MAST CELL INDEPENDENT PSEUDOINFLAMMATORY CONTRACTIONS OF URINARY BLADDER SMOOTH MUSCLE

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ABSTRACT

Lower urinary tract symptoms (LUTS) are a family of bladder symptoms that are a major negative driver of the quality of life of men and women who suffer from them. The pathophysiology is largely unknown; however, mechanisms are proposed to originate from neurogenic and myogenic changes. The neurogenic and myogenic origins of urinary bladder dysfunction are often characterized by lower urinary tract symptoms associated with inflammation, specifically resulting from mast cell degranulation, that are proposed to alter sensory nerves and urothelial cells within the urinary bladder. Interestingly, these same inflammatory mediators released from mast cells cause both short-and long-lasting contractions of urinary bladder smooth muscle (UBSM). These contractions could be viewed as initial disruptors of normal bladder function and can lead to dysregulation of bladder smooth muscle contractility. Thus, this dissertation seeks to address the following hypothesis: histamine released after mast cell activation directly increases urinary bladder smooth muscle contractility independent of nerves. We discovered that histamine-mediated contractions rapidly desensitize, whereas the mast cell activator compound 48/80 caused both a long-lasting increase in the amplitude and frequency of spontaneous phasic contractions as well as a transient increase in baseline tension in UBSM strips. Compound 48/80 also significantly augmented nerve-evoked contractions in UBSM strips, whereas histamine did not alter these contractions. Surprisingly, none of these effects were dependent on mast cells. Mast cell-deficient mice responded identically to compound 48/80, as compared to the mast cell-sufficient controls. Instead, the effects of compound 48/80 were dependent upon the release of prostaglandins from the urothelium. Together, these findings suggest that mast cells alone are not responsible for driving

myogenic changes to urinary bladder contractility. Rather, the urothelium itself responds to pseudoinflammatory insults by releasing prostaglandins that alter UBSM responses to normal neuronal input. This mast cell-independent mechanism may be useful for identifying urothelium-specific targets for the treatment of LUTS.

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“Hold fast to dreams

For if dreams die

Life is a broken-winged bird

That cannot fly.”

Langston Hughes, Insert from Dreams, The Collected Poems of Langston Hughes, 1994

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACh	Acetylcholine
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CCh	Carbachol
GAG	glycosaminoglycan
IC/BPS	Interstitial cystitis/bladder pain syndrome
ICC	Interstitial cells of Cajal
LUTS	Lower urinary tract symptoms
NGF	Nerve growth factor
OAB	Overactive bladder
P2X	Purinergic receptor type X
P2Y	Purinergic receptor type Y
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2α
SEM	Standard error of the mean
TRPV1	Transient receptor potential vanilloid family type 1
UAB	Underactive bladder
UBSM	Urinary bladder smooth muscle

AN OVERVIEW OF BLADDER STRUCTURE, FUNCTION, AND DYSFUNCTION

1. Structure of the Urinary Bladder

The urinary bladder is more than just a sack of muscle waiting to be filled with urine. It is a complex organ comprised of structural components to facilitate coordinated storage and voiding. The bladder wall consists of the following layers: glycosaminoglycan (GAG) layer, urothelium, lamina propria (suburothelium), and detrusor muscle (**Fig. 1**). In the bladder of certain species, there is also a muscularis mucosae layer [6]. Additionally, the bladder is innervated with efferent parasympathetic and sympathetic nerves, afferent sensory Ad fibers (predominantly transmit sensation of fullness), and C fibers (activated at high pressure thresholds or noxious stimuli). In addition to efferent and afferent nerves, dispersed throughout layers of the wall are interstitial cells of Cajal (ICC)-like cells, blood vessels, and extensive extracellular matrix proteins. Each component of the wall has unique functional properties. However, the focal point of this section of the dissertation will be the tissue-level function of the urothelium and detrusor.

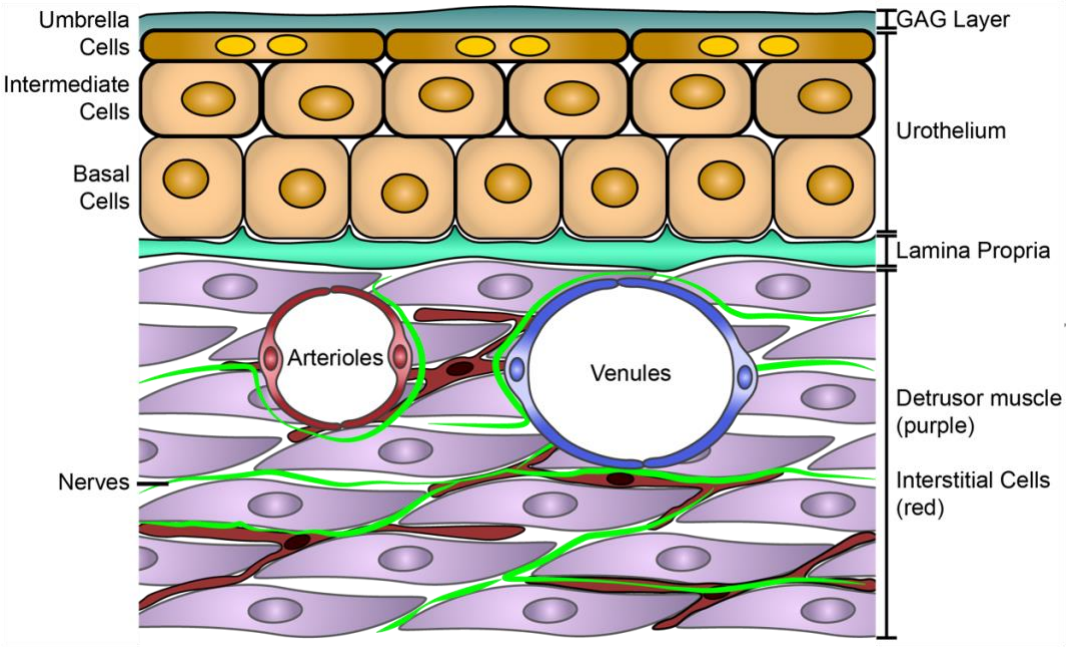

Figure 1: Components of the urinary bladder wall. GAG: glycosaminoglycan. Image courtesy of Dr. Nathan R. Tykocki, and adapted from [5].

1.1. What is the urothelial layer?

The urothelium is a transitional epithelium comprised of basal cells, intermediate cells, and umbrella cells (**Fig. 1**). Together these cells form what is arguably the most impermeable barrier of any internal organ [7]. Basal cells are small, but the most abundant cell in the urothelium. They undergo differentiation rapidly to intermediate cells and serve to connect the urothelium to the basement membrane [8]. On the other hand, intermediate cells are larger and ultimately transition into umbrella cells; however, they lack distinguishable tight junctions [9]. Multiple layers of intermediate cells may also be present, ranging from one layer in mice to five layers in humans [8]. Umbrella cells are polarized, binucleate epithelial cells expressing uroplakins on their apical membrane [8]. These uroplakins form heterotrimers that further assemble into star-shaped plaques, from which the umbrella cells gain their impermeability [8]. Though umbrella cells also contain tight junctions between each other to maintain high resistance barrier function [10, 11], they are still quite distensible. For example, when the urinary bladder fills and the urothelium stretches, umbrella cells actively insert additional membrane to maintain barrier function and tight junctions [8]. Covering the urothelium is the GAG layer, which forms a protective coating made of glycoproteins and proteoglycans that prevents the breaching of acids and toxins from the urine. Urothelial permeation due to bacterial adhesion cannot occur due to the presence of glycosaminoglycans chondroitin sulfate and hyaluronic acid, as well as heparin and dermatan [12].

As a signaling hub, the urothelium synthesizes and releases multiple signaling molecules that regulate its function and communicate with other layers within the bladder wall. Regarding signaling, the urothelium receives receptor and ion channel-mediated signals

driven by autocrine and paracrine signals. During filling, urothelial cells release adenosine, ATP, and acetylcholine in order to communicate with other neighboring umbrella cells [13]. These cells also receive input from both efferent and afferent nerves in the form of ATP, acetylcholine, norepinephrine, and Substance P [13]. Additionally, transient receptor potential (TRP) channels on the basal membrane can respond to mechanical or chemical stimuli, though the expression of TRP channels in the urothelium has been met with some controversy due to the lack of specific TRP channel antibodies [14, 15]. Urothelium-derived mediators such as ATP [16, 17] and prostaglandins [18, 19] are released under various conditions. Tanaka *et al.* elucidated a mechanism by which low and high distention of the bladder causes the release of ATP and prostaglandin E_2 (PGE_2) [20]. Purinergic signaling is proposed as the predominant control of urothelial signaling regulated by the release of ATP and subsequent activation of purinergic receptors [21]. In agreeance, Wang *et al.* determined exocytosis of ATP increases the serosal membrane surface area of umbrella cells [22]. Thus, changes to the bladder wall during filling and emptying promote feedback to and from the urothelium.

1.2. What is the detrusor layer?

The outermost layer of the urinary bladder wall is the detrusor muscle, made up of large, spindle-shaped smooth muscle cells. These fibers are positioned in longitudinal and circular orientations for organ-coordinated movements. Unlike the “tonic” nature of vasculature smooth muscle, detrusor activity is “phasic” [23]. The difference in the contractile state is due to uniformity within the vasculature, as opposed to the multifaceted movements of the detrusor myocytes. Whereas vascular smooth muscle is often in a state of constant partial contraction, independent bundles of detrusor smooth muscle phasically

contract and relax as the bladder fills. Detrusor myocytes are the key component of voiding since it is the coordinated contraction of the detrusor muscle that drives voiding. During storage, the detrusor is by and large relaxed, whereas micturition results from induced detrusor contractions. Largely, the detrusor is responsible for urinary bladder contractility in response to parasympathetic nerve innervation. The stimulated release of ATP and acetylcholine (ACh) from parasympathetic nerves activates purinergic and muscarinic receptors, respectively, on the detrusor smooth muscle cells [24]. Regarding detrusor muscarinic signaling, M2 and M3 muscarinic receptors are responsible for contractions [4]. Upon stimulation, $G_{q/11}$-coupled M3 receptors drive the release of intracellular calcium stores. Additionally, M2 receptors, coupled to $G_{i/o}$, activate nonselective cation currents and suppress K^+ channels to drive depolarization and voltage-dependent calcium influx (**Fig. 2**). [25]. From stimulatory to inhibitory the role of certain other muscarinic receptors remains unclear (M1, M5 – M2, M4) within the context of the detrusor function [26]. The role of purinergic receptors is also less clear, but $P2X_1$ receptors in the detrusor seem to facilitate calcium-dependent action potentials and calcium flashes in the detrusor during the early portions of detrusor contractions [27].

In contrast, relaxation of the detrusor occurs in part by stimulated sympathetic nerves releasing noradrenaline that activates relaxant β_3 adrenergic receptors [28]. Also at play are both large- and small-conductance calcium-activated K^+ (BK and SK) channels, which are activated by the increase in intracellular calcium during a contraction. These channels serve to hyperpolarize the membrane once again, leading to a loss of calcium influx and a relaxation of the smooth muscle [29]. There is also significant innervation of the bladder by nitrergic nerves that release nitric oxide to promote guanylate cycle activity to also

drive relaxation [30]. Together, the coordinated actions of contractile and relaxant pathways keep bladder smooth muscle tone at a minimum until the need to void.

1.3. How do these layers communicate?

Interdependent signaling between the urothelium and detrusor is necessary for proper coordination of voiding and storage. Signaling among the urothelium and detrusor is considered non-neuronal, insomuch that the mechanisms are activated in the presence or absence of nerve input. However, much of the communication between urothelium and detrusor is first instigated by parasympathetic nerves. Under normal conditions, purinergic and muscarinic signaling occurs between the layers. Purinergic signaling of the urinary bladder is regulated by P2Y and P2X receptors [31]. The ionotropic $P2X_1$ receptor involves detrusor contractility, whereas $P2X_2$ and $P2X_3$ mediate sensory signaling in the urothelium [32, 33]. Sui *et al.* demonstrated that desensitizing urothelial P2X receptors with α,β-Methylene ATP resulted in a reduced increase in phasic detrusor activity in response to the muscarinic agonist carbachol [34]. Likewise, carbachol-mediated contractions of the detrusor increased urothelial ATP release in a force-dependent manner [34]. Furthermore, urothelium-derived mediators can regulate detrusor contractility by inhibition of muscarinic receptors via an unknown urothelial-inhibitory factor [35-40]. Thus, this evidence suggests a cross-layer communication to regulate contractions.

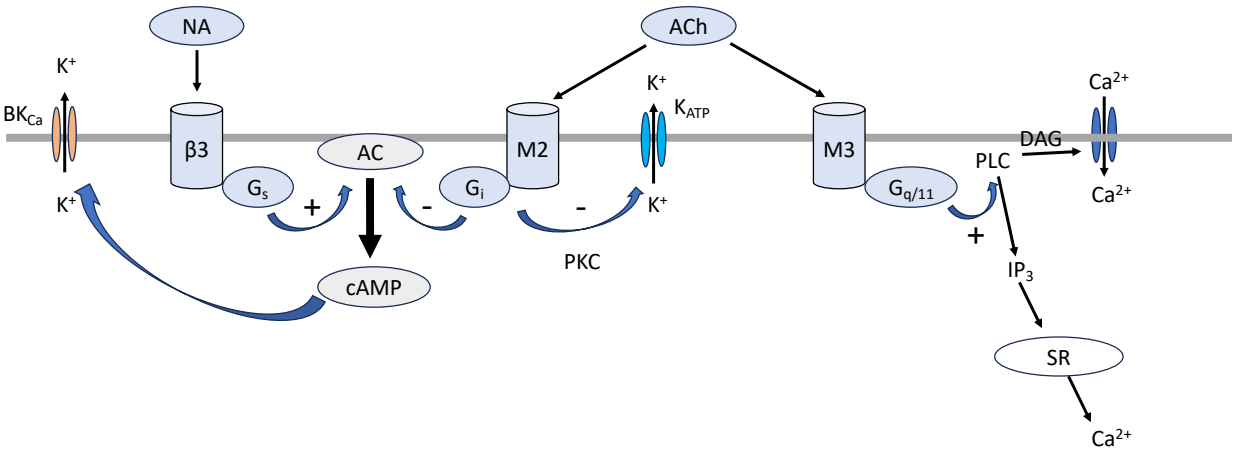

Figure 2: Simplified muscarinic signal transduction. NA: noradrenaline; AC: adenylyl cyclase; cAMP: cyclic adenosine monophosphate; PKC: protein kinase C; ACh: acetylcholine; PLC: phospholipase C; DAG: diacylglycerol; IP_3: inositol trisphosphate; SR: sarcoplasmic reticulum. Figure adapted from [4].

2. Overactive Bladder as an Outcome

2.1. Origins of overactive bladder

Overactive bladder (OAB) is historically known as an “urge” syndrome in the absence of a metabolic or pathologic condition. The term “OAB” was coined from a subjective lens by Abrams and Wein, which to date blurs the potential for new druggable targets [41]. OAB is clinically defined as urinary urgency with or without urge incontinence, which are often accompanied by increased urination frequency and nocturia [41]. Eapen and Radomski reviewed epidemiological studies (EPIC [42], NOBLE [43], EpiLUTS [44]) to compare the prevalence of OAB and LUTS in men and women [45]. EPIC and NOBLE studies reported a similar prevalence of OAB amongst women and men, whereas EpiLUTS indicated a much higher prevalence in women [45, 46]. Regarding symptoms of urgency and urinary incontinence, all studies implicated women more so than men. Based on the studies presented, the prevalence of OAB is regarded as sex-neutral bladder dysfunction, as opposed to lower urinary tract symptoms (specifically urge and stress incontinence) where the prevalence is higher in females.

There are multiple postulates pertaining to the pathophysiology of overactive bladder based on sensation and detrusor overactivity. Clinical investigations of OAB focus on urgency, whereas animal studies are unable to mimic symptoms but instead induce pathologic conditions that resemble detrusor overactivity [47]. Overactive bladder is associated with changes in the following: loss of caveolae, increased muscarinic (M2 and M3) receptor expression and altered Gq-mediated signaling. Thus, scientific investigations often examine mechanisms based on central nervous system changes (“neurogenic” hypotheses), detrusor myocyte sensitivity (“myogenic” hypotheses), and

structural input (“integrative” hypotheses) [48]. Currently, identifying druggable pathways for OAB is due to emerging experimental evidence from urodynamic animal studies of existing pathologic conditions, not of “pure” OAB as it exists in humans. Thus, hypotheses regarding the pathophysiology of OAB are based on inflammatory and obstructive urinary bladder dysfunctions such as cystitis, urinary tract infections, benign prostate hyperplasia, and aging [49].

2.2. Myogenic Hypothesis

In 1997, Brading proposed the myogenic hypothesis for OAB, which stated, “partial denervation of the detrusor alters properties of smooth muscle… resulting in coordinated myogenic contractions” [50]. The overarching outcome of the myogenic hypothesis is “detrusor spontaneity and supersensitivity” due to a lack of sympathetic nerve innervation [51]. Urodynamic studies support detrusor overactivity (DO), which is a condition that implicates spontaneous detrusor activity during the filling phase. However, the origins of this increased activity are unknown.

Elucidation of myogenic origins is investigated through the lens of smooth muscle cell ion influx and efflux. Herrera *et al.* mention the “myogenic origins of spontaneous contractions” of the guinea pig detrusor in their discovery that ryanodine receptors, BK channels, and SK channels mediate spontaneous detrusor strip contractions without any dependence on nerve input [52]. Furthermore, these spontaneous contractions drive sensory outflow from the bladder, possibly suggesting that altered contractility alone could drive symptoms [53]. Other supporting factors of the myogenic hypothesis are ischemic conditions that change detrusor function. Wang *et al.* conducted an ex vivo study with rat detrusor strips and determined the myogenic effects of reactive oxygen species on

spontaneous smooth muscle contractions were dependent on Rho kinase and SK channels [54, 55]. Current investigations of the myogenic hypothesis focus on mechanisms that not only drive sensitivity but disrupt the mobilization of Ca^{2+} and K^{+} at the tissue level as a definitive characteristic of detrusor dysregulation that leads to dysfunction [54, 55].

The myogenic hypothesis is not without its shortcomings. For example, it does not account for changes in afferent nerve innervation or efferent nerve activity: atropine and tetrodotoxin are utilized to assess detrusor strip spontaneous contractions in the absence of nerve input [52], but tetrodotoxin-resistant nerves are neglected and spontaneous activity alone is not a sign of bladder dysfunction. Another limitation of this hypothesis is that not all models of OAB result in changes to the detrusor contractile properties [47].

2.3. Neurogenic Hypothesis

The neurogenic hypothesis is based on pathologies associated with neurologic changes that cause urinary bladder incontinence [56]. In this hypothesis, one such problem is too much afferent outflow, which disrupts normal bladder function [57]. Another such problem is too much efferent nerve input to the detrusor, which drives increased contractions at much lower urine volumes than normal [58]. These pathologies implicate damage to the central nervous system (specifically the pons micturition center) and spinal cord leading to problems with the micturition reflex [59]. Pharmacologic investigations of neurogenic mediators are utilized to determine mechanisms underlying neurogenic overactive bladder [60]. Neurogenic detrusor overactivity is associated with afferent nerve signaling alterations, including a shift from A-δ fiber to c-fiber output [61]. This postulate is limited due to the complexities of neuronal signaling of the urinary bladder. Additionally, there is

no mention of cross-communication of detrusor, afferent nerves, and urothelium, which could synergistically alter bladder function through feedback and feed-forward mechanisms.

2.4. Structural Input Hypothesis

The structural components of the urinary bladder coordinate the regulation of filling and emptying with mechanosensitive elements within the urinary bladder wall itself. Certain components (e.g., interstitial cells) of the layers within the dome of the urinary bladder are believed to serve as “checkpoints”, controlling signal propagation to facilitate contractions [62]. There is also evidence that interstitial cells of Cajal (ICC)-like cells within the bladder wall serve to regulate spontaneous activity during filling [63]. Therefore, “structural input” overactivity refers to an integrative process in which the urothelium undergoes dysregulation and ICC-like cells no longer serve as regulated pacemakers of the detrusor. Loss of ICC-like cells in relation to the detrusor was found in spinal cord injury-induced overactive bladder [64]. In the absence of the urothelium, pacemaker-type spontaneous action potentials of the detrusor were also increased [65]. The integrative hypothesis implies that there is no one outcome that leads to an overactive bladder but the dysregulation of crosstalk between checkpoint elements leads to the engagement of many of the mechanisms included in the myogenic and neurogenic hypotheses [66]. The research supporting this hypothesis is still lacking, and no clear mechanism is consistently present in the literature.

3. The Molecular Basis of LUTS

The origins of OAB and Interstitial cystitis/painful bladder syndrome (IC/BPS) pathophysiology was defined and diagnosed based on a collection of symptomologies

called “lower urinary tract symptoms”, or LUTS (**Fig. 3**). LUTS are described as urgency, frequency, hesitancy, and most importantly irritability, which coincides with IC/BPS biomarkers. The most common biomarkers reviewed by Siddiqui *et al.* are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), prostaglandin E2 (PGE_2), and ATP, along with increased receptor expression changes (M3, β-adrenergic, TRPV1) [67]. Muscarinic M2 and M3 receptors are upregulated, which leads to increased contractile sensitivity [68]. Detrusor signaling under normal conditions is mediated predominately by cholinergic contractions; however, in diseased states, there is a switch to purinergic pathways [33]. Similarly, irritability is initiated by a transition from sensory information being conveyed through A∂ fibers to C fibers, due in part to TRPV1 channel sensitization and increased firing [69, 70]. However, findings regarding biomarkers of LUTS are inconclusive due to the uncertainties of the pathology. Nonetheless, these indicators correspond with the latest proposed postulates on the basis of LUTS as an inflammatory condition.

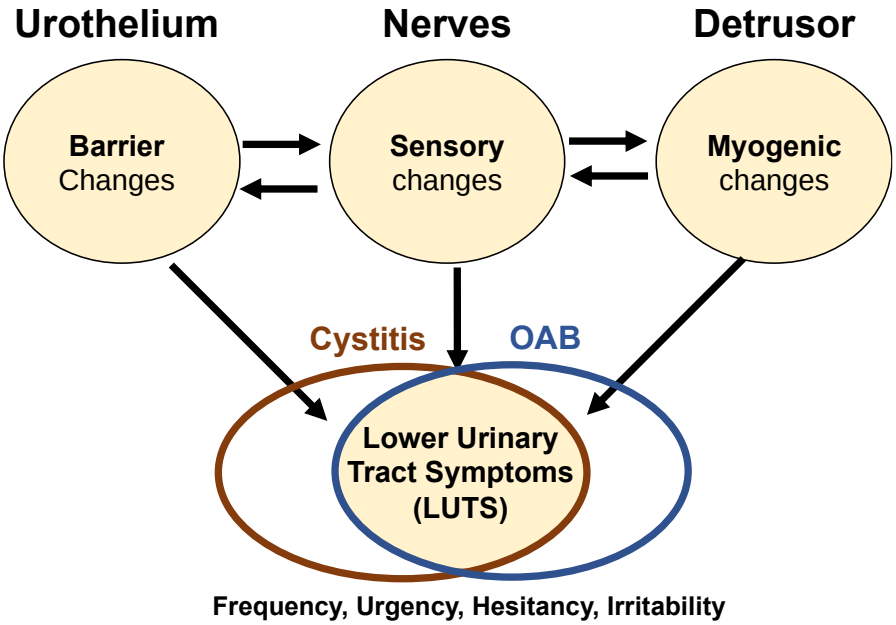

Figure 3: Intersection of the causes of LUTS. Interdependent changes to the urothelium, nerves, and muscle drive symptoms common to both OAB and IC/BPS. Modified from [3].

3.1. Neurogenic Inflammation

From theories to experimental investigations, neurogenic inflammation is implicated in changes to sensory neuronal output, vascular permeability, effector cell responses, and inflammatory mediator release [71]. Most past and present studies define neurogenic inflammation by inducing it pharmacologically, using substance P or capsaicin [72, 73]. The first theory regarding neurogenic inflammation was solely focused on the “axon reflex flare” via stimulation of sensory nerves leading to vasodilation [74]. Current investigations focus on sensitization of the nonselective cation channel transient receptor potential vanilloid type 1 (TRPV1) on C fibers, and on leakage of the vasculature within many organs including the urinary bladder [15, 75-77]. Baluk *et al.* utilized the urinary bladder to define the origins of neurogenic inflammation via baseline vascular plasma leakage in relation to neuropeptides (e.g., tachykinins) release from sensory nerves [78].

Often within the context of the urinary bladder, neurogenic inflammation is the theory used to describe LUTS associated with IC/BPS. Mediator release from the urothelium is considered a hallmark of urinary bladder neurogenic inflammation [79]. In general, the primary basis of neurogenic inflammation is the concept of nerve activation induced by inflammatory mediators. Some mediators proposed are mast cell-derived (histamine, 5-hydroxytryptamine (5-HT)) [80, 81], whereas others are neuropeptides (BNDF, NGF) [82, 83]. The most common modulator is Substance P, which is released by afferent nerve fibers. Substance P-positive nerves are found in the urinary bladder of subjects with neurogenic cystitis [84]. Thus, the cascade of neurogenic inflammation within the bladder is complex with a multitude of players supposedly responsible.

Neurogenic mediators can activate inflammatory signaling pathways that cause increased neurogenic and non-neurogenic bladder activity [85, 86]**.** Substance P, 5-HT, and histamine act on both afferent nerves and the detrusor within the urinary bladder. Substance P does not only directly contract the detrusor but also can induce histamine release from urinary bladder tissue *[87-89]*. 5-HT_{1A} agonism enhances spontaneous phasic activity found in the strips of rats with partial bladder outlet obstruction *[90]*. Histamine and 5-HT increase afferent nerve firing *[91, 92]*. Therefore, these interdependent pathways induced by inflammation call into question if nerves and muscles are involved, and how they influence one another.

3.2. "Myogenic" Inflammation?

"Myogenic inflammation**"**, an emerging concept within the context of urinary bladder dysfunction is understudied in comparison to neurogenic inflammation. In contrast to neurogenic inflammation, myogenic inflammation considers a feed-forward instead of a feedback mechanism, wherein the detrusor undergoes an inflammatory transition to change the way in which it contracts. Both concepts have commonalities, such as inflammatory mediator release, but myogenic inflammation urges a concept based on detrusor responsiveness to external inflammatory mediator release as well as neuronal changes. Unfortunately, the closest research parallels to the concept of myogenic inflammation in the bladder are with the aforementioned myogenic hypothesis of OAB. However, the myogenic hypothesis does not account for inflammatory mediator-induced contractions. Inflammatory-induced contractions are indirectly mentioned throughout the literature, wherein the detrusor contracts by forgoing spontaneous activity due to

inflammatory receptors being located on the detrusor [93, 94].Thus, the effects of inflammatory mediators directly on detrusor muscle are worthy of further investigation.

4. Mast Cells and Bladder Dysfunction

4.1. General Overview of Bladder Mast Cells

In the context of the urinary bladder, mast cells are mainly regarded as histamine-releasing granulocytes [95-97] – although mast cells release a plethora of other inflammatory mediators as well [98]. To understand the role of mast cells in the bladder, systemic or intravesical induction of inflammation is often performed, utilizing bacterial infection, chemical perturbation, psychological stress, and visceral hypersensitivity [99-101]. Infiltration and subsequent degranulation of mast cells within the urinary bladder is then measured. A resident population of mast cells in the urinary bladder is localized in proximity to nerve fibers, where they are thought to directly influence sensory outflow [100]. Other mucosal mast cells are predominately found in the urothelium and detrusor of normal and cystitis bladders [2]. It is thought that mast cell activation, recruitment, and degranulation within the bladder wall causes changes in umbrella cell barrier function [102]. In relation to the detrusor, the functional role of mast cells in the bladder has shifted due to the identification of proto-oncogene c-kit positive ICC-like cells [103]. As with TRP channel expression in the urothelium, this too is not without controversy. Others contend that c-kit positive cells are indeed mast cells, and ICC-like cells are better identified using platelet-derived growth factor alpha 9PDGFRα as a marker [104]. The role of mast cells in normal bladder function to date remains unclear, yet mast cells continue to be mentioned. This could be due to inference of mast cell involvement from bladder disease models instead of direct investigations into mast cells (**Fig.** 4).

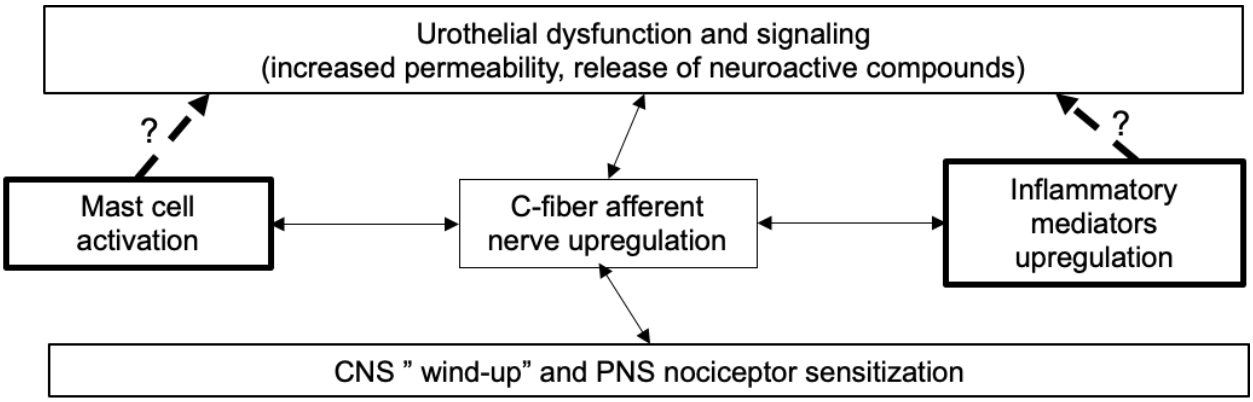

Figure 4: The proposed pathophysiology of IC/BPS. Postulated pathways of IC/BPS include mast cell activation and inflammatory mediator release, without mention of direct effects on the bladder wall. Figure adapted from [1, 2].

4.2. Mast Cell-Induced Bladder Dysfunction

Mast cell degranulation within in the urinary bladder is associated with pain due to C fiber sensitization and activation (**Fig.** 4). Historically, mast cells are implicated in interstitial cystitis (IC/BPS); recently, the significance of mast cells in IC/BPS is under cautionary surveillance as evidence against them as a biomarker for IC/BPS mounts [105]. However, there are seminal studies that support the role of mast cells in various other pathological conditions of the urinary bladder. Malik *et al.* identified mucosal mast cells are elevated in the detrusor of patients with IC/BPS [99]. Moore *et al.* conducted a study with IC patients with detrusor instability and found that only 8 out of 28 presented with detrusor mastocytosis along with decreased capacity (475 vs 725 mL) compared to the control group [106]. So, while the number of mast cells may not be a good biomarker for inflammatory bladder dysfunction, they most definitely have profound effects on bladder function when degranulated.

4.3. Mast Cells and Ex vivo Bladder Contractility

Most contractility studies ignore the presence of mast cells within the bladder and infer that application of mast cell-derived mediators exogenously can act as an appropriate surrogate for actual degranulation and release [107]. There are only a few studies that involve mast cell activation and degranulation directly as it pertains to contractility. These studies are performed with compound 48/80 or ovalbumin. An ovalbumin study was performed to determine local mast cell activation in the bladder [108]. Ovalbumin results in a rapidly desensitizing contraction, which is in alignment with the small resident population of mast cells in the absence of disease. On the other hand, compound 48/80

is used as a mast cell-depleting agent post-inflammation; the only study to date determining its direct effects on detrusor contractility can be found below in Chapter 3.

4.4. MCs Role in Neurogenic Inflammation

As described above, neurogenic inflammation as a concept mentions mast cells as modulators of hypersensitivity. This conceptualization has built a relationship between the gastrointestinal tract and the urinary bladder [109]. Comparisons of irritable bowel syndrome and detrusor overactivity implicate mast cell-induced nerve hypersensitivity. Fitzgerald *et al.* found decreased void intervals in colitis models in mast cell-sufficient mice, but not in mast cell-deficient mice [110]. Similarly, Ustinova *et al.* modeled colitis in rats and determined that increased mast cells in both the colon and bladder coincided with increased bladder afferent activity during distension [85]. Zhang *et al.* discovered visceral hypersensitivity in rats caused increased bladder mast cell infiltration along with non-voiding contractions during the bladder filling phase [101]. These studies support an indirect role of urinary bladder mast cell infiltration as it pertains to neurogenic inflammation, but do not investigate direct changes in myogenic responses.

5. Role of Histamine and Histamine Receptors in LUTS

Histamine is a biogenic amine that contributes to the innate immune response [111]. Additionally, histamine induces vasodilation, constriction, relaxation, and contraction, dependent on the receptor expression along with smooth muscle type [112-116]. In rodents, four histamine receptors exist, encoded by the following genes: *Hrh1, Hrh2, Hrh3, Hrh4*. These receptors are a part of the G protein-coupled receptor superfamily, and each receptor subtype has a different functional role throughout the body [117]. For instance, histamine receptor H1 mediates allergic responses that cause vascular changes

via $G\alpha_{q/12}$ signaling [117]. Histamine receptor H2 mainly regulates the control of gastric acids via $G\alpha_s$ signaling [117]. Receptors H3 and H4 play a role in central nervous system responses via $G\alpha_{i/o}$ signaling and immune responses via $G\alpha_{i/o}$ signaling respectively [117].

Early in the 20th century, histamine release in the urinary bladder was only associated with “detrusor mastocytosis”, wherein histamine has a direct interaction with the detrusor to influence voiding contractions [118]. To date, few studies explore the direct role of histamine in detrusor overactivity. The role of histamine in urinary bladder function is investigated by receptor genetic knockouts and pharmacological studies. Most postulates implicate histamine in dysfunctional neuronal sensory control. Others consider histamine as a biomarker for bladder cystitis and bacterial infections due to the levels of metabolites in the urine. Overall, the role of histamine and histamine receptors is largely unknown in the urinary bladder; however, some studies focus on the characterization of histamine receptors [119-121].

Histamine is now implicated in multiple bladder dysfunctions [122]. In children with allergies and OAB, Yin *et al.* report histamine as a source of overlapping symptoms, such as decreased bladder sensitivity during treatment with the histamine receptor H1 antagonist desloratadine [123]. Rudick *et al.* modeled neurogenic cystitis and found that histamine receptors H1 and H2, but not H3, were responsible for pelvic pain [124]. In contractility studies, H1 antagonism abolished the response to histamine, whereas H2 antagonism augments the response [125]. In addition to these findings, age-related changes occur at the tissue level with porcine strips, wherein histamine receptor H1-mediated responses are more sensitive in juveniles than adults [126]. Recently, there has

been a shift toward histamine indirectly driving pain through sensory nerve outflow. Grundy *et al.* found histamine receptor H1 mediates responses to urinary bladder distension [91].

To date, research into the role of histamine in urinary bladder function is still underway. Regarding the role of histamine in LUTS, the question remains: are the actions on detrusor function indirect or direct? Indirect actions of histamine are based on modulatory effects, whereas direct effects cause changes to the bladder muscle's ability to contract. Studies have indicated that histamine causes alterations to neurotransmitter signaling within the bladder [107]. Additionally, there is an increase in release of histamine and expression of the histamine-forming enzyme histidine decarboxylase in stressed rats' urinary bladder, which is linked to sensitivity as well [127, 128]. Histamine does indeed have a direct impact on the detrusor smooth muscle signaling [129-131], so it is likely that the nature of histamine as a signaling molecule involves both indirect and direct mechanisms.

6. Conclusions

The remainder of this dissertation will focus on two central questions: (1) does histamine directly alter the ability of the detrusor to contract; and (2) do mast cells within the bladder wall directly influence the ability of the detrusor to respond to contractile agonists. These studies fill a gap in knowledge in both the Structural Input Hypothesis of OAB and the concept of myogenic inflammation as an emerging driver of bladder dysfunction. Through the following studies, we learn that histamine, while able to transiently contract the detrusor, has little effect on detrusor function in the absence of disease. We will also uncover a surprising finding that a supposed mast cell activator, Compound 48/80, has

profound effects on detrusor contractility – even in mice lacking functional mast cells. My final chapter will serve to integrate my findings in the landscape of bladder function, as well as identify shortcomings in our studies and present future directions for continued research in this field.

HISTAMINE RECEPTORS RAPIDLY DESENSITIZE WITHOUT ALTERING NERVE-EVOKED CONTRACTIONS IN MURINE URINARY BLADDER SMOOTH MUSCLE

Modified from: Jones BM, Mingin GC, and Tykocki NR. “Histamine Receptors Rapidly Desensitize Without Altering Nerve-Evoked Contractions in Murine Urinary Bladder Smooth Muscle”. Am J Physiol Renal Physiol 2022 Mar 1;322(3):F268-F279. doi: 10.1152/ajprenal.00355.2021. PMCID: PMC8858670.

1. Abstract

Histamine has been implicated in urinary bladder dysfunction as an inflammatory mediator driving sensory nerve hypersensitivity. However, the direct influence of histamine on smooth muscle has not been thoroughly investigated. We hypothesized that histamine directly contracts urinary bladder smooth muscle (UBSM) independent of effects on nerves. Single cell quantitative RT-PCR determined that only histamine H_1 and H_2 receptors were expressed on UBSM cells. In isolated tissue bath experiments, histamine (200 μM) caused a highly variable and rapidly desensitizing contraction that was completely abolished by the H_1 receptor antagonist fexofenadine (5 μM) and the $G_{q/11}$ inhibitor YM254890 (1 μM). Neither the muscarinic receptor antagonist atropine (1 μM), the Na^+ channel blocker tetrodotoxin (1 μM), nor the transient receptor potential vanilloid type 1 antagonist capsazepine (10 μM) altered responses to histamine, suggesting that nerve activation was not involved. UBSM desensitization to histamine was not due to receptor internalization, as neither the cholesterol-depleting agent methyl-β-cyclodextrin (10 mM), the dynamin-mediated endocytosis inhibitor dynasore (100 μM), nor the clathrin-mediated endocytosis inhibitor pitstop2 (15 μM) augmented or prolonged histamine contractions. Buffer from desensitized tissues still contracted histamine-naïve tissues, revealing that histamine was not metabolized. Prolonged exposure to histamine also had no effect on contractions due to electrical field stimulation, suggesting that both efferent nerve and UBSM excitability were unchanged. Together, these data suggest that histamine, although able to transiently contract UBSM, does not have a lasting effect on UBSM excitability or responses to efferent nerve input. Thus, any acute effects of histamine directly on UBSM contractility are unlikely to alter urinary bladder function.

New and Noteworthy: Histamine is commonly associated with inflammatory bladder pathologies. We sought to investigate the role of histamine on urinary bladder contractility. Histamine contracts the bladder, but this response is highly variable and desensitizes completely in minutes. This desensitization is not due to internalization of the receptor or metabolism of histamine. Because nerve-evoked contractions are also not increased in the presence of histamine, our findings suggest that histamine is not directly acting to change contractility.

2. Introduction

The urinary bladder is a distensible organ with two main functions: urine storage and urine elimination [132]. Dysfunctional and/or diseased urinary bladders share common overlapping lower urinary tract symptoms (LUTS) of overactivity, hypersensitivity, and/or underactivity that are often associated with inflammatory responses that lead to histamine release [133]. The most well-studied postulate regarding histamine's role in bladder physiology involves the release of histamine from mast cells, which in turn drives central and peripheral nerve hyperexcitability in response to bladder distension [2, 70, 134]. Thus, these findings suggest that histamine indirectly augments bladder contractility through neuronal hyperexcitability, as opposed to directly acting on histamine receptors in urinary bladder smooth muscle (UBSM) that disrupt micturition coordination [135-137]. Histamine does directly contract porcine bladder urothelium/lamina propria, and histamine receptor expression is increased in detrusor muscle from patients with interstitial cystitis/painful bladder syndrome [116, 122]. However, the direct effects of histamine on UBSM contractility and excitability are less clear. Furthermore, it is unclear if histamine alters the responsiveness of UBSM to other contractile stimuli.

As a vasoactive bioamine, histamine triggers both immediate immune signaling and subsequent pleiotropic effects in various organs depending on which of the four receptor subtypes are activated [112, 138, 139]. These effects include increased neurotransmitter release [140], but also include rapidly-desensitizing smooth muscle contractions in other smooth muscle-rich organs such as trachea and uterus [141, 142]. Smooth muscle responses to histamine are typically mediated through histamine H_1 receptors (G_q-coupled contraction) and H_2 receptors (G_s-coupled relaxation) [143]. H_3 and H_4 receptors

appear to be expressed in human detrusor smooth muscle [144], though the significant phenotypic changes that smooth muscle cells undergo in culture make these data difficult to interpret. While some of the effects of histamine on UBSM contractility has been investigated [107, 116, 143], it remains unclear if these responses are due to contractions of cells in the muscularis mucosa, release of contractile compounds after sensory nerve activation, or direct actions on histamine receptors in urinary bladder smooth muscle – or for that matter, all three.

In this study, we found that histamine causes a rapidly desensitizing contraction in mouse urinary bladder strips that was mediated by H_1 receptors. Histamine-induced contractions were independent of nerve activation and were also unaffected by removal of the urothelium. This contraction was repeatable only after prolonged washout (though reduced in amplitude), suggesting receptors could recover. The rapid desensitization was not due to metabolism of histamine, as naïve tissues contracted when exposed to buffer taken from desensitized tissues. Desensitization was also not due to endocytosis; depletion of cholesterol or inhibition of dynamin-mediated endocytosis only further reduced the amplitude and AUC of histamine contractions, whereas inhibition of clathrin-mediated endocytosis had no effect. Continued exposure to histamine also had no effect on electrical field stimulated UBSM contractions or contractions in response to carbachol (200 nM). Together, these data suggest that UBSM contractile responses to histamine rapidly desensitize through a mechanism other than receptor internalization or metabolism. Also, while capable of transiently contracting UBSM directly, histamine does not alter the ability of UBSM to respond to other physiologically relevant stimuli. Thus,

any acute direct effects of histamine on urinary bladder smooth muscle appear negligible regarding normal contractile function.

3. Materials and Methods

3.1. Animal Care and Use

All animal procedures followed institutional guidelines and were approved by the Institutional Animal Care and Use Committees of Michigan State University (NIH Assurance D16-0054). Male C57BL/6 mice (9 – 17 weeks old; Jackson Laboratory, Bar Harbor, ME) were group housed in a temperature- and humidity-controlled environment with a 12 hr light/dark cycle. Mice were provided *ad libitum* access to standard chow and water. Prior to all experimental procedures, mice were euthanized by intraperitoneal injection of pentobarbital (>150 mg/kg) followed by decapitation.

3.2. Smooth Muscle Cell Dissociation

Urinary bladders were dissected and placed in ice-cold Ca^{2+}-free HEPES dissection buffer containing (in mM): NaCl (134), KCl (6), $MgCl_2$ (1.2), HEPES (10) and glucose (7); pH=7.4. Tissues were then cleaned of connective tissue, pinned flat, denuded of urothelium by blunt dissection (when appropriate), and cut into ~2mm wide strips. Bladder strips were incubated at 37°C for 18 minutes in dissociation buffer (consisting of Ca^{2+}Free-HEPES buffer with 2 mg/ml albumin), to which papain (1.0 mg/ml), and dithioerythritol (1.0 mg/ml) were added. Tissues were transferred into fresh dissociation buffer with collagenase (2.0 mg/ml) and $CaCl_2$ (100 µM) and incubated at 37°C for 10 minutes. Following incubation, UBSM myocytes were placed on ice in fresh Ca^{2+}-free dissection buffer and gently triturated with a glass Pasteur pipette. Cells were then placed

in a custom chamber on an inverted microscope, and 10 UBSM cells (determined by fusiform morphology) were collected using a suction pipette for further experimentation.

3.3. Single-Cell Quantitative RT-PCR

Isolated detrusor myocytes were used for single-cell qRT-PCR experiments, using the Ambion Cells-to-C_T qRT-PCR Kit (Thermo Fisher Scientific, Waltham, MA USA). Lysed samples were subjected to recommended thermal cycles for reverse transcription (25°C for 10 minutes; 42°C for 60 minutes; 85°C for 5 minutes) using Veriti Thermocycler (Applied Biosystems, Waltham, MA USA). Pooled TaqMan® gene expression assays/samples were then pre-amplified via the following stages: enzyme activation at 95°C for 10 minutes; 14 amplification cycles of denaturing at 95°C for 15 seconds and annealing/extending at 60°C for 4 minutes; and lastly enzyme deactivation at 99°C for 10 minutes. RT-PCR was performed using QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA USA). cDNA was amplified using recommended RT-PCR thermal cycles: Uracil-DNA Glycosylase incubation at 50°C for 2 minutes; enzyme activation at 95°C for 10 minutes; and 40 amplification cycles of denaturing at 95°C for 5 seconds and annealing/extending at 60°C for 1 minute. Expression of the following mRNAs were measured: *Hrh1* (Mm00434002_s1), *Hrh2* (Mm00434009_s1), *Hrh3* (Mm00446706_m1), *Hrh4* (Mm00467634_m1), and *Acta2* (Mm00725412_s1). All

Table 1. Context sequences used for RT-PCR.

Gene	Sequence
Hrh1	TGAGGGAGATGCCAGGGGCTCAAAG
Hrh2	GGCTCCGCAGTCTGACCAATTGCTT
Hrh3	CTTCCTCGTGGGTGCCTTCTGCATC
Hrh4	AGTTTCAAATGCTGTGTCTTATAGG
Acta2	TAGCCCTGGCCTAGCAACACTGATT

gene expression assays were obtained from Thermo Fisher Scientific, who also validated their specificity (Waltham, MA USA). Probe context sequences used are found in **Table 1**. Results are reported as raw C_T values per manufacturer recommendations.

3.4. Isometric Contractility

Whole mouse bladders were removed, placed in ice-cold Ca^{2+} free-HEPES dissection buffer, and cut into four ~2 mm wide bladder strips with urothelium denuded or intact for isometric contractility experiments. UBSM strips were hung in an 820MS Isolated Tissue Bath System (Danish Myo Technologies; Aarhus, Denmark) containing warm (37°C) bicarbonate-buffered physiological salt solution (PSS; pH=7.4) consisting of (mM): NaCl (119), $NaHCO_3$ (24), KCl (4.7), KH_2PO_4 (1.2), $MgCl_2$ (1.2), and $CaCl_2$ (2). All chambers were aerated throughout the duration of the experiments with biological atmosphere gas (25% O_2, 5% CO_2, 70% N_2) to maintain pH and tissue oxygenation. Passive tension (~10 mN) was then applied, and strips equilibrated for 1 h (exchanging fresh PSS every 15 min) before exposure to drugs. To ensure tissue strips were viable and to account for variability in strip volume, an initial contractile response was evoked by exposure to 60 mM KCl [145-147]. Similarly, responses to 200 nM carbachol were measured at the end of the experiments to verify tissue viability. Responses were recorded using a PowerLab ADC and LabChart 8 software (ADInstruments, CO USA).

For histamine concentration response experiments, increasing concentrations of histamine dihydrochloride (100 nM – 400 µM) were added directly to the bath. The next concentration was added immediately after the plateau of the previous response or after 2 minutes if no response was noted.

For histamine contractility experiments, a bolus of histamine dihydrochloride (200 µM) was applied for 15 – 20 minutes. This concentration was chosen because it caused a near-maximal contraction in our isometric contractility assay and aligned closely with previously published works [70, 116]. Following 30-minute washout/re-equilibration period, tissues were then incubated with vehicle (H_2O; 1.0% DMSO) or drug for 15-60 minutes before a second exposure to histamine. The drugs used were the H_1 receptor antagonist fexofenadine (5 µM); the H_2 receptor antagonist cimetidine (5 µM); the TRPV1 antagonist capsazepine (10 µM); the muscarinic receptor antagonist atropine (1 µM); the voltage-gated Na^+ channel blocker tetrodotoxin (1 µM); the clathrin-mediated endocytosis inhibitor pitstop2 (15 µM); the dynamin inhibitor dynasore (100 µM); or the G_q/G_s inhibitor YM254890 (1 µM). Tissue responses to carbachol (1 µM) were measured after 30-minute incubation with antagonist. Maximum responses to histamine and area under the curve (AUC) of the histamine contractions were measured using LabChart 8 software. AUC was calculated over equivalent time periods in all strips. Detrusor contractility data were normalized to the initial contraction to histamine (200 µM) or KCl (60 mM) as appropriate. For buffer exchange experiments, histamine (200 µM) was added into a tissue bath for a minimum of 5 minutes or until UBSM strips contracted and desensitized back to baseline. Buffer from this tissue bath was then manually transferred to a separate bath containing a histamine naïve UBSM strip for 10 minutes to measure the response. This protocol was repeated similarly using carbachol and buffer alone.

For desensitization experiments, urothelium-intact UBSM strips were incubated with the cholesterol depleting agent methyl β–cyclodextrin (MβCD; 10 mM) for 60 minutes prior to histamine (200 µM) exposure or cholesterol (5.1 mM) for 1.5 hours following exposure to

MβCD to measure the histamine responses. Tissue responses were normalized to an initial contraction with 60 mM KCl.

Electrical field stimulation was conducted using a CS4/CS8 stimulator and MyoPULSE software (Danish Myo Technologies; Aarhus, Denmark). Tissues received electrical field pulses (0.2 ms width, 2 sec durations, 20 V) sequentially increasing from 0.5 – 50 Hz. Responses were recorded before and after 1-hour exposure to vehicle (dH_2O) or histamine (200 μM).

3.5. Drugs and Chemicals

Fexofenadine and cimetidine were obtained from Fisher Scientific (Waltham, MA USA). Capsazepine was obtained from Cayman Chemical Company (Ann Arbor, MI USA) Tetrodotoxin was obtained from Hello Bio (Princeton, NJ USA). Unless otherwise noted, histamine, methyl-β-cyclodextrin, and all other reagents/chemicals were obtained from Sigma-Aldrich (Cleveland, OH USA). Stock solutions of methyl-β-cyclodextrin, histamine, and carbachol were made in dH_2O. Stock solutions for all other drugs were made with dimethyl sulfoxide (DMSO).

3.6. Statistical Analysis

Analyses were performed as described previously [148]. The coefficient of variation (%) was calculated as $(\sigma/mean) \times 100$. For comparisons of two unpaired samples of equal variance, statistical significance between groups was established using two-tailed, unpaired Student's *t*-tests (α = 0.05). In paired experiments, statistical significance between groups was established using two-tailed, paired Student's *t*-tests (α = 0.05). For samples of unequal variance, the Mann-Whitney *U*-test was used (α = 0.05). For comparing multiple groups, Brown-Forsythe ANOVA (if unequal SD) was used followed

by Dunnett's T3 multiple comparisons test. For EFS experiments, two-way ANOVA was used followed by Tukey's post hoc analysis to compare individual means. Calculations were performed using Microsoft Excel or GraphPad Prism (GraphPad Software, San Diego, CA). Values are expressed as means ± SEM. Differences with *P* values <0.05 were considered statistically significant**.** Where appropriate, "N" represents number of animals and "n" represents number of replicates from the same animal.

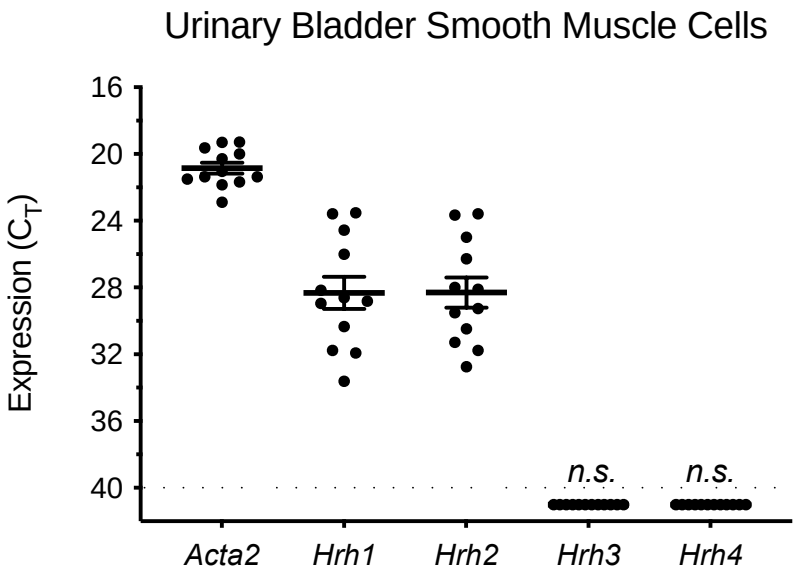

Figure 5: Urinary bladder smooth muscle cells express histamine receptors. Single-cell quantitative RT-PCR was used to measure histamine receptor expression in freshly isolated UBSM cells. Expression of histamine H_1 and H_2 receptor mRNA was detected, but no expression of H_3 or H_4 receptor mRNA was present after 40 cycles. Smooth muscle α-actin mRNA expression was used to verify that cells were smooth muscle. Data points represent each of N=12 animals (n=20 cells per animal). “n.s.”: no signal after 40 cycles.

4. Results

4.1. Histamine H1 and H2 receptor mRNA is expressed in isolated UBSM cells.

Single cell RT-PCR was used to determine which of the four subtypes of histamine receptors were expressed in freshly dissociated detrusor myocytes. UBSM cells expressed both *Hrh1* and *Hrh2* mRNA (**Fig. 5**). *Hrh3* and *Hrh4* mRNA were not detected after 40 amplification cycles. *Acta2* (smooth muscle alpha actin) mRNA was also measured to validate that samples were UBSM cells.

4.2. UBSM contracts and rapidly desensitizes to histamine.

Isometric contractility was performed to directly investigate the immediate effects of histamine on detrusor strips (**Fig. 6**). Histamine cumulative concentration response curves (100 nM – 400 µM) were indicative of rapid desensitization of the contractile response (**Fig. 6A**). Alternatively, a single bolus of 200 µM histamine rapidly contracted both urothelium-intact (**Fig. 6B**) and urothelium-denuded (**Fig. 6C**) bladder strips. As with cumulative concentration response experiments, the histamine response was also transient and highly variable (**Fig. 6D**). Responses to histamine were reproducible after washout, albeit slightly reduced in both intact and denuded strips (68.87±4.47% and 73.93±4.07% respectively; **Fig. 6E**).

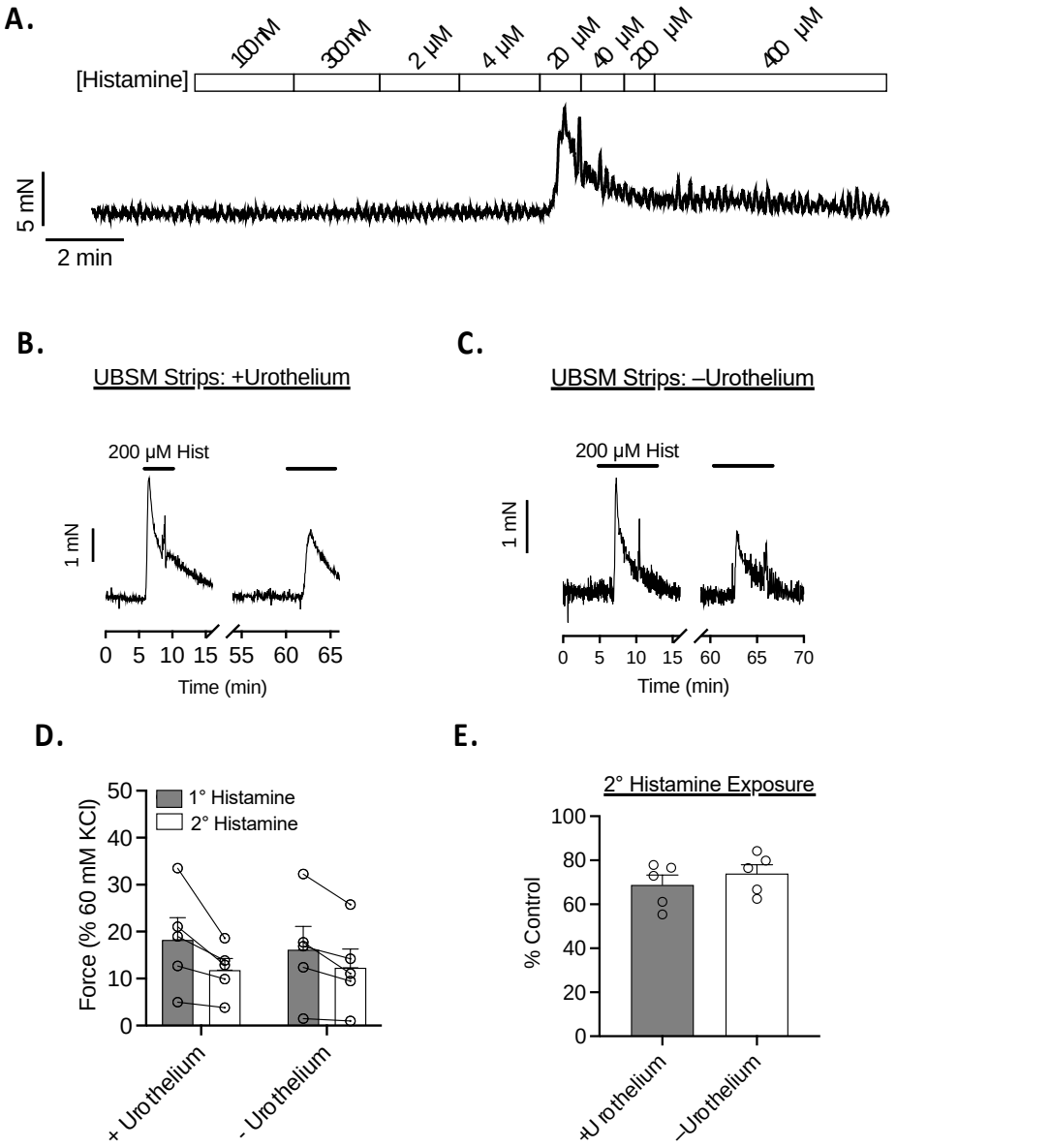

Figure 6: Histamine causes urothelium-independent transient contractions of UBSM strips. (A): Representative traces of rapid desensitization of urothelium-intact urinary bladder strips in response to increasing concentrations of histamine (100 nM – 400 μM). (B, C): Representative traces of urothelium-intact (B) and urothelium-denuded (C) urinary bladder strips during bolus administrations of histamine (200 μM) before and after washout. Repeated washes between exposures to histamine were removed from the traces for clarity. Histamine-induced contractions with and without urothelium do not significantly differ, but the second contraction to histamine tended to be reduced compared to the first (D). Results are presented as a percentage of the initial contraction to 60 mM KCl (C) or the control contraction to 200 μM histamine (E). $P > 0.05$, Brown-Forsythe ANOVA. N=5.

To assess the variability of histamine-induced contractions in UBSM strips, the coefficient of variation was calculated for responses to 60 mM KCl, 200 nM CCh, and 200 µM histamine (**Table 2**). Before normalization, both the first and second contractions to histamine showed a much larger degree of relative variability than contractions to either KCl or CCh. Normalization to the initial KCl response reduced the relative variability for CCh contractions, but the relative variability of each response to histamine was either marginally reduced or increased. This suggested that the relative variability in histamine contractions was not related to variations in strip volume or viability. When normalized to the initial histamine contraction, the relative variability of the second histamine response reduced nearly to that of the KCl contraction. Together, these data suggested that while UBSM contractions to KCl and CCh remained relatively consistent between mice, the magnitude to which histamine could contract UBSM varied between animals. Additionally, these data showed that repeated contractions to histamine in a UBSM strip from a single animal were relatively consistent. Since no differences were noted between intact and denuded tissues when compared to the initial contraction to histamine (**Fig. 6E**), urothelium-intact UBSM strips were utilized in all remaining experiments.

Table 2. Coefficient of variation of UBSM responses to agonists.

	60 mM KCl	**200 nM Carbachol**	**1° Histamine (200 µM)**	**2° Histamine**
Raw data (mN)	27.17%	36.10%	69.57%	78.71%
% KCl Response	–	23.08%	64.11%	83.63%
% Control Histamine	–	–	–	33.40%

4.3. UBSM contractions to histamine are mediated by H1 receptors.

Previous research with porcine bladders determined the H_1 receptor mediates histamine contractions [116]. Thus, we next determined which histamine receptor subtype and G protein pathway mediated histamine-induced contractions. After initial contraction with histamine, the H_2 receptor antagonist cimetidine (5 μM) did not block the subsequent histamine-induced contraction (**Fig. 7A**). The H_1 receptor antagonist fexofenadine (5 μM), however, abolished the response to histamine (**Fig. 7B**) as compared to vehicle (**Fig. 7C**). The G_q/G_s inhibitor YM254890 also abolished the response to histamine as compared to vehicle (**Fig. 7C**). The effect of fexofenadine was specific to histamine, as all tissues contracted similarly to 200 nM carbachol in the presence of either histamine receptor antagonist (**Fig. 7D**). However, YM254890 also abolished contractions to carbachol (1 μM), as muscarinic receptors are also coupled to $G_{q/11}$ (**Fig. 7D**).

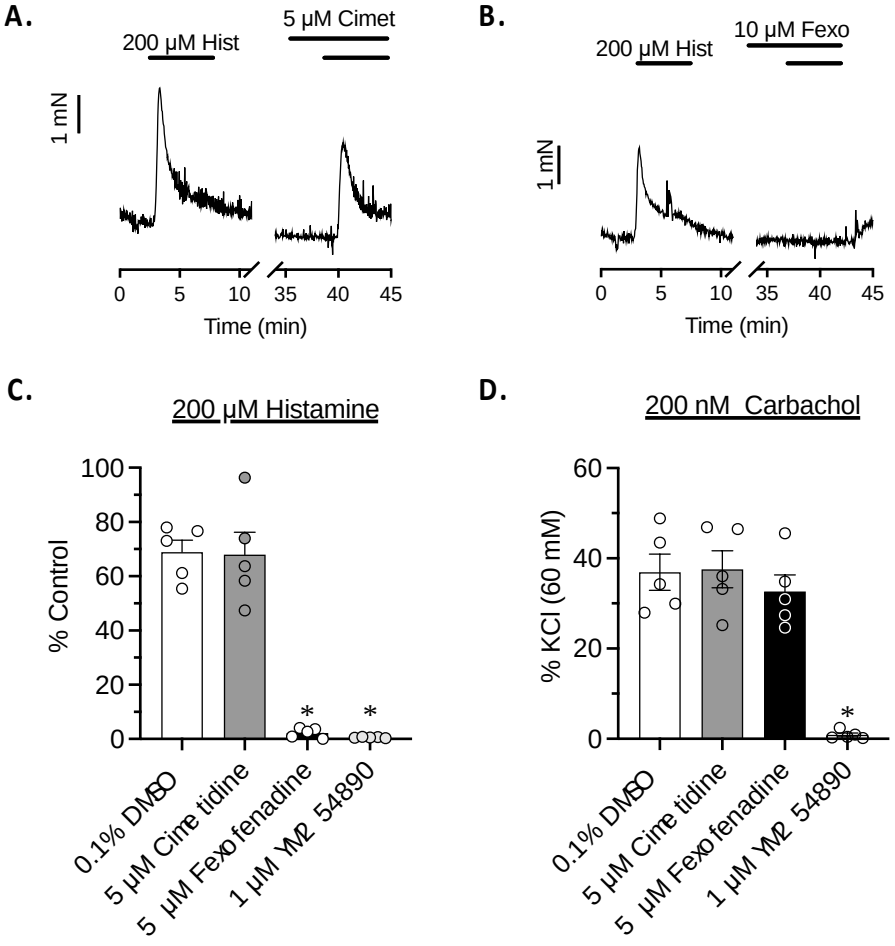

Figure 7: Histamine H_1 receptors mediate UBSM contractions to histamine. Representative traces of histamine-induced contractions from urothelium intact urinary bladder strips, before and after exposure to the H_2 receptor antagonist cimetidine (Cimet; 5 µM; A) or the H_1 receptor antagonist fexofenadine (Fexo; 5 µM; B). Histamine-induced contractions were abolished by fexofenadine (P=0.0003) and the G_q/G_s inhibitor YM254890 (P=0.0003) but were unaffected by cimetidine (C). (D) Neither histamine antagonist significantly altered responses to carbachol (CCh), indicating tissues remained viable and neither drug inhibited muscarinic receptors. Carbachol contractions were abolished by YM254890 (P=0.0022). Results shown as a percentage of the control contraction to histamine (C) or to the initial contraction to 60 mM KCl (D). * = P < 0.05, Brown-Forsythe ANOVA. N=5.

4.4. Contractions to histamine are independent of neurotransmitter release.

Histamine can drive the release of neurotransmitter from sensory nerves, which augments bladder sensory outflow and also contracts UBSM [70]. Therefore, the effects of the voltage gated Na^{2+} channel blocker tetrodotoxin (TTX; 1 µM) and the TRPV1 channel blocker capsazepine (10 µM) on histamine induced UBSM contraction were tested (**Fig. 8**). Neither TTX (**Fig. 8A, D and E**) nor capsazepine (**Fig. 8B, D and E**) had a significant effect on contractions to histamine compared to the vehicle control. The muscarinic antagonist atropine also had no effect (**Fig. 8C, D and E**).

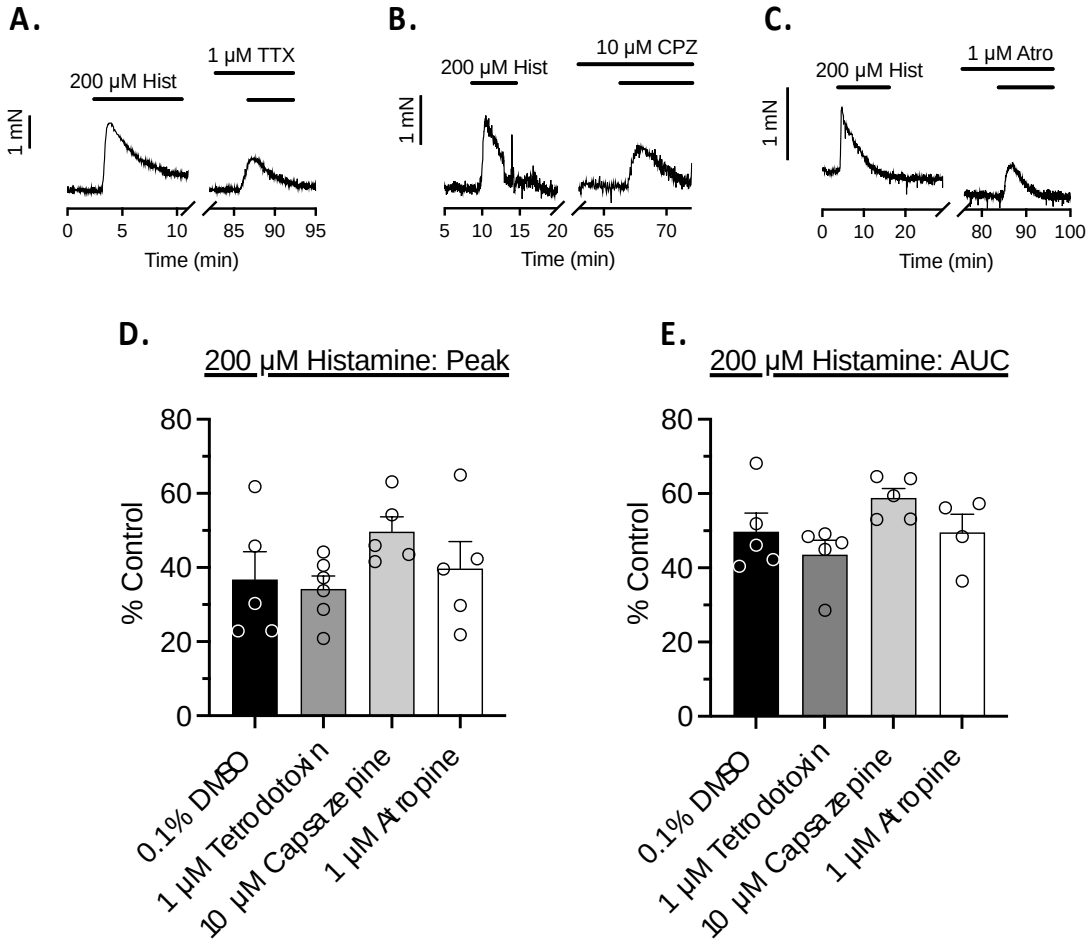

Figure 8: Histamine-dependent contractions are not driven by nerves. Representative traces of histamine-induced contractions on urothelium intact urinary bladder strips incubated with and without the Na^+ channel blocker tetrodotoxin (TTX; A), the TRPV1 channel antagonist capsazepine (CPZ; B), or the muscarinic antagonist atropine (Atro; C). None of the antagonists or blockers significantly altered the amplitude (D) or integral (E) of histamine-induced contractions. $P > 0.05$, Brown-Forsythe ANOVA. l. N=4-6.

4.5. Removal of cholesterol diminished histamine-induced contractions.

Caveolae are small, cholesterol-rich membrane invaginations that play an important role in receptor internalization for some G protein-coupled receptors [149]. In other tissues, disruption of caveolae with the cholesterol-removing agent methyl-β-cyclodextrin (MβCD) prevented rapid desensitization to contractile agonists [150]. Thus, to determine if the transient nature of histamine-induced UBSM contractions was due to caveolae-mediated receptor desensitization, contraction to a bolus of histamine (200 μM) was measured in the absence or presence of 10 mM MβCD (**Fig. 9A, B**). In the presence of MβCD, histamine contractions were significantly diminished as compared to vehicle controls (31.08±5.18% vs 5.25±0.29% respectively; **Fig. 9C**). Cholesterol (5.1 mM) replenishment recovered histamine-induced contractions after exposure to MβCD (10 mM) (**Fig. 9D**), suggesting that the effects of MβCD were specific to depletion of membrane cholesterol. These effects of MβCD were specific to the mechanism in which histamine induces contraction, as all tissues contracted similarly to 200 nM carbachol (**Fig. 9E**).

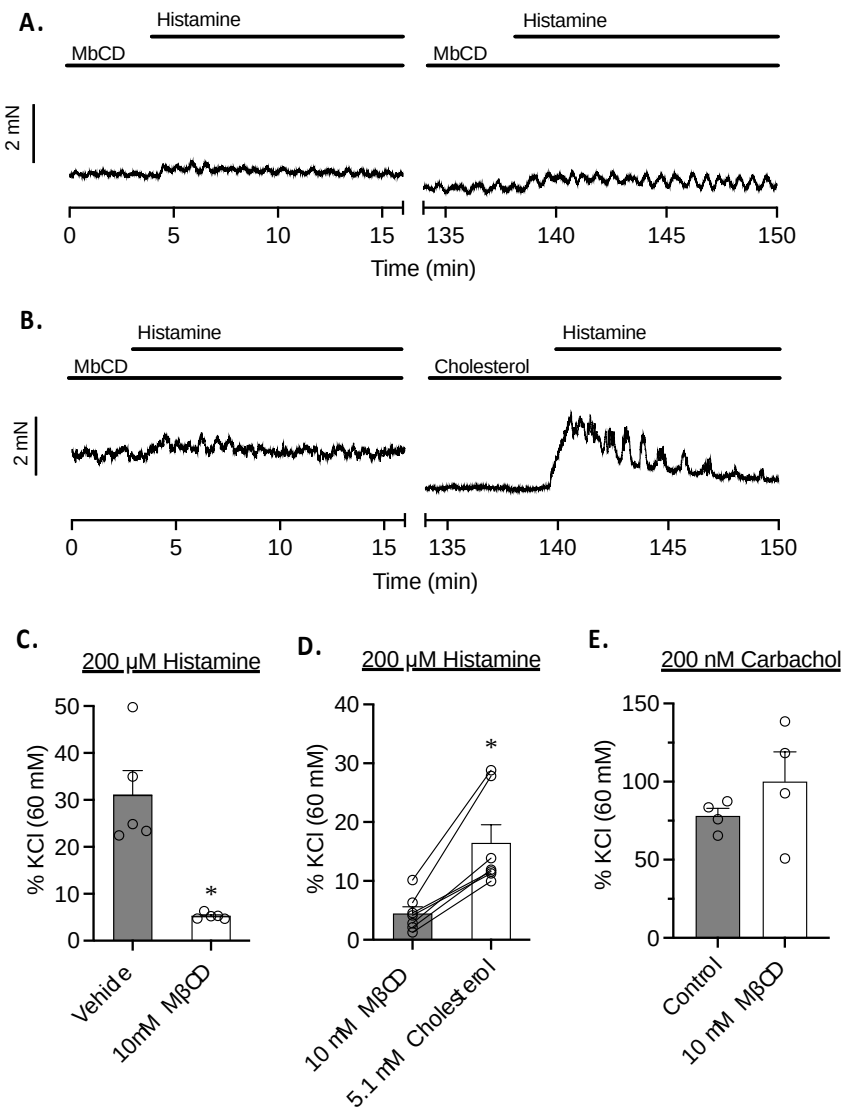

Figure 9: Methyl-β-Cyclodextrin reduces histamine-induced contractions in a cholesterol dependent manner. (A, B) Representative traces of sequential histamine-induced contractions in the presence of the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) (A), or MβCD followed by 5.1 mM cholesterol (B). (C) MβCD significantly reduced urinary bladder contractions to histamine as compared to vehicle (P=0.0075; Welch's t-test). (D) Cholesterol restores the reduced contractions to histamine induced by MβCD (P=0.0072; Welch's t-test). (E) MβCD did not significantly alter response to carbachol (CCh), indicating tissues remained viable and MβCD had no effect on muscarinic receptor-dependent contractions. * = P < 0.05. N=5-7.

4.6. UBSM desensitization to histamine is independent of dynamin-mediated endocytosis, clathrin-mediated endocytosis, and histamine metabolism.

Inhibitors of endocytosis were used to determine potential pathways by which urinary bladder smooth muscle desensitized to histamine (**Fig. 10**). Neither the dynamin-mediated endocytosis inhibitor dynasore (100 µM) nor the clathrin-mediated endocytosis inhibitor pitstop2 (15 µM) increased the the amplitude (**Fig. 10C**) or integral (**Fig. 10D**) of histamine contractions. Instead, dynasore significantly reduced both the amplitude and integral of histamine-induced contractions. Carbachol-induced contractions were reduced in the presence of dynasore as compared to vehicle control (**Fig. 10E**) but remained the same in the presence of pitstop2.

A histamine buffer exchange bioassay was performed to determine if rapid metabolism was the cause of the short-lived contractions (**Fig.** 11). Buffer transferred from desensitized tissues still contracted naïve UBSM, whereas transferred buffer alone did not change the baseline.

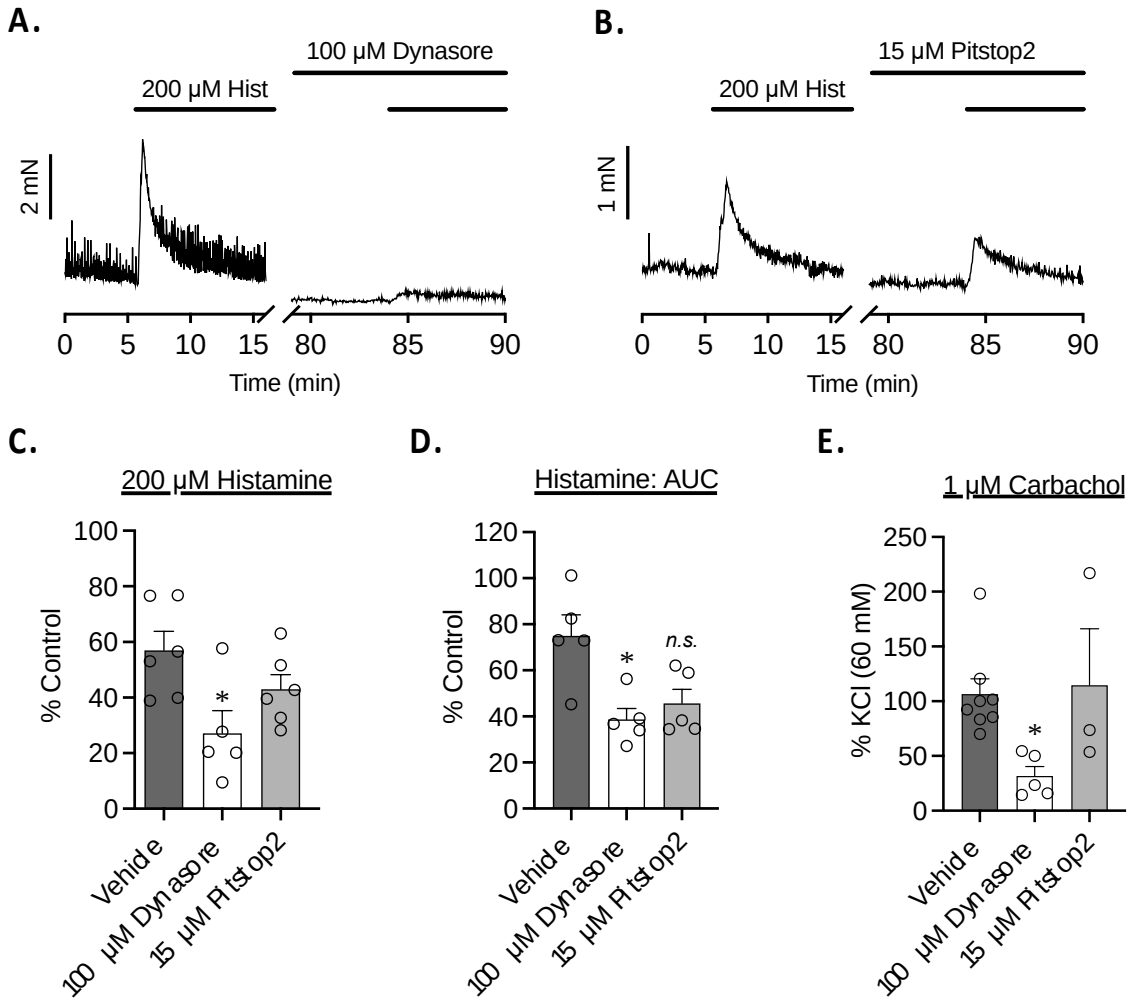

Figure 10: Histamine-induced desensitization is not dependent on dynamin- or clathrin-mediated endocytosis. Representative traces of histamine-induced contractions before and after exposure to the dynamin-mediated endocytosis inhibitor dynasore (100 µM) (A) or the clathrin-mediated endocytosis inhibitor pitstop2 (15 µM) (B). Dynasore significantly reduced both the amplitude (C) and integral (D) of contractions to histamine as opposed to augmenting them (P=0.045 and P=0.023, respectively). (E) Dynasore also reduced carbachol contractions, whereas pitstop2 did not. * = P< 0.05, Brown-Forsythe ANOVA. *n.s*. = not significant. N=5-6.

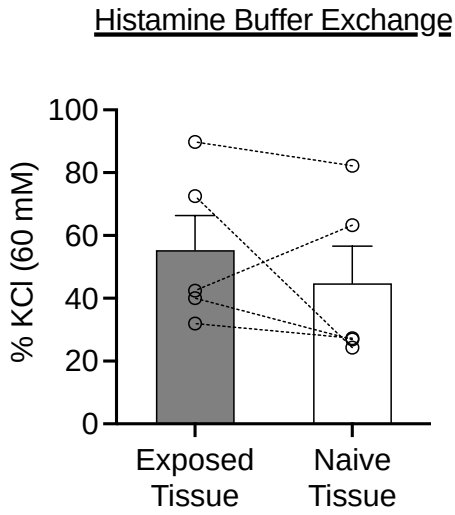

Figure 11: The transient nature of histamine-induced contractions is not due to metabolism. Summary bar graph indicates that, even after desensitizing one tissue, the same histamine-containing buffer contracted naïve UBSM strips. $P > 0.05$. Results are presented as a percentage to 60 mM KCl. Dashed lines connect exposed tissue to naïve tissue from each experiment. N=5.

4.7. Histamine does not affect UBSM contractions to electrical field stimulation.

Electrical field stimulation (EFS) mimics normal physiological stimuli to the urinary bladder by causing the release of acetylcholine and ATP from cholinergic and purinergic efferent nerve terminals in the bladder wall [151-153]. Contractions resulting from EFS are also completely blocked by TTX, suggesting the responses indeed are nerve-mediated and not due to depolarization of smooth muscle directly [154, 155]. To determine if prolonged exposure to histamine altered UBSM contractile responses to other physiological stimuli, EFS frequency-response experiments were performed before and after a 1-hour exposure to histamine (200 μM). Neither vehicle nor histamine altered contractions elicited by EFS (**Fig. 12A, B**).

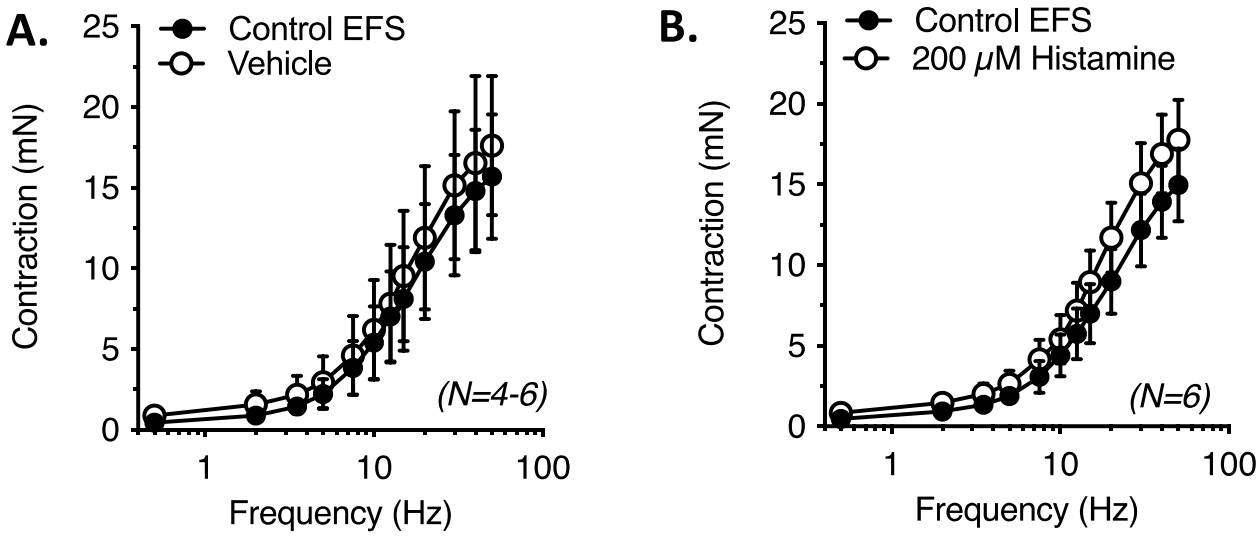

Figure 12: Prolonged histamine exposure had no effect on nerve-mediated contractions of urinary bladder smooth muscle. Frequency-response curves from urothelium-intact urinary bladder strips prior to and during incubation with vehicle (A) or histamine (B). Histamine had no effect on contractions evoked by electrical field stimulation (EFS). $P > 0.05$, 2-way ANOVA. N=4-6 for control and vehicle EFS; N=6 for control and histamine EFS.

5. Discussion

Using isometric contractility and pharmacological tools, our study thoroughly examined the role of histamine as a direct contractor of urinary bladder smooth muscle. We determined that UBSM contractions to histamine are due to direct activation of the histamine H_1 receptor on smooth muscle, and not due to release of neurotransmitter from afferent or efferent nerves. Further, that UBSM rapidly desensitizes to histamine, though not through receptor internalization or rapid histamine metabolism. Lastly, while histamine does contract UBSM directly, it does not have any profound effect on nerve-evoked contractions. Together, our findings suggest that any direct role for histamine signaling in UBSM is independent of immediate changes to contractility or excitability.

5.1. Histamine in the Pathogenesis of Bladder Dysfunction

In terms of bladder disease, histamine contributes to pelvic pain and bladder hypersensitivity in interstitial cystitis [156]. The expression of histamine receptors in the bladder wall is increased and responses to exogenous histamine are decreased in patients with interstitial cystitis, suggesting the desensitization seen in our experiments also occurs *in vivo* [122, 157]. Recently, Grundy *et al.* also found that histamine was able to sensitize sensory nerves and increase afferent outflow in response to urinary bladder distention in the absence of a disease, but without augmenting contractility [70]. Increased sensory outflow due to histamine was related to the recruitment of TRPV1-positive C-fibers normally engaged only at supraphysiological intravesical pressures. Thusly, any apparent alteration in voiding was likely to be a result of improper efferent signals driving UBSM contractions at much lower pressures due to the increase in sensory outflow falsely signaling a full bladder. Though not necessarily linked to histamine, this aberrant

recruitment of C-fibers also drives bladder overactivity in mouse models of social stress, which also strongly resembles and inflammatory response [128, 158]. Taken together, it is likely that inflammatory insults alter both the amount and type of sensory outflow relayed to the CNS during filling to significantly impact voiding. Also, these findings support our conclusion that changes in urinary bladder function due to histamine release are not related to direct alteration of UBSM contractility but are instead due to increases in sensory outflow from the bladder during filling.

Whereas many other studies focus on the additive role of histamine regarding urinary bladder pathophysiology that leads to LUTS, our findings suggest that, unto itself, histamine does not mediate UBSM contractility in the absence of disease and other pro-contractile mediators. Future experiments will assess if changes to UBSM contractility elicited by histamine differ in animal models of inflammatory bladder dysfunction (such as interstitial cystitis), or if the presence of other inflammatory cytokines in addition to histamine alters UBSM contractility directly.

5.2. Why are histamine contractions variable?

One of the more interesting findings of these experiments was the inherent variability of histamine-induced contractions between mice. Contractile responses in UBSM strips are often normalized to contractions to a depolarizing stimulus (e.g., 60 mM KCl), since this accounts for variability in strip length, width, and viability over multiple experiments [145-147]. Central to this methodology is the idea that, in the absence of disease or other treatments, a contractile response to another agonist should be consistently proportional to the contraction to the depolarizing stimulus. This indeed holds true for responses driven by carbachol in our experiments, which is expected given the role of muscarinic receptors

in driving bladder contractility and their ability to initiate calcium influx through voltage-gated Ca^{2+} channels [68]. However, our finding that histamine responses are not proportional to either KCl or carbachol contractions suggests that histamine receptor expression is extremely labile between mice and dependent on factors which are currently unclear. The mice used in these experiments were acquired from the same breeder, at the same age, and housed in the same conditions. Yet, the expression of *Hrh1* and *Hrh2* mRNA is also more variable than that of *Acta2* (**Fig.** 5). Since the tissues used for single-cell PCR and for tissue bath experiments came from different animals, we cannot correlate receptor expression with contractile responses directly; however, our overall findings suggest that this variability in histamine receptor expression may indeed match with changes in responses to histamine.

Additional experiments are needed determine if circulating histamine levels can affect histamine receptor expression in UBSM and if regional differences in histamine receptor expression exist throughout the bladder wall. Also, levels of circulating stress hormones and inflammatory cytokines should be assessed to determine if prior stresses or inflammatory insult can drive down histamine receptor expression through histamine release and receptor desensitization.

5.3. Why are histamine contractions transient?

Other studies measuring urinary bladder contractility to histamine suggest receptor desensitization plays an important role in mediating its effects [157]. To test this, we used MβCD to determine if the desensitization of histamine-induced contractions could be prevented by inhibiting caveolae-dependent receptor internalization. In rat aorta, MβCD prevents rapid tachyphylaxis and desensitization to subsequent contractions by

angiotensin-II [150]. However, instead of prolonging contraction or augmenting the maximal response, MβCD significantly reduced UBSM contractions to histamine. This reduction could be prevented by re-introducing cholesterol to the bath, suggesting that it was depletion of cholesterol mediating the effect of MβCD, and not a non-specific effect on other pathways. Our findings still suggest that caveolae (and perhaps membrane cholesterol in general) play an important role in histamine-induced UBSM contractions that is not necessary for responses to other agonists – namely, carbachol. Also, UBSM contractions induced by serotonin and angiotensin II are also reduced in the presence of MβCD and recover upon reintroduction of cholesterol [159], suggesting histamine is not alone in its requirement for membrane cholesterol to mediate a contractile response. In all of these cases, it may be the disruption of UBSM smooth cell calcium-induced calcium release pathways by MβCD that attenuates the response [160], suggesting that histamine and other such amines may also be initiating different types of calcium signals to mediate contraction as compared to muscarinic agonists. So, while caveolae-mediated receptor internalization does not appear to be responsible for the desensitization of UBSM strips to histamine, caveolae do appear to play a pivotal role in the signaling cascade required to drive the contractile response.

We also investigated other possible internalization pathways, including dynamin- and clathrin-mediated endocytosis. To investigate this, the clathrin-mediated endocytosis inhibitor pitstop2 was used as a known pharmacological inhibitor that prevents receptor endocytosis via inhibition of the clathrin terminal domain [161]. The dynamin-mediated endocytosis inhibitor was also used, as it blocks by disabling vesicle scission [162, 163]. Neither of these prolonged histamine-induced contractions. Dynasore does appear to

slightly reduce the histamine response; this could be due to the drug's ability to reduce lipid levels as well [163]. Nonetheless, if the desensitization of histamine receptors was due to dynamin-mediated endocytosis, the response to histamine should be increased or prolonged as opposed to reduced. Thus, our findings suggest that histamine contractile signaling does not depend on endocytosis via caveolae, dynamin or clathrin.

What is causing this rapid desensitization, if not caveolae-mediated internalization? One alterative hypothesis we considered was that the effects of histamine are instead dependent on rapid metabolism of histamine. Based on our results exchanging the buffer from a UBSM strip desensitized with histamine to a naïve strip, we found that histamine was still present in the bath and thus did not undergo rapid metabolism. This finding shows that rapid desensitization to histamine is not dependent on metabolizing enzymes mentioned as a cause for the desensitization trachea, gut, and kidney[164-166].

Altogether, our findings suggest it is more likely that desensitization of the histamine response is caused by β arrestin-dependent desensitization or G protein-coupled receptor kinase (GRK)-mediated phosphorylation than receptor internalization [167]. We attempted to test this with commercially available β arrestin inhibitors, but problems with solubility prevented us from thoroughly testing this hypothesis. Future experiments, either with β arrestin knockout mice or newer, more soluble β arrestin inhibitors, will be needed to determine if this mechanism is involved. Nonetheless, UBSM appears keenly capable of mitigating any contractile response initiated by histamine without altering its ability to respond to other contractile signals. Thus, the nature of histamine-induced contractions suggests it does not directly contribute to UBSM dysfunction by altering contractility, and

further suggests that the smooth muscle itself has mechanisms in place to protect against prolonged contractions driven directly by inflammatory mediators like histamine.

5.4. Species Differences in Urinary Bladder Responses to Histamine

Histamine alters detrusor contractility based on the histamine receptor subtypes in the urinary bladder of different species (or in the case of the rat, not at all [168]. In pigs, H_1 receptors drive contraction of UBSM strips, while H_2 receptors in the urothelium oppose this response [116]. In guinea pig urinary bladder, histamine also potentiates purinergic nerve-evoked contractions in addition to causing transient contractions by itself [107]. We tested both possibilities in the mouse urinary bladder and found neither to be the case. One possible explanation is the presence of a contractile muscularis mucosa in guinea pig bladders that, as of yet, is not found in mice [6]. Another possible explanation for the lack of effect on EFS-induced contraction is that mice could lack a purinergic component to nerve-mediated contractions. This is likely not the case, as both purinergic and cholinergic components of nerve-evoked UBSM contraction have been differentiated using increasing frequencies of EFS [169]. Had the purinergic component been altered, we would expect to see increased EFS contractions below 15 Hz [169]; we indeed did not. None of these prior studies investigated if any of these species-specific differences involved indirect release of contractile compounds from sensory nerves or mast cells driving contractions to histamine as opposed to direct actions on UBSM, which also may be responsible for the perceived differences.

Other studies also reported differences between histamine-induced contractions in porcine detrusor strips with and without urothelium, where responses to histamine are augmented once the urothelium was denuded [38]. In our study, removal of the urothelium

altered neither the maximum contractile response to histamine nor its desensitization (**Fig. 6**). However, the study in porcine bladder utilized strips selectively dissected from the bladder trigone; given the size of the mouse bladder, our strips encompassed the entire length from trigone to dome. Also, unlike the mouse bladder, these strips from porcine bladder trigone also were sympathetically innervated, suggesting pig trigone smooth muscle contains a very different complement of receptors as compared to other segments of the urinary bladder wall and makes any comparisons herein extremely difficult.

In summary, species-dependent differences that exist regarding the effects of histamine in the bladder may relate to muscularis mucosa contractility or the indirect effects of histamine on sensory nerves or purinergic neurotransmission. Investigation of regional differences in histaminergic contractility within the bladder may also be worthwhile, but this will require the use of larger animal models than mice and careful consideration of nerve innervation.

5.5. Limitations

Our study is not without its own limitations. We did not directly measure the histamine-specific effects on afferent or efferent nerves that could drive UBSM contractions *in vivo*. In addition, the exact mechanism by which histamine contractions desensitize remains unclear. While we also attempted to interrogate this pharmacologically, the relative insolubility of pharmacological inhibitors of β arrestin proved insurmountable; thus, this possibility requires further investigation using genetic knockout mouse models. Although this study is limited by only examining histamine effects on isolated UBSM strips instead of the whole intact bladder, these findings have revealed that the effects of histamine

alone on UBSM are short-lived and incapable of augmenting contractions induced by physiological stimuli – a finding that could otherwise be obscured in whole bladder *in vivo* experiments.

5.6. Conclusions

In closing, our findings suggest that histamine-induced UBSM contractions in mice are transient in nature, highly variable, dependent on histamine H_1 receptors and membrane cholesterol, and independent of nerve activation. The transient nature of the contraction is not due to histamine metabolism or internalization of histamine receptors via clarthin- or dynamin-mediated endocytosis, suggesting a role for receptor desensitization by β arrestins or GRK-mediated receptor phosphorylation. Furthermore, since histamine does not alter nerve-evoked contractility or sensitivity to cholinergic agonists, UBSM contractility seems largely unaffected by prolonged exposure to histamine in the absence of disease. While this study provides evidence for the non-neuronal role of histamine as a direct contractile agonist in UBSM, it also suggests that any role for histamine as a direct sensitizer or regulator of contraction in urinary bladder smooth muscle is unlikely.

5.7. Acknowledgements

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MAST CELL STIMULATOR COMPOUND 48/80 CAUSES UROTHELIUM-DEPENDENT INCREASES IN MURINE URINARY BLADDER CONTRACTILITY

Modified from: Jones BM, Mingin GC, and Tykocki NR. “Mast Cell Stimulator Compound 48/80 Causes Urothelium-Dependent Increases in Murine Urinary Bladder Contractility”. Am J Physiol Renal Physiol 2023 [In Press].

1. Abstract

Mast cells and degranulation of pre-formed inflammatory mediators contribute to lower urinary tract symptoms. This study investigates pathways by which the mast cell stimulator Compound 48/80 alters urinary bladder smooth muscle contractility via mast cell activation. We hypothesized that: (1) mast cell degranulation will cause spontaneous urinary bladder smooth muscle contractions; and (2) these contractions are caused by urothelium-derived PGE_2. Urothelium intact and denuded urinary bladder strips were collected from mast cell sufficient (C57Bl/6) and deficient (B6.Cg-*Kit*$^{w\text{-}sh}$) mice to determine if Compound 48/80 altered urinary bladder smooth muscle (UBSM) contractility. Electrical field stimulation was used to assess the effects of Compound 48/80 on nerve-evoked contractions. Antagonists/inhibitors were utilized to identify prostanoid signaling pathways activated or if direct activation of nerves was involved. Compound 48/80 caused slow-developing contractions, increased phasic activity, and augmented nerve-evoked responses in both mast cell sufficient and deficient mice. Nerve blockade had no effect on these responses; however, they were eliminated by removing the urothelium. Blocking P2 purinoreceptors, cyclooxygenases, or G protein signaling abolished Compound 48/80 responses. However, only combined blockade of prostaglandin E_2 (EP1), prostaglandin $F_{2\alpha}$ (FP), and thromboxane A_2 (TP) receptors inhibited Compound 48/80 induced responses. Thus, the effects of compound 48/80 are urothelium dependent, but independent of mast cells. Further, these effects are mediated by druggable inflammatory pathways that may be used to manage inflammatory non-neurogenic bladder hyperactivity. Finally, these data strongly suggest that great care

must be taken when using Compound 48/80 to determine mast cell-dependent responses in the urinary bladder.

2. Introduction

Mast cells are granule-containing immune cells that regulate inflammatory and physiological responses via synthesis and release of pro-inflammatory mediators [98]. Recently, mast cells were also implicated as modulators of lower urinary tract symptoms including frequent urination, urgency, nocturia, and/or urge incontinence [105, 170]. In patients, overactive bladder (OAB) and interstitial cystitis/painful bladder syndrome (IC/BPS) are associated with increased bladder wall mast cell infiltration and increased levels of pro-inflammatory mediators (e.g., histamine, prostaglandins) in urine [97, 171]. This is also true in animal models of chronic systemic inflammation: ovalbumin sensitization increased histamine release from guinea pig bladder strips [108] and trinitribenzene sulfonic acid-colon irritation caused a mast cell-dependent increase in urinary frequency in mice [110]. Systemic inflammation also increases urothelial permeability and urothelial release of ATP, prostaglandins, nitric oxide, and acetylcholine, which in turn alters visceromotor responses to bladder distention [172-176] . While these results assess how systemic inflammation and infiltrating mast cells affect the pathophysiology of bladder dysfunction, they do not account for the physiological consequence of local inflammation or for the function of mast cells normally localized in the bladder wall. Thus, it is not completely clear how local activation of mast cells alters bladder function.

The basic secretagogue compound 48/80 is a pharmacological agent first used to cause anaphylaxis-like symptoms and induce the release of histamine in plasma [177-180] . In 1951, Paton referenced not only the ability of compound 48/80 to “liberate histamine”, but to also cause the release of a “slow-contracting substance” [178]. Compound 48/80 is

now widely considered a prototypical activator of mast cells [180-183]. It is used regularly to assess how activation and degranulation of mast cells affects smooth muscle contractility in tissues with a large resident population of mast cells (e.g., trachea [184], lung [185], colon [186-188], esophagus [189]). While compound 48/80 has been used to assess changes in detrusor contractility as it relates to systemic inflammation [110], it has not been utilized to understand the effects of basic secretagogues on normal urinary bladder tissue. Additionally, the effects of compound 48/80 in the urinary bladder are most often attributed to histamine release and changes to vascular permeability *via* neuronal activation as opposed to direct effects on bladder smooth muscle [190]. Thus, it is unknown if any effects of compound 48/80 on detrusor contractility are dependent on mast cells alone or on other cell types.

Urothelial-derived mediator release can be activated through both physical and chemical stimuli. Most studies focus on the urothelium-dependent mechanical responses to stretch [17, 20], hydrostatic pressure [16, 191, 192], distension [20, 193] and urothelial trauma [19]. These studies link disrupted voiding/storage and increased transient contractions to ATP and PGE_2 derived from the urothelium. However, the findings are insufficient in understanding the direct relationship of increased UBSM excitability and changes in contractility that cause urothelium activation. While release of urothelial ATP in response to stretch or trauma can drive prostanoid synthesis, it is unclear if these mechanisms are engaged by basic secretagogues or in response to mast cell degranulation.

This study investigates the purinergic and prostanoid pathways by which the mast cell stimulator Compound 48/80 alters urinary bladder smooth muscle contractility, and if activation of these pathways is dependent on mast cells. We tested two hypotheses: (1)

that mast cell degranulation will cause spontaneous urinary bladder smooth muscle contractions; and (2) that these contractions are caused by urothelium-derived PGE_2.

3. Materials and Methods

3.1. Animal Care and Use

All animal procedures followed institutional guidelines and were approved by the Institutional Animal Care and Use Committees of Michigan State University (NIH Assurance D16-0054 2021-311), and in accordance with ARRIVE guidelines. Male C57BL/6 mice (RRID: IMSR_JAX:000664) and male B6.Cg-$Kit^{W\text{-}sh}$ mice (RRID: IMSR_JAX:030764) (8 – 12 weeks old; Jackson Laboratory, Bar Harbor, ME) were group housed in a temperature- and humidity-controlled environment with a 12 hr light/dark cycle. B6.Cg-$Kit^{W\text{-}sh}$ mice contain a mutation in the *c*-kit gene that results in mast cell deficiency from birth without affecting other hematopoietic cells [194]. Mice were provided *ad libitum* access to standard chow and water. Prior to all experimental procedures described below, mice were euthanized by intraperitoneal injection of pentobarbital (>150 mg/kg) followed by decapitation. Mice were randomly selected from cohort housing.

3.2. Isometric Contractility

As described previously [131], whole mouse bladders were removed, placed in ice-cold Ca^{2+} free-HEPES dissection buffer, and cut into approximately 2 mm wide strips with urothelium intact or denuded for isometric contractility experiments. Urothelium was removed by blunt dissection. UBSM strips were hung in an 820MS Isolated Tissue Bath System (Danish Myo Technologies; Aarhus, Denmark) containing warm (37°C) bicarbonate-buffered physiological salt solution (PSS) consisting of (mM): NaCl (119), $NaHCO_3$ (24), KCl (4.7), KH_2PO_4 (1.2), $MgCl_2$ (1.2), $CaCl_2$ (2) and glucose (7); pH = 7.4. All

chambers were bubbled throughout the duration of the experiments with biological atmosphere gas (25% O_2, 5% CO_2, 70% N_2) to maintain pH and tissue oxygenation. Passive tension (~10 mN) was then applied, and strips equilibrated for 1 h (exchanging fresh PSS every 15 min) before exposure to drugs. To ensure tissue strips were viable, an initial contractile response was evoked by exposure to 60 mM KCl. Similarly, responses to 1 µM carbachol were measured at the end of the experiments to verify tissue viability. Responses were recorded using a PowerLab ADC and Lab Chart 8 software (ADInstruments, CO USA).

Following 30-minute washout/re-equilibration period after exposure to KCl, tissues were then incubated with vehicle (dH_2O; 0.01% EtOH; 0.01% DMSO) or antagonist/inhibitor for 15-60 minutes before exposure to compound 48/80. The drugs used are listed in **Table 3**. A bolus of compound 48/80 (10 µg/mL) was then applied for 30 minutes. Detrusor contractility data were normalized to initial contraction with 60 mM KCl.

For nerve-evoked contraction experiments, electrical field stimulation (EFS) was conducted using a CS4/CS8 stimulator and MyoPULSE software (Danish Myo Technologies; Aarhus, Denmark). Tissues received electrical field pulses (0.2 ms width, 2 sec durations, 20 V) sequentially increasing from 0.5 – 50 Hz. Responses were recorded before and during exposure to compound 48/80.

To assess if bioactive substances from the urothelium were responsible for compound 48/80-induced UBSM contractions, a tissue bath urothelium bioassay was performed. The urothelium was carefully removed from UBSM, cut into two strips and placed in ice-cold Ca^{2+} free-HEPES dissection buffer. Prior to incubation with compound 48/80, urothelial strips were placed and weighted to the bottom of the bath underneath

urothelium-denuded strips. Following a 10-minute equilibration period, a bolus of compound 48/80 (10 µg/mL) was applied.

3.3. Drugs and Chemicals

SC-51089 and YM-254890 were obtained from Tocris (Bristol, United Kingdom). ^{10}Panx was obtained from ApexBio (Houston, TX USA). Cimetidine and Fexofenadine was obtained from Fisher Scientific (Hampton, NH USA). AL8810 was obtained from Cayman Chemical (Ann Arbor, MI USA). Tetrodotoxin was obtained from Hello Bio (Princeton, NJ USA). Compound 48/80, GR 32191B, Indomethacin, and pyridoxalphosphate-6-azophenyl-2',4'- disulphonic acid (PPADS), as well as all other salts and reagents, were obtained from Sigma-Aldrich (Cleveland, OH USA).

Table 3. Pharmaceutical agents and concentrations used.

Name	Type	Concentration
10Panx	Pannexin-1 mimetic inhibitory peptide	200 µM
AL8810	Prostaglandin F2 alpha receptor antagonist	5 µM
Atropine	Cholinergic receptor antagonist	1 µM
Cimetidine	Histamine H2 receptor antagonist	10 µM
Fexofenadine	Histamine H1 receptor antagonist	10 µM
GR32191B	Thromboxane A2/TP receptor antagonist	1 or 2 µM
Indomethacin	Nonselective COX inhibitor	5 µM
PPADS	Nonselective purinergic receptor antagonist	100 µM
SC-51089	Prostanoid EP1 receptor antagonist	10 µM
Tetrodotoxin	Voltage-gated Na^+ channel inhibitor	1 µM
YM-254890	$G_{aq/11}$ inhibitor	10 µM

3.4. Statistical Analysis

Tissue responses were normalized to the initial contraction with 60 mM KCl. For comparisons of two unpaired samples of equal variance, statistical significance between groups was established using two-tailed, unpaired Student's *t*-tests ($\alpha = 0.05$). Nested t-tests and nested one-way ANOVA ($\alpha = 0.05$) were used to determine significance of transient contractions measured before and after exposure to drug or urothelial strips. Otherwise, statistical significance between groups was established using a two-tailed, unpaired or paired Student's *t*-test where appropriate ($\alpha = 0.05$). For multiple sample comparisons of responses to electrical field stimulation, two-way ANOVA was used followed by Tukey's post hoc analysis to compare individual means. One-way ANOVA was used to determine if the means of three independent outcomes measured from one isolated UBSM strip were significant when compared ($\alpha = 0.05$). Calculations were performed using Microsoft Excel or GraphPad Prism 9.5 (GraphPad Software, San Diego, CA). Values are expressed as means ± SEM. Differences with *P* values <0.05 were considered statistically significant**.**

For rigor and reproducibility, replicates from the same animal were not used in our analysis. As such, "N" represents both the number of strips used and the number of individual animals. For example: "N=6" means that the experiment was repeated 6 times using a strip from a different animal each time. While four strips were collected per bladder, each strip was also considered unique and only compared to other strips using unpaired analyses. When analyzing transient contractions, n= 30 samples were taken at each time point for N=1 mouse. Sample size was determined using power analysis calculated using G*Power 3.1 software (Universität Düsseldorf, Germany). For β=0.8 with

a calculated effect size of 2.8 (derived from preliminary data), sample size should be N > 4.

4. Results

4.1. The effects of Compound 48/80 on UBSM contractility are mast cell independent but urothelium-dependent.

The effects of compound 48/80 on urothelium intact and denuded UBSM strips was determined using isometric contractility (**Fig. 13**). In wild-type C57Bl/6J mice, application of compound 48/80 caused a slowly developing contraction and an increase in phasic contractile activity that was present in urothelium-intact UBSM strips but absent in urothelium-denuded strips (**Fig. 13A, B**). To determine if mast cell activation and subsequent degranulation were responsible for compound 48/80-induced contractions, responses to compound 48/80 were measured in UBSM strips from mast cell deficient (cg-Kit$^{w\text{-}sh}$) mice. Compound 48/80 again caused a slow-developing contraction and an increase in phasic activity in urothelium-intact UBSM strips from cg-Kit$^{w\text{-}sh}$ mice that was abolished by removing the urothelium (**Fig. 13C, D**). While denudation significantly inhibited contractions to compound 48/80 in both mouse models, there were no differences between mast cell sufficient and deficient mice (**Fig. 13E**). Since compound 48/80 is considered a mast cell activator, we next tested if these responses were caused by histamine released after mast cell degranulation in C57Bl/6J mice. Contractions caused by compound 48/80 were not due to histamine release, as neither the histamine H1 receptor antagonist fexofenadine (10 µM) nor the histamine H2 receptor antagonist cimetidine (10 µM) reduced the responses (**Fig. 13F**). Because compound 48/80 also augmented phasic contractions, we then compared their amplitude before and after

exposure to compound 48/80 (10 µg/mL) in both strains, with and without urothelium. With the urothelium intact, phasic contractions were significantly augmented in both C57bl/6J and cg-Kit$^{w\text{-}sh}$ mice (**Fig. 14A, C**). In both strains, urothelial denudation nearly abolished any increase in transient contractions (**Fig. 14B, D**).

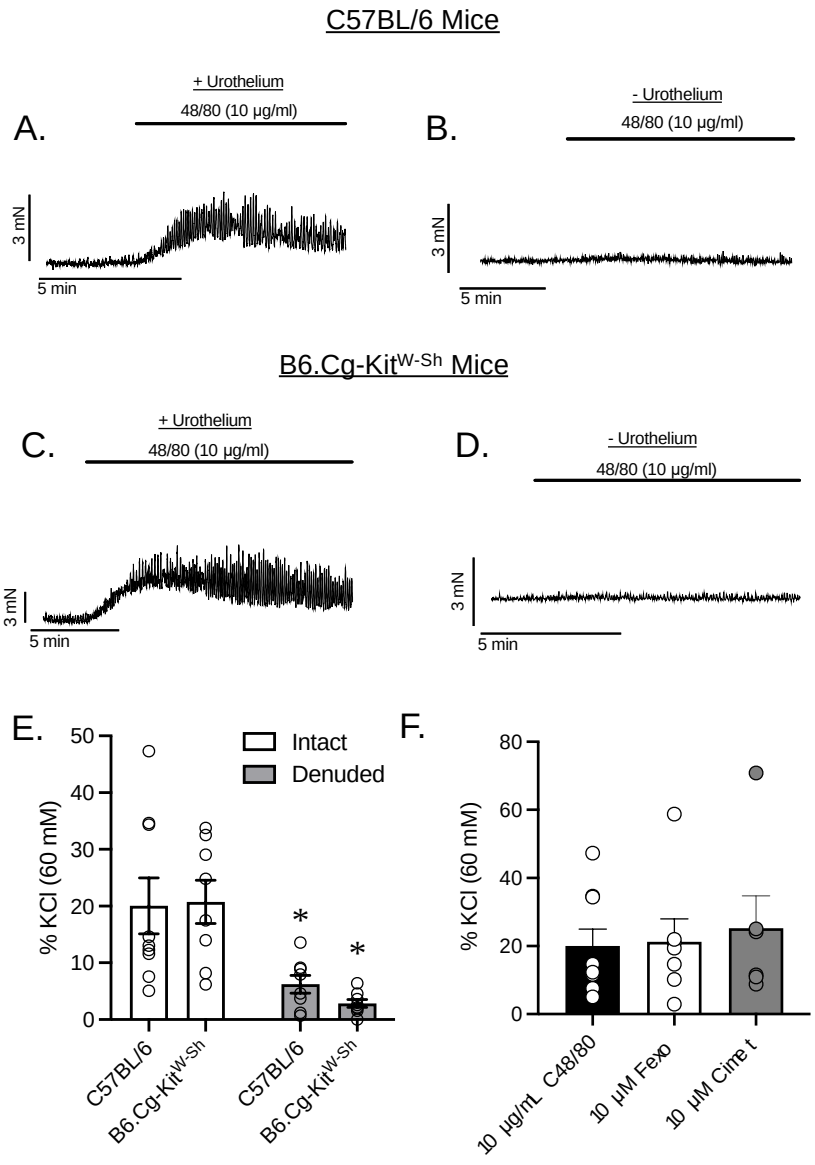

Figure 13: Compound 48/80 causes urothelium-dependent contractions in mast cell sufficient and deficient mice. *(A-D):* Representative trace of the effects of compound 48/80 (10 µg/mL) on urothelium intact (+Uro) and denuded (-Uro) UBSM strips from mast cell-sufficient C57Bl/6J mice and mast cell-deficient cg-Kit$^{w\text{-}sh}$ mice. *(E):* Compound 48/80 (10 µg/mL) significantly contracts urothelium-intact UBSM strips isolated from both C57Bl/6J and cg-Kit$^{w\text{-}sh}$ mice as compared to urothelium-denuded strips (N=8-9). Contractile responses to compound 48/80 were unchanged between mast cell sufficient and deficient mice with urothelium intact (N=8-9) or denuded (N=8). *(F):* In urothelium-intact strips from mast cell-sufficient mice, the histamine H1 receptor antagonist fexofenadine (10 µM; N=7) and H2 receptor antagonist cimetidine (10 µM; N=6) had no effect on compound 48/80-induced contractions. Results are presented as a percentage of initial contraction to 60 mM KCl or as raw mN for the force tension generated. *: P<0.05.

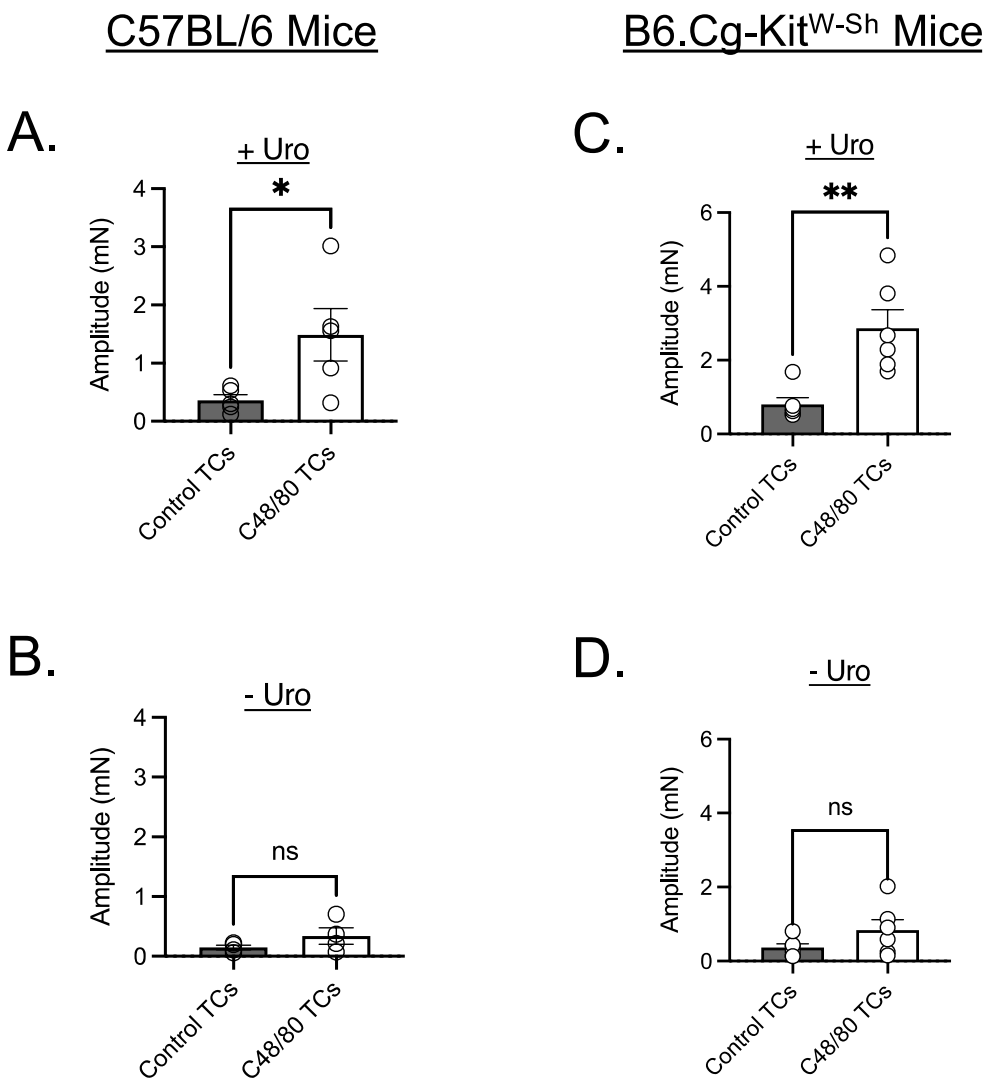

Figure 14: Compound 48/80 augments phasic contractions in both mast cell sufficient and deficient mice. *(A)* In urothelium intact UBSM strips from mast cell sufficient C57Bl/6J mice, the amplitude of phasic contractions significantly increased after exposure to compound 48/80 (10 µg/mL) (P=0.033, Nested t-test; n=30 contractions from N=6 mice). *(B)* This was increase was abolished if urothelium was removed (P=0.221, Nested t-test; n=30 contractions from N=6 mice). *(C,D)* In UBSM strips from mast cell deficient B8.Cg-Kit^{W-Sh} mice, compound 48/80 significantly increased phasic contraction amplitude in urothelium-intact strips (P=0.003, Nested t-test; n=30 contractions from N=6 mice) but had no effect when urothelium was denuded (P=0.193, Nested t-test; n=30 contractions from N=6 mice).

4.2. Compound 48/80 augments nerve-evoked contractions with and without mast cells.

We next performed electrical field stimulation (EFS) to determine if compound 48/80 also affected efferent nerve-evoked contractions. Increasing frequencies of EFS were applied to both urothelium intact and denuded UBSM strips before and after exposure to compound 48/80 (10 µg/mL). Compound 48/80 augmented EFS-mediated contractions on urothelium-intact UBSM strips from C57Bl/6J mice (**Fig. 15A**) but had no effect on urothelium-denuded strips (**Fig. 15B**). In $Kit^{w\text{-}sh}$ mice, compound 48/80 augmented EFS-induced contractions in both the presence and absence of the urothelium (**Fig. 15C-D**).

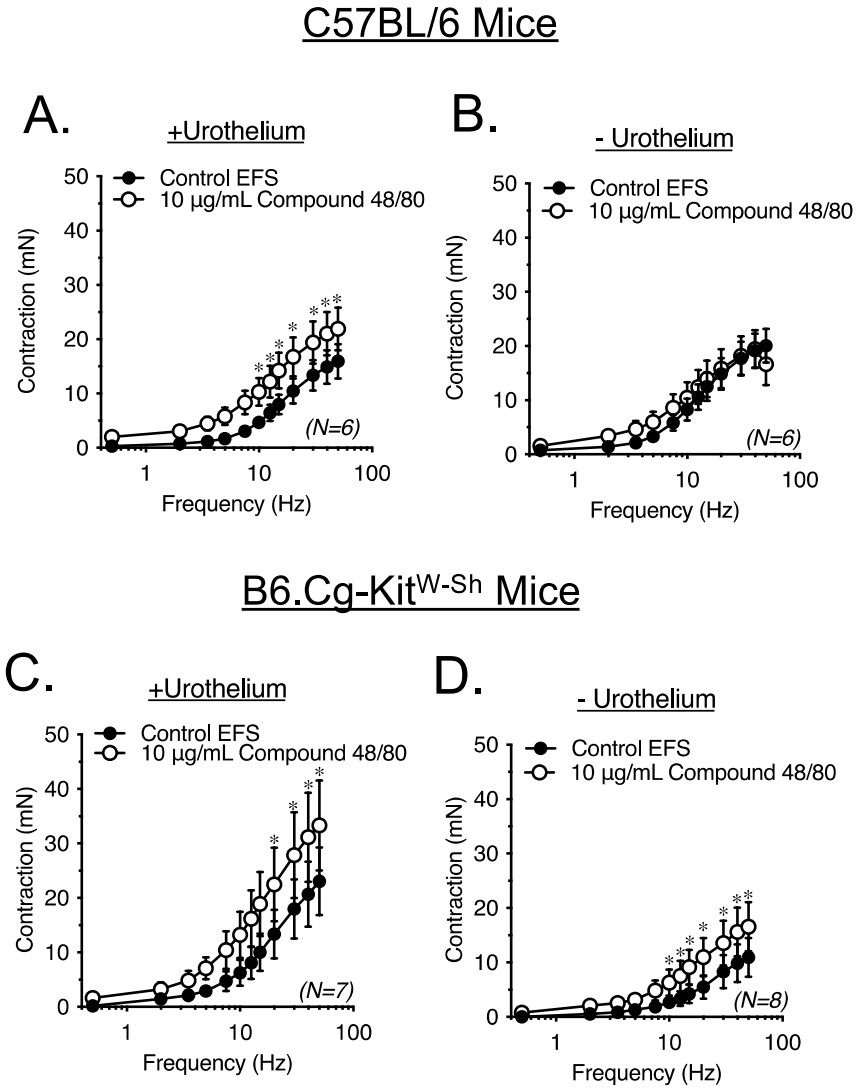

Figure 15: Compound 48/80 augments nerve-evoked contractions independent of mast cells. *(A, B):* In mast cell sufficient C57Bl/6J mice, compound 48/80 augmented contractions evoked by increasing frequencies of electrical field stimulation (EFS) in urothelium-intact strips (A) but did not affect urothelium-denuded UBSM strips (B) (two-way repeated measures ANOVA; N=6). *(C, D):* in mast cell deficient B8.Cg-Kit$^{W\text{-}Sh}$ mice, compound 48/80 augmented EFS contractions in both urothelium intact (C) and denuded (D) UBSM strips (two-way repeat measures ANOVA; N=7-8). *: $P<0.05$, Tukey's post hoc analysis.

4.3. A soluble mediator is released from the urothelium to drive UBSM contractile responses.

To determine if the urothelium was releasing a soluble mediator to cause UBSM contractions in response to compound 48/80, dissected urothelial strips were placed in a bath adjacent to urothelium-denuded strips prior to addition of 10 µg/mL compound 48/80 (**Fig. 16A**). Addition of dissected urothelial strips to the bath did not alter baseline tension or phasic activity as compared to a urothelium-denuded strips alone; only after addition of compound 48/80 did basal tension and phasic contractions significantly increase (**Fig. 16B-D**).

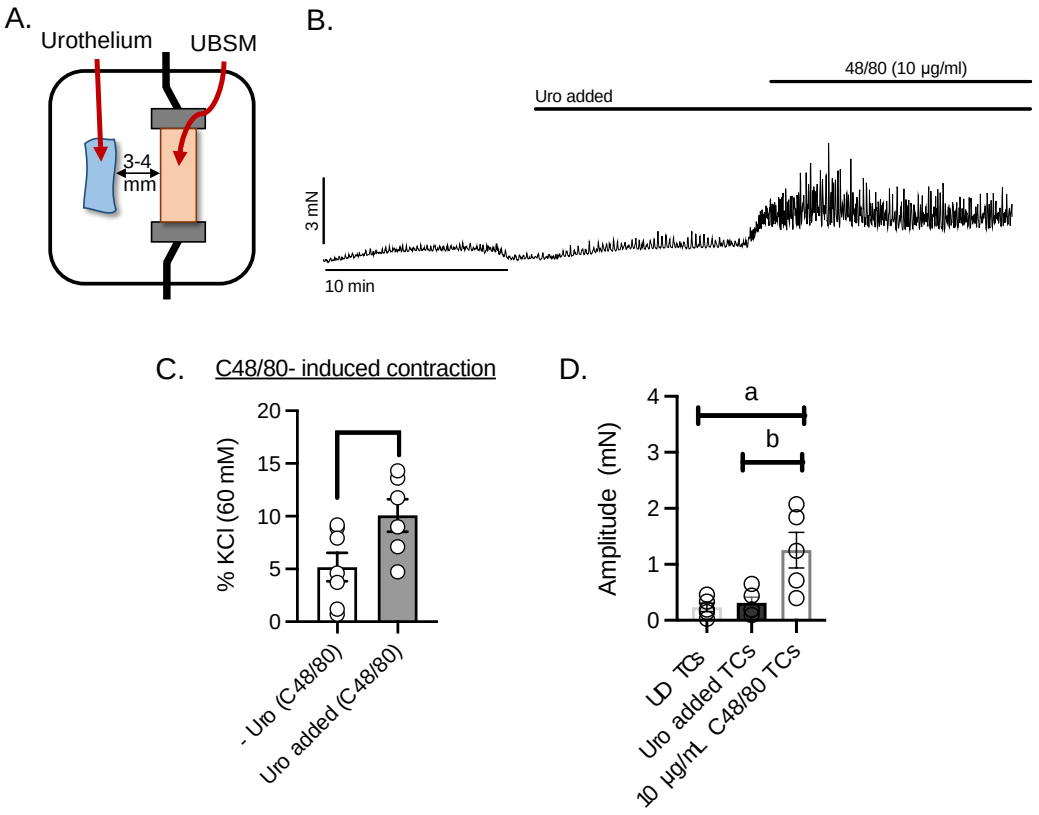

Figure 16: Contractile substances released from the urothelium are responsible for compound 48/80 induced contractions. *(A):* Cartoon depiction of the urothelium bioassay. An un-tensioned urothelial strip was placed adjacent to a denuded UBSM strip prior to exposure to compound 48/80 (10 µg/mL), and changes in force generation were measured in the UBSM strip. *(B)* Representative tracing of the denuded UBSM tension recordings before addition of urothelium, after addition of urothelium, and after exposure to compound 48/80. *(C):* As compared to urothelium-denuded strips alone, the addition of urothelial strips prior to exposure to 10 µg/mL compound 48/80 resulted in a significantly greater contraction (P=0.03, unpaired Student's t-test; N=6-7). *(D)*: The amplitude of transient contractions increased significantly after addition of compound 48/80 (10 µg/mL) in the presence of adjacent urothelium as compared to either before addition of the urothelial strip or after addition of the urothelial strip (P=0.03, nested one-way ANOVA; N=5). Results are presented as a percentage of initial contraction to 60 mM KCl or mN for the force tension generated. *: P<0.05, Student's t-test. *a*: P=0.03, urothelium-denuded (UD) strip v. strip in the presence of urothelium and compound 48/80. *b*: P=0.03, strip in the presence of urothelium (Uro).

4.4. Compound 48/80-induced contractions rely on purinergic signaling, but not cholinergic signaling or nerve depolarization.

Spontaneous contractions are associated with neurogenic urothelial-derived signals that modulate nerve activation and subsequent release of neuromodulators to drive changes in UBSM contractility [195]. Therefore, to ensure compound 48/80 did not activate cholinergic signaling, the muscarinic acetylcholine receptor antagonist atropine (2 μM) was used to block this cascade. Atropine had no effect on compound 48/80-induced contractions (**Fig. 17A, B**). Furthermore, to determine if compound 48/80 induced contractions were mediated by nerves, urothelium intact UBSM strips were exposed to the voltage gated sodium channel inhibitor tetrodotoxin (TTX, 1 μM). TTX had no effect on compound 48/80-induced contractions (**Fig. 17C-D**).

Another urothelium-derived mediator is ATP, which is released due to purinergic signaling pathway activation [196]. To prevent purinergic signaling, the nonselective purinergic P2 receptor antagonist PPADS (100 μM) was utilized. PPADS significantly reduced compound 48/80-induced contractions (**Fig. 18A, B**). Pannexin-1 channels are also highly expressed throughout the urothelium and can be responsible for ATP release [197]. To identify the source of ATP release, the pannexin-1 mimetic inhibitory peptide ^{10}Panx (200 μM) was applied prior to compound 48/80 incubation. Unlike PPADS, contractions caused by compound 48/80 were unchanged in the presence of ^{10}Panx (**Fig. 18C, D**).

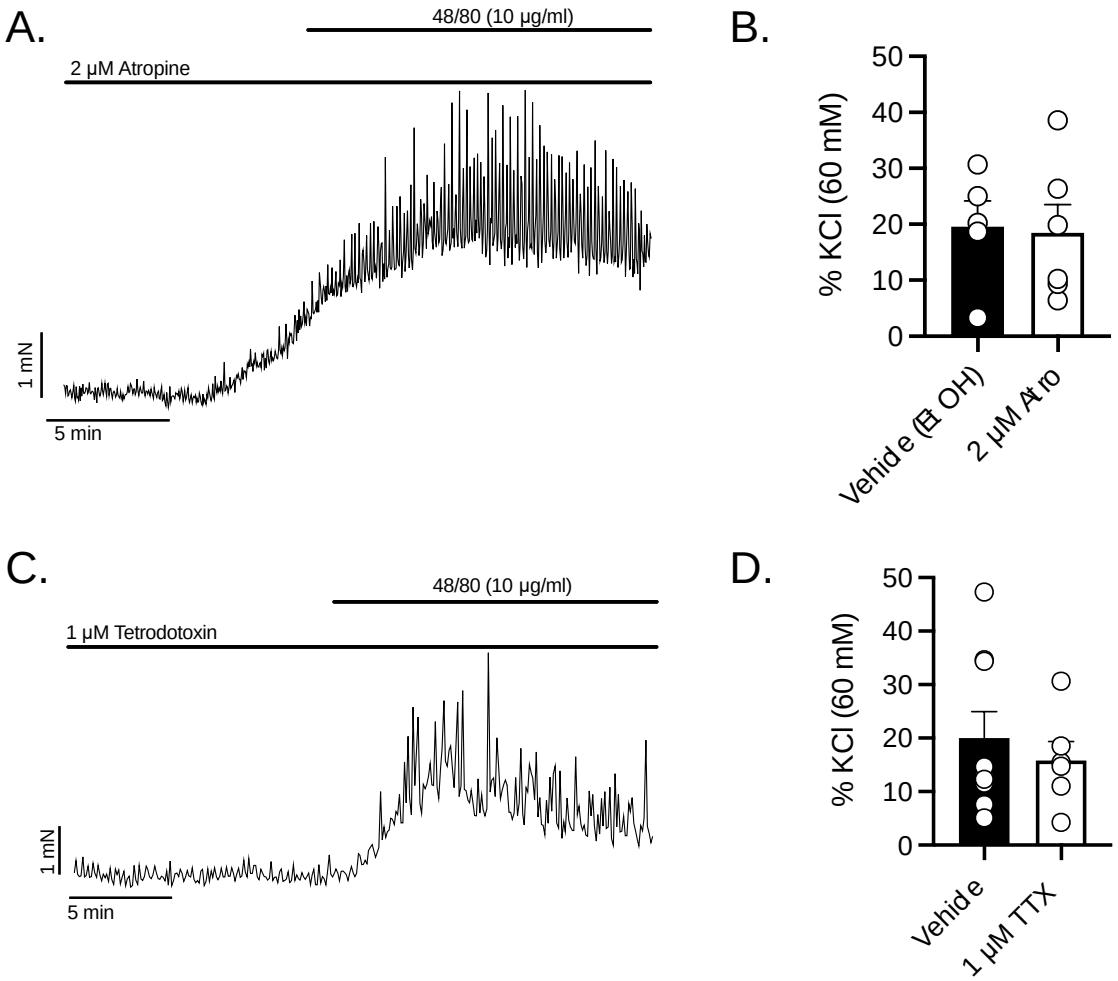

Figure 17: Effects of compound 48/80 are independent of neurogenic signaling. *(A):* Representative trace of compound 48/80-induced contractions from urothelium-intact UBSM strips after incubation with vehicle or the cholinergic antagonist atropine (Atro; 2 µM). *(B):* Atropine had no effect on responses elicited by compound 48/80 (P=0.87, unpaired Student's t-test; N=6). *(C):* Representative trace of compound 48/80-induced contractions from urothelium-intact UBSM strips after incubation with vehicle or the voltage-gated Na^+ channel inhibitor tetrodotoxin (TTX; 1 µM). *(D):* Tetrodotoxin had no effect on compound 48/80-induced contractions as compared to vehicle (P=0.49 unpaired Student's t-test; N=5-8). Results are presented as a percentage of initial contraction to 60 mM KCl.

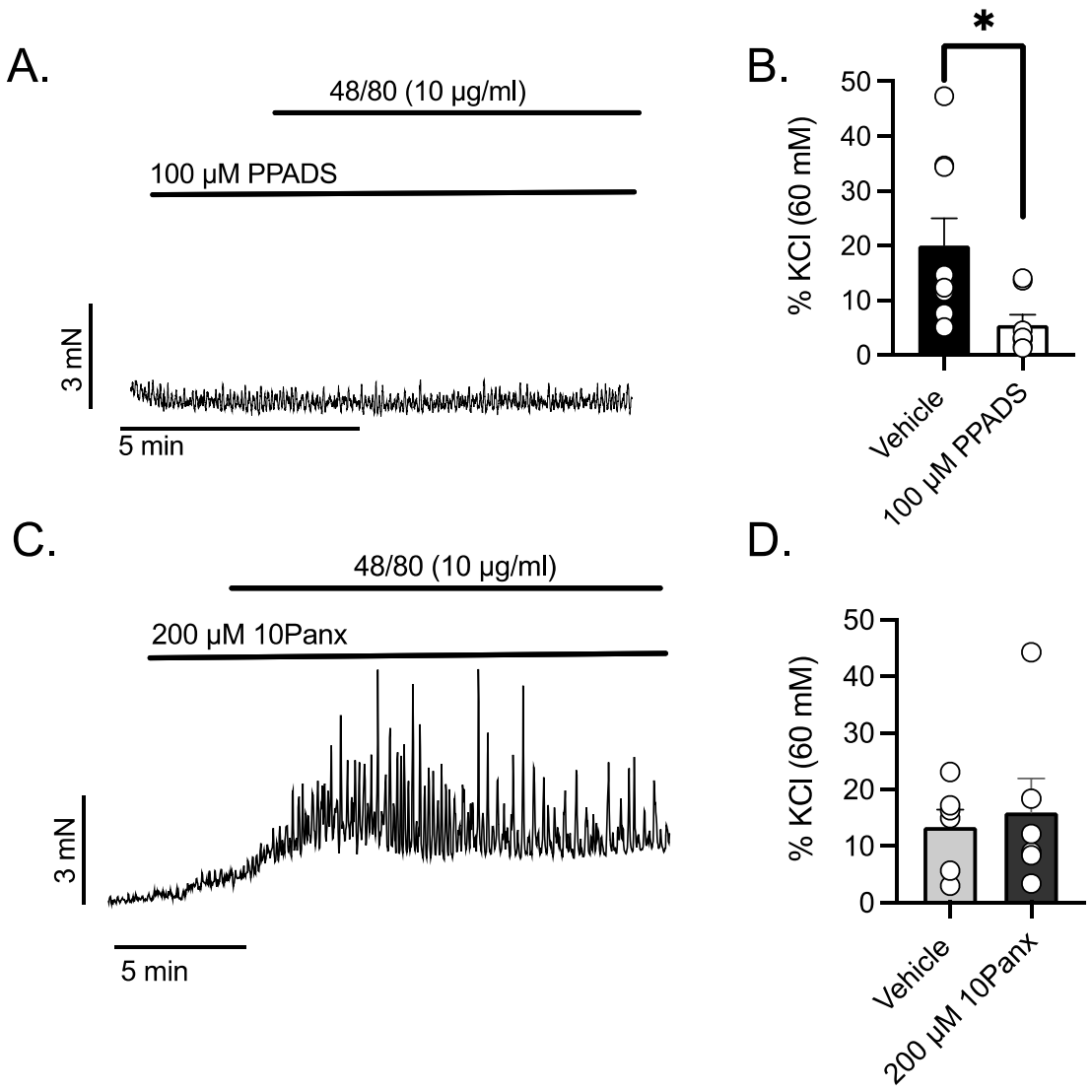

Figure 18: P2X channels mediate compound 48/80-induced contractions. *(A):* Representative trace of contractions to compound 48/80 (10 µg/mL) in urothelium-intact UBSM strips after incubation with the nonselective P2X receptor antagonist PPADS (100 µM). *(B):* Compound 48/80-induced contractions were abolished by as compared to vehicle (P=0.019, unpaired Student's t-test; N=8-9). *(C):* Representative trace of contractions to compound 48/80 (10 µg/mL) from urothelium-intact UBSM strip after incubation of the pannexin-1 mimetic inhibitory peptide ^{10}Panx (200 µM). *(D):* ^{10}Panx had no effect on compound 48/80 contractions as compared to vehicle (P=0.71, unpaired Student's t-test; N=6).

4.5. Contractions to compound 48/80 involve cyclooxygenase synthesis, G_q protein signaling, and complementary prostanoid receptor activation.

Prostanoid biosynthesis occurs in both the urothelium and detrusor, and elevated synthesis and production of prostanoids (particularly PGE_2) are often associated with bladder pathologies [198]. Since compound 48/80 causes systemic inflammation, we exposed urothelium intact UBSM strips to the nonselective cyclooxygenase inhibitor indomethacin to determine if prostaglandin synthesis via cyclooxygenase was involved in the responses to compound 48/80. Indomethacin (5 µM) drastically reduced compound 48/80 (10 µg/mL) induced contractions (**Fig. 19A, B**). Similarly, the $Ga_{q/11}$ inhibitor YM-254890 (1 µM) abolished contractions induced by compound 48/80 (**Fig. 19C, D**). However, antagonism of individual G_q-coupled prostanoid receptors, such as EP1 receptors (10 µM SC-51089; **Fig. 19E, F**), FP receptors (5 µM AL8810; **Fig. 19G, H**), and TP receptors (2 µM GR-32191B; **Fig. 19I, J**) had no effect on the increases in basal tone or phasic activity caused by compound 48/80. Only after the application of an antagonist cocktail targeting all three prostanoid receptors (EP1, FP, and TP) were the responses to compound 48/80 diminished (**Fig. 19K, L**).

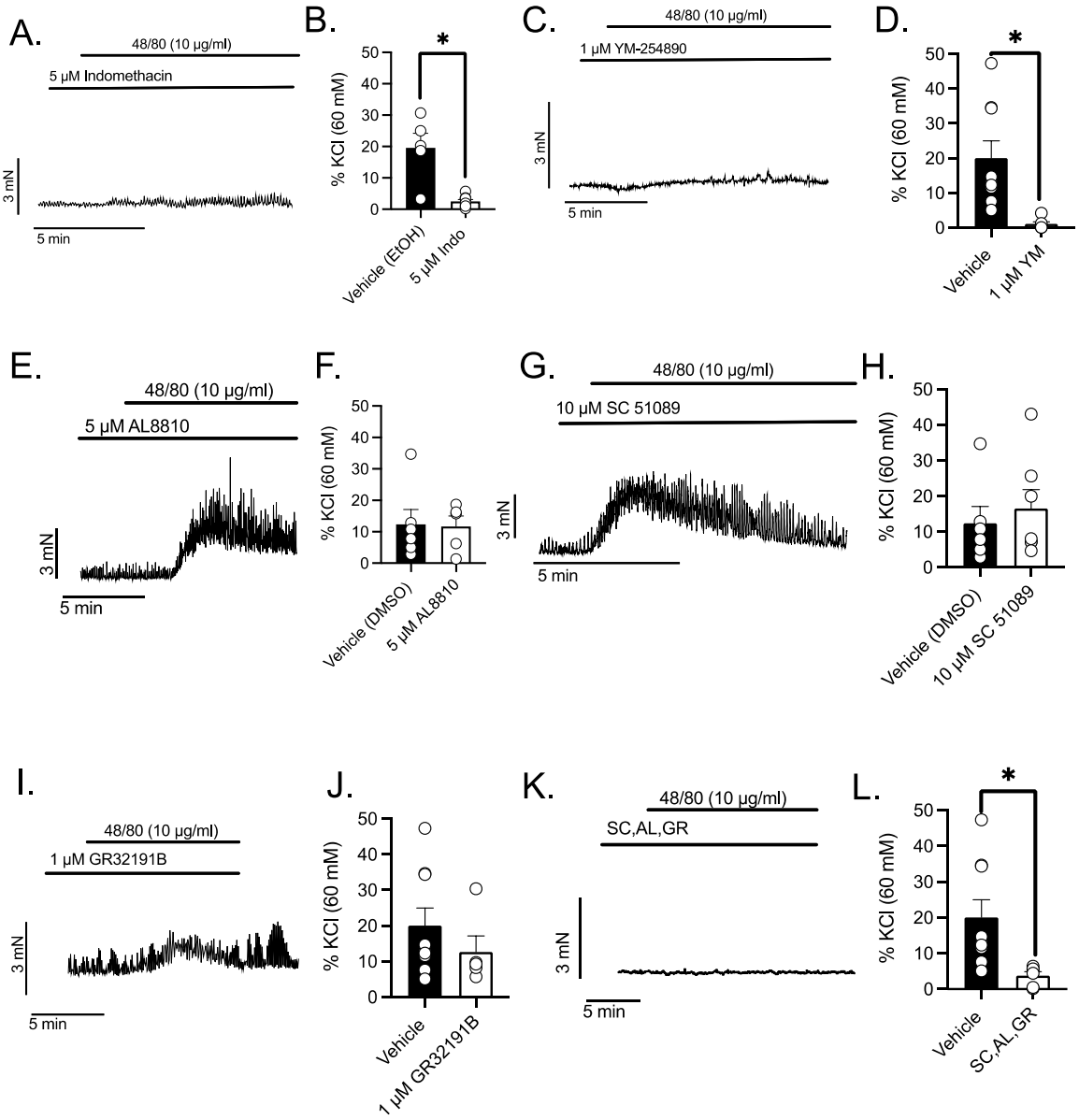

Figure 19: Inhibition of cyclooxygenases, $G_{q/11}$ signaling, and combined antagonism of prostanoid receptors (EP1, FP, TP) abolishes compound 48/80-induced contractions. *(A, B):* The non-selective cyclooxygenase inhibitor indomethacin (5 µM) abolished UBSM contractions to compound 48/80 (10 µg/mL) (P=0.019, unpaired Student's t-test; N=8). *(C,D):* The G_q/G_s inhibitor YM254890 (1 µM) also abolished compound-48/80 induced contractions as compared vehicle controls (*P=0.004, unpaired Student's t-test; N=5-6). *(E-J):* When used alone, neither the FP receptor antagonist AL8810 (5 µM), the EP_1 receptor antagonist SC-51089 (10 µM), nor the TP receptor antagonist CR32191B (1 µM) had any effect on compound 48/80-induced contractions as compared to vehicle (P=9.09, P=0.632, and P=0.288 respectively, unpaired Student's t-test; N=5-7). *(K, L):* A combination of all three prostaglandin receptor antagonists abolished compound 48/80 contractions as compared to compound 48/80 alone (P=0.011, unpaired Student's t-test; N=6-9). Results are presented as a percentage of initial contraction to 60 mM KCl. *: P<0.05.

5. Discussion

We utilized the prototypical mast cell activator compound 48/80, along with bladders from mast cell deficient mice as a negative control, to assess and understand the effects of mast cell degranulation on non-diseased UBSM strips. However, responses to compound 48/80 were unchanged in UBSM strips from mast cell deficient mice, indicating that the effects are independent of resident mast cell activation and subsequent degranulation. These findings led to subsequent pharmacological and physiological investigations to determine the factors contributing to UBSM contractions and increased phasic activity caused by compound 48/80. We found that compound 48/80 significantly augments UBSM nerve-evoked contractions, yet nerve blockade does not abolish the response. Thus, not only does compound 48/80 directly contract UBSM, it also increases UBSM contraction to efferent nerve input. Compound 48/80-induced contractions were reduced or abolished by inhibiting several relatively broad signaling pathways (COX, P2 purinoceptor, Gq), combined prostanoid receptor antagonism (EP1, FP, TP), and urothelial denudation. This suggests that the urothelium acts to directly alter UBSM responses to inflammatory insult through production of prostanoids. Our data further suggest that compound 48/80 should not be viewed nor utilized as solely a mast cell histamine releasing agent, but holistically as a basic secretagogue that can activate inflammatory disease-oriented pathways to alter the physiology of non-diseased tissue.

5.1. Mast cells implication in bladder inflammation

Studies into the effects of bladder inflammation often reference mast cell activation as an initiator of sensory changes that cause pain and local irritation, and potentiate dysfunction in the forms of UBSM hyperactivity or hypersensitivity [134]. Studies have also

determined that the number of mast cells in the detrusor increases in patients with interstitial cystitis [97, 118]. Yet, the contribution of resident and infiltrating mast cells within the urinary bladder to bladder dysfunction remains unclear. For instance, mast cells are present in the urothelium/submucosa and detrusor under normal circumstances and increase by number in the detrusor of patients with interstitial cystitis; yet these numbers remain unchanged in bacterial cystitis [199]. Whereas infiltration and 80% degranulation of mast cells occurred in the urinary bladder of rats after intravesical infusion with LPS and protamine sulfate, shams did not have a significant amount of mast cells at all [200]. Mast cells isolated from non-diseased bladder smooth muscle were also more responsive to compound 48/80 than those from bladders with interstitial cystitis [102]. Thus, we initially utilized compound 48/80 to directly to determine the effects of mast cell degranulation on UBSM contractility.

Interestingly, in our study the hyperactive effects of compound 48/80 on UBSM contractility remained in mast cell deficient mice – a finding that is not in alignment with models directed to identify the effects of mast cells on detrusor contractility. As an example, ovalbumin-induced contractions are reduced, not augmented, with the utilization of compound 48/80 as a systemic mast cell degranulating agent in isolated UBSM strips from rat bladders [201]. This suggested that degranulation of a finite population of mast cells in the bladder wall occurred with systemic exposure to compound 48/80, such that ovalbumin was no longer able to exert an effect. The results of our study challenge the utilization of compound 48/80 solely as a mast cell degranulator in the urinary bladder because augmented efferent nerve-evoked contractions and urothelium-dependent hyperactive smooth muscle responses were independent of nerve function

and persisted in mast cell deficient mice. Thus, while some responses to compound 48/80 may be nerve- and mast cell-mediated *in vivo,* significant changes to UBSM contractility still occur wholly independent of mast cells.

Typically, mast cell degranulation in the urinary bladder is co-investigated with nociceptive peptides via upregulation of genes associated with mast cells and substance P [84, 85, 109, 202]. For example, *In vivo* treatment with compound 48/80 negatively impacted the motor reflex of the bladder in rats, which could be blocked by first desensitizing sensory c-fibers [73]. Interestingly, isolated strips from rat bladders still showed an increase in rhythmic contractions that was augmented by pre-exposure to capsaicin [73]. While our experiments did not show augmented responses, the fact that rhythmic contractions to compound 48/80 persisted in mast cell-deficient mice agrees with these earlier findings. While our findings do not preclude these effects on sensory nerves as effectors of UBSM function, they do show that direct, urothelium-dependent and mast cell-independent changes to UBSM contractility can occur in response to compound 48/80.

5.2. Urothelium dependent responses in urinary bladder function and dysfunction

As described in the Introduction, urothelium-derived mediator release can be activated through both physical and chemical stimuli [16, 175, 191, 203]. In the context of this study, any mechanical insults can be ignored as a stimulus alone because tissues remain under static tension and there is no increase in phasic activity or contractile response without incubation with compound 48/80. This negation is supported through the urothelium denudation of UBSM strips in conjunction with the addition of separated urothelial sheets in the bottom of the bath (Figs. 1 and 4). Thus, Compound 48/80 stimulates the release

of a diffusible mediator from the urothelium that can alter UBSM contractility independent of effects on the urothelial barrier.

Our subsequent experiments were framed from classic pathways associated with chemical-mediated urothelium activation and mediator release in the urinary bladder to enhance UBSM excitability. Chemical induction studies (e.g., protamine sulfate, ATP, cyclophosphamide) relate inflammation-driven detrusor overactivity to changes in permeability and pharmacology of the urothelium [204-206]. An *in vivo* histamine induction study utilizing compound 48/80 also implicates changes in the urinary bladder, but instead via increased vascular permeability through activation of substance P neurons [190]. Urothelium irritation with a concentration of protamine sulfate that does not cause cellular damage causes the amplitude of UBSM contractions to increase after intravesical infusion of KCl [204]. However, this model only infers urothelial-derived substance release as the potential cause and does not investigate its identity. Similarly, our model likely increases contractility via urothelial irritation but urothelial cell damage was not assessed. Our major finding is that compound 48/80 causes urothelium-dependent UBSM hyper-responsiveness by increasing smooth muscle tone, contractility, phasic activity, and responsiveness to efferent nerve neurotransmitters. Regarding responses to efferent nerve activation, there are two possible explanations: (1) nerve hypersensitivity could be causing increased release of neurotransmitters, or (2) bladder smooth muscle has become more sensitive to the same amount of neurotransmitter release. It is possible that urothelium and smooth muscle release of prostaglandins may cause this augmentation independent of efferent nerve input, which is supported by exogenous PGE_2 effects [207].

Future studies will determine whether or not the augmented responses to EFS are indicative of UBSM or efferent nerve changes, or possibly both.

5.3. Similarities between compound 48/80 and prostaglandins

Exogenous PGE_2 significantly mitigates compound 48/80-induced acute inflammation of the hamster cheek, thus acting as an anti-inflammatory agent as opposed to an irritant [208]. Compound 48/80 also evokes significant amounts of PGD_2 as compared to PGE_2 in guinea pig model of acute allergic reaction of the skin [209]. In terms of urinary bladder, intravesical infused PGE_2 is described as the *"*unclassified irritant*"* with no clear directionality in disease [210, 211]. Targeting and dissecting pathways associated with exogenous application and endogenous release of prostaglandins pertaining to regulation of urinary bladder micturition reflex is complex [212, 213]. The challenge to targeting the role of PGE_2 is because the G protein signaling pathways to which the prostaglandin receptors are coupled (Ga_s and Ga_q) also mediate contractions of the UBSM to many other endogenous compounds and neurotransmitters (reviewed in [214]). Thus, we used the $Ga_{q/11}$ inhibitor YM-254890 and determined that compound 48/80 was activating a Ga_q receptor signaling pathway to promote prolonged contractions.

In alignment with previous investigations seeking to target PGE_2 [215, 216], our findings suggest that although synthesis of prostaglandins is involved in compound 48/80-induced contractions, individual pharmacological characterization of each contractile prostaglandin receptor subtype (EP1, TP, FP) uncovered that an entourage effect of multiple prostaglandins drives the effects of compound 48/80 on bladder contractility. Our findings additionally support the conclusion that modulation of prostaglandin synthesis relies on purinergic feedback, based on the abolishment of compound 48/80-induced

contractions in presence of the purinergic P2 receptor antagonist PPADS. This inference, however, is limited. The location of ATP release remains undetermined, although UBSM strips still responded to compound 48/80 even during blockage of pannexin-1 ATP transport channels. Also, our findings do not align with models of bladder overactivity implicating ATP [197].

Due to the various antagonistic pathways associated with the effects of compound 48/80, it is difficult to assess the origin within the bladder of contractile substance release. In the context of the urinary bladder, interstitial cells are the “localizers” of signaling pathways involving cyclooxygenase I, prostaglandins, and ATP under different conditions of smooth muscle excitability; however, the urothelium is highlighted in each model as an “inducer” [217-221]. We propose a hypothetical pathway in which compound 48/80 increases the release of ATP from the urothelium. This leads to ATP acting in a paracrine manner to initially generate a contraction to stimulate prostaglandin synthesis and release (**Fig. 20**). Overall, compound 48/80 induces a chemical feedback loop between both the urothelium and smooth muscle leading to the activation of G_q-coupled prostaglandin receptors.

5.4. Conclusions

Most pathophysiologic pathways proposed do not regard the bladder itself as an inflammatory milieu unless inflammation is induced; however, urothelial cells are first responders to noxious contents of the urine. Urothelial cells’ role in disease should not only encompass cell death but consider the directionality of combative survival strategies. Evidence provided by this study due to the utilization of compound 48/80, demonstrates the combative nature of the urothelium as a modulator of urinary bladder smooth muscle

increased phasic activity and contractility independent of immune cell recruitment and inflammatory hyperalgesia *via* mast cell degranulation.

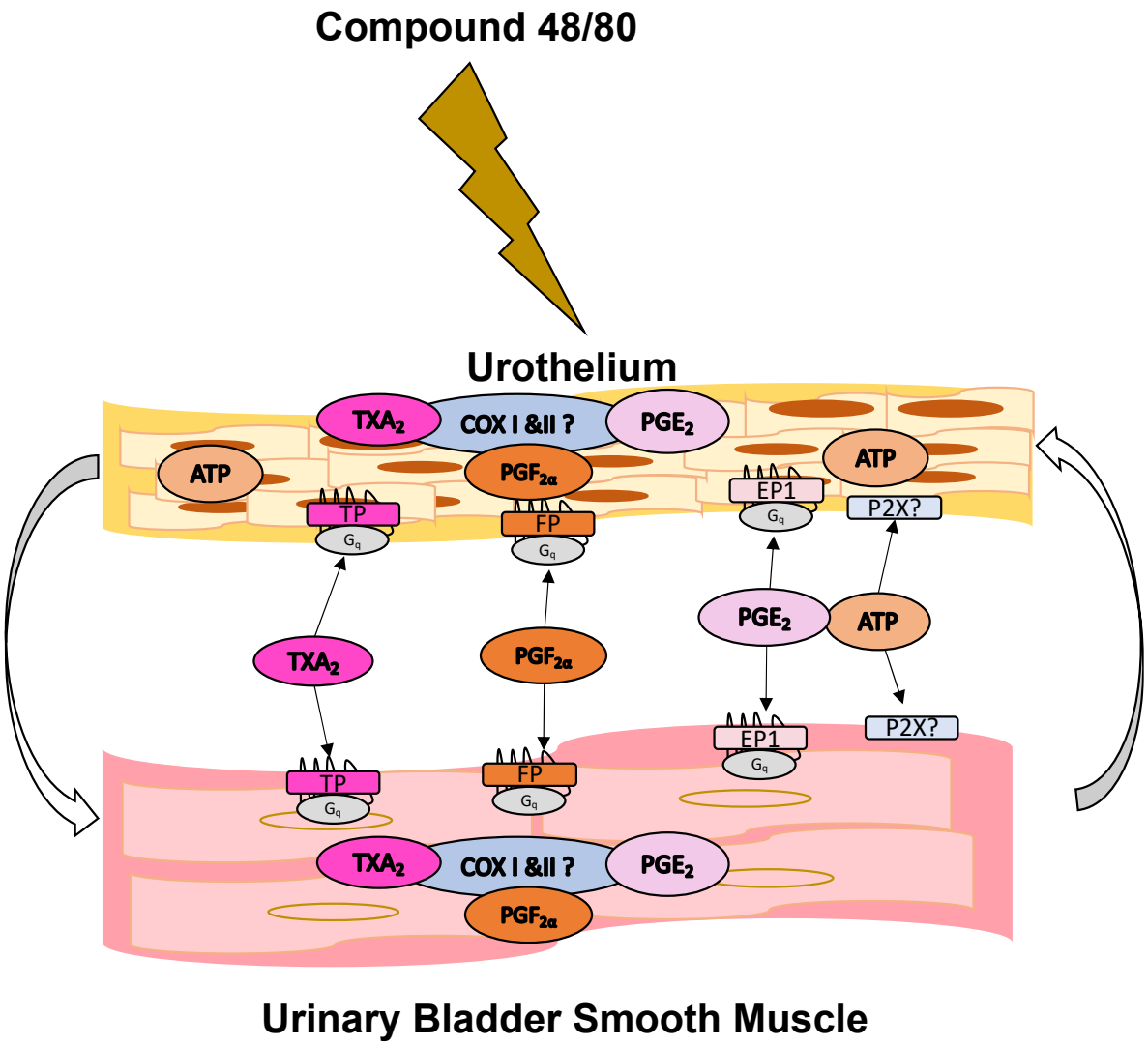

Figure 20: Hypothetical model for the effects of compound 48/80 on isolated urinary bladder strips. Cartoon depiction of a paracrine signaling pathway instigated by the release of urothelial ATP, which then causes the bidirectional release of contractile prostaglandins. Future experiments will determine which cyclooxygenases are responsible for prostanoid production, as well as purinergic receptor subtypes responsible for mediating responses to ATP. Our current findings do not establish clear directionality; ATP is assumed to be released from the urothelium, but whether urothelial cells are also the source of prostanoid release is unclear. Future experiments will investigate this mechanism to determine if it is indeed acting in an autocrine or paracrine fashion.

SUMMARY AND PERSPECTIVES

1. Summary

Neurogenic and myogenic origins of urinary bladder dysfunction are often characterized by LUTS associated with cystitis and OAB. In the context of the bladder, neurogenic refers to changes to nerve innervation, whereas myogenic refers to alterations to the detrusor smooth muscle. Perspectives regarding both “origins” focus on chemical and physical inducers of pathological states. Most inducers are proposed to derive from alterations to sensory nerves, urothelium, or mast cells within the urinary bladder. These alterations result in dysregulation of the bladder wall. Thus, this chapter addresses the intersections of bladder function and dysfunction as it pertains to the characterization of mast cell activation and histamine.

The mechanism by which mast cell degranulation and subsequent histamine release cause urinary bladder dysfunction are presumed to be through sensory c-fiber nerve engagement, wherein neuropeptides (e.g., Substance P, CGRP) activate mast cells to release histamine [222]. Reports on histamine suggest a direct functional role in non-diseased urinary bladder tissue. This evidence suggests histaminergic signaling alters urinary bladder signaling. Thus far, the elucidation of histamine’s role in bladder function points to direct dorsal root ganglion activation of afferent nerves involved in distention and increased spontaneous detrusor contractions *[91, 125]*. Examination of mast cell-derived histamine is seminal to IC/BPS pathophysiology. Whereas other mast cell-derived mediators alter the urothelium, Choi et al. determined UroPathogenic *Escherichia coli* causes mast cell protease release, which induces urothelial cell shedding *[174]*. Regarding the direct effects of histamine on the urothelium, *Grundy et al. found no evidence of activation due to a lack of* Ca^{2+} *signaling. However, urothelial cells express*

mRNA for histamine H3 receptors only, whereas H1 and H2 are expressed in detrusor. Similar to exogenous histamine, the mast cell activator compound 48/80 is documented to increase tissue levels of histamine, c-fiber firing, and mast cell count in diseased urinary bladder tissue. Few studies have focused on the effects of mast cell-derived mediators in non-diseased urinary bladder tissue. The data presented in this dissertation reveal that the effects of histamine on the detrusor are short-lived and compound 48/80 acts beyond mast cell degranulation. Exploratory investigations extended beyond the overarching hypothesis that mast cell-derived and associated inflammatory mediators directly prolong detrusor contractility. In summary, the findings of Chapter 2 determined the non-neurogenic effects of histamine on the detrusor. Chapter 3 uncovered mast cell-independent detrusor responses to compound 48/80 that mimic neurogenic inflammatory signaling. These studies originated from two aims.

The first aim was to determine if histamine directly alters the ability of the detrusor to contract. Experiments were designed to pharmacologically characterize histamine-induced urinary bladder smooth muscle contractions. Histamine receptor antagonists, along with a G protein inhibitor, were used to validate receptor signal transduction-mediated responses. Histamine H1 and H2 receptors mediate urinary bladder smooth muscle contraction and relaxation in response to histamine *[125]*. To assess the effects of histamine on purinergic and cholinergic efferent nerve-evoked contractions, electrical field stimulation was conducted. Potential indirect neurogenic origins of histamine-induced contractions were also examined through the antagonism of TRPV1 expressing afferent nerves and inhibition of voltage-gated Na+ channels on all nerves. Myogenic origins of histamine tachyphylaxis were observed by smooth muscle cholesterol

depletion. A range of pharmacological assessments was conducted for this aim due to the various reported effects of histamine on urinary bladder detrusor smooth muscle.

The second aim was to determine what inflammatory mediators are released from mast cells in the presence of compound 48/80 and if these mediators affect urinary bladder smooth muscle contractility. Experiments were designed to identify the source of contractile mediator release. Urinary bladder smooth muscle strips from mast cell-deficient mice were utilized to determine the role of mast cells in compound 48/80 induced contractions. Urothelium denudation of strips was conducted to eliminate urothelium-derived mediators. Additionally, a P2X purinoreceptor antagonist was used to elucidate urothelium-derived ATP signaling. Histamine receptor antagonism was used to determine if histamine was being released upon compound 48/80 exposure. Prostaglandins are synthesized by mast cells, urothelial cells, and detrusor myocytes; thus, prostaglandin receptor antagonists (specifically for Gq-coupled prostaglandin receptors) were utilized.

2. Conclusions and Novel Findings

The studies in this dissertation show histamine rapidly contracts the detrusor smooth muscle, whereas compound 48/80-induced contractions are prolonged with dramatic increases in spontaneous phasic activity. To my knowledge, this is the first study to report urothelium-dependent effects of compound 48/80. Several findings within the following studies are noteworthy:

1. Histamine-induced contractions are not dependent on TRPV1-expressing afferent nerve release of neuropeptides nor neurotransmission of efferent fibers (**Fig. 8**).
2. Cholesterol depletion does not prolong histamine-induced contractions but instead reduces the contractions (**Fig. 9**).

3. Compound 48/80-induced contractions do not dependent on mast cell activation and subsequent degranulation (**Fig. 13**).
4. Compound 48/80 augments nerve-evoked detrusor smooth muscle contractions (**Fig. 14**).
5. The effects of Compound 48/80 are entirely urothelium dependent (**Fig. 13**).
6. Prostaglandin receptors instead of histamine receptors are activated in response to compound 48/80-induced contractions (**Fig. 17**).

Findings presented in Chapter 2 suggest that histamine directly contracts the detrusor independent of nerves. Since nerves were not involved in the rapid desensitization of histamine, then G protein-coupled receptor signal transduction was suspected. However, blockage of the homologous GPCR desensitization did not lead to the prolongation of histamine-induced contractions. Together these data conclude the effects of histamine do not alter the ability of the detrusor to contract. This is further validated by the inability of histamine to augment nerve-evoked contractions (**Fig. 12**). Although histamine contracts the detrusor muscle in this study, myogenic origins are inconclusive. Histamine does not cause a substantial change in baseline tension or phasic activity, rendering the response's origins unclear.

Findings presented in Chapter 3 suggest that compound 48/80-induced contraction alters detrusor responsiveness to their stimuli, namely efferent nerve-evoked ATP and acetylcholine release. Physical disruption of the urothelium by denudation revealed that the origin of compound 48/80 contractions was indeed the urothelium. Other pharmacological investigations led to the discovery that three prostanoid receptors, EP1, FP, and TP, were all involved in these responses. Additionally, purinergic signaling

mediated compound 48/80 contractions. Together these data conclude that the effects of compound 48/80 on the urothelium lead to release of inflammatory mediators in a non-diseased model. Prostaglandin E2, prostaglandin F2alpha, and thromboxane A2 are proposed as the mediators, but the exact structural origin remains unknown. Overall compound 48/80 causes a plethora of effects on urinary bladder tissue strips. The directionality of these effects remains unknown. However, the activation of ATP release and prostaglandin production implicates a co-regulatory relationship between urothelial signaling and detrusor contractility.

3. Limitations

The studies presented in this dissertation are not without shortcomings. There are many questions that remain regarding the effects of histamine and compound 48/80 on the detrusor smooth muscle, though some cannot be addressed pharmacologically. All findings reported are ex vivo organ tissue bath studies without in vivo correlate experiments. In this dissertation, the shortcomings include experimental design, sex differences, detrusor muscle function, and lack of pharmacological selectivity.

3.1. Experimental Design

Regarding the experimental design, the urinary bladder tissue strips for each experiment were from non-diseased mice. Thus, it is difficult to extrapolate the role of histamine and mast cell activation in bladder dysfunction. The focus of studies in Chapters 1 and 2 was to determine the effects of histamine and compound 48/80 in the absence of bladder dysfunction. These effects were studied in vitro as opposed to ex vivo or in vivo, which strengthens the findings in relation to alterations to the ability of the detrusor to contract but limits the direct correlation of detrusor overactivity as an outcome. On the contrary,

Grundy et al. reported ex vivo application of histamine results in no change to detrusor compliance *[91]*. Similarly, unpublished histamine data (**Fig. 21**) indicates no change in intermicturition interval (IMI) and void volume. However, compound 48/80 not only increases compliance but decreases IMI and void volume in both mast cell sufficient and deficient mice *[223]*.

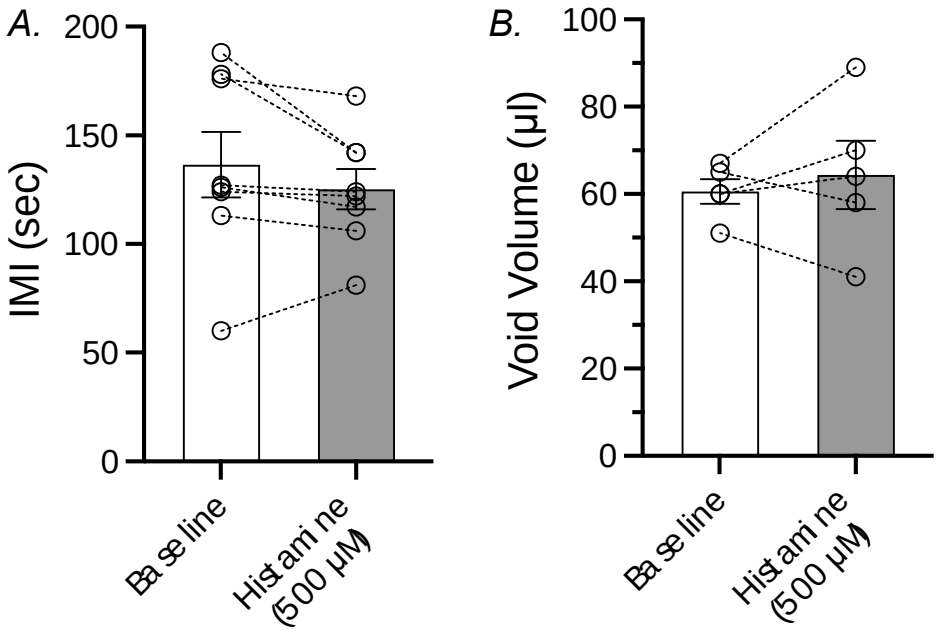

Figure 21: Intravesical histamine does not cause bladder overactivity. Intravesical instillation of histamine has no effect on intermicturition interval (IMI) (A; p=0.17) or void volume (B; p=0.54) in male C57BL/6 mice. N = 5-8.

Regarding the limitation regarding examining histamine receptor desensitization in Chapter 2, the cholesterol-depleting agent Methyl-β-cyclodextrin (MβCD) was used to target caveolae; however, no imaging analysis was done to confirm this. Due to the reduction and cholesterol restoration of the urinary bladder smooth muscle contractions to histamine, no further investigations were conducted to determine if caveolae-mediated desensitization occurred. Other minor limitations pertaining to the experimental design include the concentration of histamine applied to the bath and the spontaneous activity of strips exposed to histamine. In Chapter 2, 200 µM histamine is administered to strips, which could be considered supra-physiological. Yet, there is no response to histamine at lower concentrations (**Fig. 6**). Other recent studies also administered between 100 µM – 300 µM to examine the effects of histamine on the urinary bladder *[91, 125, 126]*. Although studies from porcine bladders report an increase in spontaneous contractions following histamine exposure *[125, 126]*, I did not measure increased spontaneous phasic activity of the detrusor in response to histamine due to the novel nature of desensitization.

3.2. Sex Differences

Inflammatory conditions of the urinary bladder (UTIs, IC/BPS) are more prevalent in women than men *[224, 225]*. Nevertheless, as stated in Chapter 1, OAB is equally prevalent in men and women. Studies in Chapters 1 and 2 were conducted on urinary bladder smooth muscle strips and detrusor myocytes from male mice in the absence of bladder pathology. The premise of studies conducted throughout this dissertation was to develop a physiological framework, wherein histamine and mast cell degranulation could mimic changes associated with inflammatory hyperalgesia mentioned in social stress-induced bladder dysfunction. Most investigations that examine the effects of

psychological social stress, conducted studies with male mice due to the lack of territorial aggression exhibited in female mice along with the possibility of male mice mounting female mice *[226]*. Alternative methods are utilized to induce social stress include female mice witnessing social defeat or experiencing defeat via male urine application *[226, 227]*. Thus, future studies will be conducted in female mice to determine if there are differences in contractility.

3.3. Detrusor Smooth Muscle Function

Detrusor function is primarily characterized by the activation of muscarinic receptors and cholinergic signaling *[152, 228]*. To assess detrusor responsiveness, CCh or ACh concentration-response curves are conducted *in vitro*. The studies presented in Chapters 1 and 2 only utilize a bolus of CCh (200 nM, 1 µM) to assess viability and electrical field stimulation was conducted to determine detrusor sensitivity to histamine and compound 48/80. This is a shortcoming because the influence of compound 48/80 on nerve-evoked contractions only implies that the smooth muscle is more sensitive to ATP and ACh, whereas determining the effects of compound 48/80 via CCh concentration-response curves would infer smooth muscle contractility has altered. Further limitations pertain to the "myogenic origins of spontaneous contractions" of compound 48/80. Calcium channels have been identified as regulators of spontaneous phasic contractions *[52, 136, 229]*. Studies conducted in this dissertation did not investigate Ca^{2+} mobilization, nor the channels involved in urothelial/detrusor calcium signaling.

3.4. Pharmacological Selectivity

The selectivity of compound 48/80 for mast cell activation has been disputed *[181]*. In Chapter 3, compound 48/80-induced contractions result in the activation of a plethora of

signaling pathways – though all were dependent on the urothelium and not mast cells. This is a limitation because the aim was to determine the effects of mast cell degranulation on the detrusor smooth muscle, so another means of degranulating mast cells is needed to truly investigate the role of mast cells as regulators of detrusor contractility. However, due to the novel finding that the profound effects of compound 48/80 were indeed mast cell-independent, further investigations into signaling pathways associated with inflammation were pursued.

Dysregulation of the urothelium due to inflammation results in the release of ATP and prostaglandins [1, 215]. Thus, the nonselective P2X purinergic antagonist PPADS and cyclooxygenase (COX) inhibitor indomethacin were utilized to determine if ATP and prostaglandins were involved. Both abolished compound 48/80-induced contractions; yet it remains unclear as to which subtype of P2X and isozyme of COX initiates detrusor contractility. P2X7 and COX-2 are implicated in inflammatory conditions of the urinary bladder *[230, 231]*. Selective drugs for these targets would allow for differentiation between normal and inflammatory responses of the bladder wall.

4. Future Directions

4.1. Histamine desensitization

Studies conducted within this dissertation need to be expanded to investigate urinary bladder myogenic and structural origins that may induce a pseudo-inflammatory state. In Chapter 2, pharmacological tools are used to examine histamine tachyphylaxis. A notable study was with MβCD, whereby cholesterol depletion reduced instead of prolonged histamine-induced contractions. To enhance this experiment further consideration should be given to specific membrane invagination such as caveoli. Caveolin-1 is implicated in

urinary bladder contractility and overactivity *[232, 233]*. To determine the effects of caveolin-1 on histamine-induced contractions, a caveolin-1 scaffolding domain peptide could be added to the tissue bath. Genetically, a caveolin-1 knockout mouse would be ideal. In each model, the expected outcome would be that histamine-induced contractions are prolonged. Thus, this proposed study would test the hypothesis that histamine desensitization is dependent on caveolin-1-mediated receptor internalization.

4.2. Nerve-independent urinary bladder smooth muscle contractions

There are tetrodotoxin-sensitive (TTX-S) and resistant (TTX-R) voltage-gated sodium (Na_V) channels on nerves that innervate the urinary bladder. Grundy et al. discovered that all Na_V channels in urinary bladder afferent nerves were sensitive to TTX, which thus abolished bladder afferent firing *[234]*. This discovery suggests that TTX-S Na_V channels are responsible for bladder afferent hypersensitivity. In Chapters 2 and 3, although histamine and compound 48/80 induced contractions were not inhibited by TTX, there should be a follow-up study examining TTX-R Na_V channels via inhibition of $Na_V1.5$ (Jingzhaotoxin-III) and $Na_V1.8$ (A88726). There are no known selective inhibitors for $Na_V1.9$.

To further ensure nerves are not involved, a pan Na_V activator could be utilized to determine if compound 48/80 and histamine-induced contractions are augmented. Additionally, this study would directly assess if afferent nerve activation via TRPV1 sensitization is involved in histamine and compound 48/80-induced contractions. To assess pharmacologically, organ tissue bath studies would be conducted in the presence of resiniferatoxin, a highly potent capsaicin analog. Prior and newly proposed experiments will seek to validate TRPV1 inhibition with capsaicin activation in the presence of

inhibitors. The expected outcomes are as follows: (1) inhibition of TTX-R $Na_V1.5$ and $Na_V1.8$ channels and (2) sensitization of TRPV1 channels will have no effect on histamine or compound 48/80 contractions. As an alternative, histamine and compound 48/80 could be applied to urinary bladder smooth muscle strips from TRPV1 knock-out mice. Thus, this proposed study would test the hypothesis that histamine and compound 48/80 induced contractions are independent of nerve activation along with TRPV1 sensitization.

4.3. Urothelial Signaling and Permeability

The urothelium is not essential for histamine-induced contractions, but compound 48/80 induced contractions are entirely urothelium dependent. This suggests that urothelial signaling as a driver of increased spontaneous phasic contractions of urinary bladder strips exposed to compound 48/80 should be further investigated. The investigations of compound 48/80 urothelium dependence should consist of the following: (1) Ca^{2+} mobilization and (2) assessment of urothelium permeability. Since activation of Ca^{2+} channels can cause spontaneous contractions, a series of experiments should be conducted measuring urothelial cell Ca^{2+} signaling in the presence or absence of activators and inhibitors [235]. Compound 48/80 also causes increased vascular permeability [190]; thus, it is possible that compound 48/80 caused urothelial cell damage and subsequent permeability. Propidium iodide could be used to assess urothelial cell viability following tissue bath experiments with compound 48/80. To assess if compound 48/80 increased permeability in the studies mentioned throughout this dissertation, an optimized Ussing chamber protocol would have needed to be carefully devised. Thus, these proposed studies would test the hypothesis that compound 48/80 increases urothelial signaling without altering urothelium permeability.

5. Perspectives

Treatments for urinary bladder dysfunction range from antimuscarinics to neurotoxins, which suggest either myogenic or neurogenic changes. Most treatments are viewed as temporarily effective. Difficulties in treating conditions of the urinary bladder could be due to a blend of LUTS or the lack of an “in-between therapeutic intervention”. An “in-between therapeutic intervention” would account for changes unrelated to myogenic and neurogenic origins. Thus, dissecting how inflammation alters urinary bladder smooth muscle contractility to identify new druggable targets for bladder dysfunction was necessary.

All the studies conducted in this dissertation are in the absence of urinary bladder dysfunction. Yet, mediators like histamine and prostaglandins are vital in understanding the pathophysiology of many bladder dysfunctions. Histamine and prostaglandin receptor expression alteration is an outcome of different bladder dysfunctions (see Chapter 1). The emergence of increased histamine and prostaglandin signaling could be indicative of changes in urinary bladder detrusor contractility. Wherein, hallmark findings in this dissertation implicate soluble mediator release from the urothelium to dictate spontaneous phasic contractions of the detrusor. Although histamine is not a driver of long-lived contractions, the chronic effects of increased bladder histamine need be explored. Further insight is needed to determine if the effects of histamine and compound 48/80 are protective or destructive.

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APPENDIX A: CONFERENCE ABSTRACTS

1. Society for Pelvic Research, 2019

Title: Histamine does not directly contract urinary bladder smooth muscle

Authors: B. Malique Jones, Osvaldo Vega Rodríguez, Nathan R. Tykocki

Introduction/Objective: Bladder dysfunction is largely associated with lower urinary tract symptoms indicative of overactive bladder (OAB) or underactive bladder (UAB). In bladder diseases such as interstitial cystitis and urinary tract infections, mast cells and mast cell degranulation are implicated as a potential cause for these symptoms. Mast cells degranulate in response to various stimuli and release histamine. Histamine is a well-studied in other tissues, but little is known about the role of histamine and histamine receptors within the bladder. Since other types of smooth muscle cells express H1 (contraction) and H2 (relaxation) histamine receptors, we tested the hypothesis that histamine causes detrusor muscle to contract.

Methods: All procedures followed institutional guidelines and were approved by the Institutional Animal Care and Use Committees of Michigan State University. 6-12-week-old C57BL/6 mice were euthanized, and bladders were removed. Dissected bladders were cut into approximately 2 mm wide bladder strips, either with or without the urothelium attached. Tissue was hung in an isolated tissue bath for isometric contractility experiments. Passive tension (1g) was applied, and tissue were exposed to an EC_{50} concentration of the cholinergic agonist carbachol (CCh; 200 nM) at the beginning and end of the experiment to verify tissue viability. Histamine concentration response curves (100 nM – 300 µM) were then performed. Other tissues were first contracted with 200 nM CCh before addition of 300 µM histamine to test for histamine-mediated relaxation.

Analyses were performed using GraphPad Prism software. Comparisons were made using two-way ANOVA with Bonferroni's post hoc analysis, or paired t-test.

Results: Histamine had no effect on CCh-induced contractions in any tissue. While histamine caused minimal contraction to bladder strips with urothelium, no contractile response occurred in the absence of the urothelium. Although bladder strips with urothelium show a small contraction, it was not statistically significant ($P > 0.05$, $N = 4\text{-}5$).

Conclusions: Histamine does not directly cause detrusor contraction in C57BL/6 mice, however further investigation is needed to determine if histamine causes release of a contractile substance from the urothelium.

2. Experimental Biology, 2020

Title: Histamine may directly contract urinary bladder smooth muscle

Authors: B. Malique Jones, Osvaldo J. Vega Rodríguez, Gerald C. Mingin, Nathan R. Tykocki

Mast cell degranulation and histamine release contribute to painful bladder syndrome, urinary tract infections, interstitial cystitis and other bladder disorders. The effects of histamine are well-studied in other tissues, but little is known about the role of histamine and histamine receptors within the bladder. Since other smooth muscle cells express H1 (contractile) and H2 (relaxant) histamine receptors, we tested the hypothesis that histamine causes contraction of the urinary bladder smooth muscle in mice. All procedures followed institutional guidelines and were approved by the Institutional Animal Care and Use Committees of Michigan State University. Six to 12-week-old C57Bl/6 mice were euthanized and dissected bladders were cut into approximately 2 mm wide bladder strips, with or without the urothelium attached, for isometric contractility experiments. Both histamine H1 and H2 receptors were expressed in bladder smooth muscle cells. Increasing concentrations of histamine (100 nM – 300 μM) did not contract strips without urothelium and minimally contracted bladder strips with urothelium. However, bolus administration of 200 μM histamine caused a rapid but transient contraction in strips with and without urothelium. The H1 receptor blocker fexofenadine (5 – 10 μM) blocked these contractions ($P<0.05$; N=5), whereas the H2 receptor blocker cimetidine (5 – 10 μM) had no effect. In addition, transient receptor potential vanilloid 1 (TRPV1) channel blocker capsazepine significantly reduced histamine-induced contractions only in bladder strips without urothelium, whereas the Na^+ channel blocker tetrodotoxin (TTX) had no effect in

any tissue ($P < 0.05$; N= 3–5). The mast cell activator compound 48/80 (10 µg/ml) contracted urinary bladder smooth muscle, and this contraction was also unaffected by TTX. These results indicate that H1 receptors on urinary bladder smooth cells likely modulate histamine-induced bladder contraction, and sufficient amounts of histamine can be released from mast cells to cause a contraction.

3. Experimental Biology, 2021

Title: The mast cell activator Compound 48/80 causes phasic urinary bladder smooth muscle contractions independent of histamine release

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

Inflammatory mediators released from mast cells cause both transient and phasic contractions of urinary bladder smooth muscle (UBSM). These contractions are viewed as initial disruptors of normal bladder function and can lead to profound bladder pathologies. However, the mechanisms responsible for each of these contractions has not been thoroughly investigated. Previously, we discovered that histamine-mediated contractions rapidly desensitize, whereas the mast cell activator compound 48/80 (10 µg/ml) caused both a long-lasting increase in the amplitude and frequency of phasic contractions in UBSM strips as well as a transient increase in baseline tension. Thus, we hypothesized that phasic contractions caused by compound 48/80 depended on the release of histamine from mast cells. Isometric contractility was performed with urothelium-intact UBSM strips from C57BL/6 mice in the presence or absence of the following drugs: the cholinergic antagonist atropine (2 µM); the $5HT_{2A}$ receptor antagonist MDL 11939 (1 µM); the histamine H1 receptor antagonist fexofenadine (10 µM), the histamine H2 receptor antagonist roxatidine (25 µM), the H2 antagonist cimetidine (10 µM); or the mast cell stabilizer cromolyn (100 µM). None of the compounds tested significantly reduced the amplitude and duration of the spontaneous phasic contractions caused by compound 48/80. However, both MDL 11939 and cromolyn significantly reduced the baseline contraction. Our data suggest that responses to the mast cell activator compound 48/80 are a superimposition of 2 components: (1) a slowly

desensitizing increase in baseline tension caused by release of 5-HT from mast cells, and (2) large-amplitude phasic contractions that are histamine- and 5HT-independent. However, the mechanism responsible for increases in the amplitude of phasic contractions remains unclear.

4. Society for Basic Urological Research, 2021

Title: Contraction and Desensitization to Histamine Require Cholesterol in Urinary Bladder Smooth Muscle

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

Background: Histamine is a vasoactive inflammatory signaling molecule that increases hypersensitivity to other inflammatory mediators and causes pleiotropic effects in various organs depending on the receptor subtype activated. Although histamine is implicated in many urinary bladder pathologies, these investigations focus heavily on nerves instead of on urinary bladder smooth muscle (UBSM) contractility. Histamine-induced contractions rapidly desensitize in the trachea, gut, and urinary bladder, but it is unknown if this is due to encocytosis, ß-arrestin mediated receptor inhibition, or histamine metabolism. We hypothesized that histamine induced UBSM contractions rapidly desensitize via caveolae mediated endocytosis.

Methods: Isometric contractility with urothelium-intact UBSM strips from male C57BL/6 mice was performed in the presence or absence of the following drugs/chemical agents: the cholesterol depleting agent methyl-ß-cyclodextrin (MßCD; 10 mM), cholesterol (5.1 mM), the dynamin inhibitor dynasore (15 µM - 100 µM), and the ß-arrestin/ß2-adaptin interaction inhibitor barbadin (100 µM). We further determined if the UBSM response to histamine was due to metabolism using the histamine-n- methyltransferase (HNMT) inhibitor SKF-91488, HNMT knockout mice, and a histamine buffer exchange bioassay.

Results: MßCD inhibited histamine induced UBSM contractions, which were recovered by reintroducing cholesterol. However, contractions to histamine remained transient.

Dynamin inhibition had no effect on histamine-induced contractions, nor did pharmacological inhibition or genetic ablation of HNMT. Also, buffer from strips already contracted and desensitized to histamine still contracted naïve UBSM strips, implying histamine was not metabolized.

Conclusion: These data suggest that histamine-induced contractions in UBSM depend on lipid rafts and/or caveolae to drive both contraction and desensitization. This desensitization is also not due to dynamin-mediated endocytosis or histamine metabolism. The transient UBSM contractile response may indicate that histamine's role pertaining to contractility is insignificant in the absence of a disease state or chronic exposure. However, future studies will examine potential genotypic changes that could occur to UBSM from prolong exposure to histamine.

5. International Continence Society, 2022

Title: The Basic Secretagogue Compound 48/80 Causes Urothelium Dependent Phasic Urinary Bladder Smooth Muscle Contractions Independent of Mast Cell Activation

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

HYPOTHESIS/AIMS OF STUDY: The urothelium serves as an active barrier to prevent pathogens and chemical stimuli within the urine from influencing urinary bladder function. However, certain pathogens can activate inflammatory signaling pathways that lead to urothelial cell damage, dysregulation of adenosine triphosphate (ATP) release, and neural activation. This is evident in urinary tract infections, interstitial cystitis, pelvic pain, and painful bladder syndrome. Current animal models used to induce interstitial cystitis or bacterial infection implicate mast cell degranulation as a leading cause of hypersensitivity that induces lower urinary tract symptoms. Thus, we hypothesized that phasic contractions caused by the mast cell activator compound 48/80 were due to mast cell degranulation and the subsequent release of prostaglandin E2 from the urothelium. Based on this hypothesis, we also examined the effects of compound 48/80 on urinary bladder smooth muscle (UBSM) strips from non-diseased mast cell deficient and C57BL/6 mice to establish a physiological mechanism by which mast degranulation can cause smooth muscle excitability.

STUDY DESIGN, MATERIALS AND METHODS: Based on our previous findings, we focused our efforts on both mast cells and the urothelium to determine the site of release of inflammatory mediators. We utilized pharmacological inhibitors and antagonists to determine the signaling molecule responsible for compound 48/80-induced contractions. The selection of each inhibitor and antagonist was based on known signaling pathways

that are responsible for inducing inflammation-driven contractility. In the urinary bladder, purinergic activation via the release of ATP causes the synthesis of prostaglandins resulting in UBSM contraction. Therefore, isometric contractility was performed with urothelium denuded and intact urinary bladder smooth muscle (UBSM) strips from C57Bl/6 and mast cell deficient c-Kit$^{W\text{-}sh/W\text{-}sh}$ (Sash) mice. UBSM strips from C57Bl/6 male mice (11 – 18 weeks old) were incubated with vehicle or the following drugs prior to exposure to compound 48/80: the nonsteroidal anti-inflammatory and cyclooxygenase (COX) inhibitor indomethacin (5 µM); the prostaglandin EP1 receptor antagonist SC51089 (10 µM); the non-selective P2 purinergic antagonist PPADS (100 µM); and the Gq/11 signaling inhibitor YM254890 (1 µM). Two urothelium-intact strip and two urothelium-denuded strips were isolated from each mouse bladder, and experiments were performed in parallel. These experiments were then repeated under the same conditions in male Sash mice (11 weeks old).

RESULTS: In UBSM strips from C57Bl/6 mice, compound 48/80 caused both an increase in basal UBSM tone as well as an increase in phasic contractions. Removal of the urothelium significantly reduced the amplitude and occurrence of phasic contractions (p=0.005 *vs* urothelium-intact). In UBSM strips from *Sash* mice, compound 48/80 still caused both an increase in basal UBSM tone as well as an increase in phasic contractions, which were unchanged vs C57Bl/6 mice. Removal of the urothelium also significantly reduced the amplitude and occurrence of phasic contractions in *Sash* mice (p=0.004 *vs* urothelium-intact). SC51089 had no effect on phasic contractions induced by compound 48/80 (p=0.420). However, indomethacin (p=0.04), PPADS (p=0.01), and

YM254890 ($p=0.028$) all significantly reduced basal tone and the amplitude and occurrence of phasic contractions.

INTERPRETATION OF RESULTS: Our data suggest that the basic secretagogue compound 48/80 is increasing phasic contractions *via* activation of urothelial cell signaling that is causing the release of ATP and activation of a cyclooxygenase-mediated pathway to increase synthesis of pro-contractile mediators. In addition, compound 48/80-induced contractions are dependent on G_q signaling, which could suggest a synergistic pathway. As compound 48/80 is known to activate G protein-coupled receptors to activate mast cells, our data further suggest the targets of compound 48/80 are not specific to mast cells. Although the exact signaling molecule and pathway responsible remains unclear, our data support a feed-forward mechanism that is increasing spontaneous mechanical behavior.

CONCLUDING MESSAGE: We have discovered that compound 48/80 induced UBSM contractions are independent of mast degranulation but depend on the urothelium. These findings show that basic secretagogues, such as compound 48/80, may not require mast cells to markedly alter urinary bladder function in response to an inflammatory insult. Our results also shed new light on the crosstalk between UBSM and urothelium and give further insight on potential therapeutic targets for inflammatory bladder dysfunction.

6. Experimental Biology, 2022

Title: Social Stress Upregulates Histamine H3 Receptor mRNA in Murine Urinary Bladder Smooth Muscle

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

In mice, social stress causes mast cell degranulation and alters voiding behavior. Histamine, a mast cell-derived biogenic amine, is hallmarked as a modulator that can drive neurogenic bladder overactivity. Additionally, histamine causes a rapidly desensitizing contraction of the urinary bladder smooth muscle (UBSM) in normal mice that is H1 receptor-dependent. However, histamine receptor expression in UBSM after social stress is unclear. We sought to determine if the expression of histamine receptors change in urinary bladder smooth muscle cells after social stress. We hypothesized that histamine H1 receptor expression would be upregulated in UBSM cells collected from stressed mice as compared to non-stressed. Prior to cell isolation and collection, 4-week-old C57BL/6 male mice were randomly housed with CD1 adult male aggressor mice for 5 minutes, after which both mice were placed in barrier housing for 1 hour. Social stress was repeated with different aggressor mice for 14 days. Afterward, bladders were enzymatically dissociated, and urinary bladder smooth muscle cells were isolated for single cell qRT-PCR. Non-stressed UBSM cells expressed only Hrh1and Hrh2 mRNA. However, UBSM cells from stressed mice also expressed Hrh3 mRNA. These data suggest that social stress alters histamine receptor expression in UBSM, specifically by upregulating histamine H3 receptor expression. Since H3 receptors are involved in inflammatory peptide release in other organ systems, the functional role of H3 receptors in stress-induced bladder dysfunction warrants further investigation.

7. CAIRIBU: Collaborating for the Advancement of Interdisciplinary Research in Benign Urology, 2022

Title: Compound 48/80 causes the release of Urothelium- Derived Mediators to Induce Urinary Bladder Smooth Muscle Contractions

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

Introduction and Objective: The urothelium serves as an active barrier to prevent pathogens and chemical stimuli within the urine from influencing urinary bladder function. However, certain pathogens can activate inflammatory signaling pathways that lead to urothelial cell damage, dysregulation of adenosine triphosphate (ATP) release, and neural activation. This is evident in urinary tract infections, interstitial cystitis, pelvic pain, and painful bladder syndrome. Current animal models used to induce interstitial cystitis or bacterial infection implicate mast cell degranulation as a leading cause of hypersensitivity that induces lower urinary tract symptoms. Thus, we hypothesized that phasic contractions caused by the mast cell activator compound 48/80 were due to mast cell degranulation and the subsequent release of prostaglandin E2 from the urothelium. Based on this hypothesis, we also examined the effects of compound 48/80 on urinary bladder smooth muscle (UBSM) strips from non- diseased mast cell deficient and C57BL/6 mice to establish a physiological mechanism by which mast degranulation can cause smooth muscle excitability.

Methods: Based on this hypothesis, we also examined the effects of compound 48/80 on urinary bladder smooth muscle (UBSM) strips from non-diseased c-*KitW-sh/W-sh* mast cell deficient and C57BL/6 mice via isometric contractility. UBSM strips were incubated with vehicle or the following drugs prior to exposure to compound 48/80: the

nonsteroidal anti-inflammatory and cyclooxygenase (COX) inhibitor indomethacin (5 μM); the prostaglandin EP1 receptor antagonist SC51089 (10 μM); the non-selective P2 purinergic antagonist PPADS (100 μM); and the Gq/11 signaling inhibitor YM254890 (1 μM).

Results: In UBSM strips from C57Bl/6 and c-*KitW-sh/W-sh* mice, compound 48/80 caused both an increase in basal UBSM tone as well as an increase in phasic contractions. Removal of the urothelium significantly reduced the amplitude and occurrence of phasic contractions in both strains. SC51089 had no effect on phasic contractions induced by compound 48/80. However, indomethacin, PPADS and YM254890 significantly reduced phasic contractions.

Conclusions: Our data suggest that the basic secretagogue compound 48/80 is increasing phasic contractions *via* activation of urothelial cell signaling that is causing the release of ATP and activation of a cyclooxygenase-mediated pathway to increase the synthesis of pro-contractile mediators.

8. American Physiological Society Summit, 2023

Title: The urothelium drives changes to urinary bladder smooth muscle contractility that mimic neurogenic inflammatory signaling

Authors: Brothely M. Jones, Gerald C. Mingin, Nathan R. Tykocki

Neurogenic inflammation disrupts urothelial homeostasis, which leads to barrier injury, cellular turnover, and dysregulated release of ATP and prostaglandins resulting in urinary bladder smooth muscle instability. Increased urothelium signaling is implicated in many bladder pathologies, such as idiopathic detrusor overactivity, interstitial cystitis, and recurrent urinary tract infections, in animal models along with patients. Previously, we utilized compound 48/80 as a pro-inflammatory agent on urinary bladder smooth muscle strips, which resulted in urothelium-dependent increases in smooth muscle phasic activity and augmented nerve-evoked contractions. Thus, we hypothesized that compound 48/80 causes spontaneous rhythmic contractions dependent on the release of urothelial-derived prostaglandins (PGE_2, PGF_{2a},Thromboxane A2) and pannexin-1-mediated ATP release, similar to neurogenic signaling pathways that drive smooth muscle hyperactivity. Tissue bath studies were performed with urothelium intact or denuded urinary bladder smooth muscle strips from 8–12-week-old male C57BL/6 mice (n=1 strip from N=5-6) to measure isometric contractility and nerve-evoked contractions in the presence of pharmacological inhibitors and compound 48/80 (10 µg/mL). Prior to compound 48/80 exposure, strips of urothelium were returned to the bottom of the organ tissue bath chambers directly underneath denuded smooth muscle strips to assess if soluble mediators were released. The non-selective cyclooxygenase inhibitor indomethacin (5 µM) diminished compound 48/80 augmentation of nerve-evoked contractions. However, the neurokinin 1 antagonist

QWF (10 μM), $PGF_{2\alpha}$ FP receptor antagonist AL8810 (5 μM), PGE_2 EP1 receptor antagonist SC51089 (10 μM), Thromboxane A2 TP receptor antagonist GR32191B (1 μM), and pannexin-1 mimetic inhibitory peptide ^{10}Panx (200 μM) had no effect on compound 48/80-induced contractions compared to respective vehicle controls (p=0.18, 0.91, 0.57, 0.41, and 0.44, respectively). A cocktail of all prostaglandin antagonists did significantly reduce contractions (p=0.01). Interestingly, despite the physical distance of the detached urothelium, exposure to compound 48/80 resulted in increased contractility (p=0.016) and phasic activity (p=0.017) as compared to a urothelium-denuded strips. These results suggest that activation of urothelial signaling via compound 48/80 mimics neurogenic inflammation to release a wide array of prostaglandins capable of altering bladder smooth muscle contractility. Thus, the urothelium should be considered as an inflammatory milieu whereby physiological and pharmacological changes contribute to urinary bladder smooth muscle dysfunction.

9. American Society for Pharmacology and Experimental Therapeutics, 2023

Title: Social stress causes the emergence of functional Histamine H3 Receptors in urinary bladder smooth muscle

Authors: B. Malique Jones, Gerald C. Mingin, and Nathan R. Tykocki

Inflammatory hyperalgesia induced by mast cell degranulation releases histamine within the urinary bladder that disrupts the micturition reflex and alters detrusor contractility. Psychological stress also causes lower urinary tract symptoms (frequency, urgency, and hesitancy) in 4-week-old mice with a pathophysiology similar to neurogenic inflammatory hyperalgesia. Most investigations have solely focused on molecular changes to nerve sensitivity instead of direct changes to urinary bladder smooth muscle activity. Recently, our lab found that urinary bladder smooth muscle of unstressed mice contracted and rapidly desensitized to histamine in an H1 receptor-dependent manner. We hypothesized that social stress increases histamine H1 receptor mRNA expression to prolong histamine-induced urinary bladder smooth contractions.4-week-old C57BL/6 male (N=6) mice were randomly housed with CD1 adult male aggressor mice for 5 minutes, after which both mice were placed in barrier housing for 1 hour. Social stress was repeated with different aggressor mice for 14 days. Afterward, bladders from 6-week-old mice were dissected and cut into strips from non-stressed (N=3) and stressed (N=3) mice to measure isometric contractile force generation and changes in contractility by histamine (200 µM). Individual bladder smooth muscle cells were also dissociated and collected for single-cell qRT-PCR. Counter to our hypothesis, histamine-induced contractions were markedly reduced in stressed as compared to non-stressed mice. Surprisingly, however, single-cell qRT-PCR performed on isolated smooth muscle cells uncovered an

upregulation of histamine H3 receptor mRNA in stressed mice that was absent in non-stressed mice. H3 receptors were present and constitutively active in the tissue, as the H3 receptor antagonist ciproxifan (10 μM) recovered the response to histamine in stressed bladders but had no effect in non-stressed bladders. In both conditions histamine-induced contractions rapidly desensitized, implying that H3 receptors do not affect this physiological mechanism. Our data aligns with previous findings that support a role for elevated histamine H3 receptor mRNA in patients with interstitial cystitis. Additionally, our data suggest that histamine H3 receptor might have a protective role in social stress by preventing histamine-induced changes to smooth muscle contractility in stressed bladders.