



Involvement of transient receptor potential melastatin 4 channels in the resting membrane potential setting and cholinergic contractile responses in mouse detrusor and ileal smooth muscles

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ABSTRACT. Here, we investigated the effects of 9-hydroxyphenanthrene (9-phenanthrol), a potent and selective transient receptor potential melastatin 4 (TRPM4) channel blocker, on the resting membrane potential and cholinergic contractile responses to elucidate the functional role of TRPM4 channels in the contractile activities of mouse detrusor and ileal longitudinal smooth muscles. We observed that, 9-phenanthrol (3–30 μM) did not significantly inhibit high K^+ -induced contractions in both preparations; however, 9-phenanthrol (10 μM) strongly inhibited cholinergic contractions evoked by electrical field stimulation in detrusor preparations compared to inhibitions in ileal preparations. 9-Phenanthrol (10 μM) significantly inhibited the muscarinic agonist, carbachol-induced contractile responses and slowed the maximum upstroke velocities of the contraction in detrusor preparations. However, the agent (10 μM) did not inhibit the contractions due to intracellular Ca^{2+} release evoked by carbachol, suggesting that the inhibitory effect of 9-phenanthrol may primarily be due to the inhibition of the membrane depolarization process incurred by TRPM4 channels. On the other hand, 9-phenanthrol (10 μM) did not affect carbachol-induced contractile responses in ileal preparations. Further, 9-phenanthrol (10 μM) significantly hyperpolarized the resting membrane potential and decreased the basal tone in both detrusor and ileal muscle preparations. Taken together, our results suggest that TRPM4 channels are constitutively active and are involved in setting of the resting membrane potential, thereby regulating the basal tone in detrusor and ileal smooth muscles. Thus, TRPM4 channels play a significant role in cholinergic signaling in detrusor, but not ileal, smooth muscles.

KEY WORDS: 9-hydroxyphenanthrene, cholinergic contraction, resting membrane potential, transient receptor potential melastatin channel 4, visceral smooth muscle

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Contraction of the detrusor smooth muscle (DSM) is necessary to completely empty urine, while abnormal contractility of DSM can lead to various diseases, including overactive bladder (OAB) [24, 41]. Urinary urgency, with or without incontinence, frequency, and nocturia are common symptoms of OAB that affect people by troubling their sleep, daily routine activities, and social interactions [1, 3, 4]. The pathophysiology of OAB is neurogenic or myogenic in origin affecting DSM contractility [7, 11, 47]. Currently, antimuscarinic drugs are used to treat OAB but their clinical use is limited due to their lack of specificity producing undesirable side effects such as dry mouth, constipation, tachycardia, ataxia, and blurred vision [3, 4]. Therefore, detailed understanding of cholinergic signal transduction pathways involved in the regulation of DSM contraction is critical for identifying novel therapeutic targets in order to improve the treatment options for OAB.

Contraction of DSM is triggered by a network of signals involving various receptors and ion channels. Targeting of the ion channel may be a promising alternative pharmacological intervention for the treatment of OAB [33, 34]. The precise mechanisms

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by which ion channels regulate the function of DSM are not known. Previous studies have reported that various types of ionic channels, including K^+ , Ca^{2+} , Cl^- , and non-selective Na^+ permeable cation channels, control the resting membrane potential and contractility of the DSM [3, 4, 21, 23, 32, 33, 35, 45]. Depolarization of the cell membrane occurs due to the activation of non-selective cation channels, which leads to the opening of L-type voltage-dependent Ca^{2+} channels (VDCCs) resulting in increased intracellular Ca^{2+} concentrations triggering contraction of the DSM [54]. Suppression of these cation channels or activation of K^+ channels leads to membrane hyperpolarization causing relaxation of DSM [33, 45]. Depolarization of cell membrane potential and increased excitability of DSM is therefore responsible for enhanced contractility of DSM.

The discovery of the transient receptor potential (TRP) protein family has been of particular interest as a candidate for molecular basis of non-selective cation channels in smooth muscle cells [31]. Recently, transient receptor potential melastatin 4 (TRPM4) channel is suggested to be involved in membrane excitability of a certain type of smooth muscle [20, 29]. This channel is Ca^{2+} activated non-selective cation channel and is permeable to Na^+ and K^+ but impermeable to Ca^{2+} [19, 29]. Experiments conducted in human embryonic kidney-293 (HEK-293) cells expressing TRPM4 channels suggest that activation of these channels causes membrane depolarization via Na^{2+} influx [10] and also in cerebral artery smooth muscle using TRPM4 antisense suggesting that TRPM4 channels are present in native cerebral artery smooth muscle cells and their activation leads to membrane depolarization, activation of DCCs, thereby causing contraction [9, 14]. On the other hand, the inhibition of TRPM4 channels causes cell membrane hyperpolarization, leading to relaxation of cerebral artery smooth muscles [15].

Recently, TRPM4 channels have also been suggested to be expressed and have a functional role in contractions of rat, guinea pig, and human DSM [22, 39, 40]. Thus, selective modulation of TRPM4 channels may also modulate membrane excitability and contractility, and may be a potential and novel target for the treatment of pathological condition of urinary bladder including OAB. However, the precise roles of TRPM4 channels in contractile responses are not fully clarified. Furthermore, there are few reports on the involvement of TRPM4 channels in the cholinergic contractile responses in mice DSM.

A few studies reported the presence of TRPM4 channels in gastrointestinal smooth muscles, such as colonic smooth muscles [8]. Dwyer *et al.* [8] recorded basally activated non-selective cation channel current in human and monkey colonic smooth muscle cells and suggested that TRPM4 channels contribute to the setting of resting membrane potential. However, there is limited information about the physiological and pathophysiological roles of TRPM4 channels in the gastrointestinal tract. If TRPM4 channels play a role in the contraction in gastrointestinal smooth muscles, the same above-mentioned side effects related to the gastrointestinal system, such as constipation, will occur if we target TRPM4 channels for the treatment of OAB. Therefore, it is important to compare the functional relevance of TRPM4 channels between intestinal and DSM preparations.

The present study was conducted to investigate the involvement of TRPM4 channels in cholinergic contractile responses in detrusor and ileal longitudinal smooth muscle preparations in mice. We used 9-hydroxyphenanthrene (9-phenanthrol) as a selective blocker for TRPM4 channels, which has been widely used in biological studies to reveal the physiological roles of these channels [20]. 9-phenanthrol effectively blocked currents generated by TRPM4 channels expressed in HEK-293 cells under whole-cell patch clamp conditions [16]. However, 9-phenanthrol exerts no inhibitory effect on the close family member of TRPM4 i.e., TRPM5 currents recorded after their expression in HEK-293 cells and also TRPM7 currents recorded in mouse interstitial cells of Cajal [16, 26]. Moreover, 9-phenanthrol showed no changes in the activity of the transient receptor potential canonical 3 and 6 (TRPC3 and TRPC6) channels, large-conductance Ca^{2+} -activated K^+ channels, voltage-gated K^+ channels, VDCCs or inwardly rectifying K^+ channels [15]. These findings endorse that 9-phenanthrol is a selective and potent inhibitor of the TRPM4 channels and is a useful tool for the investigation of the involvement of TRPM4 channels in the cholinergic contraction of detrusor and gastrointestinal smooth muscles.

MATERIALS AND METHODS

All procedures described below were performed in accordance with the guidelines approved by the local Animal Ethics Committee of the Faculty of Applied Biological Sciences, Gifu University, Japan and the Faculty of Life Sciences, Kyoto Sangyo University, Japan.

Animals and tissue preparations

Animals were housed under the standard laboratory conditions as described previously [48]. Food (MF; Oriental Yeast Co., Ltd., Tokyo, Japan; DC-8; CLEA Japan Inc., Tokyo, Japan) and water were supplied *ad libitum*.

Mice of either sex (hybrid mice of 129S4 and CF-1 strain), and ddY mice, aged 2–10 months and weighing 20–42 g, were killed by cervical dislocation. The urinary bladder and whole intestines were quickly excised and placed in a Petri dish filled with Tyrode's solution (composition described below). The urinary bladder was freed of the connective tissues and then cut open by a longitudinal incision. The mucosa of the urinary bladder was carefully removed, and DSM strips (~2–3 mm wide × 6–8 mm long) were prepared. The ileal segments of ~1.5–2 cm in length were dissected except those at distances greater than 2 cm from the ileocaecal junction. Tyrode's solution was used to flush away the intestinal content and the adhering tissues were cutoff. Then, the detrusor strips and ileal segments were kept in organ bath (see below).

Single cell preparation

Isolation of single DSM cells was performed with a modified protocol described previously [54]. The DSM strips were cut into small pieces (1–2 mm cubes) and incubated in nominally Ca^{2+} -free physiological salt solution (PSS; for composition, see below)

containing collagenase type II (1 mg/ml) and type XI (1 mg/ml), papain (1 mg/ml), dithiothreitol (1 mg/ml), and bovine serum albumin (1 mg/ml) for 30 min at 36°C. The digestive tissues were then transferred to fresh PSS containing 120 μM Ca^{2+} , and single smooth muscle cells were harvested by tissue agitation and subsequent centrifugation. Thereafter, they were re-suspended in PSS containing 0.5 mM CaCl_2 , placed on coverslips in small aliquots and stored at 4°C until use on the same day.

Single ileal longitudinal smooth muscle cells were isolated enzymatically from longitudinal muscle layers of the small intestine as described previously [37]. Briefly, longitudinal smooth muscle layer was carefully peeled off from ileal segments, cut into small pieces (1–2-mm cubes) and incubated in nominally Ca^{2+} -free PSS containing collagenase type II (91.3 U/ml), papain (0.27 mg/ml), dithiothreitol (1 mg/ml), and bovine serum albumin (1 mg/ml) for 10 min digestion successively twice at 36°C. Thereafter, single ileal smooth muscle cells were prepared similar to DSM cells.

Isometric tension recording and electrical field stimulation (EFS)

The prepared DSM strips and ileal segments were vertically mounted in an organ bath of 10-ml filled with Tyrode's solution bubbled continuously with air and kept at 37 and 30°C, respectively, as previously described [2, 18, 49]. The DSM strips were subjected to 0.5 g of tension and allowed to equilibrate for 60 min in fresh Tyrode's solution. After the equilibration period, they were exposed briefly to hypertonic 70 mM KCl in 10–15-min intervals until reproducible contractions were obtained. The purinergic receptors were inhibited by adding α,β -methylene ATP (50 μM) [12, 25], and were allowed to be present in the bathing solution throughout the experiment. The ileal tissue preparations were subjected to a load of 0.5 g and incubated for 20 min, followed by a further 60-min incubation in fresh Tyrode's solution but containing guanethidine (1 μM) to inhibit the adrenergic neuron and $\text{N}\omega$ -nitro-L-arginine methyl ester (L-NAME; 100 μM) to inhibit the nitric oxide synthase. These drugs were maintained in the bathing solution throughout the experiments in order to minimize the possible inhibitory effects due to adrenergic nerves or nitrenergic nerves. After the second incubation, hypertonic 70 mM KCl was applied for 1 min at intervals of 10–15 min until reproducible contractions were obtained.

A force-displacement transducer (T7-30-240, Orientic, Tokyo, Japan) was used to record the changes in tension of the tissues isometrically along the longitudinal axis, as previously described [48]. The transducer was coupled with a strain DC amplifier (AS1202, NEC, Tokyo, Japan). The output of the amplifier was exhibited on an ink-writing chart recorder (U-228, Nippon Denshi Kagaku, Kyoto, Japan) and captured at a sampling rate of 1 kHz using analog-digital converter (PowerLab 8/35, ADInstruments Inc., Nagoya, Japan) interfaced to a computer (CF-S9, Panasonic, Osaka, Japan) running the Chart program (version 8.0, ADInstruments Inc.).

Nerve-evoked contractions were induced by EFS using a pair of platinum ring electrodes mounted in the tissue bath placed in parallel on either side of the tissue preparations. The EFS pulses were generated using a stimulator (SEN-3301, Nihon Kohden Corp., Tokyo, Japan). The EFS pulse parameters were as follows: pulse width 0.5 msec, pulse amplitude 50 V, stimulus duration 5 sec and increasing frequency of 1–100 Hz for detrusor strips and 1–50 Hz for ileal smooth muscle tissues. The time intervals between successive trials of frequencies varied from 5–10 min as more time was required for the responses produced at higher stimulus frequencies to subside. Since the tissue preparations showed spontaneous activity throughout the experiments, the mean peak level of the spontaneous contractions ~2 min before each EFS was considered as the baseline for the measurement of contractions. After construction of a frequency-response curve as a control, 9-phenanthrol was treated for 15 min, and then EFS was applied using the same parameters.

Carbachol response

Cholinergic contractions were recorded by application of muscarinic receptor agonist carbachol (1 and 10 μM) in the absence and presence of 9-phenanthrol. In some experiments, carbachol was applied in Ca^{2+} free isotonic Tyrode's solution containing 140 mM KCl before and after application of 9-phenanthrol. Under these conditions, membrane potentials could reach to approximately 0 mV, which is near to the equilibrium potential of the non-selective cation channels and is sufficient for depolarization to inactivate VDCCs [6, 50]. Therefore, application of carbachol is expected to induce intracellularly released Ca^{2+} -mediated contractions, but not Ca^{2+} influx from the extracellular matrix through non-selective cation and VDCCs [48].

Nystatin perforated whole cell patch clamp technique

Membrane potential responses in single smooth muscle cells were recorded at room temperature (21–26°C) using nystatin-perforated patch clamp technique using glass patch pipettes with resistances of 4–6 M Ω under current clamp mode, as previously described [27]. The patch pipettes were filled with a nystatin included KCl-based solution (for composition, see below). Single detrusor or ileal smooth muscle cells were bathed in PSS and membrane potential responses were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices, LLC., CA, U.S.A.), and membrane potential signals were filtered at 1 kHz and captured at a sampling rate of 2 kHz using an analog-digital converter (DIGIDATA 1550; Molecular Devices) interfaced to a computer (ThinkPad T440p, Lenovo, NC, U.S.A.) running the pCLAMP program (version 10, Molecular Devices) for later analysis and illustration.

Data analysis

EFS- and carbachol-evoked contractile amplitudes in the DSM strips and ileal preparations were expressed as the percentage of a reproducible 70 mM KCl-induced contraction measured in the same preparation. Values in the text are given as the mean \pm standard error of the mean (S.E.M.; n =the number of tissue preparations used). The Student paired or unpaired t -test was used to

determine the statistical significance of differences between two group means. Differences were considered statistically significant when $P < 0.05$. Averaged curves representing the relationships between stimulus frequencies and contraction size were constructed by sigmoid nonlinear regression fit using GraphPad Prism version 6 (Tokyo, Japan), providing a stimulus frequency required to produce a half maximum response (50% effective frequency). The maximum upstroke velocity of the carbachol-induced contractile response was determined by differentiation of the pre-filtered original trace at 1 Hz using the Chart program (version 8.0, ADInstruments Inc.) and expressed as gram per second (g/sec). When we examined the effects of 9-phenanthrol, the changes in basal tone were calculated by subtracting the value measured after 15-min incubation of 9-phenanthrol from that immediately prior (~2 min average) under the pre-9-phenanthrol condition and was expressed as the percentage of a reproducible 70 mM KCl contraction measured in the same preparation.

Solutions

Tyrode's solution used had the following composition (mM): NaCl 136.9, KCl 2.68, CaCl_2 1.8, MgCl_2 2.1, NaH_2PO_4 0.41, NaHCO_3 11.9, and glucose 5.55. To prepare isotonic 140 mM KCl containing Tyrode's solution, NaCl (136.9 mM) was replaced with KCl (136.9 mM) in the above usual composition without adding CaCl_2 . To record high K^+ -induced contractions, 3.5 M KCl stock solution was prepared (without any other components) and applied hyperosmotically at the desired concentration (70 mM). For nystatin perforated whole cell patch clamp experiment, the physiological salt solution (PSS) had the following composition (mM): NaCl 126, KCl 6, CaCl_2 2, MgCl_2 1.2, glucose 14, and HEPES 10.5 (pH adjusted to 7.2 with NaOH). The nystatin-included KCl-based pipette solution had the following composition (mM): KCl 134, HEPES 10.5 (pH adjusted to 7.2 with KOH), to which nystatin dissolved in DMSO (4 mg/0.1 ml) was added to give a final concentration of 0.2 mg/ml.

Drugs

9-Phenanthrol was purchased from Toronto Research Chemicals (North York, Canada). Atropine, carbachol, and nystatin were purchased from Wako (Osaka, Japan). L-NAME (N ω -Nitro-L-arginine methyl ester hydrochloride), guanethidine, α , β -methylene ATP, collagenase type II and type XI, papain, dithiothreitol, and bovine serum albumin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). 9-Phenanthrol was dissolved in dimethyl sulfoxide (DMSO), where the final concentration of DMSO up to 0.03% had no effect on EFS- or carbachol-evoked contractions.

RESULTS

Effects of 9-phenanthrol on EFS-induced cholinergic contractions in DSM preparations

DSM preparations were bathed in Tyrode's solution containing α , β -methylene ATP (50 μM) to suppress the purinergic contractions. EFS (50 V in strength and 0.5 msec in duration) was applied at ascending series of frequencies of 1, 2, 5, 10, 20, 50, and 100 Hz (each for 5 sec). As shown in Fig. 1A, EFS evoked a rapid, phasic contraction in the presence of α , β -methylene ATP (50 μM). The phasic contractions appeared immediately after the initiation of EFS, reached the peak within ~5 sec, and then rapidly declined. The peak amplitude of the phasic contractions increased as the stimulus frequency was increased with a mean 50% effective frequency (EF_{50}) of 14.7 ± 0.7 Hz ($n=20$), and the maximal response was attained at 50 or 100 Hz (Fig. 1B). Application of atropine (2 μM) almost completely abolished the phasic contractions (data not shown). In our previous study [2] we reported that EFS-induced cholinergic contractile responses were reproducible in the 2nd trial of EFS, and DMSO (0.1%), used as a solvent of 9-phenanthrol had no significant effect on the mean peak amplitude.

When 9-phenanthrol was treated to detrusor muscle preparations, the spontaneous contractile amplitudes and basal tone were somewhat reduced. The extent of reduction in basal tones was $2.5 \pm 1.0\%$ ($n=6$) expressed as relative value to 70 mM KCl-induced contraction by application of 9-phenanthrol (10 μM). 9-Phenanthrol had little effect on the mean peak amplitude of 70 mM KCl-induced contractions (Fig. 1A). The mean peak amplitudes before (control) and after 9-phenanthrol treatment were 0.74 ± 0.08 and 0.78 ± 0.07 g ($n=8$) at 3 μM , 0.60 ± 0.08 and 0.52 ± 0.05 g ($n=6$) at 10 μM , and 0.78 ± 0.22 and 0.57 ± 0.21 g ($n=6$) at 30 μM respectively; the differences between the mean values were not statistically significant ($P > 0.05$). However, as shown in Fig. 1B, 9-phenanthrol (3–30 μM) significantly ($P < 0.05$) inhibited the EFS-evoked cholinergic contractions, although the agent did not alter the EF_{50} in frequency-response curves at any concentrations. The mean peak amplitudes of the cholinergic contractions estimated as percentage of 70 mM KCl-induced contractions at the highest frequency tested of 100 Hz before and after application of 9-phenanthrol were 118.2 ± 20.9 and $106.5 \pm 20.8\%$ ($n=8$) at 3 μM , 109.4 ± 19.1 and $67.9 \pm 16.2\%$ ($n=6$) at 10 μM , and 142.3 ± 22.9 and $56.7 \pm 12\%$ ($n=6$) at 30 μM , respectively, the differences between the mean values were statistically significant ($P > 0.05$). The mean EF_{50} values after the application of 9-phenanthrol were 13.4 ± 1.2 Hz ($n=8$) at 3 μM , 18.9 ± 1.9 Hz ($n=6$) at 10 μM , and 12.3 ± 0.9 Hz ($n=6$) at 30 μM , respectively, which values were comparable to the control value (14.7 ± 0.7 Hz).

These results suggest that TRPM4 channels are involved in the EFS-evoked cholinergic contractions of mice DSM, as previously reported in rat [40], guinea pig [39] and human [22] DSM.

Effects of 9-phenanthrol on EFS-induced purinergic contractions in DSM preparations

We next tested the effects of 9-phenanthrol (10 μM) on purinergic receptor-mediated DSM contractions. Purinergic contractions were evoked by application of increasing frequency of EFS in the presence of atropine (2 μM) to block the muscarinic receptors. As illustrated in Fig. 2, EFS evoked a phasic, purinergic contraction in a frequency-dependently with the mean EF_{50} of 13.2 ± 2.1 Hz ($n=6$), and 9-phenanthrol (10 μM) significantly inhibited the purinergic contractions without altering the EF_{50} value (13.1

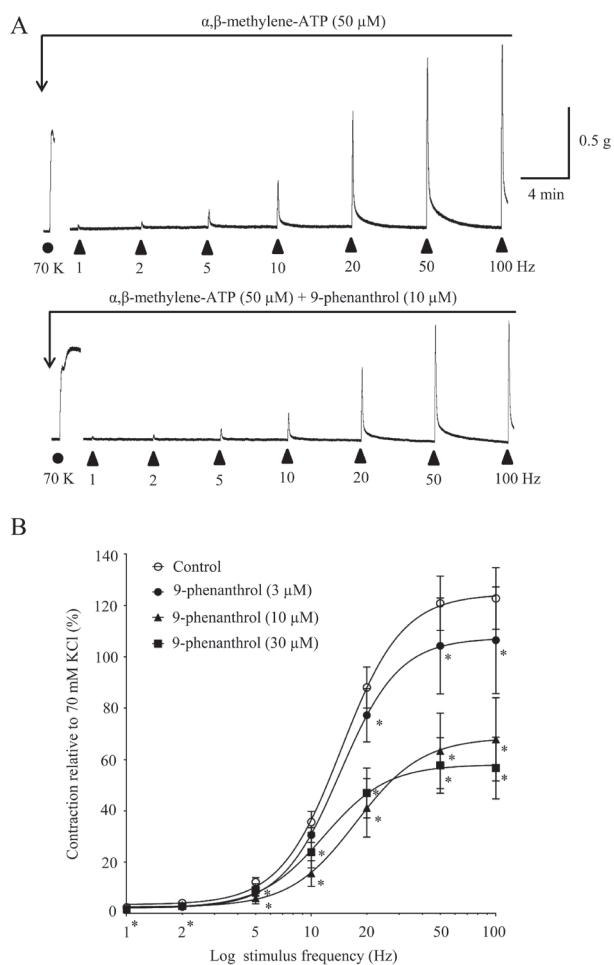


Fig. 1. Effects of 9-phenanthrol on EFS-induced cholinergic contractions in DSM preparations. (A) Representative recording of the cholinergic contractions induced by EFS (▲) and 70 mM KCl (●) before (upper trace) and after (lower trace) the application of 9-phenanthrol (10 μ M). EFS (50 V in strength, 0.5 msec in pulse duration) was applied for 5 sec at different frequencies. These experiments were performed in the presence of α,β -methylene ATP (50 μ M). (B) Summarized data showing the inhibitory effects of 9-phenanthrol (3, 10 and 30 μ M) against EFS-induced cholinergic contractile responses. The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contractions in the same preparation. Each point represents the mean \pm S.E.M. ($n=20$ for control, $n=8$ for 3 μ M, $n=6$ for 10 μ M, $n=6$ for 30 μ M 9-phenanthrol). *Significantly different ($P<0.05$) from the corresponding control values.

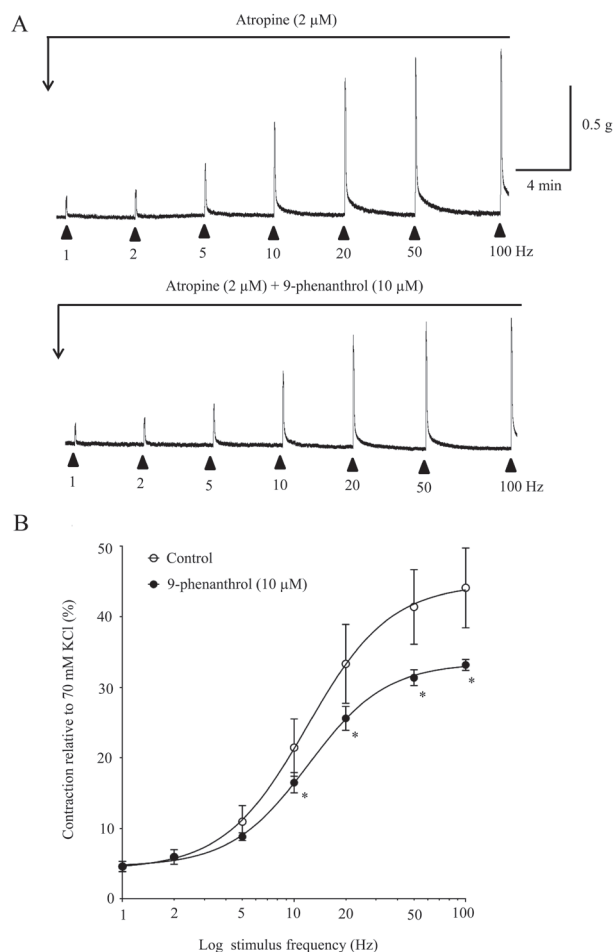


Fig. 2. Effects of 9-phenanthrol on EFS-evoked purinergic contractions in DSM preparations. (A) Representative recording of the purinergic contractions induced by EFS (▲) before (upper trace) and after (lower trace) the application of 9-phenanthrol (10 μ M). EFS (50 V in strength, 0.5 msec in pulse duration) was applied for 5 sec at different frequencies. These experiments were conducted in the presence of atropine (2 μ M) to inhibit the cholinergic contractions. (B) Summarized data showing the inhibitory effects of 9-phenanthrol (10 μ M) against EFS-induced purinergic contractile responses. The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contractions in the same preparation. Each point represents the mean \pm S.E.M. ($n=6$). *Significantly different ($P<0.05$) from the corresponding control values.

± 1.7 Hz, $n=6$, after the application of 9-phenanthrol). At the highest frequency tested of 100 Hz, the mean peak amplitudes of the purinergic contractions relative to 70 mM KCl-induced contractions before and after application of 9-phenanthrol (10 μ M) were 44.1 ± 5.7 and $33.2 \pm 0.8\%$ ($n=6$), respectively. The purinergic contractions evoked at 100 Hz was inhibited by $23.3 \pm 4.9\%$ ($n=6$) by 9-phenanthrol (10 μ M). However, the extent of inhibition in the purinergic contractions was significantly smaller than that in cholinergic contractions by 10 μ M 9-phenanthrol ($42.3 \pm 6.0\%$, $n=6$). Thus, these results suggest that the cholinergic contractions are more sensitive to 9-phenanthrol than the purinergic one in DSM.

Effects of 9-phenanthrol on EFS-induced cholinergic and non-cholinergic contractions in ileal preparations

We next examined whether 9-phenanthrol could affect the cholinergic contractile responses in ileal preparations. Ileal segment preparations were bathed in Tyrode's solution containing guanethidine (1 μ M) and L-NAME (100 μ M), and tension changes in their longitudinal directions were recorded [2, 49].

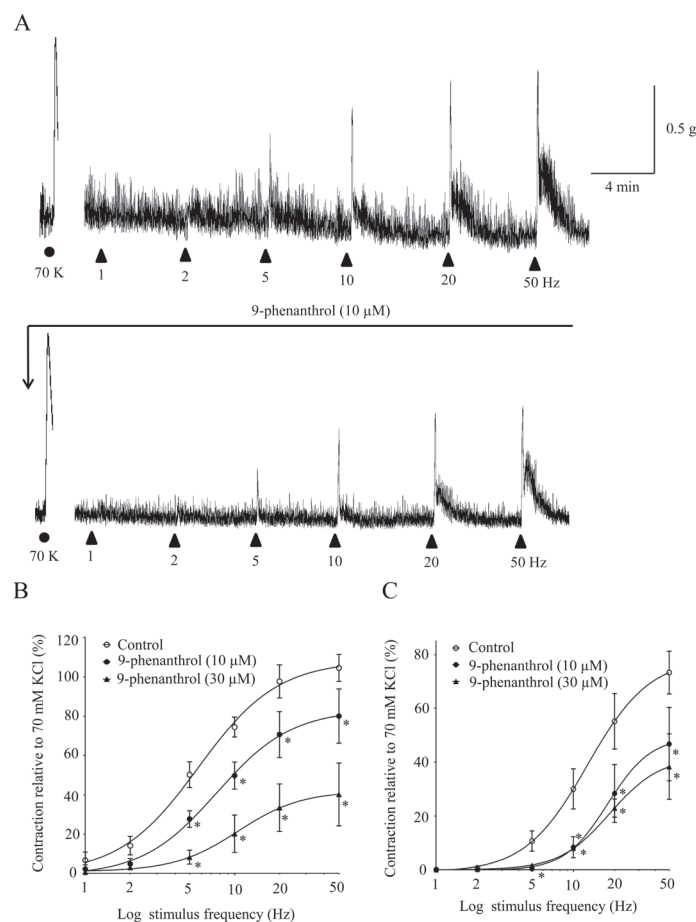


Fig. 3. Effects of 9-phenanthrol on EFS-induced cholinergic and non-cholinergic contractions in ileal preparations. (A) Representative recording of the cholinergic and non-cholinergic contractions induced by EFS (▲) and 70 mM KCl (●) before (upper trace) and after (lower trace) the application of 9-phenanthrol (10 μM). EFS (50 V in strength, 0.5 msec in pulse duration) was applied for 5 sec at different frequencies. (B and C) Summarized data showing the inhibitory effects of 9-phenanthrol (10 and 30 μM) against EFS-induced cholinergic (B) and non-cholinergic (C) contractile responses. The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contractions in the same preparation. Each point represents the mean ± S.E.M. ($n=11$ for control, $n=6$ for 10 μM, $n=5$ for 30 μM 9-phenanthrol). *Significantly different ($P<0.05$) from the corresponding control values.

All preparations showed spontaneous contractions which amplitudes were reduced by application of 9-phenanthrol (10 μM) (Fig. 3A). Like DSM preparations, 9-phenanthrol (10 μM) suppresses the basal tone by $30.4 \pm 5.5\%$ ($n=5$) in ileal preparations. The mean peak amplitudes of 70 mM KCl-evoked contractions before and after 9-phenanthrol treatment were 0.67 ± 0.08 and 0.77 ± 0.15 g ($n=6$) at 10 μM, and 0.81 ± 0.07 and 0.83 ± 0.05 g ($n=5$) at 30 μM, respectively, the differences between the two values were not statistically significant ($P>0.05$).

EFS (50 V in strength and 0.5 msec in duration) was applied at increasing frequencies of 1, 2, 5, 10, 20, and 50 Hz (each for 5 sec). As shown in Fig. 3A, EFS evoked biphasic contractions; fast, brief contractions followed by slower, sustained ones, where spontaneous contractions were superimposed [2, 49]. Both the initial fast and later slow contractions increased as the stimulus frequency was increased, and the maximal response was attained at 20–50 Hz for the initial and later contractions. The initial fast contractions appeared immediately after the beginning of EFS, reached the peak within ~5 sec, and then rapidly declined. The later slow contractions exhibited variability in amplitude and duration with different stimulus frequencies or among different preparations. At the frequency of 20 Hz, it reached the peak after 30–90 sec and then disappeared within 2–8 min. Atropine (2 μM) inhibited only the initial fast contractions (data not shown). Hence, the initial fast contractions resulted from the activation of cholinergic nerves, whereas the later slow contractions were recognized to the activation of non-cholinergic, possibly tachykinergic nerves [49].

DMSO (0.1%) has no significant effect on EFS-induced cholinergic contractions [2]. However, as shown in Fig. 3A and 3B, 9-phenanthrol treatment (10 μM) significantly ($P<0.05$) inhibited EFS-induced initial fast, cholinergic contractions, although the agent did not alter the EF_{50} in frequency-response curves (EF_{50} s before and after application of 9-phenanthrol were 4.9 ± 0.7 ($n=6$) and 7.6 ± 0.7 Hz ($n=6$), respectively). At the highest frequency tested of 50 Hz, the mean peak amplitudes relative to 70 mM KCl-induced contractions was $107.1 \pm 10.7\%$ ($n=6$) which significantly ($P<0.05$) reduced to $80.1 \pm 13.8\%$ ($n=6$) by 9-phenanthrol (10 μM) (Fig. 3B). At 30 μM, 9-phenanthrol further inhibited EFS-induced contractions; the mean peak amplitudes at 50 Hz before

and after the application of the agent were 101.6 ± 9.3 and $40.2 \pm 16.0\%$ ($n=5$), respectively. 9-Phenanthrol also significantly ($P<0.05$) inhibited the later, non-cholinergic contractions (Fig. 3C). At 50 Hz, the extent of inhibition of the cholinergic and non-cholinergic component of contractions was 27.1 ± 7.3 and $28.1 \pm 11.2\%$ ($n=6$), respectively at $10 \mu\text{M}$ 9-phenanthrol and 60.6 ± 15.4 and $55.9 \pm 13.8\%$ ($n=5$), respectively at $30 \mu\text{M}$ 9-phenanthrol; the difference between the two values was not statistically significant ($P>0.05$). The extent of % inhibitions in the cholinergic contractions in ileal preparations by 9-phenanthrol ($10 \mu\text{M}$) was smaller than that in cholinergic contractions in detrusor preparations ($42.3 \pm 6.0\%$, $n=6$).

Thus, TRPM4 channels are possibly involved in both, cholinergic and non-cholinergic contractions in the ileal smooth muscles. In the following experiments, we used $10 \mu\text{M}$ 9-phenanthrol, which is appropriate and recommended concentration without apparent side effects [20].

Effects of 9-phenanthrol on carbachol-induced contractions in detrusor and ileal smooth muscle preparations

We tested the effect of 9-phenanthrol ($10 \mu\text{M}$) on contractile responses evoked by the exogenously applied muscarinic agonist, carbachol, in detrusor and ileal smooth muscle preparations. As shown in Fig. 4A, addition of carbachol (1 or $10 \mu\text{M}$) in the bath solution rapidly produces phasic contractions. In DSM preparations, the relative % of mean peak amplitude estimated as percentage of 70 mM KCl-induced contractions was 58.3 ± 4.2 ($n=6$) and $123.7 \pm 8.1\%$ ($n=4$) after addition of 1 and $10 \mu\text{M}$ carbachol, which was significantly ($P<0.05$) reduced to 49.5 ± 3.8 ($n=6$) and $105.5 \pm 9.5\%$ ($n=4$) by 9-phenanthrol treatment ($10 \mu\text{M}$), respectively (Fig. 4B). We also analyzed the maximum upstroke velocity for carbachol-induced contractile response. The maximum upstroke velocity may reflect, at least in part, depolarizing process due to activation of non-selective cation channels after muscarinic receptor stimulation [38]. As shown in Fig. 4C, 9-phenanthrol significantly ($P<0.05$) slowed the maximum upstroke velocity from 0.20 ± 0.05 to $0.13 \pm 0.03 \text{ g/sec}$ ($n=4$).

In contrast, in ileal preparations, the relative % of mean peak amplitudes after application of carbachol (1 or $10 \mu\text{M}$) in the absence and presence of 9-phenanthrol ($10 \mu\text{M}$) were 116.6 ± 11.2 and $128.6 \pm 23.2\%$ ($n=6$), respectively, at $1 \mu\text{M}$ carbachol, and 134.1 ± 10.7 and $135.9 \pm 13.1\%$ ($n=6$) at $10 \mu\text{M}$ carbachol. The differences between the mean values in the absence and presence of 9-phenanthrol were statistically insignificant ($P>0.05$) (Fig. 5A and 5B). As shown in Fig. 5C, the maximum upstroke velocity for carbachol-induced contractile response was also unaffected by 9-phenanthrol.

These results suggest that TRPM4 channels may be involved in the muscarinic contraction induced by carbachol in DSM, while the channels seem to play a minor role in the muscarinic contractions in ileal smooth muscles.

Effects of 9-phenanthrol on intracellularly released Ca^{2+} -induced contractions in DSM preparations

It is well known that activation of M_3 muscarinic receptors induces Ca^{2+} release from the intracellular stores through the M_3/Gq type G protein/phospholipase C/inositol 1, 3, 5, trisphosphate pathway [37, 43, 44]. We tested the effects of 9-phenanthrol ($10 \mu\text{M}$) on carbachol-induced contractile responses evoked by intracellularly released Ca^{2+} in DSM preparations. The preparations were depolarized with isotonic Tyrode's solution containing 140 mM KCl and extracellular Ca^{2+} were removed. Under these conditions, carbachol ($10 \mu\text{M}$) induced phasic contractions occurred due to the release of stored Ca^{2+} [2, 48]. DSMO (0.01%) which is used as a solvent of 9-phenanthrol had little effect on these intracellularly released Ca^{2+} -evoked contractions induced by carbachol ($10 \mu\text{M}$). As shown in Fig. 6, the relative % of mean peak amplitudes evoked by carbachol ($10 \mu\text{M}$) in the absence and presence of 9-phenanthrol ($10 \mu\text{M}$) were 59.7 ± 8.1 and $54.2 \pm 7.3\%$ ($n=4$), respectively. The difference between the two values was not statistically significant ($P>0.05$).

These results suggest that the inhibitory action of 9-phenanthrol on carbachol-induced contraction in detrusor muscles may be caused by perturbations of the depolarizing process due to inhibition of TRPM4 channels but not by the disturbance of the Ca^{2+} -releasing process or other non-specific action, such as the receptor antagonistic action.

Effects of 9-phenanthrol on the resting membrane potential in detrusor and ileal smooth muscle preparations

It has been reported that TRPM4 and TRPC heteromultimer combinations may underlie basally activated non-selective cationic current regulating the resting membrane potential in isolated human and monkey colonic smooth muscle cells [8]. Here, we investigated the effects of 9-phenanthrol ($10 \mu\text{M}$) on the resting membrane potential of single detrusor and ileal smooth muscle cells held under nystatin-perforated, whole-cell current-clamp mode.

As shown in Fig. 7, 9-phenanthrol ($10 \mu\text{M}$) hyperpolarized the resting membrane potential in both detrusor and ileal preparations. In DSM preparations, 9-phenanthrol ($10 \mu\text{M}$) significantly reduced the resting membrane potential from -20.9 ± 2.4 to $-33.2 \pm 3.3 \text{ mV}$ ($n=8$). Similarly, in ileal smooth muscle preparations, 9-phenanthrol significantly reduced the resting membrane potential from -35.9 ± 1.8 to $-42.7 \pm 2.1 \text{ mV}$ ($n=8$).

These results suggest that TRPM4 channels are constitutively active and involved in the setting of resting membrane potential, and thereby regulate basal tone in detrusor and ileal smooth muscle cells.

DISCUSSION

Although TRPM4 channels have recently been detected in mouse urinary bladder whole tissue and isolated single DSM cells [28], the involvement of these channels in cholinergic responses has not been reported. Furthermore, the information about the functional role of TRPM4 channels in the gastrointestinal system is limited. To address these issues, the present study was conducted to investigate the cholinergic involvement of TRPM4 channels in detrusor and ileal longitudinal smooth muscles.

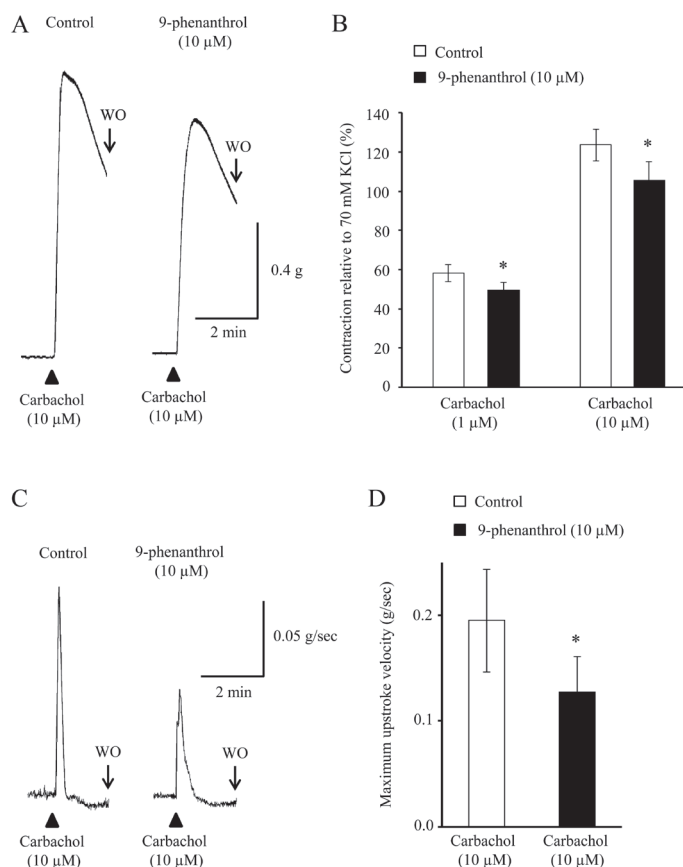


Fig. 4. Effects of 9-phenanthrol on carbachol-induced contractions in DSM preparations. Representative recording of carbachol (10 μM)-induced contractile responses (A) and the differentiated traces (C) before (left trace) and after (right trace) the application of 9-phenanthrol (10 μM). (B and D) Summarized data showing the inhibitory effects of 9-phenanthrol (10 μM) against carbachol-induced contractile amplitude (B) and the maximum upstroke velocity (D). The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contractions in the same preparation. Each column represents the mean ± S.E.M. ($n=4$). *Significantly different ($P<0.05$) from the corresponding control values.

In the present study, 9-phenanthrol hyperpolarizes resting membrane potential in both detrusor and ileal smooth muscle cells (Fig. 7), and basal tone and spontaneous phasic contractile activities were inhibited by 9-phenanthrol in both smooth muscle preparations. These results suggest that TRPM4 channels are involved in the setting of resting membrane potential and thereby in the regulation of muscle tone. Transient inward cation currents (TICCs) have been shown to be generated by the constitutive activity of TRPM4 channels in rat cerebral artery smooth muscle cells [15]. In these smooth muscles, 9-phenanthrol hyperpolarizes membrane potential causing dilation of arteries [15]. Previous studies in human, rat and guinea pig DSM cells have shown that constitutively active TICCs are inhibited by 9-phenanthrol [22, 39, 40]. Moreover, TICCs observed in human and monkey colonic smooth muscle cells are also inhibited by 9-phenanthrol [8]. Therefore, TICCs are mediated partly by constitutively active TRPM4 channels and in turn regulate the resting membrane potential [8]. To summarize, TRPM4 channels are expressed in various types of smooth muscle cell membrane and likely play a significant role in the setting of resting membrane potentials and thereby affect basal tone and spontaneous contractile activity.

9-Phenanthrol significantly inhibited both cholinergic and purinergic contractions evoked by EFS in DSM preparations (Figs. 1 and 2). Similarly, the agent significantly inhibited both cholinergic and non-cholinergic contractions in ileal smooth muscle preparations (Fig. 3). These results indicate a possibility that 9-phenanthrol can inhibit contractile responses by a non-specific action other than the inhibition of TRPM4 channels. However, the agent did not suppress 70 mM K⁺-induced contraction in both detrusor and ileal smooth muscle preparations. One plausible explanation is that 9-phenanthrol hyperpolarized the membrane due to the inhibition of constitutively active TRPM4 channels, and thereby affected EFS-induced contractile responses in both smooth muscle preparations. Because 70 mM concentration of K⁺ can strongly depolarize the membrane, it might be able to overcome such hyperpolarization induced by 9-phenanthrol and produce comparable contractile responses even in the presence of 9-phenanthrol. Alternatively, TRPM4 channels might be expressed not only in smooth muscles, but also at the nerve terminals, and be involved in the neurotransmitter release. If so, 9-phenanthrol is capable of inhibiting neurotransmitter release by altering the membrane depolarization at the nerve terminals. Further studies are warranted to understand this mechanism.

The cholinergic contractions in DSM were strongly inhibited by 9-phenanthrol (10 μM), and the extent of inhibition was much greater in comparison to purinergic contraction in DSM and cholinergic/non-cholinergic contractions in ileal muscles (Figs. 1–3).

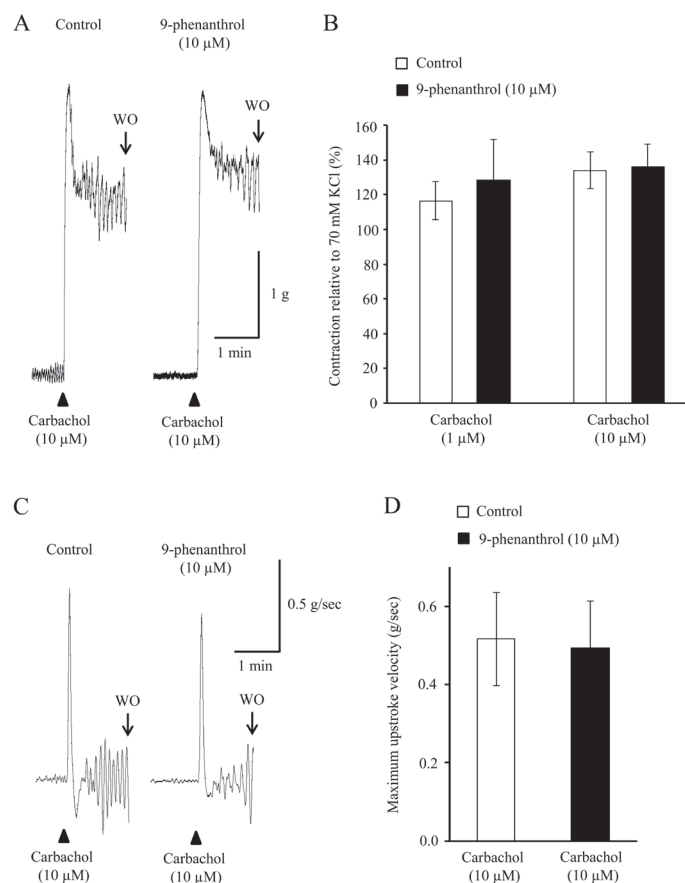


Fig. 5. Effects of 9-phenanthrol on carbachol-induced contractions in ileal smooth muscle preparations. Representative recording of carbachol (10 μ M)-induced contractile responses (A) and the differentiated traces (C) before (left trace) and after (right trace) the application of 9-phenanthrol (10 μ M). (B and D) Summarized data showing the effects of 9-phenanthrol (10 μ M) against carbachol-induced contractile amplitude (B) and the maximum upstroke velocity (D). The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contractions in the same preparation. Each column represents the mean \pm S.E.M. ($n=6$). No significant differences ($P>0.05$) observed from the corresponding control values.

9-Phenanthrol also significantly inhibited the peak amplitude and the maximum upstroke velocity of carbachol-induced contractions in detrusor, but not ileal muscle preparations. These results suggest that TRPM4 channels may be involved in the signaling process of muscarinic contraction in DSM, while the channels seem to play a minor role in such process in ileal smooth muscles. At present, the reason for such difference of the involvement of TRPM4 channels between detrusor and ileal preparations is unknown. In ileal smooth muscle cells, the major component of cation channel current activated by muscarinic receptor stimulation is believed to be TRPC4 channels [46], and the channels are suggested to compose a multi-molecular complex with muscarinic receptor and other signaling molecules [37]. Therefore, TRPM4 channels might be major component of such multimolecular complex in DSM cells. It is also known that TRP channels are capable of comprising a heteromultimer to form ionic channels [52]. If TRPC4 and TRPM4 channels form such heteromultimer ion channels, its component ratio might be different between detrusor and ileal smooth muscle cells; component ratio of TRPM4 might be much higher than that of TRPC4 in DSM cells, but vice versa in ileal smooth muscle cells. Further studies are needed to elucidate the precise molecular component and activation mechanisms for muscarinic cation channels in both tissues.

Considering that 9-phenanthrol has no effect on contraction evoked by intracellular Ca^{2+} release mediated by M_3 receptor signaling (Fig. 6), the inhibitory action of 9-phenanthrol on carbachol-induced contraction in DSM may be brought about by perturbation of depolarizing process due to inhibition of TRPM4 channels but not by disturbance of Ca^{2+} -releasing process or other non-specific action, such as M_3 receptor antagonistic action. It is well known that membrane depolarization and subsequent firing of action potentials are the primary and most important event to initiate contraction in excitatory smooth muscle cells [37, 51]. Taken together, these results suggest that TRPM4 channels may play a significant role in determining the resting membrane potential and initiation of cholinergic depolarization and contractile responses in DSM. Thus, our results provide evidence that TRPM4 channels have the potential to be a promising target for the treatment of OAB with reduced side effects on the gastrointestinal system than the antimuscarinic drugs.

TRPC4 channels have been suggested to be the major component of cation channels activated by muscarinic receptor stimulation and are involved in the muscarinic receptor-mediated contractions in intestinal smooth muscles using TRPC4 gene knockout mice

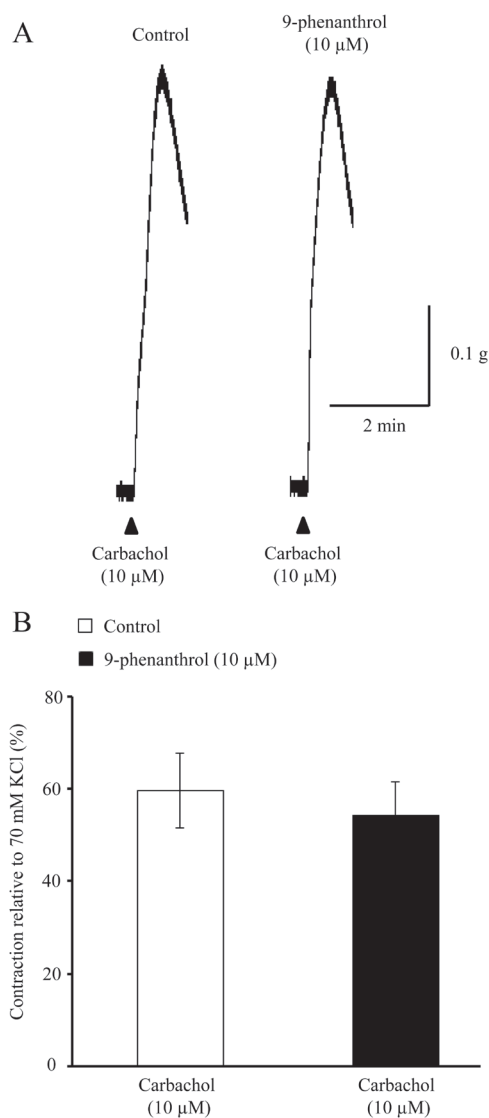


Fig. 6. Effects of 9-phenanthrol on carbachol-induced contractions evoked by intracellularly released Ca^{2+} in DSM preparations. (A) Representative recording of carbachol-induced contractions before (left trace) and after (right trace) the application of 9-phenanthrol ($10 \mu\text{M}$) in DSM preparations bathed in 140 mM KCl with removal of extracellular Ca^{2+} . (B) Summarized data showing the effects of 9-phenanthrol ($10 \mu\text{M}$) against intracellularly released Ca^{2+} -induced contractile responses. The amplitude of the contractions was expressed as percentage of the 70 mM KCl-induced contraction in the same preparation. Each column represents the mean \pm S.E.M. ($n=4$). No significant difference ($P>0.05$) observed from the corresponding control values.

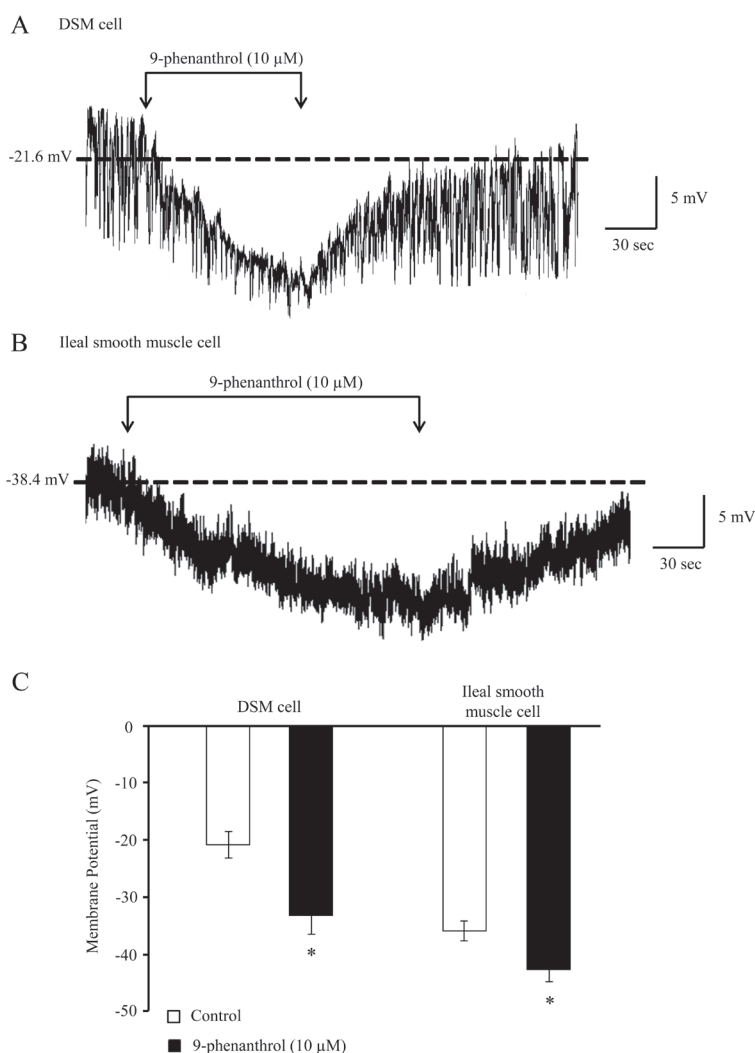


Fig. 7. Effects of 9-phenanthrol on resting membrane potential in single detrusor and ileal smooth muscle cells. (A and B) Representative recordings of the resting membrane potential responses in single detrusor (A) and ileal (B) smooth muscle cells before and after application of 9-phenanthrol ($10 \mu\text{M}$). Resting membrane potentials were recorded in current clamp mode using the nystatin-perforated whole cell patch-clamp technique. (C) Summarized data showing the hyperpolarizing effects of 9-phenanthrol ($10 \mu\text{M}$) on the resting membrane potential in single detrusor and ileal smooth muscle cells. Each column represents the mean \pm S.E.M. ($n=8$). *Significantly different ($P<0.05$) from the corresponding control values.

[46]. Recently, it was also reported that TRPC4 channels mediate muscarinic receptor-induced contractions in DSM using TRPC4 deficient mice [17]. Targeting the TRPC4 channels for the treatment of OAB may be problematic because of the associated side effects of the gastrointestinal system. Bladder contractile responses to muscarinic receptor-agonist, carbachol were found to be attenuated by 95% in M_3 receptor KO mice, suggesting that M_3 receptors play a major role in direct contraction of DSM [30]. Stimulation of M_3 receptors are known to activate diacylglycerol and protein kinase C [5]. These second messengers are capable of activating

certain types of TRP channel families, such as TRPC3 and TRPC6 [53]. Thus, it is possible that the second messengers produced by M₃ receptor stimulation activate TRPM4 channels in DSM cells. Although it has been reported that the density and function of muscarinic receptors can be changed in different urinary tract pathologies [36], M₃ receptors still play a predominant role in detrusor contractility in experimentally induced obstructed bladder. Furthermore, an increased sensitivity to muscarinic receptor stimulation has been reported in smooth muscle preparations from patients with idiopathic and neurogenic detrusor overactivity [42]. Isolated detrusor strips from patients with neurogenic detrusor overactivity were found to be supersensitive to carbachol [13]. Taken together, our results suggest that inhibition of TRPM4 channels under these pathological conditions may be useful to control detrusor overactivity with minimum influence on contractions of the gastrointestinal tract.

In conclusion, our study provides evidence that TRPM4 channels are constitutively active and involved in the setting of resting membrane potential, thereby maintaining basal tone and spontaneous contractile activity in the detrusor and ileal smooth muscles. Our results also suggest that TRPM4 channels are important for the initiation of the muscarinic receptor-induced contractions in DSM, whereas these channels appear to play a minor role in the signaling of muscarinic receptor-induced contraction in ileal smooth muscles. To summarize, TRPM4 channels could be an important and potential target to treat OAB.

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