Original Paper

Menthol Modulates Pacemaker Potentials through TRPA1 Channels in Cultured Interstitial Cells of Cajal from Murine Small Intestine

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Key Words

Interstitial Cells of Cajal • Menthol • Gastrointestinal Tract • Transient Receptor Potential Channel • TRP Channel

Abstract

Background/Aims: ICCs are the pacemaker cells responsible for slow waves in gastrointestinal (GI) smooth muscle, and generate periodic pacemaker potentials in current-clamp mode. **Methods:** The effects of menthol on the pacemaker potentials of cultured interstitial cells of Cajal (ICCs) from mouse small intestine were studied using the whole cell patch clamp technique. **Results:** Menthol (1 – 10 μ M) was found to induce membrane potential depolarization in a concentration-dependent manner. The effects of various TRP channel antagonists were examined to investigate the receptors involved. The addition of the TRPM8 antagonist, AMTB, did not block menthol-induced membrane potential depolarizations, but TRPA1 antagonists (A967079 or HC-030031) blocked the effects of menthol, as did intracellular GDP β S. Furthermore, external and internal Ca²⁺ levels were found to depolarize menthol-induced membrane potentials, whereas external Na⁺ was not. Y-27632 (a Rho kinase inhibitor), SC-560 (a selective COX 1 inhibitor), NS-398 (a selective COX 2 inhibitor), ozagrel (a thromboxane A₂ synthase inhibitor) and SQ-29548 (highly selective thromboxane receptor antagonist) were used to investigate the involvements of Rho-kinase, cyclooxygenase (COX), and the thromboxane pathway in menthol-induced membrane potential depolarizations, and all inhibitors were found to block the effect of menthol. **Conclusions:** These results suggest that menthol-induced membrane potential depolarizations occur in a G-protein-, Ca²⁺-, Rho-kinase-, COX-, and thromboxane A₂-dependent manner via TRPA1 receptor in cultured ICCs in murine small intestine. The study shows ICCs are targeted by menthol and that this interaction can affect intestinal motility.

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Introduction

Menthol (2-isopropyl-5-methyl-cyclohexanol) is an organic compound found in mint, and is widely used in the food, beverage, and cosmetic industries and as a treatment for various diseases [1, 2]. Menthol is a primary activator of the cold and menthol-sensitive transient receptor potential melastatin 8 (TRPM8) channel [3] and of TRP-ankyrin 1 (TRPA1) channel [4]. TRPM8 channels have been detected in dorsal root ganglia, vagal afferent neurons, and in the gut [3, 5, 6], and TRPA1 channels in enteric neurons and enteroendocrine cells [7, 8]. Although, enteric-coated peppermint formulations are used to treat vomiting [9, 10], dyspepsia, and irritable bowel syndrome [11, 12], the cellular and molecular targets of menthol that mediate its effects in gastrointestinal (GI) motility disorders have not been determined.

Interstitial cells of Cajal (ICCs) are the pacemaker cells of the GI tract [13–15] and participate in the generation, coordination, and propagation of electrical slow wave activity, the transduction of motor neural inputs from the enteric nervous system, and in the mechanosensation of smooth muscle cells [16–18]. C-kit [19] and anoctamin-1 (ANO1) [20] are expressed in ICCs and a selective molecular marker for all classes of ICCs in the human and mouse GI tract. In addition, it has been shown that the pacemaker activities of ICCs in the murine small intestine are mainly due to periodic activations of nonselective cation channels (NSCCs) [21, 22] or Cl[−] channels [23, 24]. Kim et al. [25] suggested transient receptor potential melastatin (TRPM) 7 is required for ICC pacemaker activity in the murine small intestine, and that a Ca²⁺-activated Cl[−] channel (CaCC) is involved in the slow waves generated by ICCs; this Cl[−] channel was later identified as transmembrane protein 16A (Tmem16A; ANO1) [24]. The role played by ANO1 in ICC pacemaker activity was identified in ANO1 knockout mice [26], and several other authors have shown that ANO1 participates in pacemaker activity [27]. Accordingly, it has been proposed that TRPM7 and ANO1 is responsible for the pacemaker activity in ICCs and may be considered the potential targets for the pharmacological treatment of GI motility disorders. The absence of ICCs in GI tract causes abnormally slow electrical waves and reduces smooth muscle cell contractility and intestinal transit [25] and ICCs also mediate or transduce inputs from the enteric nervous system [28]. Therefore, ICCs play an important role in the determination and regulation of GI motility.

The effect of menthol on pacemaker activity, receptor subtypes and signal transduction pathways in ICCs remains to be defined. Therefore, experiments were performed to determine whether menthol modulates pacemaker activities, to identify its receptor type and to determine specific signal transduction mechanisms in cultured ICCs from murine small intestine.

Materials and Methods

Preparation of cells and cell cultures

Animal care and experiments on animals were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Busan, Republic of Korea; Approval no. PNU-2015-1036). Balb/c mice were used in the studies. In each mouse, the small intestine was removed (from 1 cm below the pyloric ring to the cecum) and opened along the mesenteric border. Luminal contents were washed using Krebs-Ringer bicarbonate solution, and the tissues obtained were pinned to the base of a Sylgard dish. Mucosa was removed by sharp dissection. Small tissue strips of intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in nominally Ca²⁺ free solution (containing, in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na₂HCO₃ 0.44, glucose 10, sucrose 2.9, and HEPES 11 (pH 7.4) for 30 min. Cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical, Lakewood, NJ, USA, 1.3 mg ml^{−1}), bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, 2 mg ml^{−1}), trypsin inhibitor (Sigma-Aldrich, 2 mg ml^{−1}), and ATP magnesium salt (0.27 mg ml^{−1}). Cells were then plated onto sterile

glass coverslips coated with murine collagen ($2.5 \mu\text{g ml}^{-1}$; Falcon/BD, Franklin Lakes, NJ, USA) in a 35 mm culture dish, and cultured at 37°C in a 95% O_2 -5% CO_2 incubator in smooth muscle growth medium (SMGM; Clonetics, San Diego, CA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY) and murine stem cell factor (SCF; 5 ng ml^{-1} ; Sigma-Aldrich). ICCs were identified immunologically using anti-c-kit antibody (Santa cruz, Dallas, TX) at a dilution of 1:50 for 20 min [29]. ICCs were morphologically distinct from other cell types in culture, and were identified by phase contrast microscopy after being verified with anti-c-kit antibody.

Patch-clamp experiments

The physiological salt solution used to bathe cultured ICC cells (Na^+ -Tyrode) contained (in mM): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2, and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution used to examine pacemaking activity contained (in mM): KCl 140, MgCl_2 5, K_2ATP 2.7, Na_3GTP 0.1, creatine phosphate disodium 2.5, HEPES 5, and EGTA 0.1 (adjusted to pH 7.2 with KOH; The final pipette K^+ concentration is 145.4 mM). Patch-clamp techniques were conducted in whole-cell configuration to record membrane potentials (current clamp) of cultured ICCs using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, Foster, CA). We did not inject any current to record the resting membrane potential at current-clamp mode. Pipette resistance was usually around 4-5 megaohm and series resistance (or access resistance at Axon instrument) was around 10 megaohm. The acquisition frequency was around 2000Hz. Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1 and version 10.0; Axon Instruments). Data were filtered at 5kHz and displayed on an oscilloscope, a computer monitor, and/or a pen recorder (Gould 2200; Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin software (version 6.0, Microcal, USA). All experiments were performed at 30 – 33°C .

Immunohistochemistry

Cultured ICCs from the small intestines of Balb/C mice were fixed in cold acetone (4°C) for 5 min, washed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), and immersed in 0.3% Triton X-100 in PBS. After blocking with 1% BSA in 0.01 M PBS for 1 hour at room temperature (22 – 24°C), cells were incubated with a rat monoclonal antibody raised against c-Kit (Santa cruz; SC-168) or ANO1 (Santa cruz; SC-135235) at $0.5 \mu\text{g/ml}$ or with a rabbit polyclonal antibody against TRPA1 (Santa cruz; SC-32351) in PBS for 24 hours (4°C). After rinsing in PBS at 4°C , cells were labeled with fluorescein isothiocyanate (FITC)-coupled donkey anti-rabbit IgG secondary antibody (1:100; Jackson Immunoresearch Laboratories, Bar Harbor, MN, U.S.A.) or Texas red-conjugated donkey anti-rat IgG (1:100, Jackson Immunoresearch Laboratories) for 1 hour at room temperature. For double immunostaining, specimens were incubated with a mixture of antibodies raised against TRPA1 and antibody raised against c-kit for 24h at 4°C . After thorough washing with PBS, the mixture of labeled secondary antibodies was incubated for 1 hour at room temperature. Cells were examined under an FV 300 laser scanning confocal microscope (Olympus, Tokyo) at an excitation/emission wavelength appropriate for FITC (495/525 nm) or Texas red (590/615 nm). Final images were constructed using Flow-View software (Olympus).

Drugs

WS-12, Y-27632, NS-398, and ozagrel were purchased from TOCRIS (Minneapolis, MN, USA). SQ-29548 was obtained from Cayman (Ann Arbor, MI, USA). All other drugs were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared and stored, according to manufacturers' instructions. Chemicals were dissolved in Na^+ -Tyrode solution to their final concentrations immediately before use. For application of drugs, the bath chamber was superfused by gravity at a rate of ~ 2 – 3 ml/min constantly.

Statistical analysis

Results are expressed as means $\pm$ standard errors. Normality test was performed for all variables. The significances of differences between results were evaluated using the Student's unpaired *t*-test. *P*-values of < 0.05 were deemed significant. The *n* values reported in the text refer to the number of cells used in patch-clamp experiments.

Results

Effects of menthol on the pacemaker potentials of cultured ICC clusters

In current clamp mode, cells in cultured ICC clusters had a mean resting membrane potential of -57.3 ± 2.2 mV and produced pacemaker potentials (PPs) of amplitude 24.5 ± 3.2 mV ($n = 62$) at 30°C . Initially, we examined the effects of menthol on PPs. Menthol (1, 5 or $10\text{ }\mu\text{M}$) elicited membrane potential depolarizations in a concentration-dependent manner (Fig. 1A-1C); mean depolarizations were 0 mV at $0.03\text{ }\mu\text{M}$ ($n = 5$), 1.0 ± 0.1 mV at $0.1\text{ }\mu\text{M}$ ($n = 5$), 3.5 ± 0.2 mV at $0.3\text{ }\mu\text{M}$ ($n = 6$), 9.2 ± 0.9 mV at $1\text{ }\mu\text{M}$ ($n = 6$), 11.5 ± 0.8 mV at $3\text{ }\mu\text{M}$ ($n = 5$), 13.4 ± 1.3 mV at $5\text{ }\mu\text{M}$ ($n = 6$), 26.3 ± 1.5 mV at $10\text{ }\mu\text{M}$, 52.1 ± 2.1 mV at $100\text{ }\mu\text{M}$ ($n = 5$), and 57.3 ± 3.5 mV at 1 mM ($n = 5$) (Fig. 1D). These results showed menthol induced membrane potential depolarization in a concentration-dependent manner.

TRPA1 receptor was involved in menthol-induced membrane potential depolarization

Menthol has been reported to be a specific activator of TRPM8 [3, 30] and of TRPA1 [4]; both members of the TRP family. Therefore, we investigated the effects of TRPM8 and TRPA1 antagonists on membrane potential depolarization by menthol. Pretreatment with a TRPM8 antagonist (AMTB; [N- (3-aminopropyl)- 2- {[3-methylphenyl] methyl] oxy}- N - (2-thienylmethyl)benzamide hydrochloride salt [31]) at $10\text{ }\mu\text{M}$ for 5 min did not block menthol ($10\text{ }\mu\text{M}$)-induced membrane potential depolarization but reduce latency for recovery (Fig. 2A). However, pretreatment with a TRPA1 antagonist (A967079 $10\text{ }\mu\text{M}$ or HC-030031 $30\text{ }\mu\text{M}$) for 5 min blocked depolarization (Figs. 2B and 2C). Mean depolarizations were 26.1 ± 0.8 mV for AMTB ($n = 6$), 0.8 ± 0.5 mV for A967079 ($n = 7$) and 0.5 ± 0.4 mV for HC-030031 ($n = 7$; Fig. 2D). Furthermore, a TRPM8 agonist (WS-12 $100\text{ }\mu\text{M}$) had no effect on PPs (Fig. 3A) but that a TRPA1 agonist (Allyl isothiocyanate (AITC) $100\text{ }\mu\text{M}$ and cinnamaldehyde (CMA) $100\text{ }\mu\text{M}$) depolarized PPs (Fig. 3B and 3C). Mean depolarization was 0.5 ± 0.4 mV for WS-12 ($n = 5$), 24.6 ± 1.5 mV for AITC ($n = 6$), and 25.1 ± 1.7 mV for CMA ($n = 6$; Fig. 3D). In addition, we checked for the presence of TRPA1 channels by immunolabeling cultured ICCs. The co-localizations of c-kit (red) or ANO1 (red) and TRPA1 channels (green) in ICCs produced a yellow color (merge) (Fig. 4). Double labeling of ICCs from murine small intestine showed that these proteins were localized in ICCs. These results suggest menthol functions in ICCs via TRPA1 channels.

Fig. 1. Effects of menthol on pacemaker potentials in cultured ICCs from murine small intestine. (A-C) Pacemaker potentials of ICCs exposed to menthol (1 – $10\text{ }\mu\text{M}$) in current-clamp mode ($I = 0$). Menthol depolarized pacemaker potentials in a concentration-dependent manner. Responses to menthol are summarized in (D). Concentration-response curve with various menthol concentrations (0.03 , 0.1 , 0.3 , 1 , 3 , 10 , $100\text{ }\mu\text{M}$ and 1 mM) represents mean values $\pm$ SEs. * $P < 0.05$, ** $P < 0.01$: significantly different from non-treated controls.

Fig. 2. Effects of TRP channel antagonists on menthol-induced pacemaker potential depolarizations in cultured ICCs. (A) Pretreatment with a TRPM8 antagonist (AMTB) for 5 min did not block menthol-induced pacemaker potential depolarization. (B, C) However, pretreatment with TRPA1 antagonists (A967079 or HC-030031) blocked menthol-induced pacemaker potential depolarization. (D) A summary of responses to TRP channel antagonists. Bars represent mean values $\pm$ SEs. ** $P < 0.01$: significantly different from non-treated controls.

Fig. 3. Effects of TRP channel agonists on pacemaker potentials in cultured ICCs from murine small intestine. (A) Null effect of WS-12 (TRPM8 agonist) on pacemaker potentials. (B and C) Depolarizing effects of AITC and CMA (TRPA1 agonist) on pacemaker potentials. (D) Responses to TRP channel agonists are summarized. Bars represent mean values $\pm$ SEs. ** $P < 0.01$: significantly different from the control. AITC: Allyl isothiocyanate. CMA: cinnamaldehyde.

Effects of G proteins on menthol-induced membrane potential depolarization

To investigate the involvement of G proteins during menthol-induced membrane potential depolarizations, we applied GDP β S (a non-hydrolysable guanosine 5'-diphosphate analogue, which permanently inactivates G-protein binding proteins [32, 33]), using patch pipettes. When GDP β S (1 mM) was in the pipette solution, menthol-induced depolarizations were lower than under GDP β S-free conditions ($n = 5$; Fig. 5A). Mean depolarizations were 5.8 ± 1.0 mV in the presence of GDP β S and 78.1 % was reduced (Fig. 5B). These results

Fig. 4. Expressions of TRPA1 proteins in cultured ICCs. Double-labeling of cultured ICCs with TRPA1 (green) and c-kit (red; A) or ANO1 (red; B) antibodies. Cultured ICCs demonstrated TRPA1 and c-kit or ANO1 co-localization, as indicated by the yellow color (arrows). Scale bars = 10 μ m.

Fig. 5. Effects of GDP $_{\beta}$ S in the pipette on menthol-induced pacemaker potential depolarizations in cultured ICCs. (A) Pacemaking activities of ICCs exposed to menthol in the presence of GDP $_{\beta}$ S (1 mM) in the pipette. Under these conditions, menthol caused slight depolarization. (B) Response to menthol in the presence of GDP $_{\beta}$ S in the pipette. Bars represent mean values $\pm$ SEs. ** $P < 0.01$: significantly different from non-treated controls.

suggest that G-protein stimulation is required for menthol-induced membrane potential depolarization.

Effects of external Ca^{2+} -free solution and of Ca^{2+} -ATPase inhibitor in endoplasmic reticulum on the menthol-induced membrane potential depolarization

External Ca^{2+} influx and intracellular Ca^{2+} oscillations are required to generate PPs by ICCs [34]. To study the roles of external and internal Ca^{2+} , the effects of menthol were investigated under external Ca^{2+} -free conditions and in the presence of thapsigargin (an inhibitor of Ca^{2+} -ATPase in endoplasmic reticulum [35, 36]). In the presence of an external Ca^{2+} -free solution, pacemaking activity was completely abolished, and menthol did not depolarize PPs ($n = 7$; Fig. 6A). In addition, in the presence of thapsigargin, pacemaking activity was also completely abolished and menthol did not depolarize PPs ($n = 7$; Fig. 6B). Mean depolarizations were 0.6 ± 0.5 mV for external Ca^{2+} -free solutions, and 0.4 ± 0.5 mV for thapsigargin (Fig. 6C).

Fig. 6. Effects of an external Ca^{2+} -free solution and of thapsigargin (a Ca^{2+} -ATPase inhibitor in endoplasmic reticulum) on menthol-induced pacemaker potential depolarizations in cultured ICCs. (A) External Ca^{2+} -free solution abolished the generation of pacemaker potentials, and blocked menthol-induced pacemaker potential depolarization. (B) Thapsigargin abolished pacemaker potentials and also blocked menthol-induced depolarization. (C) Responses to menthol in external Ca^{2+} -free solution and in the presence of thapsigargin are summarized. Bars represent mean values $\pm$ SEs. $**P < 0.01$: significantly different from non-treated controls.

Fig. 7. Effects of voltage-dependent Na^+ channel and Na^+ free external solution on menthol-induced pacemaker potential depolarizations in cultured ICCs. (A) Tetrodotoxin (TTX; a voltage-dependent Na^+ channel blocker) had no effect on pacemaker potentials, and in the presence of TTX, menthol induced pacemaker potential depolarization. (B) Under the Na^+ -free external solution condition, pacemaker potentials were abolished, and under this condition, menthol induced pacemaker potential depolarization. (C) Responses to menthol in TTX and in external Na^+ -free solution are summarized. Bars represent mean values $\pm$ SEs.

These results suggest that both external Ca^{2+} and Ca^{2+} release from intracellular stores are responsible for menthol-induced membrane potential depolarization.

Effects of voltage-dependent Na^+ channel and Na^+ 5 mM external solution on menthol-induced membrane potential depolarization

To examine the characteristics of the PPs produced by menthol, we examined the effects of a voltage-dependent Na^+ channel blocker and of an external Na^+ 5 mM solution. To identify the involvement of a voltage-dependent Na^+ channel mediating menthol-induced PPs, we examined the effects of tetrodotoxin (TTX; a voltage-dependent Na^+ channel blocker).

Fig. 8. Effects of Y-27632, SC-560, NS-398, ozagrel, and SQ-29548 on menthol-induced pacemaker potential depolarizations in cultured ICCs. Menthol-induced pacemaker potential depolarizations in ICCs were blocked by: (A) Y-27632 (a Rho-kinase inhibitor); (B) SC-560 (a selective COX 1 inhibitor); (C) NS-398 (a selective COX 2 inhibitor); (D) ozagrel (a thromboxane A2 synthase inhibitor); and (E) SQ-29548 (a highly selective thromboxane receptor antagonist). (F) Summarizes responses to menthol in the presence of Y-27632, SC-560, NS-398, ozagrel, or SQ-29548. Bars represent mean values $\pm$ SEs. $**P < 0.01$: significantly different from non-treated controls.

TTX (1 μ M) had no effect on PPs and under these conditions, menthol induced membrane potential depolarization (Fig. 7A). In the presence of external Na^+ 5 mM solution (prepared by replacing external Na^+ by the same concentration of N-methyl-D-glucamine (NMDG)), PPs were abolished, and under these conditions, menthol (10 μ M) induced membrane potential depolarization (Fig. 7B). Mean depolarizations were 27.4 ± 1.1 mV ($n = 4$) for TTX and 24.8 ± 0.8 mV ($n = 5$) for external Na^+ 5 mM solution (Fig. 7C). These results suggest that external Na^+ is not an important driver of menthol-induced membrane potential depolarization.

Effects of Rho-kinase, cyclooxygenase, and thromboxane A2 inhibitors on menthol-induced membrane potential depolarization

Because TRPA1 can activate Rho-associated protein kinase (Rho-kinase) [37] and Rho-kinase can modulate smooth muscle contraction [38], we investigated whether the Rho-kinase pathway is required for menthol-induced membrane potential depolarization by measuring menthol-induced membrane potential depolarizations in the presence of Y-27632 (a Rho kinase inhibitor [37]). It was found that in the presence of Y-27632, menthol did not depolarize PPs (Fig. 8A). We also investigated the effects of a COX inhibitor and thromboxane inhibitor on menthol-induced membrane potential depolarization, because COX products have been shown to be involved in TRPA1-mediated signaling [39, 40]. In the presence of SC-560 (a selective COX 1 inhibitor [41]), NS-398 (a selective COX 2 inhibitor [41]), ozagrel (a thromboxane A2 synthase inhibitor [42]), or SQ-29548 (a highly selective thromboxane receptor antagonist [43]), blocked menthol-induced membrane potential depolarization ($n = 6$; Fig. 8B-E). Mean depolarizations were 4.1 ± 0.7 mV ($n = 6$) for Y-27632, 4.6 ± 1.0 mV ($n = 6$) for SC-560, 4.2 ± 0.8 mV ($n = 6$) for NS-398, 0.8 ± 0.7 mV ($n = 5$) for ozagrel and $1.6 \pm$

0.5 mV (n = 6) for SQ-29548 (Fig. 8F). These results suggest that Rho-kinase, COX pathways, and thromboxane A2 are involved in menthol-induced membrane potential depolarization.

Discussion

Menthol is a major constituent of peppermint (*Mentha piperita* L) oil and of the herbal preparations commonly used in traditional medicine to treat various GI disorders. In the scientific literature, peppermint oil was reported to be an effective treatment for nausea and vomiting [10], and its intraluminal administration was found to be a simple, safe, and convenient alternative for the prevention and amelioration of colonic spasms caused by endoscopy [44, 45]. In addition, peppermint oil has been used to treat irritable bowel disease [46]. Menthol has been reported to reduce gastric emptying rates *in vivo*, to relax whole stomach *in vitro* in mice [47], to relax intestinal and colonic muscle strips [48, 49], and to reduce small intestine motility [12]. However, Rogers et al. [50] suggested that peppermint oil increased colonic contraction with spasm. To date, these experiments are achieved mainly smooth muscle cells or muscle strips and the action and pathways mediating the effects of peppermint oil or menthol-containing products in GI tract remain controversial. GI motility patterns are highly integrated behaviours requiring coordination between smooth muscle cells and utilizing regulatory inputs from ICCs, neurons, and endocrine and immune cells [51]. However, the regulatory effects of menthol on ICC pacemaker potentials and mechanisms responsible have not been elucidated. For this reason, in this study, we was designed to investigate the receptors and signal pathways responsible for mediating the effects of menthol in cultured ICCs from murine small intestine.

Rho-kinase is a target molecule of RhoA, a small G protein that plays an important role in cell membrane signal transduction pathways, and the Rho-kinase pathway has been suggested to play a role in smooth muscle contraction [52]. Rho kinase is expressed in ICCs [53] and Mustafa et al. [54] reported that administration of menthol caused rat fundus contraction that was associated with the Rho kinase pathway. Also, TRPA1 can activate Rho-kinase to phosphorylate myosin light chain, ultimately lead to vasoconstriction in the mouse [36]. To investigate the involvement of Rho-kinase in menthol-induced membrane potential depolarization in ICCs, we used Y-27632, an inhibitor of Rho-kinase. Y-27632 treatment had no effect on PPs and in the presence of Y-27632, menthol-induced membrane potential depolarization was blocked (Fig. 8). These findings suggest Rho-kinase participates in the menthol-induced membrane potential depolarization. COX is a membrane-bound functional enzyme that transforms arachidonic acid to the intermediate prostaglandin (PG) H₂, which is then converted to prostanoids by specific synthases [55]. At least two distinct COX isoforms have been identified, namely COX-1 and COX-2 [56]. In isolated strips of rabbit bladder, it was previously concluded both COX isozymes participate in the spontaneous rhythmic contractions of ICCs [57]. Also, several papers have reported the involvement of COX and prostaglandins (PG) in menthol or TRPA1-induced effects [38, 39, 58, 59]. Furthermore, PG are generated through the activity of COX and play a significant role in its physiology in the GI tract [60]. In particular, PG are widely distributed in the GI tract and thromboxane A₂ (TXA₂) can regulate intestinal motility by modulating the pacemaker activities of ICCs [61]. In the present study, we think that these all pathways maybe involve in menthol-induced effects and all inhibitors involved in these pathways block menthol-induced effects and found the menthol-induced membrane potential depolarization was blocked by COX and TXA₂ inhibitors and, indicating that the production of PG is involved in stimulating menthol effects (Fig. 8).

Peppermint oil and menthol act at many molecular sites and are known to initiate several pathways [41, 62, 63]. In the intestinal tract, the various pharmacological effects of peppermint oil have been reported to involve Ca²⁺-channel [11], and in the GI tract, its effects have been associated with TRPM8 channel (a nonselective cation channel gated by cold stimuli) and its menthol content. TRPM8 channels are expressed in rat gastric and colonic

muscles [64], in mouse small and large intestine mucosa and myenteric plexus [6, 65], and in human colon muscles [6]. Menthol has also been reported to be able to modulate TRPA1 ion channels [4]. In the present study, a TRPM8 antagonist (AMTB) did not block menthol-induced membrane potential depolarization, whereas TRPA1 antagonists (A967079 or HC-030031) did (Fig. 2). Furthermore, TRPA1 protein was found to be localized in ICCs (Fig. 4), suggesting menthol depolarized PPs via TRPA1 receptor. Also, it has been known that TRPA1 interacts with G-protein-coupled receptors [66]. Therefore, we decided to test for G-protein involvement in menthol response (Fig. 5). In these experiments, when GDP β S was present in the pipette, the membrane potential depolarization by menthol was suppressed. This means that TRPA1 channels are coupled with G-proteins in ICCs.

External Ca²⁺ influx and intracellular Ca²⁺ oscillations are required to generate PPs by ICCs [34]. In the presence of an external Ca²⁺-free solution, pacemaking activity was completely abolished, and menthol did not depolarize PPs (Fig. 6). In addition, in the presence of thapsigargin, pacemaking activity was also completely abolished and menthol did not depolarize PPs (Fig. 6). We think that both external Ca²⁺ influx and internal Ca²⁺ release from endoplasmic reticulum are important in the menthol-induced membrane potential depolarization. We do not know which one prevails. In future, we will investigate intracellular Ca²⁺ concentration in each conditions. After that, we can understand which one prevails. In addition, emptying of intracellular Ca²⁺ stores by thapsigargin increased intracellular Ca²⁺ concentration [67]. Therefore, in the present study, thapsigargin maybe increase intracellular Ca²⁺ concentration and induce membrane potential depolarization (Fig. 6).

Intestinal motility depends on the intrinsic contractility of the circular and longitudinal smooth muscle layers and a superordinate coordination which is provided by the enteric nervous system (ENS). The ENS can be deactivated using TTX [68] and as reviewed by Lee et al. [69], TTX is a potent neurotoxin which binds to voltage-gated Na⁺ channels, inhibiting the initiation and propagation of action potentials in GI tract. In previous studies, by blocking the ENS using TTX, lidocaine induced contractility effects at the level of smooth muscle cells and/or ICCs [70, 71]. Also, Na(v)1.2, Na(v)1.3, and Na(v)1.6, and possibly Na(v)1.7, are the TTX-sensitive sodium channels expressed in the enteric nervous system [72, 73]. Therefore, TTX-sensitive sodium channels might be ubiquitous in the enteric nervous system, smooth muscle cells and ICCs of the GI tract. In the present study, TTX had no effect on PPs and under these conditions, menthol induced membrane potential depolarization (Fig. 7). Also, in the presence of external Na⁺ 5 mM solution, PPs were abolished, and under these conditions, menthol (10 μ M) induced membrane potential depolarization (Fig. 7). Therefore, external Na⁺ is not an important driver of menthol-induced membrane potential depolarization.

Slow waves play an important role in the regulation of GI motility by determining the frequency and timing of smooth muscle contractions, and ICCs, which are connected to smooth muscle cells by gap junctions to form a network, are the pacemaker cells that generate slow waves by producing spontaneous PPs. Accordingly, PPs generated by ICCs are directly transmitted to smooth muscles through gap junctions [13-15]. In addition, ICCs mediate inhibitory and excitatory signals from the enteric nervous system to smooth muscles, and thus, importantly regulate GI motility. Furthermore, it has been reported ICCs express muscarinic, adrenergic, tachykinin, somatostatin, and purinergic receptors, which strongly suggests they are targeted by a variety of endogenous and exogenous substances [22]. In the present study, TRPA1 antagonist did not cause any change on both pacemaking activity and membrane potential (Fig. 2), suggests that the TRPA1 channel is not participating in PP or in maintaining resting membrane potential in ICCs. Instead, our finding that TRPA1 is a cation channel involved in the menthol-induced membrane potential depolarization in the pacemaking activity of ICCs from the murine small intestine. It has been known that TRPM7 and ANO1 is responsible for the PP in ICCs [24-27].

In this study, we focused on the roles of menthol and of its TRPA1 receptors in ICCs from murine small intestine. We believe our findings substantially expand knowledge of the roles and signal pathways of menthol in cultured ICCs and of the physiological

and pathophysiological roles of menthol signaling. In particular, menthol was found to induce membrane potential depolarization in a G-protein-, Ca^{2+} -, Rho-kinase-, COX-, and thromboxane A_2 dependent manner via TRPA1 receptor, which may explain the excitatory effect of menthol on GI motility.

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Disclosure Statement

The authors have no disclosures.

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