytisine-induced calcium transients. Because PKA and PKC have numerous isoforms, which can be differentially expressed depending on tissue type, the agents we utilized in these experiments were chosen to be as general as possible, i.e. acting on as many isoforms as possible. Additionally, because protein kinases are present in the cytoplasm of the cell, we chose agents that were cell-permeable.

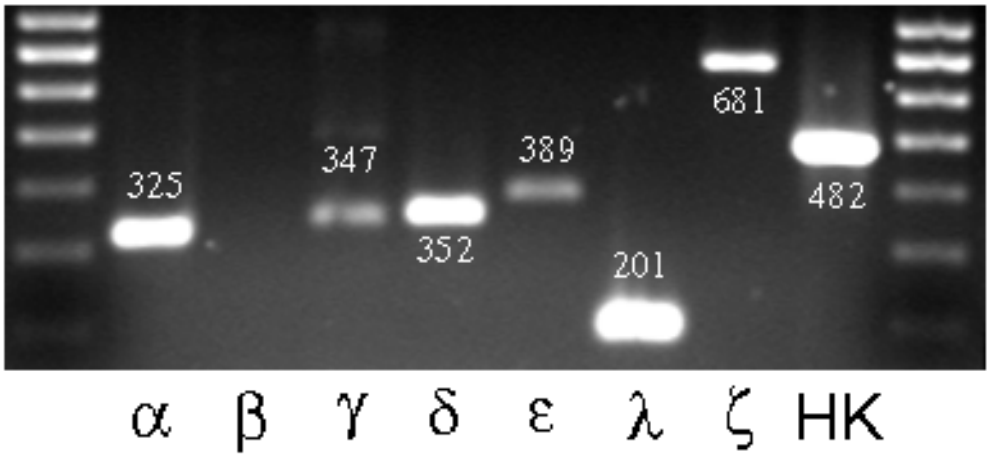

Figure 3.4 - Expression of PKC mRNA in the Urothelium

mRNA for various PKC isoforms are amplified in urothelial tissue using RT-PCR. Note positive results for the α, γ, δ, ε, λ, and ζ isoforms of PKC. "HK" indicates amplification of the housekeeping gene β-actin. 1.2% agarose gel in 1X TBE buffer stained with ethidium bromide. Results are identical from tissue taken from three separate rat bladders. Outside lanes contain a 100 base pair ladder, from 200-800 base pairs, from bottom to top.

Incubation for 15 minutes with either of the PKC activators phorbol 12-myristate, 13 acetate (PMA, 100nM in 0.1% DMSO) or Pseudo RACK1 (20μM, in HBSS) blocked cytisine-induced Ca^{+2} signals (79.1% and 83.2% decrease from cytisine alone, respectively; Figure 3.5A). These agents had no effect on the Fura-2 ratio by themselves (data not shown). 0.1% DMSO was also tested to rule out any vehicle effect on the Fura-2 ratio; no change in signal was observed (data not shown). Similar results were observed when using activators of PKA; 8-Bromo-cAMP (30μM, in HBSS) or dibutyryl-cAMP (1mM, in HBSS) blocked cytisine-induced increases in the Fura-2 ratio (86.4% and 78.0% decrease from cytisine alone, respectively; Figure 3.5B). These agents also did not influence the Fura-2 ratio by themselves. These results suggest that α3* receptors can be inhibited by phosphorylation by either PKA or PKC.

In addition to examining the effects of activating PKA or PKC on cytisine-induced calcium transients, we also studied whether inhibiting PKA or PKC could modulate the effects of

cytisine stimulation. Inhibition of PKC using either chelerythrine chloride (1μM in HBSS, 15 minute pre-incubation) or Ro 32-0432 (1μM, in 0.1% DMSO, 15 minute pre-incubation) potentiated the Ca^{+2} transient observed following cytisine stimulation (Figure 3.5A). This potentiation was evident as an increase in the peak of the calcium response (20.0% and 193% increase over cytisine alone, respectively). Similarly, PKA inhibitors PKI 14-22 (100nM, in HBSS) or PKA Inhibitor 6-22 (10μM, in HBSS) also potentiated the cytisine-induced calcium signals (54.3% and 64.0%, respectively; Figure 3.5B). None of the kinases inhibitors had an effect on the Fura-2 ratio when given by themselves. These data, considered along with our previous results, indicate that α3* receptors in the urothelium can be modulated through phosphorylation by either PKA or PKC.

While we have demonstrated that activation of PKA or PKC can inhibit α3*-mediated calcium transients, we have not yet demonstrated that the inhibition of the α3* receptor by α7 receptor stimulation is mediated through PKA or PKC. To determine if this is the case, we examined cytisine-induced calcium signals following pre-incubation with both the α7 receptor agonist PNU 282987 and inhibitors of either PKA or PKC. Pre-incubation with either 1μM chelerythrine chloride or 100nM PKI 14-22 for 15 minutes reversed the PNU 282987-induced block of the cytisine-induced transient (Figure 3.6). These data suggest that the inhibitory effects of PNU 282987 may be due to activation of PKA or PKC and subsequent phosphorylation of the α3* receptor.

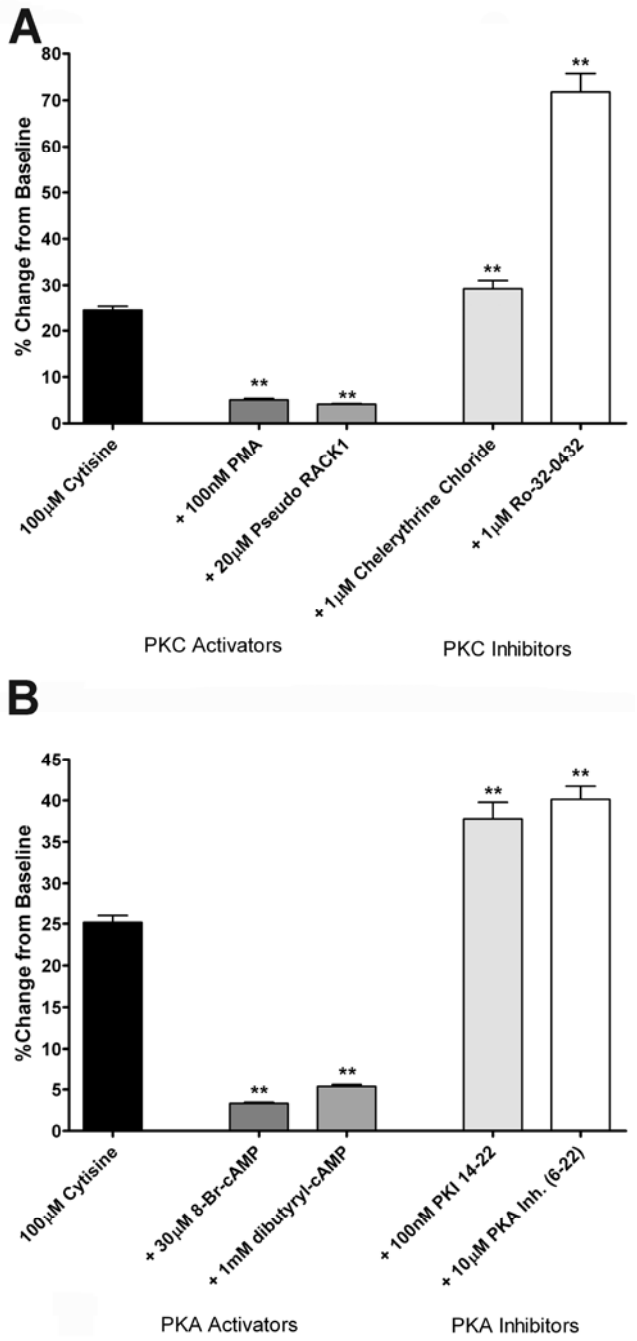

Figure 3.5 - PKA/PKC Modulation of Cytisine Induced Calcium Signals

(A) Summary graph depicting the magnitude of calcium transients following cytisine stimulation in the presence of PKC modulators, expressed as a percent change in the Fura-2 ratio. **p<0.05 compared to cytisine alone as determined by ANOVA with Dunnett's multiple post test. n = 201, 118, 52, 60 & 56 cells, respectively. **(B)** Summary graph depicting the magnitude of calcium transients following cytisine stimulation in the presence of PKA modulators, expressed as a change in the Fura-2 ratio. **p<0.05 compared to cytisine alone as determined by ANOVA with Dunnett's multiple post test. n = 201, 190, 127, 65 & 74 cells, respectively.

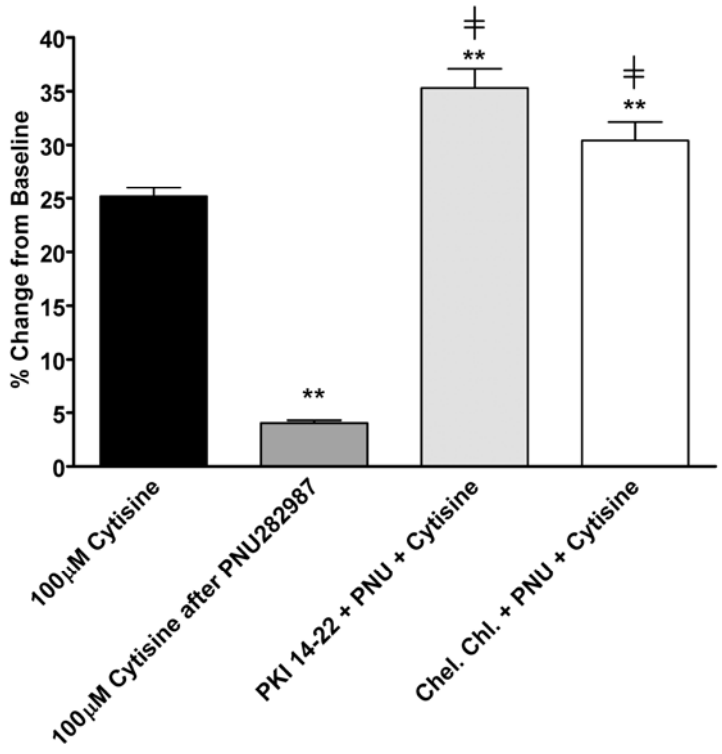

Figure 3.6 - α7 Inhibition of α3* Mediated Transients are Mediated Through Activation of PKA/PKC

Summary of the effects of the PKA inhibitor PKI 14-22 or the PKC inhibitor chelerythrine chloride on the PNU 282987 mediated inhibition of the calcium transients evoked by cytisine. 100nM PNU 282987 blocks increases in the Fura-2 ratio following 100μM cytisine (1st column), but pre-incubation of the cells with either PKI 14-22 or chelerythrine chloride removes that inhibition. **$p<0.05$ as compared to 100μM cytisine by ANOVA with Dunnett's multiple comparison post test. ⫩ $p<0.05$ as compared to 100μM cytisine after PNU 2829787 by ANOVA with Dunnett's multiple post test. n= 201, 166, 71 & 128 cells, respectively.

3.2.4 Activation of α7 Nicotinic Receptors Inhibits Basal ATP Release from Urothelial Cells

In addition to studying the ability of nAChRs to modulate intracellular calcium signals, we also examined their ability to modulate ATP release from the urothelium. Cells were cultured in the same manner as for calcium imaging, with cells being plated on glass coverslips. At the time of the experiment, coverslips were removed from the culture media and placed in a plastic dish being perfused with HBSS through the use of a peristaltic pump (flow rate: ~0.6 ml/min). During the course of the experiment, 100µl samples were taken from the dish and measured for ATP concentrations using a luciferin-luciferase kit and a luminometer.

For our experiments, readings taken during perfusion of HBSS alone were used as control. Perfusion of HBSS caused a small, but measurable amount of ATP release (average concentration: 20-40pM), which is thought to be caused by the mechanical stimulation of the cells by the flow of perfusion. This is supported by previous research that indicated that the basal release of ATP was increased in urothelial cells from cats suffering from interstitial cystitis, which are also hypersensitive to mechanical stimulation [58].

To determine the role of each nAChR subunit in modulating ATP release from urothelial cells, we examined the effects subtype-specific agents had on this mechanically-induced release of ATP. In our experiments, release of ATP could be inhibited in a concentration-dependent manner by the α7 agonist choline (Figure 3.7A&B), decreasing the average release of ATP by 23.6 pM at 1 mM of choline. To rule out any non-specific effects of choline activating muscarinic receptors, atropine (10µM, in HBSS) was included in the bath for these experiments.

This decrease in ATP release was prolonged and could only be washed out after 5 minutes of HBSS perfusion.

To rule out any possibility of choline interfering with the luciferin-luciferase assay being the cause of the decreased signal, we performed a standard curve with known concentrations of ATP, both in the presence of and in the absence of choline (Figure 3.7D). These standard curves were identical, indicating that choline does not interfere with the assay. Additionally, the possibility exists that the decreases observed following choline perfusion is due to another phenomenon, such as cell death or depletion of ATP stores over the course of the experiment. To control for this, we continuously perfused HBSS over cultured cells for 30 minutes to determine if any decrease in basal ATP release was observed. As shown in Figure 3.7B, ATP concentrations were maintained throughout the length of the experiment, indicating that the decreases in ATP release we observe during choline perfusion are not due to cell death or depletion of ATP stores.

To further determine if this decrease in ATP following choline was due to activation of α7 receptors, we attempted to block the effects of choline using the α7 antagonist MLA (100μM, in HBSS). MLA successfully blocked the effects of choline, but also increased basal ATP release by itself (Figure 3.7C). This may indicate that α7 receptors are tonically active. MLA also did not alter the standard curve, indicating that the increase in ATP release was not due to interference with the assay (Figure 3.7D).

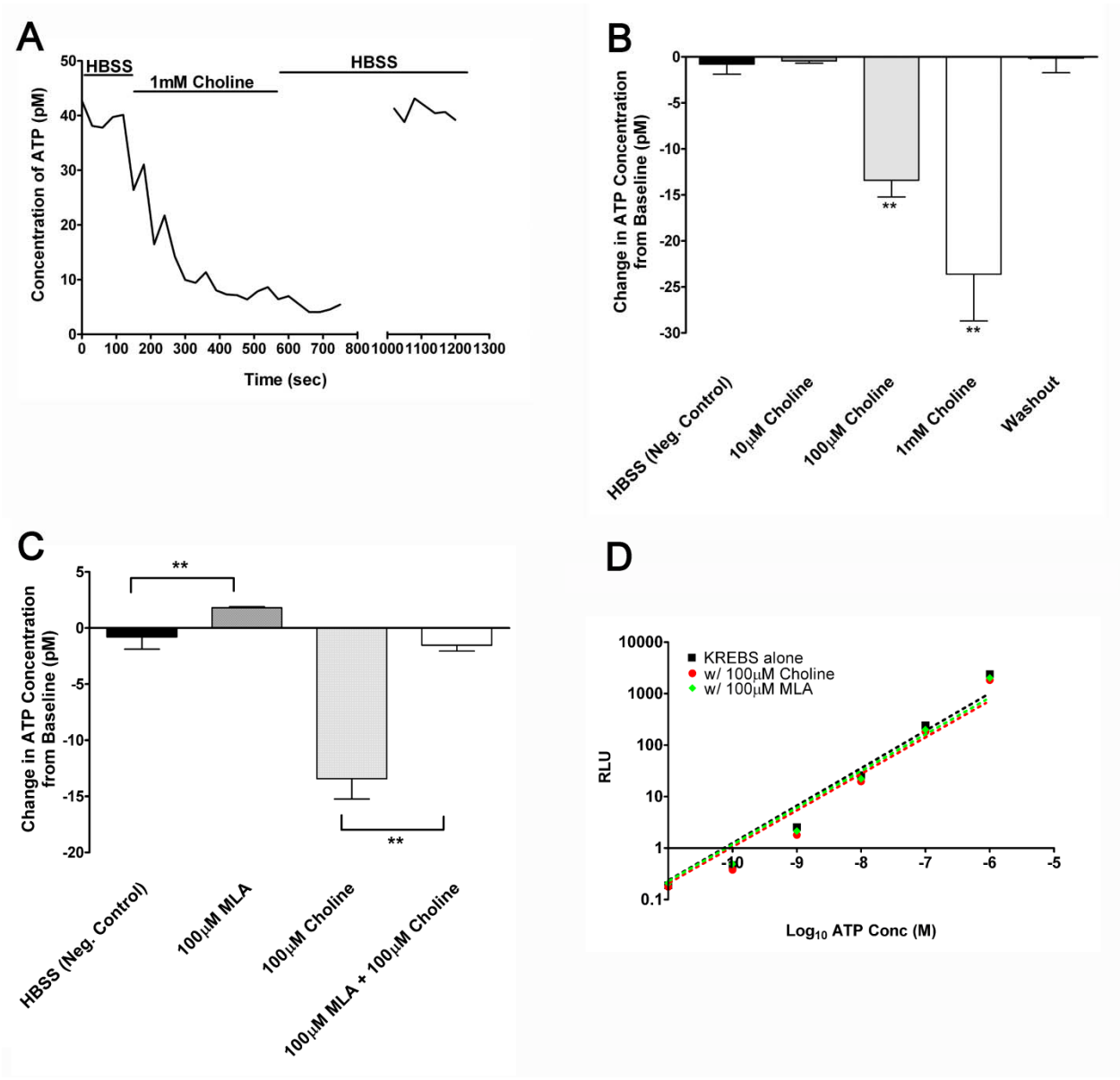

Figure 3.7 - Choline Inhibits ATP Release from Urothelial Cells

(A) Representative trace of choline's effect on ATP release from cultured urothelial cells. **(B)** Summary of ATP experiments involving choline, expressed as a change in the concentration of ATP measured. These changes were calculated in each experiment as the difference between the average of 5 readings taken prior to and during choline perfusion. Each column summarizes the changes in 6 experiments, taken from 3 separate cultures. To control for the possibility of cell death or depletion of ATP stores causing a decrease in ATP concentrations over the course of the experiment, normal HBSS was perfused over cultured cells instead of choline (first column) in 6 experiments. ** $p<0.05$ by one-way ANOVA followed by a Dunnett's post-test to compare each column to the control (HBSS alone). **(C)** Effects of the α7 antagonist MLA on ATP release from urothelial cells. **$p<0.05$ as compared by unpaired Students' t-test. **(D)** Log-Log plot of ATP standard curves performed either in HBSS alone (black squares), or in the presence of 100μM choline (red circles) or 100μM MLA (green diamonds). Dotted lines indicate a linear regression of the measured concentrations.

3.2.5 α3* Stimulation Bi-phasically Modulates ATP Release from Cultured Urothelial Cells

To determine the role α3* receptors play in the control of ATP release from urothelial cells, we examined the effects of cytisine, an α3* specific agonist, in our experimental setup. To determine if cytisine could interfere with the luciferin-luciferase assay, we first performed a standard curve with known amounts of ATP both with and without cytisine. As shown in Figure 3.8E, there was no difference in the standard curves, indicating that cytisine has no effect on the luciferin-luciferase assay.

Cytisine stimulation of cultured urothelial cells caused a bi-phasic response in ATP release. For example, basal release of ATP is diminished when low concentrations of cytisine (1-10μM in HBSS, Figure 3.8A&C) are perfused over cultured cells, much in the same manner as observed following choline stimulation. This decrease in the release of ATP (decreases from control of 4.8 pM for 1μM cytisine, and 7.8 pM for 10μM cytisine) is sustained for the length of drug application, but returns immediately following washout with HBSS, unlike the prolonged decrease observed following choline (Figure 3.8A). However, when higher concentrations of cytisine are perfused (50-100μM, in HBSS), ATP release increases above that during HBSS perfusion alone (Figure 3.8B&C). This effect (an increase over control of 18.4 pM during perfusion of 100μM cytisine) is also maintained for the duration of drug application and washes off when the perfusate is switched back to normal HBSS (Figure 3.8B).

To determine if the cytisine-induced effects were due to actions on α3* receptors and not through non-specific effects, we attempted to block the effects of cytisine with the α3* antagonist TMPH. Perfusion of the α3* antagonist TMPH (90μM, in HBSS) had no effect on ATP release by itself, but did block the response to cytisine at both high and low concentrations

(Figure 3.8D). Because both aspects of the biphasic response are blocked by the antagonist, our data suggests that both the decrease in ATP release at low concentration of agonist and the increase at high concentrations are due to activation of the α3* receptor. We will discuss possible mechanisms of this biphasic response in Section 3.3.

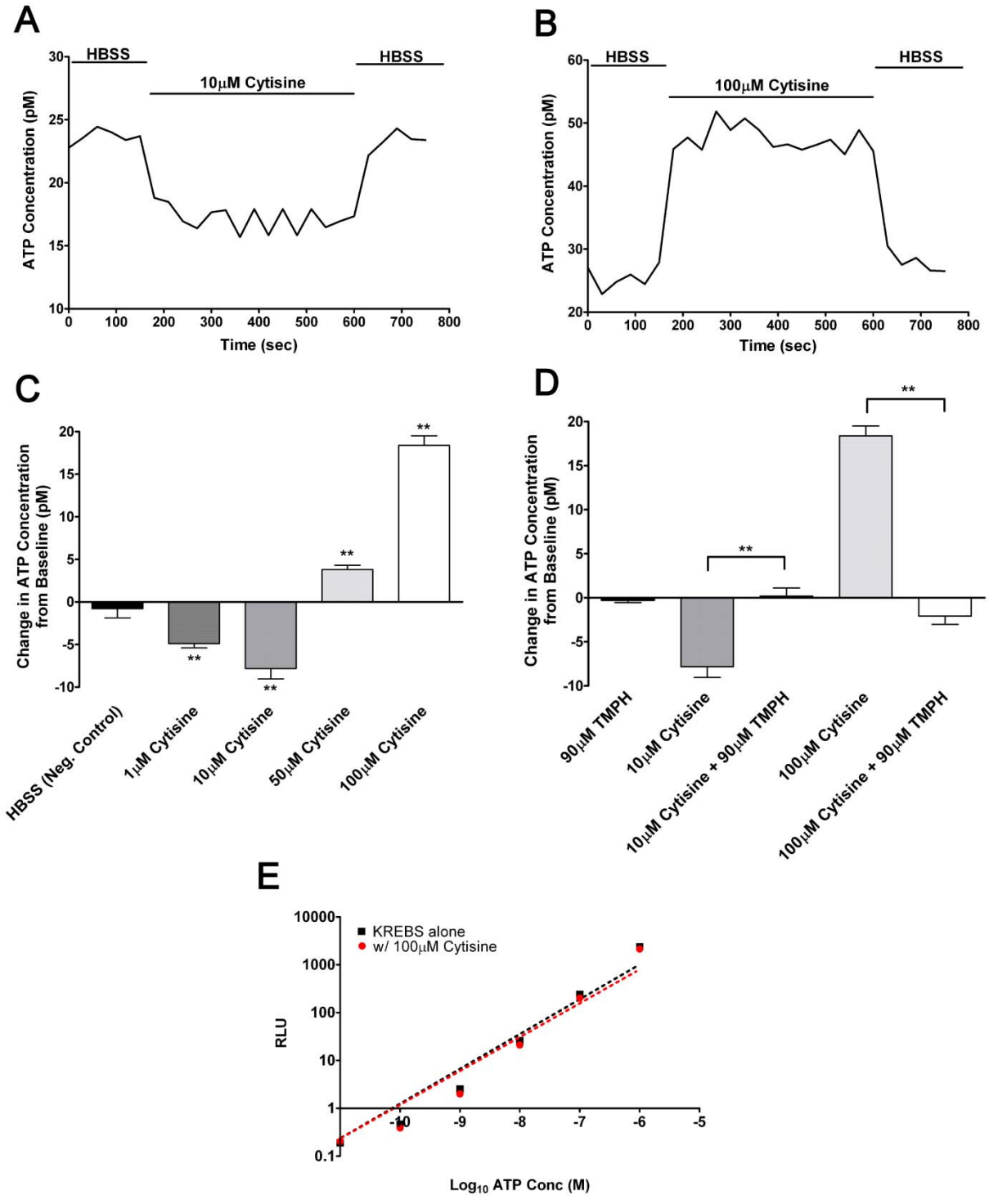
A
HBSS
10μM Cytisine
HBSS
ATP Concentration (pM)
Time (sec)
B
HBSS
100μM Cytisine
HBSS
ATP Concentration (pM)
Time (sec)
C
Change in ATP Concentration from Baseline (pM)
HBSS (Neg. Control)
1μM Cytisine
10μM Cytisine
50μM Cytisine
100μM Cytisine
D
Change in ATP Concentration from Baseline (pM)
90μM TMPH
10μM Cytisine
10μM Cytisine + 90μM TMPH
100μM Cytisine
100μM Cytisine + 90μM TMPH
E
KREBS alone
w/ 100μM Cytisine
RLU
Log10 ATP Conc (M)

Figure 3.8 - Cytisine Effects on ATP Release from Urothelial Cells

(From proceeding page) (A&B) Representative trace of ATP release from urothelial cells in response to 10μM (A) or 100μM (B) cytisine. **(C)** Summary of cytisine's effects on ATP release from cultured urothelial cells. These changes were calculated in each experiment as the difference between the average of 5 readings taken prior to and during choline perfusion. Each column summarizes the changes in 6 experiments, taken from 3 separate cultures. ** $p<0.05$ by one-way ANOVA followed by a Dunnett's post-test to compare each column to the control (HBSS alone). **(D)** Summary of the effects of the α3* antagonist TMPH on ATP release from cultured urothelial cells. **$p<0.05$ as compared by unpaired Students' t-test. **(E)** Log-Log plot of ATP standard curves performed either in HBSS alone (black squares), or in the presence of 100μM cytisine (blue triangles). Dotted lines indicate a linear regression of the measured concentrations.

3.2.6 α7 Stimulation Also Inhibits Cytisine-Induced ATP Release

Because stimulation of α7 receptors can block cytisine-induced calcium signals, we also examined if α7 stimulation could inhibit cytisine-induced ATP release. ATP release from urothelial cells is a calcium-dependent process [58], therefore the possibility exists that a treatment that can block calcium signals in response to an agonist may also block ATP release mediated by that agonist. As shown in Figure 3.9, pre-incubation of cultured cells with 100μM choline for 5 minutes resulted an inhibition in the release of ATP previously shown to be stimulated by 100μM of cytisine. Specifically, stimulation of cultured cells with 100μM of cytisine decreased ATP release 0.95 pM from control when perfused following choline pretreatment, as compared to increasing ATP release 18.4 pM from control when perfused alone.

These data, in conjunction with our earlier data demonstrating an inhibition of cytisine-induced calcium signals by α7 stimulation, suggest that the two types of may interact to influence urothelial signaling. We will further discuss what the implications of these findings might be in the next section.

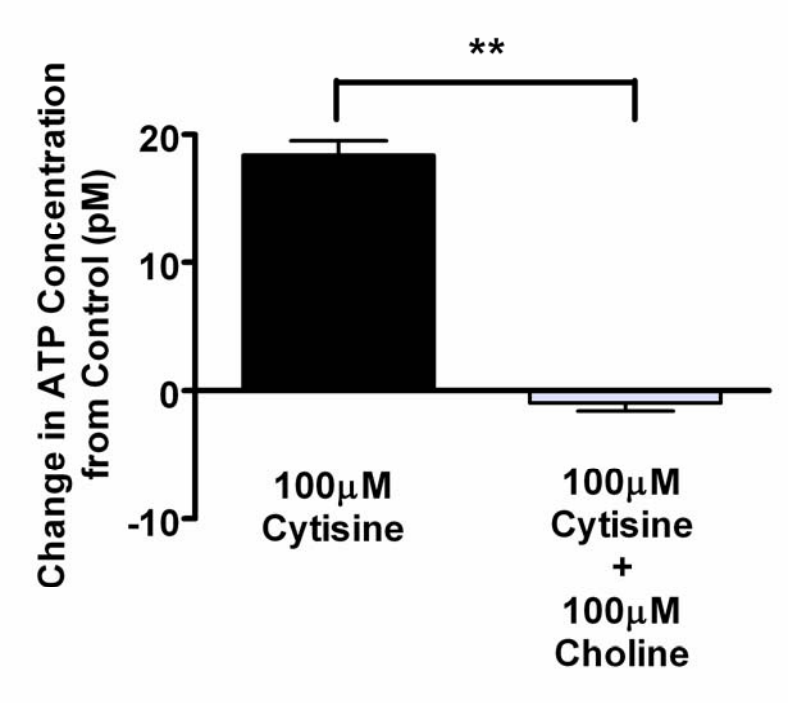

Figure 3.9 - α7 Stimulation Blocks ATP Release Evoked by α3* Stimulation

Summary of ATP release following cytisine stimulation or cytisine stimulation following choline stimulation. These changes were calculated in each experiment as the difference between the average of 5 readings taken prior to and during choline perfusion. Each column summarizes the changes in 6 experiments, taken from 3 separate cultures. **$p<0.05$ as compared by Students' t-test.

3.3 DISCUSSION

Our research indicates that both types of urothelial nAChRs can modulate intracellular calcium concentrations; however they mediate these effects through distinct mechanisms. Cytisine-induced increases in $[Ca^{+2}]_i$ are dependent on extracellular calcium, indicating a influx of Ca^{+2} ions from outside of the cell. Conversely, choline-induced $[Ca^{+2}]_i$ increases are insensitive to extracellular calcium levels, indicating that the α7 receptor mediates its effect through the release of calcium from intracellular stores. This separation of nAChR signaling between two distinct calcium pools may be the basis for the observed effects of nAChR agents

on the release of ATP from urothelial cells. In this section, we will discuss possible mechanisms for nAChR modulation of ATP and how nAChR-mediated calcium transients may play a role in this modulation.

3.3.1 nAChR Mediated Calcium Transients

Our experiments have demonstrated that stimulation of cultured urothelial cells with nicotinic agonists produce calcium transients, however through distinct mechanisms. For example, stimulation of α3* receptors with cytisine induced a calcium transient that was dependent on extracellular calcium. This data suggests that the mechanism underlying this calcium transient is the opening of the ion channel, allowing calcium to enter the cell from the extracellular space. It should be noted however, that nAChRs, such as α3* and α4*, have been known to modulate voltage operated calcium channels (VOCCs). Therefore the calcium transients we observe may not be mediated fully by influx of calcium through the nicotinic ion channel, but through the activation of VOCCs and subsequent influx of calcium through those channels. There is some evidence that urothelial cells may express VOCCs, as urothelial nitric oxide release in response to β-adrenergic stimulation is decreased following incubation with the calcium channel blocker nifedipine [61]. However, it should be noted that urothelial cells are not electrically excitable, therefore it is unclear what role, if any VOCCs, or their modulation by nAChRs might play.

In contrast to α3* receptors, α7 receptor stimulation causes a calcium transient that is completely dependent on extracellular calcium, suggesting a release of calcium from intracellular stores. This is an interesting result, as α7 receptors are widely recognized as being highly permeable to calcium, demonstrating a permeability ratio of almost 10:1 as compared to other cations (Na^{+}, Mg^{+2}) [267]. Given the receptor's high permeability to Ca^{+2}, it is strange to find

that urothelial α7 signals do not seem to be mediated by influx of extracellular Ca^{+2}. It is possible that the calcium transients we observe following α7 receptor stimulation are mediated through current-independent mechanisms. For example, it has been shown that primary cultures of rat brain microglia also demonstrate α7-mediated Ca^{+2} signals that are independent of extracellular calcium concentrations [240]. It appears as though these receptors couple to IP_3 production, possibly through direct activation of PLC, which in turn activates IP_3 receptors on the ER to release calcium from stores. Our research does not indicate a role for the IP_3 receptor pathway, instead indicating a role for ryanodine receptors; however it is possible that desensitized nAChRs may also influence this pathway through unknown mediators. It has been hypothesized that the VOCCs may couple directly to ryanodine receptors present on the ER, with direct interactions between the receptors causing activation of ryanodine receptors [268, 269]. Therefore, a similar coupling between nAChRs and ryanodine receptors may play a role in the α7 mediated transients we observe in urothelial cells.

Because our choline-induced calcium transients appear to be completely independent of extracellular calcium, we must also address the possibility that the results we observe are due to non-specific actions of choline. Choline can also activate muscarinic receptors, which have been shown to increase intracellular calcium through release from intracellular stores [270]. This raises the possibility that the calcium transients we observe following choline stimulation are due to muscarinic stimulation, not nicotinic stimulation. There are a number of reasons, however, that support nicotinic-specific actions of choline in our experiments. To begin, the concentrations of choline we use in our studies are 100-10,000 times lower than the EC_{50} of choline on muscarinic receptors (for example, the EC_{50} of choline on the M1 receptor is approximately 10mM [271]). Therefore we would expect that any effect of choline stimulation

of muscarinic receptors to be small. Second, the calcium transients observed following choline stimulation are completely blocked by α-bungarotoxin. α-BTX is an α7 specific antagonist and has no known actions on muscarinic receptors. Therefore, if choline was acting on muscarinic receptors, we would expect to observe an α-BTX resistant calcium transient in response to choline. The absence of such a transient suggests that choline's effect is mediated through nicotinic receptors. Lastly, in half of our experiments 10μM atropine was included in the bath when stimulating with choline. Atropine is a general muscarinic antagonist with low nanomolar affinities for each muscarinic receptor subtype [272]. Since no difference was observed between choline-induced transients in the presence of atropine and those in the absence of atropine, we can conclude that the effects we observe following choline stimulation are due wholly to actions on the α7 receptor.

3.3.2 nAChR Modulation of ATP Release

We have also demonstrated that nAChRs play a role in the modulation of the release of ATP from urothelial cells. Again, it appears that the two separate subtypes of nAChR modulate ATP release in distinct manners, which may be linked to their effects on intracellular calcium.

For example, our results indicate that stimulation of α7 receptors inhibits ATP release from urothelial cells and releases Ca^{+2} from ryanodine sensitive stores. Increases in intracellular calcium concentrations are generally believed to have an excitatory effect on transmitter release. However, it has also been demonstrated that release of intracellular calcium from ryanodine sensitive stores can inhibit quantal release at the neuromuscular junction through inhibition of calcium permeable ion channels [273]. What might be the mechanism behind this phenomenon? One possible mechanism may be depletion of the ryanodine sensitive stores. In feline urothelial

cells, depletion of intracellular stores using caffeine, or through a long-term application of low concentrations of ryanodine, resulted in a significant decrease in ATP released in response to hypotonic stretch [58]. Therefore, it could be possible that release of calcium from ryanodine sensitive stores drives transmitter release in urothelial cells and depletion of these stores could inhibit transmitter release.

In contrast to the effects of α7 stimulation, stimulation of α3* receptors with cytisine demonstrate a bi-phasic modulation of ATP release. Specifically, low concentrations of cytisine inhibit basal ATP release, while higher concentrations increase basal release. The mechanism for this bi-phasic response may also be mediated, in part by the calcium transients evoked by α3* stimulation. For example, when α3* receptors were stimulated with larger concentrations of cytisine, intracellular calcium levels increased. This is consistent with the nAChR signals observed in peripheral afferent nerves, which also express α3* type nAChRs [175]. In nerves, an increase in calcium is coupled to a release of transmitters from vesicles, such as GABA or dopamine [143, 274]. In urothelial cells and other non-neuronal cells, ATP release is also calcium sensitive [58, 59], indicating that, similar to the mechanism in nerves, urothelial transmitter release may be vesicular. Therefore, it is possible that ATP release can be influenced in urothelial cells through modulation of intracellular calcium levels by nAChRs.

In addition to evoking ATP release at higher concentrations, stimulation of cultured urothelial cells with lower concentrations of cytisine inhibited ATP release. It is interesting to note that this inhibition occurs at concentrations of cytisine that evoke very small calcium transients, suggesting that calcium may not play a role in the inhibitory phase of α3* stimulation. One possible mechanism that could explain this bi-phasic response could involve actions by desensitized nAChRs. Current research into heteromeric nAChRs have suggested a two-agonist

binding model, in which the receptor can enter the desensitized state from either the open, activated state or the closed, inactive state [138]. In this model, each binding site has a different affinity for agonists, and binding of an agonist to the higher affinity site (with binding constants in the nanomolar range) can force the receptor directly into a closed, inactive state without opening the receptor channel. Therefore, with this model, it is possible for nAChRs, in the presence of low concentrations of agonist, to stabilize in a desensitized state without first being activated. While it is generally accepted that a desensitized receptor is "turned off" and does not participate in cellular signaling, it has become clear through recent research that the desensitized state is a functional state for nAChRs that can mediate a number of cellular processes such as transcription, transmitter release and cross modulation of other transmitters. For example, chronic treatment of rats with nanomolar concentrations of nicotine, which have been shown to cause receptor desensitization, results in an increase in dopamine and glutamate release [185, 275] in the brain and increased expression of receptors such as the D1 dopamine receptor and the NMDA receptor [145]. Intracellular signaling proteins like PKA, PKC, MAPK1, and p38 are also increased by chronic treatment with nicotine [276, 277]. Finally, chronic application of nicotine increased the affinity of oxotremorine to muscarinic receptors [278], indicating that desensitized nAChRs can modulate muscarinic receptor binding.

Given this data, it seems possible that desensitized nAChRs may be responsible for the biphasic response in ATP release that we observe in our experiments. Desensitized α3* receptors could activate an intracellular pathway that can, in turn, inhibit the pathway responsible for mechanically evoked ATP release. While the pathway responsible for ATP release in urothelial cells has not yet been elucidated, one possible target is TRPV1. Both basal release and stretch evoked release were diminished in TRPV1 knockout mice, suggesting that TRPV1

activation is required for mechanically evoked ATP release. nAChRs have also been shown to inhibit TRPV1 currents in DRG neurons [175], further suggesting that the inhibition of ATP release in the urothelium by nAChR stimulation may be due to inhibition of the TRPV1 receptor. A number of chemical mediators can also influence ATP release from urothelial cells though, so it should be noted that nicotinic modulation of TRPV1 is just one possible mechanism.

It is interesting to note that MLA, our α7 antagonist, has an excitatory effect on ATP release. This might suggest that the inhibitory α7 receptor was being tonically activated, resulting in a reduced basal release of ATP until blocked by the antagonist. In addition to ATP, the urothelium also releases ACh in response to mechanical stimuli [93], therefore it is possible that nicotinic receptors on our cultured urothelial cells are being tonically activated in an autocrine/paracrine manner by urothelially released ACh. While this might indicate that the increased ATP release following MLA perfusion could be due to a block of the tonically activated inhibitory α7 pathway, leading to excitation, another phenomenon may play a role in the observed effects. MLA is a competitive antagonist to the α7 receptor, competing for the ACh binding site of the receptor. Therefore, when MLA is present, there would be increased ACh available in the bath, as ACh would be prevented from binding to the α7 receptor. This, in turn, could lead to increased activation of α3* receptors on the urothelial cells, resulting in increased ATP release. If this were the case, we would expect that an α3* antagonist such as TMPH, applied concurrently, would block the increase in ATP release observed during MLA perfusion. Additionally, we would expect that a non-competitive α7 antagonist, such as α-BTX, would not result in an increase in ATP release, since the non-competitive nature of the antagonist would not result in an increase in available ACh. It is also interesting to note that TMPH does not cause an

inhibitory effect; TMPH is a non-competitive inhibitor and also would not cause an increase in available ACh to act on α7 receptors.

3.3.3 Interactions Between Urothelial nAChRs

The most interesting conclusion that can be made following the experiments presented in this chapter is the inhibition of the α3* mediated effects following stimulation of α7 receptors. Both cytisine induced calcium transients and ATP release are blocked when cells are previously stimulated with α7 agonists, such as PNU 282987 or choline. This is an interaction that has not been previously described; therefore the potential implications for nicotinic receptor research may be substantial.

It is unclear what the full mechanism for this cross-modulation might be, however our research indicates that it involves PKA and PKC, presumably through phosphorylation of the α3* receptor. While we do not definitively demonstrate that α7 receptor activation results in the activation of either PKA or PKC, it is clear that inhibition of either kinase prevents the inhibition mediated through α7 receptor stimulation. This suggests a pathway where protein kinases are activated through α7 receptor stimulation, which in turn phosphorylate and inactivate α3* receptors.

It should be noted that activation of α7 receptors with PNU 282987 inhibited the cytisine induced calcium transient, however PNU 282987 elicited no calcium transient by itself. This suggests that the activation of PKA/PKC is a calcium independent process. It has been demonstrated that stimulation of α7 receptors can lead to the activation of PLC, which could, in turn activate calcium independent isoforms of PKC through the production of DAG [240]. Our RT-PCR experiments suggest that a number of these calcium-independent isoforms of PKC exist

in the urothelium, such as the δ and ε isoforms. Considered together, it is therefore possible that the inhibition of α3* receptors by α7 receptor stimulation is calcium independent and modulated through these novel PKC isoforms.

3.3.4 Influence of Urothelial nAChRs on Bladder Physiology?

We have demonstrated that stimulation of nAChRs present on urothelial cells can modulate ATP release, but what effect might this modulation have on bladder physiology? ATP has been demonstrated to be an excitatory transmitter in the bladder; ATP can increase afferent excitability and the purinergic antagonist PPADS can decrease afferent excitability. Given the excitatory nature of ATP on afferent nerves and bladder reflexes, we can hypothesize what effects nAChR stimulation might have on bladder reflexes *in vivo.* α7 receptor stimulation has been shown to decrease ATP release from the urothelium, therefore we would expect activation of urothelial α7 receptors *in vivo* would inhibit bladder reflexes, as afferent excitability would decrease. Conversely, we would expect that stimulation of α3* receptors with large concentrations of cytisine (above 50 μM) would result in increased bladder activity, as increased ATP release would sensitize afferent nerves. We might also expect that lower concentrations of cytisine may inhibit bladder reflexes, as concentrations below 10μM decreased ATP release. In order to determine if our hypotheses are correct, we will have to perform *in vivo* experiments; stimulating urothelial receptors through intravesical administration of nicotinic agents and examining the effects the drugs have on bladder reflexes. In the next chapter, we will perform these experiments, utilizing a technique known as the bladder cystometrogram to examine the effects of urothelial nAChR stimulation of bladder reflexes of the anesthetized rat.

4.0 MODULATION OF BLADDER REFLEXES IN THE ANESTHETIZED RAT THROUGH STIMULATION OF UROTHELIAL NICOTINIC RECEPTORS

Our previous research has demonstrated that the urothelium expresses nicotinic receptor subunits and that those subunits can form functional receptors capable of increasing intracellular calcium and modulating ATP release. Given the hypothesized role of urothelially-released ATP to sensitize bladder afferent nerves, our data suggests that urothelial nAChRs may be able to influence bladder activity. A number of other urothelial receptors, such as muscarinic or bradykinin receptors, have been previously shown to modulate bladder activity in rats, supporting this hypothesis. Therefore the present study was undertaken to measure the influence nAChR stimulation can have on bladder reflexes. Intravesical administration of nicotine (50nM & 1μM) resulted in an increase in the interval between bladder contractions (also known as the intercontraction interval, or ICI); indicating an inhibition of bladder reflexes. Further research with subtype selective agonists and antagonists, however, revealed the presence of two distinct, opposing effects of nAChRs on bladder reflexes. For example, stimulation of α7 receptors by intravesical infusion of choline resulted in a concentration-dependant inhibition of bladder reflexes. Additionally, the α7 selective antagonist methyllycaconitine citrate completely blocked the nicotine-induced inhibition of bladder reflexes. These data suggest that urothelial α7 nicotinic receptors mediate an inhibitory pathway in the bladder. Conversely, stimulation of α3* receptors, using intravesical cytisine, excited bladder reflexes, while inhibition of α3* receptors

using hexamethonium caused an inhibition of bladder reflexes. This suggests that α3* receptors in the urothelium mediate an excitatory bladder pathway. Further experimentation indicated that our results were due to actions of our drugs on urothelial receptors and not direct actions on those located deeper in the bladder wall. These data indicate that urothelial nAChRs may play a role in bladder physiology, possibly by controlling the release of neurotransmitters such as ATP that can ultimately influence bladder afferent excitability. Given the observed ability of nicotinic agents to modify bladder activity, our research may indicate a role for nAChRs as novel pharmacological targets to treat bladder pathophysiology.

Note: All figures in the following chapter, excepting Figure 4.5, and the text describing them have been previously published in the American Journal of Physiology [220]© 2005, however permission to reprint is not required under the American Physiological Society's rules for reprinting published material by authors of the original manuscript.

4.1 INTRODUCTION

Our earlier experiments have demonstrated that the urothelium of the rat, cat and human express the proper subunits to form proper nicotinic acetylcholine receptors. At least some of these receptors are functional, as stimulation of urothelial cells with nicotine causes increases in intracellular calcium and can also modulate urothelial ATP release.

As we have described previously in Chapter 1.2.2, the urothelium is thought to participate in the control of the urinary bladder through the release of transmitters in response to physical or

chemical stretch [10, 12, 13]. We have already demonstrated that ATP is released from the urothelium, however, another transmitter released by stretch of the urothelium is acetylcholine [16, 92, 93], the endogenous ligand for the nicotinic receptor. Given this proximity in location between nAChRs and their endogenous ligand, the possibility exists that urothelial nAChRs can be activated by urothelially-released ACh in an autocrine/paracrine manner. These autocrine/paracrine actions on nAChRs have the potential to influence bladder activity. Stimulation of other urothelial receptors, such as TRPV1, bradykinin or muscarinic receptors, have demonstrated that stimulation of the urothelium can results in the modulation of bladder reflexes [60, 63-65]. Additionally, we have already demonstrated that stimulation of urothelial cells with nicotinic agents can modulate the release of ATP, and it is thought that urothelially-released ATP can influence bladder afferent nerves to influence bladder activity. Given this data, the potential exists that urothelial nAChRs can modulate bladder activity; therefore, we examined how nicotinic stimulation of the urothelium influenced bladder reflexes.

In order to accomplish this, we examined the effects of subtype specific agonists and antagonists on bladder reflexes in the anesthetized rat, utilizing a technique known as the bladder cystometrogram (CMG). During a CMG, a saline solution is slowly and continuously infused into the bladder, mimicking physiological filling by the kidneys, while the pressure in the bladder is measured by a transducer (Figure 4.1) [279]. While many different parameters can be recorded using the CMG (such as voiding pressure and smooth muscle compliance), the main parameter important for the studies we will present is the "intercontraction interval" (ICI), which is a measure of the interval between voiding contractions. Changes in ICI during an experiment are generally regarded as indicative of a modulation in afferent excitability. This follows the idea that, in an anesthetized rat, micturition is initiated when the bladder becomes "full", i.e. the

volume of liquid in the bladder causes sufficient stretch to activate the spinobulbospinal reflex and initiate micturition. Because the infusion rate of saline into the bladder during the experiment is held constant at 0.04 ml/min, bladder contractions occur at regular intervals (normally every 8-10 minutes, depending on bladder size). Any increase or decrease in this interval following a drug treatment must then represent a shift in afferent excitability, shifting the threshold volume/pressure required to initiate micturition, resulting in either excitement or inhibition of the bladder reflex. During intravesical administration of drugs, this modulation of afferent excitability is thought to be mediated through urothelial signaling as we have previously described, i.e. activation of receptors on the urothelium resulting in the release of transmitters to influence underlying afferent nerves [10].

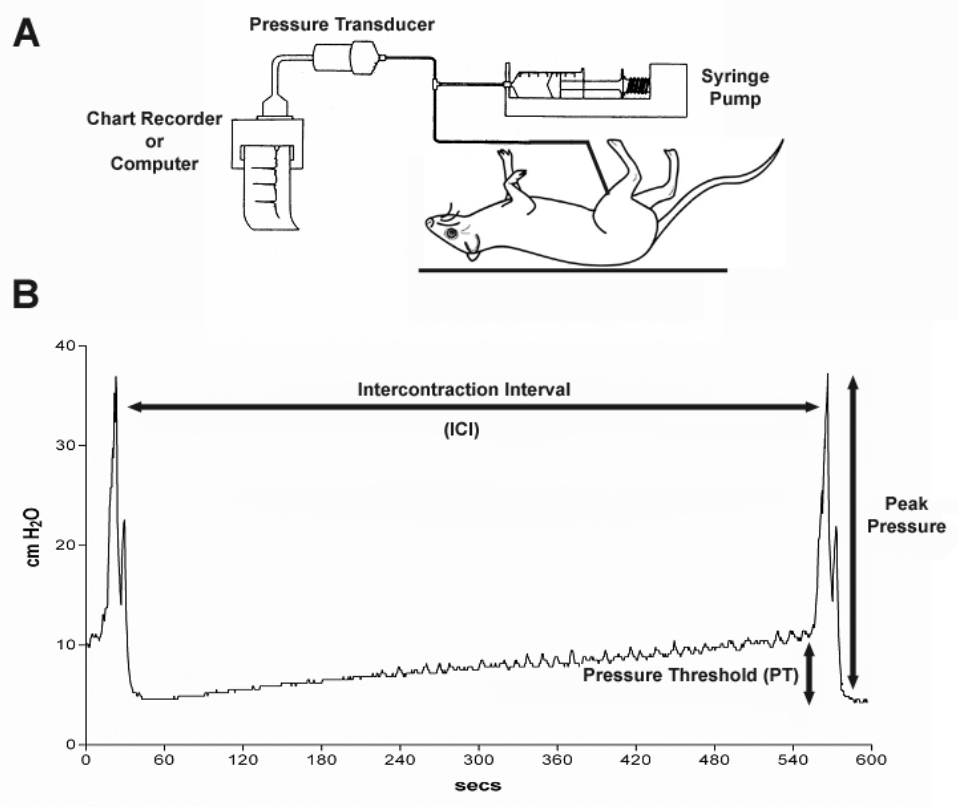

Figure 4.1- Cystometrogram Setup and Analysis

(A) The experimental setup for a cystometry experiment. The bladder of the animal (which is, for our experiments, an anesthetized rat) is attached to a syringe pump and a pressure transducer by way of a three-way stopcock. During the experiment, the bladder is filled slowly by the syringe pump (at a rate of 0.04 ml/min) and the pressure in the bladder is measured by the transducer. These readings are recorded for future analyzation through the use of a chart recorder or a data acquisition system run by a computer. **(B)** A sample cystometric recording, depicting two consecutive bladder contractions. Bladder pressure is measured in cm of H_2O (y-axis) over time (in seconds, x-axis). Common cystometric parameters are defined, such as pressure threshold, peak pressure or intercontraction interval (ICI).

In the following study, we will demonstrate that intravesical administration of nicotinic agents specific to the two types of urothelial nAChR can modulate bladder reflexes *in vivo*. Activation of urothelial α7 receptors inhibits bladder reflexes in the anesthetized rat, while activation of α3* receptors causes excitation. Further experimentation indicates that these results are due to actions on urothelial nAChRs and not receptors on underlying nerves or deeper into the bladder wall.

4.2 RESULTS

4.2.1 Nicotine Inhibits Bladder Reflexes

To determine if activation of urothelial nicotinic receptors causes changes in reflex bladder contractions, we infused a saline solution containing nicotine hydrogen tartrate into the bladder of anesthetized rats and examined changes in voiding parameters. An initial concern during our experiments is whether intravesically instilled drugs would act solely on urothelial receptors, or if they might pass through the urothelial barrier to act on receptors in the bladder wall, such as those on afferent nerves. Hence, the hydrogen bitartrate form of nicotine was chosen for these experiments based on its solubility properties; as a salt form of nicotine, the bitartrate form is water soluble, making it less likely to pass through the urothelial barrier to affect receptors deeper in the bladder wall. As shown in Figure 4.2, continuous infusion of nicotine (50nM and 1μM in normal saline, 0.04 mL/min, n=6) increased the intercontraction interval (ICI) in a concentration-dependent manner (17.4 ± 4.9% and 32.0 ± 7.5%, respectively) over saline infusion alone. These concentrations of nicotine were chosen based on earlier experiments that demonstrated these concentrations can increases intracellular calcium in urothelial cells (Birder, unpublished results). These inhibitory effects began within 2 minutes after beginning nicotine infusion and lasted for the length of the drug application (at least 30 minutes). Switching the infusate back to normal saline resulted in a complete and rapid (within 2 contractions) reversal of the increase in ICI (Figure 4.2B).

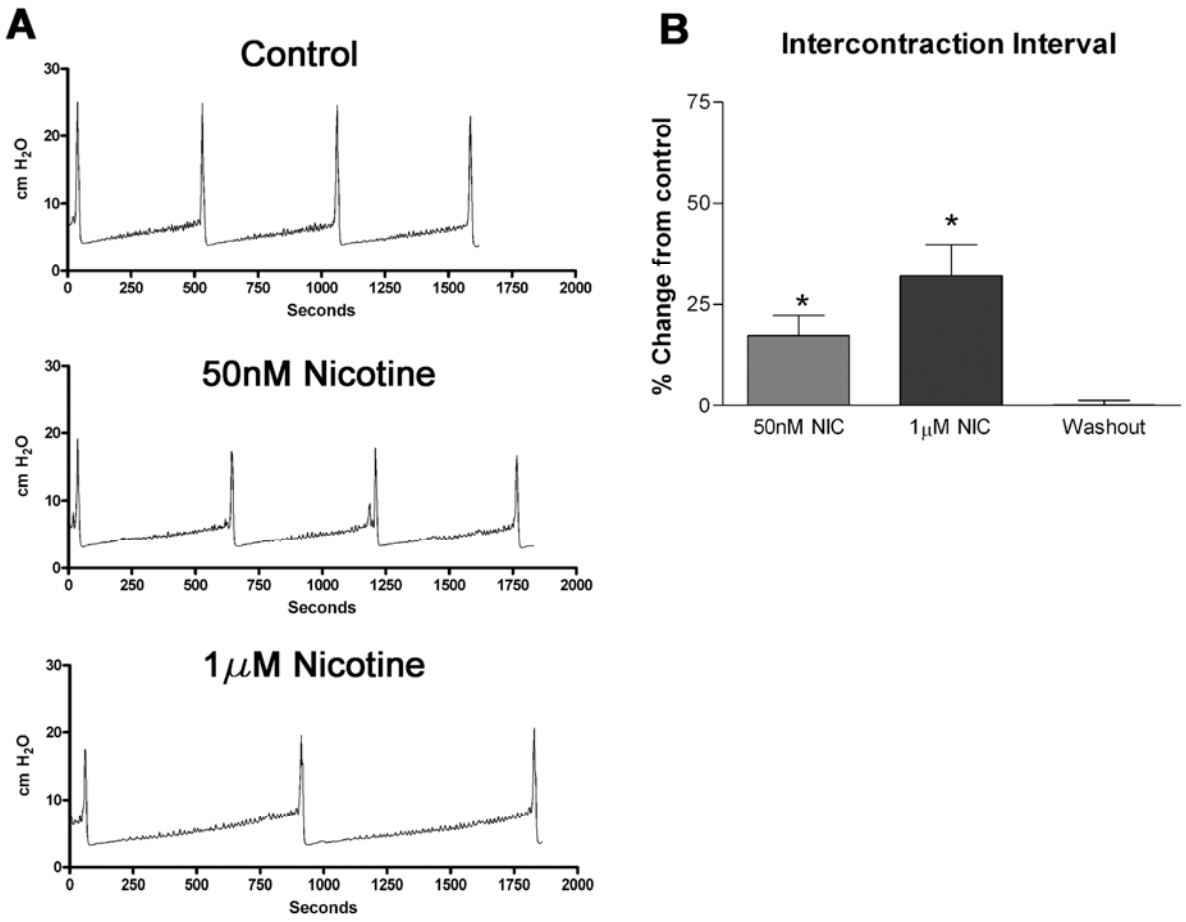

Figure 4.2 - Effects of Intravesical Nicotine on Voiding Function in the Rat.

(A) Three representative tracings of cystometry recordings. Notice the increase in the interval between contractions (referred to as the intercontraction interval or ICI) with increasing concentrations of nicotine. All traces were recorded in the same animal for comparison. The flow rate for each experiment was 0.04ml/min. (**B)** Summary of cystometry experiments. Intravesical administration of nicotine (NIC, 50nM and 1μM, n=6 each) increases the ICI in a concentration-dependent manner, which is reversed following saline washout. *$p<0.05$ as compared to a saline infused control by ANOVA.

4.2.2 Inhibition of Bladder Reflexes by Nicotine is Due to Stimulation of the α7 nAChR

Stimulation of urothelial receptors with nicotine resulted in an inhibition of bladder reflexes, however because nicotine is a non-specific agonist of nAChRs, these experiments do not elicit which subtype of nAChR is responsible for mediating the inhibitory effect. Therefore, in order to determine which nAChR is contributing to the inhibitory effects of nicotine, subtype selective agents were utilized. Choline is the major metabolite of acetylcholine and is a specific agonist of the α7 receptor (EC_{50} on human receptors expressed in oocytes: 0.4 mM). Intravesical administration of choline (1, 10 and 100 μM in saline, n=6 each) also increased ICI by 9.8 ± 1.5%, 21.0 ± 5.7% and 46.0 ± 14.8%, respectively (Figure 4.3). Additionally, the inhibitory effect of nicotine (50nM and 1μM) was blocked using the α7 specific antagonist methyllycaconitine citrate (MLA, 10μM in saline), while MLA had no significant effect by itself (1, 10 and 100 μM, Figure 4.4). This concentration was used because it was approximately 10-100 times larger than the IC_{50} as determined experimentally in other tissues [164, 175, 280], and would therefore be sufficient to block the majority of receptors.

Recent research has questioned whether choline is an appropriate agonist to use in these studies, as it can also activate certain muscarinic receptors [281]. Muscarinic receptors are also present in the urothelium [77, 218, 219], therefore the possibility exists that the inhibitory response observed following choline stimulation is due to actions on muscarinic receptors. These actions, while theoretically possible, are unlikely as most studies that demonstrate action of choline on muscarinic receptors use much higher concentrations than those used in our studies (K_i and EC_{50} for choline on the M1 receptor expressed in CHO cells: 2mM and 10mM, respectively [271]). In order to block muscarinic stimulation by choline, we could have used the

muscarinic antagonist atropine, as we did in our previous calcium and ATP experiments. However, atropine has already been shown to increase voiding frequency by itself [65], therefore any results would be difficult to interpret. In any case, our experiments demonstrate that MLA, a nicotinic antagonist that has no known actions on muscarinic receptors, fully blocks the inhibitory actions of nicotine. Therefore, we believe that our data supports the conclusion that α7 receptors in the urothelium mediate an inhibitory bladder pathway.

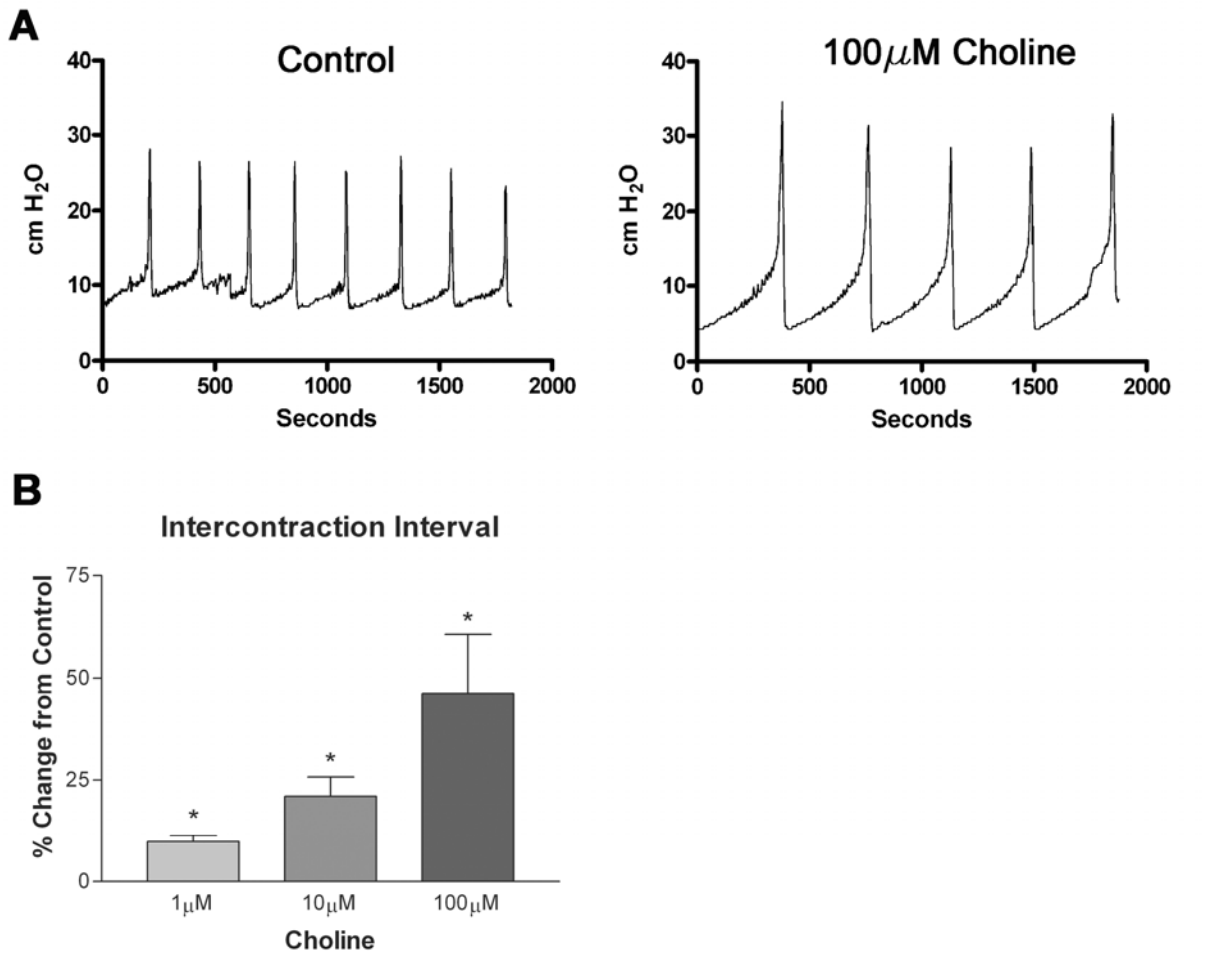

Figure 4.3 - Choline Inhibits Bladder Reflexes in the Anesthetized Rat

(A) Representative tracings of cystometrogram recordings during intravesical administration of choline. Traces shown were recorded in the same animal at a constant flow rate of 0.04ml/min for comparison. (**B)** Graph depicting the summary of the choline data presented as a percentage change in ICI over saline infused controls. *$p<0.05$ as compared to saline infused control by ANOVA. n=8 for each concentration.

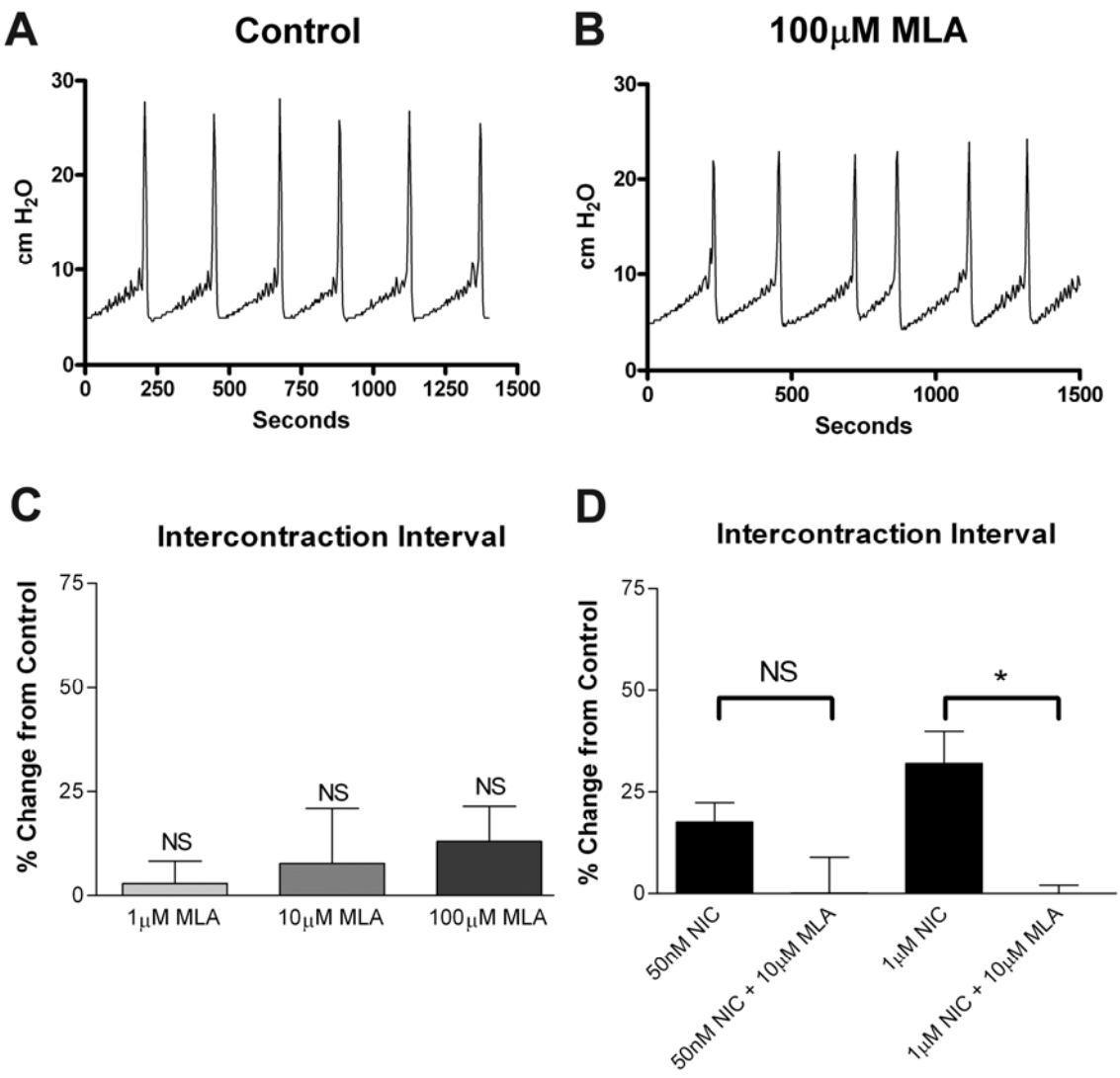

Figure 4.4 - The α7 Antagonist MLA Blocks Nicotine-Induced Inhibition of Bladder Reflexes

(A-B) Representative traces of the effects of the α7 antagonist methyllycaconitine citrate (MLA, 100μM) infusion on the bladder cystometrogram. Traces shown were recorded from the same animal at a constant flow rate of 0.04ml/min for comparison. **(C)** Graph summarizing the effects of MLA (1, 10, 100μM, n=8 each) alone. NS - not statistically significant as compared to saline-infused controls by ANOVA **(D)** Effects on ICI of nicotine infusion alone (50nM and 1μM, columns 1 and 3) as compared to simultaneous infusion of MLA (10μM) and nicotine (NIC, columns 2 & 4). n=6 for each, columns 1 & 3 are taken from different rats than columns 2 & 4. All results are shown as a percent change from saline infused controls. NS – not statistically significant as compared by students' t-test. *$p<0.05$ as compared by students' t-test.

4.2.3 α3* Stimulation Excites Bladder Reflexes in the Anesthetized Rat

To examine the role of urothelial α3* receptors in bladder function, we used the α3* specific agonist cytisine (EC_{50} on human α3β4 expressed in oocytes: 5.6 μM). As shown in Figure 4.5, intravesical administration of 1, 10 or 100 μM cytisine (in saline, n=6 for each) resulted in decreases in ICI up to 42.1% from saline infused controls. Conversely, intravesical instillation of the α3* antagonist hexamethonium (1, 10, 100 μM in saline, n=6 each) inhibited bladder reflexes, increasing the ICI in a concentration-dependent manner (10.9 ± 8.1%, 33.7 ± 8.5% and 39.3 ± 10.3%, respectively, Figure 4.6). These data suggest that urothelial α3* receptors mediate an excitatory pathway, which may be tonically active, since the antagonist inhibited bladder reflexes by itself.

These data indicate that the two distinct subtypes of nicotinic receptors present in the urothelium mediate opposing effects in the bladder. This raises the possibility that the inhibitory effects of the non-specific agonist nicotine observed earlier may be attenuated by the concurrent stimulation of the excitatory α3* pathway by nicotine. To determine if non-specific activation of α3* receptors by nicotine diminishes the inhibitory effects of α7 receptor stimulation, we concurrently instilled the bladder with nicotine (50 nM & 1 μM) and the α3* antagonist hexamethonium (20 μM). Concurrent instillation had additive effects to inhibit reflex voiding (Figure 4.6C), with hexamethonium increasing the inhibitory effects of both concentrations of nicotine (29.8 ± 7.5% and 67.2 ± 4.0% increase from control for 50nM and 1μM nicotine in the presence of 20μM hexamethonium, respectively). These data indicate that the bladder may also be inhibited by blocking the excitatory pathway mediated by the α3* receptor, and that this effect can be additive when combined with stimulation of the α7 receptor.

We have also demonstrated that the inhibition of α3* receptors results in the inhibition of bladder reflexes. This effect could be due, in part, through blocking tonic activation of α3* receptors by ACh released from the urothelium in response to stretch. However, it is also possible that prevention of ACh binding on α3* receptors by hexamethonium would lead to greater concentrations of ACh being available to bind to and activate inhibitory α7 receptors. Therefore, it is possible that the observed inhibitory effect of hexamethonium is due to a combination of thee two actions. To determine if the inhibitory effects of hexamethonium are potentiated by activation of α7 receptors, we concurrently instilled MLA (100μM) and hexamethonium (10, 100μM, n=6) into the bladder. As shown in Figure 4.7, the α7 antagonist MLA reversed the inhibition of bladder reflexes observed following hexamethonium instillation, indicating that some of this inhibition is mediated through the activation of α7 receptors. However, this reversal was not complete, suggesting that some of the hexamethonium-induced inhibition was mediated through another pathway, most likely the prevention of tonic activation of α3* receptors by urothelially released ACh.

These results observed with nicotine, hexamethonium and MLA suggest that urothelial nicotinic receptors exist in a careful balance with each other and can coordinate to influence bladder reflexes in the rat. We will discuss the implications of these results further in Section 4.3.

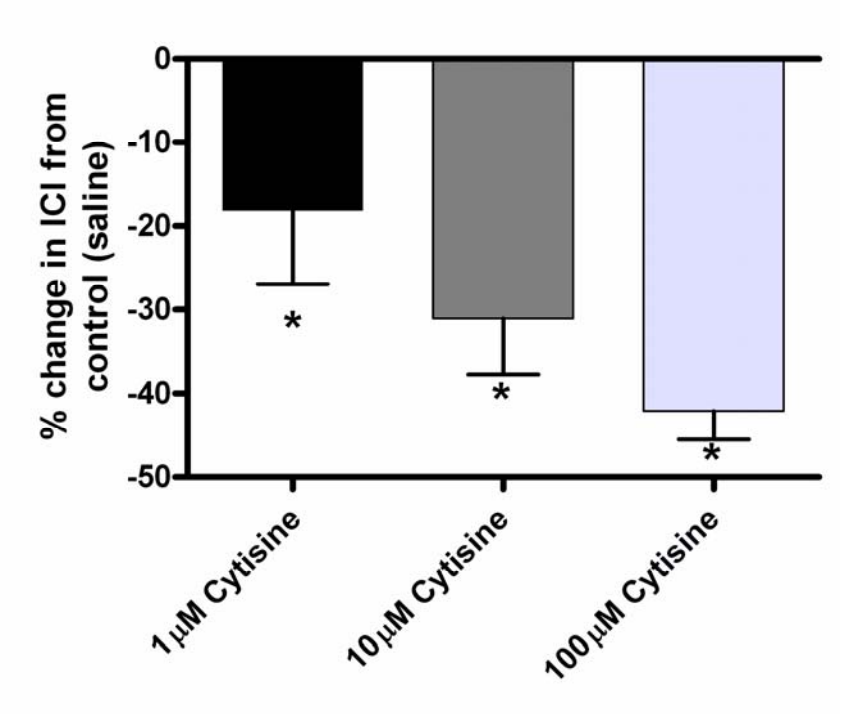

Figure 4.5 - Effects of the α3* Agonist Cytisine on Bladder Reflexes

Summary graph depicting the effects of cytisine (1, 10, 100μM, n=6 each) on bladder reflexes in the anesthetized rat. Data is expressed as a change in ICI from saline infused controls. *$p<0.05$ as compared to saline infused controls by ANOVA.

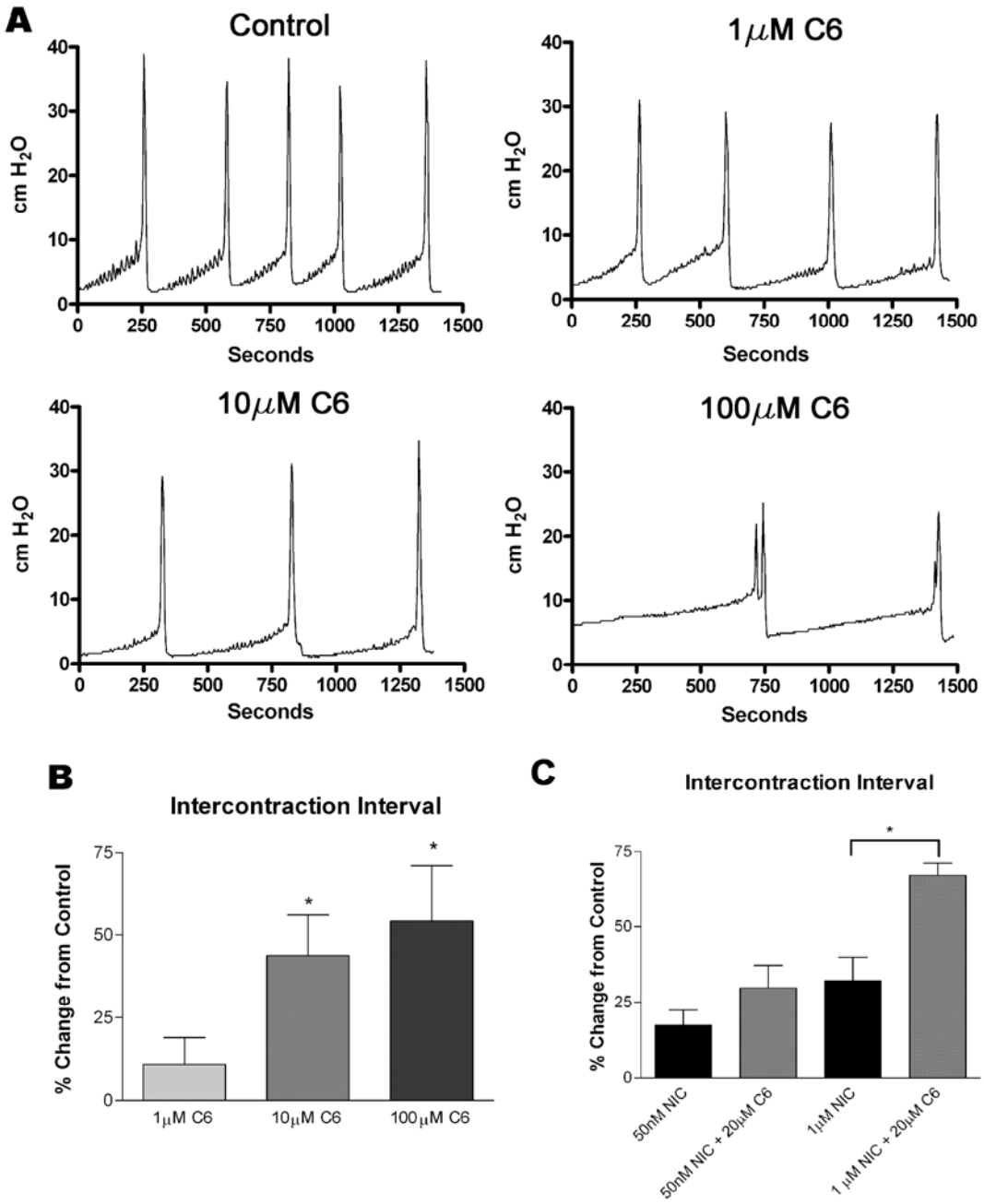

Figure 4.6 - Effect of the α3* Antagonist Hexamethonium on Bladder Reflexes in the Rat

(A) Representative traces of CMG recordings during intravesical administration of hexamethonium (C6). Traces shown were recorded from the same animal at a constant filling rate of 0.04 ml/min for comparison. **(B)** Intercontraction interval changes following instillation of hexamethonium (C6) intravesically. n=8 for each concentration. *p <0 .05 as compared to saline-infused control by ANOVA. **(C)** Effects on ICI of nicotine infusion alone (50nM and 1μM, columns 1 & 3) as compared to simultaneous infusion of C6 (20μM) and nicotine (NIC, columns 2 & 4). n=6 for each, columns 1 & 3 are taken from different rats than columns 2 & 4. *p <0 .05 as compared by students' t-test. All results are shown as a percent change from saline infused controls.

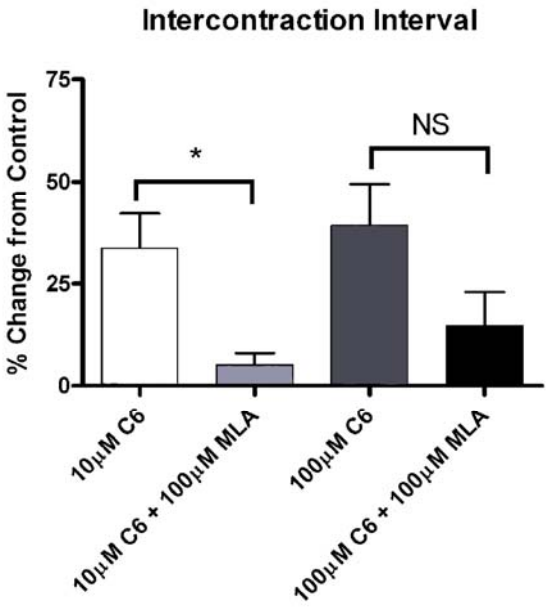

Figure 4.7 - Effect of Simultaneous Infusion of MLA and Hexamethonium on Bladder Reflexes

Comparison in the percent change in ICI from saline control following instillation of hexamethonium (C6, 10, 100μM) alone or concurrent instillation of C6 and methyllycaconitine citrate (MLA, 10, 100μM). *$p<0.05$ as compared by students' t-test. NS – not statistically significant. n=6 for each.

4.2.4 Intravesical Effects of Nicotinic Agents are Due to Actions on Urothelial Receptors

We have applied all of the agents above intravesically with the intention of specifically stimulating urothelial receptors. This follows our hypothesis that stimulation of receptors present on the luminal surface of the urothelium would activate intracellular pathways that cause the release of a transmitter that could influence bladder afferents. However, the possibility exists that agents instilled into the bladder are, in fact, permeating through the urothelial barrier to stimulate nAChRs located deeper in the bladder wall, such as those present on afferent nerves. With this in mind, it would be helpful to determine what effects stimulation of these other

receptors would cause on bladder reflexes, in order to determine if the effects we observe following intravesical administration can be attributed to actions on these deeper receptors. To this end, we performed cystometrograms where protamine sulfate was instilled intravesically prior to nicotinic stimulation in order to disrupt the urothelial barrier. Protamine sulfate treatment has been shown to allow intravesically perfused drugs to reach deeper into the bladder wall and activate receptors located on underlying afferent nerves [282, 283]. Following one hour of protamine sulfate (10 mg/ml, in saline) infusion, the infusate was switched to nicotine. Protamine sulfate treatment did not significantly alter the ICI (Figure 4.8B) by itself, suggesting that the treatment did not result in damage to bladder afferent nerves. However, nicotine (1 μM) infusion following protamine sulfate treatment decreased the ICI 38.8 ± 9.9% (Figure 4.8A&B), indicating an excitation of bladder reflexes. This effect could not be reversed by washout with normal saline.

It may also be possible to stimulate nAChRs located deeper in the bladder wall through the use of a compound that can cross lipophilic barriers. Epibatidine is an ultrapotent, highly lipophilic α3* receptor agonist, which readily crosses the blood-brain barrier. Given these properties, we can also assume that it could easily pass the urothelial barrier as well. Intravesical administration of epibatidine (250 nM in saline, 0.04 mL/min, n=4), elicited an immediate increase in voiding pressure (approximately 50%) without significantly changing the ICI (Figure 4.9A&B). Continued infusion of epibatidine for one hour resulted in complete urinary retention and overflow incontinence (Figure 4.9C), presumably through desensitization of nAChR in the autonomic ganglia. This hypothesis is supported by the actions of a systemic dose of epibatidine (0.05 μg/kg, i.p. n=4), which also suppressed reflex bladder contractions and produced urinary

retention (Figure 4.9D), however the onset occurred much more rapidly (onset of approximately 10 minutes compared to one hour following intravesical administration).

These data suggest that the actions observed earlier (in Sections 4.2.1-4.2.3) are due to activation of urothelial nicotinic receptors by our intravesically instilled agents; as our research indicates that activation of nicotinic receptors deeper in the bladder wall has an excitatory effect, contrary to the inhibitory effect observed previously.

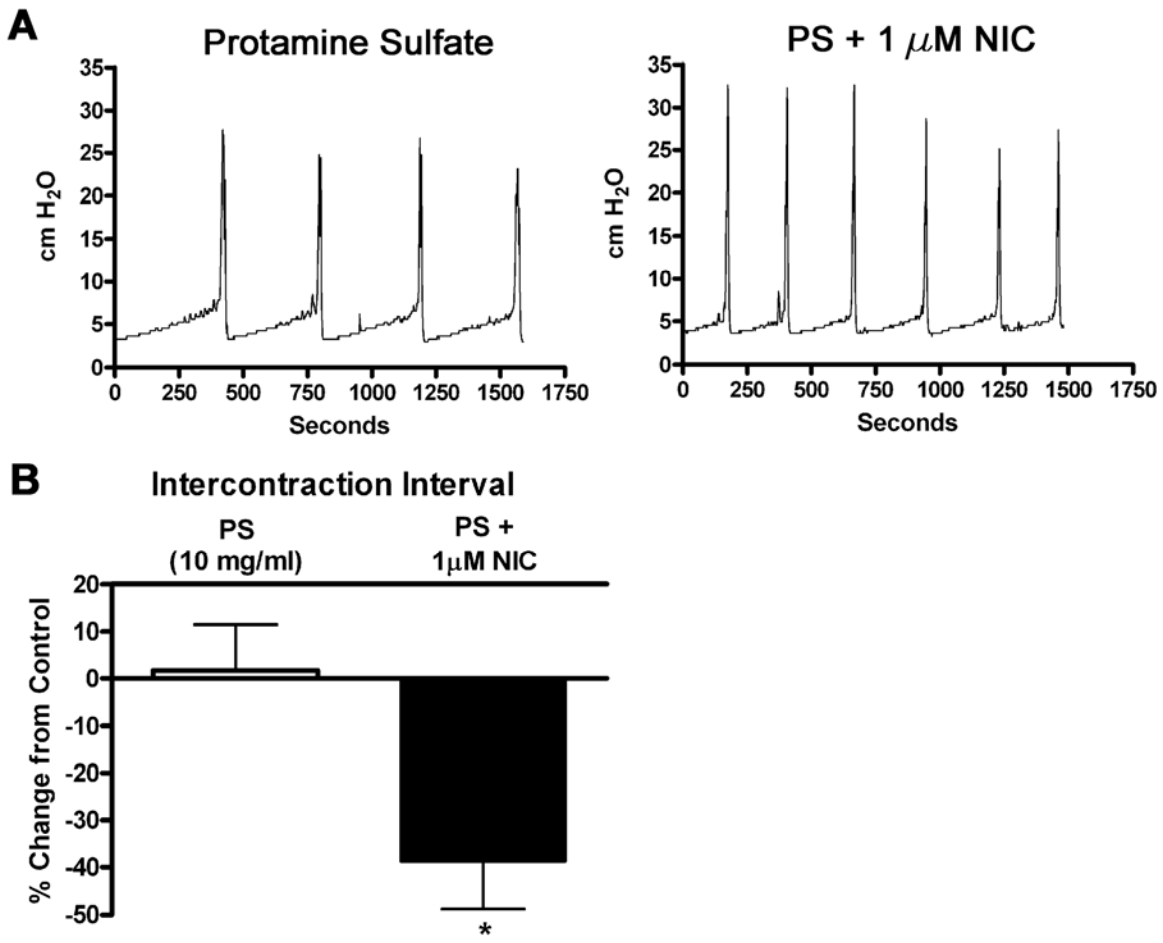

Figure 4.8 - Nicotine Excites Bladder Reflexes Following Disruption of the Urothelium with Protamine Sulfate.

Effect of permeabilization of the urothelial barrier by protamine sulfate (PS) on nicotine-induced changes in bladder reflexes. **(A)** Representative tracings of CMG recordings taken during a control period of protamine sulfate (10mg/ml) infusion and PS infusion with simultaneous nicotine (NIC, 1 μM) infusion. Traces shown were recorded from the same animal at a constant filling rate of 0.04ml/min for comparison. **(B)** Graph depicting changes in ICI during PS infusion or simultaneous PS and nicotine infusion as a change from saline infused controls. n=6 for each column. * $p < 0.05$ as compared to saline-infused control by students' t-test.

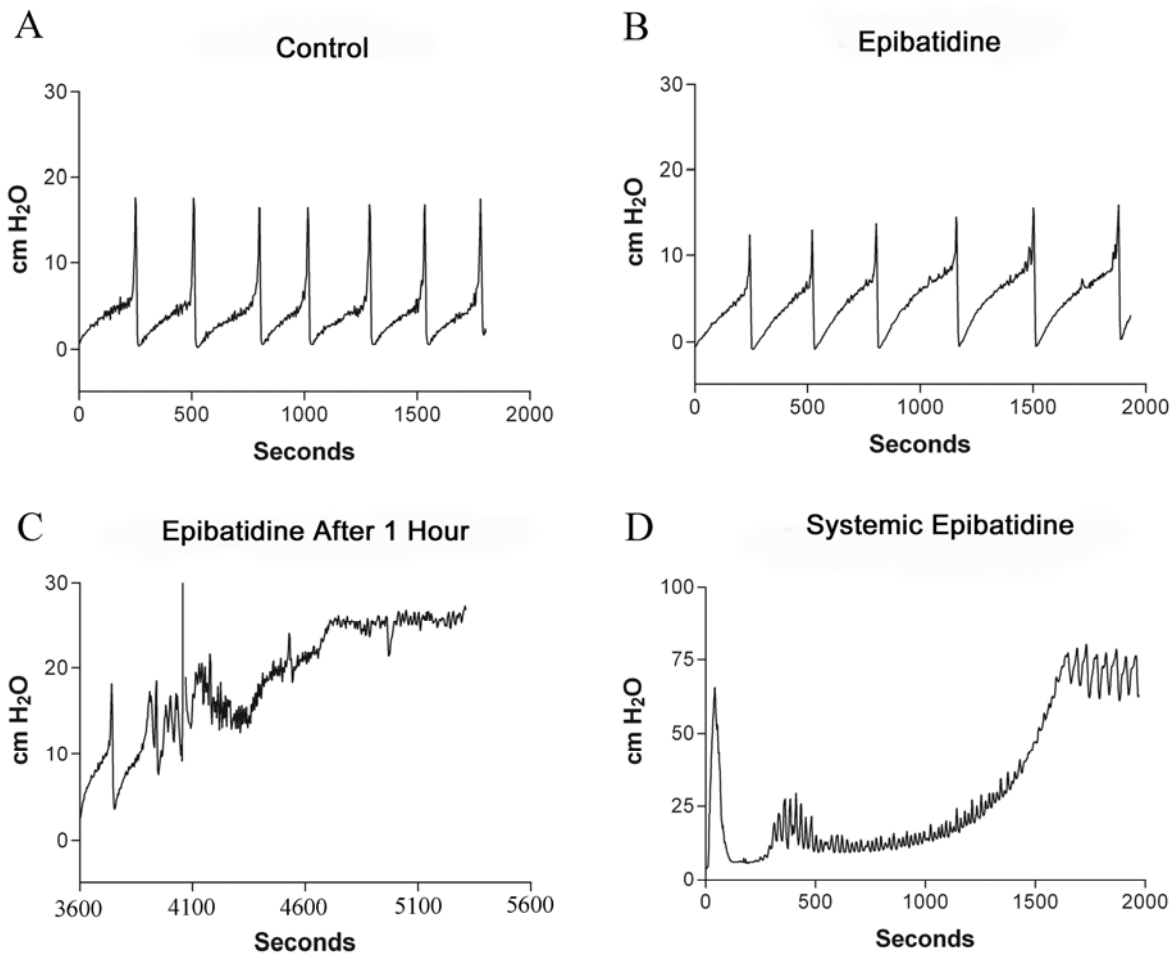

Figure 4.9 - Effect of Epibatidine, an Ultrapotent, Lipophilic α3* Agonist on Bladder Reflexes

Effect of the potent nicotinic agonist, epibatidine on bladder function. **(A)** Control recordings **(B)** After 250nM epibatidine instillation into the bladder (continuous infusion, 0.04ml/min) **(C)** After one hour of continuous instillation of epibatidine. **(D)** Cystometrogram of bladder pressure following systemic administration of epibatidine (0.01 μg, i.p.). Traces A-C were recorded from the same animal, D was recorded in a separate experiment.

4.3 DISCUSSION

Nicotinic receptor signaling is important at many levels in the neural pathways controlling bladder function; being responsible for neurotransmission in the brain, spinal cord, autonomic ganglia and detrusor smooth muscle [1, 131-134, 180]. The present study raises the possibility of an additional site for nicotinic modulation of bladder function; the urothelium. As discussed earlier, when the bladder stretches, ACh is released from the urothelium [16, 92]. Our experiments raise the possibility that this ACh can then act in an autocrine or paracrine manner on urothelial nAChRs to modulate bladder reflexes.

4.3.1 nAChR Modulation of Bladder Reflexes: Do *in vitro* Experiments Suggest Mechanism?

Our research indicates that the two different types of nicotinic receptors in the urothelium have directly opposing effects on bladder reflexes. α7 receptors, for example, mediate an inhibitory pathway in the bladder; as evidenced by the inhibitory effect of choline, as well as the ability of MLA, an α7 antagonist, to block nicotine's inhibitory effects. This inhibitory effect could be mediated through a number of different pathways. The most probable scenario, given the research performed in Sections 3.2.4-3.2.5, is the modulation of the release of ATP from the urothelium. nAChRs stimulation can alter mechanically stimulated ATP release from the urothelium; α7 stimulation inhibited ATP release, while α3 stimulation inhibited ATP release at low concentrations of agonist and evoked release at high concentrations of agonist. ATP is an

excitatory transmitter in the bladder that is thought to modulate afferent excitability through actions on P2X receptors present on afferent nerve terminals [6, 73]. Given the ability of ATP to increase afferent excitability [128], our previous *in vitro* results concerning ATP release match well with our *in vivo* data. For example, α7 stimulation inhibits mechanically-stimulated ATP release from urothelial cells. This coincides well with the observed *in vivo* inhibition of bladder reflexes; with decreased ATP release, afferent excitability would decrease, leading to an inhibition of the bladder reflexes. Additionally, stimulation of urothelial cells with large concentrations of cytisine increases ATP release, which fits well with the observed excitation of bladder reflexes. In this case, increased ATP would lead to a sensitization of bladder afferents, decreasing the amount of stretch needed to activate the micturition pathway and increasing the frequency of voiding.

It should be noted however, that while our data support the hypothesis that nAChRs mediate their effects on bladder reflexes though the release of ATP, we have not yet definitively linked the *in vivo* and *in vitro* effects. The urothelium can release a number of other transmitters, which may also be responsible for the observed effects *in vivo.* For example, it is known that stimulation of muscarinic receptors in the urothelium causes the release of an, as yet, unidentified soluble factor that can inhibit bladder smooth muscle contractions [14, 15]. It is possible then, that α7 stimulation can also cause the release of some soluble factor that inhibits bladder reflexes, most likely through actions on afferent nerves. Although research into the identity of this unidentified, muscarinically-released inhibitory has factor ruled out the involvement of nitric oxide, α7 receptors have been shown to activate nitric oxide synthase in dorsal root ganglion cells [231]. Additionally, recent studies have shown that NO is released from cultured urothelial cells following cholinergic [284] or adrenergic stimulation [61] and that

NO-donors can increase the ICI, when instilled into the bladder [66]. Oxyhemoglobin, a NO scavenger, also induces bladder overactivity in unanesthetized rats when applied intravesically [68]. It is thought that NO released from the urothelium can act on underlying afferent nerves, decreasing excitability and promoting storage. This is supported by research that demonstrates NO can modulate Ca^{+2} [97], Na^{+2} [285] and K^{+} channels [286] in afferent nerves, which could influence the resting membrane potential or transmitter release. Therefore, while our *in vitro* experiments suggest modulation of ATP release as a mechanism for our *in vivo* results, a number of other transmitters may also play a role.

The actions of nicotinic antagonists alone on bladder reflexes in the rat also give us clues on the role on cholinergic signaling in the urothelium. Hexamethonium, by itself, has an inhibitory effect on bladder reflexes, while MLA has no significant effect. Both of these antagonists are competitive, meaning that they compete with ACh to bind to their respective receptors. This raises the possibility of increased activation of the opposing nAChR following application of an antagonist. To clarify, it has been shown that ACh is released from the urothelium as the bladder stretches. When hexamethonium is instilled intravesically, it would compete with ACh, preventing binding and increasing the available concentration of ACh in the bladder lumen. This would lead to increased activation of the α7 receptor, leading to inhibition. It is curious, though, why MLA does not have an excitatory effect *in vivo*. MLA is also a competitive agonist, which would suggest that more ACh would be available in the lumen to bind and activate the excitatory α3* receptors. We demonstrated in the last chapter that MLA alone can increase ATP release from the urothelial cells, which should lead to increased afferent excitability and increased voiding frequency. It may be that an excitatory effect is not observed because ACh levels much reach a sufficient level to activate the α3* excitatory pathway. As we

demonstrated in the last chapter, stimulation of α3* receptors with low concentrations of cytisine inhibited ATP release. It is possible that low concentrations of nicotine also result in the inhibition of urothelial ATP release through α3* receptors and this is responsible for the trend towards inhibition with MLA in our *in vivo* experiments.

4.3.2 Does Intravesical Administration of nAChR Agents Activate Urothelial Receptors? Implications for Urothelial Signaling

During the course of these experiments, one question in particular becomes apparent: are the nicotinic agents perfused intravesically acting on urothelial receptors or are they passing through the barrier to act directly on receptors located on afferent nerves in the bladder wall. This is an important question to answer not only to properly analyze the data presented in this dissertation, but also to address a basic physiological question concerning urothelial signaling: does cholinergic signaling take place at the apical or abluminal surface of the urothelium? This question is of the highest importance when we consider the possible role of urothelial signaling in bladder signaling. The location of urothelial nAChRs could have profound implications on how they influence the control of the bladder. For example, receptors present on the lumen of the bladder suggest that activation of the receptor would modulate some trans-urothelial pathway, where stimulation of the receptor on the luminal surface would cause the release of a transmitter from the abluminal surface. However, nicotinic receptors are present in other locations in the bladder, such as afferent or efferent nerves. In order to determine if urothelial nAChRs can influence bladder reflexes, we must rule out the possibility that the effects we observe are not due to effects at the other sites. Our experiments, in addition to others [63, 65], indicate that our intravesically instilled agents can act on the luminal surface of the urothelium.

The first evidence that cholinergic signaling takes place at the luminal surface of the bladder involves the selection of the compounds used in our studies. The urothelium forms a highly effective barrier to the potential toxins in the urine with a permeability that is much higher than even that of the blood brain barrier [19]. Therefore, we would expect that polar, hydrophilic or large agents, such as the quaternary amine hexamethonium would not cross the urothelial barrier (Figure 4.10). We would also expect that nicotine, in its hydrogen tartrate form would be less likely to cross the urothelial barrier. This is due to the ionization state of nicotine; at a pH of approximately 7, nicotine would exist primarily in an ionized form and not in the free base form that would readily cross the urothelium. This is supported by our experiments showing that nicotine's effects can be rapidly washed out, suggesting that very little nicotine reached the inner layers of the bladder wall. The other two compounds that we utilized for this study, cytisine and MLA are lipophilic and capable of passing the blood brain barrier, which suggests that they may also pass the urothelial barrier. This may allow these agents to bind nicotinic receptors deeper in the bladder wall, influencing our results. However, in our experiments, changes in ICI were observed almost immediately after infusion of the agents began, suggesting that the drugs' actions were urothelial in nature. As we demonstrate in experiments with epibatidine, even a highly lipophilic agent took approximately an hour before systemic effects became apparent. Therefore, we believe that the results we observed with MLA and cytisine are due to actions on urothelial receptors.

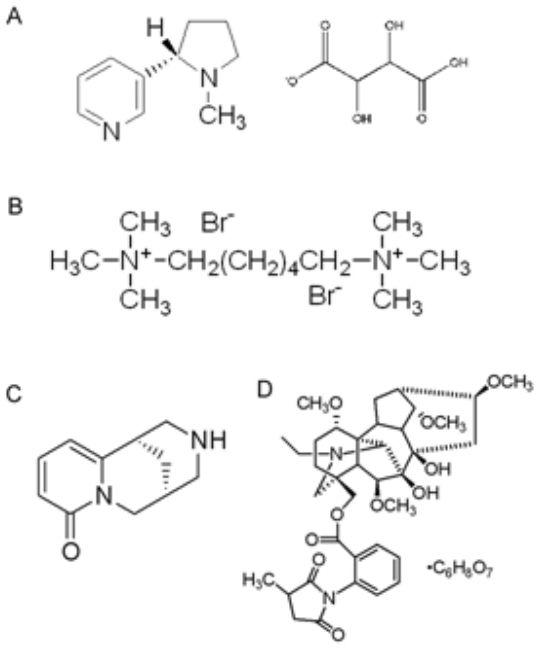

Figure 4.10 - Chemical Structures of Nicotinic Agents Used

Chemical structures of (A) nicotine hydrogen tartrate, (B) hexamethonium, (C) cytisine, and (D) methyllycaconitine citrate (MLA).

To test what might happen if a nicotinic agent did cross the urothelial barrier to work in the periphery, we examined the effect of protamine sulfate, which has been shown to disrupt the urothelial barrier and allow intravesically administered agents to pass into the underlying tissue [282, 283]. Prior to protamine sulfate treatment, nicotine has an inhibitory effect, increasing the ICI. However, when administered after protamine sulfate treatment, nicotine has the opposite effect, exciting the bladder reflex and decreasing the ICI. This switch in nicotine's effects following permeability of the urothelial barrier suggests that protamine sulfate allows nicotine to act on sub-urothelial targets such as afferent nerve terminals while nicotine infusion without protamine sulfate treatment activates receptors on the urothelium. This research is supported by studies performed by Masuda, et. al. [180], which shows that large doses of nicotine (1-10mM)

can also cause an excitation of bladder reflexes. It was hypothesized that at these concentrations, the amount of nicotine present in the free base form, and hence capable of crossing the urothelial barrier, would be sufficient to have physiological effects. Because our experiments use much lower concentrations of nicotine (50nM & 1μM), we would expect that we lack a sufficient concentration of nicotine to cross the urothelial barrier and activate afferent nerve terminals.

We also examined the effects of the nicotinic agonist epibatidine, which readily crosses the blood brain barrier and has a greater affinity for α3β4 receptors than α7 receptors (EC_{50}: 0.01-0.02 μM vs. 1.0-2.0μM) [287]. We believe that epibatidine would also cross the urothelial carrier, given its lipophilic nature. Initially, intravesically administered epibatidine had an excitatory effect on the bladder; however it did not influence the intercontraction interval, but rather altered the threshold pressure necessary to initiate a bladder contraction. These changes do not indicate actions on afferent nerves (either directly or indirectly through urothelial signaling), but instead indicate actions on bladder smooth muscle. This is most likely accomplished through actions on pre-junctional parasympathetic efferent neurons, where it is thought that nicotinic receptors can modulate ACh release and hence influence bladder smooth muscle contractility. Additionally, after two hours of continuous infusion, voiding was blocked, leading to a state of urinary retention and overflow incontinence. The onset of overflow incontinence was much more rapid following a systemic dose of epibatidine, indicating that urinary retention is a result of effects of the drug outside the bladder, most likely through desensitization of nicotinic receptors on sensory nerves or in autonomic ganglia [133]. Both of these effects are indicative of actions outside of the lumen of the bladder, therefore the lack of similar effects following intravesical nicotine, suggests that its actions are due to actions on urothelial receptors.

Therefore, given these experimental data, we believe that the nicotinic agents used in these experiments are acting primarily on urothelial receptors because: 1) the chemical structures and proposed ionization states of the compounds suggest a reduced or limited ability to diffuse across the urothelial barrier, 2) the ability to wash out the nicotinic effect and return to normal, suggesting that the compound did not cross the urothelial barrier, 3) the reversal of nicotine's effects following disruption of the urothelial barrier, allowing nicotine to penetrate to sub-urothelial afferent nerves, and 4) a lack of a biphasic response, that would indicate an early action on urothelial receptors followed by diffusion through the barrier and subsequent action at other sites.

4.3.3 Integrating nAChR Effects into the Model of Urothelial Signaling

We have demonstrated that activation of urothelial nicotinic receptors using pharmacological agents can modulate bladder reflexes, however we have not yet discussed how these results give us insight into how urothelial cholinergic signaling plays a role in normal bladder physiology. In the next chapter we will discuss in better detail how what is already known about nicotinic receptors fits together with our experimental data into a hypothetical model of nicotinic signaling in the urothelium and how nicotinic receptors might fit into urothelial signaling as a whole. We will also discuss further directions our research can take, as well as the clinical implications of the research presented.

5.0 FINAL CONCLUSIONS

In the previous chapters, we have demonstrated that the urothelium expresses functional nicotinic receptors which, when stimulated, can alter cellular processes such as calcium homeostasis and ATP release. Additionally, stimulation of nicotinic receptors *in vivo* can also modulate bladder reflexes. In this chapter, we will discuss how these results complement each other to suggest a model of urothelial signaling in the bladder and how nicotinic receptors may play a role in modulating bladder reflexes. Additionally, we will also discuss the implications these results have in regards to bladder pathology and the clinical possibilities of nicotinic receptors in the treatment of common bladder disorders. Finally, we will discuss other areas of urothelial physiology where nicotinic receptors may play an important role and why researchers will want to examine them in the recent future.

5.1 MODEL OF NICOTINIC RECEPTOR-MEDIATED MODULATION OF BLADDER REFLEXES

The research presented in this dissertation has demonstrated that stimulation of nicotinic receptors on urothelial cells can mediate a number of cellular and physiological responses. First, stimulation of urothelial nAChRs can modulate intracellular calcium transients as well as influence the release of ATP from urothelial cells. Additionally, stimulation of the urothelium *in*

vivo causes alterations in bladder reflexes. Could these effects be linked, with modulation of ATP release being the cause of the altered bladder reflexes? While a number of possibilities exist, we believe our data and that of other labs suggest a model that involves the indirect modulation of bladder afferent nerves through the release of transmitters, including ATP, from the urothelium.

ATP has been shown to be an important transmitter in the urothelial sensory pathway. For example, ATP can be released from the urothelium in response to physical stimuli such as osmotic stress [58, 59, 88] or chemical stimuli like capsaicin [13, 58, 60], bradykinin [63] or acetylcholine [77]. This ATP is thought to act on afferent nerves underlying the urothelium by acting on P2X receptors and modulating afferent excitability [73, 91]. This hypothesis is supported by a number of studies; for example, ATP applied intravesically can excite bladder reflexes [67] and the purinergic antagonist PPADS can block bladder hyperactivity caused by intravesical administration of ACh and other cholinergic agents [65]. Additionally, a recent study performed in an *in vitro* bladder-pelvic nerve preparation demonstrated that an application of ATP to bladder afferent nerves sensitized them to mechanical or electrical stimulation, lowering the threshold required to elicit an action potential [128]. Taken together, these data form a strong case for urothelially released ATP playing a role in modulating bladder afferent excitability and hence modulate bladder activity.

Our research clearly demonstrates that stimulation of cultured cells with nicotinic agents can modulate the release of ATP. Could this, then, be the mechanism for the modulation of bladder reflexes observed *in vivo* in the rat? The results we observed involving ATP release *in vitro* could suggest events that we have demonstrated *in vivo*. For example, α7 stimulation with choline results in inhibition of both ATP release in cultured cells and bladder reflexes in the

anesthetized rat. Additionally, cytisine stimulation at high concentrations results in both an increase in ATP release and an increase in the frequency of reflex bladder contractions in the anesthetized rat. These results support the hypothesis that urothelial nAChRs can modulate bladder activity through the release of ATP.

It should be noted, however, that we don't conclusively link the release of ATP from urothelial cells following stimulation with nicotinic agents with increased bladder activity or even increased afferent activity. To determine if the *in vivo* effects of nAChR stimulation are due to modulation of ATP release from the urothelium, a number of experiments could be performed. A direct experiment to test if the excitation caused by α3* stimulation during a cystometrogram is due to urothelially released ATP would be to concurrently apply a purinergic antagonist such as PPADS. We would expect PPADS to block the excitation caused by α3* stimulation. However, this result may be difficult to interpret, as the urothelium also expresses purinergic receptors [64] which may be the target for any effects observed following treatment with PPADS or other purinergic antagonists. Additionally, ATP is an important transmitter in the central nervous system [288, 289], therefore any actions of purinergic antagonists may be due to central effects, as well. It would, then, be beneficial to examine the effects of nicotinic receptor agents in afferent excitability in the bladder-nerve preparation, an *in vitro* experimental setup that removes the bladder and the pelvic nerve in order to record nerve impulses in response to stretch or chemical stimulation [128]. Using this model, it would be possible to record changes in afferent nerve activity in response to stretch with and without intravesical instillation of cytisine, to determine if activation of urothelial α3* receptors leads to an increase in afferent excitability. If our hypothesis is correct, we would expect that intravesical cytisine would increase afferent excitability and this effect would be blocked by the addition of PPADS or

another purinergic antagonist to the bath, where it could act by blocking purinergic receptors on afferent terminals. This model would eliminate the possibility of blocking purinergic signaling in the central nervous system, hence eliminating any non-specific effects.

5.1.1 A Hypothesized Role for Nicotinic Receptors in the Physiological Control of the Normal Bladder

While we have demonstrated a role for urothelial nAChRs in modulating bladder reflexes following stimulation with exogenous agents such as nicotine, our research indicates that urothelial nicotinic receptors play a physiological role in the control of micturition in the absence of any pharmacological intervention. For example, blocking α3* receptors using hexamethonium caused an inhibition of bladder reflexes, indicating the presence of an endogenous agonist that is activating the receptor. Acetylcholine is the endogenous ligand for the α3* receptor, and it has been shown that the urothelium can release ACh in response to stretch [16, 92]. It has been long hypothesized that this non-neuronal ACh could act in an autocrine/paracrine manner, stimulating cholinergic receptors on the urothelium to modulate a number of cellular functions. This kind of signaling has been shown in a number of non-neuronal tissues, such as bronchial epithelia [216, 244, 245, 290], vascular endothelial cells [221] and skin keratinocytes [205, 237]. Could urothelially released ACh act on urothelial nAChR to modulate ATP release to influence bladder physiology? If we consider our current research together with what is known about ACh release from the urothelium, as well as the pharmacological properties of the nAChRs as determined in other tissues, we can hypothesize a model of nicotinic signaling in the urothelium and how it plays a role in the normal operation of the bladder.

It is known that ACh is released from the mucosa in a stretch dependent manner; i.e. the more bladder strips are stretched, the more ACh is released [16, 92]. This suggests that a concentration gradient of ACh over time in the lumen of the bladder, when the bladder is empty, ACh levels would be low, however when the bladder is full and the urothelium is stretched, ACh levels would increase. We believe that this gradient in the concentration of ACh in the bladder over time is the basis for how nAChRs could play a role in the control of the bladder, i.e. by relaying information on the fullness of the bladder to underlying afferent nerves through controlling the release of ATP. In this manner, urothelial nAChR signaling would play a unique role in converting physical conditions in the bladder (i.e. stretch) into chemical signals that can modulate bladder afferent nerves; promoting storage when the bladder is empty and promoting voiding when the bladder is full.

In order to explain this hypothesis, let us first examine the effects of cytisine stimulation on ATP release and relate them to their perceived effects *in vivo*. Low concentrations of cytisine inhibit basal ATP release in cultured cells. Assuming that ACh acts on α3* receptors in a similar manner to cytisine, this would mean that urothelial production of ATP would be diminished when ACh levels were low. If ATP release is diminished then it would be less likely to sensitize afferent nerves, leading to higher thresholds of activation and promoting storage. We also demonstrated in our experiments that higher concentrations of cytisine elevated ATP release from cultured cells. ACh release from the urothelium increases as the bladder stretches, therefore we would expect concentrations of ACh in the bladder to rise as the bladder fills. Based on the effects of cytisine, we would expect that as ACh levels increase, urothelial cells would switch from inhibiting ATP release to promoting it. This ATP could then act on afferent

nerves to increase their excitability and increase sensations felt from the bladder (in a conscious patient) or activate bladder reflexes (in the anesthetized animal) (Figure 5.1B).

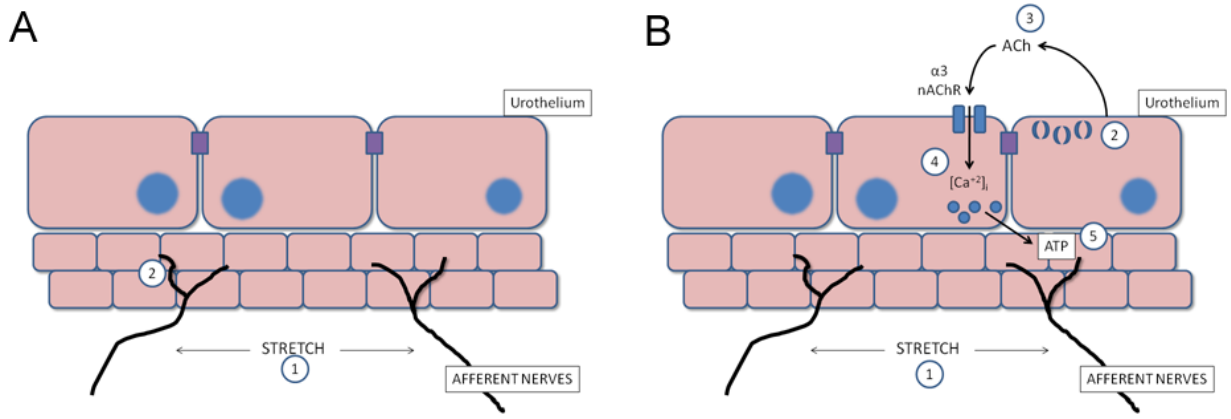

Figure 5.1 - Hypothetical Model of α3 Modulation of Bladder Reflexes

(A) Depiction of the classical hypothesis on stretch-activation of bladder afferent nerves. As the bladder fills, the bladder wall stretches (1), which activates stretch-sensitive receptors on afferent nerve terminals (2), causing increased afferent activity. **(B)** Hypothetical model depicting how we believe urothelial α3 nAChRs modulate this response. When the bladder is full and the bladder is stretched (1), discoidal vesicles in the umbrella cells of the urothelium fuse with the apical surface (2), releasing ACh (3). This ACh can then act in an autocrine/paracrine manner on urothelial α3 receptors, increasing intracellular calcium (4). Increased $[Ca^{+2}]_i$ leads to increased fusion of vesicles and hence increased release of ATP. ATP can then act on P2X3 receptors on underlying afferent nerves (5), increasing their excitability and decreasing the stimulus needed to activate them. This would lead to a decrease in the threshold volume needed to initiate a micturition contraction and therefore decrease the intercontraction interval in a cystometrogram.

How, then, does the α7 receptor fit into this model? Our research demonstrates that stimulation of the α7 receptor results in a decrease in ATP release. Because ATP excites bladder afferent nerves [6, 73, 128], the inhibition of release could decrease afferent excitability and hence inhibit bladder reflexes. As discussed in Chapter 3.3.2, it is unclear what the mechanism of this inhibition might be; it could involve the inhibition of the α3* receptor of be mediated through an, as yet, undiscovered pathway. We have demonstrated that stimulation of α7 receptors also releases calcium from ryanodine sensitive stores. Could this then be a mechanism

for the inhibition of ATP release? This seems unlikely, as ATP release from urothelial cells in response to stretch is inhibited if intracellular calcium is chelated using BAPTA-AM [58], suggesting an excitatory role for intracellular calcium in ATP release. Therefore, the possibility exists that the inhibition of ATP release we observe following α7 stimulation is independent of the changes in intracellular calcium also elicited by α7 stimulation, much in the same way it appears that the inhibition of α3* receptor signaling following α7 receptor stimulation appears to be calcium-independent. Despite the fact that more experimentation must be completed to fully understand how α7 receptors mediate their inhibitory effects on ATP release, this inhibition fits well with the observed effects during our *in vivo* rat experiments. Whether α7 receptors mediate their effect through direct inhibition of transmitter release, through phosphorylation and subsequent desensitization of excitatory α3* receptors or through some other, unknown mechanism, the result is inhibition of bladder reflexes. This inhibitory nature of the α7 receptor fits well with what is known about nAChR pharmacology and urothelial physiology.

In contrast to α3β4 receptors, α7 receptors are activated by lower concentrations of ACh (EC_{50}: ~20μM) and desensitized by higher concentrations [169, 173]. These properties allow for a steady state current in the continuous presence of ACh that increases with growing concentrations of ACh up to approximately 20μM and decreasing from there. These properties suggest that urothelial α7 receptors, then, would be active in the presence of low concentrations of ACh and inactivate when levels increase. This coincides with the inhibitory nature of the receptor, as when the bladder is empty and ACh levels are low, promotion of storage would be desirable. Therefore, the urothelium could have a two-fold mechanism in place to modulate bladder activity in the rat: when the bladder is empty and ACh levels are low, α7 receptors would be activated and α3* receptors desensitized, resulting in a prevention of ATP release (Figure

5.2). However, as the bladder fills and stretches, higher ACh levels would desensitize α7 receptors and activate α3* receptors, leading to the removal of inhibition and increased ATP release. As mentioned previously, this release of ATP would increase afferent excitability, promoting voiding.

In addition to acetylcholine, choline can also activate α7 receptors [169, 170]. While choline is much less efficacious on α7 than ACh (EC_{50}: ~2mM), it is much more selective for α7 receptors, exhibiting very little activity on other nicotinic receptors. Because urothelial cells express cholinesterase enzymes [291], urothelially released ACh could be metabolized into choline, which could also activate the α7 inhibitory pathway. With this is mind, we believe that when the bladder is empty and ACh concentrations are low, urothelially released ACh can be metabolized by acetylcholinesterase in the urothelium to produce choline, which would activate the α7 inhibitory pathway, decreasing ATP release. As the bladder stretches and ACh release increases, acetylcholinesterase could become saturated, allowing for a buildup of ACh and the eventual desensitization of α7 receptors and the activation of α3* receptors, leading to increased ATP release and excitation of afferent nerves. Therefore acetylcholinesterase may act as the enzymatic "switch" that would determine which nicotinic pathway would be activated, depending on the concentration of ACh in the lumen of the bladder.

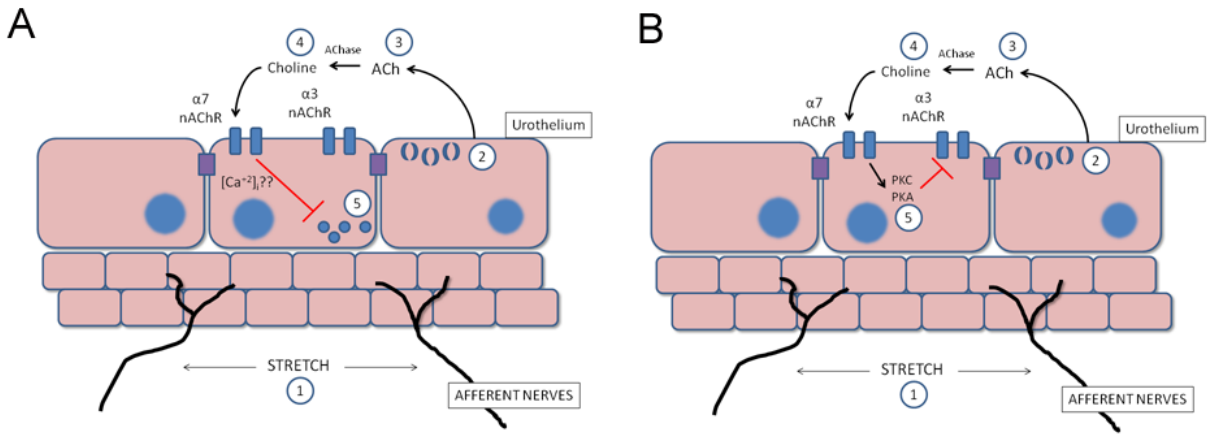

Figure 5.2 - Hypothetical Model of α7 Signaling in the Urothelium

(A) When the bladder is filling, the bladder stretches (1), vesicles fuse to the plasma membrane in the umbrella cells (2) and ACh is released (3). The urothelium expresses acetylcholinesterase (ACh), which degrades ACh into choline (4) a specific agonist for the α7 nAChR. Stimulation of α7 receptors causes an inhibition of ATP release, possibly through a mechanism involving release of intracellular calcium from ryanodine sensitive stores. Without the action of ATP on bladder afferent nerves, excitability would be decreased, resulting in inhibition of the bladder reflex. **(B)** Our research also suggests that the inhibitory effect of α7 receptors could be mediated through an inhibition of α3* receptors. This could happen through phosphorylation of the a3* receptor through the activation of protein kinases, such as PKA or PKC (5).

It is important to realize that this hypothesis proposes only that urothelial nAChRs can, through modulation of ATP release, influence bladder reflexes. In other words, urothelial nicotinic receptor stimulation is not required to initiate a bladder contraction. This is indicated by our experiments using hexamethonium; even though blocking the α3* receptor resulted in an inhibition of bladder reflexes, a contraction did eventually occur. We then hypothesize that the nicotinic signaling pathway represents a mechanism for modulating the micturition reflex mediated by stretch-sensitive afferents in the bladder wall. In this way, urothelial nAChR signaling could modulate excitability of the stretch-activated nerves controlling micturition, increasing or decreasing the threshold necessary to activate micturition depending on conditions in the lumen of the bladder. In other words, this type of signaling could transform physical

stimuli stretch of the urothelium) into a chemical signals that could coordinate cellular responses throughout the bladder lumen.

5.1.2 Future Directions

While the research presented in this dissertation significantly furthers what is known about nicotinic signaling in the urothelium, we should point out that there exist some discrepancies in the research presented that will require additional experimentation in order for our model to be confirmed. For example, while it is known that the urothelium releases ACh in a stretch-dependent manner, no study to date has measured the levels of ACh in the bladder of the rat *in vivo* during filling to determine the range of ACh or choline concentrations present in the lumen. Therefore, the question of whether ACh concentrations in the lumen of the bladder could ever reach physiological relevance remains. To determine the feasibility of our model, it would be necessary to measure the levels of ACh near the urothelium while the bladder is stretched in varying amounts. Classically, ACh release is measured in one of two ways: 1) by incubating cells in radiolabeled choline and examining the amount of radiolabeled ACh released following a stimulus, or 2) through the use of high-performance liquid chromatography. Neither of these techniques, however, is desirable to use *in vivo*, as they would measure the levels in the lumen as a whole and may not give an accurate account of what ACh or choline concentrations are close to the urothelium. Recent advances in technology, however, have allowed for the development of ACh-specific microsensors, capable of measuring ACh and choline levels in real time [292]. The development of these sensors may now allow sensitive readings of ACh and choline levels near the urothelium, which would allow us to determine the feasibility of our hypothetical model.

Additionally, while we can make assumptions on the pharmacological properties of nAChRs in urothelial cells based on properties exhibited by those receptors expressed in other tissues or heterologous expression systems, no study as yet as examined the properties of these receptors in urothelial cells. Attempts have been made to patch clamp urothelial cells, with some limited success [293], however to date no group has examined nAChR-mediated currents. Until such experiments are performed, it is impossible to conclusively state that properties such as agonist affinity, steady-state current, peak current, desensitization rate and recovery from desensitization rate in urothelial nAChRs is the same as they are in other tissues or heterologous expression systems. Differences in these properties between urothelial cells and the cells already studied may necessitate a revision of our hypothetical model.

While our research indicates that nicotinic receptor stimulation modulates both intracellular calcium and ATP release, it should be noted that we have not definitively linked the two phenomena together. For example, while choline evokes release of calcium from ryanodine sensitive stores and inhibits ATP release, we have not demonstrated that the inhibition of ATP release is due to the intracellular calcium transients. Additionally, we did not demonstrate that the increase in ATP release in response to higher concentrations of cytisine is dependent on the increase in intracellular calcium that cytisine also produces. In order to fully develop our hypothetical model, these links must be made experimentally. If these links exist, we would expect that stimulation of ryanodine receptors using low concentrations of ryanodine would result in a decrease in ATP release from cultured urothelial cells. We would also expect that removing extracellular calcium from our ATP experiments would block cytisine induced ATP release.

It should also be noted that while we have shown that α3*-mediated calcium transients can be modulated by kinases such as PKA and PKC, we have not shown that these kinases can alter α3*-mediated ATP release. This is due to the fact that kinase activation has been shown to increase vesicular transmitter release in nerve cells, which may be due to phosphorylation of SNAP-25, a member of the SNARE complex responsible for vesicle docking and fusion [294]. Activation of PKC or PKA with the non-specific agents we use in Chapter 3.2.3 also increases ATP release from urothelial cells (unpublished data), which would make any effect on cytisine-induced ATP release difficult to interpret. Therefore, in order to determine if α3*-mediated ATP release can be influenced by protein kinases, other techniques would have to be utilized. The urothelium expresses a number of PKC isoforms, and general stimulation would activate multiple isoforms which may have multiple effects on cellular signaling. To better elucidate which isoform could phosphorylate and inhibit the α3* receptor, we could either utilize more subtype specific pharmacological agents or genetic techniques to knockout specific isoforms of PKC.

Nicotinic receptors have also been shown to affect a number of cellular processes that could affect bladder function, but for which we have not tested. It is possible that one of these processes also plays a role in urothelial signaling and will have to be addressed before a comprehensive model of urothelial nAChR signaling can be formed. For example, nicotinic receptors have been shown to modulate gap junction function in a number of experimental models [295, 296]. Urothelial cells also express gap junctions, composed mainly of the protein connexin 26 [297, 298]. These gap junction proteins are upregulated in the neonatal rat as well as in the spinal cord- transected rat, where they appear to play a role in coordinating calcium signals in the urothelium [297]. This increased expression of gap junctions, both in the

urothelium and in the suburothelium, are thought to drive the spontaneous muscle activity observed in neonatal and spinal cord transected rats, as this activity disappears when 18β-glycyrrhetinic acid is applied to the preparation. Nicotinic agents have also been shown to modulate gap junction functionality; in adrenal chromaffin cells nicotinic antagonists increase the flow of dye between cells, indicating an opening of gap junctions [295]. This suggests that nAChR activation tonically inhibits gap junction function. Could the same mechanism be at work in the urothelium, and could nAChR modulation of gap junctions play a role in influencing smooth muscle activity? nAChR expression in a number of tissues are commonly altered during development [151, 182, 202, 228] as well as in adults following pathology [162, 299, 300]; therefore if nicotinic receptors can modulate gap junctions, nAChR plasticity in neonates and pathology may help explain the differences in spontaneous activity in bladders taken from those animals. Further studies would have to be completed to determine if nAChRs can alter gap junction communication in urothelium, however these studies would help elucidate the entire role of urothelial nicotinic receptor signaling.

While our combined experiments suggest the hypothetical model we have proposed, a number of inconsistencies between our in vivo and in vitro studies exist that must be addressed. Table 4 lists the effects each of our nicotinic agents had in our experiments. As denoted by the asterisks, there are some discrepancies between the effect of an agent *in vitro* and its effect *in vivo*. For example, MLA increases ATP release *in vitro*, however the same concentration (100μM) has no effect on bladder cystometrograms. Additionally, choline effectively inhibits bladder reflexes *in vivo* at concentrations as low as 1μM, while choline's effect on ATP release does not begin until a concentration of 100μM. Given our hypothetical model, we would expect changes in ATP release to mirror the effects seen *in vivo*.

It would be easy to attempt to explain these incongruities with a simple "cultured cells never behave completely the same as cells *in vivo"* comment. However, it should also be possible to hypothesize specifically what the cause of the differences between the two experimental models might be and design experiments to determine if these differences could be explained. For example, urothelial cells resident in the bladder *in vivo* are subjected to stretch during the course of the experiment (as the bladder fills and expands), whereas cultured cells are not. Stretch of urothelial cells results in a number of physiological changes, such as increases in vesicle trafficking (both endo- and exocytsis) [56, 57, 72, 266, 301, 302], increases in expression of urothelial proteins [53, 54] and release of a number of transmitters, such as ACh [16, 93]. All of these stretch-induced processes could influence bladder reflexes *in vivo* in ways that could lead to results inconsistent with our *in vitro* results. For example, it could be hypothesized that ACh, released from the urothelium in response to stretch, could act in synergy with the nicotinic agents we perfused during the cystometrogram, lowering the concentration needed to evoke a response. Since the urothelial cells used in our *in vitro* experiments aren't being stretched, a higher concentration of agent would be needed. Additionally, it is also possible that stretch could induce changes in nAChR expression in urothelial cells in the rat, which could alter the concentrations needed to affect a result.

If either of these scenarios is correct, simply subjecting cultured urothelial cells to stretch should alter nAChR signaling in our *in vitro* experiments to more closely match the results observed *in vivo.* Urothelial cells can be grown on stretchable supports, which would allow us to examine how nAChR signaling could be affected by stretch. Additionally, some researchers have used hypotonic stress as a model of stretch [58, 59, 93], based on the fact that urothelial cells swell when presented with hypotonic conditions. We would expect the addition of either

hypotonic or physical stretch of urothelial cells would lower the concentrations of agonist needed to elicit a response.

Table 4 - Summary of *in vivo* and *in vitro* Experiments

Agent	Concentration	Effect *in vivo*	Effect *in vitro*
Choline	1 μM	Increase ICI	Ca^{+2}: none * ATP: none *
	10 μM	Increase ICI	Ca^{+2}: increase (intracellular stores) ATP: none *
	100 μM	Increase ICI	Ca^{+2}: increase (intracellular stores) ATP: decrease
MLA	1 μM	No change in ICI	Ca^{+2}: none ATP: N/D
	10 μM	No change in ICI Blocks nicotine	Ca^{+2}: none ATP: N/D
	100 μM	No change in ICI	Ca^{+2}: none ATP: increase *
Cytisine	1 μM	Decrease ICI	Ca^{+2}: none * ATP: N/D
	10 μM	Decrease ICI	Ca^{+2}: increase (extracellular) ATP: decrease
	100 μM	Decrease ICI	Ca^{+2}: increase (extracellular) ATP: increase
C6/ TMPH	1 μM / -	Increase ICI (not significant)	Ca^{+2}: N/D ATP: N/D
	10 μM / 30 μM	Increase ICI	Ca^{+2}: none (blocks cytisine) * ATP: no effect *
	100 μM / 90 μM	Increase ICI	Ca^{+2}: N/D ATP: none (blocks cytisine)

Summary of the effects of nicotinic agents in our experiments. An * denotes a discrepancy between *in vivo* and *in vitro* effects.

5.1.3 Interactions Between nAChRs and Other Signaling Pathways: Possible Implications for Urothelial Signaling

In the future, we would also like to examine the interactions urothelial nAChRs may have with other signaling pathways in the urothelium. It is well known that nicotinic receptors can interact to modulate and be modulated by a number of different cellular pathways, some of which are also important players in bladder physiology. Interactions with some of these pathways could

have significant effects on nicotinic receptor-mediated signaling in the urothelium and allow us to better understand how nicotinic receptors fit into the whole of urothelial physiology.

For example, nicotinic receptors are not the only receptors present in the urothelium that respond to ACh. It has been well established that the urothelium also expresses muscarinic receptors, metabotropic receptors that are also activated by acetylcholine [14, 15, 31, 32, 65, 74, 77, 123, 124, 218, 219]. Muscarinic receptors in the urothelium were first deemed important to bladder physiology when muscarinic antagonists began to be studied as a possible treatment of overactive bladder syndrome [7, 32, 74, 123, 124]. The rationale behind anti-muscarinics for the treatment of OAB was simple; if bladder overactivity was being caused by increased parasympathetic activation of bladder smooth muscle, then anti-muscarinics should be effective in blocking this overstimulation. However, it became clear that anti-muscarinics were mediating their effect during the filling phase of micturition when parasympathetic activity is virtually non-existent. This suggested a second site of muscarinic modulation of the bladder. Attention was turned to the urothelium when research by Chapple and Chess-Williams discovered that muscarinic stimulation of the urothelium led to the release of a diffusible factor that inhibited bladder smooth muscle contractions [14, 15]. As a result of these experiments, and the efficacy of anti-muscarinics in the treatment of OAB, many researchers have begun to study muscarinic signaling in the urothelium.

A number of recent studies have examined the role of muscarinic receptors in urothelial signaling. For example, RT-PCR has indicated that the urothelium of the rat, mouse and human express all five muscarinic receptors (M1-M5) [218, 219]. Stimulation of these receptors, using Oxotremorine-M (Oxo-M) results in increases in intracellular calcium, release of ATP, and alteration of bladder reflexes [65, 77]. Much in the same manner as nicotinic receptors, it

appears as though different receptor subtypes mediate different effects; for example, low concentrations of Oxo-M have an inhibitory effect on bladder reflexes in the anesthetized rat, while large concentrations have an excitatory effect [65]. Additionally, it appears as though each muscarinic receptor subtype influences intracellular calcium signals uniquely, as some subtype specific antagonists block increases in intracellular calcium following stimulation with a non-specific muscarinic agonist, while others potentiate the increases [77]. These data, interestingly, are very similar to those we have reported above with the nicotinic receptors in that they demonstrate opposing physiological effects depending on which receptor is stimulated.

While these data involving muscarinic signaling in the urothelium have garnered a lot of attention in the bladder research community due to the use of anti-muscarinics in the treatment of bladder hyperactivity, our current research may open up a new area of interest for clinical researchers. Given that both muscarinic and nicotinic receptors modulate intracellular calcium homeostasis as well as ATP release, the potential for the two related classes of receptors to interact is great. Indeed, muscarinic and nicotinic receptors are present together in a number of tissues, including neurons in the brain [277] and the spinal cord [132], as well as in the periphery, such as the enteric nervous system [303]. Both types of receptor are also present in a number of inflammatory cell types, such as neutrophils, mast cells, and macrophages [304]. In many of these tissues, it has been shown that interactions between muscarinic and nicotinic receptors exist, sometimes leading to an increase in cholinergic signaling in the target tissue and sometimes inhibiting it. For example, in the human brain, simultaneous antagonism of muscarinic and nicotinic receptors impaired cognitive abilities to a greater extent than antagonism of either class alone [277]. This research suggests that the two types of cholinergic receptor act in concert to affect selective cognitive processes, such as working memory and

visual attention. However, in the enteric nervous system, it has been demonstrated that muscarinic receptor stimulation can result in an increase in the rate and length of nicotinic receptor desensitization [303]. In enteric neurons, M1 receptor stimulation leads to the activation of phospholipase C, and hence the production of IP_3 and DAG. DAG can activate PKC, which in turn acts on nAChRs to phosphorylate and desensitize them. As we have demonstrated in our research, α3* receptors can also be inhibited through actions by kinases such as PKC. Could this suggest that α3* receptors in the urothelium may also be modulated through M1 stimulation and subsequent PKC activation? Since M1 receptors also increase intracellular calcium though IP_3-sensitve stores, could M1 receptors also play a role in modulating the intracellular calcium changes produced by nicotinic receptor stimulation? Could the other muscarinic receptors modulate nicotinic receptors in different ways? For example, M2 receptors have been known to inhibit adenylate cyclase production, decreasing levels of cAMP [31]. Could this lead to a downregulation in the activity of PKA in the urothelium and increase α3* signals, as our research suggests might occur when PKA activity is blocked? The answers to these questions will take further study, however they will lead to a greater understanding of cholinergic signaling in the urothelium and perhaps elucidate more effective treatments for bladder pathologies than the current treatment of anti-muscarinics alone.

In addition to muscarinic receptors, nicotinic receptors have also been shown to interact with other receptor pathways that are important players in urothelial physiology. The first is TRPV1, the capsaicin- and proton-sensitive ion channel found in both urothelial cells and bladder afferent nerves [13, 60, 105, 305, 306]. As we discussed in Chapter 1.2.2.3, TRPV1 has an important sensory role in the urothelium, as TRPV1 KO mice exhibit decreases in stretch-evoked ATP release from the urothelium [60]. TRPV1 is also expressed in C-fiber nerves

innervating the bladder, and is thought to play a role in the propagation of urgency or painful sensations felt during bladder pathology [105]. This hypothesis led to the development of capsaicin or resiniferatoxin (RTX), two specific TRPV1 agonists, as treatments for overactive bladder disorders, as discussed later in Section 5.2 [62]. It is thought that this treatment is due to actions of capsaicin and RTX on afferent nerves to desensitize them to noxious stimuli. Nicotinic receptors also reside on C-fiber neurons and activation of these receptors with agonists can also block nociception [194, 199, 307]. One hypothesis on how this might occur is through modulation of TRPV1 channels by nAChRs. Studies have demonstrated that TRPV1 mediated currents exhibit both acute desensitization and tachyphylaxis in response to α7 nAChR stimulation [175]. It is thought that this decrease in sensitivity of TRPV1 channels to further stimuli is mediated through nAChR-mediated Ca^{+2} influx, possibly through a second messenger, such as PKC. The urothelium also expresses nAChR and TRPV1 channels, therefore a question presents itself: Can nAChRs in the urothelium modulate TRPV1 channels? Since TRPV1 channels seem to play an important role in ATP release due to physical stimulation, such as stretch, could the inhibition of ATP release observed in our experiments be due to nAChR-mediated inhibition of TRPV1? Given the role of TRPV1 in urothelial signaling, these questions will be important to answer.

Another potential interaction with nicotinic receptors in the urothelium could be the ionotropic nucleotide receptors (P2X receptors). P2X receptors are activated by ATP and are commonly found co-localized with nicotinic receptors in the spinal cord and brain [308]. In these neurons, nicotinic and purinergic receptors cross-modulate each other in an inhibitory fashion. For example, currents elicited by co-stimulation of sympathetic neurons with ATP and ACh were smaller than currents elicited by either stimulus alone [309-311]. The currents elicited

by co-stimulation were also larger when an antagonist for either receptor was included, indicating that each receptor has an inhibitory effect on the other. Because P2X receptors have been shown to be important in the transmission of nociception [73], these interactions may represent a mechanism for the anti-nociceptive properties of nicotinic agonists such as epibatidine [194, 199]. The urothelium of the rat, cat and human also expresses a number of P2X receptors (e.g. $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ in the cat [79]). Therefore, the possibility of cross-modulation between nicotinic and purinergic receptors in the urothelium playing a significant role in bladder signaling exists. One hypothesis into a possible role for cross-modulation in the urothelium could be as a feedback mechanism to inhibit ATP release. We have demonstrated that nicotinic receptor stimulation can release ATP, therefore it is plausible to hypothesize that, if P2X receptors in the urothelium do inhibit nicotinic receptors, that this could represent a mechanism to control extracellular ATP release.

Given these possible interactions, it is clear that nicotinic receptor signaling in the urothelium has the potential to become much more complicated than the model we have outlined above. Further studies must be performed in order to fully elucidate the role of nicotinic receptors in the sensor/transducer properties of the urothelium.

5.2 CLINICAL POSSIBILITIES FOR UROTHELIAL NICOTINIC RECPTORS

The possibility that nicotinic receptors can influence afferent excitability through the release of ATP has important clinical implications. A number of bladder disorders exist, such as overactive bladder or interstitial cystitis, which present symptoms of urgency or even bladder pain [1, 8, 12,

124]. It is thought that these feelings are driven by increased afferent activity, however it is not yet known how the increased activity in generated. While we have not examined urothelial nicotinic receptors in the human, if we make the assumption that they behave in a similar fashion to the rat, we can make a few reasoned hypotheses on the clinical applications of nicotinic agents as related to bladder pathologies.

One possibility of how afferent activity is increased in pathological bladder conditions is the overproduction of excitatory transmitters that could lower the threshold required to activate afferents. For example, cats that suffer from a similar disorder to interstitial cystitis in humans (known as feline interstitial cystitis or FIC) exhibit increased ATP release from the urothelium in response to physical stimuli such as stretch [58]. ATP is an important transmitter in nociception [73], therefore it is possible that bladder pathology in humans is driven by increased release of ATP from the urothelium, increasing afferent activity and driving the urge to urinate. Another possibility could be plasticity in the purinergic receptors present on afferent nerves that bind urothelially-released ATP. Increased expression of P2X receptors on bladder afferents, or increased sensitivity of these receptors to ATP could also result in increased afferent activity. This hypothesis is supported by research that indicates increased $P2X_3$ immunostaining on afferent nerves in patients suffering from overactive bladder [54, 55]. Additionally, DRG cells taken from rats with chemically induced cystitis exhibit increased purinergic signaling [122].

Given the apparent importance of purinergic signaling in bladder pathology, researchers have focused on this system in the search for potential pharmacological targets to treat bladder disorders. The research presented here adds another potential set of targets to modulate purinergic signaling in the bladder. Our data suggests that stimulation of the urothelium with α7 agonists inhibits the release of ATP from urothelial cells and inhibits bladder reflexes. Therefore

pharmacological agents that could selectively activate urothelial α7 receptors may have beneficial effects against the urgency exhibited by patients with OAB or IC.

In addition to α7 agonists, α3* antagonists may also be beneficial to treat bladder pathology. While TMPH failed to decrease ATP release in cultured cells by itself, hexamethonium successfully inhibited bladder reflexes *in vivo*. While these two data may at first seem inconsistent, as we discussed in the previous section, the cells in the *in vitro* preparation are not subject to stretch as is the urothelium *in vivo.* Stretch of the urothelium causes ACh release, therefore while the α3* antagonist has no effect on ATP release in unstretched cultured cells, it may prevent activation of the receptor from stretch-induced ACh release, resulting in inhibition of the ATP release exhibited by α3* receptors. This could explain the inhibitory effect hexamethonium has *in vivo.*

While our research suggests that modulation of urothelial nicotinic receptors may benefit patients with bladder pathologies, a number of difficulties exist that may complicate their practical use as clinical treatments. The most important of these would be the lack of a drug that could specifically act on an urothelial receptor. For example, one would expect an orally administered drug to be biologically available to activate nicotinic receptors in a number of locations throughout the body. This could be especially true for nicotinic agents currently in clinical development, mostly for treatment of psychological disorders such as schizophrenia [161] or Alzheimer's Disease [163], since these agents are designed to cross the blood-brain barrier in order to reach their targets. Given the prevalence of nicotinic receptors throughout the body and the wide range of effects they mediate, the possibility for non-specific effects becomes significant. According to earlier research, some of the non-specific effects may be desirable. For example, stimulation of α3 receptors in the brain and the spinal cord excites bladder reflexes

[133]. Therefore, α3 antagonists acting centrally should also have inhibitory effects on bladder reflexes. However, α3 receptors are prevalent throughout autonomic ganglia [130, 131, 167, 181, 312], therefore inhibition of these receptors may have significant effects on other autonomic functions, such as the digestive or respiratory systems.

Activation of α7 receptors could have similar beneficial effects towards bladder pathologies, but also result in undesirable side effects. α7 receptor agonists are beginning to be classified as effective analgesics, presumably through actions on the central terminals of afferent nerves, preventing transmitter release in the spinal cord and preventing propagation of the nociceptive signal [280, 313]. It could be hypothesized that these central actions of α7 agonists could benefit patients suffering from overactive bladder disorders, as they could block the propagation of nociceptive signals from bladder afferent nerves into the spinal cord. Similar to α3 receptors though, α7 receptors also mediate a number of physiological effects throughout the body which could be influenced by a systemic dose. For example, α7 agonists are also being studied as potent anti-inflammatory agents, as macrophages express α7 receptors and respond to nicotinic stimulation by down-regulating the release of inflammatory cytokines and chemokines [304, 314-316]. While these anti-inflammatory properties of nicotinic agonists may be desirable as an acute treatment to block sepsis [208], for example, chronic treatment may lead to the undesirable side effect of increased susceptibility to infection.

Keeping these possible side effects in mind, it becomes apparent that treatment of hyperactive bladder disorders through the use of systemic nicotinic agents may not be the most desirable choice clinically. Given that the nature of the bladder is to contain harmful waste apart from the rest of the body, an intriguing way to get around non-specific actions would be to target the drug specifically to the bladder. The easiest way to accomplish this would be to inject agents

directly into the bladder. This is already being done with certain treatments for bladder disorders, such as capsaicin, resiniferatoxin (RTX) and botulinum toxin type A (BOTOX-A) in a clinical setting. Capsaicin and RTX, instilled intravesically, act by desensitizing the TRPV1 receptor on C-fiber bladder afferent nerves, effectively destroying the nociceptors driving bladder pathology while sparing the A-δ fibers that drive normal bladder activity [62]. BOTOX-A treatment differs in that it is injected directly into the detrusor muscle, where it is thought to act on efferent nerves, preventing the release of ACh and ATP that drives urinary bladder contractions [90, 317]. BOTOX-A may also have effects on afferent nerve terminals, preventing the release of substance P or glutamate, reducing their effects on the sensory pathway. Application of these agents directly to the bladder allows them to act specifically on bladder nerves, without any non-specific actions on receptors outside of the bladder. These agents have long-lasting effects due to their actions on nerves, where stimulation of axonal terminals can result in a desensitization that can last up to 6 months, depending on the dose used.

While intravesical treatments with nicotinic agents could also avoid actions on receptors outside of the bladder, it is doubtful that they could have the same long lasting actions on micturition. Capsaicin and RTX are thought to act through numerous pathways, including phosphorylation of TRPV1 receptors, inhibition of voltage gated sodium channels through the activation of second messenger systems and depolarization of mitochondria, resulting in long term disruption of the nerve's ability to produce an action potential [62]. Our research indicates, that while it is possible that urothelial nicotinic receptors also cause phosphorylation through the activation of PKA or PKC, these effects do not seem to last very long. In our ATP experiments, stimulation of urothelial cells with either choline or cytisine resulted in a sustainable modulation of ATP release, however ATP levels rapidly return to normal following washout of the agent.

This is mirrored *in vivo,* as intravesical administration of nicotine causes inhibition of bladder reflexes that can be rapidly washed out. Given this data, it seems as though any nicotinic agent used intravesically to treat bladder disorders would have to be continuously infused, a limitation that would seriously reduce its desirability as a treatment for bladder pathologies. Therefore, it appears as though another route of administration would have to be taken for urothelial nicotinic receptors to become a viable pharmacological target in the treatment of bladder disorders.

Another possibility to specifically target urothelial nicotinic receptors could be to design drugs that would be active predominately in the bladder and inactive in other parts of the body. This might be accomplished by designing a drug as an inactive, precursor form that would become active following metabolism in the liver. This utilization of the "first-pass effect" would maximize the percentage of the drug being filtered by the kidneys and minimize the percentage in the blood stream, where it could have non-specific effects.

It may also be possible to take advantage of urine's lower pH in order to develop an urothelium-specific agent. Urine has a pH of between 4.5 and 8, as compared to the pH of blood plasma, which is tightly controlled at 7.4. Urine pH can be influenced by a number of factors, such as diet, bladder infections or other pathologies such as diabetes or respiratory problems. The pH of urine can be modified through changing one's diet or through the use of drugs, such as potassium citrate, which increases the alkalinity of urine [318], or chlorothiazide diuretics, which can increase the acidity of urine [319]. This manipulation of urine pH is an important factor in the treatment of urinary tract infections, as antibiotics such as kanamycin or neomycin exhibit increased activity when the pH of urine is alkaline. Therefore, it may be possible to design a drug that would be relatively inert in the blood stream at pH 7.4, but activate in either an

alkaline or acidic environment in the urine. This would then potentially allow the drug to only act on urothelial receptors, instead of other nicotinic receptors located around the body.

While these two hypothetical means of specifically targeting urothelial receptors may give us a glimpse of what may be in the future of pathological bladder treatments, they will require years of research and trial before becoming reality. The development of a drug precursor that could be activated by modification by metabolic enzymes is an enormous task, given that the liver contains a number of enzymes that could modify any drug precursor in unpredictable, and possibly undesirable ways. Could there, then, be a way of activating urothelial nicotinic receptors that minimizes the risk of activating non-urothelial receptors already in our pharmacological arsenal? A promising possibility lies with a new class of compounds termed α7 nAChR positive allosteric modulators (PAMs). These compounds do not act as agonists on the α7 receptor, but rather change the receptors conformation upon binding, increasing their affinity for agonists [320]. These compounds include PNU-120596, genistein, 5-hydroxyindole (5-HI) and 4-napthalene-1-yl-3*a*,4,5,9*b*-tetrahydro-3-*H*-cyclopenta[*c*]quinoline-8-sulfonic acid amide (TQS) (see Figure 5.3). These compounds can left-shift the concentration response curve to a number of nicotinic agonists such as ACh or PNU-282987, as well as reactivate desensitized receptors. However, none of the reported α7 PAMs evoked a current by themselves and none exhibited any type of action (either as an agonist or a PAM) or other nicotinic receptors such as α3β4 [320]. This, then would make α7 PAMs the ideal compounds to increase α7 signaling in response to endogenous stimulation by ACh, but limit side effects due to non-specific activation of α7 receptors elsewhere in the body. Because ACh is released from the urothelium during the filling phase, we would expect that an α7 PAM would increase the activity of urothelial α7 receptors, leading to increased inhibition of bladder reflexes (in the anesthetized rat) or decreased

sensations of urgency (in the human). However, as we mention in Chapter 3, it is possible that the effects we observe following α7 stimulation may actually due to the receptor in its desensitized form, not due to activation of the receptor. If this is the case, then we would expect PMAs to have no beneficial effect on bladder reflexes, since they stabilize α7 receptors in their open state, not the desensitized state.

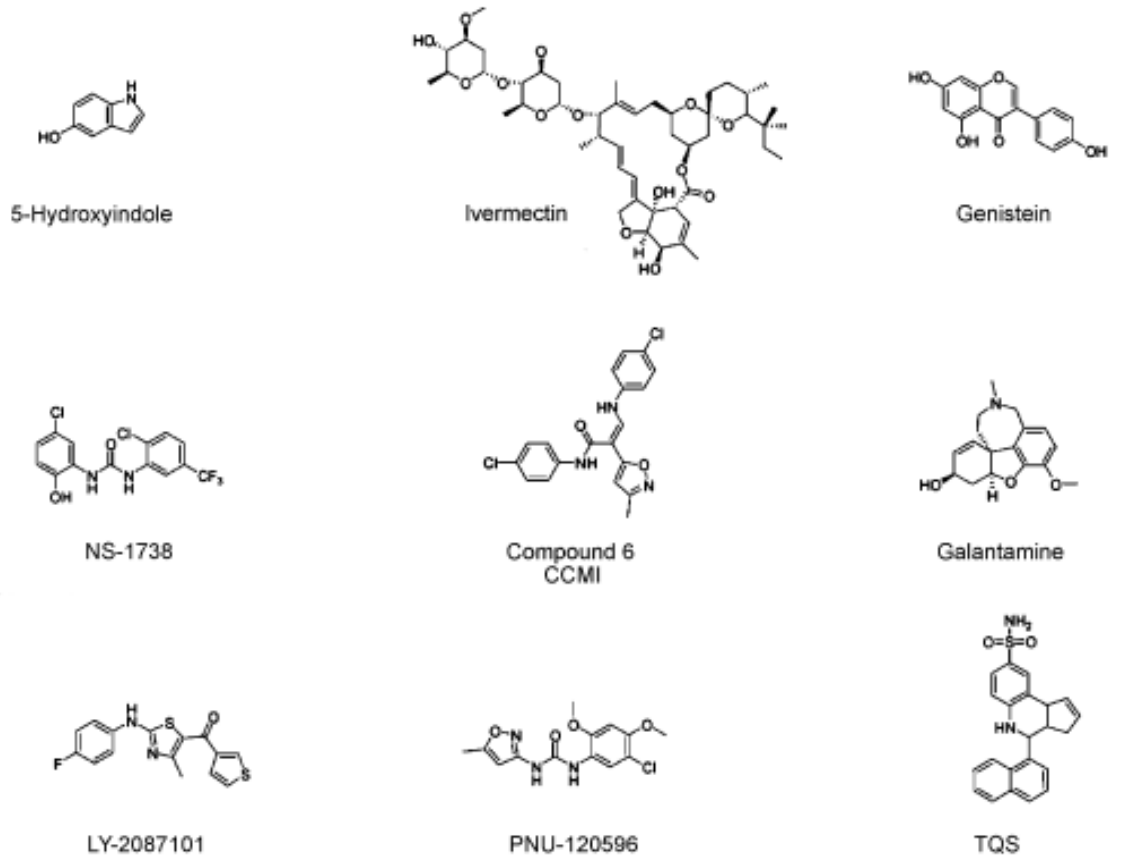

Figure 5.3 - Positive Allosteric Modulators of α7 nAChRs

Structures of some current positive allosteric modulators of the α7 nAChR.

5.3 FINAL THOUGHTS

In conclusion, we believe that our current research, presented in this dissertation, indicates an important role in the sensor/transducer role of urothelial signaling. The urothelium expresses two main classes of nicotinic receptor which, when stimulated, have opposing effects on bladder reflexes in the rat. These effects may be mediated through a modulation of extracellular ATP release, as our *in vitro* experiments with cultured urothelial cells suggest. These data reveals another possible mechanism concerning how the urothelium can sense and transmit information about conditions in the bladder to underlying afferent nerves, influencing bladder function. This mechanism is important clinically, as modulation of urothelial nicotinic receptors can influence bladder reflexes and may represent a novel target in the treatment of bladder hyperactivity as a result of pathology.

APPENDIX A

MATERIALS AND METHODS

A.1.1 Animals

All experiments used female Sprague-Dawley (SD) rats (250-300g) that were fed a standard diet with free access to water prior to experimentation. Tissues were harvested from rats that were euthanized by inhalation of 100% CO_2. All studies were carried out with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee and maintained according to the standards set forth in the American Physiological Society's handbook on the care and use of laboratory animals.

A.1.2 Rat Urothelial Cell Culture

Rat urinary bladders were excised and stored in oxygenated Krebs solution. The bladder was cut open, gently stretched with the epithelial side up and pinned in a sylgard coated dish. The bladder was incubated overnight in minimal essential medium, penicillin/streptomycin/fungizone and 2.5 mg/ml dispase (all from Invitrogen). The epithelium was then gently scraped from the underlying tissue using a spatula, placed in a culture flask and treated with enzyme (0.25%

trypsin) for 10 minutes with periodic tituration to dissociate urothelial cells. Following dissociation, the cell suspension was placed in MEM containing 10% FBS to inhibit trypsin and centrifuged at 416xg for 15 minutes. The pellet was then re-suspended in keratinocyte medium (Invitrogen) and centrifuged again. This pellet was again suspended in keratinocyte media (~1ml) and 0.1 ml of the cell suspension (50,000-250,000 cells/ml) was added to the surface of collagen-coated plates. Cells were used within the first 1-2 days following plating.

A.1.3 RNA Extraction and RT-PCR

mRNA for RT-PCR experiments was obtained from both cultured urothelial cells and whole urothelial tissue. To collect whole urothelial tissue, bladders were obtained from three female SD rats, cut open and pinned flat on Sylgard dishes. The urothelium was then gently teased away from the underlying tissue using fine forceps and scissors and placed in RNase free buffer. The RNeasy RNA Extraction Kit from Qiagen was used to extract RNA following the published protocol for tissue samples or cultured cells. The RNA was resuspended in RNAse free water and tested for quality, purity and concentration by UV spectrophotometry and gel electrophoresis (1.2% formaldehyde agarose gel). First-strand cDNA synthesis was performed using the Roche Molecular Biochemicals First-Strand cDNA Synthesis Kit for RT-PCR, using an oligo-dT15 primer. PCR was performed using Qiagen's *Taq* PCR Core Kit, using the optional Q-solution, sequence-specific primers designed for each subunit, and annealing temperatures determined by the sequence of the primers. Primers for PCR are shown in Table 5. Rat primers were designed in-house using online primer design software Primer3 (http://primer3.sourceforge.net); cat primers were taken from Bairam, et. al. [228] and human primers were taken from Maus, et. al. [227].

Table 5 - Primer Sets for RT-PCR of nAChRs

Subunit Species	Primer Pair (5'→3')	T_m (°C)	Expected Product Size (bp)
α2 Rat	(L) CGAGTCGGGGAGTATGGTAA (R) AGGAAGTGGCTTCTCAGTCG	60	300
α3 Rat	(L) GTGAATTCAGCCGTGCAGACTCCA (R) ATAAGCTTGGCAACGTACTTCCAATCATC	60	271
α4 Rat	(L) GTGAATTCCACAGGTCGTACACGGGTCG (R) ATAAGCTTGCGAGCCCGGCATCTTGAGT	62	257
α5 Rat	(L) AGTGGGCTGGACCTAAATCTCG (R) CAAAAAGCCCTAAAGTCCCAATGA	61	286
α7 Rat	(L) GTGAATTCAAGAGGCCCGGAGAGGACAA (R) ATAAGCTTCGCCACATACGACCCCAGAG	67	190
β2 Rat	(L) GTGAATTCAGGGCGAGGCGGTTTTCTT (R) ATAAGCTTGCGTACGCCATCCACTGCT	65	180
β3 Rat	(L) GTGAATTCTGGGTGAAGAGGCTGTT (R) ATAAGCTTATCGCTGGCGGGAGTCTGTT	63	157
β4 Rat	(L) GTGAATTCCATGGCATCCTGGGTCAAG (R) ATAAGCTTCTGGGGAGGCCTGCTGTGT	65	258
α1 Human	(L) CGTCTGGTGGCAAAGCT (R) CCGCTCTCCATGAAGTT	55	580/505
α2 Human	(L) CCGGTGGCTTCTGATGA (R) CAGATCATTCCAGCTAGG	55	466
α3 Human	(L) CTGGTGAAGGRGGATGAAGT (R) CTCGCAGCAGTTGTACTTGA	58	464
α4 Human	(L) GGATGAGAAGAACCAGATGA (R) CTCGTACTTCCTGGTGTTGT	58	444
α5 Human	(L) GATAATGCAGATGGACGT (R) TGATGGTATGATCTCTTC	55	525
α6 Human	(L) GTGGCCTCTGGACAAGACAA (R) CCTGCAGTTCCAAATACACA	58	372
α7 Human	(L) GAGGCAGATATCAGTGGCTA (R) GATGATGGTGAAGACCGAGA	55	893
β2 Human	(L) CAGCTCATCAGTGTGCA (R) GTGCGGTCGTAGGTCCA	55	347
β3 Human	(L) TGGAGAGTACCTGCTGTTCA (R) CGAGCCTGTTACTGACACTA	58	439
β4 Human	(L) CTGAAACAGGAATGGACT (R) CCATGTCTATCTCCGTGT	55	310
α3 Cat	(L) ATCATCCCCTGCCTGGTCA (R) GAGGTGGAAGGGATTGTCTCG	66	149
α4 Cat	(L) GTCCACTTCGGGCTGTCCAT (R) GCTTGTAGTCGTGCCATTCCTG	66	103
α7 Cat	(L) CAGCCGCTCACCGTCTACTT (R) CCATCTGGGAAACGAACAGTCT	66	170
β2 Cat	(L) AGCACTTCCCCTTCGACCA (R) TCCACGAGCGGAACTTCATG	66	51

To amplify PKC isoforms in Chapter 3, the following primers were designed in-house using online primer design software Primer3 (http://primer3.sourceforge.net):

Table 6 - Primers for PKC Isoforms

Primer	Sequence (5'-3')	T_m (˚C)	Expected Product Size
α	(L) TGAACCCTCAGTGGAATGAGT (R) GGCTGCTTCCTGTCTTCTGAA	59	325
β	(L) TGTGATGGAGTGTGTGAACGGGGG (R) TCGAAGTTGGAGGTGTCTCGCTTG	59	639
γ	(L) TTGATGGGGAAGATGAGGAGG (R) GAAATCAGCTTGGTCGATGCTG	59	347
δ	(L) CACCATCTTCCAGAAAGAACG (R) CTTGCCATAGGTCCCGTTGTTG	59	352
ε	(L) CGAGGACGACTTGTTTGAATCC (R) CAGTTTCTCAGGGCATCAGGTC	59	389
λ	(L) GCTTATGTTTGAGATGATGGCGG (R) TGACAACCCAATCGTTCCTTTG	59	201
ζ	(L) CGATGGGGTGGATGGGATCAAAA (R) GTATTCATGTCAGGGTTGTCTG	59	681
β-actin	(L) ATGGTGGGTATGGGTCAGAA (R) GCTGTGGTGGTGAAGCTGTA	59	482

PCR was performed using the GeneAmp 9700 thermocycler from Applied Biosystems. PCR products were run on a 1.2% agarose gel using ethidium bromide to visualize bands. Results were analyzed using the Eagle Eye II digital imaging system (Stratagene). Positive results were sequenced using ABI PRISM 3100 Automated Genetic Analyzer in the University of Pittsburgh DNA Sequencing Core Facility.

Quantitative RT-PCR was also performed to determine the relative expression of nAChR subunit message in the rat. Following first-strand synthesis, as described above, the iQ SYBR Green Supermix kit (Bio-Rad, Inc.) was utilized to perform qPCR. For each reaction, 12.5µl of the Supermix were added to 0.5µl of cDNA and 0.25µl of the subunit-specific primers listed above (at a final concentration of 100nM each) and 11.25µl of sterile water. Each reaction was

set-up in triplicate, plated in a 96-well plate and run in a MyiQ Quantitative thermocycler (Bio-Rad, Inc.), using the following protocol: 95.0ºC for 3 minutes, 35 cycles of 95.0ºC for 15 seconds and the annealing temperature listed in Table 5 for 45 seconds. Quantitative readings were taken each cycle during the annealing temperature hold. Following completion of amplification cycles, a melting temperature curve was performed to determine specificity of primer annealing. This was accomplished by first holding the reactions at 95.0ºC for 1 minute to denature the reaction products, then rapidly cool the reaction to 55ºC to force annealing. The temperature of the reaction was then raised by 0.5ºC every 10 seconds and fluorescence readings taken. Because the iQ SYBR only fluoresces when combined with double-stranded DNA, the signal drops significantly when the temperature reaches a significant level to break apart the two strands of the product. Primers are assumed to be amplifying the proper product if only one drop in fluorescence is observed; if two or more drops are present then some non-specific amplification must have taken place. To double check the specificity of our primers, even following a favorable annealing temperature curve, each reaction product was also sent for DNA sequencing as described above and matched against a BLAST search for the subunit in question.

A.1.4 Immunohistochemistry:

Bladders were removed from female Sprague Dawley rats (200-250g), cut open longitudinally and snap frozen in OCT compound using liquid nitrogen. These bladders were then sectioned at 6μm, placed on slides and air dried. Slides were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. Following washes in PBS, the slides were incubated in 0.3M glycine for 20 minutes to reduce auto-fluorescence. The slides were then permeabilized in 0.1% Triton X-100 and then blocked for 30 minutes. The blocking solution

contained 0.1% Triton X-100, 0.5% bovine serum albumin and 10% normal donkey serum. The slides were then incubated with Alexa-488 α-bungarotoxin (for α7 receptors, 1μM) or goat anti-α3 polyclonal antibody (1:100 dilution) for 2 hours at 25˚C. After primary incubation, α3 slides were washed with PBS and incubated with a donkey anti-goat-FITC secondary antibody (1:250 in PBS) for 1 hour. For co-localization studies, slides were also incubated with mouse monoclonal antibodies against either cytokeratin -17 or -20 (1:50 dilution each) followed by a donkey anti-mouse-Cy3 (1:250) secondary antibody. All slides were also incubated with DAPI (1:5000 in PBS for 5 minutes) to provide a nuclear counterstain. Following final washes with PBS, the slides were mounted and viewed. Results shown are representative of experiments performed from bladders of three separate animals. Antibodies for the cytokeratins were obtained from Dako; the anti-α3 polyclonal antibody, Alexa labeled α-bungarotoxin and all secondary antibodies were obtained through Invitrogen.

A.1.5 ATP Release:

Urothelial cells were cultured as described above, plated on 35mm plastic culture dishes and allowed to grow for 24-48 hours until they became approximately 30-50% confluent. These dishes were then perfused with HBSS using a peristaltic pump at 0.6ml/min for 10 minutes to completely wash off the culture media. Following wash, the cells continued to be perfused with HBSS and 100μl samples of the perfusate was taken every 30 seconds for 5 minutes from the dish using a pipetteman. These samples were read in a luminometer (Turner Designs, TD 20/20 Sunnyvale, CA) using the ATP luminescence kit (Sigma Aldrich, St. Louis, MO) to establish the basal levels of ATP release. For all samples, the luminometer was set at a sensitivity of 49.2% and an integration time of 10 seconds. The cells were then stimulated by switching the perfusate

to the appropriate nicotinic agent and samples taken every 30 seconds for 10 minutes. For antagonist studies, the appropriate antagonist was perfused over the cells for 10 minutes prior to as well as during agonist stimulation. Results reported were obtained using cells cultured separately from three rats.

A.1.6 Calcium Imaging (Fura-2):

Urothelial cells grown on collagen coated coverslips for 24-48 hours were washed with Hanks Balanced Saline Solution (HBSS) then loaded with Fura-2 AM dye (1μM dye, 30 min incubation at 37˚C). Following loading the coverslips were placed into a flow chamber specifically designed to fit the microscope stage (Olympus IX70 inverted microscope). Cells were maintained in HBSS throughout the experiment by use of a gravity-fed perfusion system with a flow rate of approximately 2.4ml/min. To record changes in $[Ca^{+2}]_i$ the cells were alternatively illuminated at 340 and 380nm by using a xenon lamp and imaged at 510nm with a Dage-MTI cooled CCD camera with 640x480 pixel resolution. A Dage-MTI Gen. II system image intensifier and software package from Compix Inc. (Cranberry, PA, USA) was used to collect data. The cells were then stimulated by switching the perfusate to the appropriate nicotinic agent and samples taken every 5 seconds for the duration of stimulation. For antagonist studies, the appropriate antagonist was perfused over the cells for 10 minutes prior to as well as during agonist stimulation. Kinase agents were perfused for 15 minutes prior to and during agonist stimulation. Results reported were obtained using cells cultured from three separate rats.

A.1.7 Rat *In Vivo* Bladder Cystometrogram:

Female SD rats (weight 250-275g) were anesthetized with urethane (1.2 g/kg, s.c.). A midline incision was made and a catheter (intramedic tubing, PE 50) was inserted into the bladder lumen through a small incision in the bladder dome and secured with a suture. The catheter was connected by way of a three-way stopcock to a syringe pump for fluid infusion and a pressure transducer connected to a computer to record changes in bladder pressure. The ureters were also tied and cut to prevent filling of the bladder from the kidneys. A wick of sterile gauze was inserted into the midline incision and sewn in place to drain urine from the abdominal cavity. Saline was infused intravesically (0.04 ml/min) for 1 hour in order to allow the bladder to recover from surgery. Saline infusion continued for an additional hour while bladder pressure was recorded to obtain control data. The bladder infusate was then switched to a drug solution and recorded for at least another hour before switching to another drug solution. In 4 animals, epibatidine was administered (0.05 μg/kg i.p. injection) at the end of the experiment and bladder pressure recordings were obtained for 2 hours.

A.1.8 Solutions and Reagents

All nicotinic agents used in this study were obtained by Tocris Bioscience, Ellisville, MO. All other reagents, unless otherwise noted were obtained from Sigma-Aldrich, Inc., St. Louis, MO. The composition of Krebs solution was (in mM): NaCl: 113, KCl: 4.7, $MgSO_4$: 1.2, $NaHCO_3$: 25.0, KH_2PO_4: 1.2, Glucose: 11.5, $CaCl_2$: 2.5, HEPES: 10.0. The composition of HBSS was (in mM): KCl: 5.0, KH_2PO_4: 0.3, NaCl: 138, $NaHCO_3$: 4.0, Na_2HPO_4: 0.3, Glucose: 5.6, $CaCl_2$: 2.0, $MgCl_2$: 1.0, HEPES 10.0. In experiments where extracellular calcium was to be removed, $CaCl_2$

was omitted, the concentration of NaCl was increased to 140mM and 0.5mM EGTA was added. All solutions were adjusted to pH 7.4 with 10N NaOH and 300-310 mOsM using NaCl.

A.1.9 Statistical Analyses

Statistical significance was assessed in all experiments using either unpaired, two-tailed Student's *t*-tests or one-way ANOVA with Tukey's multiple comparison post test to compare all columns to each other or Dunnett's post test to compare all columns to a control, when appropriate. Statistical significance was accepted when $p < 0.05$.

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