Novel role for the transient potential receptor melastatin 4 channel in guinea pig detrusor smooth muscle physiology

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Smith AC, Hristov KL, Cheng Q, Xin W, Parajuli SP, Earley S, Malysz J, Petkov GV. Novel role for the transient potential receptor melastatin 4 channel in guinea pig detrusor smooth muscle physiology. Am J Physiol Cell Physiol 304: C467–C477, 2013. First published January 9, 2013; doi:10.1152/ajpcell.00169.2012.—Members of the transient receptor potential (TRP) channel superfamily, including the Ca2+-activated monovalent cation-selective TRP melastatin 4 (TRPM4) channel, have been recently identified in the urinary bladder. However, their expression and function at the level of detrusor smooth muscle (DSM) remain largely unexplored. In this study, for the first time we investigated the role of TRPM4 channels in guinea pig DSM excitation-contraction coupling using a multidisciplinary approach encompassing protein detection, electrophysiology, live-cell Ca2+ imaging, DSM contractility, and 9-phenanthrol, a recently characterized selective inhibitor of the TRPM4 channel. Western blot and immunocytochemistry experiments demonstrated the expression of the TRPM4 channel in whole DSM tissue and freshly isolated DSM cells with specific localization on the plasma membrane. Perforated whole cell patch-clamp recordings and real-time Ca2+ imaging experiments with fura 2-AM, both using freshly isolated DSM cells, revealed that 9-phenanthrol (30 μM) significantly reduced the cation current and decreased intracellular Ca2+ levels. 9-Phenanthrol (0.1–30 μM) significantly inhibited spontaneous, 0.1 μM carbachol-induced, 20 mM KCl-induced, and nerve-evoked contractions in guinea pig DSM-isolated strips with IC50 values of 1–7 μM and 70–80% maximum inhibition. 9-Phenanthrol also reduced nerve-evoked contraction amplitude induced by continuous repetitive electrical field stimulation of 10-Hz frequency and shifted the frequency-response curve (0.5–50 Hz) relative to the control. Collectively, our data demonstrate the novel finding that TRPM4 channels are expressed in guinea pig DSM and reveal their critical role in the regulation of guinea pig DSM excitation-contraction coupling.

9-phenanthrol; TRP channel; urinary bladder; patch-clamp

Overactive bladder (OAB) is characterized with symptoms that include urgency, frequency, and nocturia, with or without incontinence (1). Among factors contributing to OAB, the most common factor is detrusor overactivity, which is caused by altered myogenic or neurogenic factors affecting detrusor smooth muscle (DSM) contractility (8, 14). The primary pharmacotherapy for OAB aimed at reducing detrusor overactivity is based on antimuscarinics; however, the reports of their clinical use demonstrate a lack of efficacy and many adverse side effects including dry mouth, tachycardia, ataxia, constipation, and blurred vision (3, 4, 6). Therefore, the current research efforts, directed towards identifying alternative pathways with the potential to reduce DSM contractility, hold promise for validating novel therapeutic targets such as ion channels for the treatment of OAB.

The mechanisms by which ion channels regulate DSM function have not yet been completely elucidated. This lack of knowledge hinders the efforts aimed at identifying suitable ion channel modulators and suitable targets for bladder disorders. Studies so far have shown that the resting membrane potential and contractility of DSM cells are regulated by various types of K+, Ca2+, and nonselective Na+ permeable cation channels (4, 6, 22, 31, 32, 34, 38). The activation of nonselective cation channels leads to depolarization of the cell membrane, activation of L-type voltage-dependent Ca2+ channels (VDCC), an increase in intracellular Ca2+ concentration, and subsequent contraction of the DSM. Blockade of these cation channels or activation of K+ channels has the opposite effect causing DSM relaxation (32, 38). The depolarized membrane potential and increased DSM excitability, hence, contribute to the enhanced DSM contractility.

Several members of the transient receptor potential (TRP) superfamily of cation channels have been identified in the urinary bladder (5). Most of these channels, however, show expression in the urethra or afferent sensory neurons, as demonstrated by molecular biology and immunostaining methods (5, 11). One such member is the transient receptor potential melastatin 4 (TRPM4) channel (42). Its functional role and physiological significance in the urethra remain unknown, and its expression and function in DSM have never been studied. In contrast, the function of the TRPM4 channel has been extensively studied in non-DSM myocytes and reports have identified them as important mediators of vascular and colonic smooth muscle cell membrane potential and contractility (9, 10, 17). The TRPM4 channel is a Ca2+-activated cation channel and highly selective for monovalent cations such as Na+ and K+ but impermeable to anions and divalent cations including Ca2+ (19). TRPM4 channels exhibit Ca2+ dependency, monovalent cation selectivity with the rank order of Na+ > K+ > Cs+ > Li+, single-channel conductance of ~25 pS, and voltage dependency (18, 29, 30, 39, 41). Activation of the TRPM4 channels is thought to lead to cell depolarization via the net entry of Na+ into the cell, which in turn enhances the activity of VDCC favoring Ca2+ entry, and thus modulating Ca2+ signaling (12). Further investigation into the regulation of the TRPM4 channels and how they influence smooth muscle function has been hampered so far by the lack of selective pharmacological modulators. In a recent study, it was demonstrated that the hydroxytrycyclic compound 9-hydroxyphenanthrene (9-phenanthrol) selec-
tively inhibited human TRPM4 channel but not its close family member the TRPM5 cationic channels or the cystic fibrosis transmembrane conductance regulator chloride channels (18). In human embryonic kidney-293 (HEK-293) cells expressing recombinant human TRPM4 channels, 9-phenanthrol effectively blocked these channels with the potency of ~15–20 μM (IC50 value) determined using conventional whole cell patch-clamp and single-channel recordings from excised patches (18). In freshly isolated cerebral artery myocytes, 9-phenanthrol (30 μM) did not alter the activity of the canonical (C) TRP channels TRPC3 and TRPC6, voltage-gated K+ (Kv), large conductance Ca2+/activated K+ (BK), inwardly rectifying K+ (Kir), and VDCC (17). 9-phenanthrol was also shown to have no effect on TRPM7 currents in mouse interstitial cells of Cajal (26). Furthermore, 9-phenanthrol has been shown to block TRPM4 currents in cerebral blood vessels and evoke substantial hyperpolarization of vascular smooth muscle cell membrane potential and dilation of arteries with myogenic tone (17). In addition, 9-phenanthrol produced an antiarrhythmic effect in murine ventricle by reducing the frequency of early afterdepolarizations when applied for 10 min at a concentration of 10 μM; this effect was attributed to TRPM4 channels (36). Collectively, these findings confirm that 9-phenanthrol is an important novel pharmacological tool that could be used to investigate the TRPM4 channel physiological role in DSM excitation-contraction coupling.

Here, we examined the expression of TRPM4 channels in guinea pig DSM and revealed their functional role using the TRPM4 channel-selective inhibitor 9-phenanthrol. To achieve this aim, we applied a multidisciplinary approach using Western blot, immunocytochemistry, live-cell Ca2+ imaging, patch-clamp electrophysiology, and isometric DSM tension recordings of guinea pig DSM-isolated strips.

MATERIALS AND METHODS

**Tissue preparation and single DSM cell isolation.** Fifty-three male Harley Albino guinea pigs (Charles River Laboratory, Raleigh, NC), with an average weight of 424.6 ± 11.0 g were used in this study. All animals were kept under standard laboratory conditions (12:12-h light/dark). Guinea pigs were euthanized with CO2 followed by a thoracotomy according to the protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of South Carolina (Animal Use Protocol No. 1747). The dissection of guinea pig DSM, preparation of tissue strips for contractility studies, and isolation of fresh DSM single cells followed the methods previously described by our group (2, 23, 31, 40).

**Western blot.** Guinea pig DSM-isolated strips (30–50 mg) were placed in TBS and sonicated using a model 100 dismembrator (Thermo Fisher Scientific, West Grove, PA) overnight in the dark at 4°C. After being labeled, cells were washed three times for 15 min with 1% BSA/PBS and were again blocked and permeabilized in 1% BSA/PBS containing 5% normal donkey serum for 10 min. DSM cells were then incubated with the rabbit polyclonal primary antibody anti-TRPM4 (1:500 dilution, ab63080; Abcam) diluted in 1% BSA/PBS at 37°C for 1 h. After incubation with the primary antibody, cells were rinsed two times for 15 min with 1% BSA/PBS and were again blocked and permeabilized in 1% BSA/PBS containing 5% normal donkey serum for 10 min. DSM cells were then labeled with secondary antibody Cy3-conjugated anti-rabbit IgG at 1:500, 1% BSA/PBS; Jackson ImmunoResearch, West Grove, PA) overnight in the dark at 4°C. After being labeled, cells were washed three times for 15 min with 1% BSA/PBS and then washed with PBS for 15 min. After being washed, the cells were incubated with Alexa Fluor 488 phalloidin dye (diluted in PBS, 1:50) for 2 h in the dark. After incubation with Alexa Fluor 488 phalloidin, DSM cells were then washed two more times with PBS and incubated with 4',6-diamidino-2-phenylindole (diluted in PBS 1:5,000) for 15 min and washed again with PBS. DABCO was placed onto the slides and the slides were coverslipped. Images at ×63 oil objective were acquired with a LSM 510 META confocal microscope (Carl Zeiss, Göttingen, Germany). The cells for each group were imaged with the same laser power, gain settings, and pinhole for the controls and antibody treatment.

**Patch-clamp recordings.** We employed the amphotericin-B-perforated whole cell configuration of the patch-clamp technique, which preserves the native environment and the signal transduction pathways of the cells. TRPM4 currents were recorded using two different protocols. The first protocol included the pipette and bath solutions used followed the method described previously (2, 7, 24, 31, 33). The second set of experiments were that we recorded the transient inward currents (TICCs) at a holding potential of -70 mV (corrected for the junction potential), and the currents were analyzed as open channel probability (NPo) before, after the addition of 9-phenanthrol, and after washout of 9-phenanthrol. The second protocol involved voltage-step-induced currents recorded in Cs+-containing pipette and bath solutions in the presence of 10 mM tetrodoylamine (TEA; see Solutions and drugs). Between steps, the voltage (corrected for junction potential) was held at -74 mV and stepped for 400 ms from -94 to +96 mV in 10-mV increments.

**Live-cell Ca2+ imaging.** Measurement of DSM cell intracellular Ca2+ levels was performed as previously described (21, 25). 9-phenanthrol was applied in the absence or presence of nifedipine (1 μM) or carbachol (0.1 μM) as indicated.

**Isometric DSM tension recordings.** Isometric DSM tension recordings were conducted as previously described (2, 23, 31). The experimental details are provided in these reports except for the following modifications. In this study, after the equilibration period DSM strips were separated into four experimental groups. The first group consisted of DSM-isolated strips that exhibited substantial spontaneous phasic contractions after the initial stretch of tension following the equilibration period. In the second, third, and fourth groups, DSM contractions were induced by carbachol (0.1 μM), a cholinergic agonist; by KCl, a depolarizing agent (20 mM or 60 mM); or by electrical field stimulation (EFS), respectively. Increasing concentrations of 9-phenanthrol (0.1–30 μM), added cumulatively, were applied at 10-min intervals directly into the bath solution. In an additional experiment, we tested the effects of 30 μM 9-phenanthrol and

incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3,000) in blocking buffer for 1 h at room temperature. Bound antibodies were detected by a Pierce Fast Echom veniaminescence Substrate Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The primary antibody specificity was verified by preincubation of the antibody with a competing peptide.

**Immunocytochemistry.** For immunocytochemical studies, freshly isolated DSM cells were dropped on a poly-L-lysine-coated slide and allowed to settle for 30 min at room temperature. The DSM cells were fixed with 4% paraformaldehyde for 10 min. DSM cells were then washed three times for 15 min each with PBS/0.01 M glycine/0.1% Triton-X solution and blocked and permeabilized in 1% BSA/PBS containing 5% normal donkey serum for 10 min. DSM cells were then incubated with the rabbit polyclonal primary antibody anti-TRPM4 (1:500 dilution, ab63080; Abcam) diluted in 1% BSA/PBS at 37°C for 1 h. After incubation with the primary antibody, cells were rinsed two times for 15 min with 1% BSA/PBS and were again blocked and permeabilized in 1% BSA/PBS containing 5% normal donkey serum for 10 min. DSM cells were then labeled with secondary antibody Cy3-conjugated anti-rabbit IgG at 1:500, 1% BSA/PBS; Jackson ImmunoResearch, West Grove, PA) overnight in the dark at 4°C. After being labeled, cells were washed three times for 15 min with 1% BSA/PBS and then washed with PBS for 15 min. After being washed, the cells were incubated with Alexa Fluor 488 phalloidin dye (diluted in PBS, 1:50) for 2 h in the dark. After incubation with Alexa Fluor 488 phalloidin, DSM cells were then washed two more times with PBS and incubated with 4',6-diamidino-2-phenylindole (diluted in PBS 1:5,000) for 15 min and washed again with PBS. DABCO was placed onto the slides and the slides were coverslipped. Images at ×63 oil objective were acquired with a LSM 510 META confocal microscope (Carl Zeiss, Göttingen, Germany). The cells for each group were imaged with the same laser power, gain settings, and pinhole for the controls and antibody treatment.

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nifedipine (3 μM) on 60 mM KCl-induced DSM tonic contractions. DSM strips were incubated with 60 mM KCl and subsequently washed out after the 60 mM KCl contractions reached a stable steady state. Following the washout of 60 mM KCl, DSM strips were preincubated with either 3 μM nifedipine or 30 μM 9-phenanthrol for 10 min and then stimulated again with 60 mM KCl.

Nerve-evoked contractions were induced by EFS using the following parameters: 0.75-ms pulse width, 20-V pulse amplitude, 3-s stimulus duration, and either increasing frequency of 0.5–50 Hz or continuous 10 Hz as specified in the text; polarity was reversed for alternating pulses.

Statistical analysis. MiniAnalysis software (Synaptosoft, Decatur, GA) was used to analyze the DSM contraction parameters. Specifically, the DSM contractile activity was quantified by measuring the average phasic contraction amplitude (the difference between the force-time baseline curve and the maximum peak of the contractions), the frequency (contractions per minute), muscle force integral (calculated by integrating the area under the force-time baseline curve), the phasic contraction duration (defined as width of the individual phasic contraction at 50% of the amplitude), and the DSM tone (the difference between the baseline and the force-time baseline curve). Statistical analysis was performed with GraphPad Prism 4.03 software (GraphPad Software, La Jolla, CA) and CorelDraw Graphic Suite X3 software (Corel, Ottawa, Canada) was used to illustrate the data. IC50 values were obtained using a sigmoidal fitting function in GraphPad Prism 4.03 software and are reported as means (95% confidence interval). To compare the phasic contraction parameters for spontaneous, 0.1 μM carbachol, or 20 mM KCl-induced contractions, data were normalized to the last 5 min of the contractions before the addition of the first concentration of 9-phenanthrol (taken to be 100%) and expressed as percentages. In the 60 mM KCl-induced DSM contraction experiments, the steady-state of the 60 mM KCl-induced DSM tone (~10-min interval after the addition of 60 mM KCl) was taken to be 100% before the addition of 30 μM 9-phenanthrol or 3 μM nifedipine. For the EFS-induced contractions, the contraction amplitude at EFS frequency of 50 Hz under control conditions was taken to be 100% and the data were normalized. To evaluate the effect of the cumulative concentrations of 9-phenanthrol (0.1–30 μM), the last 5 min before the addition of each concentration to the bath was analyzed. In electrophysiology experiments using K+-containing pipette and Na+ -containing bath solutions, NPo values were analyzed in the absence and presence of 9-phenanthrol as described previously (16, 17). The TICCs activity at ~70 mV was calculated as the sum of the NPo of multiple open states using the build-in single-channel analysis feature of Clampfit software. The single-channel TRPM4 current of 1.75 pA was chosen in the data analysis based on the reported unitary conductance of TRPM4 (25 pS; Ref. 27). In voltage step experiments using Cs+-containing pipette and bath solutions steady-state currents measured as averages over the last 50–200 ms of 400-ms steps were obtained and divided by the cell capacitance for each cell. For normalization purposes in each DSM cell, the responses for each voltages were further normalized to the current density measured at +96 mV before the addition of 9-phenanthrol (taken as the value of 1 for this condition). Fura-2 Ca2+- imaging measurements were expressed as ratios of 340- to 380-nm emission. When we examined the effects of 9-phenanthrol in the absence or presence of nifedipine or carbachol, the change in the ratio was calculated by subtracting the value measured in 9-phenanthrol from that just prior (~5-min average) in the pre-9-phenanthrol condition; the negative response indicates a decrease in fura-2 (340/380 nm) ratio. Data were summarized as means ± SE for the n (the number of DSM strips or cells) isolated from N (the number of guinea pigs). Data were compared using a two-way ANOVA followed by Bonferroni posttest or two-tailed paired Student’s t-test. A P value <0.05 was considered statistically significant.

Solutions and drugs. The Ca2+-free dissection solution had the following composition (in mM): 80 monosodium glutamate, 55 NaCl,
6 KCl, 2 MgCl₂, 10 HEPES, and 10 glucose, adjusted to pH 7.3 with NaOH. For DSM contraction studies, the physiological saline solution was prepared daily and had the following composition (mM): 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄ and 11 glucose, aerated with 95% O₂-5% CO₂ to obtain pH 7.4. The extracellular (bath) solution used in the patch-clamp and Ca²⁺ imaging experiments contained (in mM) either: 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH; or 10 TEA, 6 CsCl, 124 NaCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 0.003 nifedipine and 10 glucose, pH 7.4 with CsOH. The K⁺ patch-clamp pipette solution contained the following (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, pH adjusted to 7.2 with NaOH. The composition of Cs⁺-containing pipette solution was as follows (in mM): 110 CsOH, 110 aspartic acid, 10 NaCl, 1 MgCl₂, 10 HEPES, 0.05 EGTA, and 30 CsCl, adjusted to 7.2 with CsOH. Pipette solutions were supplemented with freshly dissolved (every 1–2 h) 200 μg/ml amphotericin-B. BSA and amphotericin-B were obtained from Thermo Fisher Scientific. All other drugs were obtained from Sigma-Aldrich, (St. Louis, MO). 9-Phenanthrol was dissolved daily in DMSO. Carbachol was dissolved in double-distilled water and tetrodotoxin (TTX) was dissolved in citrate buffer. Nifedipine was dissolved in ethanol. The final concentration of DMSO in the bath solutions did not exceed 0.1%.

RESULTS

Guinea pig DSM expresses TRPM4 channel. Protein expression of TRPM4 channel in whole DSM tissue was detected using Western blot and a TRPM4 channel-specific antibody (Fig. 1A). A band consistent with the expected molecular mass of the TRPM4 channel protein of ~134 kDa was detected. In control experiments, preabsorption of the primary antibody with its antigenic competing peptide resulted in the loss of the band (Fig. 1A), indicating the specificity of the antibody for their intended epitope. The Western blot experiments, thus, confirmed the presence of the TRPM4 channel protein in guinea pig DSM whole tissue.

To demonstrate the expression of the TRPM4 channel in isolated single DSM cells, immunocytochemistry was performed with the same specific TRPM4 channel antibody as used in the Western blot experiments. As illustrated in Fig. 1B, immunolabeling showed the specific expression of the TRPM4 channel protein in freshly isolated guinea pig DSM cells and localization of the TRPM4 channel to the cell membrane. Control treatments were carried out by omission of the primary antibody for confirming the specificity of the secondary antibody.

The selective TRPM4 channel inhibitor 9-phenanthrol inhibits cationic current activity in freshly isolated guinea pig DSM cells. The role of TRPM4 channel in guinea pig DSM cell excitability was investigated by the amphotericin-B-perforated patch-clamp technique and the selective TRPM4 channel inhibitor 9-phenanthrol. The average DSM cell capacitance in this experimental series was 21.0 ± 2.5 pF (n = 5; N = 4) and remained unchanged during the course of the recordings. At the holding potential of −70 mV, DSM cells exhibited TICCs as illustrated in Fig. 2. The application of 30 μM 9-phenanthrol decreased the TICCs activity by ~36% from NPo of 4.0 ± 1.3 in the absence of the compound to 2.5 ± 0.9 in its presence (n = 5; N = 4; P < 0.05; Fig. 2A). Washout of DSM cells with fresh bath solution removing 9-phenanthrol completely reversed the TICC activity to the control level 4.0 ± 1.3 NPo (n = 5; N = 4; P < 0.05; Fig. 2B). This suggests that TRPM4 channels are active in guinea pig DSM and function as important regulators of DSM cell excitability.

To further investigate the physiological role of TRPM4 channel in the regulation of DSM cell excitability, the current-voltage relationship was measured using Cs⁺-containing solutions and TTX to minimize the contribution of selective K⁺ currents and thus more optimally isolate the cationic currents. The current-voltage responses were obtained in the presence or absence of 30 μM 9-phenanthrol as exemplified by Fig. 2C and summarized in Fig. 2D. As illustrated, 9-phenanthrol reduced...
the cationic currents at voltages greater than or equal to +6 mV and less than or equal to −34 mV (P < 0.05; n = 10; N = 5). At +96 mV, the highest voltage tested, the control current density of 2.24 ± 0.50 pA/pF (n = 10; N = 5) was reduced by 9-phenanthrol to 0.740 ± 0.056-fold of the control (n = 10; N = 5; P < 0.005). The inhibitory effects were reversible upon washout of 9-phenanthrol with current density returning back to 1.84 ± 0.43 pA/pF at +96 mV (n = 7; N = 4; P > 0.05 washout vs. control).

Selective inhibition of TRPM4 channel with 9-phenanthrol reduces the intracellular Ca\textsuperscript{2+} in guinea pig freshly isolated DSM cells. To study the effects of TRPM4 channel inhibition with 9-phenanthrol on intracellular Ca\textsuperscript{2+}, real-time live-cell Ca\textsuperscript{2+} imaging was carried out using the ratiometric dye fura 2-AM. The control resting Ca\textsuperscript{2+} level was measured for at least 10 min, and then 9-phenanthrol (30 μM) was added to the bath solution, and recordings continued for at least 30 min. The intracellular steady-state Ca\textsuperscript{2+} level was significantly decreased following 9-phenanthrol application. Under control conditions, the fluorescent ratio (340/380 nm) was 0.871 ± 0.022, and in the presence of 9-phenanthrol for 15 min or longer, the steady-state fluorescent ratio decreased by 0.253 ± 0.023 (n = 49; N = 8; P < 0.0001; Fig. 3, A and D). 9-Phenanthrol (30 μM) induced a transient increase in the fura-2 fluorescence ratio before reaching the decreased steady-state level (Fig. 3A). This unexpected transient increase in the fluorescence ratio caused by 9-phenanthrol was also observed in the presence of nifedipine (Fig. 3B) but was not associated with any increase in DSM contractility (Fig. 4). When the cells were treated with 0.1 μM carbachol, the fura-2 fluorescent steady-state ratio increased from the precarbachol value of 0.713 ± 0.017 to 1.000 ± 0.023 (n = 24; N = 6; P < 0.0001; Fig. 3C). Subsequent addition of 30 μM 9-phenanthrol in the continued presence of carbachol reduced the ratio by 0.376 ± 0.023 (n = 24; N = 6; P < 0.0001; Fig. 3D). The decrease in intracellular Ca\textsuperscript{2+} levels by 9-phenanthrol in the presence of carbachol was significantly greater than in its absence likely related to higher intracellular Ca\textsuperscript{2+} levels evoked by muscarinic receptor activation. In contrast, 9-phenanthrol-induced much lower decrease in the steady-state ratio in the presence of nifedipine of 0.101 ± 0.014 from the pre-9-phenanthrol control of 0.722 ± 0.014 than either in the presence of carbachol or without any compound (n = 25; N = 6; Fig. 3B and D). This suggests that nifedipine-sensitive VDCCs play a role in the 9-phenanthrol-evoked reduction in the steady-state intracellular Ca\textsuperscript{2+} levels. The responses of the TRPM4 channel-selective inhibitor 9-phenanthrol on decreasing intracellular Ca\textsuperscript{2+} are most likely due to membrane hyperpolarization resulting from diminished DSM excitability.

The selective TRPM4 channel inhibitor 9-phenanthrol attenuates spontaneous phasic and tonic DSM contractions in guinea pig DSM-isolated strips. In this experimental series, functional studies were carried out to elucidate the effect of the TRPM4 channel inhibitor 9-phenanthrol on spontaneous contractile activ-

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**Fig. 3.** Selective inhibition of TRPM4 channel with 9-phenanthrol reduces the intracellular Ca\textsuperscript{2+} levels in guinea pig DSM-isolated cells. A: representative tracing of arbitrary ratio (340/380 nm) fluorescence units demonstrating the effect of 9-phenanthrol causing decrease in intracellular Ca\textsuperscript{2+} level after 10 min. In this example, the steady-state level was obtained at ~20 min after the addition of 9-phenanthrol. B: example depicting the effect of 9-phenanthrol in the continued presence of the selective voltage-dependent Ca\textsuperscript{2+} channels (VDCC) inhibitor nifedipine (1 μM). C: an original tracing of arbitrary ratio (340/380 nm) fluorescence units showing an increase in intracellular Ca\textsuperscript{2+} levels by carbachol and subsequent reduction by 9-phenanthrol. D: summary of the effects of 9-phenanthrol in the absence or presence of nifedipine (1 μM) or carbachol (0.1 μM). Data are expressed as changes in arbitrary ratio fluorescence units with respect to the pre-9-phenanthrol values (taken as 0). Responses were analyzed as steady-state levels of at least 10 min after the addition of 9-phenanthrol. Negative values indicate a decrease in the arbitrary ratio fluorescence units indicating a reduction in intracellular Ca\textsuperscript{2+} levels. ***P < 0.001, for the comparison shown; each data-point is n = 24–49; N = 6–8.
ity of guinea pig DSM-isolated strips. After initial spontaneous phasic contractions stabilized (30–60 min), cumulative addition of 9-phenanthrol (0.1–30 μM) to guinea pig DSM-isolated strips was carried out as exemplified in Fig. 4A. Cumulative addition of 9-phenanthrol (0.1–30 μM) decreased the amplitude, muscle force integral, and duration of the spontaneous phasic contractions of guinea pig DSM-isolated strips (n = 12; N = 9; Fig. 4B). The IC50 value and maximum inhibition for spontaneous phasic DSM contraction parameters by 9-phenanthrol were as follows: phasic contraction amplitude, 1.65 (0.6–4.5, 95% confidence interval) μM and 64.4 ± 8.7%; muscle force integral, 0.72 (0.12–4.3) μM and 74.9 ± 7.3%; and contraction duration, 2.69 (0.27–25.8) μM and 40.4 ± 6.0%, respectively. In comparison, phasic contraction frequency was reduced to a lesser extent with a maximum inhibition value of 21.7 ± 11.1%.

In the next experimental series, we used 20 mM KCl to depolarize guinea pig DSM and induce phasic contractions. As illustrated in Fig. 5A, carbachol (0.1–30 μM), caused a significant inhibition of the 20 mM KCl-induced contractions of DSM strips. Cumulative addition of 9-phenanthrol significantly decreased the amplitude, muscle force integral, duration, and frequency of the 20 mM KCl-induced DSM phasic contractions (n = 12; N = 9; Fig. 5B).

![Cumulative addition of 9-phenanthrol significantly inhibited the spontaneous phasic contractions of guinea pig DSM-isolated strips.](image)

The selective TRPM4 channel inhibitor 9-phenanthrol (0.1–30 μM) to guinea pig DSM-isolated strips was carried out as exemplified in Fig. 4A. Cumulative addition of 9-phenanthrol (0.1–30 μM) decreased the amplitude, muscle force integral, and duration of the spontaneous phasic contractions of guinea pig DSM-isolated strips (n = 7; N = 7; Fig. 4B). The IC50 value and maximum inhibition for spontaneous phasic DSM contraction parameters by 9-phenanthrol were as follows: phasic contraction amplitude, 1.65 (0.6–4.5, 95% confidence interval) μM and 64.4 ± 8.7%; muscle force integral, 0.72 (0.12–4.3) μM and 74.9 ± 7.3%; and contraction duration, 2.69 (0.27–25.8) μM and 40.4 ± 6.0%, respectively. In comparison, DSM phasic contraction frequency was reduced to a lesser extent with a maximum inhibition value of 25.4 ± 15.8%.

The selective TRPM4 channel inhibitor 9-phenanthrol attenuates pharmacologically induced DSM contractions in guinea pig DSM-isolated strips. In the next set of experimental series, we sought to test the effect of cumulative addition of 9-phenanthrol under conditions of either muscarinic receptor stimulation with carbachol or sustained membrane depolarization with 20 mM KCl. Carbachol (0.1 μM) rapidly increased the spontaneous phasic contractions of DSM strips (Fig. 5A). Cumulative addition of 9-phenanthrol (0.1–30 μM) decreased the amplitude, muscle force integral, and duration of the carbachol-induced phasic contractions of guinea pig DSM-isolated strips (n = 12; N = 9; Fig. 5B). The IC50 value and maximum inhibition for carbachol-induced DSM contraction parameters by 9-phenanthrol were as follows: phasic contraction amplitude, 3.0 (1.1–8.7, 95% confidence interval) μM and 72.6 ± 6.8%; muscle force integral, 2.3 (1.0–5.4) μM and 86.1 ± 2.8%; and contraction duration, 6.7 (3.3–13.4) μM and 52.9 ± 4.8%, respectively. In comparison, phasic contraction frequency was reduced to a lesser extent with a maximum inhibition value of 21.7 ± 11.1%.

In the next experimental series, we used 20 mM KCl to depolarize guinea pig DSM and induce phasic contractions. As illustrated in Fig. 6A, cumulative addition of 9-phenanthrol (0.1–30 μM), caused a significant inhibition of the 20 mM KCl-induced contractions of DSM strips. Cumulative addition of 9-phenanthrol significantly decreased the amplitude, muscle force integral, duration, and frequency of the 20 mM KCl-induced DSM phasic contractions (n = 12; N = 9; Fig. 6B).

![Cumulative addition of 9-phenanthrol significantly inhibited the carbachol-induced contractions of guinea pig DSM-isolated strips.](image)

The selective TRPM4 channel inhibitor 9-phenanthrol attenuates pharmacologically induced DSM contractions in guinea pig DSM-isolated strips. In the next set of experimental series, we sought to test the effect of cumulative addition of 9-phenanthrol under conditions of either muscarinic receptor stimulation with carbachol or sustained membrane depolarization with 20 mM KCl. Carbachol (0.1 μM) rapidly increased the spontaneous phasic contractions of DSM strips (Fig. 5A). Cumulative addition of 9-phenanthrol (0.1–30 μM) decreased the amplitude, muscle force integral, and duration of the carbachol-induced phasic contractions of guinea pig DSM-isolated strips (n = 12; N = 9; Fig. 5B). The IC50 value and maximum inhibition for carbachol-induced DSM contraction parameters by 9-phenanthrol were as follows: phasic contraction amplitude, 3.0 (1.1–8.7, 95% confidence interval) μM and 72.6 ± 6.8%; muscle force integral, 2.3 (1.0–5.4) μM and 86.1 ± 2.8%; and contraction duration, 6.7 (3.3–13.4) μM and 52.9 ± 4.8%, respectively. In comparison, phasic contraction frequency was reduced to a lesser extent with a maximum inhibition value of 21.7 ± 11.1%.
The IC₅₀ and maximum inhibitory effects on the 20 mM KCl-induced contraction parameters in the presence of cumulative addition of 9-phenanthrol were: phasic contraction amplitude, 2.0 (0.8–5.5, 95% confidence interval) μM and 73.8 ± 6.1%; muscle force integral, 1.2 (0.6–2.6) μM and 79.5 ± 3.7%; and contraction duration, 0.9 (0.1–5.2) μM and 23.8 ± 4.5%, respectively. In comparison, the contraction frequency was reduced to a lesser extent with a maximum inhibition value of 18.0 ± 6.6%. Furthermore, at 30 μM 9-phenanthrol reduced the 20 mM KCl-induced DSM tone to 78.4 ± 5.8%, whereas 60 mM KCl-induced DSM tone was reduced to only 89.1 ± 5.6% (n = 11; N = 8; P < 0.005).

Since Ca²⁺ entry via the VDCC is responsible for the initial depolarization phase of the DSM action potential and thus activation of the DSM contraction, the VDCC blocker nifedipine (3 μM) was used to investigate the effects of 9-phenanthrol on VDCC activity. As illustrated in Fig. 7A, nifedipine was added to the bath after cumulative addition of 9-phenanthrol (0.1–30 μM) on DSM tonic contractions induced by 60 mM KCl. 9-phenanthrol at a concentration of 30 μM reduced the 60 mM KCl-induced DSM tone by 34.7 ± 5.9% compared with the control. However, in the presence of the VDCC blocker nifedipine the 60 mM KCl-induced DSM tone significantly decreased by 85.8 ± 5.7% compared with the control (n = 11; N = 6; P < 0.005; Fig. 7B).

In an additional experimental series, DSM strips were incubated with 60 mM KCl and subsequently washed out after the 60 mM KCl-induced DSM tone reached a stable steady state. Following the washout of 60 mM KCl, DSM strips were preincubated with either 3 μM nifedipine or 30 μM 9-phenanthrol for 10 min and then stimulated again with 60 mM KCl. There was no significant difference between the steady-state level of the 60 mM KCl-induced DSM tone in the presence of 9-phenanthrol vs. control (103.3 ± 13.2%; n = 33; N = 12; P > 0.05). However, the steady state of the 60 mM KCl-induced DSM tone in the presence of nifedipine significantly decreased down to 2.3 ± 2.2% (n = 13; N = 8). Taken together, these experiments indicate that VDCCs are not directly blocked by 30 μM 9-phenanthrol whereas nifedipine causes complete DSM relaxation.

The selective TRPM4 channel inhibitor 9-phenanthrol reduces nerve-evoked contractions in guinea pig DSM-isolated strips. Activation of parasympathetic nerves causes DSM contractions during bladder voiding. EFS triggers activation of excitatory neurotransmitter release and subsequently induces high-amplitude DSM phasic contractions. In this experimental series, we sought to explore the ability of cumulative addition of 9-phenanthrol (0.1–30 μM) to reduce DSM neurogenic contractions evoked by continuous repetitive EFS of 10-Hz frequency. A representative myograph recording in Fig. 8A shows that cumulative addition of 9-phenanthrol (0.1–30 μM) reduced the EFS-induced contractions at a stimulation frequency of 10 Hz in guinea pig DSM-isolated strips. Figure 8B summarizes the mean-concentration responses and demonstrates significant inhibitory effects on nerve-evoked contraction amplitude and muscle force integral at a stimulation frequency of 10 Hz with IC₅₀ values and maximum inhibition of 1.8 (1.2–2.6, 95% confidence interval) and 84.7 ± 3.2%; and 1.8 (1.3–2.6) and 84.4 ± 3.1%, respectively (n = 12; N = 8).

In another group of experiments, guinea pig DSM-isolated strips were stimulated by increasing EFS frequencies (0.5–50 Hz). Following a frequency-response curve under control conditions, DSM strips were incubated with 3 μM 9-phenanthrol for 10 min and a frequency-response curve in the presence of 9-phenanthrol was generated. As illustrated in Fig. 9A, 9-phenanthrol significantly decreased the contraction amplitude generated in response to EFS frequencies of 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz (n = 21; N = 13; P < 0.05; Fig. 9B). At the highest frequency tested of 50 Hz, addition of 9-phenanthrol (3 μM) to the bath solution inhibited the EFS-induced contraction amplitude in guinea pig DSM-isolated strips to 52.3 ± 4.8%, compared with the control responses (Fig. 9B). This experimental series further indicates that inhibition of the TRPM4 channel can reduce nerve-evoked contractions of guinea pig DSM-isolated strips.

**DISCUSSION**

This study shows for the first time the expression of TRPM4 channels in guinea pig DSM and reveals their contribution to DSM excitation-contraction coupling. The results of our study showed the following: 1) TRPM4 channels were identified at the protein level in DSM whole tissue and DSM single cells by Western blot and immunocytochemistry, respectively; 2)
TRPM4 channel inhibitor 9-phenanthrol reduced TICCs and voltage-evoked cation steady-state currents consistent with the blockade of the TRPM4 channels causing attenuation of DSM cell excitability and associated reduction of intracellular Ca²⁺ in freshly isolated DSM cells; and 3) 9-phenanthrol decreased the spontaneous phasic, pharmacologically induced and nerve-evoked contractions in guinea pig DSM-isolated strips. Collectively, these observations support that TRPM4 channels are critical regulators in guinea pig DSM excitation-contraction coupling.

Western blot and immunocytochemistry experiments demonstrated for the first time, the expression of the TRPM4 channel protein in both whole DSM tissue and isolated DSM single cells from guinea pigs (Fig. 1, A and B). In the immunocytochemistry studies, we identified the presence of TRPM4 channels within the vicinity of the DSM cell membrane, which is consistent with previous reports showing increased TRPM4 channel labeling near the plasma membrane in cerebral artery smooth muscle cells (15, 16).

Here, for the first time we revealed the critical role of the TRPM4 channels in guinea pig DSM excitability by conducting perforated patch-clamp experiments using freshly isolated DSM cells. At the holding potential at −70 mV, freshly isolated guinea pig DSM cells exhibited 9-phenanthrol-sensitive TICCs (Fig. 2, A and B). Voltage-step protocols utilizing Cs⁺-containing solutions also revealed 9-phenanthrol sensitive steady-state currents, similar to the observation in HEK-293 cells expressing recombinant TRPM4 channels (18). In the cerebral artery smooth muscle cells, TICCs have been shown to be generated by the activity of TRPM4 channels (10, 15, 17). Also, in human and monkey colonic smooth muscle cells the TICCs, which determine the constitutively active nonselective cation conductance and regulate the resting membrane potential of these cells, are thought to be at least in part mediated by TRPM4 channels (9). Murine DSM cells also showed constitutively active, Na⁺ permeable currents that control the membrane potential and DSM contractility (38). Until now, the molecular identity of this cation current in guinea pig DSM cells was unknown. We addressed the involvement of TRPM4 channels by examining the effects of 9-phenanthrol, a recently characterized selective inhibitor of TRPM4 channels (17, 18) on guinea pig DSM cells. The results showed that 9-phenanthrol, at the concentration of 30 μM,
Which is approximately threefold higher than its reported IC\textsubscript{50} value determined using recombinant cells (18), decreased TICC activity in guinea pig DSM cells. 9-Phenanthrol diminished TICC activity by $\sim$40%, suggesting that other conductances contribute to these currents in DSM cells. The present study did not address the identity of the channels contributing to the 9-phenanthrol-insensitive component, but possible candidates include Ca$^{2+}$-activated Cl$^{-}$ channels or other TRP nonselective cation channels such as TRPC channels. Their specific functions and roles in regulation of DSM excitability and contractility remain to be elucidated. Our observations with 9-phenanthrol are comparable with the effects of this compound reported for cerebral artery and colonic smooth muscle cells inhibiting TICCs or TRPC channels, their respective roles in regulation of DSM excitability and contractility. After the TRPM4 channels are activated, this increases the net influx of Na$^{+}$, which depolarizes the cell membrane and activates the VDCC channels, ultimately leading to an increase in intracellular Ca$^{2+}$ and subsequent contraction (15). Most likely the activation of the TRPM4 channels in DSM contributes to enhancement of contractility by a similar mechanism. Future studies are needed to fully elucidate and characterize whether this pathway operates in DSM cells. Our observations with 9-phenanthrol provide the initial line of evidence.

The biophysical profile of TRPM4 channels, primarily determined using recombinant systems, situates these proteins as ideal candidates for the regulation of DSM function. They are also sensitive to stretch (28), the key parameter that allows the urinary bladder to expand to accumulate and store urine. These mechanosensitive ion channels, including TRPM4 channels, are expected to be key determinants of DSM function. TRPM4 channels, similar to other TRP family members, demon-
strate thermosensitivity. Their activity increases over the range 15–35 °C (37), implying that our patch-clamp and live-cell Ca\(^{2+}\) imaging experiment both conducted at room temperature of ~22 °C likely underestimate the contribution that these channels present at higher physiological temperatures (~37 °C) such as the conditions of our in vitro contractility studies. At the resting membrane potential of DSM cells of approximately conditions of our in vitro contractility studies. At the resting membrane potential of DSM cells of approximately 40 mV (20, 35), the TRPM4 channels potentially activated by increases in intracellular Ca\(^{2+}\) (15) directly or indirectly mediated by stretch or mechanostimulation are expected to be conducting higher flux of net Na\(^+\) than K\(^+\) and thus provide a depolarizing influence. Their blockade via pharmacological means such as the application of 9-phenanthrol would lead to attenuation of DSM contractility. Future studies are needed to determine how stretch and other intracellular signaling pathways, including Ca\(^{2+}\) and protein kinase C, regulate DSM function by targeting TRPM4 channels.

In conclusion, this is the first study to identify the expression and to address the functional role of the TRPM4 channels in guinea pig DSM. We identified TRPM4 channel expression in whole DSM tissue and isolated single DSM cells at the protein level. We showed that the selective TRPM4 channel inhibitor 9-phenanthrol reduced TICCs or TRPM4-like current activity, decreased the level of global intracellular Ca\(^{2+}\), and inhibited spontaneous phasic, pharmacologically induced and nerve-evoked contractions in guinea pig DSM. Thus our data provide strong evidence that TRPM4 channels are fundamental regulators of DSM excitation-contraction coupling and may represent an attractive therapeutic target for the treatment of OAB.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


