Novel regulatory mechanism in human urinary bladder: central role of transient receptor potential melastatin 4 channels in detrusor smooth muscle function

Kirl L. Hristov,1 Amy C. Smith,1 Shankar P. Parajuli,1 John Malysz,1 Eric S. Rovner,2 and Georgi V. Petkov1,2

1Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, South Carolina; and 2Medical University of South Carolina, Charleston, South Carolina

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Overactive bladder (OAB) remains a poorly understood condition that presents a significant medical challenge (19). Although some therapeutic options are available for the treatment of OAB, there is no universally effective OAB therapy. While antimuscarinics are the primary pharmacological treatment for OAB, the clinical use of these agents provides limited efficacy and undesirable side effects (3, 37, 46). The long-term effectiveness of newer therapies such as mirabegron, a selective β3-adrenoceptor agonist (6, 9), and botulinum toxin (8, 26, 33) remains uncertain, and in some cases their use presents safety concerns (26, 28). The lack of safe and universally effective OAB treatments continues to spur the scientific community to seek novel therapeutic approaches to control OAB. Detrusor smooth muscle (DSM) cells represent the primary functional unit of the urinary bladder, and their dysregulation is responsible for a significant portion of lower urinary tract dysfunction. Ion channels expressed in DSM control urinary bladder function and, therefore, represent promising alternative targets for the pharmacological intervention of OAB (40, 41). Selective manipulation of individual ion channel subtypes in DSM could substantially alleviate various types of bladder dysfunction, such as OAB, urinary incontinence, or detrusor underactivity, while potentially minimizing collateral adverse effects elsewhere in the body.

A group of ion channels recently attracting significant interest is the transient receptor potential (TRP) cation channel family (25). As a large superfamily of 28 members, these channels are classified into seven related subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPN (no mechanopotentential (NOMP)), and TRPA (ankyrin) (34). The physiological roles of TRP channels include responding to physical and chemical stimuli such as temperature, pH, osmolality, pressure, stretch, light, and alkaloids, as well as intracellular stimuli such as Ca2+ (49).

A particularly interesting and important member of the TRP channel superfamily is the TRPM4 channel (18, 32). This channel is highly permeable to monovalent cations, such as Na+ and K+, but impermeable to anions and divalent cations, including Ca2+ (17, 32). TRPM4 channels, with a single-channel conductance of ~25 pS, exhibit Ca2+ and voltage dependency (35, 36, 48, 50). These channels are expressed in various mammalian tissues (21, 27, 32), including smooth muscle (10, 13–17, 29, 39), and are implicated in many diverse physiological and pathophysiological conditions (7, 24, 30, 31, 42, 43). Activation of TRPM4 channels causes cell membrane depolarization via Na+ influx, which in turn activates L-type voltage-gated Ca2+ (CaV) channels (11). Alternatively, inhibition of TRPM4 channels promotes substantial cell hyperpolarization and smooth muscle relaxation (15, 16). Because of these unique channel properties, selective modulation of

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Address for reprint requests and other correspondence: G. V. Petkov, Dept. of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, Univ. of South Carolina, Coker Life Sciences Bldg., Rm. 609D, 715 Sumter St., Columbia, SC 29208 (e-mail: petkov@cop.sc.edu).
TPRM4 channels may have profound effects on cell excitability and may be an attractive, novel approach for the pharmacological treatment of various conditions, including OAB.

Recent studies have defined the expression and function of TRPM4 channels in rat and guinea pig DSM (39, 44, 45). These innovative studies revealed that TRPM4 channels are important regulators of DSM excitability and contractility in these species. However, the expression and function of TRPM4 channels in human DSM are completely unknown (25). Significant species-related differences (animal vs. human) in DSM excitability and contractility have been well documented (20). Thus, it is critically important to investigate the physiological role of TRPM4 channels directly in human DSM cells and tissues under physiological conditions (25).

Here, we provide the first detailed examination of TRPM4 channels in human DSM by applying a multidisciplinary experimental approach, including RT-PCR, Western blotting, immunohistochemistry, immunocytochemistry, patch-clamp electrophysiology, isometric DSM tension recordings, and native freshly isolated human DSM cells and tissue strips, as well as examination of the effects of a novel TRPM4 channel inhibitor 9-phenanthrol. 9-Phenanthrol specifically targets TRPM4 channels and has been used in a variety of biological preparations to reveal the contribution of these channels in physiological processes (18). The results from the current study suggest a critical role for the TRPM4 channels in human DSM excitability and contractility. Pharmacological inhibition of TRPM4 channels with 9-phenanthrol leads to DSM cell hyperpolarization, thus reducing the excitability and contractility of DSM cells. This study validates the TRPM4 channels as key regulators of human DSM excitability and contractility.1

MATERIALS AND METHODS

Human DSM tissue collection. Human DSM tissue samples were obtained from donor patients undergoing open bladder surgeries, as previously described (22, 23). All procedures involving human tissue collection were reviewed and approved by the Institutional Review Board of the Medical University of South Carolina (protocol no. Pro00045202). Thirty-six patients (23 men and 13 women; 30 Caucasian, 4 African-American, and 2 other; 67.3 ± 1.3 (range 51–82) yr of age) without OAB symptoms and American Urological Association symptom score <8 were used in this study. Two types of DSM samples were collected from each patient. The first sample was stored in ice-cold Ca2+-free HEPES-buffered dissection solution (see Solutions and drugs) and used to conduct patch-clamp, Western blot, immunocytochemistry, immunohistochemistry, and functional studies on DSM contractility. The second sample was stored in RNAlater solution (Qiagen, Hilden, Germany) and used for RT-PCR experiments. The samples were immediately transported to the laboratory after surgery and further processed for DSM single-cell isolation and functional studies on DSM contractility.

Human DSM single-cell isolation. Human DSM single cells were enzymatically isolated as previously described (22, 23). The freshly isolated DSM cells were used for patch-clamp recordings, single-cell type RT-PCR, and immunocytochemistry within 12 h after isolation. RNA extraction, RT-PCR, and sequencing. The RNeasy Mini Kit (Qiagen) was used to extract total RNA from human DSM whole tissue, and a pool of enzymatically freshly isolated DSM single cells was extracted as previously described (23). The extracted total RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo(dT) primers. The TRPM4 channel-specific primer pair sequences were designed on the basis of the cDNA complete sequences of human genes in GenBank and synthesized by Integrated DNA Technologies (Coralville, IA). The forward and reverse primers were 5’-ACCTCAGGAAATTCGAGAAGGGA-3’ and 5’-GGAACCGCGCGTAGG-3’, respectively. The cDNA product was amplified in the presence of GoTaq Green Master Mix (Promega) and specific primers for the TRPM4 channel using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). The cDNA products from human DSM whole tissue, brain tissue, prostate tissue, and isolated DSM cells were heated for 5 min at 95°C and then amplified by 40 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 30 s) followed by a 5-min extension at 72°C. PCR products were loaded onto a 2% agarose-telium-bromide-stained gel and allowed to migrate via electrophoresis. Total RNA samples, extracted from human brain and prostate, were purchased from Clontech Laboratories (Mountain View, CA) and used as positive controls. Negative control experiments were performed in the absence of reverse transcriptase. PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma, St. Louis, MO) and sequenced at the University of South Carolina Environmental Genomic Core Facility for sequence confirmation.

Western blotting. Western blot experiments were conducted on freshly isolated mucosa-free human DSM tissues as previously described (23, 44, 45). Briefly, ~50 μg of total protein extracted from human DSM tissue were loaded into adjacent lanes, subjected to 7.5% precast SDS-PAGE for 50 min at 150 V, and transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) in transfer buffer using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) for 110 min at 75 V. The membrane was blocked with 5% dry milk-Tris-buffered saline-Tween 20 buffer for 1 h at room temperature and then incubated with the primary antibody anti-TRPM4 (1:200 dilution; catalog no. ACC-044, Alomone Labs, Jerusalem, Israel) overnight at 4°C. The membrane was washed four times with Tris-buffered saline-Tween 20 and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000 dilution; Jackson ImmunoResearch, West Grove, PA) in blocking buffer for 1 h at room temperature. Bound antibodies were detected by a Pierce enhanced chemiluminescence Western blotting substrate kit (Thermo Fisher Scientific, Fair Lawn, NJ) according to the manufacturer’s instructions. The antibody specificity was verified by preincubation of the antibody with the respective competing peptide. Human embryonic kidney (HEK-293) cell lysate (TRPM4 overexpression lysate; catalog no. NBL1-17336, Novus Biologicals, Littleton, CO) was used as a positive control.

Immunohistochemistry and immunocytochemistry. Immunohistochemistry and immunocytochemistry were carried out using mucosa-free human DSM tissues or freshly isolated human DSM single cells, as previously described (44, 45). Human DSM whole tissue, fixed with 4% paraformaldehyde, was sliced into 120-μm-thick sections using a tissue slicer (model G, Vibratome, Oxford Laboratories, Foster City, CA). Tissue sections or DSM single cells were stained with primary antibodies specific for TRPM4 (1:250 dilution; catalog no. ACC-044, Alomone Labs) and α-smooth muscle actin (1:100 dilution; catalog no. ab21027, Abcam). Nuclei were stained with 4’,6-diamidino-2-phenylindole (1:5,000 dilution). Secondary antibodies were tagged with Cy3-conjugated anti-rabbit IgG (1:200 dilution; Jackson ImmunoResearch) to stain for TRPM4 and Alexa Fluor 488 donkey anti-goat IgG (1:100 dilution; Life Technologies) to stain for α-smooth muscle actin. Tissue sections or single cells were mounted with 1,4-diazabicyclo[2.2.2]octane and visualized under a confocal microscope (LSM 700 META, Carl Zeiss, Oberkochen, Germany) with a ×63 oil-immersion objective.

Patch-clamp recordings. The amphotericin B-perforated whole cell patch-clamp technique was used in all electrophysiological recordings. Transient inward cationic currents (TICCs) and voltage step-induced whole cell TRPM4 currents were recorded in voltage-clamp...
mode, as previously described (39, 44, 45). Briefly, TICCs were recorded at a holding potential of -70 mV (corrected for the junction potential), and the currents were analyzed as total open channel probability (NP), before and after the addition of 9-phenanthrol. Voltage step-induced whole cell currents were recorded in Cs⁺-containing pipette and bath solutions in the presence of 10 mM tetraethylammonium chloride. Human DSM cells were held at -74 mV (corrected for junction potential), and then 400-ms steps from -94 to +96 mV in 10-mV increments were applied. Membrane potential of human DSM cells was recorded in current-clamp mode (current = 0). An Axopatch 200B amplifier (Digidata 1322A) and pCLAMP 10.2 software (Molecular Devices, Union City, CA) were used, and the currents were filtered using an eight-pole Bessel filter (model 900CT/9L8L, Frequency Devices, Ottawa, IL). The patch-clamp pipettes were made from borosilicate glass (Sutter Instruments, Novato, CA) and pulled using a vertical puller (model PP-830, Narishige, Tokyo, Japan). The pipettes were then polished with a fire polisher (Micro Forge MF-830, Narishige). All patch-clamp experiments were conducted at room temperature (22–23°C).

Isometric DSM tension recordings. Isometric DSM tension was recorded as previously described (22, 23, 44, 45). DSM strips were separated into three experimental groups. In the first group, DSM strips exhibiting spontaneous phasic contractions were allowed to stabilize for ≥30 min before application of 30 μM 9-phenanthrol. In the second group, DSM contractions were induced by the cholinergic agonist carbachol (0.1 μM) and allowed to reach a stable level before application of 9-phenanthrol (30 μM). To minimize the potential effects of neurotransmitter release, the DSM strips exhibiting spontaneous phasic contractions and carbachol-induced contractions were treated in the presence of 1 μM tetrodotoxin, a selective blocker of neuronal voltage-gated Na⁺ channels. In the third group, nerve-evoked contractions were induced by electrical field stimulation (EFS) using a pair of platinum electrodes mounted in the tissue bath parallel to the DSM strip. The EFS pulses were generated using a PHM-152I stimulator (Med Associates, Georgia, VT); the EFS pulse parameters were 0.75-ms pulse width, 20-V pulse amplitude, and 3-s stimulus duration, with polarity reversed for alternating pulses. DSM strips were subjected to continuous repetitive stimulation with a frequency of 10 Hz at 1-min intervals or increasing frequencies from 3.5 to 50 Hz at 3-min intervals. Isometric tension was recorded at 37°C using the Myomed myograph system (Med Associates).

Solutions and drugs. For the functional studies on human DSM contractility, the physiological saline solution consisted of (in mM) 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄, and 11 d-glucose, aerated with 95% O₂-5% CO₂ (pH 7.4). The composition of Cs⁺-free dissection solution was (in mM) 80 monosodium glutamate, 55 NaCl, 6 KCl, 2 MgCl₂, 10 HEPES, and 10 d-glucose, with pH adjusted to 7.3 with NaOH. The extracellular (bath) solution used in the patch-clamp experiments in the gap-free mode (voltage and current clamp) contained (in mM) 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 d-glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH. The patch-clamp pipette solution for these experiments contained (in mM) 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, with pH adjusted to 7.2 with NaOH, and was supplemented with freshly dissolved (every 1–2 h) amphotericin B (200 μg/ml) in dimethyl sulfoxide (DMSO). The extracellular (bath) solution used in the voltage step-induced whole cell patch-clamp experiments contained (in mM) 10 tetraethylammonium chloride, 6 CsCl, 124 NaCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 d-glucose, with pH adjusted to 7.3 with CsOH. The composition of Cs⁺-containing pipette solution was (in mM) 110 CsOH, 110 aspartic acid, 10 NaCl, 1 MgCl₂, 10 HEPES, 0.05 EGTA, and 30 CsCl, with pH adjusted to 7.2 with CsOH, and was supplemented with freshly dissolved (every 1–2 h) amphotericin B (200 μg/ml) in DMSO. BSA was obtained from Thermo Fisher Scientific (Fair Lawn, NJ). All other compounds were obtained from Sigma-Aldrich. 9-Phenanthrol and carbachol were dissolved in double-distilled water. The final concentration of DMSO in the bath solutions did not exceed 0.05%.

Data analysis and statistics. MiniAnalysis software (SynaptoSoft, Decatur, GA) was used to analyze the DSM phasic contraction parameters. The DSM contractile activity was quantified from measurement of average phasic contraction amplitude (the difference between the force-time baseline curve and the maximum peak of the contractions), frequency (contractions/min), muscle force integral (calculated by integrating the area under the force-time baseline curve), phasic contraction duration (defined as width of the individual phasic contraction at 50% of the amplitude), and DSM tone (the difference between the zero line and the force-time baseline curve). Statistical analyses were performed with Prism 4.03 software (GraphPad, La Jolla, CA), and CorelDRAW Graphics Suite X3 software (Corel, Ottawa, ON, Canada) was used to illustrate the data. For the EFS-induced contractions, the contraction amplitude at the EFS frequency under control conditions was taken to be 100%, and the data were normalized. For evaluation of the effect of cumulative addition of 9-phenanthrol (0.1–30 μM) on 10-Hz EFS-induced contractions, the last 5 min before addition of each concentration to the bath were analyzed. The TICCs were analyzed as NP, as previously described (39, 44). In voltage step experiments, the last 200-ms interval was analyzed and averaged before, after, and following washout of 9-phenanthrol. Five minutes of ≥10 min of stable current-clamp recordings prior to application of 9-phenanthrol were analyzed for control data, and the last 5 min of each 10-min continuous recording after application of 9-phenanthrol were analyzed to evaluate the effect of 9-phenanthrol on the membrane potential. Data are summarized as means ± SE for the number of DSM strips or cells (n) isolated from the number of patients (N). Data were compared using a two-way ANOVA followed by Bonferroni’s post test or paired or unpaired Student’s t-test where appropriate. P < 0.05 was considered statistically significant.

RESULTS

TRPM4 channel mRNA detection in human DSM. Previously, we demonstrated TRPM4 channel expression in rat and guinea pig DSM (39, 44, 45). Here, we took an additional step...
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TRPM4 CHANNELS AND HUMAN BLADDER FUNCTION

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to elucidate TRPM4 channel expression at the cell and tissue levels in human DSM. Utilizing RT-PCR, we detected mRNA transcripts for TRPM4 channels in DSM whole tissues \( (n = 4, N = 4); \) Fig. 1. Since mRNA transcripts in DSM whole tissue may not have originated from only DSM cells, but may also have originated from other cell types in the detrusor muscle layer (such as neurons, interstitial cells, fibroblasts, and vascular and endothelial cells), additional RT-PCR experiments were carried out on RNA samples collected only from freshly isolated human DSM cells. The single-cell RT-PCR experiments on isolated DSM cells, which avoid plausible additional sources of mRNA from non-DSM cells, clearly confirmed the presence of TRPM4 channel mRNA directly in human DSM cells \( (n = 4, N = 4); \) Fig. 1. Negative control experiments performed in the absence of the reverse transcriptase enzyme did not detect the band, indicating an absence of genomic DNA contamination (Fig. 1).

TRPM4 channel protein expression at the protein level in human DSM. To further investigate if the TRPM4 channels are expressed at the protein level in native human DSM tissue, we performed Western blot experiments using a TRPM4 channel-specific antibody (see Materials and Methods). A \( \sim 134\)-kDa band, consistent with the expected molecular mass of the TRPM4 channel protein, was detected (Fig. 2A). In control experiments, preabsorption of the primary antibody with its antigenic competing peptide resulted in the loss of the protein band, thereby indicating the specificity of the antibody for its intended epitope \( (n = 4, N = 4); \) Fig. 2A. The slightly different bands might be due to posttranslational modifications of native human DSM tissue and HEK-293 cells (Fig. 2A). Thus the Western blot experiments confirmed the presence of the TRPM4 channel protein in human DSM tissue.

Immunohistochemistry with confocal microscopy also detected the expression and localization of TRPM4 channel protein in human DSM whole tissue (Fig. 2B), further confirming the results from the Western blot experiments. Our data reveal that TRPM4 channels are expressed in DSM cells, as evidenced by our findings that TRPM4 channels and \( \alpha \)-smooth muscle actin are colocalized spatially within the same cell.

To determine further whether DSM cells express TRPM4 channel proteins, immunocytochemistry was carried out with TRPM4 channel-specific antibodies. As illustrated in Fig. 2C, colocalization with TRPM4 channel-specific antibodies and the \( \alpha \)-smooth muscle actin marker revealed the expression of TRPM4 channel protein in DSM single cells. Control experiments were further conducted by preabsorption of the primary antibody with its antigenic competing peptide to verify the specificity of the antibody for its intended epitope (Fig. 2, D–E).

**Pharmacological inhibition of TRPM4 channels with 9-phenanthrol decreases TICC activity in freshly isolated human DSM cells.** The role of TRPM4 channels in human DSM cell excitability was investigated using the amphotericin B-perforated patch-clamp technique and the novel TRPM4 channel inhibitor 9-phenanthrol (18). The average cell capacitance of all human DSM cells used in this study was 21.9 \( \pm \) 2.0 pF \( (n = 37, N = 19) \). At the holding potential of \(-70 \) mV, human DSM cells exhibited TICCs (Fig. 3A). Application of 30 \( \mu \)M 9-phenanthrol decreased TICC activity by \(-50\% \) from \( N_P \) of 2.7 \( \pm \) 0.6 to 1.3 \( \pm \) 0.4 \( (n = 15, N = 9, P < 0.05); \) Fig. 3B. These results support the novel concept that TRPM4 channels are functionally active in human DSM. By regulating TICC activity, TRPM4 channels are key determinants of human DSM excitability.

**Pharmacological inhibition of TRPM4 channels with 9-phenanthrol decreases the amplitude of voltage step-induced whole cell currents in human DSM cells.** To further investigate the role of TRPM4 channels in human DSM excitability, we measured the current-voltage relationship of the cationic current in human DSM isolated cells at \(-94 \) to \(+96 \) mV in 10-mV increments for 400 ms, as previously described (44). Between steps, the holding potential was \(-74 \) mV (corrected for junction potential). In this experimental series, \( K^+ \) channel currents were blocked by 10 mM extracellular tetroethylammonium and
Cs⁺-containing solution (see Solutions and drugs). 9-Phenanthrol (30 μM) significantly inhibited the amplitude of the voltage step-induced whole cell currents in human DSM cells, and the effect was reversed by washout with 9-phenanthrol-free extracellular bath solution (n = 7, N = 7, P < 0.05; Fig. 4). For the depolarization step to +96 mV, 9-phenanthrol inhibited the outward current by 43.4 ± 8.2% (n = 7, N = 7, P < 0.05; Fig. 4). These results reveal the inhibitory effect of 9-phenanthrol on the whole cell currents in human DSM cells, providing further insight into TRPM4 channel properties in human DSM cells.

**TRPM4 channels regulate the membrane potential in human DSM cells.** The role of TRPM4 channels in controlling the human DSM cell resting membrane potential was investigated using the current-clamp mode of the patch-clamp technique. 9-Phenanthrol (30 μM) significantly hyperpolarized the membrane potential in human DSM cells by 24.3 ± 7.7 mV (n = 4, N = 3, P < 0.05; Fig. 5). Under control conditions, the DSM cell membrane potential was -22.7 ± 6.1 mV, which was hyperpolarized to -46.9 ± 11.9 mV after application of 9-phenanthrol (n = 4, N = 3, P < 0.05; Fig. 6). These results strongly support a key functional role for the TRPM4 channels in regulating the resting membrane potential in human DSM cells.

**The TRPM4 channel inhibitor 9-phenanthrol significantly decreases spontaneous phasic and tonic contractions in human DSM isolated strips.** In this experimental series, in vitro functional studies were carried out to elucidate the effects of the TRPM4 channel inhibitor 9-phenanthrol on spontaneous contractile activity of human DSM isolated strips. 9-Phenanthrol (30 μM) significantly decreased the spontaneous phasic contraction amplitude by 99.3 ± 0.7%, muscle force integral by 99.4 ± 0.5%, contraction duration by 94.2 ± 5.8%, contraction frequency by 97.3 ± 2.7%, and muscle tone by 64.8 ± 8.7% (n = 11, N = 5, P < 0.005; Fig. 6). These results suggest that TRPM4 channels are key regulators of human DSM spontaneous phasic and tonic contractions under physiological conditions.

**The TRPM4 channel inhibitor 9-phenanthrol attenuates carbachol-induced contractions in human DSM isolated strips.** Initiation of bladder voiding contractions is associated with activation of parasympathetic nerves releasing acetylcholine, which subsequently activates muscarinic receptors in DSM cells (2). To study the role of the TRPM4 channels under...
conditions of muscarinic receptor stimulation, we performed functional studies on human DSM contractility in the presence of the muscarinic receptor agonist carbachol. Specifically, we sought to determine the effects of 9-phenanthrol on carbachol-induced contractions in human DSM isolated strips. The muscarinic receptor agonist carbachol (0.1 M) rapidly increased the spontaneous phasic contractions of DSM strips (Fig. 7A). 9-Phenanthrol (30 μM) significantly decreased the amplitude of carbachol-induced contraction by 45.7% ± 7.7%, muscle force integral by 89.6% ± 2.9%, contraction duration by 64.1% ± 8.8%, contraction frequency by 68.9% ± 7.7%, and muscle tone by 56.9% ± 4.6% (n = 17, N = 5, P < 0.005; Fig. 7B).

The TRPM4 channel inhibitor 9-phenanthrol reduces nerve-evoked contractions in human DSM isolated strips. During bladder voiding, activation of parasympathetic nerves causes release of the excitatory neurotransmitter acetylcholine, which induces forceful DSM contractions (2). In this experimental series, we sought to explore how the cumulative addition of 9-phenanthrol (0.1–30 μM) reduces DSM nerve-evoked contractions induced by continuous 10-Hz EFS in the absence of tetrodotoxin. A representative myograph recording in Fig. 8A illustrates that the cumulative addition of 9-phenanthrol (0.1–30 μM) reduced the 10-Hz EFS-induced contractions in human DSM isolated strips. Figure 8B illustrates the concentration responses and demonstrates significant inhibitory effects of 9-phenanthrol on nerve-evoked contraction amplitude and muscle force integral, with IC50 values and maximum inhibition of 3.3 (95% confidence interval = 1.1–10.1) μM and 40.5 ± 5.4% and 3.5 (95% confidence interval = 1.3–9.1) μM and 27.3 ± 7.1%, respectively (n = 16, N = 11, P < 0.05; Fig. 8).

In the next series of experiments, human DSM isolated strips were stimulated by increasing EFS frequencies (3.5–50 Hz). After a frequency-response curve under control conditions,
DSM strips were incubated with 30 μM 9-phenanthrol and a second frequency-response curve was generated. 9-Phenanthrol significantly decreased the contraction amplitude generated in response to EFS (n = 22, N = 16, P < 0.05; Fig. 9A). At the highest frequency of 50 Hz, 9-phenanthrol (30 μM) inhibited EFS-induced contraction amplitude in human DSM isolated strips by 30.3 ± 3.7% compared with the control responses (Fig. 9B). This experimental series further indicates that inhibition of TRPM4 channels reduces nerve-evoked contractions of human DSM isolated strips to a lesser extent than spontaneous phasic and tonic contractions (Fig. 7).

**DISCUSSION**

This study used clinically characterized human DSM tissues and isolated DSM cells obtained from donor patients without a preoperative history of OAB to investigate the functional role of TRPM4 channels in the human urinary bladder under physiological conditions. The data revealed novel findings that TRPM4 channels are important and physiologically relevant regulators of human DSM.

The physiological role of the TRPM4 channels is facilitation of membrane depolarization via nonselective influx of monovalent cations through the cell membrane (11). Given the greater driving force for Na⁺ than K⁺ under physiological conditions, pharmacological inhibition of TRPM4 channels can reduce the net Na⁺ influx to promote membrane potential hyperpolarization (15, 16). On the other hand, activation of TRPM4 channels would favor an increase in Na⁺ influx, causing membrane depolarization (11). Therefore, unlike K⁺ channels, which are responsible primarily for membrane hyperpolarization, TRPM4 channels contribute to the depolarization component of the ionic currents in excitable cells (15, 17, 29, 30).

Recently, TRPM4 channels have been identified in rat and guinea pig DSM, suggesting important roles for these channels in the rodent urinary bladder (39, 44, 45, 47). However, there was a lack of knowledge regarding the TRPM4 channel expression and function in human DSM (25). Since humans are the primary species of interest for therapeutic intervention, investigations on human DSM are critically important to validate previous findings in animal studies and to fully characterize TRPM4 channel properties in humans while determining key interspecies differences. Here, we revealed TRPM4 channel expression in human DSM by utilizing a combination of molecular biological techniques, which demonstrated TRPM4 channel expression in human DSM whole tissue and single cells. Our single-cell RT-PCR, Western blot, immunohistochemistry, and immunocytochemistry with confocal microscopy experiments (Figs. 1 and 2) provided the first direct evidence for TRPM4 channel expression at mRNA and protein levels in human DSM cells and further demonstrated TRPM4 channel localization at the DSM cell membrane.

The abundant TRPM4 channel protein expression in human DSM may infer an important functional role for these channels in the human bladder. In rat and guinea pig DSM cells, inhibition of the TRPM4 channels with 9-phenanthrol attenuated TICC activity, thereby decreasing intracellular Ca²⁺ levels to promote DSM relaxation (39, 44, 45). The TRPM4 channel inhibitor 9-phenanthrol reduced cellular excitability to a much larger extent in human than rodent DSM (Fig. 10A–D), indicating important species differences. The voltage-clamp recordings revealed that 9-phenanthrol significantly attenuated TICC activity at ~70 mV (Fig. 3) and inhibited voltage step-induced whole cell outward currents in human DSM cells (Fig. 4). Furthermore, 9-phenanthrol substantially hyperpolarized the human DSM membrane potential by ~24 mV (Fig. 5). The combined results from our patch-clamp studies (Figs. 3–5) support the concept that TRPM4 channels are functionally active in human DSM and play a key role in controlling human DSM excitability.

Hyperpolarization of DSM cell membrane potential promotes inhibition of L-type Cav channels and leads to DSM...
relaxation (41). Previous studies on DSM isolated from guinea pigs and rats showed that inhibition of TRPM4 channels with 9-phenanthrol reduced the intracellular Ca\(^{2+}\) levels in DSM cells to induce DSM relaxation (44, 45). We were intrigued by our finding of a higher degree of 9-phenanthrol-induced attenuation of the spontaneous phasic contraction amplitude, muscle force integral, duration, frequency, and tone of human DSM isolated strips (Fig. 6) than inhibition of DSM contractility in rats and guinea pigs (44, 45) (Fig. 10B). Furthermore, inhibition of the TRPM4 channels with 9-phenanthrol attenuated carbachol-induced (Fig. 7) and nerve-evoked (Figs. 8 and 9) contractions in human DSM isolated strips. Consistent with previous findings in rodents (39, 44, 45), our functional studies clearly support a key regulatory role of TRPM4 channels in human DSM contractility. One important observation is that 9-phenanthrol more effectively inhibited the spontaneous phasic DSM contractions than nerve-evoked DSM contractions, suggesting that TRPM4 channel inhibition may effectively control the nonvoiding contractions associated with detrusor overactivity while having little or no effect on normal voiding.

To investigate the functional role of TRPM4 channels in human DSM, we used 9-phenanthrol as a selective pharmacological inhibitor. This agent has been successfully used to study TRPM4 channel activity in various preparations (18), including rodent DSM (39, 44, 45). However, recent studies indicated that 9-phenanthrol inhibits transmembrane protein 16A (TMEM16A) channels (5) in vascular smooth muscle and activates intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) (IK) channels (12) in endothelial cells. These nonspecific effects of 9-phenanthrol raised an important question about the plausible involvement of TMEM16A and/or IK channels in the inhibitory effects of 9-phenanthrol on human DSM. Theoretically, 9-phenanthrol could affect TMEM16A and IK channel activity and, thus, reduce human DSM excitability and contractility. However, although we cannot completely exclude this possibility, our experimental data support the concept that the majority of the inhibitory effects of 9-phenanthrol in human DSM are mediated by TRPM4 channel inhibition. The substantial hyperpolarizing effect of 9-phenanthrol on the DSM membrane potential (~24 mV; Fig. 5) is consistent with a relatively large ionic gradient for Na\(^{+}\) (\(E_{\text{Na}} = +65 \text{ mV}\)) in our experimental conditions. This significant shift (Fig. 5) could be explained by TRPM4 channel inhibition by 9-phenanthrol and subsequent reduction of Na\(^{+}\) conductance, but not by TMEM16A channel inhibition. TMEM16A channels are Ca\(^{2+}\)-activated Cl\(^{-}\) channels, contributing to Cl\(^{-}\) conductance in smooth muscle cells (4). Because of the relatively small driving force for Cl\(^{-}\) (\(E_{\text{Cl}} = -32 \text{ mV}\)), with the assumption that pipette Cl\(^{-}\) replaces all the intracellular Cl\(^{-}\) under our experimental conditions, we expect that inhibition of TMEM16A channels would cause only a minor, if any, shift in DSM resting membrane potential. However, the physiological role of TMEM16A channels in human DSM is largely unknown, and clarification of these important issues requires further investigation. On the other hand, it is very unlikely that 9-phenanthrol inhibitory effects are mediated by IK channel activation, since previous studies reported no functional role of IK channels in the bladder (1, 38). Most importantly, our molecular biology data clearly demonstrate an abundant TRPM4 channel mRNA and protein expression in human DSM cells (Figs. 1 and 2). Therefore, our data support the concept that the majority of 9-phenanthrol inhibitory effects in human DSM are caused by TRPM4 channel inhibition.

In the present study, we demonstrated significant species-related differences in TRPM4 channel activity in human DSM compared with rat (45) and guinea pig (44) DSM (Fig. 10). Pharmacological inhibition of TRPM4 channels with 9-phenanthrol leads to a larger decrease in human than rodent DSM excitability and contractility. Figure 10A illustrates that the whole cell 9-phenanthrol-sensitive current is much higher in human than guinea pig DSM cells. We also compared the effects of 9-phenanthrol on the spontaneous phasic contractions in human, guinea pig, and rat DSM isolated strips. Consistently, the data analysis revealed a greater inhibitory effect of 9-phenanthrol on DSM contractility in human than guinea pig and rat DSM isolated strips (Fig. 10B). However, no significant differences were noted between rats and guinea pigs (Fig. 10B). These intriguing results indicate important inter-
species-related differences and reveal that TRPM4 channels have a more prominent physiological role in the regulation of DSM function in humans than rodents (Fig. 10).

A critical step for development of novel, safe, and more effective therapies for OAB is a better understanding of the mechanisms that control DSM excitability and contractility in OAB patients. Abnormal functionality of TRPM4 channels could result in substantial pathological changes in DSM and lead to OAB and associated detrusor overactivity. Dysfunction in expression and activity of certain ion channels or their regulatory mechanisms may affect DSM contractility and result in OAB (40, 41). However, the physiological role of TRPM4 channels in OAB etiology requires future investigations on DSM tissue and cells obtained from patients with confirmed OAB symptoms.

Given the prominent role of the TRPM4 channels in human DSM excitability and contractility, these channels may represent novel therapeutic targets for pharmacological or genetic treatment of OAB. The challenge of TRPM4 channel expression in other tissues (32) is not unique to therapeutic targets affecting lower urinary tract function, and, fortunately, this obstacle has been pharmacologically overcome in a number of disease states. Considering the relatively higher TRPM4 channel mRNA expression in DSM than vasculature (39), as well as the important functional roles of TRPM4 channels in DSM, pharmacological manipulation of TRPM4 channels in OAB patients may provide effective treatment with minimal adverse collateral cardiovascular effects. The potential clinical application of TRPM4 channel modulation for OAB therapy should be considered as a novel approach for OAB management and further validated in clinical trials.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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C611

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