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Effect of Sulprostone, a Prostanoid EP_3 Receptor Agonist, on Colonic Motility

朝鮮大學校 大學院

醫學科

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김 석 원

지도교수: 김 만 우

조선대학교 대학원 의학과

Prostaglandin E_2 (PGE_2)는 PGs 생성 과정의 한 종류로서 세포막에 존재하는 4 종류의 EP-수용체 (EP_1 ~ EP_4)을 통하여 위장관 평활근에 직접 작용하거나 장관 신경계를 통하여 평활근의 수축을 조절함으로서 운동성을 조절하고 있다. 일반적으로 EP_2 와 EP_4 수용체는 수축억제작용, EP_1 와 EP_3 수용체는 수축항진 작용으로 알려져 있다. 카할 사이질 세포 (Interstitial Cells of Cajal; ICCs)는 위장관내 향도잡이 세포로 평활근에 직접 지배하여 위장관 운동을 조절하고

있는 매우 중요한 역할을 하고 있다. 그러나 이 세포에 대한 EP-수용체의 분포와 그 기능에 대한 연구는 매우 미진하다. 본 연구는 대장 ICCs의 자발적 활동도에 대해 EP_3-수용체 촉진제인 sulprostone의 효과를 연구하여 다음과 같은 실험 결과들을 얻었다.

1. Sulprostone는 ICCs에서 세포막 전압의 저분극과 함께 자발적 활동의 발생 빈도를 증가시켰다.
2. EP_3-수용체 길항제인 L798106는 ICCs의 자발적 활동도에 대한 sulprostone의 효과를 차단하였다.
3. RT-PCR 실험에서 EP_3 수용체가 Ano-1 양성세포인 대장 ICCs에서 발현 되었다.
4. Sulprostone에 의한 작용은 phospholipase C 억제제인 U-73122와 세포내 내형질 세망에서 칼슘분비를 억제하는 thapsigargin에 의해서 차단되었다.
5. Clonidine과 ZD7288 (HCN channel 억제제)는 sulprostone의 작용을 차단하였다.
6. 소장 ICCs의 자발적 활동도에 대해 sulprostone의 효과는 없

었으며 또한 EP_3 수용체가 발현되지 않았다.

이상의 실험결과들로부터 대장 ICCs의 자발적 활동도에서 sulprostone에 의한 EP_3 수용체의 작용은 phospholipase C-의존성, 세포 내 칼슘을 분비하여 HCN channel의 활성화를 통한 흥분성 반응을 나타낸다. 따라서 EP_3 활성화에 의한 대장 운동의 항진기능은 ICCs를 통하여 발생될 수 있으며, 대장 운동기능이 억제된 운동성 질환에서 EP_3 수용체 조절을 통한 질환 개선에 있어 약물학적 표적 부위로서의 가능성이 있음을 나타낸다.

핵심단어: sulprostone, EP_3 수용체, 자발적 활동도, 카할 사이질 세포, 대장 운동

INTRODUCTION

Prostaglandins (PGs) are biologically active substances that synthesized from arachidonic acid through the activities of cyclooxygenase (COX)-1 and COX-2 enzymes in almost tissues (Calder, 2001). PGE_2 is the main form of COX-derived PGs. The biological actions of PGE_2 are mediated by G-protein coupled prostanoid EP receptors that are comprised of four subtypes, namely EP_1, EP_2, EP_3 and EP_4 receptors (Breyer et al., 2001). EP receptors also widely distributed to gastrointestinal (GI) tract and mediate diverse physiological functions in mucosal protection, secretion and motility by PGE_2 (Mosa et al., 2008; Takeuchi et al., 1999; Dey et al., 2006). Especially, it has been known that PGE_2 plays an important role in regulating GI motility by affecting GI smooth muscle contractility through EP receptors located in smooth muscle cells and myenteric neuronal cells (Grass et al., 2006; Morimoto et al., 1997). Generally, EP_2 and EP_4 receptors activation caused smooth muscle relaxation and decreased peristalsis, while EP_1 and EP_3 receptors activation caused smooth muscle contraction and increased peristalsis in various species (Fairbrother et al., 2011; Botella et al., 1993; Shahbazian et al., 2002).

GI smooth muscles show spontaneous phasic contractions that are mediated by electrical cyclic depolarization, termed as slow waves (Szurszewski, 1987). Interstitial cells of Cajal (ICCs) are GI pacemaker cells that generate slow waves in smooth muscles by producing spontaneous inward currents (pacemaker currents) (Thomsen et al,. 1998). ICCs are connected with smooth muscle cells by gap junctions. Thus, spontaneous pacemaker currents by ICCs are transmitted to smooth muscles through gap junctions directly and followed to evoke smooth muscle contractions. ICCs also mediate enteric motor neurotransmission in smooth muscles (Sanders et al., 2006). The disruption of ICCs networks and reduction of ICCs numbers implicated in various motility disorders, indicating that ICCs are basic modulator of GI motility and therapeutic targets in altered motility disorders (Farrugia, 2008; Jain et al., 2003).

Although EP_3 receptors are expressed by postjunctional cells in mouse ileum and by myenteric neurons in mouse colon, the identity of EP_3 receptor had not been elucidated in colonic ICCs. Therefore, this study was undertaken to investigate whether EP_3 receptor activation by sulprostone can modulate colonic motility through modulating pacemaker activity of colonic ICCs.

MATERIALS AND METHODS

Preparation of cells

The protocols used, and treatment of animals, in this study were in accordance with the guiding principles approved by the ethics committee of Chosun University, and the National Institutes of Health Guide, South Korea for the Care and Use of Laboratory Animals. Mice had free access to water, and they were fed a standard mouse diet until the day of experimentation. Balb/C mice (5–8 days old) of either sex were anesthetized with ether and euthanized by cervical dislocation. The small intestine, from 1 cm below the pyloric ring to the cecum, was removed from each mouse and opened along the mesenteric border. The colon from below the cecum to the rectum was removed, and the middle portion of the colon was used. The colon was opened along the mesenteric border. The luminal contents were washed with Krebs–Ringer bicarbonate solution. Tissues were pinned to the base of a Sylgard dish and the mucosa was removed by dissection. Small strips of the colonic muscle were equilibrated in Ca^{2+}-free Hank's solution for 30 min. Cells were dispersed with an enzyme solution comprising 1.3 mg/ml

collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma, St. Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma), and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml Falcon/BD) in 35-mm culture dishes. Cells were cultured in smooth muscle growth medium (SMGM; Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 ng/ml urine stem cell factor (SCF, Sigma) at 37°C/5% CO_2.

Electrical activity recording

Current clamp mode of patch clamp was applied to record pacemaker potentials in colonic ICCs that showed a network-like structure in cultures (2–3 days). Pacemaker potentials were amplified using Axopatch 200B (Axon Instruments, Foster, CA, USA). Data were filtered at 5 kHz and displayed on a computer monitor. Results were analyzed using pClamp and GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). All experiments were performed at 30°C.

Separation of ICCs and RT-PCR

Digested muscle by collagenase solution was chopped finely to make a single-cell suspension. The large clumps of cells were removed by spinning down in 3000 rpm for 1 min, and the supernatant containing single cells was transferred to a new tube for the separation. A Robosep Cell separating machine (StemCell Technologies Inc., Vancouver, Canada) was used for this purpose. The cells were incubated with mouse CD117 phycoerythrin (PE) labeling antibody, Magnetic nanoparticles positive selection reagent, and PE selection cocktail according to the protocol stored (manually changed) in the automated machine. After washing with PB, the result was pure, separated ICCs. Total RNA was isolated from c-Kit-positive cells using TRIzol reagent according to manufacturer specifications (Invitrogen). cDNAs were produced from the total RNA using Superscript^{TM} One-Step RT-PCR with Platinum Taq (Invitrogen). Primers used are shown in Table 1. The thermal cycler was programmed such that cDNA synthesis was followed immediately by PCR amplification automatically. The cDNA synthesis were carried out at 45°C for 30 min for reverse transcription reaction followed by 94°C for 5 min for the denaturation of cDNA hybrid. The three-step cycling process was carried out for 38 cycles at 94°C for 30 s for denaturation,

59°C for 30 s for annealing, and 72°C for 30 s for the extension. The same PCR protocol was used for myosin and CD14 except the annealing temperature was changed to 55°C. The PCR products were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

Table 1. Nucleotide sequences of the primers used for RT-PCR

Gene Name	Sequences	Size (bP)
ANO-1	(Forward) AGGCCAAGTACAGCATGGGTATCA	213
	(Reverse) AGTACAGGCCAACCTTCTCACCAA	
Myosin	(Forward) GAGAAAGGAAACACCAAGGTCAAGC	264
	(Reverse) AACAAATGAAGCCTCGTTTCCTCTC	
PGP 9.5	(Forward) GCCAACAACCAAGACAAGCTGGAA	213
	(Reverse) GCCGTCCACGTTGTTGAACAGAAT	
EP_1	(Forward) CTGGGCTCAAGGGTATAGAG	290
	(Reverse) GGTGACAGAGATGGGAAGTG	
EP_2	(Forward) CAGGAGAGGAGAGAGGACTT	261
	(Reverse) GAAGTCCGACAACAGAGGAC	
EP_3	(Forward) CAGGGAAGGATGACTGAGTA	225
	(Reverse) GGTTCTGAGGCTGGAGATA	
EP_4	(Forward) GTT GGT GGA TGA GGT TAG TG	220
	(Reverse) CTA GTG GGA GTC CAG ATG AA	

Reagents

Cells were bathed in a buffer comprising 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM, and 10 mM HEPES, with the pH adjusted to 7.2 using Tris. The pipette solution was composed of 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM Na_2GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA, adjusted to pH 7.2 with Tris. The drugs used were sulprostone, L798106, U-73122, chelerythrine, thapsigargin, clonidine, and ZD7288. All drugs were purchased from Sigma except L798106, which was purchased from Cayman Chemicals.

Statistical analysis

Data are expressed as the means ± standard errors. Differences in the data were evaluated using the Student *t* test. A P-value < 0.05 was considered statistically significant. The *n* values reported in the text refer to the number of cells used in the patch-clamp experiments.

RESULTS

Effects of sulprostone on pacemaker potentials in colonic ICCs

The patch clamp technique was tested with ICCs that exhibited network-like structures in culture (2–3 days). Under a current clamp, colonic ICCs generated pacemaker potentials and pacemaker currents at -70 mV of holding potential in voltage clamp (Fig. 1A and B). In colonic ICCs, the resting membrane potential, amplitude, and frequency were –55.8 ± 3.1 mV, 38.2 ± 6.7 mV, and 11.3 ± 2.4 cycles/5min, respectively (n = 24). The amplitude of spontaneous inward pacemaker currents was -700 ± 120 pA (n = 12). Treatment with sulprostone (10 nM, 100 nM, and 1 μM) depolarized the membrane and increased the pacemaker potential frequency, dose-dependently (Fig. 2). The summarized values and a bar graph of the effects of sulprostone on pacemaker potentials are shown in Figure 3A and B (n = 8).

Inhibition of sulprostone effects by EP_3 receptor antagonist on pacemaker potentials in colonic ICCs

To investigate whether sulprostone effects are mediated through EP_3 receptor activation, a specific EP_3 receptor antagonist (L798106) was tested. Pretreatment with L798106 (5 μM) itself blocked the generation of pacemaker potentials and under this condition, sulprostone-induced effects on pacemaker potentials were almost blocked (Fig. 4A), suggesting that sulprostone action on pacemaker potentials of colonic ICCs was mediated by EP_3 receptor. The summarized values and a bar graph of the blocking effects of L798106 on pacemaker potentials are shown in Figure 4B and C (n = 5~6).

Molecular expression of EP receptors in colonic ICCs

To support the above pharmacological effects of sulprostone on EP_3 receptor, RT-PCR with ANO-1 positive cells were performed. RT-PCR analysis revealed the mRNA transcripts for EP_3, and EP_4 receptor subtypes in ANO-1 positive cells (Fig. 5). This result indicates that prostanoid EP_3 receptors are exist in colonic ICCs and may be involved in regulating pacemaker potentials.

Effects of PLC inhibitor in EP_3-induced actions in colonic ICCs

To investigate whether phosphoinositide hydrolysis by PLC or protein kinase C is involved in sulprostone-induced effects on pacemaker potentials, U-73122 (a PLC inhibitor) and chelerythrine (a protein kinase C inhibitor) were tested. Treatment with U-73122 (10 μM) blocked the sulprostone-induced effects on pacemaker potentials (Fig. 6A). However, chelerythrine (10 μM) did not block the sulprostone-induced effects (Figure 6B). The summarized values and a bar graph of the effects of U-73122 (n = 5) and chelerythrine (n = 6) on the sulprostone-induced effects are shown in Figure 7A, B, C, and D. These results indicate that PLC mediates EP_3 receptor activation.

Effects of thapsigargin in EP_3-induced actions in colonic ICCs

Inositol(1,4,5) triphosphate-dependent periodic intracellular Ca^{2+} release from endoplasmic reticulum is a primary pacemaking mechanism in ICCs. Thus, to investigate whether intracellular Ca^{2+} release from endoplasmic reticulum is involved in sulprostone-induced effects on pacemaker potentials, thapsigargin (a Ca^{2+}-ATPase inhibitor from endoplasmic

reticulum) was tested. Treatment with thapsigargin (10 μM) itself abolished the generation of pacemaker potentials and in this condition, sulprostone-induced effects were blocked (Fig. 8A). The summarized values and a bar graph of the effects of thapsigargin (n = 5) on the sulprostone-induced effects are shown in Figure 8B and C.

Effects of hyperpolarization-activated cyclic nucleotide channel blockers in EP_3-induced actions on pacemaker channels in colonic ICCs

Recently, it was suggested that hyperpolarization-activated cyclic nucleotide (HCN) channels could possibly regulate the pacemaker activity of mouse colonic ICCs (Shahi *et al.,* 2014). To examine the role of HCN channels in sulprostone-induced effects on pacemaker channels, specific HCN channel blockers (ZD7288 and clonidine) were tested. Both pretreatment with ZD7288 (20 μM *n* = 6) or clonidine (100 μM, *n* = 5) abolished the generation potentials and in these conditions, sulprostone did not induced the excitatory effects on pacemaker potentials (Fig. 9A and B), suggesting that sulprostone-induced action is

may be mediated by activating HCN channels. The summarized values and a bar graph of the effects of clonidine and ZD7288 on the sulprostone-induced effects are shown in Figure 10A, B, C, and D.

Effects of sulprostone on pacemaker potentials in small intestinal ICCs

To evaluate whether EP_3 receptors also involves in small intestinal ICCs, sulprostone was treated. However, sulprostone (1 μM, n = 5) had no effects on pacemaker potentials of small intestinal ICCs (Fig. 11A). In RT-PCR analysis, the mRNA transcript of EP_2 receptor only detected in cultured c-kit and Ano-1 positive small intestinal ICCs (Fig. 11B).

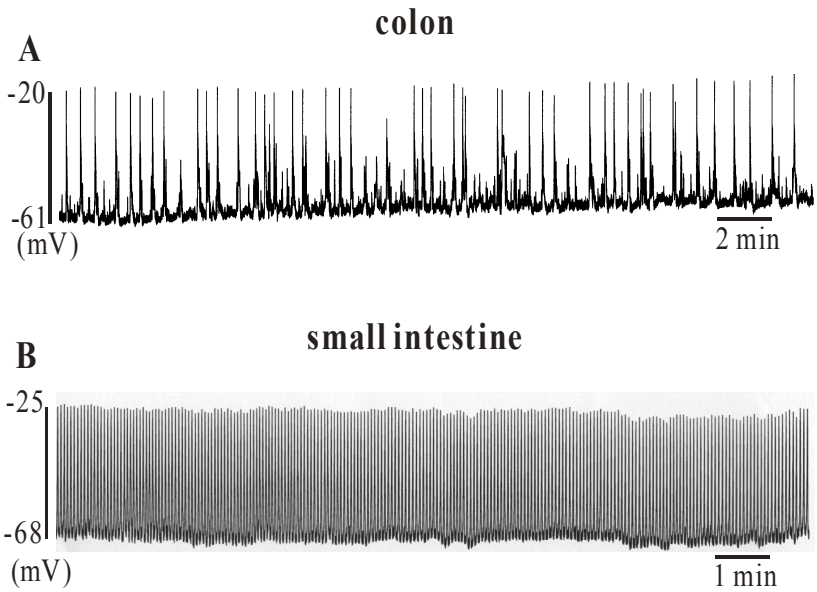

Figure 1. Typical traces of pacemaker potentials in current clamping mode in colonic (A) and small intestinal (B) ICCs from mouse.

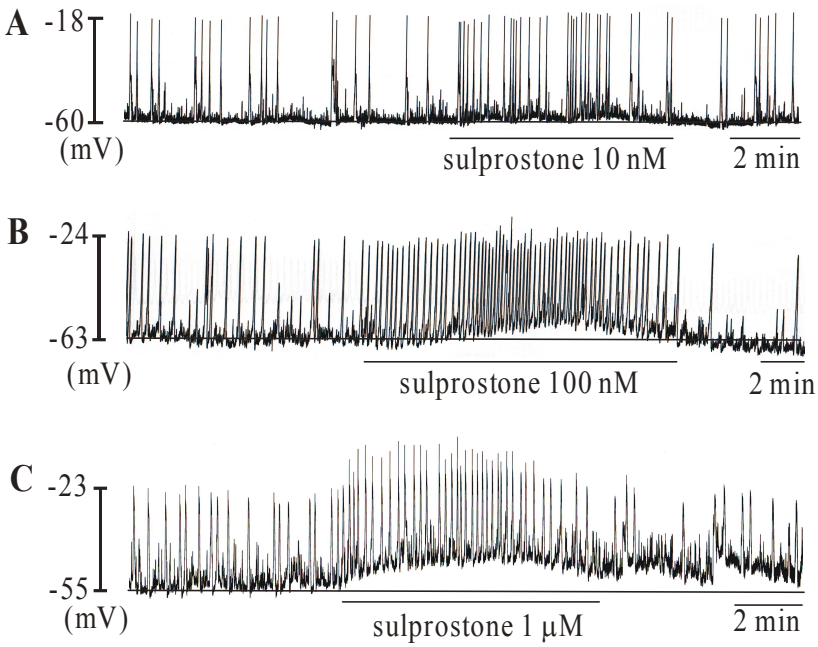

Figure 2. Effects of sulprostone on pacemaker potentials of ICCs from mouse colon. (A)-(C) The treatment of sulprostone from 1 nM to 1 μM depolarized membrane and increased pacemaker potential frequency, dose-dependently.

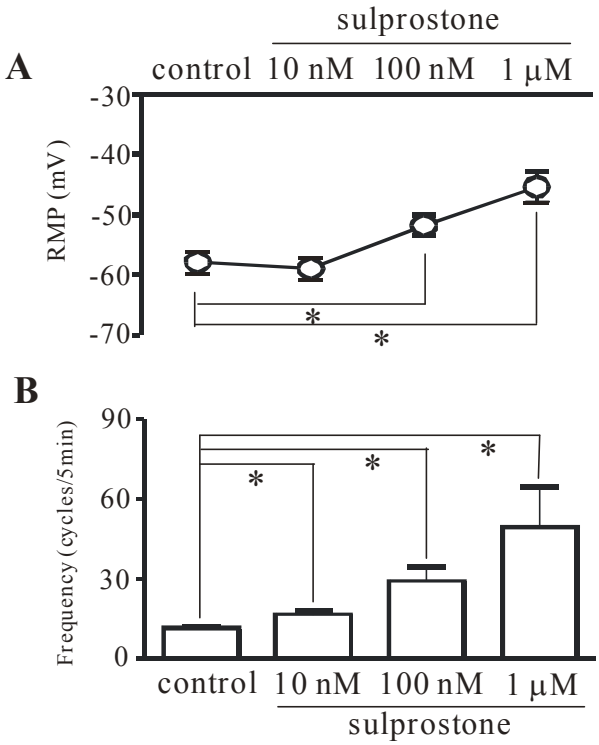

Figure 3. The summarized effects of sulprostone on pacemaker potentials of ICCs from mouse colon. (A) and (B) represent membrane potential and pacemaker frequency, respectively. Bars represent mean ± SE values. *P < 0.05: significantly different from control. RMP: resting membrane potential.

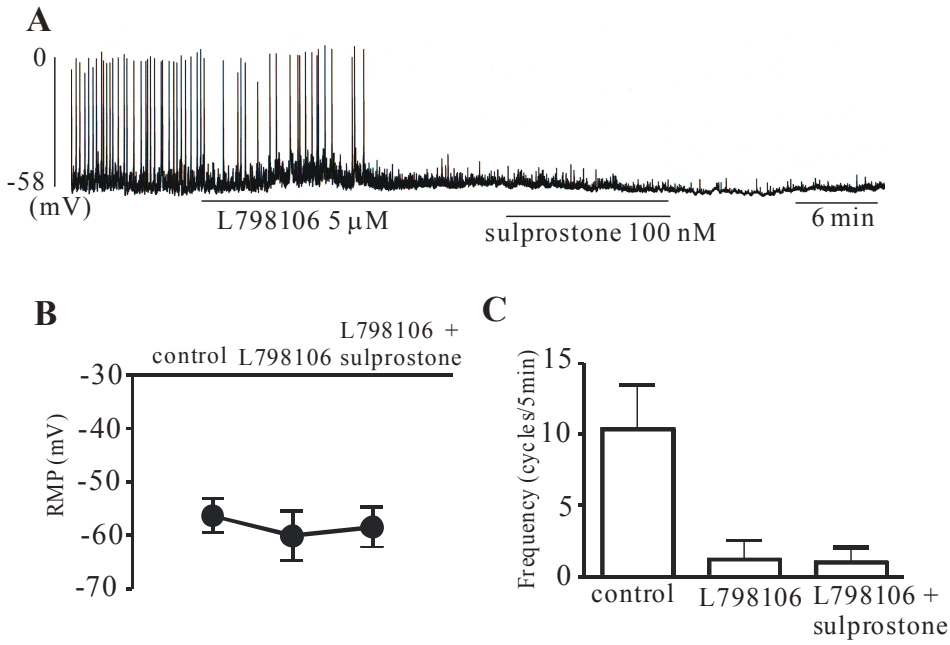

Figure 4. Effects of EP_3 receptor antagonist on the sulprostone-induced effects in pacemaker potentials of ICCs from mouse colon. (A) L798106 (100 nM) itself abolished the generation of pacemaker potentials. In this condition, the depolarization of resting membrane potential and the increase pacemaker potential frequency by sulprostone were almost blocked. Responses to sulprostone in the presence of L798106 were summarized in (B) and (C). Bars represent mean ± SE values. *$P < 0.05$: significantly different from control. RMP: resting membrane potential.

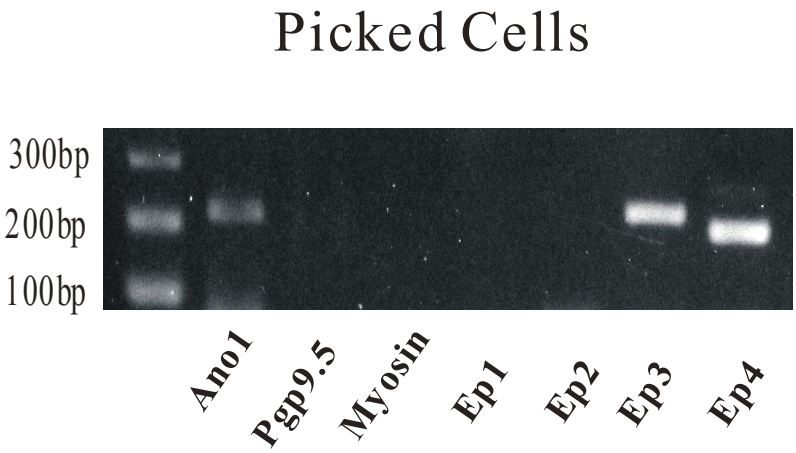

Figure 5. RT-PCR detection and expression of EP_3 receptor subtypes in isolated ICCs from mouse colon. The mRNA transcripts of EP receptors are expressed in picked ICCs EP_3 and EP_4 receptors are only expressed in ANO-1 positive picked ICCs.

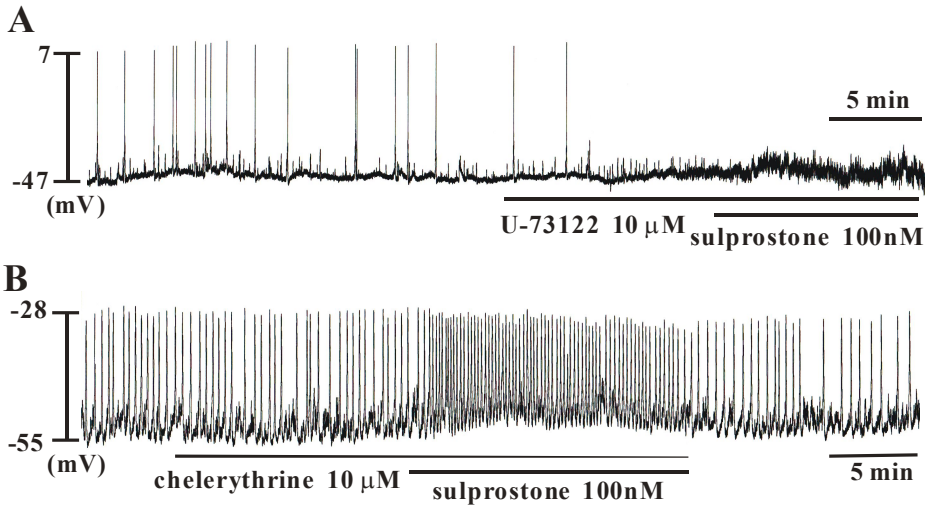

Figure 6. Effects of PLC inhibitor and protein kinase C inhibitor on the sulprostone-induced actions in pacemaker potentials of ICCs from mouse colon. PLC inhibitor U-73122 (10 μM) (A) itself abolished the generation of pacemaker potentials. However, protein kinase C inhibitor chelerythrine (10 μM) (B) had no effects on pacemaker potentials. In the pretreatment of U-73122, the depolarization of resting membrane potential and the increase pacemaker potential frequency by sulprostone were almost blocked. In contrast, in the pretreatment of chelerythrine, the depolarization of resting membrane potential and the increase pacemaker potential frequency by sulprostone were still induced.

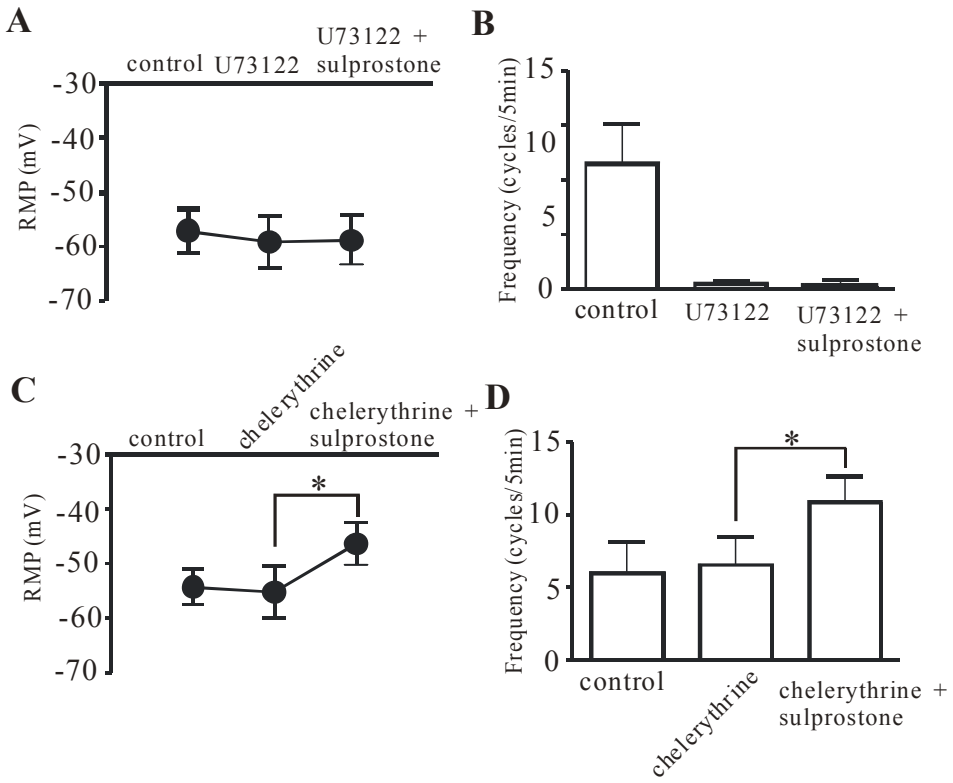

Figure 7. The summarized effects of PLC inhibitor and protein kinase C inhibitor on the sulprostone-induced effects in pacemaker potentials of ICCs from mouse colon. Responses to sulprostone in the presence of U-73122 or chelerythrine were summarized in (A), (B), (C), and (D). Bars represent mean ± SE values. *P < 0.05: significantly different from control. RMP: resting membrane potential.

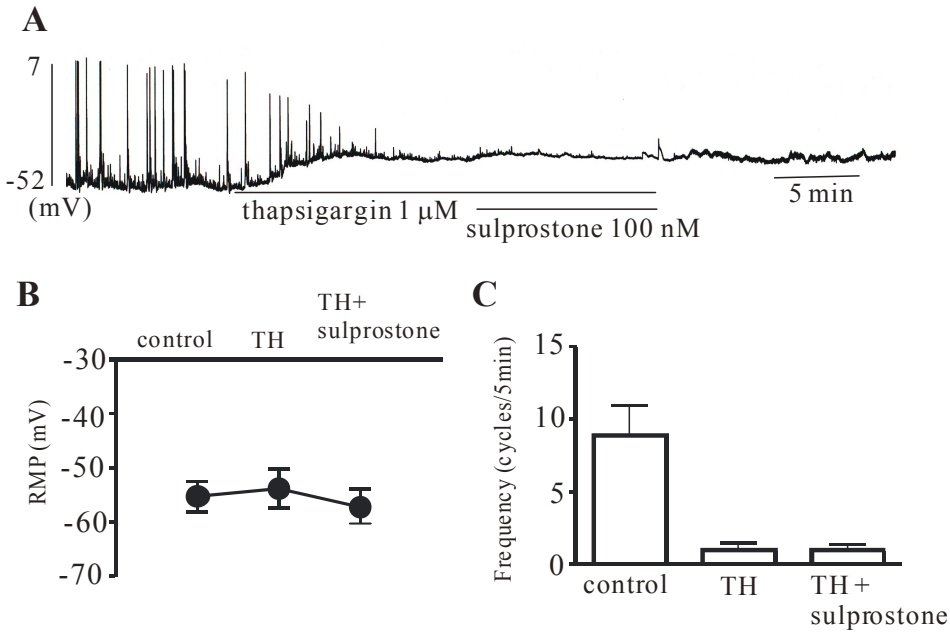

Figure 8. Effects of Ca^{2+}-ATPase inhibitor from endoplasmic reticulum on the sulprostone-induced actions in pacemaker potentials of ICCs from mouse colon. (A) thapsigargin (10 μM), a Ca^{2+}-ATPase inhibitor from endoplasmic reticulum, itself abolished the generation of pacemaker potentials. In this condition, the depolarization of resting membrane potential and the increase pacemaker potential frequency by sulprostone were almost blocked. Response to sulprostone in the presence of thapsigargin was summarized in (B) and (C). Bars represent mean ± SE values.

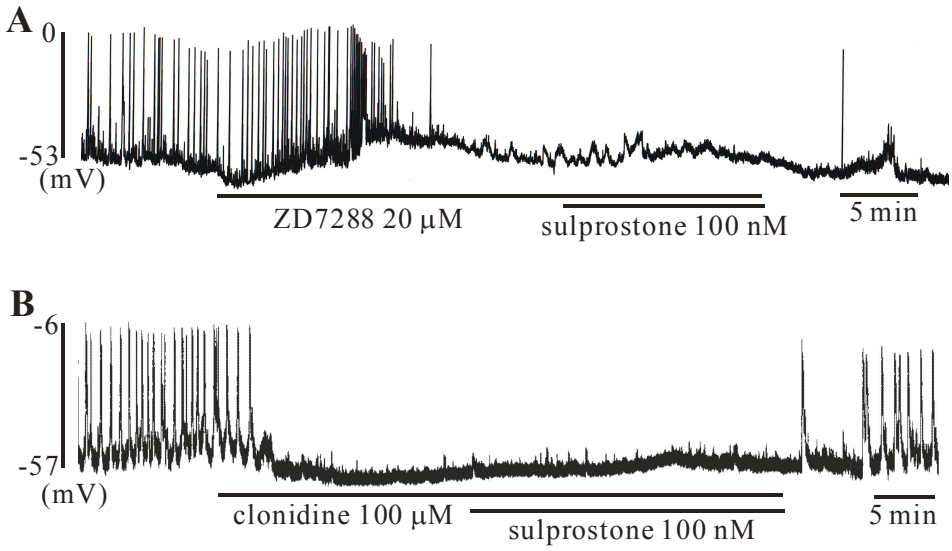

Figure 9. Effects of HCN channel blockers on the sulprostone-induced actions in pacemaker potentials of ICCs from mouse colon. (A) ZD7288 (20 μM) and (B) clonidine (100 μM) itself abolished the generation of pacemaker potentials. In this condition, the depolarization of resting membrane potential and the increase pacemaker potential frequency by sulprostone were almost blocked.

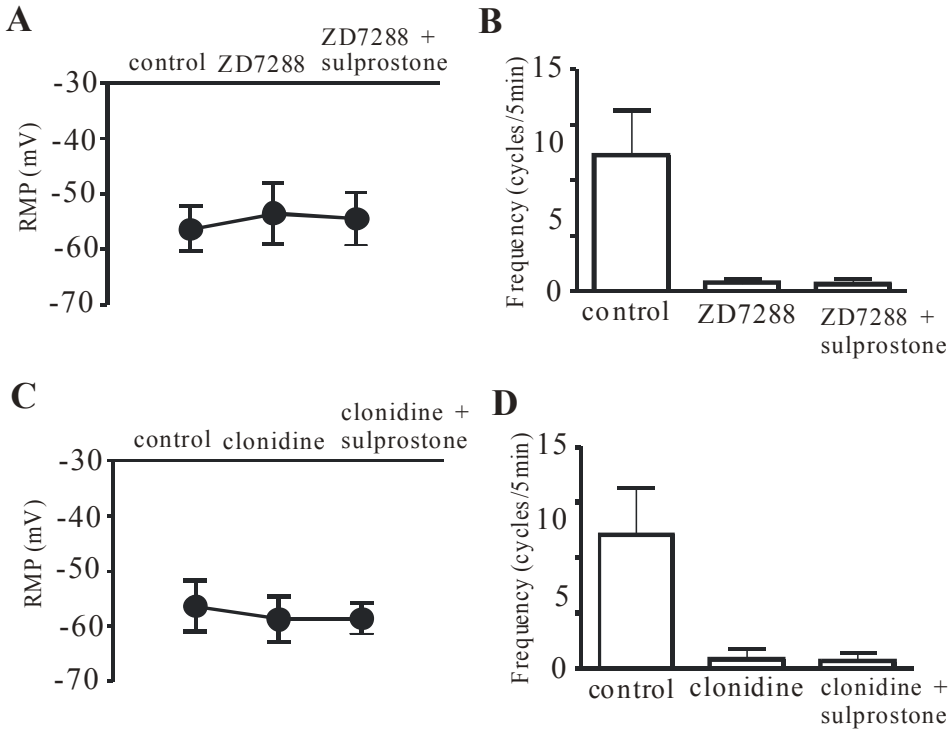

Figure 10. The summarized effects of HCN channel blockers on the sulprostone-induced actions in pacemaker potentials of ICCs from mouse colon. Responses to sulprostone in the presence of ZD 7288, or clonidine (100 μM) were summarized in (A), (B), (C), and (D). Bars represent mean ± SE values. *P < 0.05: significantly different from control. RMP: resting membrane potential.

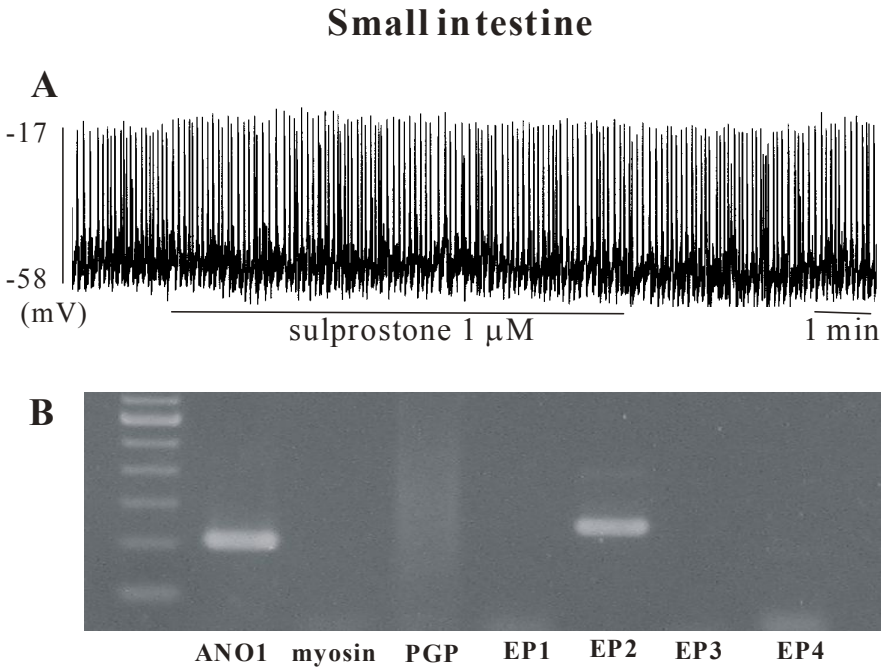

Figure 11. Effects of sulprostone on pacemaker potentials and RT-PCR in isolated ICCs from mouse small intestine. (A) Sulprostone 1 μM had no effect on pacemaker potentials in small intestine. (B) The mRNA transcript of EP_2 receptor only expressed in ANO-1 positive picked ICCs.

DISCUSSION

In the present study, EP_3 receptor activation by sulprostone depolarized the membrane and increased pacemaker potential frequency of interstitial cells of Cajal in mouse colon. The sulprostone-induced effects may be mediated by the activation of HCN channels through phospholipase C (PLC)-dependent intracellular Ca^{2+} release.

PGE_2 has an important role in regulating GI smooth muscle contractility by binding to prostanoid $EP_{1\text{-}4}$ receptors that are distributed to smooth muscles and enteric neurons (Breyer et al. 2001; Dey et al., 2006). EP_1 and EP_3 receptor mediate contractile effects, while EP_2 and EP_4 receptor mediate relaxing effects in small intestine and colon in various species (Iizuka et al., 2014; Martinez-Cutillas et al., 2014). PGE_2 increased slow wave frequencies and integrated peristalsis in mouse stomach through EP_3 receptors and EP_3 receptors expressed by interstitial cells of Cajal (ICCs) (Forrest et al., 2009), suggesting that EP_3 receptor activation can control contractile activity of GI smooth muscle by modulating pacemaker activity of ICCs, indirectly. In this study, sulprostone, a specific EP_3 receptor agonist, enhanced the pacemaker activity by inducing depolarization of the resting membrane and increasing

pacemaker potential frequency in colonic ICCs. And the sulprostone-induced effects were almost blocked by in the pretreatment of L798106 (EP_3 antagonist). Together with, in RT-PCR analysis, EP_3 receptor was expressed in cultured colonic ICCs, which was correlated with functional data. Therefore, these results suggest that EP_3 receptors are present in colonic ICCs and can modulate colonic contractility by indirectly through ICCs. However, EP_3 receptors are not detected and sulprostone did not affect the pacemaker potential activity in small intestinal ICCs, suggesting the regional difference of EP_3 receptor expression. Iizuka et al (2014) reported that EP_3 receptors were not expressed in rat colonic ICCs, suggesting the species difference of EP_3 receptor expression.

Prostanoid EP receptors couple to G-proteins, lead to different signaling pathways, respectively. EP_2 and EP_4 receptors coupled to Gs subunits and stimulate cAMP production through activation of adenylate cyclase. EP_3 receptors coupled to Gi subunits and reduce cAMP production, or coupled to Gq subunits and evoke phosphatidylinositol hydrolysis (Narumiya et al., 1999). The activation of phospholipase C leads to the formation of inositol (1,4,5) triphosphate and diacylglycerol. Inositol (1,4,5) triphosphate release Ca^{2+} from endoplasmic reticulum into cytosol

and diacylglycerol activates protein kinase C (Grasa et al., 2006). PGE_2-induced contractions of longitudinal smooth muscle were mediated by protein kinase C (Iizuka et al, 2014). In cerebral artery, EP_3 receptor mediated PGE_2-induced contraction by the activation of protein kinase C (Jadhav et al., 2004). In myometrial cells, EP_3 receptor activated phospholipase C and increased intracellular Ca^{2+} (Asbóth et al., 1996). Moreover, inositol (1,4,5) triphosphate-dependent periodic intracellular Ca^{2+} release from endoplasmice reticulum is primary pacemakeing mechanism of ICCs (Ward et al., 2000). In the present study, sulprostone-induced action on pacemaker potentials was blocked by phospholipase C inhibitor U-73122 and thapsigargin, a Ca^{2+}-ATPase inhibitor from endoplasmic reticulum, but not by protein kinase C inhibitor chelerythrine, implicating that the depolarization of the membrane and the increase frequency of pacemaker potential by EP_3 receptor is mediated via the activation of phospholipase C and the increase of intracellular Ca^{2+} in colonic ICCs through protein kinase C-independent manner. The chronotropic effects of pacemaker activity by muscarinic receptor in gastric ICCs also mediated by phospholipase C- and intracellular Ca^{2+} -dependent (Forrest et al., 2009).

Until know, transient receptor potential (TRP) channels and ANO-1 (Ca^{2+}-activated Cl^- channels) have been considered candidate pacemaker channels in ICCs (Kim et al., 2005; Zhu et al., 2009). Recently, hyperpolarizing cyclic nucleotide-gated (HCN) channels are suggested as another possible pacemaker channels in mouse colonic ICCs but not in small intestinal ICCs. HCN channels are activated by intracellular cAMP, directly. The generation of pacemaker potentials was abolished by SQ-22536 and dideoxyadenosine (an adenylate cyclase inhibitor), whereas rolipram (a cAMP-specific phosphodiesterase inhibitor) increased the pacemaker potential frequency in colonic ICCs. Together with, HCN channel inhibitors (ZD7288, clonidine, and CsCl) blocked the generation of pacemaker potentials (Shahi et al., 2014). In the present study, clonidine and ZD7288 blocked the sulprostone-induced effects on pacemaker potentials. Therefore, it seems that HCN channels are may involve in the sulprostone-induced actions on pacemaker potentials of colonic ICCs.

In summary, EP_3 receptors for PGE_2 are distributed to colonic ICCs but not in small intestinal ICCs. The activation of EP_3 receptor by sulprostone depolarized the membrane and increased pacemaker potential

frequency through activation of HCN channels by PLC-dependent intracellular Ca^{2+} release from endoplasmic reticulum. Therefore it seems that EP_3 receptors may play an important role in regulating colonic motility and can be target of depressed colonic motility disorders.

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