Sphingosine and FTY720 Modulate Pacemaking Activity in Interstitial Cells of Cajal from Mouse Small Intestine

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Interstitial cells of Cajal (ICCs) are the pacemakers of the gastrointestinal tract, and transient receptor potential melastatin type 7 (TRPM7) and Ca²⁺ activated Cl⁻ channels (ANO1) are candidate the generators of pacemaker potentials in ICCs. The effects of D-erythro-sphingosine (SPH) and structural analogues of SPH, that is, N,N-dimethyl-D-erythro-sphingosine (N,N-DMS), FTY720, and FTY720-P on the pacemaking activities of ICCs were examined using the whole cell patch clamp technique. SPH, N,N-DMS, and FTY720 decreased the amplitudes of pacemaker potentials in ICC clusters, but resting membrane potentials displayed little change. Also, perfusing SPH, N,N-DMS, or FTY720 in the bath reduced both inward and outward TRPM7-like currents in single ICCs, and inhibited ANO1 currents. The another structural analogue of SPH, FTY720-P was ineffective at the pacemaker potentials in ICC clusters and the TRPM7-like currents in single ICCs. Furthermore, FTY720-P had no effect on ANO1. These results suggest that SPH, N,N-DMS, and FTY720 modulate the pacemaker activities of ICCs, and that TRPM7 and ANO1 channels affect intestinal motility.

INTRODUCTION

Interstitial cells of Cajal (ICCs) are the pacemaker cells of the gastrointestinal (GI) system and generate rhythmic oscillations in membrane potentials known as slow waves (Huizinga et al., 1995; Sanders, 1996; Ward et al., 1994). The pacemaker activities of ICCs in the murine small intestine are mainly due to periodic activations of nonselective cation channels (NSCCs) (Koh et al., 2002) or Cl⁻ channels (Huizinga et al., 2002; Zhu et al., 2009). As a primary molecular candidate for NSCC responsible for pacemaking activity in ICCs, we previously suggested that transient receptor potential (TRP) melastatin 7 (TRPM7) is required for pacemaking activity in murine small intestine (Kim et al., 2005). Also, Zhu et al. (2009) suggested Ca²⁺-activated Cl⁻ channel is involved in slow wave current in ICCs and that

this channel is the transmembrane protein 16A (*Tmem16a*), which encodes anoctamin 1 (ANO1) channels expressed in human embryonic kidney (HEK)-293 cells (Yang et al., 2008). Therefore, TRPM7 and ANO1 are potentially promising new targets for the pharmacological treatment of GI motility disorders.

Recently, Qin et al. (2013) suggested that D-erythro-sphingosine (SPH) and structural analogues of SPH, that is, N,N-dimethyl-D-erythro-sphingosine (N,N-DMS) and FTY720, are potent inhibitors of TRPM7 channels, proposed a mechanism for the modulation of TRPM7, and suggested that TRPM7 serves as a direct target of them.

However, the effect of SPH and its structural analogues on the pacemaking activities of ICCs and on the ANO1, the latter of which (like TRPM7) is an important channel for pacemaking activity in ICCs, has not been fully investigated. Therefore, to identify the roles played by TRPM7 and ANO1 channels on the pacemaking activities of ICCs, we undertook to investigate the effect of SPH and its structural analogues on the pacemaking activities of ICCs and on ANO1 currents.

MATERIALS AND METHODS

Preparation of cells and cell cultures

The mice were treated ethically according to the Guidelines for the Care and Use of Animals approved by Pusan National University. Balb/c mice (3–5 days old) of either sex were anaesthetized with ether and sacrificed by cervical dislocation. Small intestines were removed from 1 cm below the pyloric ring to the cecum and opened along the mesenteric border. Luminal contents were washed out with Krebs-Ringer bicarbonate solution. The tissues so obtained were pinned to the base of a Sylgard dish and mucosa was removed by sharp dissection. Small strips of intestinal muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca²⁺ free Hank's solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na₂HCO₃, 10 mM glucose, 2.9 mM sucrose, and 11 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)

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for 30 min, and the cells obtained were dispersed in a solution containing 1.3 mg/ml collagenase (Worthington Biochemical, USA), 2 mg/ml bovine serum albumin (Sigma-Aldrich, USA), 2 mg/ml trypsin inhibitor (Sigma-Aldrich) and 0.27 mg/ml ATP. Dispersed cells were plated onto sterile glass coverslips coated with 2.5 mg/ml murine collagen (Falcon/BD, USA) in 35 mm-diameter culture dishes and cultured at 37°C in a 95% O₂-5% CO₂ incubator in smooth muscle growth medium (SMGM; Clonetics, USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and 5 ng/ml murine stem cell factor (SCF; Sigma-Aldrich). ICCs were identified immunologically by incubation with anti-*c-kit* antibody [phycoerythrin (PE)-conjugated rat anti-mouse *c-kit* monoclonal antibody; eBioscience, USA] at a dilution of 1:50 for 20 min. Since the morphology of ICCs differed from those of other cell types in culture, it was possible to identify them by phase contrast microscopy after incubation with anti-*c-kit* antibody.

Patch-clamp experiments

The physiological salt solution used to bathe cells (Na⁺-Tyrode) contained 5 mM KCl, 135 mM NaCl, 2 mM CaCl₂, 10 mM glucose, 1.2 mM MgCl₂ and 10 mM HEPES, and was adjusted to pH 7.4 with NaOH. The pipette solution contained 140 mM KCl, 5 mM MgCl₂, 2.7 mM K₂ATP, 0.1 mM NaGTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES and 0.1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), adjusted to pH 7.2 with KOH. For single ICCs, cells were placed in a bath containing 2.8 mM KCl, 145 mM NaCl, 2 mM CaCl₂, 10 mM glucose, 1.2 mM MgCl₂ and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 145 mM Cs-glutamate, 8 mM NaCl, 10 mM Cs-2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, and 10 mM HEPES-CsOH, adjusted to pH 7.2 with CsOH. The whole-cell configuration of the patch-clamp technique was used to record membrane potentials (current clamp) of cultured ICCs, and an Axopatch 1-D (Axon Instruments, UK) was used to amplify membrane currents and potentials. Command pulses were applied using an IBM-compatible personal computer running pClamp software (version 6.1; Axon Instruments). Data were filtered at 5 kHz and displayed on an oscilloscope and on a computer monitor equipped with a Gould 2200 pen recorder (Gould, USA), and analyzed using pClamp and Origin (version) software. All experiments were performed at 30°C.

Ca²⁺ activated Cl⁻ channel expression in human embryonic kidney-293T cells

The mammalian expressible plasmid for mouse ANO1/TMEM16A (pEGFP-N1-mANO1, ac isoform) was provided by Dr. Min Goo Lee (Yonsei University, Korea). Plasmids were transiently transfected into human embryonic kidney (HEK) 293T cells by using Lipofectamine Plus Reagent (Life technologies, USA) based on the manufacturer's recommended transfection protocols. Briefly, HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin at 37°C in a 90% O₂-10% CO₂ incubator. One day before the transfection, HEK293T cells were transferred on glass coverslips. For electrophysiological experiments, HEK293T cells were transfected with 0.9 µg of mANO1 plasmid and 0.1 µg of a plasmid expressing green fluorescent protein (pEGFP-N1). The cells transfected with 1 µg of pEGFP-N1 were used as negative controls. HEK-293T cells were routinely studied at 21-25°C, 24 h after transfection. The bath solution contained 146 mM HCl, 10 mM HEPES, 10 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, and 150 mM N-methyl-D-glucamine (NMDG), adjusted to pH 7.4.

The pipette solution contained 150 mM NMDG-Cl, 1 mM MgCl₂, 3 mM MgATP, 10 mM EGTA, 5 mM CaCl₂ and 5 mM HEPES at pH 7.2 (titrated with NMDG). The free calcium concentration was fixed at 200 nM by using WEBMAX-C software (C. Patton, Stanford University, www.stanford.edu/~cpatton/maxc.html).

TRPM7 Expression in HEK-293T cells

HEK-293T cells transfected with the Flag-murine LTRPC7/pCDNA4-TO construct were grown on glass coverslips with Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, blasticidin (5 µg/ml), and zeocin (0.4 mg/ml). TRPM7 (LTRPC7) expression was induced by adding 1 µg/ml tetracycline to the culture medium. Whole-cell patch-clamp experiments were performed at 21-25°C 24 h after induction by using cells grown on glass coverslips. The solutions were the same as those used for recording whole-cell currents in single ICCs.

Western blot analysis

The protocols for Western blot analysis described elsewhere. Briefly, the cells were transfected with 1 µg of mANO1. ANO1 transfected HEK293T cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer containing protease inhibitor cocktail (Thermo Scientific, USA). The protein samples were electrophoresed in 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was blocked for an hour at 4°C using 5% blocking solution. And then, the membrane was incubated with ANO1 antibody (1:1,000, Santa Cruz Biotechnology, USA) or aldolase A (1:1,000, Santa Cruz Biotechnology, USA) in 2% blocking solution overnight at 4°C. Aldolase A was used as an internal control. Next, the blots were washed and incubated with HRP-conjugated secondary antibody for an hour at room temperature. ANO1 proteins were detected by enhanced chemiluminescence (Amersham Biosciences). The intensities of protein bands were analyzed using Multi Gauge software (Fuji Film, Japan).

Drugs

D-erythro-sphingosine (SPH) was purchased from Sigma (USA), whereas N,N-dimethyl-D-erythro-sphingosine (DMS), FTY720, and FTY720-phosphate were obtained from Cayman Chemicals (USA). All stock solutions were prepared and stored, according to suppliers' instructions. Chemicals were dissolved in Na⁺-Tyrode solution to their final concentrations immediately before they were used to perfuse cells.

Statistics

Results are expressed as means ± SEs. The student's *t*-test for unpaired data was used to compare control and experimental groups. Statistical significance was accepted for *p* values < 0.05.

RESULTS

D-erythro-sphingosine decreased the amplitude of pacemaker potentials in ICC clusters

To understand the relationship between D-erythro-sphingosine (SPH) and modulation of pacemaker activity in ICCs, we examined the effects of SPH on pacemaker potentials in ICC clusters. In current clamp mode (*I* = 0), ICC had a mean resting membrane potential of -51.4 ± 1.1 mV and produced electrical pacemaker potentials (*n* = 45) of mean frequency 28 ± 2 cycles/min and mean amplitude 21.2 ± 1.5 mV (*n* = 45; Fig. 1A).

Fig. 1. D-erythro-sphingosine (SPH) decreased the amplitude of pacemaker potentials in ICC clusters. (A) In current clamp mode ($I = 0$), the addition of SPH (5–20 μM) decreased the amplitudes of pacemaker potentials. (B) Histograms showing the relation between pacemaker potential amplitudes and SPH concentrations. (C) Histograms summarizing the relation between resting membrane potentials and SPH concentrations. Bars represent mean values $\pm$ SEs. $**P < 0.01$: significantly different from the control.

The addition of SPH (5–20 μM) decreased pacemaker amplitudes, but resting membrane potentials exhibited little change (Fig. 1). Mean amplitudes were 21.5 ± 1.3 mV at 5 μM SPH ($n = 5$), 11.8 ± 1.0 mV at 10 μM ($n = 5$), and 1.3 ± 0.9 mV at 20 μM ($n = 5$) (Fig. 1B). Mean resting membrane potentials were -51.6 ± 1.1 mV at 5 μM , -50.2 ± 1.1 mV at 10 μM , and -51.2 ± 1.3 mV at 20 μM (Fig. 1C). SPH in the concentration range 5–20 μM effectively inhibited the amplitudes of the pacemaker potentials of ICCs. Concentrations of SPH > 20 μM produced no further inhibition. The IC_{50} of SPH on pacemaker activity in ICC clusters was 9.98 μM (Fig. 2A). To investigate the effect of SPH on pacemaking activity, we performed whole-cell voltage-clamp recordings on cultured single ICCs. Single ICCs were identified using phycoerythrin-bound anti-c-kit antibody. A voltage ramp from +100 mV to -100 mV evoked an outward-rectifying cation current at positive potentials when standard bath solution and pipette solutions lacked magnesium adenosine triphosphate (Fig. 2B), which are similar to those associated with the recently cloned TRPM7 channel (Nadler et al., 2001). This TRPM7 like outwardly rectifying currents are Mg^{2+} -dependent. Basal TRPM7 activity is regulated by millimolar levels of intracellular MgATP and Mg^{2+} , and hence, TRPM7 is activated by depletion of intracellular MgATP and Mg^{2+} , and inhibited by high concentrations of MgATP and Mg^{2+} (Nadler et al., 2001). To provide evidence that inhibition by internal Mg^{2+} was involved in this current, various internal Mg^{2+} concentrations were included in the pipette solution and currents were recorded. These currents in ICCs was elicited a rapid large increase in outward whole-cell currents but only a slight increase in inward currents in the absence of intracellular Mg^{2+} . At a lower Mg^{2+} concentration, the amplitude of this current was indeed larger than at a higher concentration. Furthermore, the presence of 3 mM Mg^{2+} in the pipette solution significantly inhibited this current (Kim et al., 2013a; Fig. 2Ba). These ion channels are consist of TRPM6 and TRPM7 channels. However, these currents in single ICC are inhibited by 2-APB. 2-APB has broad inhibitory effects on the TRP superfamily (Hanano et al., 2004; Kim et al., 2013a; Ma et al., 2000; Schindl et al., 2002; Trebak et al., 2002). Also 2-APB is a useful tool for differentiating TRPM6 and TRPM7. At micromolar concentrations, 2-APB enhanced TRPM6 but inhibited TRPM7 currents (Kim et al., 2013a; Montell-Zoller et al.,

2003). In the presence of 100 μM 2-APB, the amplitude of the currents were inhibited by $73.5\% \pm 1.8\%$ outwardly and $85.3\% \pm 2.1\%$ inwardly (Fig. 2Bb). The inhibition by 2-APB suggests that TRPM7 channels are involved in this current, and not TRPM6 channels. Also, we changed the chloride concentrations 145 mM Cs-glutamate to 145 mM Cs-Cl in the pipette solution. In this conditions, we got the outwardly rectifying currents (Fig. 2C). However, these currents did not have the characteristics of Mg^{2+} -dependence (data not shown) and did not showed the I-V curve of overexpressed TRPM7 in HEK 293 cells (Nadler et al., 2001). Perfusing SPH (20 μM) into the bath reduced both inward and outward TRPM7-like currents in single ICCs ($n = 5$) (Fig. 2D). Recently, Zhu et al. (2009) suggested that Ca^{2+} -activated Cl^- conductance is involved in ICC slow wave currents and that ANO1 participates in ICC pacemaking activity. Therefore, we investigated the effect of SPH on ANO1. ANO1 channels were overexpressed in HEK293T cells, and whole cell currents were recorded using patch-clamp techniques (Fig. 2E). To determine the current-voltage (I-V) relationships, a ramp pulse was applied from -100 mV to +100 mV for 2 s. ANO1 currents were not activated and perfusing SPH into the bath had no effects in mock transfected cells (Figs. 2F and 2G). However, perfusing SPH (1 or 10 μM) into the bath reduced both inward and outward currents in ANO1 overexpressed in HEK293T cells ($n = 10$) (Figs. 2H and 2I).

N,N-dimethyl-D-erythro-sphingosine decreased the amplitude of pacemaker potentials in ICC clusters

To understand the relationship between N,N-dimethyl-D-erythro-sphingosine (N,N-DMS) and the modulation of pacemaking activity in ICCs, we examined the effects of N,N-DMS on pacemaker potentials in ICC clusters. The addition of N,N-DMS (5–20 μM) decreased the amplitude of pacemaker potentials, but resting membrane potentials exhibited little change (Fig. 3A). The amplitudes were 21.3 ± 1.2 mV at 5 μM N,N-DMS ($n = 5$), 14.7 ± 0.9 mV at 10 μM ($n = 5$), and 1.2 ± 0.8 mV at 20 μM ($n = 5$) (Fig. 3B), and resting membrane potentials were -51.2 ± 0.8 mV at 5 μM , -50.6 ± 0.9 mV at 10 μM , and -50.8 ± 0.8 mV at 20 μM (Fig. 3C). N,N-DMS at concentrations from 5–20 μM effectively inhibited the amplitudes of ICC pacemaker potentials. However, N,N-DMS concentrations > 20 μM produced no fur-

Fig. 2. Effects of SPH on pacemaker potentials in ICC clusters, on TRPM7-like currents in single ICCs, and on Ca^{2+} -activated Cl^- channels overexpression in HEK 293T cells. (A) Concentration-dependent inhibition of pacemaker potentials in ICC clusters. The estimated median inhibitory concentration value for SPH was 9.98 μM . (B) (a) Representative TRPM7-like currents in single ICCs at 0, 1, or 3 mM $[Mg^{2+}]_i$. A voltage ramp from +100 to -100 mV was applied from a holding potential of -60 mV. (b) Effect of 2-APB on TRPM7-like currents. I-V curves in the absence (■) and presence (▲) of 100 μM 2-APB. (C) Representative outwardly rectifying currents in the conditions of CsCl in pipette solutions in single ICCs. (D) Representative TRPM7-like currents in single ICCs at an SPH concentration of 20 μM . (E) Protein samples from HEK293T cells transfected with pCMV-hANO1 and mock plasmids were used as positive and negative controls, respectively. Aldolase A was used as a loading control. (F) Representative trace of the effect of SPH on Mock transfected HEK 293T cells. SPH had no effects on Mock transfected HEK 293T cells. (G) Representative I-V relationships for (F). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV. (H) Representative trace of the effect of SPH on Ca^{2+} -activated Cl^- currents in HEK 293T cells. SPH inhibited Ca^{2+} -activated Cl^- currents in HEK 293T cells. (I) Representative I-V relationships for (H). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV.

Fig. 3. N,N-dimethyl-D-erythro-sphingosine (N,N-DMS) decreased the amplitudes of pacemaker potentials in ICC clusters. (A) In current clamp mode ($I = 0$), the addition of N,N-DMS (5–20 μM) decreased the amplitudes of pacemaker potentials. (B) Histograms showing the relation between pacemaker potential amplitudes and N,N-DMS concentrations. (C) Histograms showing the relation between resting membrane potentials and N,N-DMS concentrations. Bars represent mean values $\pm$ SEs. ** $P < 0.01$: significantly different from the control.

ther inhibition. The IC_{50} of N,N-DMS on pacemaking activity in ICC clusters was 9.06 μM (Fig. 4A). To investigate the effect of N,N-DMS on pacemaking activity, we performed whole-cell voltage-clamp recordings in cultured single ICCs. A voltage ramp from +100 mV to -100 mV evoked an outward-rectifying cation current at positive potentials when a standard bath solution and pipette solutions lacking magnesium adenosine triphosphate were used (Fig. 4B). Perfusing N,N-DMS (20 μM) into the bath reduced both inward and outward TRPM7-like currents in single ICCs ($n = 5$) (Fig. 4B). Next, we investigated the effect of N,N-DMS on ANO1 currents. We overexpressed ANO1 in HEK293T cells, and recorded whole cell currents using patch-clamp techniques. To determine the current-voltage (I - V) relationships, we applied a ramp pulse from -100 mV to +100 mV for 2 s. ANO 1 currents were not activated and perfusing N,N-DMS into the bath had no effects in mock transfected cells (Fig. 4C and 4D). However, perfusing N,N-DMS (1 or 10 μM) into the bath reduced both inward and outward currents in ANO1 overexpressed in HEK293T cells ($n = 10$) (Figs. 4E and 4F).

FTY720 decreased the amplitude of pacemaker potentials in ICC clusters

To understand the relationship between FTY720 and the modulation of pacemaking activity in ICCs, we examined the effects of FTY720 on pacemaker potentials in ICC clusters. The addition of FTY720 (1–10 μM) decreased the amplitudes of pacemaker potentials, but resting membrane potentials exhibited little change (Fig. 5). Mean amplitudes were 21.0 ± 0.8 mV at 1 μM FTY720 ($n = 5$), 11.7 ± 1.0 mV at 5 μM ($n = 5$), and 1.2 ± 0.7 mV at 10 μM ($n = 5$) (Fig. 5B), and mean resting membrane potentials were -51.2 ± 0.8 mV at 1 μM , -51.6 ± 0.9 mV at 5 μM , and -53.2 ± 0.9 mV at 10 μM (Fig. 5C). FTY720 at concentrations from 1–10 μM effectively inhibited the amplitudes of pacemaker potentials of ICCs. Concentrations of FTY720 of > 10 μM produced no further inhibition. The IC_{50} of FTY720 on pacemaker activity in ICC clusters was 4.82 μM (Fig. 6A). To investigate the effect of FTY720 on pacemaking activity, we performed whole-cell voltage-clamp recordings in cultured single ICCs. A voltage ramp from +100 mV to -100 mV evoked an outward-rectifying cation current at positive potentials when standard bath solution and pipette solutions lacking magnesium

adenosine triphosphate were used (Fig. 6B), which is similar to that described for the recently cloned TRPM7 channel (Nadler et al., 2001). Perfusing FTY720 (10 μM) into the bath reduced both inward and outward TRPM7-like currents in single ICCs ($n = 5$) (Fig. 6B). Next, we investigated the effect of FTY720 on ANO1. We overexpressed ANO1 in HEK 293T cells. Whole cell currents were recorded using patch-clamp techniques. To determine the current-voltage (I - V) relationship, we applied a ramp pulse from -100 mV to +100 mV for 2 s. In addition, perfusing FTY720 (1 or 10 μM) into the bath reduced both inward and outward ANO1 currents ($n = 10$) (Figs. 6C and 6D). Qin et al. (2013) suggested that among the structural analogues of phospholipids, FTY720-P had no effect on TRPM7 channel. In the present experimental systems, FTY720-P also had no effect on pacemaking activity in ICCs (Fig. 7). Perfusing FTY720-P into the bath did not inhibit TRPM7-like currents in single ICCs (Fig. 8A). ANO 1 currents were not activated and perfusing FTY720-P into the bath had no effects in mock transfected cells (Figs. 8B and 8C). Furthermore, FTY720-P had no effect in ANO1 overexpressed in HEK293T cells ($n = 10$) (Figs. 8D and 8E).

Sphingosine 1-phosphate (S1P) had no effects on TRPM7 or ANO1 currents

Recently, Kim et al. (2013b) suggested that S1P depolarized the resting membrane and increased tonic inward pacemaker currents in ICCs. However, there were no results about the effects of S1P on TRPM7 or ANO1 currents. Perfusing S1P (10 μM) into the bath had no effects in TRPM7 overexpressed HEK293T cells ($n = 5$) (Fig. 9A). Next, we investigated the effect of S1P on ANO1 currents. We overexpressed ANO1 in HEK293T cells, and recorded whole cell currents using patch-clamp techniques. To determine the current-voltage (I - V) relationships, we applied a ramp pulse from -100 mV to +100 mV for 2 s. ANO 1 currents were not activated and perfusing S1P into the bath had no effects in mock transfected cells (Figs. 9B and 9C). Also, perfusing S1P (1 or 10 μM) into the bath had no effects in ANO1 overexpressed in HEK293T cells ($n = 10$) (Figs. 9D and 9E). These results suggest that these effects may be dependent on intracellular signaling molecules and pathways. Taken together, our data suggest that SPH, N,N-DMS, and FTY720 play impor-

Fig. 4. Effects of N,N-DMS on pacemaker potentials in ICC clusters, on TRPM7-like currents in single ICCs, and on Ca^{2+} -activated Cl^- channel overexpression in HEK 293T cells. (A) Concentration-dependent inhibition of pacemaker potentials in ICC clusters. The estimated median inhibitory concentration value for N,N-DMS was $9.06 \mu\text{M}$. (B) Representative TRPM7-like currents in single ICCs at a N,N-DMS concentration of $20 \mu\text{M}$. (C) Representative trace of the effect of N,N-DMS on Mock transfected HEK 293T cells. N,N-DMS had no effects on Mock transfected HEK 293T cells. (D) Representative I-V relationships for Mock transfected HEK 293T cells. A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV. (E) Representative trace of the effect of N,N-DMS on Ca^{2+} -activated Cl^- currents in HEK 293T cells. N,N-DMS inhibited Ca^{2+} -activated Cl^- currents in HEK 293T cells. (F) Representative I-V relationship of HEK 293T cells. A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV.

Fig. 5. FTY720 decreased the amplitudes of pacemaker potentials in ICC clusters. (A) In current clamp mode ($I = 0$), the addition of FTY720 (1 - $10 \mu\text{M}$) decreased the amplitudes of pacemaker potentials. (B) Histograms showing the relation between pacemaker potential amplitudes and FTY720 concentrations. (C) Histograms showing the relation between resting membrane potentials and FTY720 concentrations. Bars represent mean values $\pm$ SEs. ** $P < 0.01$: significantly different from the control.

Fig. 6. Effects of FTY720 on pacemaker potentials in ICC clusters, on TRPM7-like current in single ICCs, and on overexpressed Ca^{2+} -activated Cl^- channels in HEK 293T cells. (A) Concentration-dependent inhibition of pacemaker potentials in ICC clusters. The estimated median inhibitory concentration value for FTY720 was 4.82 μM . (B) Representative TRPM7-like currents in single ICCs at a FTY720 concentration of 10 μM . (C) Representative trace of the effect of FTY720 on Mock transfected HEK 293T cells. FTY720 had no effects on Mock transfected HEK 293T cells. (D) Representative I-V relationships for Mock transfected HEK 293T cells. A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV. (E) Representative trace of the effect of FTY720 on Ca^{2+} -activated Cl^- currents in HEK 293T cells. FTY720 inhibited Ca^{2+} -activated Cl^- currents in HEK 293T cells. (F) Representative I-V relationships of HEK 293T cells. A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV.

Fig. 7. FTY720-P has no effect on pacemaker potentials in ICC clusters. (A) In current clamp mode ($I = 0$), the addition of FTY720-P (1-10 μM) had no effect on pacemaker potentials.

tant roles in the modulation of pacemaking activity through TRPM7 and ANO1 in ICCs.

DISCUSSION

We present several new findings. First, we found that SPH, N,N-DMS and FTY720 decrease the amplitude of pacemaker potentials in ICC clusters, whereas FTY720-P does not. Second, SPH, N,N-DMS, and FTY720 inhibited TRPM7-like currents in single ICCs, whereas FTY720-P had no effect. Third, SPH, N,N-DMS, and FTY720 inhibited the overexpression of ANO1 in HEK 293T cells, whereas FTY720-P did not. These results suggest SPH, N,N-DMS, and FTY720 play important roles in the modulation of pacemaking activity in ICCs and that they inhibit ANO1 channels.

Koh et al. (2002) suggested that ICCs possess Ca^{2+} -inhibited NSCCs for pacemaking activity. Goto et al. (2004) studied freshly isolated ICCs from murine intestine and concluded that pacemaker currents were due to a Ca^{2+} -dependent NSCCs. Subsequently, we found that TRPM7 might be responsible for NSCCs in ICCs (Kim et al., 2005). Also, Ca^{2+} -activated Cl^- channel was suggested to involve in slow wave in ICCs (Zhu et al., 2009) and this channel is made up the *Tmem16a*, which encodes ANO1 (Yang et al., 2008). Therefore, both TRPM7 and ANO1 have important roles in modulation of pacemaking activity on ICCs and GI motility.

Recently, Qin et al. (2013) suggested that D-erythro-sphingosine (SPH) and its structural analogues, N,N-dimethyl-D-

Fig. 8. Effects of FTY720-P on TRPM7-like currents in single ICCs and on Ca^{2+} -activated Cl^- channel overexpression in HEK 293T cells. (A) Representative TRPM7-like currents in single ICCs at FTY720-P 10 μM . (B) Representative trace of the effect of FTY720-P on Mock transfected HEK 293 cells. FTY720-P had no effects on Mock transfected HEK 293 cells. (C) Representative I-V relationships for (B). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV. (D) Representative trace of the effect of FTY720-P on Ca^{2+} -activated Cl^- currents in HEK293T cells. FTY720-P had no effect on Ca^{2+} -activated Cl^- currents. (E) Representative I-V relationships of (D). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV.

Fig. 9. Effects of S1P on TRPM7 and on Ca^{2+} -activated Cl^- channel overexpression in HEK293T cells. (A) Representative effects of S1P on TRPM7 overexpressed HEK293T cells. (B) Representative trace of the effect of S1P on Mock transfected HEK293T cells. S1P had no effects on Mock transfected HEK293T cells. (C) Representative I-V relationships for (B). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV. (D) Representative trace of the effect of S1P on Ca^{2+} -activated Cl^- currents in HEK293T cells. S1P had no effect on Ca^{2+} -activated Cl^- currents. (E) Representative I-V relationships of (D). A voltage ramp from +100 to -100 mV was applied from a holding potential of 0 mV.

erythro-sphingosine (N,N-DMS) and FTY720, are potent inhibitors of TRPM7 channels, and proposed a modulation mechanism. In addition, they suggested that TRPM7 is directly targeted by these entities. They demonstrated that SPH and FTY720 efficiently inhibit TRPM7 by reducing single-channel open probability in excised inside-out patches, which suggests that SPH and FTY720 block TRPM7 channel activity independently of intracellular signaling molecules and pathways, and that TRPM7 may serve as an important mediator of the physiological/pathological functions of SPH and FTY720. Furthermore, they found that SPH also strongly blocks TRPM6, but not other TRPM channels, such as, TRPM2 and TRPM4.

In this study, we found that SPH, N,N-DMS, and FTY720 decreased pacemaker potential amplitudes in cultured ICCs, but had little effect on resting membrane potentials (Figs. 1, 3, and 5). Also, perfusing SPH, N,N-DMS, or FTY720 into the bath reduced both inward and outward TRPM7-like currents in single ICCs and ANO1 currents, respectively (Figs. 2, 4, and 6). Furthermore, the other structural analogue of phospholipid sphingosine, FTY720-P, had no effect on pacemaker activity in ICCs or ANO1 currents (Figs. 7 and 8). Therefore, we believe that both TRPM7 and ANO1 are involved in pacemaking activity in ICCs (Goto et al., 2004; Kim et al., 2005; Zhu et al. 2009).

The exact functions of TRPM7 and Ca^{2+} -activated Cl^- conductances remain unclear. Mazzone et al. (2011) suggested that changes in Ca^{2+} -activated Cl^- currents expression in ICCs might contribute directly to human diabetic gastroparesis. However, Kim et al. (2008) suggested TRPM7 has an important role in gastric cancer cell death. It is evident that the exact functions of TRPM7 and Ca^{2+} -activated Cl^- conductances in the GI tract need further study. In a previous study, 5-lipoxygenase (LOX) inhibitors inhibited TRPM7 and pacemaking activity in ICCs (Kim et al., 2011). However, these inhibitors did not inhibit ANO1. Although the mechanisms responsible are not understood, TRPM7 might have a more important role in pacemaking activity than ANO1.

ICCs are involved in physiological GI motility, and therefore, are of clinical importance in many bowel disorders, which include inflammatory bowel disease, chronic idiopathic intestinal pseudo-obstruction, intestinal obstruction with hypertrophy, achalasia, Hirschsprung's disease, juvenile pyloric stenosis, juvenile intestinal obstruction, and anorectal malformation (Sanders et al., 1999). Discovering the molecules involved in the generation of pacemaker activity in ICCs may lead to dramatic new therapies for chronic GI diseases that currently result in lifelong suffering.

Recently, Kim et al. (2013b) suggested that sphingosine 1-phosphate (S1P) depolarized the resting membrane and increased tonic inward pacemaker currents in ICCs. However, S1P had no effects on TRPM7 or ANO1 (Fig. 9), and therefore, these effects may be dependent on intracellular signaling molecules and pathways. Qin et al. (2013) suggested that SPH and its analogues inhibit TRPM7 by reducing channel open probability without changing single channel conductance in inside-out patches. Moreover, although the positively charged FTY720 and SPH potentially inhibit TRPM7, their phosphorylated analogues FTY720-P and S1P do not have any direct effect on TRPM7, which could be due to the fact that the net positive charge is internally neutralized as the zwitterions in S1P and FTY720-P (Qin et al., 2013; Zheng et al., 2006). In previous studies, activation of TRPM7 requires the availability of PIP_2 (Runnels et al., 2002; Xie et al., 2011), and depletion of PIP_2 inactivates TRPM7 (Qin et al., 2013). Moreover, it has been demonstrated that positive charge screening of PIP_2 inhibits TRPM7 activity (Ko-

zak et al., 2005). Therefore, it is plausible that SPH and FTY720 may inhibit TRPM7 channel activity by masking negative charges of PIP_2 , thereby inactivating TRPM7 by reducing single channel open probability (Qin et al., 2013). Also, intracellular Mg^{2+} and Mg-ATP have been demonstrated to play important roles in regulating TRPM7 channel functions (Demeuse et al., 2006). As the experiments were conducted with Mg^{2+} -free pipette solutions, it seems that Mg^{2+} is not required for SPH and FTY720 to block TRPM7 (Qin et al., 2013). In ANO1, SPH and its analogues significantly inhibited ANO1 currents like TRPM7 (Figs. 2, 4, and 6). However, there are no other experiments about the effects of SPH and its analogues on ANO1 and we think that the mechanisms of the inhibition of SPH and its analogues on ANO1 might be the same with those of TRPM7. Nevertheless, further investigation is required to understand the detailed mechanism by which SPH and its analogues inhibit TRPM7 and ANO1.

TRPM7 has been suggested to play a central role in cellular Mg^{2+} homeostasis (Schmitz et al., 2003), in defecation rhythm in *Caenorhabditis elegans* (Vriens et al., 2004), in cholinergic vesicle fusion with the plasma membrane (Brauchi et al., 2008), in phosphoinositide-3-kinase signaling in lymphocytes (Sahni et al., 2008), and in cell death in gastric cancer (Kim et al., 2008). On the other hand, ANO1 has been suggested to play an important role in breast cancer progression (Britschgi et al., 2013), in stroke (Zhang et al., 2013), in pulmonary hypertension (Forrest et al., 2012) and in head and neck squamous cell carcinoma migration (Ruiz et al., 2012). However, until now, the effects of SPH and its structural analogues on TRPM7 and ANO1 currents and on several physiological functions have not been investigated.

The present study shows that both TRPM7 and ANO1 channels modulate intestinal motility, and thus, potential pharmacologic targets for the treatment of motor disorders of the gut.

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