Activation of Urothelial Transient Receptor Potential Vanilloid 4 by 4α-Phorbol 12,13-Didecanoate Contributes to Altered Bladder Reflexes in the Rat

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ABSTRACT

The ion channel transient receptor potential vanilloid (TRPV) 4 can be activated by hypo-osmolarity, heat, or certain lipid compounds. Here, we demonstrate expression of functional TRPV4 protein in the urothelium lining the renal pelvis, ureters, urinary bladder, and urethra. Exposure of cultured rat urothelial cells from the urinary bladder to the TRPV4-selective agonist 4α-phorbol 12,13-didecanoate (4α-PDD) promoted Ca2+ influx, evoked ATP release, and augmented the ATP release evoked by hypo-osmolarity. In awake rats during continuous infusion cystometrograms, intravesical administration of 4α-PDD (10–100 μM) increased maximal micturition pressure by 51%, specifically by augmenting the portion of each intravesical pressure wave that follows high-frequency urethral oscillations and voiding. This unusual pharmacological effect was prevented by intravesical pretreatment with the nonselective ATP receptor antagonist, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (100 μM), systemic treatment with the selective P2X3 purinergic antagonist 5-[(3-phenoxybenzyl)[1S]-1,2,3,4-tetrahydro-1-naphthalenyl]amino[carbonyl]-1,2,4-benzenetricarboxylic acid (A317491) (250 μmol/kg), or urethane anesthesia, but was unaffected by capsaicin pretreatment (100 mg/kg s.c.) or denervation of the urethral sphincter. 4α-PDD (1–100 μM) did not alter the contractility to electrical stimulation of excised bladder strips. We conclude that activation of urothelial TRPV4 by 4α-PDD and release of mediators such as ATP trigger a novel neural mechanism that regulates the late phase of detrusor muscle contraction after micturition. These data raise the possibility that TRPV4 channels in the urothelium could contribute to abnormal bladder activity.

Distention or chemical irritation of the urinary bladder results in the activation of voiding reflexes that are regulated by supraspinal mechanisms (de Groat, 1993). Historically, most attention has been focused on the afferent sensory neurons and parasympathetic motor neurons that contribute to these reflex responses. A number of recent studies, however, have provided evidence that non-neuronal cells within the bladder wall, most notably the transitional epithelial cells of the urothelium (urothelial cells), also participate in detection of responses to physical and chemical stimuli in the bladder (Downie and Karmazyn, 1984; Ferguson et al., 1997; Birder et al., 2001; Sun et al., 2001; Birder and de Groat, 2007).

In the intact bladder and ureter, mechanical stretch evokes the release of ATP from urothelial cells (Ferguson et al., 1997; Sun et al., 2001; Birder et al., 2002). Mechanically evoked ATP release from urothelial cells can also be demonstrated in the excised bladder and is accompanied by an increase in urothelial membrane capacitance (Birder et al., 2002). Exposure of primary cultured urothelial cells to...
rheric, cholinergic, or vanilloid agonists results in the release of ATP, nitric oxide and other mediators from these cells (Birder et al., 2001, 2003; Birder and de Groat, 2007). Hypotonic media, which cause the cells to swell and may act as a surrogate for mechanical stretch, have the same effect (Birder et al., 2002, 2003). It has been proposed that ATP released from the urothelium diffuses to nearby sensory nerve terminals, where it activates the ATP-gated cation channel, P2X3, resulting in membrane depolarization, action potential generation, and signaling to the spinal cord (Burnstock, 2007). Evidence for this interaction comes from the observation that in mice lacking P2X3 receptors, stretch-evoked reflex bladder contractions are diminished (Cockayne et al., 2000). Together, these findings suggest that the urothelium in addition to being a passive barrier is a sensory structure that is capable of responding to its chemical and physical environment and in turn sends chemical signals to adjacent afferent neurons.

One of the molecules that contribute to the physical and chemical responsiveness of the urothelium is the nonselective cation channel, transient receptor potential vanilloid (TRPV) subtype 1. This channel, first identified in small-caliber nociceptive neurons, can be gated by the pungent chemical responsiveness of the urothelium is the nonselective cation channel, transient receptor potential vanilloid (TRPV) subtype 1. This channel, first identified in small-caliber nociceptive neurons, can be gated by the pungent TRPV1 agonist capsaicin and also by a number of other noxious stimuli, including intense heat (>43°C), protons (pH <5), and certain lipid molecules (e.g., anandamide, N-arachidonyl dopamine, 5-hydroperoxyeicosatetraenoic acid) (Caterina et al., 1997). Studies in humans and laboratory animals have demonstrated the importance of capsaicin-sensitive neurons in certain forms of pathological bladder hyperreactivity (Abelli et al., 1988; Chancellor and de Groat, 1999). Accordingly, patients suffering from neurogenic bladder hyperreactivity experience relief from both urinary frequency and urgency after intravesical desensitization with TRPV1 agonists (Barbanti et al., 1993; Chancellor and de Groat, 1999; Fowler, 2000). Previously, we reported that TRPV1 is expressed not only in bladder sensory neurons but also in urothelial cells (Birder et al., 2001). Disruption of the TRPV1 gene in mice results in the elimination of bladder responsiveness to capsaicin, attenuation of stretch-evoked reflex bladder contractions under anesthesia, as well as profound deficits in both stretch-evoked ATP release from the excised bladder and hypotonicity-evoked ATP release from cultured urothelial cells (Birder et al., 2002). These results suggest a role for TRPV1 in bladder mechanosensation that had not been anticipated from the responses of heterologously expressed TRPV1 and further suggest the involvement of the urothelium in this process.

The mammalian genome encodes five ion channels closely related to TRPV1 (Clapham et al., 2001). One of these, TRPV4, is highly expressed in the renal nephron, skin, respiratory epithelium, and hypothalamus and can be activated by hypo-osmotic cell swelling (Liedtke et al., 2000), heat (Guler et al., 2002), 5′,6′-epoxyeicosatrienoic acid (5′, 6′-EET) (Watanabe et al., 2003), or the synthetic phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD) (Watanabe et al., 2002; Chung et al., 2003; Nilius et al., 2003). Indeed, mice lacking TRPV4 exhibit deficits in osmoregulation, mechanically evoked paw withdrawal responses, and floor temperature selection behavior (Liedtke and Friedman, 2003; Lee et al., 2005). In the present study, we examine the expression of TRPV4 within the lower urinary tract of the rat and the effect of 4α-PDD activation of TRPV4 on urothelial cells and reflex bladder contractions.

**Materials and Methods**

**Animals.** All experiments were approved by the University of Pittsburgh and Johns Hopkins University Institutional Animal Care and Use Committees. Male and female Sprague-Dawley rats (150–600 g) were used in the experiments.

**Immunohistochemistry/Immunoblot/Reverse Transcription-PCR.** For immunohistochemistry, rats were deeply anesthetized with ketamine (80 mg/kg i.p.) and xylazine (8 mg/kg i.p.), then perfused transcardially with phosphate-buffered saline (PBS), followed by 3.7% formaldehyde in PBS. Tissues were dissected and postfixed overnight at 4°C in 3.7% formaldehyde, followed by cryoprotection in 30% sucrose, and embedding in orinthine carbamyl transferase matrix (Ted Pella, Redding, CA). Sixteen-micron cryostat sections, mounted on glass slides (Superfrost Plus; Fisher Scientific Co., Pittsburgh, PA), were blocked for 30 min 25°C with 10% normal goat serum in PBS supplemented with 0.3% Triton X-100, pH 7.4, incubated overnight at 4°C with affinity-purified rabbit anti-TRPV4 (Guler et al., 2002) diluted 1:1000 in PBS supplemented with 0.3% Triton X-100, pH 7.4, containing 1% normal goat serum washed with PBS supplemented with 0.3% Triton X-100, pH 7.4, containing 1% normal goat serum (3 × 5 min), incubated for 2 h at 25°C in the dark with Cy3-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA), washed with PBS (3 × 5 min), and then dehydrated with ethanol and xylene and coverslipped using DPX (Electron Microscopy Science, Washington, PA). Immunofluorescence was visualized using an inverted Nikon Diaphot microscope (Nikon, Tokyo, Japan) and Sutter DG4 illumination source (Sutter Instrument Company, Novato, CA) or a Zeiss confocal microscope (Carl Zeiss GmbH, Jena, Germany). For immunoblot analysis, rat bladder urothelial cells were harvested and cultured overnight as described in the next section and lysed using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) containing a protease inhibitor mixture (Complete Protease Inhibitor Tablets; Roche, Palo Alto, CA). Lysates (2.3 μg protein/lane) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Billerica, MA), and immunoblotted using anti-TRPV4 (generous gift of Dr. Stephan Heller, Department of Otolaryngology, Stanford University School of Medicine, Palo Alto, CA) at 1:5000, followed by horseradish peroxidase-conjugated donkey anti-rabbit secondary antibodies and enhanced using chemiluminescence, as described previously (Chung et al., 2003). Whole-cell lysates derived from HEK293 cells stably transformed with control vector (pCDNA3) or the rat TRPV4 cDNA (Guler et al., 2002) were loaded on the same gels as negative and positive controls, respectively.

For the reverse transcription-polymerase chain reaction, total RNA was isolated from microdissected rat urothelium and detrusor smooth muscle and from dissociated urothelial cells and smooth muscle cells using TRIzol reagent (Invitrogen, Carlsbad, CA), and first strand cDNA was synthesized as described previously (Birder et al., 2001). Complementary DNA (1.6 μg) was subjected to the polymerase chain reaction amplification for TRPV4 or glyceraldehyde phosphate dehydrogenase (GAPDH; input control) in a 50-μl reaction containing: 2.5 units of Taq polymerase (QIAGEN, Valencia, CA), 1× Q-solution, 1× PCR buffer, 200 μM dNTP and 0.15 μM of primers. Reactions were incubated at 94°C/5 min, then cycled 30 times at 94°C/1 min, 53°C/1 min, and 72°C/2 min 40 s before a final extension step of 72°C/10 min. Primers used were as follows: for TRPV4, 5′-agagctgatgatgct-3′ and 5′-cggagatccagctgcccgct-3′; and for GAPDH, 5′-ccacagctctgggtg-3′ and 5′-tcac-ccaccttggtgcttc-3′. Primers were chosen to span intron-exon boundaries and thereby avoid contaminating amplification from genomic
DNA. Samples were electrophoresed on 1% agarose/ethidium gels, and amplified bands were extracted and subjected to sequencing to confirm that they encoded TRPV4. Negative controls were performed with no template.

**Cell Culture and ATP Release.** Preparation and characterization of urothelial cultures have been described in previous reports (Birder et al., 2001, 2002). In brief, bladders were excised from deeply anesthetized (1.2 g/kg s.c. urethane; euthanized with CO₂) rats, cut open, and gently stretched (urothelial side up). The tissue was incubated overnight in minimal essential medium (Cellgro; Mediatech, Herndon, VA), penicillin/streptomycin/fungizone, and 2.5 mg/ml dispase (Invitrogen, Rockville, MD). The urothelium was gently scraped from underlying tissue, treated with 0.25% trypsin, and resuspended in keratinocyte medium (Invitrogen). The dissociated cell suspension (0.1 ml, 0.5–1.5 × 10⁶ cells/ml) was plated on the surface of collagen-coated dishes, 18 to 72 h before testing. All cells in culture were cytokeratin positive (DAKO, Carpinteria, CA), and therefore, were presumably of epithelial origin. For ATP release, cells were superfused with oxygenated Krebs containing 4.8 mM KCl, 120 mM NaCl, 1.1 mM MgCl₂, 2.0 mM CaCl₂, 11 mM glucose, KH₂PO₄, and 11.7 mM glucose, pH 7.4. The initial tension was set at 10 mN, and contractions were evoked by electrical field stimulation (100 Hz, 500 μsec). The detection limit was 10 fmol ATP/sample. Values for each condition were normalized by comparison with peak response of the calcium ionophore ionomycin (3 μM). Pooled data results are given as mean ± S.E.M., and statistical significance was determined using unpaired Student’s t test. Each figure represents data collected from a minimum of three independent cultures. Statistical significance was accepted when p < 0.05.

**Measurement of [Ca²⁺]i.** Cultured rat urothelial cells (18–72 h after plating) were incubated with the fluorescent Ca²⁺ indicator, fura-2-acetoxymethyl ester (5 μM; Molecular Probes, Eugene, OR) in Hanks’ balanced salt solution containing bovine serum albumin (5 mg/ml) for 30 min at 37°C in an atmosphere of 5% CO₂. Cells were washed in Hanks’ balanced salt solution (containing 138 mM NaCl, 5 mM KCl, 0.3 mM KH₂PO₄, 4 mM NaHCO₃, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 5.6 mM glucose, pH 7.4, and 295 mOsm), transferred to a perfusion chamber, and mounted onto an upright epifluorescence microscope (IX70; Olympus, Tokyo, Japan). Measurement of [Ca²⁺]i was performed by ratiometric imaging of fura-2 at 340 and 380 (100 Hz), and the emitted light was monitored at 510 nm. The fluorescence ratio, F₃₄₀/F₃₈₀, was calculated and acquired by C-imaging systems (Compix Inc., Sewickley, PA), and background fluorescence was subtracted. All test agents were bath applied (flow rate, 1.5 ml/min). Data were obtained from at least three independent cultures and analyzed using Student’s t test for unpaired samples.

**Bladder Strip Contractile Responses.** Longitudinal bladder strips (1.5 × 10 mm) were prepared from adult female Sprague-Dawley rats and mounted in a vertical double-jacketed organ bath (room temperature) in oxygenated Krebs: 118 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄, 24.9 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.7 mM glucose, pH 7.4. The initial tension was set at 10 mN, and contractions were evoked by electrical field stimulation using platinum electrodes inserted at the top and bottom of the organ bath. Stimuli consisting of trains (10-s duration, 25 pulses/s, 0.25-s pulse duration, 100-V amplitude) were delivered every 90 s using a Grass S88 stimulator (Grass, Astromed, RI). Stimulus-evoked contractions were measured with a force displacement transducer (Grass, Doylestown, PA). Data were recorded and analyzed using Windag software (DATAQ Instruments Inc., Akron, OH) and Excel (Microsoft, Redmond, WA).

**Cystometry.** Female Sprague-Dawley rats (200–250 g) were anesthetized with halothane, and upon the absence of a pedal reflex, the urinary bladder was exposed by a midline abdominal incision. A catheter (PE-50) was inserted through the apex of the bladder dome, secured with surgical silk, and connected with a T stopcock to an infusion pump and a pressure transducer. The cystometries were done within 2 h after placement of the bladder catheter. For awake cystometry (CMG), rats were placed in a restraining cage (Broome Style Rodent Restrainters; Kent Scientific, Litchfield, CT). Variations in intraluminal pressure were recorded in response to continuous infusion (0.1 ml/min) of saline or drug solutions to elicit repeated voiding responses in either awake or anesthetized (1.2 g/kg s.c. urethane) rats. For isovolumetric cystometry, saline or drug solutions (0.1 ml/min) were infused until peak voiding pressures were reached, when the infusion was discontinued and maintained under isovolumetric conditions to record repeated reflex bladder contractions. In all animals, control CMGs were recorded for 1 h to establish baseline before drug administration. Capsaicin (100 mg/kg s.c.; Sigma) in a solution of 10% ethanol, 10% Tween 80, and 80% physiologic saline, was administered (under anesthesia, s.c.) in divided doses on 2 consecutive days (experiments were performed 4 days later). Effectiveness of capsaicin desensitization was confirmed by the lack of an eye wipe test response to ocular capsaicin administration.

**Results**

**TRPV4 Protein Is Expressed at Multiple Locations along the Urinary Tract.** Using a polyclonal antiserum specific for TRPV4, we performed immunofluorescence histochemistry on fixed tissue sections derived from a number of structures within the upper and lower urinary tracts. In the kidney, we observed specific TRPV4-like immunoreactivity (TRPV4-LI) in the distal convoluted tubule and ascending thin limb of the loop of Henle, as reported by others (Tian et al., 2004) (Fig. 1, a and b). Strong, specific TRPV4-LI was also observed in the urothelium lining the renal pelvis, ureters, urinary bladder, and urethra (Fig. 1, c–f). At higher magnification, confocal microscopy revealed that urothelial TRPV4-LI extended throughout all cell layers of the urothelium and was most prominent in the vicinity of the plasma membrane (Fig. 1e). No obvious nerve fiber staining was observed in these tissues. In all locations, antibody preincubation with antitrPV4 peptide substantially attenuated the immunofluorescence signal, whereas preincubation with an unrelated peptide did not (data not shown). Consistent with these results, anti-TRPV4 immunoblot analysis of primary cultured urothelial cells revealed three bands between 96 and 120 kDa (Fig. 1g). A very similar pattern was observed in lysates of HEK293 cells stably expressing rat TRPV4. This complex pattern is consistent with previous reports of native and recombinant TRPV4 and probably reflects alternative glycoforms of this protein (Xu et al., 2003). No immunoreactive bands were observed in lysates derived from HEK293 cells stably transfected with control vector (pCDNA3). To further confirm the expression of TRPV4 in urothelial cells, we performed the reverse transcription-polymerase chain reaction on isolated rat urothelial cells using oligonucleotide primers specific for TRPV4. A product with the predicted size and nucleotide sequence could be amplified from urothelium or dissociated urothelial cells. Only a very weak band of the same size could be amplified from the underlying nonurothelial tissue or isolated smooth muscle.
Ca\(^{2+}\) solution (no added Ca\(^{2+}\)) kinase C (Watanabe et al., 2002). In addition, it fails to esters, this compound has extremely low activity on protein primary cultured urothelial cells. Unlike many other phorbol effects of ruthenium red (RR; 10\(^{-7}\)) Ca\(^{2+}\) (less than 0.2 change in fura ratio), demonstrating a role for TRPV4 by bladder urothelial cells.

Fig. 1.

TRPV4 Produces Ca\(^{2+}\) Influx and ATP Release in Urothelial Cells. To determine whether the TRPV4 expressed in urothelial cells is functionally competent, we examined the effect of the TRPV4-selective agonist, 4\(\alpha\)-PDD, on primary cultured urothelial cells. Unlike many other phorbol esters, this compound has extremely low activity on protein kinase C (Watanabe et al., 2002). In addition, it fails to activate any other ion channel tested to date. 4\(\alpha\)-PDD (1 \(\mu\)M) evoked an increase in [Ca\(^{2+}\)] in 247 of 369 urothelial cells (Fig. 2a) that typically reached a peak within 2 to 3 min after drug application (mean \pm S.E.M. change in fura-2 fluorescence ratio, 1.0 \pm 0.1 compared with 2.1 \pm 0.6 evoked by the Ca\(^{2+}\) ionophore, 3 \(\mu\)M ionomycin). At a lower concentration of 4\(\alpha\)-PDD (50 nM), increases in [Ca\(^{2+}\)] could also be observed, albeit less frequently (in 25 of 135 cultured cells). In experiments where we first perfused iso-osmotic Ca\(^{2+}\)-free solution (no added Ca\(^{2+}\); 100 \(\mu\)M EGTA), none of the cells tested (0 of 163) showed a 4\(\alpha\)-PDD-evoked increase in [Ca\(^{2+}\)] (less than 0.2 change in fura ratio), demonstrating a role for Ca\(^{2+}\) influx in this response (Fig. 2a). We next evaluated the effects of ruthenium red (RR; 10 \(\mu\)M) as well as hyperosmotic (327 mOsm) bath solution, both of which have been demonstrated to reduce TRPV4-mediated responses in other cells (Fig. 1h). These findings confirm the expression of TRPV4 by bladder urothelial cells.

4\(\alpha\)-PDD Enhances Bladder Voiding Pressure in Vivo. The possible influence of TRPV4 activation on urinary bladder function in vivo was evaluated in awake rats using continuous cystometry. In these experiments, the urethral outlet remained open, and luminal contents were therefore eliminated during each void. Continuous (0.1 ml/min) intravesical infusion of 4\(\alpha\)-PDD (100 \(\mu\)M; \(n = 9\)) significantly increased the amplitude of reflex bladder contractions (51 \pm 9\% increase; \(p < 0.05\)) (Fig. 3, a, b, and e). In contrast, there was no obvious change in baseline intravesical pressure (Fig. 3, a and b). The filling phase of the bladder pressure traces was also normal, with no evidence of nonvoiding contractions. There was a trend toward an increase in the frequency of voiding [i.e., a decrease in the intercontraction interval (ICI)] during 4\(\alpha\)-PDD infusion; however, this apparent difference was not statistically significant (Fig. 3f). Similar increases in bladder contraction amplitude (40 \pm 5\% and 44 \pm 4\% increase, \(n = 8\) total) were detected using lower concentrations of 4\(\alpha\)-PDD (10 and 25 \(\mu\)M, respectively, not shown). However,
the higher (100 μM) concentration produced more consistent responses and, thus, was used for all subsequent experiments. The onset of 4α-PDD-evoked increases in bladder contraction amplitude was rapid (within 3–5 min after start of infusion), completely reversible upon drug washout, and repeatable (Fig. 3c). In contrast to 4α-PDD, which does not activate protein kinase C (PKC), intravesical infusion of the structurally related PKC activator, phorbol 12-myristate 13-acetate (100 μM; n = 4), which is a far less potent activator of TRPV4, did not facilitate bladder reflexes (data not shown).

To explore the mechanism of 4α-PDD action on bladder contraction amplitude in the rat, we tested the effect of this compound on the contraction of isolated bladder strips. After a 2-h equilibration period, electrical stimulation (trains of 10-s duration, 25 pulses/s, 100 V) of bladder strips elicited reproducible large amplitude contractions (Fig. 4a). Application of muscarine (0.1–1 μM) to the organ bath significantly increased both baseline amplitude (22.7 ± 6.7% above control; n = 7; p < 0.05), and the amplitude of electrically evoked contractions (29.4 ± 3.3% above control; n = 7; p < 0.05) (Fig. 4, b and c). This effect was reversible upon muscarine washout. In contrast, 4α-PDD (1–100 μM) did not significantly change either baseline tension or amplitude of electrically evoked contractions. These results argue against either a direct excitatory action of 4α-PDD on detrusor smooth muscle or enhancement of neurally evoked contractions by a facilitatory effect on efferent or afferent nerve terminals within the bladder strip.

Another possible mechanism for 4α-PDD enhancement of intravesical pressure during voiding would be reflex constriction (or inadequate relaxation) of the urethral outlet, which would increase outflow resistance (de Groat, 1993; Cheng et al., 1997). To test this possibility, we first examined the effects of 4α-PDD on micturition after bilateral surgical interruption of the pudendal nerves, which contain the motor nerves to the external urethral sphincter and mediate reflex urethral contractions and, thereby, regulate outflow resistance. Bilateral pudendal nerve transection (n = 5) did not alter the increase in amplitude of bladder contractions evoked by 4α-PDD infusion (Fig. 3e versus Fig. 5a). A second approach toward evaluating the outflow resistance hypothesis was to examine the amplitude of high-frequency oscillations (HFOs) in bladder pressure (Fig. 5, b and c) that occur during micturition and that reflect the contractile activity of the urethral sphincter (de Groat, 1993). HFOs that occur during the period when the bladder neck is open and fluid flows from the bladder through the urethra were significantly decreased in amplitude following bilateral pudendal nerve transection (Fig. 5, b and c). 4α-PDD treatment did not alter the frequency or amplitude of HFOs, nor did it change the basal intravesical pressure before or during the HFOs (Fig. 5, c and d). Rather, this drug increased the peak pressure in the period following the HFOs, when the bladder neck is typically closed (Fig. 5, a and b). As an additional experiment, we examined several animals (n = 4) in which a cannula (PE-50) was placed in the bladder via the urethra and tied in place. Under these isovolumetric conditions, intravesical application of 4α-PDD still elicited a robust increase in bladder contraction amplitude (38 ± 4%; data not shown) compared with control contractions elicited during distension of the bladder with saline. Together, these data argue strongly against enhancement of urethral tone as an explanation for the effects of 4α-PDD on bladder contraction amplitude.
4α-PDD Augments Bladder Contraction via a Capsaicin-Insensitive, Purinergic Signaling-Dependent Pathway. The results outlined above suggest that the effect of 4α-PDD on contraction amplitude is most probably mediated by an action on the afferent limb of the bladder reflex pathway. We therefore sought to explore the nature of the afferent fibers responsible for this effect. The TRPV1 agonist, capsaicin (trans-8-methyl-N-vanilly-6-nonenamide), has been used to desensitize a subpopulation of small-diameter, unmyelinated C-fiber afferents that innervate the bladder, leav-
ing the other afferent unaffected (Barbanti et al., 1993; de Groat, 1993). In animals pretreated with capsaicin (100 mg/kg s.c.), 4 days before the experiment, there was no significant change in bladder contraction amplitude or intercontraction interval in the cystometrograms of rats perfused with saline as previously reported (Cheng et al., 1999). In these rats, 4α-PDD still significantly increased peak intravesical voiding pressures (41 ± 6%, n = 5) (Fig. 5e) but did not significantly affect either frequency or amplitude of HFOs (Fig. 5, c and d). Because anesthesia apparently increases the relative contribution of capsaicin-dependent afferents as opposed to capsaicin-independent reflex voiding responses, we also conducted cystometry experiments in urethane-anesthetized rats (n = 4). Under these conditions, 4α-PDD elicited no significant change in peak intravesical voiding pressures, suggesting that the effects of this compound involve the capsaicin-insensitive voiding mechanisms that dominate in the awake state.

Given that TRPV4 activation evokes ATP release from urothelial cells, we sought to determine whether such release might underlie the cystometric effects of 4α-PDD. Intravesical administration of the relatively nonselective purinergic antagonist PPADS (100 μM; n = 4) had no effect on either bladder contraction amplitude or ICI, suggesting that ATP released endogenously in the region of the urothelium does not affect micturition under basal conditions (Fig. 3, e and f). However, when either PPADS (100 μM intravesical, n = 5) or A317491, a selective P2X₃ antagonist (250 μmol/kg s.c.; n = 4; Sigma), was administered before 4α-PDD, the facilitatory response to the latter compound was prevented (Fig. 3, d–f). Thus, intercellular ATP signaling within the bladder, possibly on P2X₃ sensory fibers, seems to be essential for the cystometric effects of 4α-PDD.

**Discussion**

Several findings presented in this study demonstrate that rat urothelial cells express functional TRPV4 channels: 1) TRPV4 mRNA and protein are both detectable; 2) exposure to the selective TRPV4 agonist, 4α-PDD, results in Ca²⁺ influx and ATP release, both of which can be blocked by RR; 3) the endogenous TRPV4 agonist, 5,6-EET, evokes ATP release; 4) hypo-osmolarity-evoked ATP release from urothelial cells is potentiated by 4α-PDD and reduced by inhibitors of EET synthesis, as previously reported for hypo-osmolarity-evoked activation of native and recombinant TRPV4 (Liedtke et al., 2000); and 5) 4α-PDD-evoked [Ca²⁺]ᵢ responses are inhibited by hyperosmolarity. Our data also indicate that intravesical application of 4α-PDD in vivo increases intravesical pressure following voluntary voiding in awake rats without significantly affecting voiding frequency. This effect is probably to arise from 4α-PDD action on TRPV4, rather than on PKC, because intravesical application of the more potent PKC-activating phorbol ester, phorbol 12-myristate 13-acetate, failed to influence voiding pressure. In contrast to the results in the rat, we were unable to observe robust effects of 4α-PDD on intravesical pressure during cystometry in the awake mouse (unpublished data). These findings, which may

![Image](https://via.placeholder.com/150)
reflect differences between these species in bladder control mechanisms, precluded us from evaluating 4α-PDD in TRPV4 knockout mice. Given that the 4α-PDD-evoked enhancement of bladder pressure in the rat is observed after the high-frequency oscillations produced by rhythmic urethral contractions and that this effect persists following bilateral pudendal nerve transection or cannulation of the urethral outlet, we conclude that this cystometric alteration arises from enhancement of bladder wall smooth muscle contraction, as opposed to urethral outlet constriction.

Several observations further indicate that the TRPV4-mediated bladder response most probably stems from this channel’s influence on the initiation of voluntary bladder contractions, rather than a direct effect on bladder smooth muscle or neurotransmission at the parasympathetic neuroeffector junction in the bladder. First, the effect of TRPV4 activation by 4α-PDD on intravesical pressure is confined to perivoiding periods, with no apparent effect on baseline or intervoid bladder pressure. Second, 4α-PDD has no effect on stimulus-evoked contractions in isolated bladder strips. Third, the effect of 4α-PDD on bladder contractions is prevented by intravesical administration of the nonselective purinergic antagonist, PPADS, or systemic treatment with a P2X3-selective antagonist, A317491, strongly supporting the participation of TRPV4-evoked intercellular ATP signaling. The release of bioactive mediators such as ATP, most probably from urothelial cells, has been demonstrated to facilitate reflex voiding via activation of purinergic afferent receptors from urothelial cells, has been demonstrated to facilitate release of bioactive mediators such as ATP, most probably participation of TRPV4-evoked intercellular ATP signaling. The release of bioactive mediators such as ATP, most probably from urothelial cells, has been demonstrated to facilitate reflex voiding via activation of purinergic afferent receptors (Andersson, 2002). In support of this view are studies showing that intravesical administration of ATP activates bladder afferent nerves and in turn triggers bladder hyperactivity (Zhang et al., 2003), and mice lacking the ATP-gated channel, P2X3, exhibit diminished reflex voiding responses (Cockayne et al., 2000). We cannot completely rule out an effect of 4α-PDD mediated by TRPV4-dependent release of ATP from sensory afferents or release from the urothelium of other bioactive mediators such as acetylcholine, which can in turn modulate or trigger the release of ATP. However, the robust expression of TRPV4 in rat urothelial cells and our failure to detect TRPV4 immunoreactivity in bladder afferents all argue against this possibility.

What type of bladder afferent is likely to convey TRPV4-mediated responses to the spinal cord? It has been suggested that capsaicin-insensitive Aδ-fiber afferents in awake rats are responsible for voluntary voiding (de Groat, 1993). The lack of a significant change in the ICI or voiding pressure in capsaicin-pretreated awake animals reported in this and another study (Chuang et al., 2001) further supports this view. Two recent reports have provided different views regarding the effect of ATP on subpopulations of bladder afferent nerve fibers. It was shown that the excitatory effect of ATP persists after resiniferatoxin treatment (Zhang et al., 2003), consistent with a role for capsaicin-insensitive afferents in certain reflex responses mediated by purinergic signals. In contrast, another study (Nishiguchi et al., 2005) was not able to detect a facilitatory effect of intravesically administered ATP until the urothelial barrier was reduced by protamine sulfate treatment, suggesting that the effects of intravesical ATP can be influenced by the experimental conditions. In the present study, the persistence of 4α-PDD-evoked augmentation of voiding pressure after capsaicin-induced desensitization of C-fibers suggests that capsaicin-resistant Aδ or C-fibers are responsible for the cystometric effects of TRPV4 activation in awake rats.

Another implication of the present study is that the roles of TRPV1 and TRPV4 in bladder function are apparently distinct. Both channels are widely expressed in the urothelium, and activation of either results in urothelial cell ATP release. However, capsaicin-evoked TRPV1 activation in the rat and mouse in vivo leads to a decrease in ICI, in both awake and anesthetized states, with no change in contraction amplitude (Maggi et al., 1984). In the excised rat bladder, TRPV1 activation increases contraction amplitude (Gevaert et al., 2007). The distension-evoked reflex bladder contraction seems to be reduced selectively in the anesthetized state in mice lacking TRPV1. In contrast, 4α-PDD-induced TRPV4 activation in the rat enhances reflex bladder contraction amplitude, with only a tendency toward a decreased ICI, and does so only in the awake state. TRPV1 immunoreactivity, unlike TRPV4, has been readily observed in bladder afferent nerves (Tominaga et al., 1998; Birder et al., 2001). Although we cannot exclude the possibility that some TRPV4 protein is expressed in bladder afferents, as reported for rat sciatic nerve and mouse trigeminal ganglia (Liedtke et al., 2003; Alessandri-Haber et al., 2004), the expression of TRPV4 in urothelium might account, in part, for the differential results of activating TRPV1 versus TRPV4. Alternatively, the complement of signaling molecules released from urothelial cells in response to activation of these two channels may be different, resulting in the activation of distinct neuronal populations. The TRPV4-mediated release of multiple mediators could also explain why TRPV4 activation increases the amplitude of the postmicturition contractions, whereas intravesical administration of ATP produces only a decrease in ICI, with no change in amplitude.

This difference between the effects of intravesical ATP administration and the effects of intravesical 4α-PDD that are apparently mediated in part by ATP release from the urothelium is difficult to explain unless the two types of stimuli activate different populations of afferent nerves. This seems likely because the bladder excitatory effect of intravesical ATP is suppressed by pretreatment with systemic capsaicin (Nishiguchi et al., 2005) after the urothelial barrier was disrupted by protamine sulfate pretreatment, whereas the effect of 4α-PDD was unaffected. This indicates that afferent nerves below the urothelium might be responding to ATP, whereas 4α-PDD seems to act directly on the urothelial cells to release ATP and therefore might activate only a subpopulation of afferent nerves located within the urothelium.

4α-PDD had a very selective effect on bladder activity. It did not alter intravesical pressure during: 1) the storage phase in between voluntary voiding bladder contractions, 2) the isometric contraction before opening of the urethral outlet, or 3) voiding. However, it did markedly increase intravesical pressure during the postvoid isovolumetric contraction, suggesting that distinct neural mechanisms are involved in prevoiding/voiding responses and postvoiding responses. The neurological basis of this late phase of micturition, at a time when the bulk of urine flow has stopped, remains poorly understood, but that may be important for the evacuation of residual urine from the bladder lumen. Selective enhancement of the postvoid isovolumetric contractions has been noted during cold saline infusion into the
bladder (Cheng et al., 1997). This effect was blocked by capsaicin pretreatment or transection of pudendal nerves, indicating that it was due to activation of cold-sensitive, capsaicin-sensitive bladder afferents triggering reflex contraction of the external urethral sphincter via efferent pathways in the pudendal nerves. However, this mechanism is clearly not involved in the effect of 4α-PDD and raises the possibility that 4α-PDD activates a distinct afferent pathway that triggers the postvoid isovolumetric contraction.

Under what physiological or pathophysiological conditions might TRPV4 activation be important for bladder function? Because the effect of 4α-PDD only occurred in the awake rat, it seems likely that the effect is due to facilitation of a voluntary bladder contraction triggered by stimulation of bladder afferent nerves and the initiation of an abnormal bladder sensation. Afferent input, indicating that the bladder is not empty, could induce a maintained bladder contraction, reflecting an attempt in the rat to void completely. In some patients with lower urinary tract dysfunction (e.g., men with urethral outlet obstruction due to benign prostatic hypertrophy and women with multiple sclerosis or idiopathic voiding disorders), sensations of incomplete bladder emptying can occur (Chute et al., 1993; Al-Shahrani and Lovatissy, 2005; Durufle et al., 2006). In women, the sensations are not associated with increased residual urine and therefore are not triggered by incomplete emptying. Thus, a postvoiding bladder contraction as noted during TRPV4 stimulation in the urethra of rats might contribute to the abnormal postvoiding sensations observed in these patients. A greater understanding of the role of urethral TRPV4 in bladder function might lead to development of new treatments of lower urinary tract disorders.

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