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Urinary Bladder Function and Somatic Sensitivity in Vasoactive Intestinal Polypeptide (VIP)^{-/-} Mice

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Abstract

Vasoactive intestinal polypeptide (VIP) is an immunomodulatory neuropeptide widely distributed in neural pathways that regulate micturition. VIP is also an endogenous anti-inflammatory agent that has been suggested for the development of therapies for inflammatory disorders. In the present study, we examined urinary bladder function, hindpaw and pelvic sensitivity in VIP^{-/-} and littermate wildtype controls. We demonstrated increased bladder mass and fewer but larger urine spots on filter paper in VIP^{-/-} mice. Using cystometry in conscious, unrestrained mice, VIP^{-/-} mice exhibited increased void volumes and shorter intercontraction intervals with continuous intravesical infusion of saline. No differences in transepithelial resistance or water permeability were demonstrated between VIP^{-/-} and WT mice; however, an increase in urea permeability was demonstrated in VIP^{-/-} mice. With the induction of bladder inflammation by acute administration of cyclophosphamide (CYP), an exaggerated or prolonged bladder hyperreflexia, hindpaw and pelvic sensitivity were demonstrated in VIP^{-/-} mice. The changes in bladder hyperreflexia and somatic sensitivity in VIP^{-/-} mice may reflect increased expression of neurotrophins and/or or proinflammatory cytokines in the urinary bladder. Thus, these changes may further regulate the neural control of micturition.

Keywords

neurotrophins; cytokines; cystometry; pelvic pain; bladder permeability

Introduction

Neuropeptides are potential mediators or modulators of inflammation and are found in human micturition pathways (Chapple et al., 1992; Lasanen et al., 1992; Morgan et al., 1999). Changes

in the expression of neuropeptides have been observed with bladder hyperreflexia (Chapple et al., 1992; Lasanen et al., 1992) and in animal models of bladder inflammation (Vizzard, 2000b, 2001; Zvarova and Vizzard, 2006). VIP is a member of the glucagon/secretin superfamily of hormones (Dickinson et al., 1999) and acts through two high affinity receptors, the VPAC₁ and VPAC₂ receptors (Harmar et al., 1998). VIP exerts, species-specific, excitatory or inhibitory actions in neural pathways controlling micturition and these functions may be altered with neural injury, disease or inflammation (Erol et al., 1992; Igawa et al., 1993; Uckert et al., 2002; Hernandez et al., 2006). VIP-immunoreactivity has been demonstrated throughout the neural pathways of the lower urinary tract (Keast and de Groat, 1989, 1992; Wanigasekara et al., 2003). Previous studies have demonstrated increased expression of phosphorylated cAMP response element binding protein (p-CREB) in bladder afferent cells in the lumbosacral dorsal root ganglia, increased nerve growth factor (NGF) content in the urinary bladder, and urinary bladder hypertrophy in VIP^{-/-} mice compared to littermate, WT mice and after CYP-induced cystitis (Vizzard et al., 2007; Jensen et al., 2008). Increased NGF bladder content after urinary bladder inflammation (Vizzard, 2000a), spinal cord injury (Vizzard, 2000a) or urethral outlet obstruction (Steers et al., 1991; Tuttle et al., 1994; Zvara et al., 2002) has been demonstrated and an involvement of urinary bladder NGF in contributing to urinary bladder hyperreflexia has been suggested (Lamb et al., 2004; Hu et al., 2005; Yoshimura et al., 2006; Zvara and Vizzard, 2007). In addition to roles as neurotransmitter or neuromodulator in autonomic nervous system pathways, VIP exhibits considerable anti-inflammatory properties (Said, 1991; Delgado et al., 2000; Voice et al., 2002; Szema et al., 2006). Recent studies (Girard et al., 2008) from our laboratory have demonstrated a proinflammatory shift in cytokines, chemokines and associated receptors after induction of urinary bladder inflammation by cyclophosphamide (CYP) in VIP^{-/-} mice.

Studies with a chemically (cyclophosphamide, CYP)-induced bladder inflammation model have demonstrated alterations in neurochemical (Vizzard, 2000b, 2001), electrophysiological (Yoshimura and de Groat, 1999) and organizational (Vizzard and Boyle, 1999) properties of micturition reflex elements. These changes may be mediated by chemical mediators produced in the bladder, spinal cord or dorsal root ganglia with cystitis (Vizzard, 2000a, 2000b, 2001; Malley and Vizzard, 2002; Braas et al., 2006). Possible mechanisms underlying the neural plasticity following chronic CYP-induced cystitis (Vizzard and de Groat, 1996; Jennings and Vizzard, 1999; Yoshimura and de Groat, 1999; Vizzard, 2000b, 2000d, 2001) may involve alterations in neurotrophic factors, neural activity arising in the urinary bladder (Vizzard, 2000a) and neuroimmune activation (Cominelli and Pizarro, 1996; Wong et al., 1997; Hill et al., 1999; Anderson and Rao, 2001; Mason et al., 2001; Samad et al., 2001; Winkelstein et al., 2001).

Due to the previous demonstration of increased NGF bladder content, urinary bladder hypertrophy and enhanced expression of proinflammatory mediators with urinary bladder inflammation, the present studies compared urinary bladder function and somatic sensitivity in VIP^{-/-} mice and littermate, WT mice. Studies involved: (1) the characterization of urinary bladder function in conscious, unrestrained mice with or without CYP-induced cystitis; (2) somatic sensitivity testing of hindpaw and pelvic region and nociceptive behavioral scoring after CYP-induced cystitis and (3) the characterization of transepithelial resistance, permeabilities and capacitance of urinary bladder.

Methods

Animals

VIP^{-/-} mice (Dr. James Waschek, University of California, LA, USA) (Colwell et al., 2003) were used in these experiments. The VIP^{-/-} mouse model was prepared using a VIP gene disruption strategy with confirmation of targeted mutation in mice and subsequent

backcrossing to the C57BL/6 strain for at least six generations (Colwell et al., 2003). Mice used were bred locally at The University of Vermont and lack of the VIP gene was confirmed by PCR genotyping of tail snips. Mice were housed (12-hr light/dark cycle) in groups (5) in the UVM animal vivarium with water and food provided ad libitum. VIP^{-/-} and wildtype (WT) controls from the same litter were analyzed in most cases (Jensen et al., 2008). When a WT littermate was unavailable, an age-matched control mouse from another C57BL/6 litter was used. Previous studies (Girard et al., 2006) have demonstrated that the closely related neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), does not compensate for VIP in VIP^{-/-} mice. Thus, the VIP and PACAP systems appear distinct. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC # 06-014). Animal care was under the supervision of the University of Vermont's Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress.

Acute cyclophosphamide (CYP)-induced cystitis

To produce an acute inflammation of the bladder, the cytostatic drug, cyclophosphamide (CYP; Sigma-Aldrich, 40 mg/ml in saline), was injected (150 mg/kg; i.p.) (Qiao and Vizzard, 2002a, 2004; Zvarova and Vizzard, 2006). After CYP-treatment, animal health status was observed daily. Control rats received volume-matched injections of saline (0.9%; i.p.) or no treatment and no difference among the control groups was observed. All injections were performed under isoflurane (2%) anesthesia. The CYP model of bladder inflammation produces an increase in voiding frequency with small micturition volumes and is associated with inflammatory cell infiltrates in the urinary bladder including mast cells, macrophages and neutrophils (Vizzard, 2000c; Hu et al., 2003; LaBerge et al., 2006). The animals were evaluated for bladder function or mechanical sensitivity of the hindpaw or pelvic region 4 hours (h) after treatment.

Bladder Catheter Implant

A lower midline abdominal incision was performed under general anesthesia (isoflurane 2.5-3.5%) and PE-10 tubing with the end flared by heat was inserted into the dome of the bladder and secured in place with a 6-0 nylon purse string suture (Hu et al., 2005; Braas et al., 2006; Zvara and Vizzard, 2007). The distal end of the tubing was sealed, tunneled subcutaneously and externalized. Animals were maintained for 48 h to ensure complete recovery. Postoperative analgesics were given for a period of 48 h.

Bladder Function: Cystometrograms

Animals were placed conscious and unrestrained in recording cages with a balance and pan for urine collection and measurement placed below (Hu et al., 2005; Braas et al., 2006; Zvara and Vizzard, 2007). Intravesical pressure changes are recorded using a Small Animal Cystometry System (Med Associates, Inc.). Saline at room temperature is infused at a rate of 25 µl/min to elicit repetitive bladder contractions. At least four reproducible micturition cycles were recorded after an initial stabilization period of 25 - 30 minutes. Voided saline was collected to determine voided volume. After void volumes are collected, the infusion was stopped and residual volume was determined: residual saline was withdrawn through the intravesical catheter. Intercontraction interval, maximal voiding pressure, pressure threshold for voiding and baseline resting pressure were measured (Maggi et al., 1986). The number of non-voiding bladder contractions (NVC) per voiding cycle and maximal NVC pressure were assessed. For these studies, NVC were defined as rhythmic intravesical pressure rises (greater than 5 cm H₂O from baseline pressure) without a release of fluid from the urethra. *Exclusion*

criteria Mice were removed from study when adverse events occurred that included: $\geq 20\%$ reduction in body weight post surgery, a significant post-operative event, lethargy, pain or distress not relieved by our IACUC-approved regimen of post-operative analgesics or hematuria in control rodents. In the present study, no mice were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable (Streng et al., 2006). Experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements (Dorr, 1992). Mice were euthanized at the conclusion of study by isoflurane (4%) and thoracotomy.

Urination Patterns

WT and VIP^{-/-} mice with and without CYP treatment (4 h) were placed individually in standard cages for 1 h with the bedding replaced with Whatman Grade 3 filter paper. Food and water were provided ad libitum. Urine spots were photographed under UV light (Thor et al., 1989; Birder et al., 2002) and counted. Mice were placed into cages and data analyzed by an individual blinded to treatment and mouse strain. Groups were decoded after data analysis.

Mechanical sensitivity testing and nociceptive behavior observation

Referred (secondary) hyperalgesia and tactile allodynia was tested using calibrated von Frey hairs with forces of 0.04, 0.16, 0.4, 1, and 4 g to the abdomen (Laird et al., 2001; Laird et al., 2002; Rudick et al., 2007) and hindpaw (Chaplan et al., 1994; Guerios et al., 2006; Rudick et al., 2007) before and after CYP treatment. Mice were tested in individual Plexiglas chambers with a stainless steel wire grid floor. Mice were acclimated to the chambers for a period of 2 h. Pilot studies determined that this period of acclimation was necessary. Mechanical sensitivity testing was performed in separate groups of mice not used for bladder function determination. The von Frey hairs were applied in an up-down method for 1–3 sec with an interstimulus interval of 15 sec. For pelvic region stimulation, stimulation was confined to the lower abdominal area overlying the urinary bladder. Testing of the plantar region of the hindpaw and lower abdominal area was performed by perpendicular application of the von Frey hairs to the indicated areas until the hair bent slightly. The following behaviors were considered positive responses to pelvic region stimulation: sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area (Rudick et al., 2007). A positive response to hindpaw stimulation was sharp withdrawal of the paw or licking of the tested hindpaw (Rudick et al., 2007). In addition to mechanical sensitivity testing in mice, we also observed mice for nociceptive behaviors beginning 30 min after CYP treatment (Laird et al., 2000; Hu et al., 2005) for WT and VIP^{-/-} mice. After treatment, rodents were observed every 30 min for 5 min for a total duration of 4 h. The number and type of the following behaviors was recorded, assigned points and a cumulative point score of all observed behaviors was quantified for each time point (Laird et al., 2000; Shin et al., 2006): (1) normal (score 0); (2) piloerection (score 2); (3) strong piloerection (score 3); (4) labored breathing (score 4); licking of the abdomen (score 5) and (6) stretching or contraction of the abdomen (score 6). All somatic testing and behavioral scoring was performed in a blinded manner with respect to treatment and mouse strain. The groups were decoded after data analysis.

Permeability measurements

Permeability studies were performed as previously described (Lavelle et al., 2002). Briefly, WT and VIP^{-/-} mouse bladders were rapidly excised after the animals were euthanized with 100% CO₂ inhalation and thoracotomy. The bladders were bisected and placed immediately into Ringer solution (containing (mM): NaCl 111.2, NaHCO₃ 25, KCl 5.8, CaCl₂ 2, MgSO₄ 1.2, KH₂PO₄ 1.2 and glucose 11). The solution was oxygenated and maintained at 37°C and

pH 7.2-7.5. The bladders were placed on a rack with the epithelium facing downward, stretched on a 0.73 cm² ring and held in place by a set of pins located away from the area of investigation. The tissue was mounted between two halves of an Ussing chamber as described (Lavelle et al., 2002) and the chamber filled with Ringer solution. The temperature of the chamber was maintained at 37°C and the hemichambers were constantly stirred. Electrical measurements of transepithelial resistance (TER) were performed throughout the experiments using a four-electrode current/voltage clamp (Warner Instruments, Hamden, CT, USA) as previously described (Truschel et al., 1999) to determine epithelial integrity. The membranes were allowed to stabilize for 1 h prior to addition of isotopes and measurements.

[³H]Water (1 µCi ml⁻¹) and [¹⁴C]urea (0.25 µCi ml⁻¹) were added to the apical side of the membrane and both hemichambers were sampled (2 × 100 µl per hemichamber) at 15 min intervals throughout the experiment. To determine the contribution of unstirred layers to the measured permeabilities, the apical membranes were destroyed by the addition of Triton X-100 (100 µl). In all experiments, Triton X-100 abolished the TER. Experimental results for each mouse were determined by averaging the four flux measurements during each experimental stage. Flux rates were obtained before and after permeabilization of the epithelium by Triton X-100. The electrical resistance values represent the means of at least five recordings taken during the course of each experiment.

Tissue Capacitance Measurements

Mouse bladders were excised, and after careful dissection of the muscularis, the bladder mucosa was placed on tissue rings that exposed 2 cm² of tissue as described previously (Wang et al., 2003). Mouse tissue was mounted on rings that exposed 0.35 cm² of tissue. The tissue rings were mounted between 2 halves of a custom Ussing stretch chamber, each hemichamber was filled with 13 ml Krebs solution, and the tissue was equilibrated for 30 minutes as described previously (Truschel et al., 2002; Wang et al., 2003). Bladder filling was mimicked by increasing hydrostatic pressure across the mucosal surface of the tissue by filling the mucosal hemichamber to a volume of 14 ml (hemichamber capacity). We measured capacitance (where 1 µF ≈ 1 cm² of membrane surface area) by monitoring the voltage response to a square current pulse, as described previously (Truschel et al., 2002). Although the tissue preparations used in this study contained multiple cell layers, previous analysis confirmed that changes in capacitance primarily reflect changes in the apical surface area of the umbrella cell layer (Clausen et al., 1979; Lewis and de Moura, 1984; Wang et al., 2003).

Materials

All standard chemicals were obtained from Sigma-Aldrich or Fisher and were either analytical or laboratory grade.

Statistical Analyses

All values represent mean ± S.E.M. Data were compared using Student's t-test, one-way analysis of variance (ANOVA) or two-way ANOVA, where appropriate. When F ratios exceed the critical value ($p \leq 0.05$), the Newman-Keul's post-hoc test was used to compare means.

Results

General properties of WT and VIP^{-/-} mice

Female WT and VIP^{-/-} mice were of similar body mass but consistent with previous studies (Girard et al., 2008), the urinary bladder of VIP^{-/-} mice exhibited significantly increased mass (Table 1). Fluid intake measured over a 24 h period was similar in both WT and VIP^{-/-} mice (Table 1). Urine spots on filter paper quantified over a 1 h period were significantly fewer in

number but greater in area ($12.2 \pm 4.7 \text{ cm}^2$) in $\text{VIP}^{-/-}$ mice compared to urine spots made by WT mice that were numerous but smaller in area ($4.3 \pm 3.5 \text{ cm}^2$) (Table 1).

Somatic sensitivity and nociceptive behavioral scores in WT and $\text{VIP}^{-/-}$ mice with CYP-induced cystitis

Increased sensitivity to somatic stimuli (referred hyperalgesia and tactile allodynia) has been noted in the presence of visceral inflammation (Laird et al., 2001; Laird et al., 2002; Guerios et al., 2006; Rudick et al., 2007). In the present study in separate groups of mice, we evaluated mechanical somatic sensitivity using a calibrated series of von Frey hairs on the plantar region of the hindpaw and pelvic region overlying the urinary bladder in WT and $\text{VIP}^{-/-}$ mice with CYP-induced cystitis. Behavioral scores were also generated in mice every 30 min for a duration of 4 h after CYP administration. The cumulative behavioral scores of $\text{VIP}^{-/-}$ mice were less than those observed in WT mice treated with CYP from time 0 to 1.5 h after CYP administration (Fig. 1A). This decreased behavioral score reached significance ($p \leq 0.01$) at 1 h after CYP treatment whereas other time points (0 h, 0.5 h, 1.5 h) exhibited a trend toward decrease (Fig. 1A). After CYP treatment for 1.5 h, cumulative pain scores for WT and $\text{VIP}^{-/-}$ overlapped and reached a maximum at 2 h with only a slight reduction in cumulative scores from 2.5 h to 4 h after CYP treatment (Fig. 1A). The 4 h time point was used in subsequent somatic sensitivity testing of the hindpaw and pelvic region after induction of bladder inflammation.

Somatic sensitivity in the pelvic region as evidenced by sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area was significantly ($p \leq 0.001$) increased in $\text{VIP}^{-/-}$ mice with CYP treatment with von Frey hairs of all forces tested (0.1 - 4 g) compared to WT with CYP treatment (Fig. 1B). In the plantar region of the hindpaw, a significant ($p \leq 0.01$) decrease in paw pressure threshold that elicited sharp withdrawal of the paw or licking of the tested hindpaw was observed in both WT and $\text{VIP}^{-/-}$ mice 4 h after CYP treatment (Fig. 1C). This significant ($p \leq 0.01$) decrease in paw pressure threshold was maintained in $\text{VIP}^{-/-}$ mice 24 h after CYP treatment (Fig. 1C). In contrast, paw pressure threshold returned to normal 24 h after CYP treatment in WT mice (Fig. 1C).

Bladder function in WT and $\text{VIP}^{-/-}$ mice without bladder inflammation

Consistent with fewer urine spots of increased area (Table 1), bladder function studies demonstrated significantly ($p \leq 0.001$) increased voided volume in $\text{VIP}^{-/-}$ mice compared to WT mice (Fig. 2Aa, Ab, Ba, Bb) (Fig. 3A). Both $\text{VIP}^{-/-}$ ($7.3 \pm 2.5 \text{ cm H}_2\text{O}$) and WT ($6.3 \pm 2.5 \text{ cm H}_2\text{O}$) mice exhibited non-voiding contractions in the absence of bladder inflammation consistent with previous studies (Lagou et al., 2006; Streng et al., 2006) (Fig. 2Aa, Ab, Ba, Bb). Non-voiding contractions in both mouse strains occurred throughout the filling period but seemed to more obvious in the $\text{VIP}^{-/-}$ mice prior to a micturition event (Fig. 2Aa, Ab, Ba, Bb). Residual volume in both the WT and $\text{VIP}^{-/-}$ mice was minimal ($< 5 \mu\text{l}$). Intercontraction intervals in $\text{VIP}^{-/-}$ mice were significantly longer compared to WT mice (Fig. 3B). No differences in maximum voiding pressure, micturition threshold pressure or baseline resting pressure were observed in WT or $\text{VIP}^{-/-}$ mice (Fig. 4A).

Bladder function in WT and $\text{VIP}^{-/-}$ mice CYP-induced cystitis (4 h)

WT and $\text{VIP}^{-/-}$ mice were treated with CYP (4 h) and bladder function assessed. In some instances, bladder function data was obtained in the same mice both under control (non-inflamed) and inflamed bladder conditions. After CYP treatment, void volume and intercontraction interval significantly ($p \leq 0.001$) decreased in both WT and $\text{VIP}^{-/-}$ mice (Fig. 2Ac, Ad, Bc, Bd) (Fig. 3A, B). However, the magnitude of the reduction of the void volume (6.5-fold decrease in $\text{VIP}^{-/-}$ mice vs. 2.2-fold decrease in WT mice) and intercontraction interval (6.7-fold decrease in $\text{VIP}^{-/-}$ mice vs. 2.2-fold decrease in WT mice) was significantly

($p \leq 0.001$) greater in VIP^{-/-} mice (Fig. 3A, B). Non-voiding contractions continued to be exhibited in WT and VIP^{-/-} mice with CYP-induced cystitis. Non-voiding contractions were increased in amplitude in both VIP^{-/-} (15.3 ± 3.5 cm H₂O) and WT (14.7 ± 4.2 cm H₂O) mice treated acutely with CYP. No changes in maximum voiding pressure, micturition threshold pressure or baseline resting pressure were observed between WT and VIP^{-/-} mice after CYP treatment (Fig. 4B). No changes in bladder pressures were observed in either strain under control (no inflammation) or CYP (4 h) conditions (inflamed) (Fig. 4A, B).

Transepithelial resistance, permeability and capacitance in urinary bladder

Transepithelial resistance (TER) is a measure of the ability of the epithelium to prevent ionic movement across the tissue and represents a critical component of urothelial barrier function (Kanai et al., 2002). Permeabilities to water and urea also relate to the integrity of the apical membrane and tight junctions (Kanai et al., 2002). In the present study, no differences in TER (Fig. 5A) or permeability to water (Fig. 5B) were demonstrated in urinary bladder of WT and VIP^{-/-} mice. In contrast, an increase in urea permeability was revealed ($p \leq 0.05$) in VIP^{-/-} urinary bladder compared to WT (Fig. 5C). No changes in capacitance of urinary bladder of WT or VIP^{-/-} mice with or without stretch were observed (Fig. 6A, B).

Discussion

The present study demonstrates several novel findings concerning the bladder function and somatic sensitivity of VIP^{-/-} mice. We demonstrated increased bladder mass and fewer but larger urine spots on filter paper in VIP^{-/-} mice. VIP^{-/-} mice exhibit increased void volumes and shorter intercontraction intervals with continuous intravesical infusion of saline in conscious, unrestrained mice. No differences in transepithelial resistance or water permeability were demonstrated between VIP^{-/-} and WT mice. However, an increase in urea permeability was demonstrated in VIP^{-/-} mice. With the induction of bladder inflammation by acute (4 h) administration of CYP, an exaggerated or prolonged bladder hyperreflexia, hindpaw and pelvic sensitivity were demonstrated in VIP^{-/-} mice. The changes in neural control of bladder function that manifest as bladder hyperreflexia and increased somatic sensitivity in VIP^{-/-} mice may reflect increased expression of nerve growth factor (NGF) (Jensen et al., 2008) or proinflammatory cytokine production in urinary bladder (Girard et al., 2008) as previously demonstrated.

A number of diverse and conflicting roles for VIP have been demonstrated in the urinary bladder from numerous species. VIP has been shown to relax urinary bladder from human (Uckert et al., 2002) or pig (Hernandez et al., 2006), and contract or produce no effects on urinary bladder from the rat (Erol et al., 1992; Igawa et al., 1993). These contradictory findings might be attributable to species differences (Uckert et al., 2002) and differential VIP receptor distribution. The majority of VIP in the lower urinary tract is located in postganglionic efferent neurons of the pelvic ganglia (Chapple et al., 1992; Smet et al., 1997; Wanigasekara et al., 2003). Although the functional effects of VIP on the bladder seem diverse, our cystometry results do not demonstrate differences in basal tone of the urinary bladder as resting baseline pressures were similar in both VIP^{-/-} and WT mice with and without bladder inflammation. Future studies could address contractility using isolated bladder strips and electrically- and agonist-induced contractions (Braas et al., 2006). Consistent with our previous demonstration (Jensen et al., 2008) of an increased bladder to body weight ratio and increased detrusor smooth muscle thickness in VIP^{-/-} mice, the present study also demonstrates increased urinary bladder mass in VIP^{-/-} mice.

Hypertrophy of the urinary bladder smooth muscle is associated with increased NGF expression. Partial urethral obstruction, spinal cord injury and CYP-induced cystitis are associated with increases in bladder mass, altered expression of urinary bladder NGF (Steers

and de Groat, 1988; Steers et al., 1991; Tuttle et al., 1994; Steers et al., 1996) and the NGF receptor, TrkA (Qiao and Vizzard, 2002b; Qiao and Vizzard, 2004). In previous studies, VIP^{-/-} mice exhibited increased basal and bladder inflammation-induced expression of NGF. A recent study (Lelievre et al., 2007) of intestinal morphology and function in VIP^{-/-} mice has also demonstrated a significant increase in intestinal smooth muscle thickness. A regulatory role for VIP in intestinal smooth muscle proliferation has been suggested (Lelievre et al., 2007) given the ability of VIP to inhibit airway smooth muscle proliferation (Maruno et al., 1995). The results of the present study may support a similar regulatory role for VIP in detrusor smooth muscle. The increased bladder mass of the VIP^{-/-} mice was consistent with the conscious cystometry and urine spot data that demonstrated increased voided volume with continuous intravesical infusion of saline as well as fewer but significantly larger urine spots. Both WT and VIP^{-/-} mice demonstrated evidence of phasic, non-voiding contractions during intravesical saline infusion. The functional significance and origin (myogenic vs. neurogenic) of these non-micturition contractions is not known (Herrera et al., 2003; Lagou et al., 2006; Streng et al., 2006). It has been suggested that non-voiding contractions are associated with mechanoreceptor activation in the detrusor and may contribute to sensations related to bladder volume (Streng et al., 2006).

With acute CYP induced bladder inflammation, VIP^{-/-} mice exhibited a more dramatic reduction in void volume and intercontraction interval compared to WT mice. A change in barrier function or integrity of the apical membrane or tight junctions of the urothelium could contribute to an exaggerated response to urinary bladder inflammation (Truschel et al., 1999; Kanai et al., 2002; Parsons, 2007). In this study, no differences in barrier function or permeability to water were demonstrated between VIP^{-/-} and WT mice. However, a significant increase in urea transport was demonstrated in VIP^{-/-} mice. Increased urea transport may reflect a different distribution of urea transporters or increased rate of urea transport in VIP^{-/-} mice that may impact urine concentration mechanisms (Parsons, 2007). Previous studies have demonstrated VIP-mediated effects on epithelial function including stimulation of CFTR-dependent chloride secretion via VPAC₁ receptors (Derand et al., 2004). Thus, there may be additional effects of VIP mediated by VPAC₂ urothelial receptors (Braas et al., 2006) on urothelial function not currently known that could contribute to altered bladder function.

Previous studies have suggested an involvement of NGF in bladder hyperreflexia (Lamb et al., 2004; Hu et al., 2005; Yoshimura et al., 2006; Zvara and Vizzard, 2007) and neurochemical changes in micturition reflexes (Zvara and Vizzard, 2007). VIP^{-/-} mice exhibit increased basal and inflammation-induced NGF content of the urinary bladder (Jensen et al., 2008). CYP-induced cystitis alters NGF and receptor expression in urinary bladder, dorsal root ganglia and major pelvic ganglia (Vizzard, 2000a). NGF scavenging (Dmitrieva et al., 1997; Hu et al., 2005) reduces bladder hyperreflexia in rats with bladder inflammation. Painful bladder syndrome (PBS)/Interstitial Cystitis (IC) is a chronic inflammatory bladder syndrome characterized by urinary frequency, urgency, suprapubic and pelvic pain (Driscoll and Teichman, 2001; Sant and Hanno, 2001). Numerous theories including; infection, autoimmune disorder, toxic urinary agents, deficiency in bladder wall lining and neurogenic causes have been proposed. Pain and altered bladder function in PBS/IC may involve a change in visceral sensation/bladder sensory physiology. Altered visceral sensations (Driscoll and Teichman, 2001; Sant and Hanno, 2001) may be mediated by changes in peripheral bladder afferent and central pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). Neurotrophins (e.g., nerve growth factor) have been implicated in the peripheral sensitization of nociceptors (Dray, 1995). Elevated levels of neurotrophins are in urine (Okragly et al., 1999) or in urinary bladder (Lowe et al., 1997) of women with PBS/IC. However, a recent study did not demonstrate an association of increased urothelium/suburothelium NGF with detrusor overactivity (Birder et al., 2007). In the present

studies, increased NGF bladder content in VIP^{-/-} mice may contribute to the exaggerated bladder hyperreflexia compared to WT mice.

In addition to roles as neurotransmitter or neuromodulator in autonomic nervous system pathways, VIP exhibits considerable anti-inflammatory properties (Said, 1991; Voice et al., 2002; Szema et al., 2006), mediated through VPAC₁ receptors on inflammatory cells (Delgado et al., 2000). It has been shown that VIP inhibits the production of pro-inflammatory compounds including, TNF α , iNOS, IL-1 and IL-12 (Chorny et al., 2006). Conversely, VIP upregulates production of the anti-inflammatory cytokine, IL-10 (Delgado et al., 1999). In diverse experimental models of inflammation, VIP has shown to improve symptoms and survival (Delgado et al., 1999; Abad et al., 2003; Bik et al., 2004; Juarranz et al., 2005; Martinez et al., 2005; Newman et al., 2005; Gonzalez-Rey and Delgado, 2006). We have recently demonstrated the increased expression of inflammatory gene transcripts and protein in urinary bladder of VIP^{-/-} mice treated with CYP as demonstrated with a superarray and confirmed with enzyme-linked immunoassays (Girard et al., 2008). A positive shift in the balance of proinflammatory mediators in urinary bladder of VIP^{-/-} mice with bladder inflammation (Girard et al., 2008) may contribute to the exaggerated response (reduced void volume and intercontraction interval) to urinary bladder inflammation demonstrated in the present study. In addition, the increased somatic sensitivity of the pelvic region overlying the urinary bladder and the prolonged hindpaw hypersensitivity in VIP^{-/-} mice with CYP-induced cystitis may also involve the increased expression of inflammatory mediators. Thus, proinflammatory mediators can influence the neural control of bladder function.

Both neurotrophins (e.g., NGF) and cytokines/chemokines may contribute to urinary bladder hyperreflexia (Lamb et al., 2004; Vera and Meyer-Siegler, 2004; Hu et al., 2005; Yoshimura et al., 2006; Zvara and Vizzard, 2007) and increased somatic sensitivity (Abbadie, 2005; Bielefeldt et al., 2006; Guerios et al., 2006; Martinez and Melgar, 2008) in the presence of visceral inflammation. Current studies are determining if increased NGF expression drives cytokine/chemokine expression in the urinary bladder using an NGF-overexpressing mouse line under the control of the uroplakin II promoter. Additional studies are evaluating the contribution of cytokines/chemokines in the observed urinary bladder hyperreflexia and somatic sensitivity in VIP^{-/-} mice with CYP treatment using subtraction techniques.

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Figure 1.

Nociceptive behavioral scoring and somatic sensitivity testing in WT and VIP^{-/-} mice after cyclophosphamide (CYP) treatment. **A.** Cumulative nociceptive scores for wildtype (WT) and VIP^{-/-} mice. Mice were observed every 30 min for 5 min after CYP treatment for a period of 4 hours (h). Cumulative scores were assigned based upon the following behaviors: (1) normal (score 0); (2) piloerection (score 2); (3) strong piloerection (score 3); (4) labored breathing (score 4); licking of the abdomen (score 5) and (6) stretching or contraction of the abdomen (score 6). Data represent the mean $\pm$ S.E.M. of $n = 10$ mice per group. **B.** Pelvic region testing with calibrated von Frey hairs in WT and VIP^{-/-} mice after CYP treatment (4 h). The von Frey hairs were applied in an up-down method for 1-3 sec with an interstimulus interval of 15 sec.

For pelvic region stimulation, stimulation was confined to the lower abdominal area overlying the urinary bladder. The following behaviors were considered positive responses to pelvic region stimulation: sharp retraction of the abdomen, jumping, or immediate licking or scratching of the pelvic area (Rudick et al., 2007). $VIP^{-/-}$ mice exhibited significantly (*, $p \leq 0.001$) increased pelvic response frequency with all von Frey hairs (0.1 - 4 g) tested. Data represent the mean $\pm$ S.E.M. of $n = 14$ mice per group. **C.** Hindpaw sensitivity testing with calibrated von Frey hairs in WT and $VIP^{-/-}$ mice after CYP treatment (4 h). $VIP^{-/-}$ mice exhibited a significant (*, $p \leq 0.01$) decrease in the paw pressure threshold required to elicit a positive response at 4 h and 24 h after CYP treatment; sharp withdrawal of the paw or licking of the tested hindpaw (Rudick et al., 2007). Data represent the mean $\pm$ S.E.M. of $n = 10$ mice per group. All somatic testing and behavioral scoring was performed in a blinded manner with respect to treatment and mouse strain.

Figure 2.

Representative cystometrograms from conscious, unrestrained $VIP^{-/-}$ (**A**) and wildtype (WT; **B**) mice with continuous intravesical infusion (25 μ l/min) of room temperature saline under control (no inflammation; **Aa, b; Ba, b**) conditions or after acute treatment with cyclophosphamide (CYP; **Ac, d; Bc, d**). Bladder pressure (cm H₂O) and voided volume (ml) are shown. Data presented are from the same WT mouse or $VIP^{-/-}$ mouse in the absence and presence of CYP-induced cystitis. Arrows indicate non-voiding contractions.

Figure 3.

Summary bar graphs of the voided volume (μl; **A**) and intercontraction interval (seconds, s; **B**) using conscious cystometry in conscious, unrestrained WT and VIP^{-/-} mice with continuous infusion of saline in non-inflamed mice or mice treated with CYP (4 h). **A.** Voiced volumes in VIP^{-/-} mice were significantly ($p \leq 0.001$) greater in control mice without bladder inflammation (white bars). Both WT and VIP^{-/-} mice exhibited a significant ($p \leq 0.001$) decrease in voided volume after bladder inflammation with CYP (black bars). The magnitude of reduction in the voided volume was significantly greater in VIP^{-/-} mice. Data represent the mean $\pm$ S.E.M. of $n = 10$ mice per group. **B.** Intercontraction intervals were significantly longer in VIP^{-/-} mice compared to WT mice in control mice without bladder inflammation (white bars). Both WT and VIP^{-/-} mice exhibited a significant decrease in intercontraction interval duration after CYP treatment (black bars). The magnitude of reduction in the intercontraction interval was significantly greater in VIP^{-/-} mice. Data represent the mean $\pm$ S.E.M. of $n = 10$ mice per group.

Figure 4.

Bladder pressures in WT and VIP^{-/-} mice under control (no CYP treatment; **A**) and inflamed urinary bladder (CYP; 4 h; **B**) conditions as determined with conscious cystometry and continuous intravesical infusion of saline. No differences in maximum voiding pressure, threshold pressure or baseline resting pressure were determined in WT and VIP^{-/-} mice without CYP treatment (**A**) or after CYP treatment (**B**). Bladder pressures were not different between mouse strains or after CYP treatment. Data represent the mean $\pm$ S.E.M. of $n = 10$ mice per group.

Figure 5.

Transepithelial resistance (TER) and permeability measurements in urinary bladder from WT and VIP^{-/-} mice. **A.** No difference in TER was demonstrated in WT or VIP^{-/-} mice. **B.** No change in permeability to ³H-water permeability was demonstrated in WT or VIP^{-/-} mice. **C.** A significant ($p \leq 0.01$) increase in ¹⁴C-urea permeability was demonstrated in VIP^{-/-} mice. Bar graphs in **A-C** represent the mean $\pm$ S.E.M. of $n = 16$ VIP^{-/-} mice and $n = 18$ WT mice.

Figure 6. Capacitance of urinary bladder from WT (**A**) and VIP^{-/-} mice (**B**) in response to bladder stretch or no stretch. WT and VIP^{-/-} mouse bladder tissue was equilibrated in Krebs solution. No differences in capacitance were observed between urinary bladder from WT and VIP^{-/-} mice with or without stretch. Line graphs in **A-B** represent the mean $\pm$ S.E.M. of $n = 8$ VIP^{-/-} mice with stretch; $n = 4$ VIP^{-/-} mice without stretch; $n = 3$ WT mice with stretch; $n = 4$ WT mice without stretch.

Table 1

Comparison of urinary tract parameters between female wildtype (WT) and VIP^{-/-} littermates

	WT (<i>n</i> = 8)	VIP ^{-/-} (<i>n</i> = 8)
Body weight (g)	25.8 ± 1.3	24.2 ± 1.1
Bladder weight (mg)	39.2 ± 3.5	54.5 ± 4.5*
Fluid intake (ml/24 h)	7.2 ± 0.8	7.4 ± 1.0
Urine spots (number/h)	10.4 ± 2.5	4.2 ± 1.2*

Values represent mean ± S.E.M. for the number of animals analyzed (*n* = 8) in each group. Statistical significance (*p* ≤ 0.01) is denoted by *.