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Transient Receptor Potential A1 (TRPA1) Activity in the Human Urethra—Evidence for a Functional Role for TRPA1 in the Outflow Region

Christian Gratzkeab, Tomi Strenga,c, Eginhard Waldkirchd, Katja Siglb, Christian Stiefb, Karl-Erik Anderssona,*, Petter Hedlundaf

aDepartment of Clinical Chemistry and Pharmacology, Lund University Hospital, Lund, Sweden
bDepartment of Urology, Ludwig-Maximilians University Hospital, Munich, Germany
cDepartment of Pharmacology, Drug Development and Therapeutics, University of Turku, Turku, Finland
dDepartment of Urology, Hannover Medical School, Hannover, Germany
eWake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC, USA
fDepartment of Clinical Pharmacology, Linköping University Hospital, Linköping, Sweden

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Abstract

Background: A role for the transient receptor potential (TRP) A1 ion channel in rat lower urinary tract (LUT) sensation and disease has been proposed, but in the human LUT no information on TRPA1 activity is available.

Objectives: To investigate the distribution of TRPA1 in the human urethra and to study the effect of TRPA1 agonists on isolated urethral strip preparations.

Design, settings, and participants: Urethral specimens were obtained preoperatively from 10 patients and were freshly prepared for Western blot, immunohistochemistry, and functional in vitro investigations.

Measurements: The expression patterns of TRPA1 were studied with Western blot and immunohistochemistry. The effects of allyl isothiocyanate (AI), cinnamaldehyde (CA), and NaHS (donor of H2S) on tension of urethral strips were investigated in tissue baths.

Results and limitations: TRPA1 immunoreactivity (-IR) was found in nerve fibres in the suburothelial space and was also located to nerve fibres of the muscle layer. Single TRPA1-IR nerves extended into the urothelium. A majority, but not all TRPA1-IR nerves also expressed immunoreactivity for CGRP or TRPV1. In the urothelium, TRPV1 was located to the outer layers whereas TRPA1 was observed in basal urothelial cells. Interspersed between strands of smooth muscle cells of the urethral wall, TRPA1- and vimentin-IR cells containing central nuclei and slender cytoplasmatic extensions were observed.

In functional experiments, TRPA1-agonists had no contractile effect in urethral preparations. After precontraction with phenylephrine, AI, CA, and NaHS caused concentration-dependent relaxations of urethral strip preparations.

Conclusions: The localization of TRPA1 to nerves that also express TRPV1 and CGRP, and in urothelial cells and interstitial cells, as well as the findings that TRPA1 agonists can modify tone of urethral preparations, propose a role for TRPA1 in afferent and efferent sensory signaling of the human outflow region.

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1. Introduction

The main function of the lower urinary tract (LUT), storage and voiding of urine from the urinary bladder, requires coordination of smooth muscles in the bladder and urethra by a complex neural control system [1]. Sensory nerves from the lamina propria and the bladder wall convey information about urine volume and bladder pressure during urine storage to the central nervous system (CNS) [2]. Myelinated Aδ-fibres respond to passive distension and active contraction, and C-fibres, which increase in density towards the outflow region and the urethra, have a high mechanical threshold and respond to chemical irritation of the LUT mucosa as well as to changes in temperature [1].

Urothelial cells exhibit similar properties to sensory neurons through the expression of sensory receptors and ion channels [3]. Such a sensory receptor that is located on urothelial- and suburothelial cells and on C-fibres is the transient receptor potential (TRP) cation channel subfamily V member 1 (TRPV1). TRPV1 ion channels are activated by vanilloids such as capsaicin, moderate heat, and protons [3]. A role for TRPV1 in stretch-evoked urothelial signals to afferent nerves has been proposed [4]. Anandamide, an endogenous lipid metabolite that also activates TRPV1, has been suggested to contribute to detrusor overactivity (DO) during cystitis [4]. Bladder instillations with capsaicin and resiniferatoxin cause desensitization of C-fibres and have been used for the treatment of the overactive bladder (OAB) syndrome and DO [5].

In sensory nerves, TRPV1 is coexpressed with TRPA1, another member of the TRP superfamily [6]. TRPA1 is the only mammalian member of the Ankyrin TRP subfamily and generally known to be present on capsaicin-sensitive primary sensory neurons [6], which upon activation elicit pain, protective reflexes, and local release of neurotransmitters in the periphery [7,8]. TRPA1 can be activated by plant-derived irritants such as allyl isothiocyanate (AI), cinnamaldehyde (CA) [9–11], H2S [12], menthol [13], and formalin [14]. We have recently demonstrated the presence of TRPA1 ion channels on capsaicin-sensitive primary sensory neurons in the rat bladder; furthermore, we showed the coexistence of TRPA1 and TRPV1 on C-fiber bladder afferents and the expression of TRPA1 on urothelial cells [12]. Together with the findings that intravesical administration of activators of TRPA1 ion channels such as CA, AI, and the inflammatory mediator H2S induced DO, a role of TRPA1 in detrusor sensation and inflammatory disease of the bladder was postulated [12].

To the best of our knowledge, there is currently no information available on the distribution and function of TRPA1 in human LUT tissues. The aim of the present study was to examine the expression of TRPA1 in relation to other sensory markers in the human urethra and to investigate the effects of TRPA1-activators on human urethral specimens.

2. Methods

2.1. Patients and tissues

In accordance with the regulations of the Ethics committees of Lund (Sweden) and Munich (Germany), proximal urethral specimens were obtained peroperatively from 10 patients (mean age 62 yr) undergoing cysturethrectomy (3 females) due to bladder cancer or radical prostatectomy (7 male patients) due to localized carcinoma of the prostate.

2.2. Western blot

Human urethral specimens (n = 4) were snap frozen, homogenized, and lysed in RIPA buffer supplemented with 1:10 protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using Bio-Rad’s DC protein assay. One to 3 μg/ml of total protein for samples was separated on the 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis, transferred to a PVDF membrane (Millipore Corporation, Bedford, MA, USA), probed overnight in 5% w/v skim milk/1 X PBST (0.5% v/v Tween-20) with TRPA1 antibodies (1:2000; Alomone Labs, Jerusalem, Israel), followed by horseradish peroxidase-labelled IgG. Images were obtained using LAS3000 Imager. β-actin was used as a standard. Broad range molecular marker (Bio-Rad Laboratories, Hercules, CA, USA) and biotinylated protein ladder (Cell Signaling Technology, Danvers, MA, USA) were used to determine molecular masses. Controls without primary antibodies were performed for all samples.

2.3. Immunohistochemistry

Urethral specimens were fixed and processed for immunohistochemistry as previously described [12]. Sections were incubated overnight (room temperature) with antibodies for TRPA1 (rabbit; 1:500, Alomone Labs, Jerusalem, Israel), TRPA1 (goat; 1:250, Alomone Labs, Jerusalem, Israel), TRPV1 (goat; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CGRP (guinea-pig; 1:1000; Euro-Diagnostica, Malmö, Sweden), tyrosine hydroxylase (TH; mouse; 1:2000, Diasorin AB, Bromma, Sweden), nitric oxide synthase (sheep; 1:2000, P.C. Emson, Babraham Institute, Cambridge, UK), or vimentin (goat; 1:200, Sigma Biosciences, St Louis, MO, USA).

For simultaneous demonstration of two antigens, primary antibodies were incubated as cocktails. After rinsing [12], species-directed secondary antibodies (1:600; Alexa Fluor, Molecular Probes Inc., Leiden, The Netherlands) were applied to the sections (60 min). Sections were analyzed using a laser fluorescence microscope with single and double filter settings at 488 and 522 nm (Olympus Corp., Osaka, Japan). Images were
acquired using Viewfinder Lite version 2.0 (Pixera Corp, Egham, UK). Control stainings without primary antibodies were performed. The amounts of immunoreactive structures per regional area in sections were assessed with SigmaScan (Systat Software Inc, San Jose, CA, USA).

No signs of malignant cells were noted in any of the urethral sections.

2.4. Functional investigations

Full wall preparations (3 × 3 × 6 mm) were dissected from the proximal portion of the urethra. Silk ligatures were applied at both ends of the preparations, which were mounted in 5 ml aerated (95 % O₂ and 5 % CO₂) tissue baths (37 °C, pH 7.4), containing Krebs solution. Mechanical activity was registered by exposure to a 60 mM K⁺ Krebs solution, which produced contractions amounting to 4.5 ± 0.8 mN (n = 8). Viability of the preparations was assessed by exposure to a 60 mM K⁺ Krebs solution, which produced contractions amounting to 4.5 ± 1.8 mN (n = 8).

The effects of capsaicin (1 nM–10 μM), allyl isothiocyanate (Al; 10 nM–10 μM), cinnamaldehyde (CA; 10 μM–1 mM), and sodium hydrogen sulfide (NaHS; a donor of H₂S; 10 μM–1 mM) on basal tone or after precontraction with phenylephrine (Phe, 4.3 μM) were investigated.

2.5. Drugs and solutions

The following drugs were used: capsaicin, Al, CA, and NaHS. Stock solutions (10⁻¹ M; ethanol) were made of capsaicin, Al, and CA, which were kept in –20 °C until use. NaHS was prepared fresh for each experiment. Subsequent dilutions of the drugs were made in saline on the day of the experiment. The Krebs solution had the following composition (mM): NaCl 119, KCl 4.6, CaCl₂ 1.5, MgCl₂ 1.2, NaHCO₃ 15, NaH₂PO₄ 1.2, glucose 5.5, K⁺ (60 mM) Krebs solution was prepared by replacing NaCl with semimolar amounts of KCl.

2.6. Calculations and statistical analysis

Values are given as mean ± standard error of mean (SEM). The Student’s two-tailed t-test was used for paired or unpaired observations and for multiple comparisons, a one-way analysis of variance (Holm-Sidak) was used. A probability of p < 0.05 was regarded as significant; n denotes the number of patients used.

3. Results

3.1. Western blot

Western blot analysis using TRPA1-selective antibodies detected characteristic bands at the expected molecular weight of 128 kDa in the human urethra (Fig. 1A). Bands for β-actin were characteristically visualized at 42 kDa. The mean normalized TRPA1 band intensity amounted to 89 ± 5 % of β-actin.

3.2. Immunohistochemistry

The suburothelial space was supplied by TRPA1-immunoreactive (-IR) varicose and nonvaricose nerve fibres (Fig. 1B), which were distributed among smooth muscle cells of the lamina propria and which circumpassed the suburothelial vasculature. TRPA1-IR nerve fibres were also running along smooth muscle bundles and surrounding arteries of various sizes in the wall of the human urethra (Fig. 1C). Compared to the suburothelial region and the inner muscular layer of the urethra, TRPA1-IR nerve fibres were less numerous in the outer muscular urethral wall. The amount of TRPA1-IR nerve fibres in the wall of the urethra was 30 ± 5.0 % lower than in the suburothelial space (Fig. 1D).

A majority, but not all TRPA1-IR nerve fibres also expressed immunoreactivity for CGRP. In terminal nerve varicosities, colocalization between immunoreactivities for TRPA1 and CGRP was verified (Fig. 2A–C). Nerve fibres expressing TRPV1 had a similar distribution pattern as TRPA1-IR nerve structures of the human urethra. In double stained sections, TRPA1-IR nerve fibres also expressed immunoreactivity for TRPV1 (Fig. 2D–F). However, all TRPV1-IR nerve fibres did not contain immunoreactivity for TRPA1 and larger amounts of TRPV1-IR nerve fibres than TRPA1-IR nerve fibres were detected in within, for example, coarse nerve trunks (Fig. 2G–I).

Immunoreactivity for TH, a marker for adrenergic nerves, was observed throughout the human urethral wall. TRPA1-IR and TH-IR nerve fibres exhibited separate distribution patterns but were on occasion found to run close in parallel between smooth muscle cells (data not shown). As assessed in double stained sections, TRPA1- and NOS-IR nerve fibres did not exhibit similar profiles (data not shown). Single nerve fibres expressing immunoreactivity for NOS or TRPA1 were occasionally found in close proximity of each other.

Immunoreactivities for both TRPA1 and TRPV1 were distributed in the urothelium but only rarely in the same cellular layers or in the same urothelial cells (Fig. 3A, D, and E). Single TRPA1-IR nerve fibres extended into the basal and middle portions of the urothelium (Fig. 3B). Continuous strands of TRPV1-IR cells were mainly located to the outer layers the urothelium. In apposition, noncontinuous strands of TRPA1-IR cells were mainly located to the inner layers of the urothelium. Single TRPA1-IR cells that occasionally were located to the middle or outer urothelial layers also expressed immunoreactivity for TRPV1, and vice versa.
TRPA1 immunoreactivity was also detected in single cells that were evenly distributed and interspersed between bundles of smooth muscle cells of the inner and outer muscular layers of the human urethra (Fig. 4A and B). These cells which also expressed immunoreactivity for vimentin exhibited a central nucleus and slender cytoplasmatic extensions that covered groups of urethral smooth muscle cells (Fig. 4C–E).

There were no gender differences in the distribution of immunoreactivities for investigated proteins.

3.3. Functional investigations

At baseline, none of the TRPA1-agonists, allyl isothiocyanate (AI, 10 nM–100 μM), cinnamaldehyde (CA, 1 μM–1 mM) and NaHS (1 μM–1 mM), or capsaicin (1 nM–10 μM) affected the level of tension of the preparations (data not shown).

In phenylephrine (Phe, 10 μM)-contracted preparations, AI, CA, and NaHS caused concentration-dependent relaxations (Fig. 5A–D). Maximal relaxant effects were obtained at the highest investigated concentrations of the compounds and amounted to 85 ± 9.5%, 59 ± 5.9%, and 30 ± 6.9% for CA (1 mM), NaHS (1 mM), and AI (100 μM), respectively. In comparison, capsaicin produced concentration-dependent relaxant responses with a mean maximum of 86 ± 14.0% at 10 μM. The $-\log IC_{50}$ values for CA, NaHS, and capsaicin amounted to 5.0 ± 0.24, 3.2 ± 0.13, and 6.3 ± 0.41. A $-\log IC_{50}$ value could not be calculated for AI, but a $-\log IC_{30}$ value amounted to 3.3 ± 0.10. No differences in functional responses were recorded for male or females urethral preparations.
4. Discussion

The current study demonstrates for the first time the existence of TRPA1 in the human lower urinary tract and shows expression of TRPA1-ion channels on sensory nerves that also express TRPV1 and CGRP in the human urethra. These results correspond to observations in the trigone of the mouse and in rat bladder where TRPA1 has been located to nonmyelinated C-fibres or dorsal root ganglion (DRG) neurons that also expressed TRPV1, CGRP, or substance P [12,15,16]. The present findings that TRPA1 was located on nerve fibre afferents extending into the urothelium, that the suburothelium exhibited a higher density of TRPA1-IR than the muscular urethral wall, and that TRPA1-IR nerves were distinct from markers of autonomic nerves, further support a role for TRPA1 in chemosensation and nociception of the human urethra. Still, the occasional, close relation of TRPA1-IR nerves and adrenergic or NOS-containing nerves may support interactions between TRPA1-mediated mechanisms and efferent nerve functions. A complete colocalisation of TRPA1 and TRPV1 has been described for the rat detrusor [12]. In contrast, not all TRPV1-positive terminal afferents or nerve fibres were immunoreactive for TRPA1 in the human urethra. These findings may reflect species differences or differences in the sensory innervation patterns of the outflow region and the detrusor. However, in accordance to the current observations that not all CGRP-IR terminal afferents expressed immunoreactivity for TRPA1, only 50–60% of CGRP-containing rat bladder afferents or retrograde labelled CGRP-containing DRG neurons expressed immunoreactivity for TRPA1 [16].

Besides a barrier function, the urothelium is recognized as an active structure that expresses various receptors and that in response to physical or chemical stimuli can release transmitters that in turn may affect adjacent urothelial cells, afferent neurons, smooth muscle, or other components of the interstitium [3]. Immunoreactivity for TRPA1...
was located to the urothelium suggesting that urothelial cells of the human urethra have the capacity to interact with molecules or transmitters that have been shown to bind and activate TRPA1. Similar to findings in the rat detrusor, TRPA1-IR cells were abundant in the basal urothelial layers of the human urethra. As described for the human and rat detrusor [12,17], TRPV1 was mainly located to the superficial urothelial layers of the human urethra. Similar distinct regional localisation patterns within the urothelium have been described for other interrelated receptors or transmitter systems that have been proposed involved in urothelial-afferent nerve interactions that affect reflex voiding [1,18,19].

The distinct organisation of related transmitters or receptors in separate urothelial cell layers may also imply different functions for different cell-types not only in sensory functions but also in, for example, growth, proliferation, and repair [3]. In LUT disease, including OAB/DO, cystitis, or urethritis, the urothelium undergoes structural or functional changes [3,24]. The basal layers of the urothelium of the detrusor from patients with neurogenic DO have been described to exhibit increased expression of TRPV1 and increased amounts of mRNA for TRPV1 in the trigonal urothelium from women with urge incontinence have been reported [25,26]. Further studies are needed to understand if altered TRPA1 activity in urethral or detrusor tissues occurs in response to LUT diseases.

The distribution pattern of TRPA1 in basal cells and terminal afferents of the urothelium seems like an appropriate arrangement for TRPA1 to function as a sensor for noxious urinary constituents in conditions that affect the integrity or permeability of the urothelium of the human urethra. Hydrogen sulphide (H₂S) is an endogenous modulator of leukocyte-mediated inflammation, which has been shown to stimulate the micturition reflex by activation of TRPA1 after disruption of the urothelium in awake rats [12,20]. Escherichia coli, Proteus mirabilis, or Klebsiella, which are involved in the development of LUT infections also produce H₂S and symptoms such as dysuria may partially be explained through TRPA1-mediated mechanisms in the urethra [21,22]. In addition, H₂S is a mediator
Fig. 4 – TRPA1 (Alexa green) immunoreactivity was observed in cells with a central nuclei and slender cytoplasmic processes which were interspersed between bundles of smooth muscle cells of the wall of the urethra (A, 100× magnification; B–E, 1000× − oil). TRPA1 immunoreactive interstitial cells (C, E, Alexa green) also expressed immunoreactivity for vimentin (D, E, Alexa red).

Fig. 5 – In precontracted tissue (phenylephrine; 1 µM), cinnamaldehyde, NaHS, allyl isothiocyanate, and capsaicin induced concentration-dependent relaxations in the order of potency: capsaicin > cinnamaldehyde > NaHS > allyl isothiocyanate (n = 5–8, A). Original tracings describing the relaxant effects by cinnamaldehyde (B), NaHS (C), and allyl isothiocyanate (D) in three separate isolated preparations of the human urethra.
of neurogenic inflammation, which may be involved in the pathogenesis of interstitial cystitis or pelvic pain syndrome [23].

TRPV1 plays an important role in the detection and integration of noxious stimuli through afferent signals to the CNS and is also involved in triggering appropriate efferent signals at a spinal level, that is, autonomic reflexes [4]. Upon activation of TRPV1 on sensory nerves, direct or axonal release of peptides or amines from terminals cause local responses, which in pathologic conditions act to sensitize the tissue to sensory and motor stimuli [4]. In the isolated detrusor, both capsaicin and the TRPA1-activators AI and CA have been shown to produce contractions, and contractile effects by AI and CA were suggested to be dependent on the release of tachykinins and cyclooxygenase products from C-fibres [27]. Administration of AI, CA or NaHS had no effect on the basal tone of the human urethra but produced concentration-dependent relaxant responses in phenylephrine-activated preparations. In accordance with previous reports in dogs [28], capsaicin also produced relaxations in the precontracted human urethra. These findings suggest that TRPA1 and TRPV1 are involved in efferent functions of sensory nerves in the human urethra, but the exact mechanism for TRPA1-induced relaxation needs to be further investigated. It may be speculated if TRPA1 and TRPV1 are involved in axonal and autonomic reflexes that prevent damage to the LUT function by expelling any noxious constituent of the urine through contraction of the detrusor and relaxation of the urethra.

Recent findings describe the presence in the LUT of cells with similar characteristics as the Interstitial cells of Cajal that are involved in regulation of gastrointestinal motility [29]. These cells are distinguished by the expression of vimentin and c-kit but are also reported to contain cGMP [29,30]. In the bladder, networks of interstitial cells (ICs) in the suburothelial region and in the muscular wall of the detrusor have been suggested to be involved in integrating signals from the urothelium, sensory nerves and bladder wall [1]. Suburothelial ICs have also been described to express TRPV1 [7]. ICs have been located to the human, pig, rabbit, and guinea pig urethra and are known to exhibit spontaneous firing activity. The close structural relation between ICs, smooth muscle, and nerves form a basis for the involvement of theses cells in the regulation of urethral tone and the continence mechanism [30,31]. In our study, TRPA1-IR was detected in vimentin-positive elongated cells that were evenly distributed in the muscular wall and specifically located on the boundaries of urethral smooth muscle cell bundles. Interestingly, the H2S-producing enzyme cystathionine γ-lyase has been located to c-kit-positive interstitial cells of Cajal of the guinea pig and H2S has been proposed as a neuromodulator of the guinea pig and human colon [32]. Taken together with the current results, a modulatory role of TRPA1 and H2S for urethral ICs activity may be considered.

5. Conclusion

The current study for the first time describes TRPA1 receptors on nerve fibres, urothelium, and ICs and also shows that TRPA1-agonists can reduce tone of isolated preparations of the human urethra. The distribution of TRPA1 on TRPV1- and CGRP-positive nerve fibres, and in the urothelium suggests a role for TRPA1-mediated functions in afferent signals from the human outflow region. Functional data with AI, CA, and NaHS also suggest that the TRPA1 ion channel is involved in efferent functions of the human urethra. The demonstration of immunoreactivity for TRPA1 on vimentin-IR cells further strengthens a putative interactive role for ICs in afferent-efferent modulator activity of human urethral functions.

Author contributions: Karl-Erik Andersson had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Andersson, Hedlund.
Acquisition of data: Gratzke, Streng, Waldkirch, Sigl, Hedlund.
Analysis and interpretation of data: Gratzke, Streng, Waldkirch, Sigl, Hedlund.
Drafting of the manuscript: Gratzke, Streng.
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