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PACAP38-mediated bladder afferent nerve activity hyperexcitability and Ca^{2+} activity in urothelial cells from mice

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP; *AdcyaP1*) and its cognate PAC1 receptor *(Adcyap1r1)*, have tissue-specific distributions in the lower urinary tract (LUT). The afferent limb of the micturition reflex is often compromised following bladder injury, disease and inflammatory conditions. We have previously demonstrated that PACAP signaling contributes to increased voiding frequency and decreased bladder capacity with cystitis. Thus, the present studies investigated the sensory components (e.g., urothelial cells, bladder afferent nerves) of the urinary bladder that may underlie the pathophysiology of aberrant PACAP activation. We utilized bladder-pelvic nerve preparations and urothelial sheet preparations to characterize PACAP-induced bladder afferent nerve discharge with distention and PACAP- induced Ca^{2+} activity, respectively. We determined that PACAP38 (100 nM) significantly ($p \leq 0.01$) increased bladder afferent nerve activity with distention that was blocked with a PAC1/VPAC2 receptor antagonist PACAP6–38 (300 nM). PACAP38 (100 nM) also increased Ca^{2+} activity in urothelial cells over that observed in control preparations. Taken together, these results establish a role for PACAP signaling in bladder sensory components (e.g., urothelial cells, bladder afferent nerves) that may ultimately facilitate increased voiding frequency.

Keywords

neuropeptides; urinary bladder distension; nerve activity; micturition

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Conflicts of Interest

The authors declare that the research described from the Vizzard laboratory were conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funding entity, NIH, had no role in the studies described including: design, data collection and analysis of studies, decision to publish or preparation of the review. The contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

Authors and Contributions

Conceived, discussed and outlined the study: TJH, GWH, VM, MTN, MAV. Performed experiments: TH, GWH. Drafted and revised paper: TJH, GWH, VM, MTN, MAV.

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Introduction

The storage and elimination functions of the micturition reflex involve the coordination of the structural features of the urinary bladder and complex neural pathways organized in the central nervous system (CNS) and peripheral nervous system (PNS) (Andersson and Arner, 2004; Merrill et al., 2016). The mature micturition reflex is a spinobulbospinal reflex pathway activated by mechanoreceptors in the urinary bladder wall (Beckel and Holstege, 2011). The lower urinary tract (LUT) reflex mechanisms, organized at the level of the lumbosacral spinal cord, are modulated predominantly by supraspinal control (de Groat, 1990; 1993). The switch between the storage and voiding phases of the micturition reflex occurs when mechanoreceptor activity in the urinary bladder exceeds a threshold. As the urinary bladder fills, slowly adapting mechanoreceptors in the bladder wall increase their activity, signaling to initiate elimination through activation of sensory afferents (e.g., Aδ and C-fibers) (Fowler et al., 2008).

Bladder afferents contain a variety of neuroactive compounds including neuropeptides: calcitonin-gene related peptide (CGRP), substance P (Sub P), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), cholecystokinin and enkephalins (Donovan et al., 1983; De Groat, 1986; Keast and De Groat, 1992; Vizzard et al., 1994; Vizzard, 2000; 2001). All of these neuropeptides, except CGRP, are predominantly expressed in small diameter (presumably C-fiber) afferents in dorsal root ganglia (DRG) (Donovan et al., 1983; De Groat, 1986; Keast and De Groat, 1992; Vizzard et al., 1994; Vizzard, 2000; 2001). PACAP is expressed in peripheral autonomic and sensory neurons and can exert differential downstream effects depending on the receptor subtype expression in the target tissue. PACAP (*Adcyap1*) and its cognate receptor, PAC1 *(Adcyap1r1),* have tissue-specific distributions in the LUT (Girard et al., 2017). Dense PACAP expression is present in LUT pathways in the CNS and PNS including expression in the urinary bladder (Braas et al., 2006; Girard et al., 2008; Girard et al., 2016; Gonzalez et al., 2016a; Girard et al., 2017). PACAP- immunoreactivity (IR) and PAC1 receptor-IR is exhibited throughout the urinary bladder in nerve fibers in the urinary bladder smooth muscle, urothelium, suburothelial nerve plexus, DRG, and surrounding blood vessels (Fahrenkrug and Hannibal, 1998a; b; Braas et al., 2006; Girard et al., 2008; Girard et al., 2016; Gonzalez et al., 2016a; Girard et al., 2017).

Increases in PACAP expression in lumbosacral DRG are observed after nerve injury, inflammation, spinal cord injury or bladder inflammation induced by cyclophosphamide (Zhang et al., 1995; Zhang et al., 1996; Larsen et al., 1997; Moller et al., 1997b; Vizzard, 2000). Chronic pathological conditions inducing tissue irritation or inflammation can alter the properties of sensory pathways leading to a reduction in pain threshold (allodynia) and an amplification of painful sensations (hyperalgesia) (Raja et al., 1988). Urinary bladder inflammation can also increase afferent nerve activity to noxious and non-noxious stimuli (Habler et al., 1990; Sengupta and Gebhart, 1994) elicit painful sensations and result in altered urinary bladder function. Previous studies have demonstrated PACAP modulation of ionic conductances that underlie neuronal excitability or PACAP facilitation of spinal reflexes (Xu and Wiesenfeld-Hallin, 1996). Activation of the PACAP-selective PAC1 receptor produces different plasma membrane and endosomal signals that can integrate to

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produce changes in neuronal excitability (May and Parsons, 2017). In this brief communication, we hypothesized that PACAP signaling contributes to bladder afferent nerve excitability and Ca^{2+} signaling in the urothelium that may ultimately affect urinary bladder function (e.g., bladder hyperreflexia).

Materials and Methods

Animals and Ethical Approval

Male C57BI/6 mice (4–6 months old) purchased from Jackson Laboratories (Bar Harbor, ME) were housed with littermates and maintained in standard laboratory conditions with food and water available ad libitum. The University of Vermont Institutional Animal Care and Use Committee approved all experimental protocols involving animal use. 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 in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8^{th} edn). All efforts were made to minimize the potential for animal pain, stress or distress.

Bladder-pelvic nerve electrophysiology

Male C57BI/6J mice (n=12) were euthanized with isoflurane (4–5% in O_2) followed by decapitation. The urinary bladder and surrounding tissues were isolated as previously described (Gonzalez et al., 2016b). Briefly, the urinary bladder, urethra, ureters, postganglionic nerves, major pelvic ganglia, and pelvic nerves were excised and transferred to ice-cold HEPES dissection solution consisting of (mM): 134 NaCl, 6 KCI, 10 glucose, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and adjusted to pH 7.3 with NaOH. The ureters were tied close to the bladder wall and the pelvic nerves were isolated and cleaned of connective tissue before placing the preparation into a recording chamber. All experiments were conducted in physiological saline solution (PSS) (35° C - 37° C) consisting of (mM): 119 NaCI, 4.7 KCI, 24 $NaHCO_3$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 7 glucose and constantly bubbled with Biological Gas (95% O_2, 5% CO_2) to maintain pH at 7.4. One arm of a triple lumen cannula was inserted through the urethra into the bladder, ligated and the cannula attached to a remote-controlled syringe pump and pressure transducer to monitor intravesical pressure. The remaining arm of the cannula was used to empty the bladder. One of the pelvic nerves was attached to a suction electrode to record distention-evoked afferent nerve activity.

The bladder was filled with PSS at a rate of 30 μl/min up to 26 mmHg and then manually emptied. There was a 10 min rest period between the emptying phase and the start of the next filling phase. For the co-administration studies, bladders were first pretreated with the PAC1 receptor antagonist followed by PACAP38. Administration of drugs began once bladder afferent nerve activity to the vehicle plateaued for two consecutive filling phases (usually after 5–6 filling cycles). Bladder afferent nerve activity was collected and amplified with a Neurolog head stage (NL104, Digitimer), filtered (band pass 200–4000 Hz) using a Digitimer NL125/NI126 filter and digitized with a Power 1401 analog to digital interface (Cambridge Electronic Design, Cambridge, UK). The acquisition rates for nerve activity and

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bladder pressure were 25,000 Hz and 100 Hz, respectively. Data were analyzed offline via Spike 2 software (version 5.11, Cambridge Electronic Design, Cambridge, UK).

Analysis of afferent nerve activity

The threshold for action potential detection was set at twice the root mean square of the recorded signal in the absence of action potentials. Baseline afferent activity was measured by binning afferent activity in 1-s bins and taking the average of the lowest bins of afferent activity within 10 s of either side of the selected pressure. A two-way repeated measures ANOVA followed by Sidak's multiple comparisons test was then performed to compare these frequency group means. Percentage of control PACAP38 baseline afferent nerve activity was graphed but statistical analyses were performed on the mean data as described above. Linear regression analyses of control and PACAP38 afferent frequency were performed to determine the relationship between low pressure (< 8 mm Hg) distention, high pressure (≥ 8 mm Hg) distention and afferent nerve activity (Hz). We performed linear analyses around a bladder distention pressure of 8 mm Hg because PACAP38 significantly increased baseline afferent nerve activity at ≥ 8 mm Hg.

Urothelial sheet preparation and Ca^{2+} imaging

Urinary bladders were removed from adult male mice (n = 5) and placed in cold HEPES solution. The urothelium was removed from the detrusor and carefully cleaned of lamina propria with sharpened forceps until all visible lamina propria was removed. The urothelial sheets were loaded for 90 minutes (37° C) with the Ca^{2+} sensitive fluorescent dye, Cal 520 (AAT Bioquest, Inc.,) + pluronic acid (2.5 mg/ml) in HEPES solution and placed in a specialized chamber for imaging. The urothelium was superfused with PSS and visualized with a 60X water immersion (NA 1.2) fluorescent objective. Images were collected with a Noran Oz laser scanning confocal microscope at a rate of 16 images/s for 88–125 s. Cal 520 was excited at 488 nm, and the emitted fluorescence collected at >500 nm. Imaging fields were 133 × 133 pm (512 × 512 pixels). Ca^{2+} events were initially visualized offline using software developed in our laboratory by Dr. Adrian Bonev.

Ca^{2+} event analysis

Detailed analysis of Ca^{2+} events in urothelial cells was made using custom-written software (Volumetry G9: Grant Hennig) as previously described (Heppner et al., 2017). To localize cells that had resolvable Ca^{2+} activity, movies were differentiated ($\Delta t = \pm 1.6$s), then frame averaging (± 0.19 s) and Gaussian smoothing (3×3 pixels SD = 1.0 [0.8 × 0.8 μm]) were applied. Particles less than 30 pixels (2μm^2) in total area were filtered out and calcium transient particle (PTCL) files were created. Ca^{2+} transient PTCL areas were integrated to show the degree of activity in active cells both spatially and temporally (see Fig. 3).

Figure preparation

Images were imported into Photoshop 7.0 (Adobe Systems, San Jose, CA) or PowerPoint (Microsoft PowerPoint for Mac 2011, Version 14.7.3, Microsoft Corporation) where groups of images were assembled and labeled.

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Materials

All standard chemicals were obtained from Sigma-Aldrich or Fisher and were either analytical or laboratory grade. PACAP38 and the PAC1 receptor antagonist, PACAP(6–38) were purchased from Bachem, Torrance, CA. Before its use, stock solutions of PACAP38 and PACAP(6–38) were diluted with PSS to a working concentration of 100 nM and 300 nM, respectively.

Statistics

All values represent means ± SEM. For the of events, The Gaussian filter used in Ca^{2+} analyses required the standard deviation (SD) to set the broadness of the filter. Data were compared on GraphPad Prism (v. 6.07, La Jolla, CA) with one-way or two-way repeated measures ANOVA and Student's unpaired or paired *t* test where appropriate. For categorical variables, distribution of frequency of cell populations with different activity patterns in urothelial sheets was compared using the chi-square test. All statistical analyses were 2-tailed, used an *a priori* alpha level of 0.05 to determine statistical significance, and were performed using SAS statistical software (SAS Institute Inc., Cary, NC).

Results

PACAP38 increased distention-evoked bladder afferent nerve discharge

In *ex vivo* preparations, slow filling with physiological saline resulted in an increase in bladder pressure consistent with a thin-walled elastic sphere that can be simplified into two relatively linear phases (Fig. 1A); the first phase was gradual during the initial filling (low pressure), but as the bladder became increasingly distended (≥ 8 mm Hg, high pressure), there was a rapid rise to peak bladder pressure (~ 24 mm Hg). Afferent nerve activity, as measured in the same preparation, appeared to follow a similar two-phase pattern. The activity appeared relatively quiescent during the initial filling but became increasingly active, especially upon bladder high pressure distention. Under baseline control conditions, afferent nerve activity during the slower initial low pressure fill to ~ 8 mm Hg increased gradually from 0 to ~ 100 Hz, whereas activity in the rapid high pressure second phase increased rapidly to ~ 190 Hz.

The application of PACAP38 had no apparent effects on the pressure profiles during bladder filling compared to vehicle instillations alone (compare pressure profiles, Fig. 1A and 1B). In twelve (n=12) preparations that were evaluated, PACAP38 (100 nM) increased mean baseline afferent nerve frequency in 7 of 12 preparations from 0–24 mm Hg (Fig. 1B, C). Notably, the continuous superfusion of PACAP38 (100 nM) had a significant ($p = 0.034$) treatment effect on mean bladder afferent nerve frequency (Hz). Whereas nerve activity during baseline and PACAP38 treatment did not appear different in the initial low pressure fill phase, PACAP38 infusions significantly increased nerve firing compared to baseline responses approximately 1.5-fold during the high pressure phase (8 – 24 mmHg) (baseline, 100 – 190 Hz versus PACAP38, 150 – 250 Hz, $n = 7$, $p \leq 0.01$). The changes in activity are expressed as percent increase from control in Fig. 1C. Given that bladder pressure profiles were unchanged with PACAP38 compared to baseline controls (Fig. 2A, C), then an increase in nerve activity as a function of pressure implicated an increase in frequency over the same

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temporal window. Accordingly, afferent frequency was plotted as a function of time (Fig. 2A-D). Although there was a small apparent increase in afferent frequency from regression analyses during the low pressure phase after PACAP38 treatment, the changes did not appear statistically different (Fig. 2B; red regression line, $p = 0.27$) in agreement with activity/pressure data above. However PACAP38 (100 nM) superfusions produced a 1.6-fold increase in afferent nerve activity frequency during the high urinary bladder distention phase (Fig. 2D; ≥ 8 mm Hg, blue regression line, $p \leq 0.04$; Fig. 2E). The superfusion of the preparation with the PAC1/VPAC2 receptor antagonist PACAP6–38 (300 nM) for 20 minutes followed by PACAP38 (100 nM) addition, blocked the increase in afferent nerve excitability; superfusion of VIP (100 nM) had no effect on bladder afferent nerve activity (284.3 ± 56 to 274.1 ± 44 impulse/s, control and VIP, respectively; data not shown).

PACAP38 effects on Ca^{2+} events in urothelial cells

Previous studies in rats demonstrated PAC1R-IR in urothelial cells and PACAP38 application to urothelial sheets resulted in increased ATP secretion (Girard et al., 2008). We evaluated the effects of PACAP38 (100 nM) superfusion on Ca^{2+} activity in cells within urothelial sheets isolated from the murine bladder. Continuous recordings (6 min) of Ca^{2+} activity in cells in urothelial sheets (n=4 preparations, cells=81, range 11–34 cells per field of view) revealed three subpopulations of urothelial cells with differing Ca^{2+} responses to PACAP (Fig. 3A-D). The largest subpopulation of urothelial cells (50.52 ± 3.21%) was quiescent during control (no drug) conditions but demonstrated heightened Ca^{2+} signals upon PACAP38 infusions. The PACAP- mediated Ca^{2+} responses were maximal within 2 min of PACAP application and diminished to a lower but elevated plateau by 4 – 6 min (Fig. 3A-D). These temporal parameters were comparable to responses seen previously in HEK cells stably expressing PAC1Hop1 receptors (May et al., 2014). Another urothelial cell population (31.08 ± 5.00%) demonstrated basal Ca^{2+} activity in control (no drug) conditions and was unresponsive to PACAP. The last and smallest population (18.40 ± 5.68%) demonstrated basal Ca^{2+} activity but became quiescent upon PACAP exposure. The damping effects of PACAP are not understood but may reflect preferential PAC1 receptor conformations in these cells that promote receptor internalization rather than G protein signaling (May and Parsons, 2017). Chi-square analyses demonstrated a statistical difference ($p \leq 0.001$) in the incidence of quiescent cells increasing activity after PACAP38 addition and of cells that ceased activity after PACAP38 addition in urothelial preparations assuming a null distribution of cells in three subpopulations.

Discussion

The current studies show that PACAP signaling contributes to bladder afferent nerve hyperexcitability with bladder distention; the PACAP responses were blocked by the PAC1/VPAC2 receptor antagonist, PACAP(6–38) and not stimulated by VIP, suggesting that the effects were mediated by PAC1 receptor signaling. Further PACAP38 also increased Ca^{2+} activity in a large population of urothelial cells. Hence in aggregate, these studies demonstrate that the sensory components (e.g., bladder afferent nerves, urothelial cells) of the urinary bladder can respond to PACAP38 application and may contribute to micturition reflex function.

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PACAP peptides exhibit many diverse functions in endocrine, nervous, gastrointestinal, and cardiovascular systems and the lower urinary tract (LUT) and are expressed in many CNS neurons and sensory and autonomic ganglia (Koves et al., 1990; Arimura et al., 1991; Koves et al., 1991; Ghatei et al., 1993; Masuo et al., 1993; Tatsuno et al., 1994; May and Braas, 1995; Portbury et al., 1995; Shiotani et al., 1995; Braas and May, 1996; Holgert et al., 1996; Klimaschewski et al., 1996; Sundler et al., 1996; Brandenburg et al., 1997; Moller et al., 1997a; Moller et al., 1997b; Nogi et al., 1997; Arimura, 1998; Beaudet et al., 1998; Braas et al., 1998; May et al., 1998; Braas and May, 1999; Beaudet et al., 2000; Cheppudira et al., 2009). PACAP facilitates neuronal calcium influx, induces depolarization of the membrane, activates AC and PLC, and stimulates neurotransmitter secretion (Murase et al., 1993; Tatsuno et al., 1994; May and Braas, 1995; Braas and May, 1996; Beaudet et al., 1998; May et al., 1998; Braas and May, 1999; Beaudet et al., 2000). In the LUT, widespread PACAP-IR has been demonstrated in nerve fibers within the urinary bladder smooth muscle, suburothelial plexus and surrounding blood vessels (Fahrenkrug and Hannibal, 1998a; b; Braas et al., 2006; Girard et al., 2008; Girard et al., 2016; Gonzalez et al., 2016a; Girard et al., 2017). Neonatal capsaicin treatment significantly reduced PACAP suggesting these fibers are derived from sensory neurons (Fahrenkrug and Hannibal, 1998b). These results are consistent with the expression of PACAP in DRG and its neurochemical plasticity following nerve injury or inflammation (Zhang et al., 1995; Zhang et al., 1996; Larsen et al., 1997; Moller et al., 1997a; Vizzard, 2000).

Bladder dysfunction and altered somatic sensation have previously been demonstrated in mice with a genetic disruption or deletion to PACAP or VIP (May and Vizzard, 2010). Both PACAP$^{-/-}$ mice and VIP$^{-/-}$ mice exhibited an increase in bladder mass with hypertrophy specific to the lamina propria and detrusor smooth muscle in PACAP$^{-/-}$ mice or only the detrusor smooth muscle in VIP$^{-/-}$ mice (Jensen et al., 2008; May and Vizzard, 2010). Functionally, PACAP$^{-/-}$ and VIP$^{-/-}$ mice have increased bladder capacity, void volumes, and longer intercontraction intervals (Studeny et al., 2008; May and Vizzard, 2010). Pharmacological studies targeting PACAP/receptor signaling wildtype mice or rats have demonstrated its role in bladder dysfunction with inflammation. Intrathecal (L6-S1) or intravesical administration of a PAC1 receptor antagonist, PACAP(6–38), increased bladder capacity but not intravesical pressure with intermediate (48 hr) CYP-induced cystitis (Braas et al., 2006; Girard et al., 2016). The different routes of administration with similar functional effects suggest PACAP(6–38) may have multiple sites of action. No effects on *in vivo* bladder function were observed with intrathecal or intravesical administration of PACAP(6–38) in control (no inflammation) conditions suggesting a role(s) for PACAP/PAC1 signaling in micturition reflexes only following inflammation (Braas et al., 2006). However, intravesical PACAP(6–38) effects on bladder afferent nerve activity should be evaluated in the *ex vivo* bladder-nerve preparation in future studies to complement in *vivo* studies (Braas et al., 2006). Although the specific site(s) of action of PAC1 receptor antagonist, PACAP(6–38) is unknown, the inhibition of aberrant PACAP signaling may be a promising target to reduce voiding frequency with cystitis.

In the present study, we determined that PACAP38, identified previously as one of the inflammatory mediators of cystitis (Vizzard, 2000; Braas et al., 2006), was able to stimulate an increase in distention-evoked bladder afferent nerve activity. The PACAP38-induced

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increase in distention-evoked afferent nerve activity was specific to ligand/receptor activation because PACAP38 co-administration with a PAC1/VPAC2 receptor antagonist, PACAP(6–38), attenuated the increase in bladder afferent nerve activity. As described above, many components of the LUT express PACAP or PAC1R (Fahrenkrug and Hannibal, 1998a; b; Braas et al., 2006; Girard et al., 2008; Girard et al., 2016; Gonzalez et al., 2016a; Girard et al., 2017); how PACAP infusions in the current studies activate the afferent fibers remains to be further investigated. PACAP38 may have direct actions on afferent terminals to increase activity following bladder distention. However, PCR transcript analyses suggest that PAC1 receptor expression levels in sensory neurons are relatively low with uncertain functional attributes (Braas et al., 2006). The urothelial cell layer that lines the bladder wall express significant levels of PAC1 receptor transcripts and immunoreactivity (Braas et al., 2006), and hence, the observed increase in bladder afferent nerve activity may represent indirect PACAP/PAC1 receptor-mediated activation via ATP release from the urothelium (Girard et al., 2008). The current studies also demonstrate Ca^{2+} activity in different populations of cells in urothelial sheet preparations. Approximately fifty-percent of urothelial cells exhibited no Ca^{2+} activity during control conditions (no drugs) but became active after the addition of PACAP38. Following stimulation, the urothelium can release signaling mediators to produce localized vascular changes (Birder and de Groat, 2007; Fowler et al., 2008) and to influence adjacent tissues and cells, including: detrusor smooth muscle, afferent nerve fibers in the suburothelial nerve plexus, inflammatory cells and interstitial cells within the bladder (Birder and de Groat, 2007; Fowler et al., 2008; Birder and Andersson, 2013; Merrill et al., 2016). Upon stimulation, the urothelium can release ATP to activate purinergic receptors on underlying sensory nerve fibers (Girard et al., 2008). We previously demonstrated that ATP release was evoked by PACAP27, PACAP38 and VIP application to cultured urothelial cells with PACAP27, and that PAC1 receptor antagonism blocked ATP release (Girard et al., 2008). These previous results suggest PACAP and PAC1 signaling may regulate micturition reflex function at the level of the urothelium (Girard et al., 2008). However, the effects of PACAP38 co-administration with a PAC1/VPAC2 receptor antagonist, PACAP(6–38), on Ca^{2+} activity should be evaluated in urothelial sheet preparations to confirm this finding (Girard et al., 2008). Future studies should examine if PACAP38 signaling contributes to bladder afferent nerve excitability through purinergic mechanisms that may ultimately facilitate increased voiding frequency. The release of signaling molecules from the urothelium can be altered with injury, inflammation and disease (Birder, 2005; Birder and de Groat, 2007; Arms and Vizzard, 2011; Birder and Andersson, 2013; Merrill et al., 2013; Gonzalez et al., 2014a; Gonzalez et al., 2014b; Merrill et al., 2016) and affect overall bladder afferent nerve activity. Thus, a comprehensive understanding of the downstream signaling effectors that interact in the afferent limb of the micturition reflex in control situations and following bladder dysfunction induced by injury, inflammation or disease may provide insights into novel therapeutic approaches.

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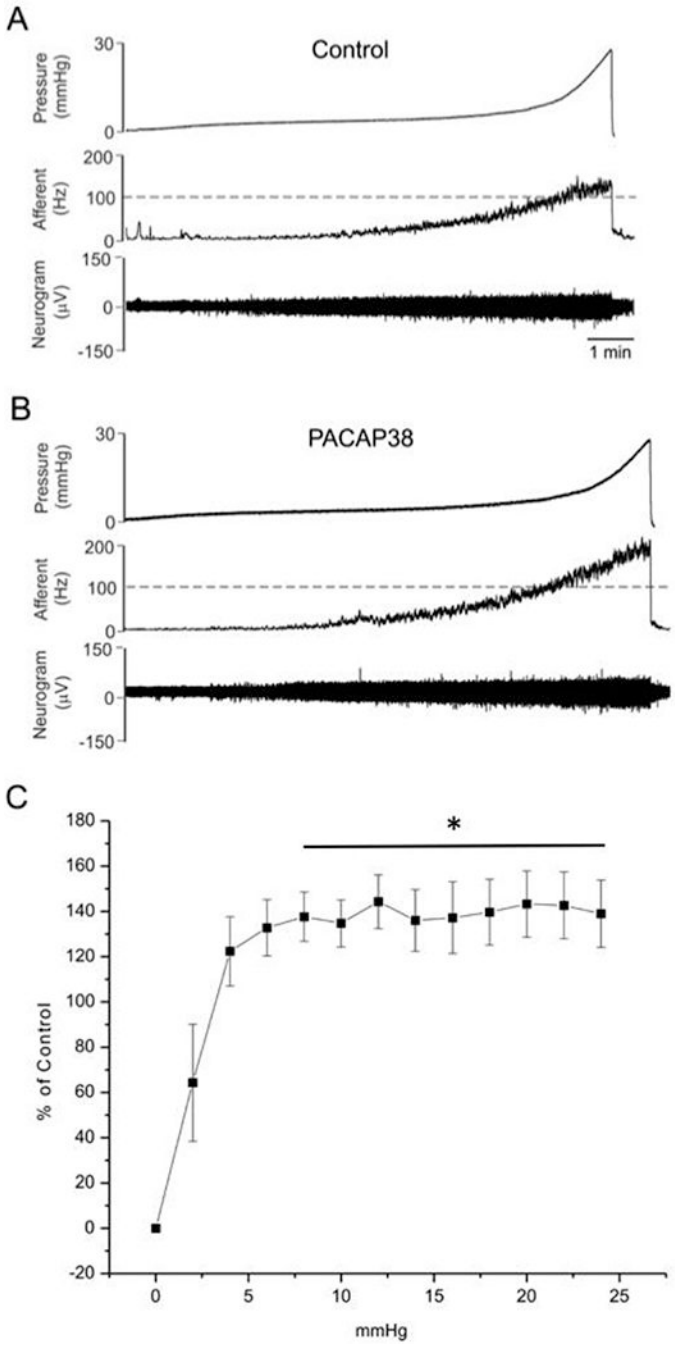

Figure 1: PACAP38 increased distention-evoked bladder afferent nerve discharge.

Representative traces of control (vehicle)(A) and PACAP38 (100 nM)(B) instillation from the same preparation. (B) PACAP38 significantly increased mean bladder afferent nerve frequency (impulse/sec) (n=7); values are mean ± SEM. PACAP38 infusions significantly increased nerve firing compared to baseline responses approximately 1.5-fold during the high pressure phase (8 – 24 mmHg) (baseline, 100 – 190 Hz versus PACAP38,150 – 250 Hz, $n = 7$, $p \leq 0.01$)(C). Data are graphed as the PACAP-induced increase in bladder afferent nerve activity over control (% control)(C). Statistical analyses were performed on mean data

(mean ± SEM); *, $p \leq 0.01$. Group data were compared using a repeated measures ANOVA followed by Sidak's multiple comparisons test.

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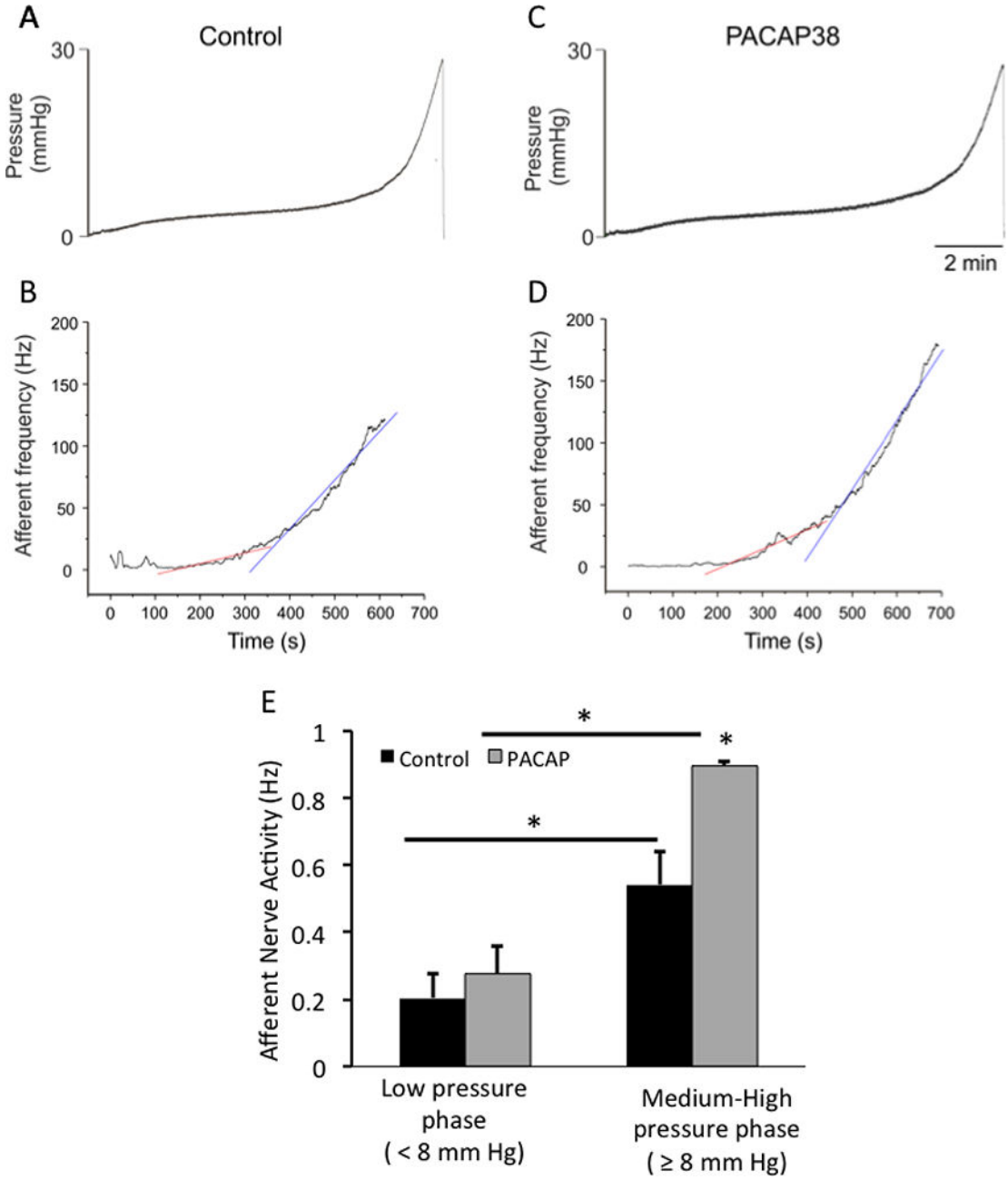

Figure 2: Bladder afferent nerve frequency plotted as a function of time with PACAP38 application.

Although there was a small apparent increase in afferent frequency from regression analyses during the low pressure phase after PACAP38 treatment, the changes were not statistically different (B; red regression line, $p = 0.27$) in agreement with activity/pressure data (see Fig. 1). PACAP38 (100 nM) superfusions produced a 1.6-fold increase in bladder afferent nerve activity frequency during the high urinary bladder distention phase (D; ≥ 8 mm Hg, blue regression line, $p \leq 0.04$; E). Group data were compared using ANOVA followed by Sidak's multiple comparisons test.

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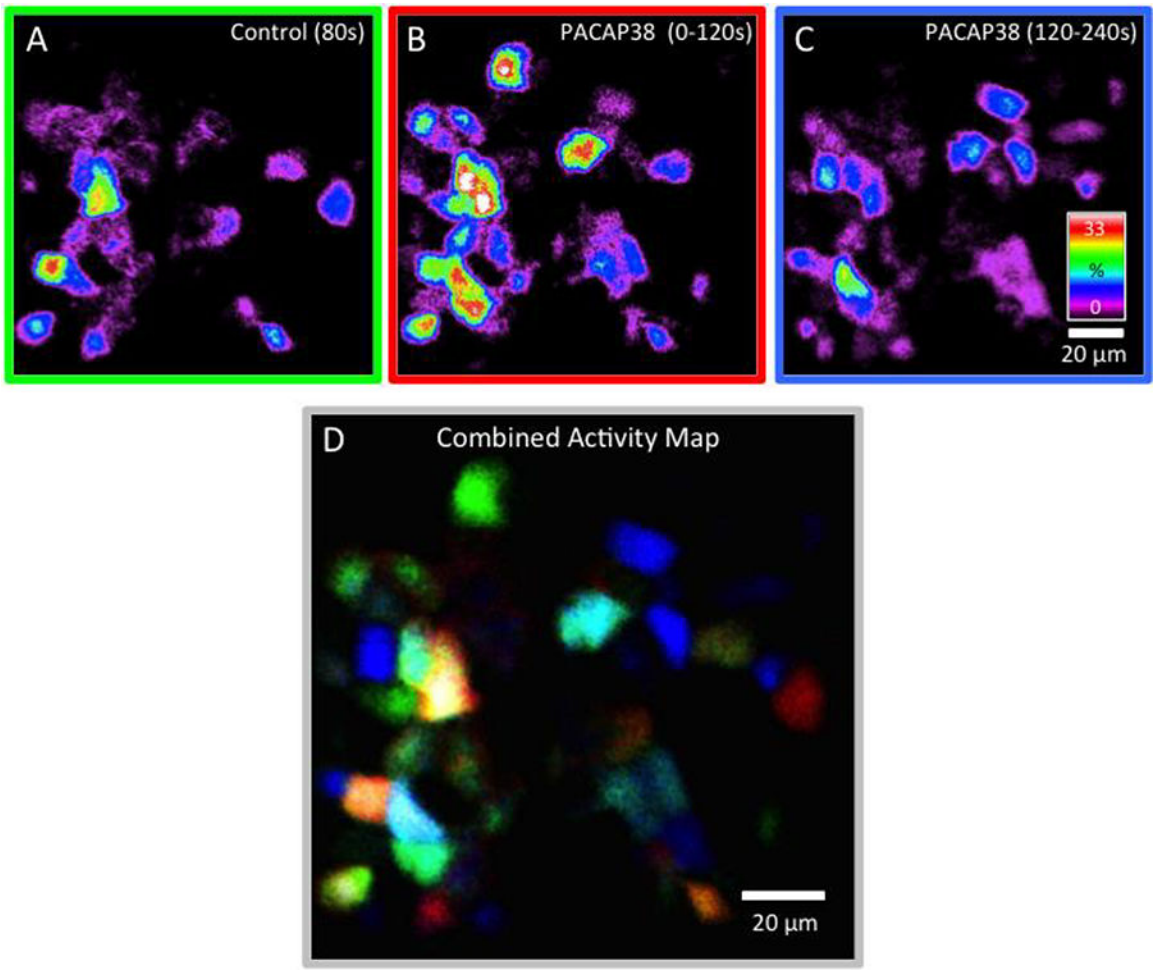

Figure 3: Integrated Ca^{2+} activity in urothelial cells before and after the addition of PACAP38 (100 nM) during a continuous recording.

A) Integrated Ca^{2+} activity in urothelial cells during the first 80s under control conditions (no drugs) reveals Ca^{2+} activity in approximately half of the urothelial cells. B) After the addition of PACAP38 (100 nM), a substantial number of previously quiescent cells became active (0–120s). C) The response to PACAP38 waned over time (120–240s). D) To visualize the populations of cells that were active during control conditions and after the addition of PACAP38, each of the top 3 panels was converted to grayscale and assigned a color channel (A = red, B = green & C = blue) and combined to create an RGB image. Calibration bar in C and D represents 20 µm. Integrated Ca^{2+} activity is presented on a spectrum scale with white representing Ca^{2+} transients in cells that were active for the equivalent of $1/3^{rd}$ of duration of the movie segment. Chi-square analyses demonstrated a statistical difference ($p \leq 0.001$) in the incidence of quiescent cells increasing activity after PACAP38 addition and of cells that ceased activity after PACAP38 addition in urothelial preparations.