Effects of Sphingosine-1-Phosphate on Pacemaker Activity of Interstitial Cells of Cajal from Mouse Small Intestine

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Interstitial cells of Cajal (ICC) are the pacemaker cells that generate the rhythmic oscillation responsible for the production of slow waves in gastrointestinal smooth muscle. Spingolipids are known to present in digestive system and are responsible for multiple important physiological and pathological processes. In this study, we are interested in the action of sphingosine 1-phosphate (S1P) on ICC. S1P depolarized the membrane and increased tonic inward pacemaker currents. FTY720 phosphate (FTY720P, an S1P receptor agonist) and SEW 2871 (an S1P receptor agonist) had no effects on pacemaker activity. Suramin (an S1P antagonist) did not block the S1P-induced action on pacemaker currents. However, JTE-013 (an S1P antagonist) blocked the S1P-induced action. RT-PCR revealed the presence of S1P receptor 1 (S1P1, an S1P receptor agonist) and S1P receptor 3 (S1P3, an S1P receptor agonist) in ICC. Calphostin C (a protein kinase C inhibitor), NS-398 (a cyclooxygenase-2 inhibitor), PD 98059 (a p42/44 mitogen-activated protein kinase kinase [MEK] inhibitor), or SB 203580 (a p38 inhibitor) had no effects on S1P-induced action. However, c-jun N-terminal kinase (JNK) inhibitor II suppressed S1P-induced action. External Ca²⁺-free solution or thapsigargin (a Ca²⁺ ATPase inhibitor of endoplasmic reticulum) suppressed action of S1P on ICC. In recording of intracellular Ca²⁺ ([Ca²⁺]i) concentration using fluo-4/AM S1P increased intensity of spontaneous [Ca²⁺]i oscillations in ICC. These results suggest that S1P can modulate pacemaker activity of ICC through S1P₁ by regulation of external and internal Ca²⁺ and mitogen-activated protein kinase activation.

INTRODUCTION

Bioactive sphingolipid metabolites include ceramide, sphingosine-1-phosphate (S1P), and sphingosine phosphocholine (Anliker and Chun, 2004). Various biological roles of sphingolipids include stimulation of cell survival and growth, regulation of migration and differentiation, apoptosis, modulation of cardiovascular functions, and smooth muscle contractions (Watterson et al., 2005). As a normal constituent of plasma, S1P is present at concentrations ranging from 0.4 to 1.5 μM (Murata et al., 2000). Growth factors, inflammatory cytokines, and G-protein-coupled receptor agonists, such as muscarinic and purinergic agonists, can stimulate an increase in the concentration of S1P (Alemany et al., 2000; Alvarez et al., 2007; Pfaff et al., 2005; van Koppen et al., 2001). S1P is generated from sphingosine as a result of phosphorylation by sphingosine kinase; its action as an intracellular second messenger occurs through mobilization of intracellular calcium and an extracellular ligand via binding of G-protein coupled receptors. Five of these receptors have been identified: S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ (Sanchez and Hla, 2004; Young and Nahorski, 2002). Presence of S1P receptors has been detected in the neuronal system, pulmonary system, endocrine organs, reproductive system, immune system, cardiovascular system, and gastrointestinal system, indicating their involvement in physiological regulation of many organ systems (Hla, 2004). In the gastrointestinal tract, S1P induces contraction of gastric, esophageal smooth muscle and is involved in postoperative intestinal dysmotility (Dragusin et al., 2006; Song et al., 2006; Zhou and Murthy, 2004), suggesting its ability to modulate gastrointestinal motility.

Interstitial cells of Cajal (ICC), which function as pacemaker cells of the gastrointestinal tract, serve as regulators of gastrointestinal motility by generation of slow waves in smooth muscle cells and by translation of enteric neuronal signals (Huizinga et al., 1995; Sanders, 1996; Ward et al., 1994). Disruption of ICC has been reported to evoke abnormal patterns of motility in several pathological conditions, including achalasia, obstructive intestinal disorders, pyloric stenosis, constipation, and diarrhea (Mostafa et al., 2010). As a result of these findings, ICC are now considered therapeutic targets for treatment of gastrointestinal motility disorders.

No physiological data describing the effects of S1P on pacemaker activity of ICC is currently available; therefore, we conducted this study in order to examine the effects of S1P on pacemaker currents and its signal transduction mechanisms in cultured ICC from mouse small intestine.
MATERIALS AND METHODS

Preparation of cells and tissues
All animals received ethical treatment, in accordance with the guiding principles for care and use of animals in the field of physiologic sciences, as approved by the Institutional Animal Use and Care Committee at Chosun University College of Medicine (approval number, CIACUC2012-A0002). For anesthetization, Balb/C mice (3-7 days old) of either sex received diethyl ether, and were then sacrificed by cervical dislocation. Following its excision 1 cm below the pyloric ring to the cecum, the small intestine was opened along the mesenteric border. Krebs-Ringer bicarbonate solution was used to wash away luminal contents. Isolated tissue was pinned to the base of a Stygard dish, and sharp dissection was performed for removal of mucosa. Small strips of intestinal muscle were equilibrated in calcium-free Hank’s solution containing the following constituents (in mM): KCl, 5.38; NaCl, 125; NaOH, 0.336; Na2HCO3, 0.44; glucose, 10; sucrose, 2.9; and HEPES, 11. Using Tris buffer, pH was adjusted to 7.4 for 30 min. To disperse the cells, they were incubated in a collagenase solution containing collagenase (1.3 mg/ml; Worthington Biochemicals, USA), bovine serum albumin (2 mg/ml; Sigma, USA), trypsin inhibitor (2 mg/ml; Sigma), and ATP (0.27 mg/ml) for 15 min at 37°C. After the cells had been finely chopped they were placed on sterile glass coverslips coated with poly-L-lysine in 35-mm culture dishes and incubated in smooth muscle growth medium (SMGM; Gibco, USA) and murine stem cell factor (SCF, 200 ng/ml; Sigma) at 37°C in a 95% O2-5% CO2 incubator.

Solutions and drugs
To bathe the cells, they were placed in a standard solution containing the following (in mM): KCl, 5; NaCl, 135; CaCl2, 2; glucose, 10; MgCl2, 1.2; and HEPES, 10. Using Tris buffer, the standard solution was adjusted to pH 7.4. The pipette solution contained the following (in mM): K-aspartate, 120; KCl, 20; MgCl2, 5; K-ATP, 2.7; NaGTP, 0.1; creatine phosphate disodium, 2.5; EGTA, 0.1; and HEPES, 5. Using Tris buffer, the solution was adjusted to pH 7.4.

Sphingosine 1-phosphate, suramin, SEW2871, JTE-013, calphostin C, PD 98059, SB 203580, NS-398, and thapsigargin were purchased from Sigma (USA). FTY720 phosphatase was purchased from Cayman Chemicals (USA). JNK inhibitor II (SP600125) was purchased from Calbiochem Co. (USA). Drugs used in this study were dissolved in an appropriate solvent, according to the product information.

Patch clamp experiments
The whole cell patch-clamp technique was used in recording of membrane currents (voltage clamp) and membrane potentials (current clamp). The Axopatch 1D-4 (Axon Instruments, USA) was used for amplification of currents or potentials. An IBM-compatible personal computer and pClamp software (version 9.2; Axon Instruments) were used for application of command pulse. Data were filtered at 5 KHz. All experiments were performed at 30°C.

ICC picking and RT-PCR
After 2 days of culture, an even distribution of single cells was observed. The distinct morphology of ICC was easily identified among the mixture of other cells. Patch clamp microelectrodes with a slightly larger pore size were used for collection of single cells. Following placement of the microelectrode near the single isolated cell, slight negative pressure was applied, resulting in entry of the cell into the electrode. Each cell collected was then transferred to a tube containing PBS and maintained in a chilled condition. A total of 5-10 cells were added to a single tube.

Following the manufacturer’s protocol, total RNA were extracted from the isolated ICC using Trizol reagent (Generay Biotech). During each step, care was taken to avoid loss of RNA. SuperScript one-step RT-PCR with Platinum Taq (Invitrogen, USA) was used for reverse transcription polymerase chain reaction (RT-PCR) and cDNA amplification of isolated RNA. For amplification, sense and antisense primers were selected according to the mouse sequence for indicated genes: S1P1 (NM_007901), 5'- ACC TTT GGC ACT TTT ATT GA -3' and 5'- TAA AGT AAT GCT TTT GGG GA -3' (227 bp); S1P2 (NM_010333), 5'- CCG TCA TCT TAC TGG CTA TC -3' and 5'- TTG AGC AGT GAG TAA GTG GT -3' (245 bp); S1P3 (NM_010101), 5'- AAA CCA GTG ATC CCA CCA GAC AC -3' and 5'- GAT TGG GCA TCA AAT GTA GT -3' (255 bp); S1P4 (NM_010102), 5'- TTT CCT GTT ATG CTC AAG GT -3' and 5'- ATA CAG TTG GAA CAG TTG GG -3' (259 bp); S1P5 (NM_053190), 5'- CCA ACC CCA ATA AAT AAA CA -3' and 5'- AGA GCT GTG GAC AAA GGT AA -3' (247 bp); c-Kit (AV569430), 5'- GCA CAG AAG GGA GCA CTT ATA CCT -3' and 5'-TGA AGG AGG AGG ACT TTT ATT GA -3' (240 bp); Myosin (NM_013607), 5'- AGG CAG ACC TCA TGC AGC TCC AAG A -3' and 5'- CCT CAT TCT GTT CAT CCC GAG CTT G -3' (340 bp) and PGP 9.5 (NM_011670), 5'-GCC AAC AAC CAA AAC CTG GAA -3' and 5'-GCC GTC CAC GTT GAA CAG AAT -3' (213 bp). Using these primer pairs, reverse transcription PCR was performed at 45°C for 30 min on cDNA templates derived from mRNA isolated from the separated ICC. Cycling parameters included: initial denaturation at 95°C for 5 min, then 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles followed by a 10-min final extension period at 72°C; 2 μl of product resulting from the procedure described above was used for re-amplification for another 30 cycles, followed by electrophoresis of 5 μl of this product on a 2% agarose gel and staining with ethidium bromide for visualization of DNA fragments.

Measurement of intracellular Ca2+ concentration
Fluo-4/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20°C, was used for monitoring of changes in the intracellular Ca2+ ([Ca2+]i) concentration. Cultured ICC on coverslips (25 mm) were rinsed twice using a bath solution, followed by incubation in bath solution containing 1 μM fluo-4/AM with 5% CO2 at 37°C for 5 min, and rinsed 2 more times with the bath solution. They were then mounted on a perfusion chamber, and scanned every 0.4 s using a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (× 200). Fluorescence excitation occurred at a wavelength of 488 nm, and emitted light was observed at 515 nm. The temperature of the perfusion chamber containing the cultured ICC was maintained at 30°C during scanning of Ca2+ imaging. Variations of [Ca2+]i fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging.

Statistical analysis
Data were expressed as means ± standard errors. The Student’s t and one way ANOVA followed by Dunnett’s test were applied for evaluation of differences. P values of < 0.05 were considered statistically significant. N values reported in the text.