Salvia miltiorrhiza induces depolarization of pacemaker potentials in murine small intestinal interstitial cells of Cajal via extracellular Ca^{2+} and Na^{+} influx

DONGHUN HAN[1], JEONG NAM KIM[1], MIN JI KWON[1], TAEWON HAN[2], YUN TAI KIM[2] and BYUNG JOO KIM[1]

[1]Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan, Gyeongsangnam-do 50612; [2]Korea Food Research Institute, Wanju-gun, Jeollabuk-do 55365, Republic of Korea

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Abstract. Interstitial cells of Cajal (ICCs) are pacemaker cells that control smooth muscle contraction in the gastrointestinal (GI) tract. The present study investigated the effects of *Salvia miltiorrhiza* (SM) on the pacemaker potentials of ICCs from the mouse small intestine *in vitro* and on GI motility *in vivo*. The whole-cell patch-clamp configuration was used to record pacemaker potential in ICCs *in vitro*, and GI motility was investigated *in vivo* by recording intestinal transit rate (ITR). Using the whole-cell patch-clamp configuration, SM depolarized the pacemaker potentials of ICCs in a dose-dependent manner. Fulvestrant blocked SM-induced effects but 1,3-dihydro-3,3-bis(4-hydroxyphenyl)-7-methyl-2H-indol-2-one did not. Additionally, 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl] phenol blocked SM-induced effects. Intracellular guanosine 5'-O-(2-thiodiphosphate), and pretreatment with extracellular Ca^{2+}- and Na^{+}-free solutions also blocked SM-induced effects. Furthermore, ITR values were increased by SM *in vivo* and SM elevated the levels of motilin (MTL). The SM-induced increase in ITR was associated with increased protein expression levels of c-kit and the transmembrane protein 16A (TMEM16A) channel. In addition, SM induced pacemaker potential depolarization through estrogen receptor β in a G protein-dependent manner via extracellular Ca^{2+} and Na^{+} regulation in the murine small intestine *in vitro*. Moreover, SM increased the ITR *in vivo* through the MTL hormone via c-kit and TMEM16A-dependent pathways. Taken together, these results suggested that SM may have the ability to control GI motility and could be used as a GI motility regulator.

Correspondence to: Professor Byung Joo Kim, Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, 49 Busandaehakro, Yangsan, Gyeongsangnam-do 50612, Republic of Korea
E-mail: vision@pusan.ac.kr

Key words: Salvia miltiorrhiza, interstitial cells of Cajal, pacemaker potential, gastrointestinal motility, intestinal transit rate

Introduction

Salvia miltiorrhiza (SM), known as Danshen in China (1-3), is a popular traditional herbal medicine owing to its anti-inflammatory (1), antioxidative (2), and antiosteoporotic (3) activity. SM is often used alone or in conjunction with other traditional herbal medicines to treat dementia and cardiovascular diseases, such as stroke, myocardial infarction, and angina pectoris (4,5). SM also affects the gastrointestinal (GI) tract. SM pretreatment inhibits the increase in cholecystokinin (CCK) and vasoactive intestinal peptide (VIP), thereby promoting the recovery of impaired GI motility from digestive diseases caused by liver ischemia (6). SM suppresses the colon inflammation induced by dextran sulfate sodium in rats (7) and alleviates the pathological changes in the intestine, thymus, and spleen and promotes recovery in rats with acute pancreatitis (8). SM causes intestinal contraction owing to an intracellular Ca^{2+} concentration- and Ca^{2+}-calmodulin-dependent mechanism (9) and the contraction of the lower esophageal sphincter via an extracellular Ca^{2+} influx-dependent mechanism (10).

Estrogen and estrogen receptors (ERs) control various GI activities (11). Additionally, SM is involved in the activation of ERs (12). SM is clinically used to increase estrogen-like efficacy and treat postmenopausal symptoms (13). Subtypes of the ER include ERα and ERβ (14). ERα is mainly present in the uterus, mammary glands, adipose tissue, and bone and ERβ in the ovary, prostate, and cardiovascular and central nervous systems (15). Many studies are currently investigating the role of estrogen and ERs in the GI tract (11,12,16-19) but there is still a lack of knowledge on the physiological, pharmacological, and molecular biological processes involved.

Interstitial cells of Cajal (ICCs) are pacemaker cells located throughout the GI tract (20,21). Slow waves are generated by ICCs, which are electrically connected to nearby ICCs and smooth muscle cells via gap junctions; thus, the slow waves are conducted (22). However, few studies on the effect of SM on ICCs and GI motility have been conducted to date.

In the present study, we investigated the effects of SM on the pacemaker potentials of ICCs *in vitro* and GI motility *in vivo*.

Materials and methods

Preparation of the sample and high-performance liquid chromatography (HPLC) analysis. Dried SM was purchased from Boncho Co. Tanshinone I (T), tanshinone IIA (TA), and cryptotanshinone (CT) were purchased from Sigma-Aldrich; Merck KGaA. The plant sample was identified by Dr Yun Tai Kim according to a previous study (23) and a voucher specimen (#NP-0103) was deposited with the Research Group of Innovative Functional Foods, Korea Food Research Institute. Dried SM (600 g) was extracted with 70% ethanol (6,000 ml) for 4 h at 80˚C. The extract was filtered through a membrane filter (0.45 μm; EMD Millipore). After removing the solvents via rotary evaporation, the remaining extracts were freeze-dried, yielding 33.6% of the dried weight (w/w). The freeze-dried extract powder (100 mg) was dissolved in 5 ml of methanol:dimethyl sulfoxide (DMSO; 1:1, v/v), before it was filtered through a 0.45-μm regenerated cellulose membrane filter (Sartorius Stedim) and diluted in methanol/DMSO (1:1, v/v) to a final concentration of 100 μg/ml prior to the injection of 10 μl of the solution for HPLC. Analytical HPLC was performed using a Jasco HPLC system (Jasco), comprising a PU-980 pump, an AS-950-10 autosampler, and an MD-2010 Plus multi-wavelength detector. Chromatographic separation was conducted at 30˚C using a Waters Symmetry® C18 (4.6x250 mm, particle size 5 μm) column with gradient elution using a mobile phase composed of 100% methanol (mobile phase A) and water containing 0.5% (v/v) glacial acetic acid (mobile phase B). The mobile phase change was carried out with a linear gradient system from 20:80 (mobile phase A:mobile phase B, v/v) to 80:20 (mobile phase A:mobile phase B, v/v) over 30 min with a 1 ml/min flow rate, before the samples were detected at 254 nm. Quantitative analysis was replicated four times. The regression equation and correlation coefficient (r^2) of each standard curve were automatically determined using the Jasco HPLC system. The regression equations for T, TA, and CT were y=70277.0913x + 103899.0348 (R^2 was 0.99847), y=65183.0492x + 76181.5418 (R^2 was 0.99937), and y=85154.2548x + 84310.5739 (R^2 was 0.99981), respectively, indicating that a high linear correlation was achieved for all standard curves. The concentrations of T, TA, and CT were determined to be 2.61±0.131, 30.84±0.324, and 2.92±0.096 mg/g, respectively, using the peak area in the chromatogram and regression equation (Fig. 1).

Animals. A total of 82 mice (40 male and 42 female; 4-8 days old; weighing 2.0-2.3 g) of the Institute of Cancer Research (ICR) mice from the Samtako Bio Korea Co., Ltd. (Osan) were used for the ICCs experiments, 32 mice (male; 5-6 weeks old; weighing 20-25 g) for the intestinal transit rate (ITR) experiments, and 21 mice (male; 5-6 weeks old; weighing 20-25 g) for the intestinal hormones and protein expression experiments. ICCs experiments were completed within 12 h after culture and ITR experiments within 1 h. In the hormone measurement experiments, it took about 1 min to draw blood from the tail vein after immobilizing the mouse using a holder. Also, in the protein expression experiments, the mice were anesthetized and then sacrificed. Subsequently, the small intestine was removed. The whole process took ~3 min. All mice were housed in a specific pathogen-free laboratory environment under a controlled temperature (21-23˚C) and humidity (50-60%) with day and night cycles (light on at 7:00 a.m. and light off at 7:00 p.m) and *ad libitum* access to normal diet and autoclaved water. During the study, indicators of the general condition of the mice were observed daily, such as fur brightness, food and water intake, defecation and behavior. Furthermore, body weight was measured every day. According to the cellular survival status and experimental repetitions, 135 ICR mice were sacrificed in this research. There were no other causes of mortality of mice other than execution for the experiment. Before execution, their skin condition and autonomous movements were monitored, and a warm environment was required. Euthanasia of mice was performed by decapitation after anesthesia. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Brain palsy was considered effective when spontaneous or stimulating movements and squeaks were not detected, and then, the mice were decapitated. The Institutional Animal Care and Use Committee at Pusan National University (approval no. PNU-2019-2462) approved all animal care and experiments (Busan, Republic of Korea). Additionally, animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (24).

Preparation of ICCs and ICC clusters. The small intestine was removed, and the luminal contents were removed using Krebs-Ringer bicarbonate solution. As reported previously, mucosae were removed by sharp dissection and small tissue strips of intestinal muscle were equilibrated for 30 min in Ca^{2+}-free Hank's solution. Then, cells were dispersed in an enzyme solution and cultured at 37˚C in a 95% O_2-5% CO_2 incubator in a smooth muscle growth medium (Clonetics). Finally, ICCs were identified (25). The patch-clamp technique was used on ICCs that showed the network-like structures in culture.

Patch-clamp experiments. We used the whole-cell patch-clamp method to record the effects of SM on the pacemaker potentials of ICCs. For the bath solution, 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4) were used and, for the pipette solution, 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM NaGTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA (pH 7.2) were used. Patch-clamping was conducted using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1 and version 10.0; Axon Instruments). All experiments were performed at 30-31˚C. In case of Ca^{2+} free experiments, the experiment was conducted after removing 2 mM of Ca^{2+} in bath solution. In case of Na^+ 5 mM experiments, the experiment was carried out by changing 130 mM Na^+ to 130 mM *N*-Methyl-D-glucamine (NMDG).

Drugs. 1,3-Dihydro-3,3-bis(4-hydroxyphenyl)-7-methyl-2H-indol-2-one (BHPI; selective ERα antagonist) and MA2029 (MTL receptor antagonist) were purchased from Tocris Bioscience and fulvestrant (ERα and ERβ antagonist),

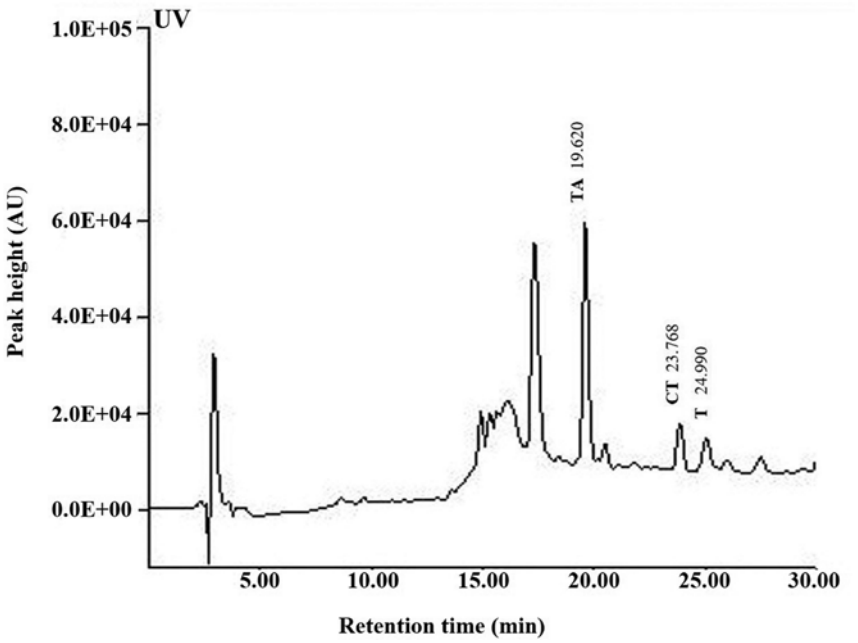

Figure 1. High-performance liquid chromatography chromatogram of the composition of an ethanol extract of *Salvia miltiorrhiza*. TA, tanshinone IIA; CT, cryptotanshinone; T, tanshinone I.

4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP; selective ERβ antagonist), guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S; for the inactivation of G protein-binding proteins) and all other drugs were obtained from Sigma-Aldrich; Merck KGaA.

ITR measurement using Evans blue in vivo. Evans blue (5%, w/v; 0.1 ml/kg) was administered through an orogastric tube 30 min after the intragastric administration of SM to ICR mice. After 30 min of Evans blue administration, the ITR was measured (the distance that Evans blue traveled from the pylorus to the most distal point).

Measurement of serum gut hormone levels. After SM (0.5 g/kg) was fed once a day for 5 days, serum levels of gut hormones, such as MTL, substance P (SP), somatostatin (SS), and vasoactive intestinal polypeptide (VIP), were detected by radioimmunoassay using commercial kits purchased from Abbkine Scientific Co., Ltd.

Western blotting. After feeding SM (0.5 g/kg) for 5 days, small intestine samples were collected. The samples were prepared by incubation with in RIPA buffer containing protease and phosphatase inhibitor cocktail (Calbiochem). The total protein extracted from the samples was quantified using the Bradford method (Bio-Rad Laboratories, Inc.). An equal amount of protein (35 μg per lane) from the samples was resolved using 8% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in TBS + 0.1% Tween-20 for 1 h at room temperature and probed with the indicated antibodies. Anti-transmembrane protein 16A (TMEM16A; Abcam), anti-c-kit (Cell Signaling Technology, Inc.), anti-transient receptor potential melastatin 7 (TRPM7; Abcam), and anti-β-actin (Santa Cruz Biotechnology, Inc.) antibodies were used. An enhanced chemiluminescence reagent kit (Advansta) was used for detection. All other procedures were carried out as previously described (26).

Statistical analysis. Results are expressed as mean ± SEM. For multiple comparison analysis, we used one-way analysis of variance (ANOVA) with Bonferroni's post hoc comparison and when only two groups were compared, Student's t-test for unpaired data was used. For statistical analyses, we used Prism 6.0 (GraphPad Software Inc.) and Origin version 8.0 (OriginLab Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Functional constituents of SM. The presence of T, TA, and CT in SM was established by HPLC and their levels were quantified using calibration curves obtained from purchased standards (Fig. 1). Validation of the method used confirmed its reliability and stability.

Effects of SM on the pacemaker potentials of ICCs. ICC clusters generated spontaneous rhythmic contractions (21,22,27). Under the current clamp mode (I=0), ICCs generated pacemaker potentials with a mean resting membrane potential of -57.6±1.4 mV and mean amplitude of 24.6±1.1 mV (Fig. 2). SM (0.5-10 mg/ml) depolarized pacemaker potentials and decreased their amplitudes in a concentration-dependent manner (Fig. 2A-D). The mean degrees of depolarization by SM were 9.8±1.5 mV ($P<0.01$) at 0.5 mg/ml, 12.6±1.9 mV ($P<0.01$) at 1 mg/ml, 24.6±1.8 mV ($P<0.01$) at 5 mg/ml, and 42.2±1.9 mV ($P<0.01$) at 10 mg/ml (Fig. 2E, n=13) and the mean amplitudes were 13.3±0.8 mV ($P<0.01$) at 0.5 mg/ml, 6.9±0.7 mV ($P<0.01$) at 1 mg/ml, 2.9±0.9 mV ($P<0.01$) at 5 mg/ml, and 1.6±0.7 mV ($P<0.01$) at 10 mg/ml (Fig. 2F, n=13). These results show that SM dose-dependently depolarizes ICC pacemaker potentials.

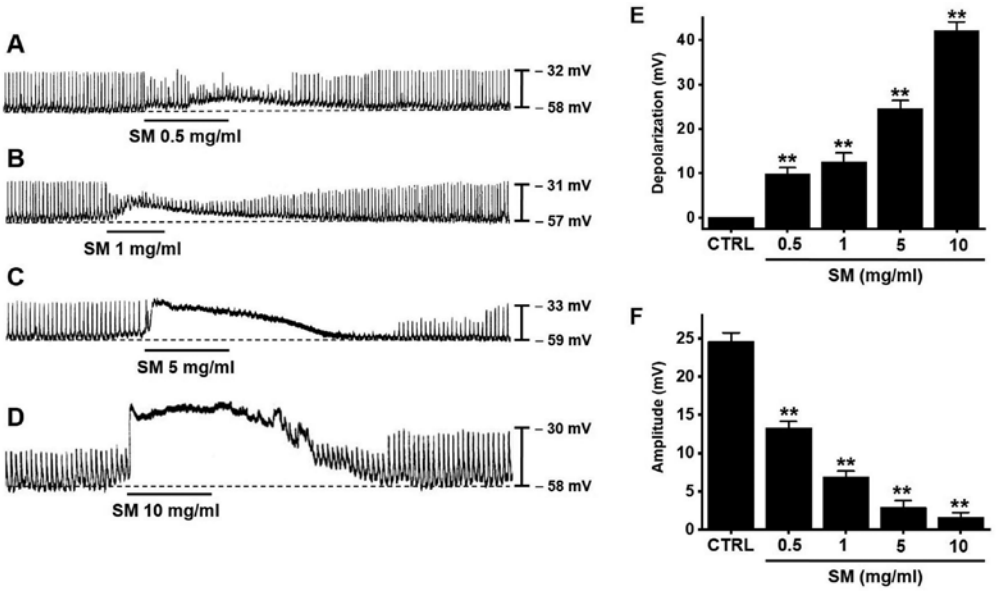

Figure 2. Effects of SM on the pacemaker potentials of ICCs. (A-D) SM depolarized pacemaker potentials and suppressed the pacemaker potential amplitude. Responses to SM are summarized in (E) and (F). Results represent mean ± SEM. **P<0.01 vs. CTRL. CTRL, control; SM, *Salvia miltiorrhiza;* ICCs, interstitial cells of Cajal.

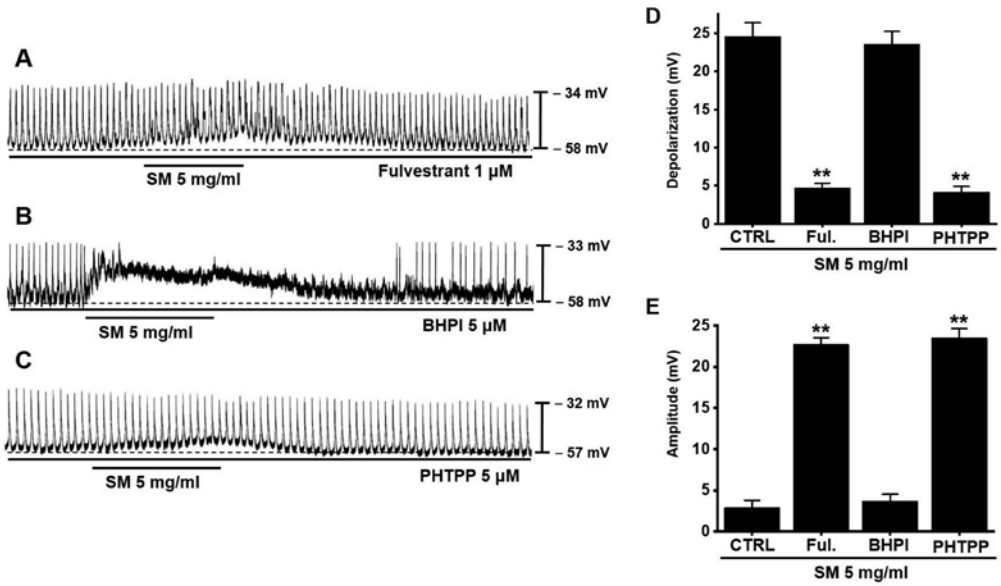

Figure 3. Effects of the estrogen receptor agonist and antagonists on SM-induced pacemaker potential depolarization in ICCs. (A) Fulvestrant blocked the SM-induced depolarization of pacemaking activity. (B) BHPI did not inhibit the SM-induced depolarization of pacemaking activity. (C) PHTPP blocked the SM-induced depolarization of pacemaking activity. (D and E) Responses to SM in the presence of fulvestrant, BHPI, and PHTPP are summarized. Results represent mean ± SEM. **P<0.01 vs. CTRL. CTRL, control; SM, *Salvia miltiorrhiza*; ICCs, interstitial cells of Cajal; Ful., fulvestrant.

Effects of ER antagonist on SM-induced pacemaker potential depolarization in ICCs. SM is involved in the activation of ERs (12). Various ER agonists and antagonists were used to confirm the relevance of the ER in the response of ICCs to SM. Daidzein (ER agonist) depolarizes pacemaking activity (25). Pretreatment with fulvestrant (both an ERα and ERβ antagonist) blocked SM-induced effects (Fig. 3A). However, pretreatment with BHPI (ERα antagonist) did not (Fig. 3B). Additionally, pretreatment with PHTPP (ERβ antagonist) blocked SM-induced effects (Fig. 3C). The mean degrees of depolarization by SM were 4.7±0.7 mV (P<0.01) with fulvestrant, 23.6±1.7 mV with BHPI, and 4.1±0.8 mV (P<0.01) with PHTPP (Fig. 3D) and the mean amplitudes were 22.7±0.8 mV (P<0.01) with fulvestrant, 3.7±0.8 mV with BHPI, and 23.5±1.1 mV (P<0.01) with PHTPP (Fig. 3E). The results show that SM affects ICC pacemaker potentials via ERβ.

Effects of GDP-β-S and extracellular Ca^{2+} and Na^{+} on SM-induced pacemaker potential depolarization in ICCs. GDP-β-S was used to identify the relevance of the G protein in the response of ICCs to SM. GDP-β-S disables G protein-binding proteins (28,29). When GDP-β-S (1 mM) was injected intracellularly, SM had a slight pacemaker potential depolarization reaction (Fig. 4A). The mean degree

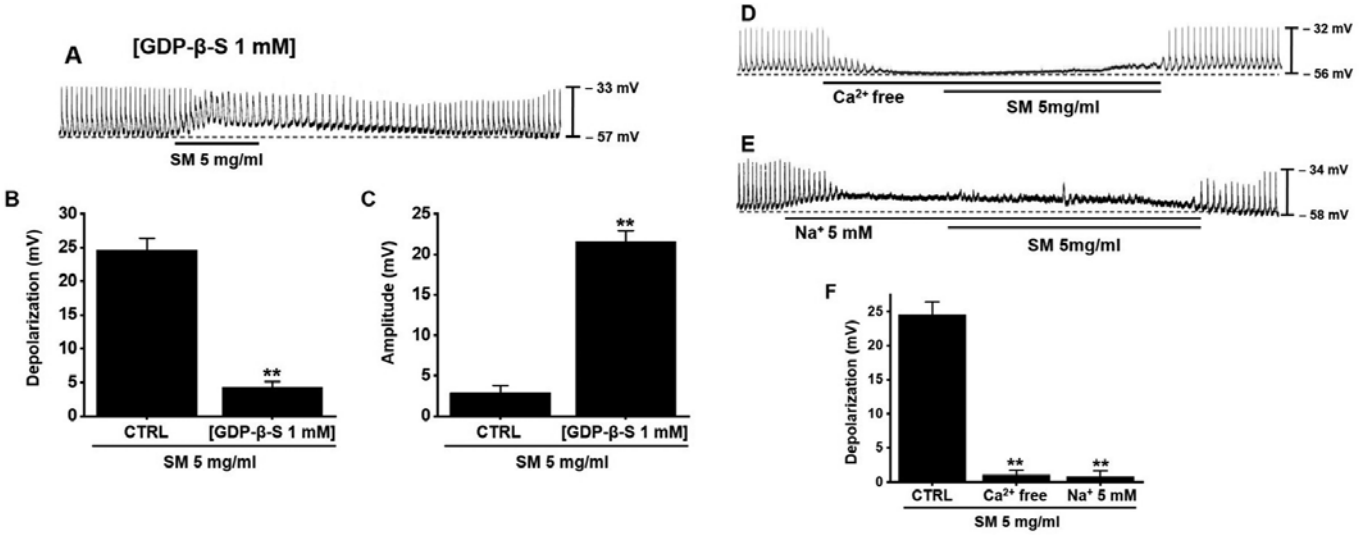

Figure 4. Effects of GDP-β-S and extracellular Ca^{2+} and Na^{+} regulation on SM-induced pacemaker potential depolarization in ICCs. (A) After the intracellular application of GDP-β-S, SM slightly depolarized ICC pacemaker potentials. (B and C) SM-induced responses in the presence of GDP-β-S are summarized. (D) SM did not induce depolarization under extracellular Ca^{2+}-free solution conditions. (E) SM did not induce depolarization under extracellular Na^{2+}-free solution conditions. (F) SM-induced responses are summarized. Results represent the mean ± SEM. **P<0.01 vs. CTRL. CTRL, control; SM, *Salvia miltiorrhiza;* ICCs, interstitial cells of Cajal.

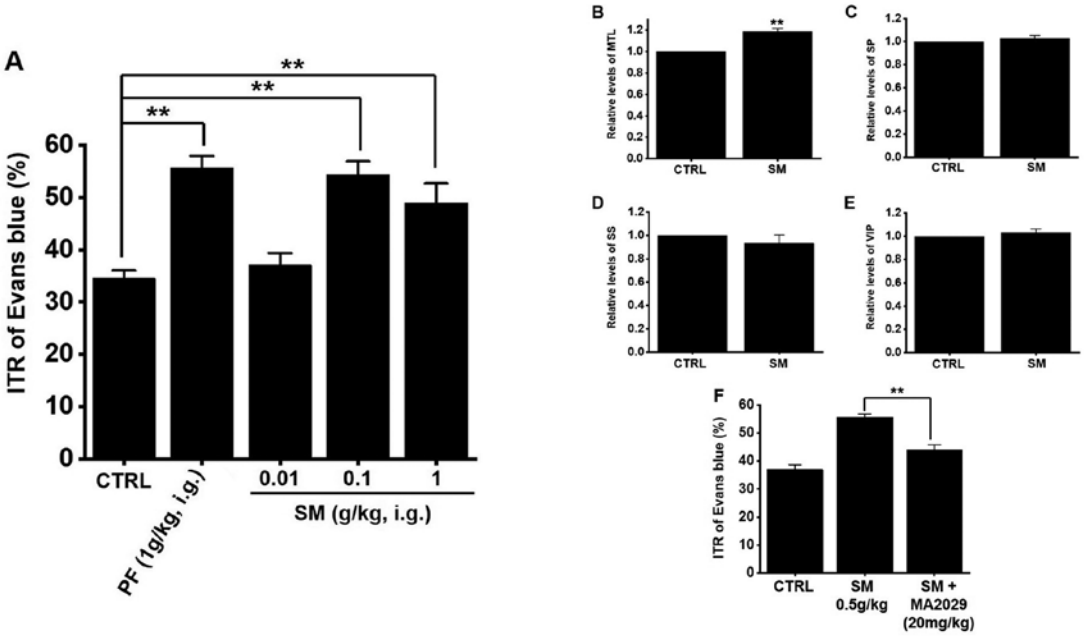

Figure 5. Effects of SM on ITR and intestinal hormones in mice. (A) SM increased ITR. Levels of GI hormones such as (B) MTL, (C) SP, (D) SS, and (E) VIP in the serum were measured using a radioimmunoassay technique. (F) Feeding SM and MA2029, motilin receptor antagonist together for 5 days reduced ITR. Results represent the mean ± SEM. **P<0.01 vs. CTRL or as indicated. SM, *Salvia miltiorrhiza*; ITR, intestinal transit rate; GI, gastrointestinal; MTL, motilin; SP, substance P; SS, somatostatin; VIP, vasoactive intestinal peptide; CTRL, control; PF, *Poncirus trifoliata* Raf.

of depolarization by SM was 4.3±0.8 mV (P<0.01) with GDP-β-S (Fig. 4B) and the mean amplitude was 21.6±1.3 mV (P<0.01) with GDP-β-S (Fig. 4C). Extracellular Ca^{2+} and Na^{+} have important roles in GI motility (30,31). To investigate the involvement of extracellular Ca^{2+} and Na^{+} in the SM-induced response in ICCs, we conducted experiments with no extracellular Ca^{2+} or Na^{+}. Pre-treatment with no extracellular Ca^{2+} solution (Fig. 4D) or no extracellular Na^{+} (Fig. 4E) abolished the pacemaker potentials and inhibited the SM-induced response in ICCs. The mean degree of depolarization by SM was 1.1±0.7 mV (P<0.01) with no extracellular Ca^{2+} and 0.8±0.7 mV (P<0.01) with no extracellular Na^{+} (Fig. 4F). The results suggest that G protein and extracellular Ca^{2+} and Na^{+} influx are involved in the SM-induced response in ICCs.

Effects of SM on ITR and intestinal hormones of normal mice. ITR was measured 30 min after the administration of Evans blue in normal mice. In normal mice, the average ITR was 34.5±1.5% (Fig. 5A). After the administration of SM, ITR was 37.1±2.3% at 0.01 g/kg, 54.4±2.5% (P<0.01) at 0.1 g/kg,

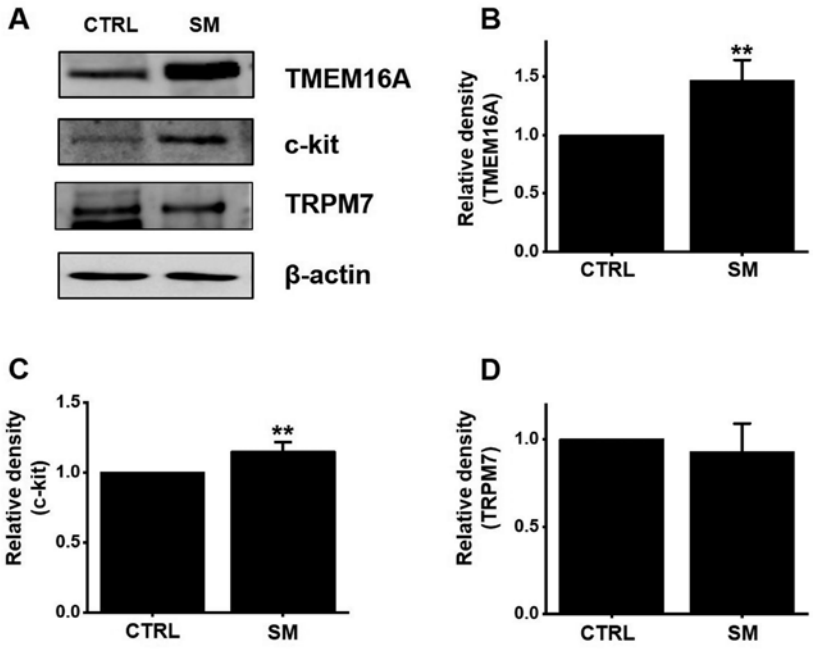

Figure 6. Effects of SM on the expression of c-kit, TMEM16A, and TRPM7 in mice. (A) Western blotting showed that the expression of c-kit and TMEM16A increased considerably but that of TRPM7 was almost unchanged. (B-D) Expression of c-kit, TMEM16A, and TRPM7 is presented as band density relative to CTRL. Results represent the mean ± SEM. **P<0.01 vs. CTRL. CTRL, control; SM, *Salvia miltiorrhiza*; TMEM16A, transmembrane protein 16A; TRPM7, transient receptor potential melastatin 7.

and 48.9±3.8% (P<0.01) at 1 g/kg (Fig. 5A). In comparison, *Poncirus trifoliata* Raf., which is another herbal medicine commonly used in GI motility studies, showed an average ITR of 55.7±2.2% (P<0.01), similar to the results of a past study (32). GI hormone levels in the mouse serum were evaluated by radioimmunoassay. The level of MTL in the GI was significantly elevated (Fig. 5B) but the levels of SP (Fig. 5C), SS (Fig. 5D), and VIP (Fig. 5E) showed no significant changes after SM administration. Furthermore, feeding SM (0.5 g/kg) and MA2029, MTL receptor antagonist together for 5 days reduced ITR (Fig. 5F). These results suggest that the SM increases ITR through an increase in MTL.

Effects of SM on the protein expression of TMEM16A, c-kit, and TRPM7. The TMEM16A (33,34) and TRPM7 (21) channels are involved in ICC activity. Furthermore, c-Kit is a transmembrane protein associated with the density of ICCs (35). Therefore, TMEM16A, TRPM7, and c-Kit may play a role in the treatment of GI motility disorders. After treatment with SM, the expression of TMEM16A, TRPM7, and c-Kit was evaluated using western blotting. The expression of TMEM16A and c-kit increased considerably after SM treatment (Fig. 6A). TMEM16A and c-kit expression significantly increased by 46.7 and 15.3%, respectively (P<0.01) after SM treatment (Fig. 6B and C). The expression of TRPM7 was almost unchanged (Fig. 6D). The results suggest that the SM-induced ITR increase is mediated through an increase in TMEM16A and c-kit expression.

Discussion

SM, a plant belonging to the Lamiaceae family, is widely used as a traditional herbal medicine to promote blood circulation, slow blood congestion, suppress edema, and maintain cognitive equilibrium (36). Additionally, it has anti-inflammatory (1), antioxidative (2), and antiosteoporotic (3) activities. Furthermore, SM is often used alone or in conjunction with other traditional herbal medicines to treat dementia and cardiovascular diseases, such as stroke, myocardial infarction, and angina pectoris (4,5). SM also affects the GI tract. SM pretreatment inhibits the increase in CCK and VIP levels, thereby promoting the recovery of impaired GI motility from digestive diseases caused by liver ischemia (6). SM suppresses colon inflammation induced by dextran sulfate sodium in rats (7) and alleviates the pathological changes in the intestine, thymus, and spleen and promotes recovery in rats with acute pancreatitis (8). SM causes intestinal contraction owing to intracellular Ca^{2+} concentration- and Ca^{2+}-calmodulin-dependent mechanisms (9) and contraction of the lower esophageal sphincter via an extracellular Ca^{2+} influx-dependent mechanism (10). Although there are many reports that SM is effective in the treatment of many digestive tract diseases, few have reported the effect of SM on GI motility.

ICCs provide the pacemaker activity needed for electrical slow waves in GI muscles. Slow waves are generated by ICCs, which are electrically connected to nearby ICCs and smooth muscle cells via gap junctions; thus, the slow waves are conducted (22). Slow waves are caused by phasic contraction in most areas of the GI tract. In the present study, we elucidated the mechanism by which SM depolarizes murine small intestinal ICC pacemaker potentials. We demonstrated that SM depolarized pacemaker potentials of ICCs from the murine small intestine in a dose-dependent manner (Fig. 2). We then showed that this occurred via ERβ, as this effect was inhibited by the ERβ antagonists fulvestrant and PHTPP (Fig. 3). We

also demonstrated that intracellular GDP-β-S and extracellular Ca^{2+} and Na^{+} inhibited SM-induced depolarization (Fig. 4). Moreover, ITR values were increased by SM *in vivo* (Fig. 5A) and SM elevated the level of MTL but had no effect on SP, SS, and VIP levels (Fig. 5B-E). Additionally, the SM-induced ITR increase was related to the increase in the protein expression of c-kit and the TMEM16A channel (Fig. 6). Therefore, SM may have the ability to control GI motility and could be used as a GI motility regulator in the future.

SM increases estrogenic effects and is a safe and effective complementary or alternative treatment for menopause (3,12). In this study, fulvestrant (both an ERα and ERβ antagonist) and PHTPP (ERβ antagonist) blocked SM-induced effects but BHPI (ERα antagonist) did not. SM could play a major role in the regulation of estrogen-related GI motility through the pacemaking activity of ICCs. Gender-related differences in GI motility have been broadly studied in the clinical and in numerous animal models (16,17). Female hormones, mainly estrogens, were found to affect visceral sensitivity, GI motility, and intestinal permeability leading to the conclusion that female sex hormones may play an important role in the pathophysiology of GI dysmotility diseases such as irritable bowel syndrome (IBS) (18,19). The physiological effects of female sex hormones on the body systems and on the regulation of GI homeostasis are predominantly moderated *via* ERs (37). G protein-coupled estrogen receptor (GPER) belongs to the seven transmembrane G protein-coupled receptor (GPCR) family and mediates the effects of estrogen on GI motility (38). The classical nuclear ERs (ERα and ERβ) appear to have the function of cellular and physiological responses (growth, differentiation, and proliferation) (39,40). Therefore, ERs are involved in the regulation of GI motility and they may be an important pharmacological target in the GI dysmotility therapy.

In this study, pre-treatment with no extracellular Ca^{2+} solution abolished the ICCs pacemaker potentials (Fig. 4D). It is well known that the underlying pacemaker potential in ICCs is spontaneous transient inward currents (STICs) and STICs generate depolarization, activate Ca^{2+} entry, and synchronize the openings of channels responsible for STICs (34,41). Therefore, reduced extracellular Ca^{2+} concentrations and T-type Ca^{2+} channel blockers decreased the number of STICs and firing probability of Ca^{2+} transients in ICCs (42). Also, intracellular Ca^{2+} release from endoplasmic reticulum depend on ryanodine receptors as well as amplification from IP_3 receptors and is important in generating pacemaker activity in ICCs (22,43). Therefore, when there is no extracellular Ca^{2+}, the pacemaker potential of ICCs does not occur. At this time, there was no effect by SM in this study (Fig. 4D). In addition, Tsai *et al* (9) suggested that Ca^{2+}-free Krebs solution plus EGTA also had no significant effect on SM-induced rat ileal segment contractions. There are many cells such as ICCs, smooth muscles, and platelet-derived growth factor receptor-α positive ($PDGFR\alpha^{+}$) interstitial cells in the GI tissue (35). Therefore, the effects of SM in the small intestine segments are thought to be the results of the reactions of these various cells, although it is not known exactly which cell reactions among these many cells. However, in this paper, it can be seen that when there is no extracellular Ca^{2+}, pacemaker potential does not occur in ICCs and at this time, there is no response of SM. In the future, it is thought that in-depth studies are needed on the effects of SM in each of these various cells in GI tract. In addition, both Gd^{3+} and flufenamic acid, nonselective cation channel blockers, abolished pacemaker current generation, as did a reduction in external Na^{+} concentrations (to 5 mM) (Fig. 4E) (44). These results strongly suggest that nonselective cationic channels are involved in generating the pacemaker potentials of ICCs. Furthermore, the bidirectional nature of Na^{+}/Ca^{2+} exchanger (NCX) is exploited during the slow wave cycle in ICCs (45). NCX facilitates removal of Ca^{2+} during the inter-slow wave interval and provides Ca^{2+} for sustained activation of ANO1 during the slow wave plateau phase (45). Therefore, extracellular Na^{+} also is involved in generating the pacemaker potentials of ICCs.

SM depolarized the pacemaker potentials in ICCs and increased ITR (Figs. 2 and 5A). ICCs have a pacemaker function to generate the GI motility and signaling through the tyrosine kinase receptor c-Kit in ICCs is important for development and differentiation in ICCs (20-22). GI motility patterns are achieved through coordinated contractions and relaxations of smooth muscle in the gut wall which are controlled by a number of intrinsic and extrinsic mechanisms including various humoral factors such as GI hormones (20-22). The GI hormones can be divided into main groups based upon their chemical structure. i) Gastrin-cholecystokinin family: Gastrin and cholecystokinin. ii) Secretin family: Secretin, glucagon, and VIP. iii) Somatostatin family. iv) Motilin family. v) Substance P (46). We selected 4 representative GI hormones such as MTL, SP, SS, and VIP (47,48) and conducted the experiments. These hormones are secreted by endocrine cells and islet cells. They stimulate smooth muscle cells and play key roles in the control of GI motility (49,50). In GI abnormal motility states, ICCs dysfunction may result in abnormal motility and GI hormones, leading to further GI dysfunction. Thus, altering the hormone level would promote GI motility. Therefore, changes in hormone levels are involved in the control of GI motility. In this study, MTL level increased considerably (Fig. 5B) but the levels of SP (Fig. 5C), SS (Fig. 5D), and VIP (Fig. 5E) remained unchanged after the administration of SM. In addition, feeding SM and MA2029, MTL receptor antagonist together for 5 days reduced ITR (Fig. 5F). Therefore, we think that SM may increase MTL and increased MTL may stimulate c-kit to increase ICCs activity, resulting in increased ANO1 activity rather than TRPM7 in ICCs cell membranes. Therefore, the increase in the secretion of the GI hormone MTL could be a key mechanism involved in the SM-mediated control of intestinal motility.

Organized smooth muscle contraction, such as peristalsis and segmentation in GI motility, is caused by the integrated regulation of smooth muscle cells, ICCs, enteric motor neurons, and hormones (22,35). Slow waves in ICCs are caused by Ca^{2+}-activated Cl^{-} channels, such asTMEM16A (34,51,52) and TRPM7 channels (21). Additionally, the expression of *c-kit*, a proto-oncogene, and the mechanism through the receptor kinase gene product, KIT, are essential to the conductance of the electrical rhythm (53,54). In the present study, western blotting revealed the higher expression of TMEM16A and c-kit in the murine small intestine after SM treatment (Fig. 6A-C). However, the expression of TRPM7 was almost unchanged following exposure to SM (Fig. 6A and D). Therefore, we

believe that the SM-induced ITR increase may be associated with the upregulation of TMEM16A and c-kit in ICCs.

In summary, the results of this study show that: i) SM depolarized the pacemaker potentials of ICCs; ii) PHTPP, an ERβ antagonist, inhibited SM-induced depolarization; iii) Intracellular GDP-β-S inhibited SM-induced depolarization; iv) Extracellular Ca^{2+}- and Na^{+}-free solutions blocked SM-induced depolarization; v) ITR values were increased by SM *in vivo*; vi) SM elevated the level of MTL but had no effect on SP, SS, and VIP levels; vii) SM-Induced ITR increase was related to the increase in the protein expression of c-kit and the TMEM16A channel. Taken together, the results show that SM induces pacemaker potential depolarization through ERβ in a G protein-dependent manner via extracellular Ca^{2+} and Na^{+} regulation in the murine small intestine *in vitro*. Moreover, SM increased the ITRs *in vivo* through MTL hormone via c-kit- and TMEM16A-dependent pathways. SM may control GI motility through ICCs; thus, SM could be used as a GI regulator in the future, although further research is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DH and BJK designed the research. DH, JNK, MJK, TH and YTK conducted experiments. DH, JNK and BJK analyzed the data. BJK wrote the manuscript. DH and BJK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee at Pusan National University (approval no. PNU-2019-2462) approved all animal care and experiments (Busan, Republic of Korea). Additionally, animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (23).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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