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Expression of Phosphorylated cAMP Response Element Binding Protein (pCREB) in Bladder Afferent Pathways in VIP^{-/-} Mice with Cyclophosphamide (CYP)-induced Cystitis

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Abstract

The expression of phosphorylated cAMP response element binding protein (pCREB) in dorsal root ganglia (DRG) with and without CYP-induced cystitis (150 mg/kg, i.p; 48 hr) was determined in VIP^{-/-} and wildtype (WT) mice. p-CREB-immunoreactivity (IR) was determined in bladder (Fastblue) afferent cells. Nerve growth factor (NGF) bladder content was determined by ELISAs. Basal expression of pCREB-IR in DRG of VIP^{-/-} mice was ($p \leq 0.01$) greater in L1, L2, L5-S1 DRG compared to WT mice. CYP treatment in WT mice increased ($p \leq 0.05$) pCREB-IR in L1, L2, L5-S1 DRG. CYP treatment in VIP^{-/-} mice ($p \leq 0.01$) increased ($p \leq 0.01$) p-CREB-IR in L6-S1 DRG compared to WT with CYP. In WT mice, bladder afferent cells (20–38%) in DRG expressed pCREB-IR under basal conditions. With CYP, pCREB-IR increased in bladder afferent cells (60–65%; L6-S1 DRG) in WT mice. In VIP^{-/-} mice, bladder afferent cells (12–58%) expressed pCREB-IR under basal conditions and CYP increased pCREB expression (78–84%) in L6-S1 DRG. Urinary bladder NGF expression in VIP^{-/-} mice under basal conditions or after cystitis was significantly greater than WT. Detrusor smooth muscle thickness was significantly increased in VIP^{-/-} mice. Bladder NGF expression may contribute to differences in pCREB expression.

Index entries

inflammation; urinary bladder; afferent neurons; growth factors; cytokines

Introduction

Patients with interstitial cystitis (IC) or painful bladder syndrome (PBS), a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency and suprapubic and pelvic pain and pain at low to moderate bladder pressure (Petrone et al., 1995). Although the etiology and pathogenesis of IC/PBS are unknown, numerous theories including; infection, autoimmune disorder, toxic urinary agents, deficiency in bladder wall lining and neurogenic causes have been proposed (Petrone et al., 1995; Ho et al., 1997; Johansson et al., 1997; Driscoll

and Teichman, 2001; Sant and Hanno, 2001). We have hypothesized that pain associated with IC/PBS involves an alteration of visceral sensation/bladder sensory physiology. Altered visceral sensations from the urinary bladder (i.e., pain at low or moderate bladder filling) that accompany IC/PBS (Petrone et al., 1995; Ho et al., 1997; Johansson et al., 1997; Driscoll and Teichman, 2001; Sant and Hanno, 2001) may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder.

Neuropeptides are potential mediators or modulators of inflammation and are found in human micturition pathways (Chapple et al., 1992; Lasanen et al., 1992; Smet et al., 1997; Morgan et al., 1999; Uckert et al., 2002). Changes in the expression of neuropeptides have been observed with bladder overactivity (Chapple et al., 1992; Lasanen et al., 1992; Smet et al., 1997) and in animal models of bladder inflammation (Vizzard, 2000d, 2001; Zvarova and Vizzard, 2006). In this study, we have examined the contribution of vasoactive intestinal polypeptide (VIP) in afferent pathways to the urinary bladder by using wildtype and VIP^{-/-} mice under control conditions or after induction of acute bladder inflammation. VIP is a 28 amino-acid peptide, belonging to 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 may exert excitatory or inhibitory actions in neural pathways controlling micturition and these functions may be altered with neural injury, disease or inflammation. 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.

Numerous studies involving a chemically (cyclophosphamide; CYP)-induced bladder inflammation (Cox, 1979; Maggi et al., 1992; Lantéri-Minet et al., 1995; Vizzard, 2000a; Vizzard, 2000d; Zvarova and Vizzard, 2006) model have demonstrated alterations in neurochemical (Vizzard, 2000a; Vizzard, 2000d, 2001; Zvarova and Vizzard, 2006), electrophysiological (Jennings and Vizzard, 1999; Yoshimura and de Groat, 1999), organizational (Vizzard and Boyle, 1999; Vizzard, 2000b) and functional properties of bladder afferent neurons in dorsal root ganglia (DRG) and in central reflex micturition pathways as well as changes in the urinary bladder (xx). Neurotrophins, including nerve growth factor (NGF) have been implicated in mediating some of these changes. In vitro studies demonstrate that activation of downstream intracellular signaling molecules, especially transcription factors CREB (camp-response element binding protein), c-Jun and Elk-1 are important steps in neurotrophin-signaling cascades (Hawley et al., 1992; Gold et al., 1993; Riccio et al., 1999; Avelino et al., 2002; Arthur et al., 2004). Previous studies have demonstrated that CYP-induced cystitis in the rat induces the expression of phosphorylated cAMP response element binding protein (p-CREB) in bladder afferent cells in the lumbosacral DRG (Qiao and Vizzard, 2004). In the present study, we: (1) examined the contribution of VIP to the expression of p-CREB in micturition afferent pathways under control or inflamed conditions by using antibodies that specifically recognize the phosphorylated form of CREB (p-CREB) in VIP^{-/-} and wildtype mice; (2) determined whether p-CREB expression is associated with bladder afferent cells in the DRG by retrograde labeling of bladder afferent neurons with a conventional tracing dye, Fast blue (FB) and (3) determined NGF bladder content in VIP^{-/-} and WT mice under basal (no inflammation) conditions and after CYP-induced cystitis.

Materials and 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 tailsnips. 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. When a WT littermate was unavailable, an age-matched control mouse from another C57BL/6 litter was used. The Institutional Animal Care and Use Committee at The University of Vermont approved all experiments.

Retrograde labelling of bladder afferents

To label dorsal root ganglion (DRG) cell bodies with an axonal projection to the bladder, the fluorescent dye Fast Blue (FB) (EMS-Chemie, Germany, 4% in water), was injected into the bladder wall of anesthetized mice using a slight modification of a published procedure (Qiao and Vizzard, 2002a; Qiao and Vizzard, 2002b; Qiao and Vizzard, 2004). Briefly, a total volume 20 µl, divided into 5–10 injections, was injected into the entire surface of the bladder wall using a Hamilton syringe with particular care to avoid injections into the bladder lumen, major blood vessels, or overlying fascial layers. At each injection site, the needle (28 gauge) was kept in place for several seconds after injection, and the site was washed with saline to minimize contamination of adjacent organs with FB. FB was injected 5–6 days before CYP treatment as described below.

Acute cyclophosphamide (CYP)-induced cystitis

To produce an acute inflammation of the bladder, the cytostatic drug, cyclophosphamide (CYP; Sigma, 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. The animals were euthanized 48 hr after treatment.

Euthanasia, tissue harvesting and preparation

Mice were anesthetized with isoflurane (0.4%), euthanized by thoracotomy and urinary bladders harvested. Bladder weight and animal weights were recorded. Dorsal root ganglia (DRG) were identified, harvested and immersion-fixed in paraformaldehyde solution (4%) overnight at 4 °C. Spinal cord segments were identified based upon at least two criteria: (1) the T13 DRG was present after the last rib, and (2) the L6 vertebra was the last moveable vertebra followed by the fused sacral vertebrae. Another less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest DRG, L5. Tissue was then placed overnight in sucrose (30%) in 0.1 M PBS for cryoprotection at 4 °C. DRG (L1, L2, L5–S1) were sectioned parasagittally at a thickness of 20 µm on a cryostat. Every third section (1:2 series) of DRG was thaw-mounted on gelled (0.5%) slides. Some DRG (L1, L2, L6, S1) were specifically chosen for analysis based upon the previously determined segmental representation of urinary bladder circuitry (Donovan et al., 1983; Keast and de Groat, 1992; Nadelhaft and Vera, 1995). Bladder afferents are not distributed within the L4–L5 DRG (Donovan et al., 1983; Keast and de Groat, 1992) that contain only somatic afferents nor are neurons that are involved in urinary bladder function observed in the L4–L5 spinal segments (Donovan et al., 1983; Keast and de Groat, 1992; Nadelhaft and Vera, 1995). Thus, the L5 DRG served as an internal control for these studies.

Immunostaining for phosphorylated cAMP response element binding protein (p-CREB)

DRG sections for WT and VIP^{-/-} mice were processed for pCREB-immunoreactivity (IR) using an on-slide processing technique (Vizzard, 1997). Groups of control (no inflammation) and CYP-treated WT and VIP^{-/-} mice were processed simultaneously to decrease the possible

incidence of variation in staining and background between tissues and between animals. DRG sections were incubated overnight at room temperature with rabbit anti-pCREB antibody (Cell Signaling Technology, MA, USA, 1: 1000) in 1% goat serum and 0.1 M KPBS (Phosphate Buffer Solution with potassium), and then washed (3×15 min) with 0.1 M KPBS, pH 7.4. Tissue was then incubated with Cy3-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 2 hr at room temperature. After several rinses with 0.1 M KPBS, tissues were mounted with Citifluor (Citifluor, London, UK) on slides and coverslipped. Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed.

Hematoxylin and Eosin Staining of Urinary Bladder

Sections of urinary bladder (25 μ m) from VIP^{-/-} and WT mice were stained with hematoxylin/eosin (H/E). Urinary bladders were harvested from animals euthanized as described but not subjected to any other protocol. Bladders were blotted dry and the weights recorded. Subsequently, the tissue was postfixed in 4% paraformaldehyde, placed in ascending concentrations of sucrose (10–30%) in 0.1M PBS for cryoprotection, sectioned and counterstained using the Mayer's technique for hematoxylin and eosin (Bancroft and Stevens, 1990). Bladder sections (1:2 series) were thaw-mounted on gelled (0.5%) microscope slides.

Assessment of positive staining in lumbosacral DRG

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Tissues exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained.

Data analysis of pCREB and FB-labeled DRG

Tissues were examined under an Olympus fluorescence photomicroscope for visualization of Cy3 and FB. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range of 610–655 nm. In DRG from control and CYP-treated rats, pCREB-immunoreactive (IR) cell profiles were counted in 5–8 sections of each selected DRG (L1, L2, L5–S1). Only cell profiles with a nucleus were quantified and only cells with nuclear expression of pCREB-IR were quantified. DRG sections with FB-labeled cells were viewed with a filter with an excitation wavelength of 340–380 nm and an emission wavelength of 420 nm. Cells colabeled with FB and pCREB-IR were similarly counted. Numbers of pCREB-IR cell profiles per DRG section are presented (mean $\pm$ SEM). The percentage of presumptive bladder afferent cells (FB-labeled) expressing pCREB-IR in each DRG examined is also presented (mean $\pm$ SEM). The results are not corrected for double-counting. pCREB-IR and FB-labeled cells were quantified by two individuals. Comparisons between control and CYP-treated groups were made by using analysis of variance (ANOVA). Percentage data were arcsin-transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. Thus, day was treated as a blocking effect in the model. Two variables were tested in the analysis: (1) inflammation versus no inflammation (control), and (2) mouse strain (WT versus VIP^{-/-}). When F ratios exceeded the critical value ($p \leq 0.05$), the Newman-Keuls test was used for multiple comparisons among means.

Enzyme-linked immunoassays for NGF in urinary bladder

In separate groups of VIP^{-/-} and WT mice with and without CYP-induced cystitis (48 hr), mice were euthanized as described above and the urinary bladder was dissected and weighed. Individual bladders were solubilized in Tissue Protein Extraction Reagent (Pierce Biotechnology, Woburn, MA) with protease inhibitors (Roche Diagnostics GmbH, Germany).

As previously described (Vizzard, 2000c; Murray et al., 2004), tissue was homogenized and centrifuged (10,000 rpm for 5 min); supernatants were used for NGF quantification. Total protein was determined (Pierce). The NGF E-max immunoassay system (Promega Corp., Madison, WI) demonstrates very low cross-reactivity with structurally related growth factors. The NGF standard generated a linear standard curve from 7.8–500 pg/ml ($R^2 = 0.996$, $p \leq 0.001$). Absorbance values of standards and samples were corrected by subtraction of background value. Samples were diluted to bring the absorbance values onto the linear portion of the standard curve. Curve fitting of standards and evaluation of NGF content of samples was performed using a least squares fit. Comparisons of NGF bladder content were made using ANOVA. When F ratios exceeded the critical value ($p \leq 0.05$), the Newman-Keuls test was used to compare means. All values are expressed as mean $\pm$ S.E.M.

Detrusor smooth muscle thickness determination

Grayscale images acquired in tiff format were imported into Meta Morph image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA). The opened image was first calibrated for pixel size by applying a previously created calibration file. In VIP^{-/-} and WT mice, 4–5 bladder sections for each mouse ($n=5-7$) were used for smooth muscle measurements. Using the free hand drawing tool, a tangent was dropped perpendicular to the serosal surface of the bladder section. Measurements were performed using an established calibration. This process was repeated 4–5 times obtaining thickness measurements throughout an individual bladder section. Comparisons of detrusor smooth muscle thickness were made using ANOVA. When F ratios exceeded the critical value ($p \leq 0.05$), the Dunnett's test was used to compare means. All values are expressed as mean $\pm$ S.E.M.

Figure Preparation

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis Corp., Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis Corp.). Exposure times were held constant when acquiring images from VIP^{-/-} and WT mice processed and analyzed on the same day. Images were imported into Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA) where groups of images were assembled and labeled.

Results

Basal p-CREB immunoreactivity in lumbosacral DRG is increased in VIP^{-/-} mice

Basal expression of p-CREB-IR in lumbosacral DRG in VIP^{-/-} mice was significantly ($p \leq 0.01$) greater than that observed in DRG from WT mice (Fig. 1 A, C). Significantly increased basal pCREB expression in VIP^{-/-} mice was observed in all lumbosacral DRG (L1, L2, L5-S1) examined (Fig. 2). There was no difference in the number of p-CREB-IR DRG cells observed among different DRG levels (range 23.2–28.4 cells/section) in VIP^{-/-} mice (Fig. 3). Numbers of pCREB-IR cells in WT mice under basal condition were similar among all DRG levels examined (range 10.3–15.4 cells/section) (Fig. 3).

Increased p-CREB immunoreactivity in lumbosacral DRG in WT and VIP^{-/-} mice with CYP-induced cystitis

Acute CYP-induced cystitis resulted in a significant ($p \leq 0.01$) increase in p-CREB-IR in DRG levels (L1, L2, L5-S1) examined in WT mice (Fig. 1–Fig. 3). The magnitude of change in pCREB expression in WT mice with CYP-induced cystitis was greatest in the L2 (2.5-fold), L6 (2.1-fold) and S1 (3.7-fold) DRG (Fig. 2, 3). In contrast, CYP-induced cystitis significantly increased pCREB-IR only in L6 and S1 DRG in VIP^{-/-} mice (Fig. 1–Fig. 3). The magnitude of change in pCREB expression in VIP^{-/-} mice with CYP-induced cystitis was less than that

(L6, 1.9-fold; S1, 1.8-fold) observed for WT mice (Fig. 2). No changes in pCREB-IR in L1, L2 or L5 DRG were observed in VIP^{-/-} mice with CYP-induced cystitis (Fig. 2).

p-CREB immunoreactivity in bladder afferent cells in the DRG

To determine if pCREB-IR was present in bladder afferent cells in the lumbosacral DRG under basal conditions or after CYP-induced cystitis, bladder afferent cells were identified by retrograde transport of Fastblue after injection into the detrusor smooth muscle (Fig. 4).

Basal expression—Under basal conditions in WT mice, p-CREB-IR was observed in 20–38% of FB labelled bladder afferents in L1, L2, L6 and S1 DRG (Fig. 4C). A significantly ($p \leq 0.05$) greater percentage of bladder afferents cells exhibiting p-CREB-IR in L6, S1 DRG compared to L1, L2 DRG was observed in WT mice (Fig. 4B, C). In VIP^{-/-} mice, 12–58% of bladder afferent cells expressed p-CREB-IR with L6-S1 DRG exhibiting a significantly ($p \leq 0.05$) greater percentage than that observed in L1-L2 DRG (Fig. 4C). Under basal conditions, the percentage of bladder afferent cells exhibiting pCREB-IR in L6, S1 DRG in VIP^{-/-} mice was significantly ($p \leq 0.01$) greater than that observed in WT L6, S1 DRG (Fig. 4C).

CYP-induced cystitis—CYP treatment in WT or VIP^{-/-} mice did not affect the percentage of bladder afferent cells exhibiting pCREB-IR in the L1 or L2 DRG (Fig. 4C). In contrast, acute CYP-treatment in both WT and VIP^{-/-} mice significantly increased the percentage of bladder afferent cells expressing pCREB-IR in L6 and S1 DRG (Fig. 4A–C). The magnitude of change in the L6 DRG induced by CYP treatment was similar in WT (1.8-fold) and VIP^{-/-} (1.4-fold) mice (Fig. 4C). In contrast, in the S1 DRG, the magnitude of change in WT mice (2-fold) was greater than that observed in VIP^{-/-} mice (1.3-fold) (Fig. 4C). Not all p-CREB-IR cells in the DRG were bladder afferent cells (Fig. 4A, B).

NGF Bladder Content in VIP^{-/-} and WT mice with or without CYP-induced cystitis

Basal expression of NGF in whole urinary bladder was significantly ($p \leq 0.001$) greater in VIP^{-/-} mice compared to WT (Fig. 5). Both VIP^{-/-} (2.3-fold increase) and WT (1.94-fold increase) mice exhibited a significant ($p \leq 0.001$) increase in NGF bladder content with CYP-induced cystitis (48 hr) (Fig. 5); however, the magnitude of change (2.1-fold) observed was significantly greater in VIP^{-/-} mice (Fig. 5).

Increased detrusor smooth muscle thickness in VIP^{-/-} mice

In VIP^{-/-} mice, the urinary bladder/body mass ratio (Fig. 6A) and the thickness of the detrusor smooth muscle (Fig. 6B–D) were significantly ($p \leq 0.01$) increased compared to littermate WT mice.

Discussion

These studies demonstrate several novel findings with respect to pCREB expression in lumbosacral DRG and specifically in bladder afferent pathways and NGF expression in the urinary bladder of VIP^{-/-} mice. First, pCREB expression in lumbosacral DRG was greater in VIP^{-/-} mice under basal conditions and after bladder inflammation induced by cyclophosphamide (CYP). However, the magnitude of change in pCREB expression induced by CYP-induced cystitis was greater in WT mice. A similar finding was demonstrated when bladder afferent cells labeled with the retrograde tracing agent, Fastblue, were examined for pCREB expression in VIP^{-/-} and WT mice with and without cystitis. To begin to understand what factors may contribute to altered pCREB expression in VIP^{-/-} mice, we examined NGF expression in the urinary bladder because previous studies (Vizzard, 2000c; Murray et al., 2004) have implicated NGF in contributing to micturition reflex plasticity following inflammation. Previously, studies from our laboratory have demonstrated that pCREB

expression and not c-jun expression is increased in bladder afferent cells after CYP-induced cystitis in rats (Qiao and Vizzard, 2004). Urinary bladder from VIP^{-/-} mice exhibited greater mass, increased thickness of detrusor smooth muscle and increased NGF content compared to WT mice.

VIP^{-/-} mice exhibit higher basal level of p-CREB-IR in DRG

A significantly higher basal level of p-CREB expression in lumbosacral DRG was observed in VIP^{-/-} mice. This may be accounted for by a number of factors including, changes in (1) urinary bladder activity; (2) urinary bladder afferent activity or (3) expression in bladder-derived neurotrophic factors, including NGF. Greater basal activity in the primary bladder afferents in the DRG or in the target organ itself (i.e., urinary bladder) could result in increased pCREB expression in the absence of inflammation by increasing Ca⁺⁺ and/or cAMP in VIP^{-/-} mice. Primary afferent cells in the DRG express VIP (Morgan et al., 1999; Hernandez et al., 2006) and VIP receptors (Yashpal et al., 1991; Braas et al., 2006). Thus, absence of VIP in the VIP^{-/-} mice could produce a direct effect on primary afferent cells. However, VIP acting through VIP receptors activates adenylate cyclase (Ishihara et al., 1992), increasing cAMP and inducing phosphorylation of CREB by cAMP dependent protein kinase A (Delgado et al., 1999; Dickinson et al., 1999; Delgado et al., 2000; Mayr and Montminy, 2001). Thus, in VIP^{-/-} mice, one might have expected a decrease in pCREB expression in DRG cells if VIP at the level of the DRG directly mediated changes in pCREB expression. Therefore an effect of VIP deletion on the target organ (i.e., urinary bladder) may be more likely. This may also explain the regional differences in DRG expression of pCREB observed in the present study with DRG levels innervating the urinary bladder being specifically affected.

VIP functions in the lower urinary tract

A number of diverse and conflicting roles for VIP have been demonstrated in 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 (Igawa et al., 1993; Erol et al., 1992). These contradictory findings might be attributable to species differences (Uckert et al., 2002). 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 results may be attributed to absence of VIP-mediated relaxation in VIP^{-/-} mice. Changes in basal tone or contractility of the urinary bladder may increase pCREB expression in the lumbosacral DRG.

VIP^{-/-} mice show increased p-CREB-IR in DRG in response to CYP-induced cystitis but magnitude of change is less than that in WT

CYP-induced cystitis increased pCREB expression in both VIP^{-/-} and WT mice and although the absolute increase in numbers of pCREB-IR cells in DRG or percentages of bladder afferent cells expressing pCREB were greater in VIP^{-/-} mice the magnitude of this change from basal conditions was less than that observed in WT mice. This could reflect saturation at the level of the DRG. In the future, it would be of interest to determine if pCREB is expressed in both A-δ and C-fiber bladder afferents or in only one population of bladder afferent cell. This could account for a maximum percentage of bladder afferent cells expressing pCREB. pCREB expression was also observed to increase in L5 DRG with CYP-induced cystitis. This likely represents viscerosomatic convergence or cross-talk among somatic and autonomic pathways (Amir and Devor, 1996; Jaggar et al., 1999).

Increased NGF expression in urinary bladder of VIP^{-/-} mice with and without inflammation compared to WT

Both neurotrophins and cytokines may affect pCREB expression (Hawley et al., 1992; Singh et al., 1996; Riccio et al., 1999; Arthur et al., 2004). This possibility has been suggested in studies addressing pCREB involvement with peripheral nerve injury (Gold et al., 1993), somatic (Ji and Rupp, 1997; Messersmith et al., 1998), and visceral inflammation (Qiao and Vizzard, 2004) and sympathetic neuron survival (Riccio et al., 1999). The influence of target organ-neuron interactions in the adult animal has been extensively demonstrated (Steers et al., 1991; Vizzard, 2000c; Qiao and Vizzard, 2002a; Murray et al., 2004; Steers and Tuttle, 2006). Under basal and inflamed conditions, VIP^{-/-} bladders expressed greater NGF content compared to WT bladders. Altered urinary bladder NGF expression in VIP^{-/-} mice may contribute to altered pCREB expression in DRG under both basal and inflamed bladder conditions. Regulation of neuropeptide expression by NGF in DRG has been demonstrated (Verge et al., 1995). NGF suppressed VIP as well as neuropeptide Y, galanin, and cholecystokinin expression in rat DRG. Future studies will determine neuropeptide expression (i.e., PACAP, galanin) in DRG from VIP^{-/-} mice and the potential contribution to altered pCREB expression in DRG.

Hypertrophy of the urinary bladder 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; Vizzard, 2000c) and the NGF receptor, TrkA (Qiao and Vizzard, 2002b; Qiao and Vizzard, 2004). In the present study, VIP^{-/-} mice exhibited increased basal and inflammation-induced expression of NGF, increased bladder/body weight ratio and increased detrusor muscle thickness under basal conditions. 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). A similar regulatory role for VIP in detrusor smooth muscle may be possible.

Anti-inflammatory role of VIP in bladder reflex pathways

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 (Karin et al., 1997; 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; Newman et al., 2005; Juarranz et al., 2005; Martinez et al., 2005; Gonzalez-Rey and Delgado, 2006). We have recently demonstrated (Vizzard et al., 2007b) that production of pro-inflammatory compounds including IL-1 β , IL-6 and monocyte chemoattract protein (MCP-1; CCL2) is increased in urinary bladder of VIP^{-/-} mice treated with CYP compared to WT. Thus, changes in expression of inflammatory compounds as well as NGF expression in VIP^{-/-} urinary bladder may contribute to altered pCREB expression with inflammation. With respect to bladder function in VIP^{-/-} mice, we have recently demonstrated (Vizzard et al., 2007a) increased urinary bladder hyperreflexia (smaller void volumes and increased voiding frequency) in VIP^{-/-} mice treated with CYP.

In summary, these studies demonstrate increased basal expression of pCREB in unidentified lumbosacral DRG cells as well as bladder afferent cells in DRG in VIP^{-/-} mice. Under basal conditions, NGF expression was significantly increased in urinary bladder of VIP^{-/-} mice and VIP^{-/-} exhibited increased bladder/body weight ratio. With inflammation, NGF bladder content further increased in the urinary bladder compared to WT with inflammation. Although bladder inflammation increased the absolute number of pCREB-IR DRG cells in VIP^{-/-} mice, the magnitude of change was less than that observed in WT mice. pCREB expression under basal or inflamed conditions in VIP^{-/-} mice in DRG cells may be related to changes in bladder-derived factors, including NGF.

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Figure 1. p-CREB-IR in S1 dorsal root ganglia (DRG) from wildtype (WT; A, B) or $VIP^{-/-}$ (C, D) mice

$VIP^{-/-}$ mice exhibit greater numbers of p-CREB-IR DRG cells under basal condition (A vs. C). $VIP^{-/-}$ mice exhibit greater numbers of p-CREB-IR DRG cells after induction of acute cyclophosphamide (CYP)-induced cystitis (B vs. D). Arrows indicate examples of DRG cells counted as p-CREB-IR with a clearly stained nucleus or nucleus and cytoplasm.

Figure 2. Expression of pCREB-IR in lumbosacral DRG with and without CYP treatment in $VIP^{-/-}$ and wildtype (WT) mice

Summary histogram of p-CREB-IR cells in lumbosacral dorsal root ganglia (DRG) under basal conditions or after acute induction of cyclophosphamide (CYP)-induced cystitis in WT and $VIP^{-/-}$ mice. #, $p \leq 0.01$ compared to WT without CYP; *, $p \leq 0.01$; 'n' = 4 for each group.

Figure 3. p-CREB-IR in L6 dorsal root ganglia (DRG) from wildtype (WT; A, B) or VIP^{-/-} (C, D) mice

CYP-induced cystitis (48 hr) induces greater numbers of p-CREB-IR DRG cells in VIP^{-/-} mice compared to WT (A vs. C). Higher power magnification of p-CREB-IR DRG cells in WT (B) or VIP^{-/-} mice (D) induced after CYP-induced cystitis. Arrows indicate examples of DRG cells counted as p-CREB-IR with a clearly stained nucleus or nucleus and cytoplasm.

Figure 4. Bladder afferent cells (Fastblue, FB-labeled) exhibit p-CREB-IR in WT and VIP^{-/-} mice under basal conditions and after CYP-induced cystitis

A, B: Fastblue labelled cells (blue) in L6 from WT (A) and VIP^{-/-} (B) with CYP-induced bladder inflammation. White arrows demonstrate examples of bladder afferent cells with p-CREB-IR. Yellow arrows demonstrate examples of bladder afferent cells without p-CREB-IR. C: In WT and VIP^{-/-} mice, a greater percentage of bladder afferent cells express p-CREB-IR in the L6 and S1 DRG under basal or inflamed conditions compared to L1 and L2 DRG. VIP^{-/-} mice exhibit a greater percentage of bladder afferent cells exhibiting p-CREB-IR under basal or inflamed conditions compared to WT (C). #, p ≤ 0.05 compared to WT without CYP; *, p ≤ 0.01. 'n' = 4 for each group.

Figure 5. Nerve growth factor (NGF) bladder content is significantly increased in VIP^{-/-} mice under basal conditions and after induction of CYP-induced cystitis (48 hr) compared to wildtype (WT) mice

Summary histogram of total urinary bladder NGF as detected with ELISA with no treatment (basal) and after CYP-induced cystitis (48 hr) in VIP^{-/-} and littermate WT mice. *, $p \leq 0.001$. 'n' = 6 for each group.

Figure 6. Increased bladder mass and detrusor muscle thickness in VIP^{-/-} mice

A. Summary histogram of bladder/body weight ratio for VIP^{-/-} and WT mice. B. Detrusor muscle thickness was significantly increased in VIP^{-/-} mice. C. Brightfield images of hematoxylin and eosin stained bladder sections from VIP^{-/-} (C) and WT (D) mice demonstrating increased thickness in detrusor smooth muscle in VIP^{-/-} mice. U, urothelium. Calibration bar represents 750 μm. *, $p \leq 0.01$. 'n' = 6 for each group.