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Ph.D. Thesis

The Functional Study of Prostaglandin E_2 - EP Receptor on Pacemaker Activity in Interstitial Cells of Cajal

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ABBREVIATIONS

GI - gastrointestinal tract

SMC - smooth muscle cells

ICC - interstitial cells of Cajal

Ca^{2+} - calcium

ER - endoplasmic reticulum

IP_3 - inositol 1,4,5-trisphosphate

ANO1 channel - Anoctamin1 channel

CACC - Ca^{2+} activated Cl^- channel

VDCC - voltage-dependent Ca^{2+} channel

PGs - prostaglandins

PGE_2 - prostaglandin E_2

EP receptor - prostaglandin E2 receptor

RT-PCR - reverse transcription polymerase activated reaction

초록

위장관 카할 사이질 세포의 향도잡이 활동도에 대한 프로스타글랜딘 E_2-EP receptor의 효과와 기전 연구

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카할 사이질 세포는 위장관 평활근에서 발생되는 서파 (slow waves)를 생산하는 향도잡이 활동도를 가진 특수 세포이다. 더불어 PGE_2는 위장관 운동성에 다양한 생리학적 그리고 병리학적 기능을 담당하는 것으로 알려져 있다. 하지만 아직까지 PGE_2의 대장 카할 사이질 세포에 대한 연구는 밝혀지지 않아 본 연구를 통해 확인해 보았다.

연구 결과 도출을 위해 본 연구자는 whole cell patch clamp, RT-PCR 그리고 세포내 칼슘 분석 방법을 실시하였다.

전류 고정 모드에서 PGE_2는 농도에 따라 카할 사이질 세포에 두 가지 효과를 보여주었다. 낮은 농도에서는 향도잡이 전압의 빈도수를 높여주었고, 높은 농도에서는 빈도수를 억제하였다. RT-PCR 결과를 통해 본인은 EP3 와 EP4 수용체가 대장 카할 사이질 세포에 존재하는 것을 확인 하였다. 낮은 농도의 PGE_2는 SQ22536 (a adenylate cyclase inhibitor) 전처치를 통해 억제되었고 EP3 수용체 agonist인 sulprostone은 낮은 농도의 PGE_2와 동일한 결과를 보여주었다. 대장 카할 사이질 세포의 향도잡이 활동도에 대한 Sulprostone의 효과는 PLC inhibitor, Ca^{2+}-ATPase inhibitor 그리고 ANO1 channel blocker에 의해 억제되었다. 세포내 칼슘 분석 방법을 통해 sulprostone은 세포내 칼슘 주지적인 진동을 증가시켰다.

높은 농도의 PGE_2는 카할 사이질 세포막의 전압을 과분극 시켰고 향도잡이 전압의 빈도수를 억제하였다. 더불어 향도잡이 전압에 대한 고농도 PGE_2의 억제 효과는 ATP 민감성 K^+ channel 억제제에 의해 억제되었다.

본 연구는 대장 카할 사이질 세포의 향도잡이 활동도에 대한 PGE_2의 효과를 확인하였다. 낮은 농도의 PGE_2는 cAMP, ANO1 channels 그리고 PLC와 세포내 칼슘 분비 조절을 통해 카할 사이질 세포의 향도잡이 활동도를 활성화 시켰다. 하지만 높은 농도의 PGE_2는 카할 사이질 세포의 향도잡이 활동도를 억제 시켰고 이는 세포 막전압 조절 이온통로인 ATP 민감성 K^+ 통로가 관여하였다.

1. INTRODUCTION

1.1. The Motility of Gastrointestinal Tract

The gastrointestinal (GI) tract is a muscular tube which consists of mouth, pharynx, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum and anus. To absorb nutrients and water into our body and eliminate waste products, the GI tract mainly occurs four physiological processes: motility, secretion, digestion and absorption. Motility of the muscular wall of the GI tract move the contents along the tract to digestion and absorption, and mix the food with secretion.

1.2. Slow Wave

GI motility caused by the contractions of the gut smooth muscle. GI smooth muscle cells (SMC) shows spontaneous and rhythmic depolarizations which was termed slow wave. These partial depolarizations are equivalent to fluctuations in membrane potential of 5 - 15 mV and the frequency depends on the section of GI tract: 12 to 20 times per munite in the small intestine and 3 to 8 times per minute in the colon. Slow wave is a intrinsic property of smooth muscle but do not elicit smooth muscle contractions by themselves. When the membrane potential of slow wave passes over the calcium (Ca^{2+}) channel open threshold, voltage dependent Ca^{2+} channels activated, resulting in Ca^{2+} enter the SMC and triggers smooth a muscle cells to contract (Thorneloe *et al*., 2005). The generally accepted mechanism for slow wave generation has arisen based on the key finding that peacemaking activity was generated in Interstitial Cells of Cajal (Thuneberg, 1982; Huizinga *et al*., 1995).

1.3. Interstitial Cells of Cajal

Interstitial Cells of Cajal (ICC) were first identified by Spanish anatomist Santiago Ramon Y Cajal (Cajal, 1911). He described a network of cells in GI tissues and hypothesized these cells as accessory components of nervous system that

modify the smooth muscle contraction. These network of cells were later assumed as act a pacemaker system for the intestinal musculature by Sir Arthur Keith (Keith, 1914). ICC originate mainly from kit-positive mesenchymal mesodermal precursor (Sanders *et al*., 1999; Young *et al*., 1996). Kit-positive precursor cells receive kit signaling to develop into functional ICC, whereas that without kit-signaling via this pathway become SMC.

1.3.1. Identification of ICC

For many years ICC were difficult to investigate because there were no specific labels for these cells. ICC was previously characterized by morphological features with various histological staining methods till the discovery that ICC express the proto-oncogene c-Kit (Maeda *et al*., 1992). The c-kit receptor is a type 3 receptor tyrosine kinase, which also includes PDGFR-α, -β and SCF-1. It acts a important role on the development of ICC, mast cells, melanocytes, gametocytes and blood cells. The distribution of several ICC subpopulation throughout the GI tract was specially identified by using antibodies against the c-kit (Burns *et al*., 1997). However, not all ICC express c-kit such as ICC-DMP in the human small intestine (Torihashi *et al*., 1999). And also there are some other cells types such as mast cells, melanocytes, glia do express c-kit (Zhang *et al*., 1997).

Transmembrane protein 16A (TMEM16a) which encodes anactomin1 (ANO1), a calcium activated chloride channel (CACC), was emerged as one of the most selective maker for ICC in human and mouse GI tract (Gomes-Pinilla *et al*., 2009). Actually, ANO1 provide more selectively labeling for ICC than c-Kit.

1.3.2. Morphology and Classification of ICC

Several morphological types of ICC have been described depends on their location (Fig. 1) (Sanders, 1996; Sanders *et al*., 1999). There are at least two classes of ICC:

a. Intracellular ICC (ICC-IM) is a collective term of ICC of the circular muscle (ICC-CM) and ICC of the longitudinal muscle (ICC-LM). These are mainly bipolar cells or spindle-shaped cells intermingled within muscle bundles of the circular and longitudinal muscle layers. They do not form their own network.

b. ICC of the myenteric plexus (ICC-MY) are multipolar cells with branched processes connecting to each other and arranged in a dense network around the myenteric plexus between the circular and longitudinal muscle layers. Cells located on the submucosa surface of circular muscle (ICC-SM), found in the deep muscular plexus (ICC-DMP), occur in the connective tissue septa (ICC-SEP) and located on subserosal where between the serosa and longitudinal muscle (ICC-SS) are all form loose network with each other.

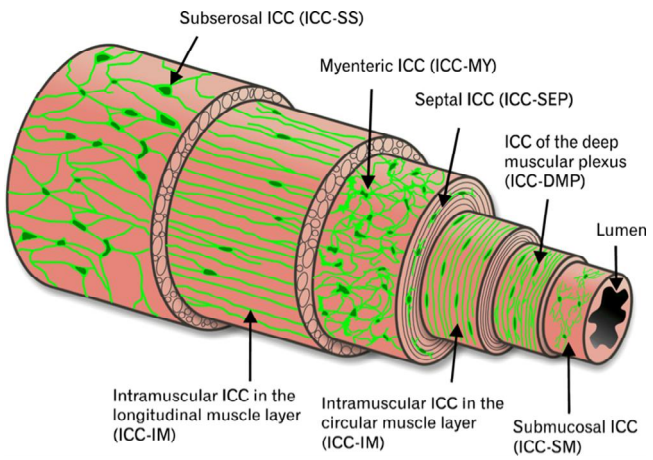

Fig. 1. The various subpopulation of ICC in GI tract. (Blair *et al.*, 2014)

1.3.3. Ultrastructure of ICC

Even though there are some variations between different species, the ultrastructure features of ICC are regarded as a valuable method for their identification (Rumessen *et al.*, 1992; Huizinga *et al.*, 1997):

1) numerous large mitochondria in processes, moderately well-developed Golgi

apparatus, rough and smooth endoplasmic reticulum (ER), and caveolae at cell surface;

2) thin and intermediate filaments;

3) differently developed basal lamia;

4) synapse-like contacts with nerve bundles and the formation of gap junction, both with each other and smooth muscles. The gap junction is an essential feature required to propagate slow wave between ICC and SMC and to transduce neurotransmitter from nerves system.

1.3.4. Functions of ICC

GI motility is regulated by the cooperation of ICC, smooth muscles and enteric neurons (Fig. 2).

1) ICC act as pacemaker cells of GI tract.

ICC produce spontaneous pacemaker activity. Due to the electrically connection between ICC and smooth muscles, ICC network directly transmitte pacemaker currents to the circular and longitudinal muscle layers via gap junction.

2) ICC mediate enteric motor neurotransmission in smooth muscles.

Junctions between ICC and nerve varicosities can be found with less than 20 nm space. Therefore, ICC can also transduce neurotransmitter signals from the enteric nervous system and modulating excitation-contraction coupling between slow wave and contraction (Sanders *et al*., 2006; Beckett *et al*., 2005)

3) ICC provide mechano-sensitive regulation of pacemaker activity (Won *et al*., 2013).

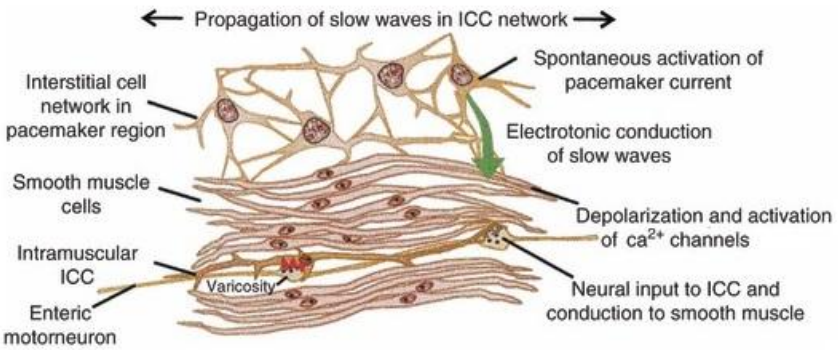

Fig. 2. GI motility is regulated by the cooperation of ICC, smooth muscles and enteric neurons. ICC act as pacemaker cells. It generates slow wave and propagates to SMC via gap junctions. ICC also mediates neurotransmission to SMC (Sanders *et al.*, 1999)

1.3.5. Pacemaker Mechanism

Morphological and ultrastructural characteristics of ICC shown an abundance of mitochondria near to the ER and plasma membrane. ER and Mitochondria are main intracellular Ca^{2+} stores. Variations in cytosolic Ca^{2+} concentration modulate a variety of intracellular function such as muscle contraction. Researchers have demonstrated Ca^{2+} release from intracellular stores is the basic event responsible for pacemaker activity (Helden *et al.*, 2000; Malysa *et al.*, 2001; Ward *et al.*, 2000). Ward *et al* (Ward *et al.*, 2000) suggested that the activation of pacemaker currents derived from the periodic release of Ca^{2+} from ER through inositol 1,4,5-trisphosphate (IP_3) receptors. Released Ca^{2+} triggers uptake by mitochondria. The pacemaker cycle may be completed and reset by uptake of Ca^{2+} into the ER.

This Ca^{2+} oscillation periodically activates Ca^{2+}- dependent membrane pacemaker ion channels to generate pacemaker activity. Non-selective cation channels (such as transient receptor potential (TRP) channels and hyperpolarizing cyclic nucleotide-gated (HCN) channels) and Ca^{2+}-activated Cl^- channels (CACC channels) were candidates as pacemaker channels in ICC (Kim *et al.*, 2005; Shahi *et al.*, 2014; Zhu *et al.*, 2009).

HCN channels are a family of non-selective cation channels that activated by hyperpolarization of the membrane potential and by direct binding of intracellular cAMP. These channels were found in a variety of spontaneously active cells such as cardiocytes and act as pacemaker channels to regulate cell excitability (DiFrancesco, 1991). Recently, it was suggested as possible pacemaker channel in mouse colonic ICC. The generation of pacemaker potentials in colonic ICC was abolished by adenylate cyclase inhibitors (SQ-22536 and dideoxyadenosine), meanwhile cAMP-specific phosphodiesterase inhibitor (rolipram) increased pacemaker potential frequency. Moreover, HCN channel inhibitors (ZD7288 and CsCl) blocked the generation of pacemaker potentials (Shahi *et al*., 2014).

Recently, researchers proposed an idea for the activation and propagation of slow wave via activation of ANO1 channels (Fig. 3; Blair *et al*., 2014):

1) Ca^{2+} randomly released from ER in ICC results in transient activation of ANO1 channels.

2) Chloride ions flow out of cell via these Ca^{2+} activated chloride channels, producing spontaneous transient inward currents (STICs). STICs causing spontaneous transient depolarizations (STDs) of the cell membrane.

3) STDs activates voltage-dependent Ca^{2+} channels (VDCC), Ca^{2+} flow into the cell. Massive Ca^{2+} entry results Ca^{2+} release from IP_3 receptors and activation of whole cell ANO1 channels, generating slow wave. slow wave propagates to neighboring ICC by depolarizing the cell membrane and triggering activation of VDCC. Ca^{2+} entry in adjacent ICC activates ANO1 channels and regenerates slow waves which can propagate throughout ICC networks. slow wave depolarize the membrane potential of adjacent SMC and activates voltage-dependent Ca^{2+} channels triggering contraction of SMC.

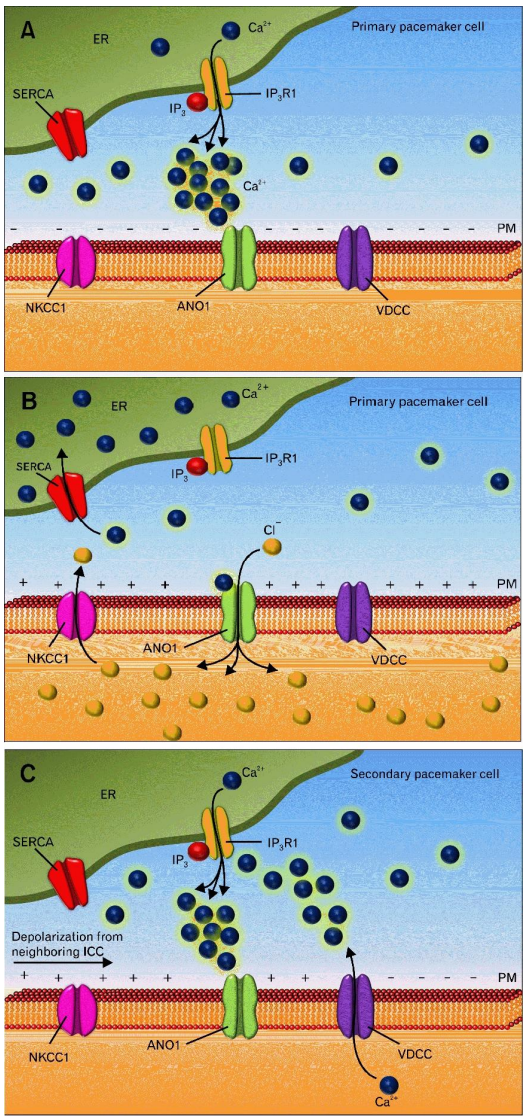

Fig. 3. The mechanism responsible for activation and propagation of pacemaker activity in ICC. See text for details (Blair *et al*., 2014).

1.4. Prostaglandin E_2

1.4.1. The Synthesis of Prostaglandin E_2

Prostaglandins (PGs) are ubiquitous in all tissues and mediate variety of physiological and pathological functions. The synthesis of PGE_2 initiates from the

hydrolysis of cell-membrane phospholipids by phospholipase A_2 (Murakami *et al*., 1997; Murakami and Kudo, 2002). Next, arachidonic acid (AA) catalyzed by cyclooxygenase enzymes (COX-1 and COX-2). Upon release, AA is converted into unstable intermediates, PGG_2 and PGH_2 (Hamberg *et al*.,1974). The PGH_2 is inturn metabolized into PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and thromboxane A_2 (TXA_2) (Fig. 4) (Calder, 2001).

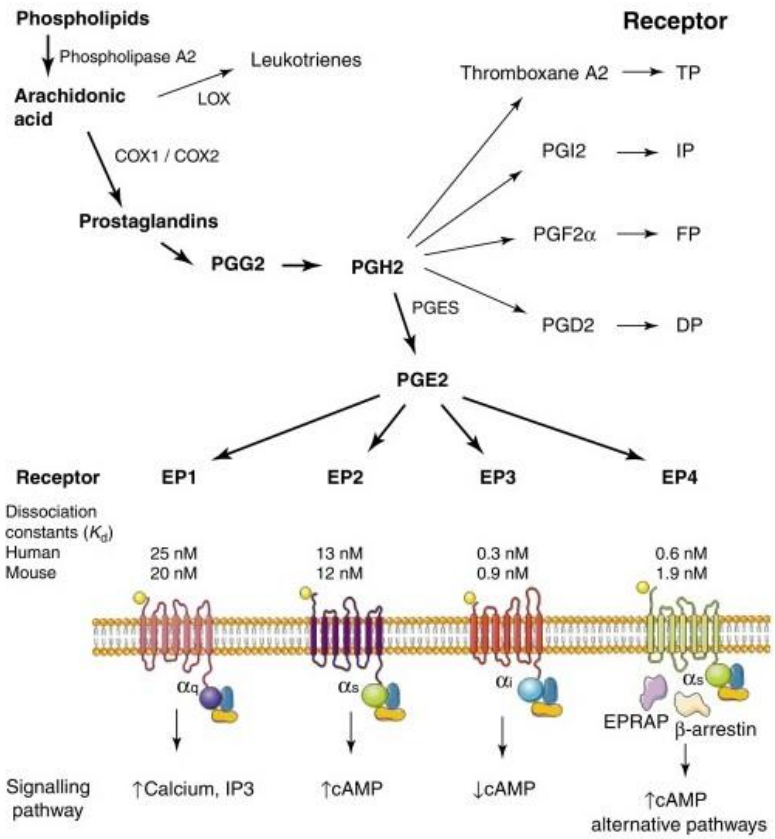

Fig. 4. The synthesis of PGE_2 and its binding affinity to EP receptors (Markovič et al., 2017)

1.4.2. EP receptors and downstream signaling pathway

Among these different PGs, PGE_2 is found to be the most widely produced substance within the body. PGE_2 exerts its actions by acting four G protein coupled EP receptors namely EP1, EP2, EP3 and EP4. The EP receptors are composed of seven transmembrane domains. Each receptor subtype being intracellularlly coupled to different subunits of G protein and initiate specific downstream signaling pathways (Fig. 5) (Breyer *et al*., 2001). Activation of EP1 receptors results into enhancement of intracellular Ca^{2+}. EP2 and EP4 receptors are coupled through G_s subunit to increase cAMP. It was reported that the phosphoinositide-3-kinase (PI3K) signaling pathway was activated by the EP4 receptor (Fujino *et al*., 2003). EP3 receptor is unique among the EP receptor family cause it has different C-terminal tail isoforms, which are generated by alternative splicing (Namba *et al*., 1993). In the mouse, three isoforms of the EP3 receptor have been identified (Paul *et al*., 1998). Each isoform has a unique amino acid composition in the C-terminal region which coupling with various G proteins. The major signaling pathway of EP3 receptor is coupled to G_i subunits, which induce the inhibition of adenylate cyclase. However, EP3 were also reported coupled to G_s and act to increase levels of cAMP (Sugimoto and Narumiya, 2007; Hatae *et al*., 2002). Moreover, it is also shown that EP3 activate phospholipase C (PLC) to release intracellular Ca^{2+} via $G_{\alpha q}$ (Yang *et al*., 1994; Schmid *et al*., 1995). Due to signaling through different EP receptor determines the multiple effects of PGE_2, the expression of these receptors in GI tract is critical for determine its biological functions.

Subtype	Isoform	Amino acid	G protein	Signaling
EP2		362	G_s	cAMP ↑
EP4		513	G_s (G_i)	cAMP ↑, PI3K
EP1		405	Unknown	Ca^{2+} ↑
EP3	EP3α	366	G_i, G_{12}	cAMP ↓, IP_3/Ca^{2+} ↑,Rho
	EP3β	362	G_i, G_{12}	cAMP ↓, IP_3/Ca^{2+} ↑,Rho
	EP3γ	365	G_i, G_s	cAMP ↓, cAMP ↑, IP_3/Ca^{2+} ↑

Fig. 5. Signal transduction properties of EP receptor subtypes and EP3 isoforms (Sugimoto and Narumiya, 2007)

1.4.3. PGE_2 in the GI tract

PGE_2 is one of the most important prostanoids which produced throughout the GI tract. It is not only regulates various physiological functions including mucosal protection, secretion and motility, but also plays various pathophysiologic roles in the GI disfunction, such as inflammatory bowel diseases and colorectal neoplasia. The motility of the GI tract is maintained through contraction and relaxation of GI smooth muscle. In rat middle colon, PGE_2 enhances the contractions by activating the EP1 and EP3 receptors directly on longitudinal smooth muscle cells (Iizuka *et al*., 2014). In rabbit small intestine, PGE_2 evoked contractions of smooth muscle cells through EP1 and EP3 receptors (Grasa *et al*., 2006). PGE_2-induced contraction of longitudinal smooth muscle is mediated by EP1 receptors in human colon and by a combination of EP1 and EP3 receptors in mouse intestine, whereas EP2 receptors modulate relaxation in all three preparations (Fairbrother *et al*., 2011). Studies on EP receptor knockout mice shown the importance of EP1 and EP3 receptors in the contraction and EP4 receptors in the relaxation of longitudinal smooth muscles of gastric fundus and ileum (Okada *et al*., 2000). On the whole, activation of EP1 and EP3 receptors leads to smooth muscle contraction, while activation of EP2 and EP4 receptors results into relaxation of smooth muscle on GI tract.

1.5. Thesis Rationale and Objectives

The contraction and relaxation of smooth muscle cells determined by ICC pacemaker activity. This means PGE_2 may mediate smooth muscle contraction through ICC. Meanwhile, reports found that the frequency of peristaltic contraction and the appearance of ectopic sites of waves increased by PGE_2 was not observed in ICC deficient mice, suggesting a possible effect of PGE_2 on stomach ICC (Forrest *et al*., 2009). Furthermore, previous study has shown that PGE_2 regulates pacemaker activity through EP2 receptor in small intestinal ICC (Choi *et al*., 2006). Even through there are many studies related to the PGE_2 effect on GI tract and small intestinal ICC, the functions of PGE_2 on colonic pacemaker activity has been still unknown. This study examined the effect of PGE_2 on pacemaker potential of colonic ICC and possibly related signaling pathway through EP receptors.

2. MATERIALS AND METHODS

2.1. Experimental Animals

All animal care and handling protocols used in these experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, and approved by the ethics committee in Chosun University and the National Institutes of Health Guide, South Korea.

2.2. Preparation of Cells and Tissues

Either sex of ICR mice aged 4 - 7 days were anesthetized with diethyl ether and killed by cervical dislocation. An abdominal midline incision was made to afford optimal exposure. Small intestine (from 1 cm below the pyloric ring to the cecum) and colon (from below the cecum to the rectum) were cut and pinned to the base of sylgard dish which full with ice-cold Ca^{2+} free Hank's solution (see solutions). Tissues were opened along the mesenteric border. After remove luminal contents, the mucosa and submucosa of tissues were peeled away by sharp dissection. Stripes of intestinal or colonic muscle were equilibrated in Ca^{2+} free Hank's solution for 30minutes. The muscle strips were transferred into enzymic solution (see solutions) and incubated in 37 ℃ water bath for 14 minutes. Then tissues were washed out 3 times with Ca^{2+} free solution and triturated with ablunt pipettes to disperse cell lumps. Cells were plated on poly-L-lysine (200 μl, sigma) coated sterile glass coverslips in 35 mm culture dishes and incubated at 37 ℃ in a 95% O_2 - 5% CO_2 incubator in smooth muscle growth medium (SMGM, Lonza, Walkersville, MD, USA) supplemented with 2% antibiotic - antimycotics (Gibco, Grand Island, NY, USA) and stem cell factor (SCF, 5 ng/ml, sigma). After 1 day incubation, the medium was replaced with SMGM without SCF, then incubated further 24 hours with same condition till performing the following experiments.

2.3. Whole-Cell Patch Clamp

2.3.1. Patch Clamp Equipment

Whole-cell patch clamp technique was used to measure the alterations of membrane potentials from cultured intestinal and colonic ICC. The basic elements of patch clamping set-up including: an anti-vibration table (Daeil systems) to minimize mechanical interference, a faraday cage to exclude electromagnetic interference, an inverted microscope (Olympus IX70) to observe the preparations, a macro and micromanipulator to position the micropipette tip onto the cell membrane. A super fusion system with temperature controller (Harvard apparatus) was used to perfume solutions and electronic equipments such as head stage (CV203BU headstage, Axon instruments, Foster, CA, USA), amplifier (Axopatch 200B, Axon instruments), digitizer (Digidata 1322A, Axon instruments), computer and software (pClamp 9.2, Axon instruments) to record and analysis data.

2.3.2. Micropipettes

Micropipettes (GC150TF-7.5, Harvard apparatus) were made from a rod of borosilicate glass with 1.5 mm outer diameter and 75 mm length. After local melting and pulling by micropipette puller (Narishige PP-83, Japan), the tip was fire polished by micro forge (MF-830, Narishige, Japan) to make it rounded and smooth. Finally, the resistance of micropipette tip was adjusted to 3 - 5 mV.

2.3.3. Current-Clamp Recording

The cells in 35 mm dishes were taken out from the CO_2 incubator after 48 hours of culture and placed into the recording chamber on the inverted microscope. The bath solution was perfused into dishes and equilibrate at least 15 minutes. The temperature of cells were maintained at 30 ℃ by temperature controller.

Micropipettes were filled with pipette solution (see solutions) and tightened by the pipette holder. The headstage was angled between 45° to 60° and dipped into the

bath solution (see solutions), then the resistance were displayed on the Clampex. Adjusted the current pulse to zero by turning the pipette offset on Axon 200B amplifier. Micropipettes touched the surface of cells and negative pressure was applied to help forming the giga ohm (GΩ) seal. Continuously gave negative pressure or pressing the zap switch on Amplifier to break the cell membrane, applied the -70 mV holding voltage and turned the mode to I = 0. The alteration of cell membrane potential were recorded by using the gap-free protocol in the Clampex software.

2.4. Measurement of Intracellular Ca^{2+} Concentration

After 24 to 30 hours of culture, the ICC cultured on coverslips (25 mm) were well growth and ready for the measurement of intracellular Ca^{2+} concentration. The medium in culture dishes were removed. The cells were washed twice and incubated at 37℃ for 10 minutes with bath solution. Next, the cells was stained by Fluo-4/AM (1 mM, Invitrogen) at 37 ℃ for 15 minutes and then rinsed for 3 times with bath solution. After mounted on the perfusion chamber, the cells were scanned every 0.4 seconds with Nikon Eclipse TE2000-U inverted microscope equipped with a PerkinElmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (200x; Hamamatsu Instrument, Hamamatsu, Shizuoka, Japan). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. The temperature of perfusion chamber was remaining at 30 ℃ during the experiment. Relative alterations of intracellular Ca^{2+} fluorescence emission intensity were expressed as the ratio (F1 / F0), where F0 was taken from the fluorescence of first image.

2.5. RNA Isolation, DNA Synthesis and PCR

2.5.1. Single Cell RNA Isolation

The culture dish was rinsed with 1x PBS 3 times and mounted on the chamber. Micropipette with relatively little bigger pore size was used to pick up the single ICC. The pipette was filled with 1x PBS and moved closer to the single ICC. Applied negative pressure to suck the cell into pipette. The pipette contents were subsequently eject into a sterile 1.5 ml eppendorf tube containing sterile and chilled 1x PBS. The tube was rapidly placed into ice and the RNA was isolated using the Trizol reagent according to the manufacturer's instruction.

2.5.2. Whole Dish Total RNA Isolation

After enzymatic digestion, tissue of small intestine and colon were cultured on 100 mm culture dish separately. 48 hours later, the culture dish was washed twice with ice-cold 1x PBS. Add 1 ml 1xPBS into dish and scraped cells off, then transferred it into an eppendorf tube. The tube was centrifuged at 8000 rpm for 5 minutes. The pellets was then used for total RNA isolation by Trizol reagent. RNA isolation was according to the manufacturer's instruction.

2.5.3. DNA Synthesis and PCR

Isolated RNA from single cell and whole dish were performed cDNA synthesis by using the PrimeScript™ 1st strand cDNA synthesis kit (6110A, TaKaRa) according to the manufacturer's instructions with minor modifications. The reultant cDNA was purified with the Maxime PCR PreMixi-Star Taq (iNtRON Biotechnology Inc., Korea) and amplified with specific primers (Table. 1) by PCR, using the following amplification conditions: 94 ℃ for 5 minutes to activate i-Star Taq DNA polymerase and then 30 cycles at 94 ℃ for 30s to desaturation, 59 - 63 ℃ for 30s depends on different primers to annealing, 72 ℃ for 30s to extension. Followed by a

final extension at 72℃ for 10 minutes. The PCR products (5 μl) were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.

Official Symbol for mRNA	GenBank Accession No.	Primer Sequences (5'-3')	Size (bp)
c-Kit	AY536430	(F)-GCACAGAAGGAGGCACTTATACCT (R)-TGAGACAGGAGTGGTACACCTTTG	215
Ano-1	NM_178642	(F)-AGGCCAAGTACAGCATGGGTATCA (R)-AGTACAGGCCAACCTTCTCACCAA	213
PGP 9.5	AF172334	(F)-GCCAACAACCAAGACAAGCTGGAA (R)-GCCGTCCACGTTGTTGAACAGAAT	213
Myosin	NM_013607	(F)-GTACCATCGAGGTCATGGAG (R)-GGTCTCTCTCATCCGCATAC	264
EP1	NM_013641	(F)-CTGGGCTCAAGGGTATAGAG (R)-GGTGACAGAGATGGGAAGTG	290
EP2	NM_008964	(F)-CAGGAGAGGAGAGAGGACTT (R)-GAAGTCCGACAACAGAGGAC	261
EP3	NM_011196	(F)-CAGGGAAGGATGACTGAGTA (R)-GGTTCTGAGGCTGGAGATA	225
EP4	NM_008965	(F)-GTTGGTGGATGAGGTTAGTG (R)-CTAGTGGGAGTCCAGATGAA	220

Table. 1. Primers used for RT-PCR.

2.6. Solutions

2.6.1. Ca^{2+} free Hank's Solution

135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, adjusted PH to 7.4 with Tris-base.

2.6.2. Enzymatic Solution

2 mg collagenase (Worthington, Lakewood, NJ, USA), 2 mg Bovine serum albumin (Sigma), and 1mg trypsin inhibitor from Glycine max were mixed with 2 mL Ca^{2+} free Hank's solution.

2.6.3. Bath Solution

Cells were bathed in a buffer containing 135 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 1.8 mM $CaCl_2$, adjusted PH to7.4 with Tris-base.

2.6.4. Pipette Solution

The pipette solution contained 140 mM KCl, 2.5 mM $MgCl_2$, 2.7 mM MgATP, 0.1 mM Na_2GTP, 2.5 mM Creatine phosphate disodium, 0.1 mM EGTA, 5 mM HEPES, adjusted PH to 7.2 with Tris-base.

2.7. Drugs and Chemicals

The drugs used in this study were showed as follows (Table. 2):

Drug Names	Companies	Catalog No.
Prostaglandin E_2 (PGE_2)	sigma	5640
Sulprostone	cayman	14765
CAY10580	cayman	16835
Glibanclamide (GBC)	sigma	G0639
tetraethylammonium (TEA)	sigma	T1515
4-aminopyridine (4-AP)	sigma	A78403
Apamin	sigma	A1289
Nickel chloride ($NiCl_2$)	sigma	N5756
Mibefradil	cayman	15037
SQ22536	tocris	1435
L798106	sigma	L4545
U-73122	sigma	U6756
Chelerythrine	sigma	C2932
Thapsigargin	sigma	T9033
TMEM16A inhibitor	calbiochem	613551
MONNA	sigma	AML0902

Table. 2. Summarized informations about drugs and chemicals used in this study.

2.8. Statistical Analysis

Data are expressed as means±standard errors (SEM). Student's t-test and ANOVA was used for statistical comparison. P values < 0.05 indicated statistically significant. The n values stand for the number of cells used in patch clamp.

3. RESULTS

3.1. PGE_2 shows dual effect with different concentrations in colonic ICC

Previous study have described that the effect of PGE_2 in ICC from small intestine (Choi *et al*., 2006). However, there are many differences involved in the peacemaker activity between small intestinal and colonic ICC. In this study, I checked the action of PGE_2 on pacemaker potential by using current clamp in cultured colonic ICC. Under control conditions, the membrane potential and pacemaker potential frequency were -55.7 ± 0.7 mV, 9.7 ± 0.4 cycles/5min, respectively (n = 50). Treating PGE_2 with low concentration (0.05 nM to 0.3 nM, Fig. 6A ~ 6C) slightly increased membrane potential and frequency. Membrane potentials were -52.4 ± 1.3 mV at 0.05 nM, -49.5 ± 1.8 mV at 0.1 nM, -49.8 ± 2.5 mV at 0.3 nM. Pacemaker potential frequencies were 12.3 ± 2.9 cycles/5min (n = 4) at 0.05 nM, 12.7 ± 1 cycles/5min (n = 6) at 0.1 nM, 17.3 ± 6.4 cycles/5min (n = 4) at 0.3 nM. However in the presence of high concentration (0.5 nM to 10 nM, Fig. 6D ~ 6F), PGE_2 hyperpolarized the membrane potential and inhibited the generation of pacemaker potentials in a concentration dependent manner. Membrane potentials were -61.2 ± 1.9 mV at 0.5 nM, -60.8 ± 1.6 mV at 1 nM, -64.8 ± 0.8 mV at 10 nM (Fig. 6G). Frequencies were 5.7 ± 1.4 cycles/5min (n = 6) at 0.5 nM, 1 ± 0.6 cycles/5min (n = 7) at 1 nM, 0.9 ± 0.3 cycles/5min (n = 23) at 10 nM (Fig. 6H). Theses results suggests that PGE_2 activates pacemaker potentials with low concentrations and inhibits pacemaker potentials with high concentrations.

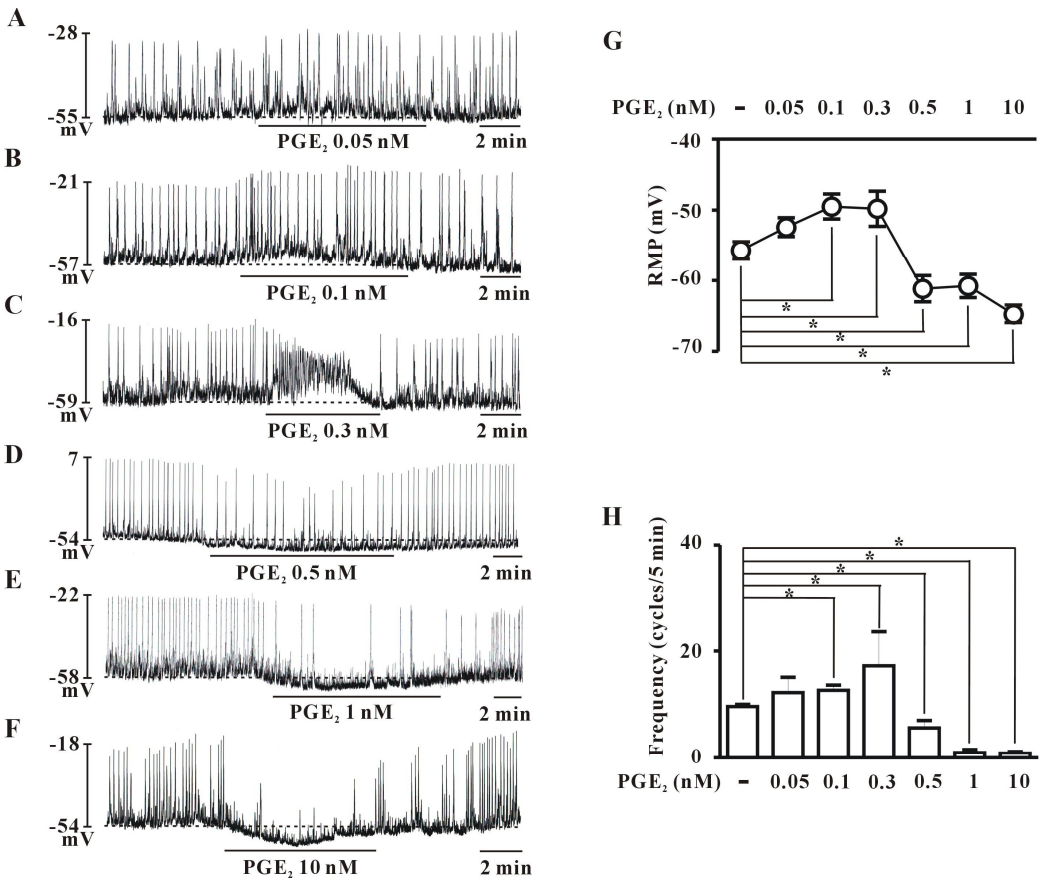

Fig. 6. Effects of PGE_2 on pacemaker potentials in colonic ICC. Pacemaker potential from ICC exposed to PGE_2 in current clamping. Low concentration of PGE_2 (0.05 nM ~ 0.3 nM) depolarized membrane potential and increased frequencies (A ~ C). Conversely, high concentration of PGE_2 (0.5 nM ~ 10 nM) hyperpolarized membrane potential and inhibited pacemaker potential frequencies (D ~ F). Changes in the resting membrane potentials were represented in (G), the variation of control is 0 mV. (H) are the summarized data of frequency in response to PGE_2. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.2. Prostanoid EP3 and EP4 receptors are involved in PGE_2-induced effects on colonic ICC

PGE_2 targets four EP receptors: EP1, EP2, EP3 and EP4. The expression of EP receptor varies depending on the tissues and cell types. In order to understand the existence of EP receptor subtype in colonic ICC, I examined the expression of EP receptor subtypes in the total mRNA extracted from the whole cultured colonic cells and single colonic ICC cells respectively by performing RT-PCR. Total RNA extracted from cultured whole colonic cells detected all four subtypes of EP receptor (Fig. 7A). Total RNA extracted from single colonic ICC expressed mRNA encoding for EP3 and EP4 receptor. Only ANO1 but not PGP9.5 and myosin expression in single colonic ICC implying that there were no neuron cells and smooth muscle cells, but only ICC in the sample (Fig. 7B).

To determine whether the EP3 and EP4 involved in pacemaker activity, I tested the effects of selective EP3 receptor agonist (sulprostone, 100 nM) and EP4 receptor agonist (CAY10580, 1 μM) by performing current clamping (Fig. 8A and 8B). Sulprostone depolarized membrane potential from -56.6 ± 1.5 mV to -51 ± 2.1 mV and increased frequency from 11.2 ± 1.1 cycles/5min to 29.3 ± 5.3 cycles/5min. CAY10580 almost totally blocked the generation of pacemaker potentials, with the frequency decrease from 11.2 ± 1.1 cycles/5min to 0.3 ± 0.2 cycles/5min. But the membrane potential did not change too much (from -56.6 ± 1.5 mV to -57.9 ± 1.7 mV) (Fig. 8C and 8D). These RT-PCR and pharmacological results indicated that PGE_2 may mediate pacemaker activity through EP3 and EP4 receptor in colonic ICC. EP3 agonist mimic the PGE_2 effect with low concentration. Also, the effect of EP4 agonist very similar to the effect of PGE_2 with high concentration. It may indicates that EP3 and EP4 receptor activated by PGE_2 with differ concentration.

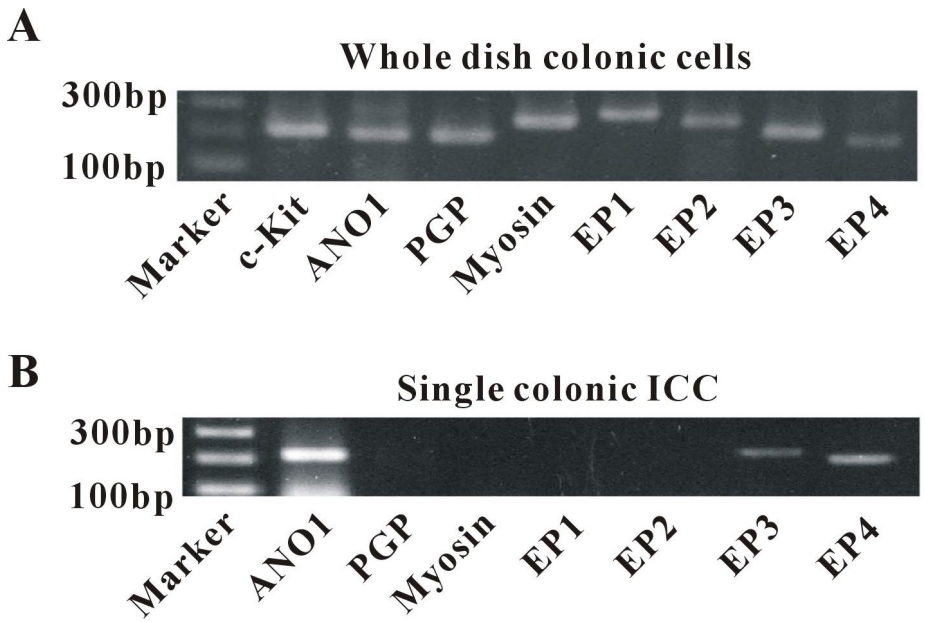

Fig. 7. Agarose gels of the RT-PCR products of four subtypes of prostaglandin EP receptor by using cultured whole colonic cells and single colonic ICC. 2% agarose gel was loaded with 10 μl of PCR products and stained with ethidium bromide. The markers shown in lane indicated product size. C-kit and ANO1 are makers of ICC. PGP9.5 and myosin were makers of neuron cells and smooth muscle cells, respectively. (A) All of four EP receptors primers produced the expected products in whole colonic cells (lanes 6 to 9). (B) EP3 and EP4 receptor primers produced the expected products in single colonic ICC (lanes 7 and 8).

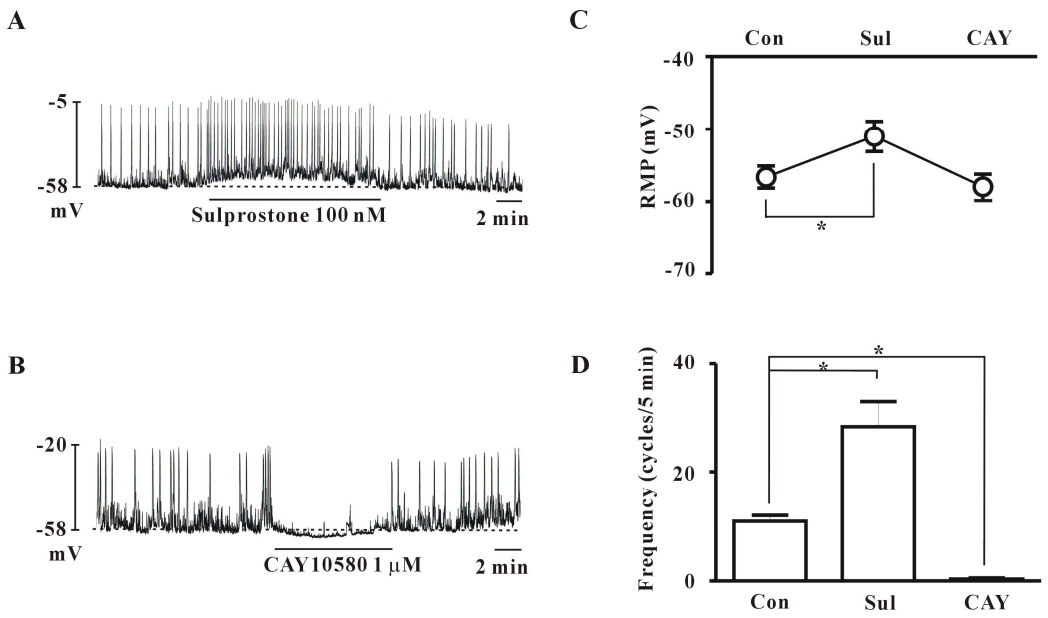

Fig. 8. Effects of EP3 receptor agonist (sulprostone) and EP4 receptor agonist (CAY10580) on pacemaker potentials in colonic ICC. (A) Sulprostone (100 nM) depolarized membrane potential and increased the pacemaker potential frequency. (B) CAY10580 (1 μM) inhibited the generation of pacemaker potentials. (C and D) The summarize data of changes in membrane potentials and pacemaker potential frequency. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.3. Low dose PGE_2 stimulates cAMP accumulation

It's well known that activation of EP receptors leads to changes in the production of cAMP. To explore whether low dose PGE_2 stimulates production of cAMP or not, SQ22536, an adenylate cyclase inhibitor was pre-treated. PGE_2 0.1 nM depolarized the membrane potential (4.8 ± 0.9 mV) and increased the pacemaker potential frequencies (from 8.6 ± 0.9 cycles/5min to 12.7 ± 1 cycles/5min) (n = 6). However, in the presence of SQ22536 100 μM, PGE_2 0.1 nM induced effects were almost abolished. The change of membrane potential and frequency were recorded as 0.5 ± 0.6 mV and 1.5 ± 0.6 cycles/5min (n = 4) (Fig. 9). These results suggest that low concentration of PGE_2 induced effect through activating adenylate cyclase and increasing cAMP production in colonic ICC.

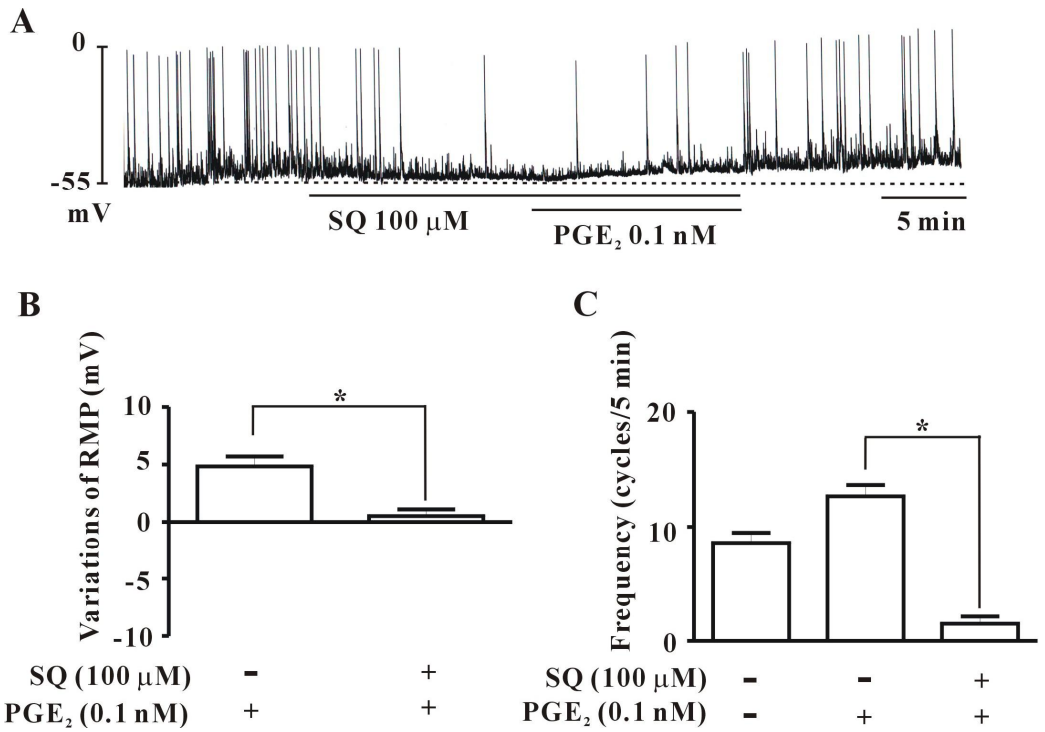

Fig. 9. Effects of PGE_2 (0.1 nM) on pacemaker potentials in the presence of an adenylate cyclase inhibitor (SQ) in colonic ICC. (A) After SQ inhibited the generation of pacemaker activity, co-treatment with PGE_2 0.1 nM did not regenerated the pacemaker potential. (B and C) Graphical presentation on the variations of membrane potential and frequency on PGE_2 0.1 nM and co-treatment with SQ and PGE_2 0.1 nM. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.4. Selective EP3 agonist sulprostone enhanced pacemaker activity with a concentration dependent manner

To rule out the effects induced from PGE_2 via EP4 receptor, I used a selective EP3 agonist sulprostone to mimic the effect of low dose PGE_2 via EP3 receptor. Cultured colonic ICC were exposed to sulprostone in a range of concentration from 10 nM to 1 μM. Sulprostone depolarized membrane potential and increased frequencies in a concentration dependent manner under current clamp mode at holding potential of -70 mV (Fig. 10A ~ 10C). Under the control condition, pacemaker potentials and frequencies generated from ICC were recorded as -57.9 ± 1.2 mV and 11.4 ± 0.7 cycles/5min (n = 18). When treated with 10 nM, 100 nM and 1 μM, the membrane potentials were measured as -59.0 ± 1.5 mV, -51.8 ± 1.7 mV and -45.3 ± 2.5 mV (Fig. 10D). In the mean time, the mean control values of pacemaker frequency were changed respectively to 16.8 ± 1.5 cycles/5min, 29.3 ± 5.3 cycles/5min and 49.3 ± 15.4 cycles/5min in treatment of sulprostone with 10 nM (n = 8), 100 nM (n = 6) and 1 μM (n = 4) (Fig. 10E). This results suggested sulprostone mimic the effects of low concentration of PGE_2 on the pacemaker potentials of colonic ICC.

To confirm whether sulprostone induced enhancement of pacemaker activity was activated through EP3 receptor or not, I pre-treated a selective EP3 antagonist L798106 (5 μM). Compared to treat sulprostone alone, L798106 almost completely blocked sulprostone induced depolarization and enhancement of frequency. The depolarization of membrane potentials and frequency were decreased from 5.6 ± 1.4 mV, 29.3 ± 5.3 cycles/5min to 0.6 ± 0.2 mV, 0.7 ± 0.7 cycles/5min (n = 5) (Fig. 11). This result suggested that EP3 receptor mediated the effects of sulprostone on the pacemaker potentials of colonic ICC.

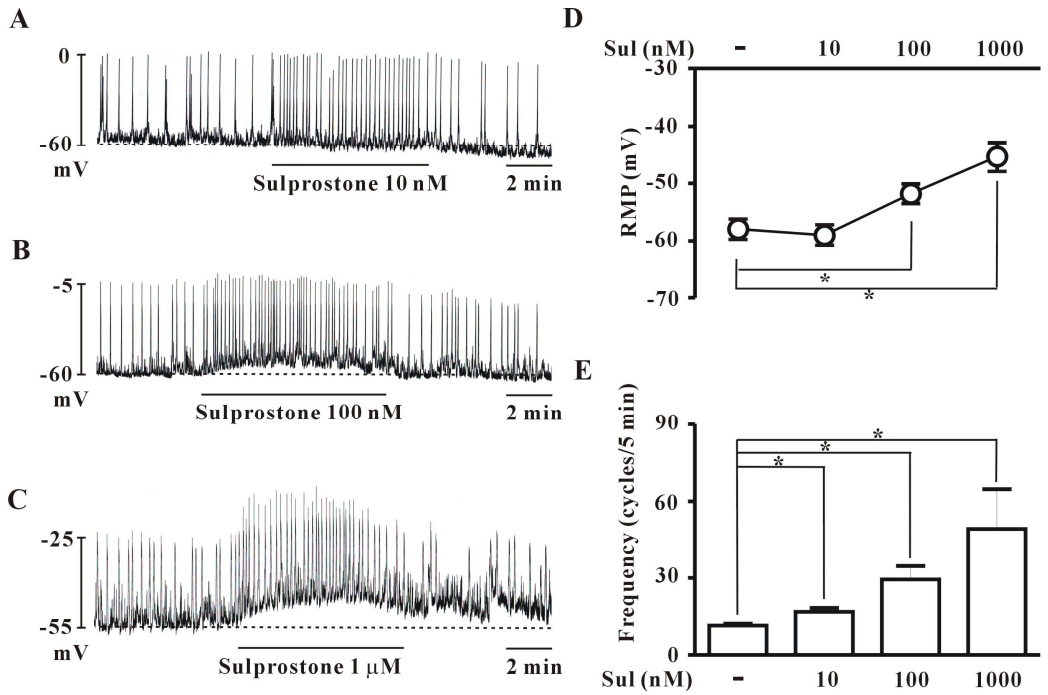

Fig. 10. Effects of sulprostone (a selective EP3 agonist)on pacemaker potentials in colonic ICC. (A ~ C) Colonic ICC exposed to sulprostone in a range of 10 nM, 100nM and 1uM concentration respectively at holding potential of -70 mV under current clamp. Sulprostone depolarized membrane potential and increased frequencies with a concentration-dependent manner. (D and E) Graphical presentation of sulprostone induced effects on membrane potentials and frequencies of pacemaker potentials produced from colonic ICC. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

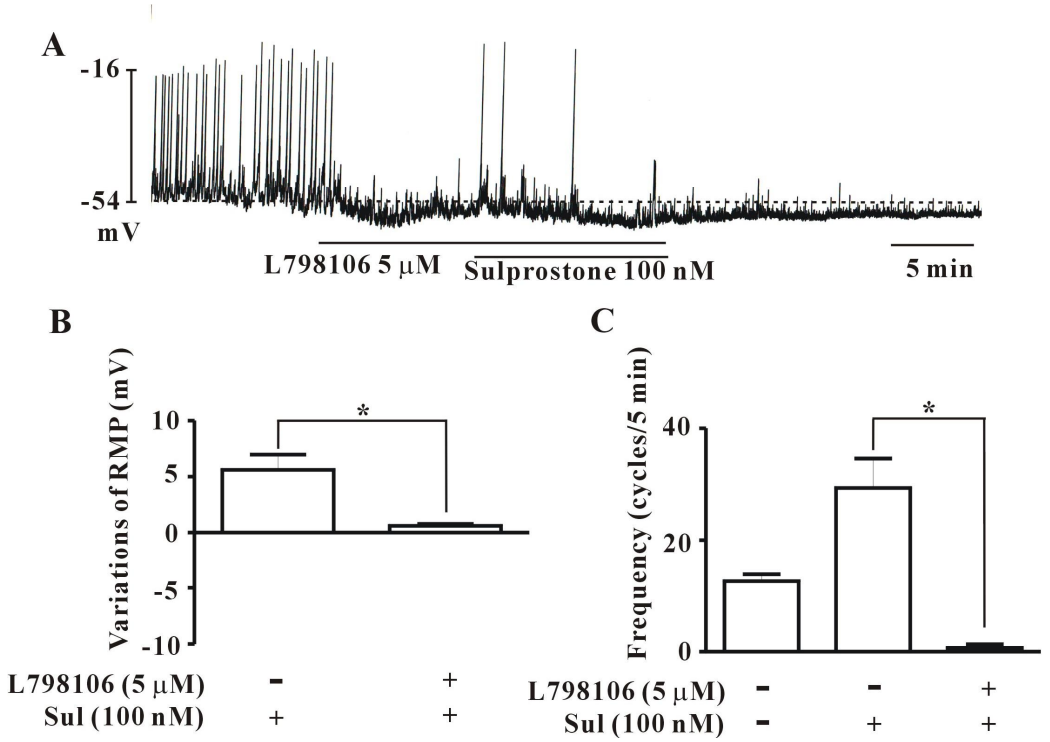

Fig. 11. Effects of sulprostone on pacemaker potentials in the presence of a selective EP3 receptor antagonist (L798106) in colonic ICC. (A) Pre-treatment of L798106 5 μ M followed by co-treatment of sulprostone 100 nM. Sulprostone induced effect was almost completely blocked by L798106. (B and C) Graphical comparison of sulprostone and co-treatment with L798106 and sulprostone induced variation of pacemaker potentials and frequency. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.5. Sulprostone enhanced pacemaker activity via phospholipase C but not protein kinase C in colonic ICC

To examine whether the regulation of sulprostone induced effects on pacemaker potentials is mediated by phospholipase C (PLC) or not, U-73122, a specific inhibitor of PLC was used. U-73122 (5 μM) was applied to colonic ICC inhibited the generation of pacemaker potentials by itself. In presence of U-73122, the effect induced by sulprostone was almost blocked (Fig. 12A). The membrane potnetial and pacemaker potential frequency induced co-treatment of U73122 and sulprostone was decresed from 5.6 ± 1.4 mV to 0.7 ± 0.2 mV, 29.3 ± 5.3 cycles/5min to 3 ± 3 cycles/5min (n = 4), comparing to treated sulprostone alone (Fig. 12B and 12C). The result suggested that PLC involved in sulprostone-mediated EP3 receptor activation.

PLC cleaves the phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Since IP_3 releases Ca^{2+} from the endoplasmic reticulum periodically is a fundamental pacemaker mechanism of ICC. DAG activates protein kinase C (PKC). To investigate whether sulprostone activated DAG - PKC pathway or not, a PKC inhibitor (chelerythrine) was treated. Compare to treat sulprostone alone, pre-treated chelerythrine in colonic ICC did not blocked the effect induced by sulprostone (Fig. 13A). The variation of membrane potentials (from 5.6 ± 1.4 mV to 6.3 ± 1.7 mV) and frequency (from 29.3 ± 5.3 cycles/5min to 28.75 ± 4.8 cycles/5min) did not altered significantly (Fig. 13B and 13C). This result indicated that IP_3 but not DAG - PKC pathway is involved in the sulprostone induced enhancement of pacemaker potentials.

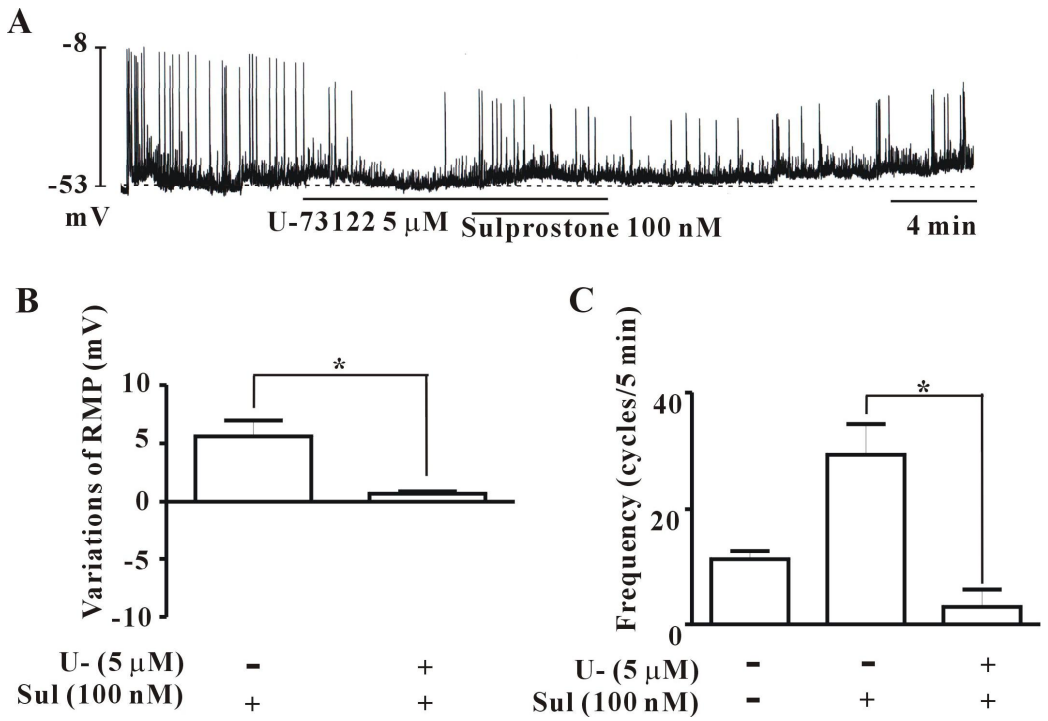

Fig. 12. Effects of sulprostone on pacemaker potentials in the presence of phospholipase C inhibitor (U-73122) in colonic ICC. (A) Pre-treatment of U-73122 (5 μM) followed by co-treatment of sulprostone (100 nM). Sulprostone induced effect was blocked by U-73122. (B and C) Graphical comparison of sulprostone and co-treatment with U-73122 and sulprostone induced variation of pacemaker potentials and frequency. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

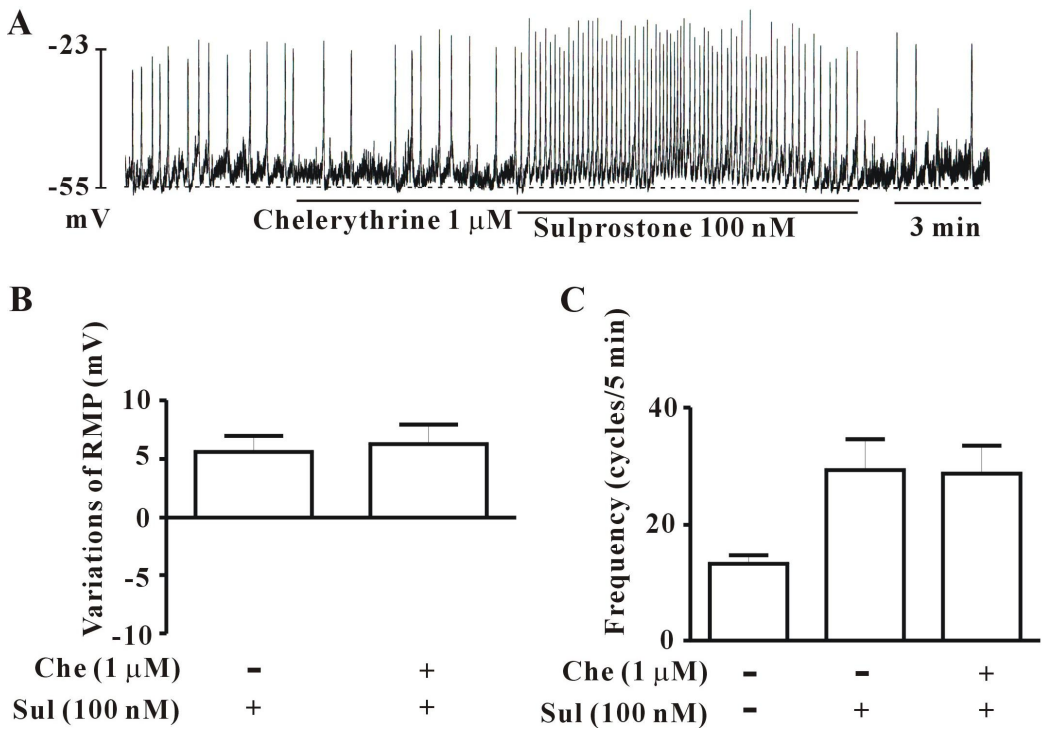

Fig. 13. Effects of sulprostone on pacemaker potentials in the presence of protein kinase C inhibitor (chelerythrine) in colonic ICC. (A) Pre-treatment of chelerythrine 1uM followed by co-treatment of sulprostone 100 nM. Sulprostone induced effect was not blocked by chelerythrine. (B and C) Graphical comparison of sulprostone and co-treatment with chelerythrine and sulprostone induced variation of pacemaker potentials and frequency. Bar represent mean values ± standard error (SE).

3.6. Sulprostone induced activation of pacemaker potentials depends on the variation of intracellular Ca^{2+} concentration

Periodic release and uptake of Ca^{2+} from endoplasmic reticulum is a primary pacemaker mechanism of ICC. To explore the variation of intracellular Ca^{2+} concentration after treating sulprostone, ICC were exposed to thapsigargin, a Ca^{2+} ATPase inhibitor of endoplasmic reticulum. Thapsigargin (1 μM) itself depolarized membrane potential and inhibited the generation of pacemaker potentials. ICC were further co-treated thapsigargin and sulprostone together. Thapsigargin significantly abolished the sulprostone induced activation of pacemaker activity (Fig. 14A). Compared to treatment of sulprostone alone, the change of membrane potentials and frequency were decreased from 5.6 ± 1.4 mV, 29.3 ± 5.3 cycles/5min to -0.7 ± 0.9 mV, 0.5 ± 0.5 cycles/5min (Fig. 14B and 14C). This result suggest that sulprostone induced effect on pacemaker activity related to Ca^{2+} uptake by endoplasmic reticulum.

To confirm whether effects induced by sulprostone related to the variation of intracellular Ca^{2+} concentration, we stained colonic ICC with Fluo-4/AM and measured intracellular Ca^{2+} concentration after treated the culture dish with sulprostone 100 nM. The Ca^{2+} wave frequency significantly increased after treating sulprostone 100 nM (Fig. 15).

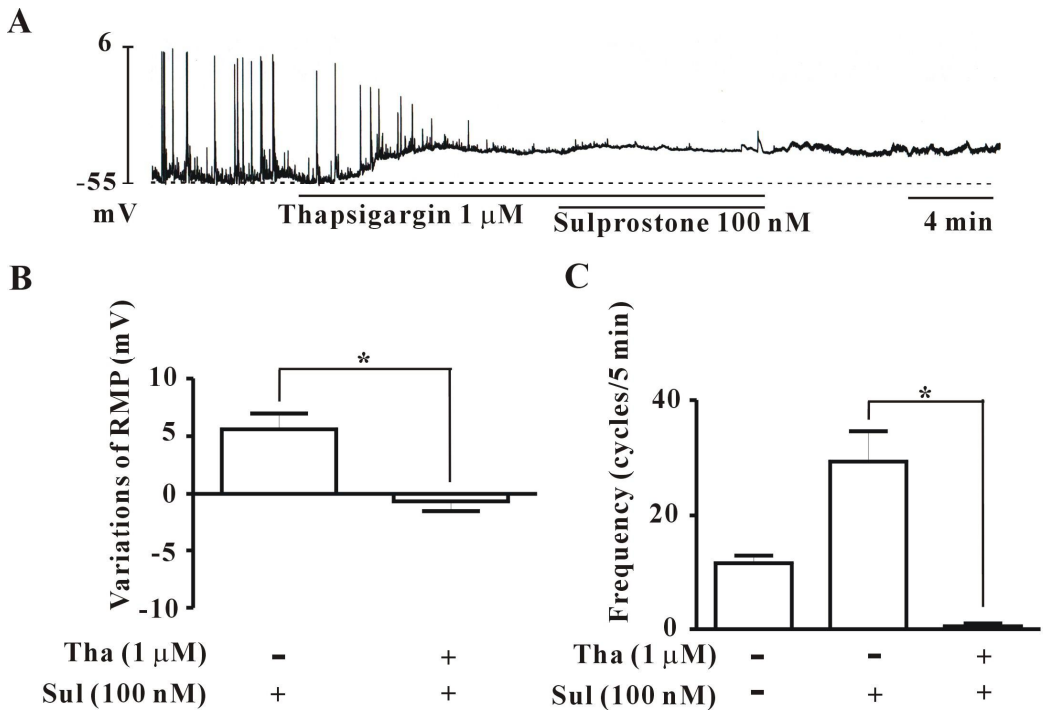

Fig. 14. Effects of sulprostone on pacemaker potentials in the presence of Ca^{2+}-ATPase inhibitor (thapsigargin) in colonic ICC. (A) Pre-treatment of thapsigargin (1 μM) followed by co-treatment with sulprostone (100 nM). Sulprostone induced enhancement of pacemaker potentials were blocked by thapsigargin. (B and C) Graphical comparison of sulprostone and co-treatment with thapsigargin and sulprostone induced variation of pacemaker potentials and frequency. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

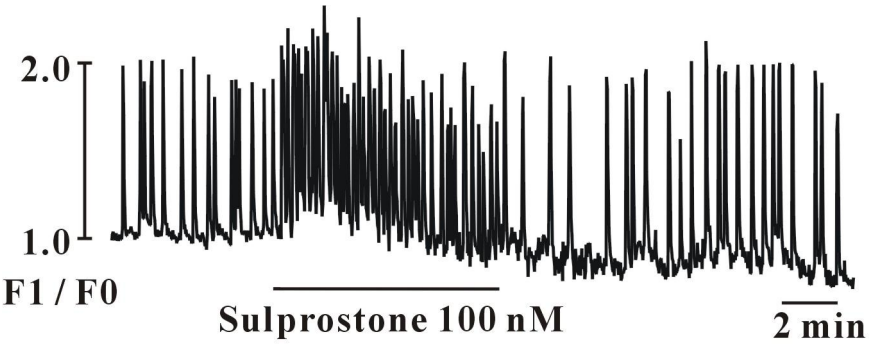

Fig. 15. Variation of Intracellular calcium waves after treating sulprostone in colonic ICC. Upon treatment of sulprostone 100 nM, the calcium oscillation frequency increased.

3.7. Sulprostone activated pacemaker potentials through ANO1 channels

Studies suggested that activation of ANO1 channel by periodically released Ca^{2+} from endoplasmic reticulum is a primary pacemaker mechanism of ICC. To confirm if ANO1 channel involved in sulprostone induced effect or not, ICC were exposed to TMEM16A inhibitor (5 μM) or MONNA (5 μM). Both two ANO1 channel blockers hyperpolarized membrane potential and inhibited the generation of pacemaker potentials. ICC were further co-treated with ANO1 channel blockers and sulprostone together. TMEM16A inhibitor and MONNA significantly abolished the sulprostone induced augment of pacemaker activity (Fig. 16A and 16B). Compared to treatment of sulprostone alone, the change of membrane potentials and frequency were decreased from 5.6 ± 1.4 mV, 29.3 ± 5.3 cycles/5min to -0.7 ± 0.9 mV, 0.5 ± 0.5 cycles/5min and -0.7 ± 0.9 mV, 0.5 ± 0.5 cycles/5min, respectively (Fig. 16C and 16D). This result suggest that ANO1 channel involved in sulprostone induced effect on colonic ICC.

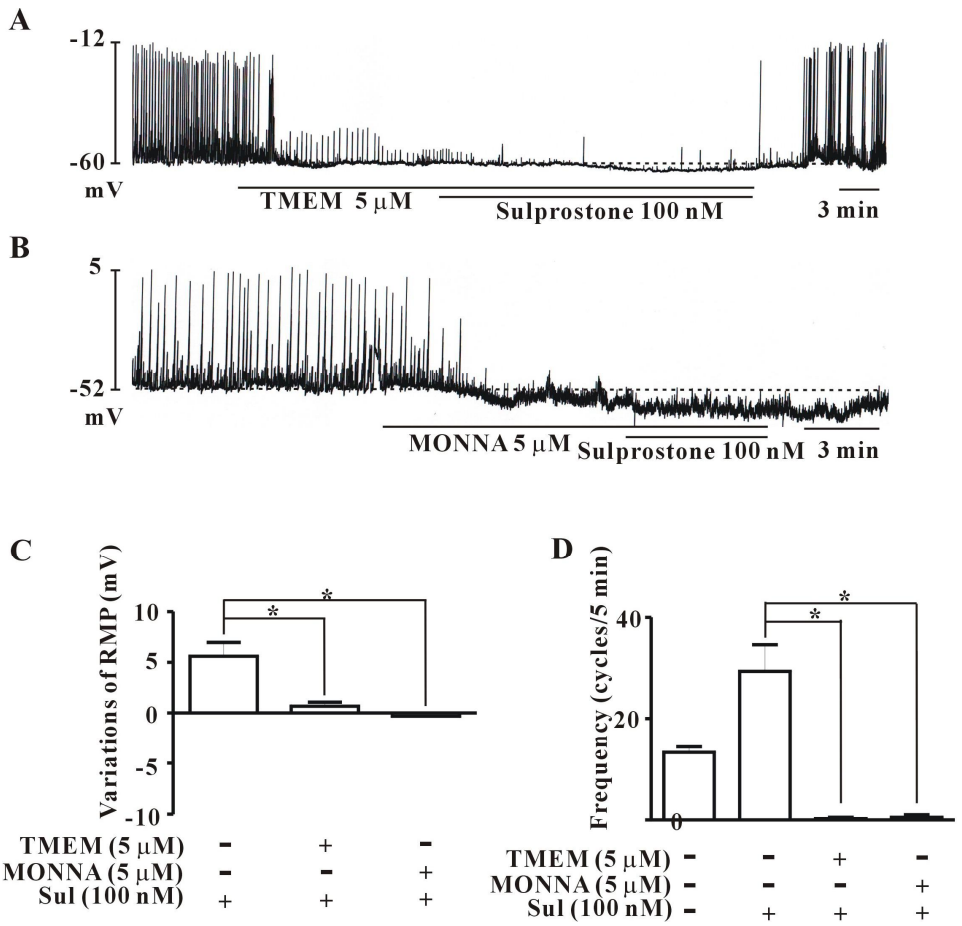

Fig. 16. Effects of sulprostone on pacemaker potentials in the presence of ANO1 channel blockers (TMEM16A inhibitor and MONNA) in colonic ICC. (A) Pre-treatment of TMEM16A inhibitor (5 μM) followed by co-treatment with sulprostone (100 nM). Sulprostone induced effect was blocked by TMEM16A inhibitor. (B) ICC exposed to MONNA (5 μM) followed by co-treatment with sulprostone (100 nM). Sulprostone induced effect was blocked by MONNA. (B and C) Graphical comparison of sulprostone and co-treatment with ANO1 channel blockers and sulprostone induced variation of pacemaker potentials and frequency. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.8. High concentration of PGE_2 activates ATP-sensitive K^+ channels in colonic ICC

It was reported that ATP-sensitive K^+ channels were activated in colonic ICC in resting state and contribute to maintain the resting membrane potential. Due to the effects of PGE_2 (10 nM) hyperpolarized membrane potential, I pre-treated glibenclamide, an ATP-sensitive K^+ channels blocker, to determine whether high concentration of PGE_2 affects ATP-sensitive K^+ channels in colonic ICC. ICC cell was exposed to glibenclamide (10 μM) for 10 minutes and PGE_2 (10 nM) was treated in continuous presence of glibenclamide as shown (Fig. 17A). Compare to the control, PGE_2-induced inhibitory effects was significantly blocked by glibenclamide. After treating glibenclamide, the variation of membrane potentials and frequency of PGE_2 were increased from -9.6 ± 0.8 mV, 0.9 ± 0.3 cycles/5min to -2.5 ± 1.0 mV, 5.7 ± 1.5 cycles/5min (Fig. 17B and 17C). The results suggest that PGE_2 (10 nM) activates ATP-sensitive K^+ channels in colonic ICC.

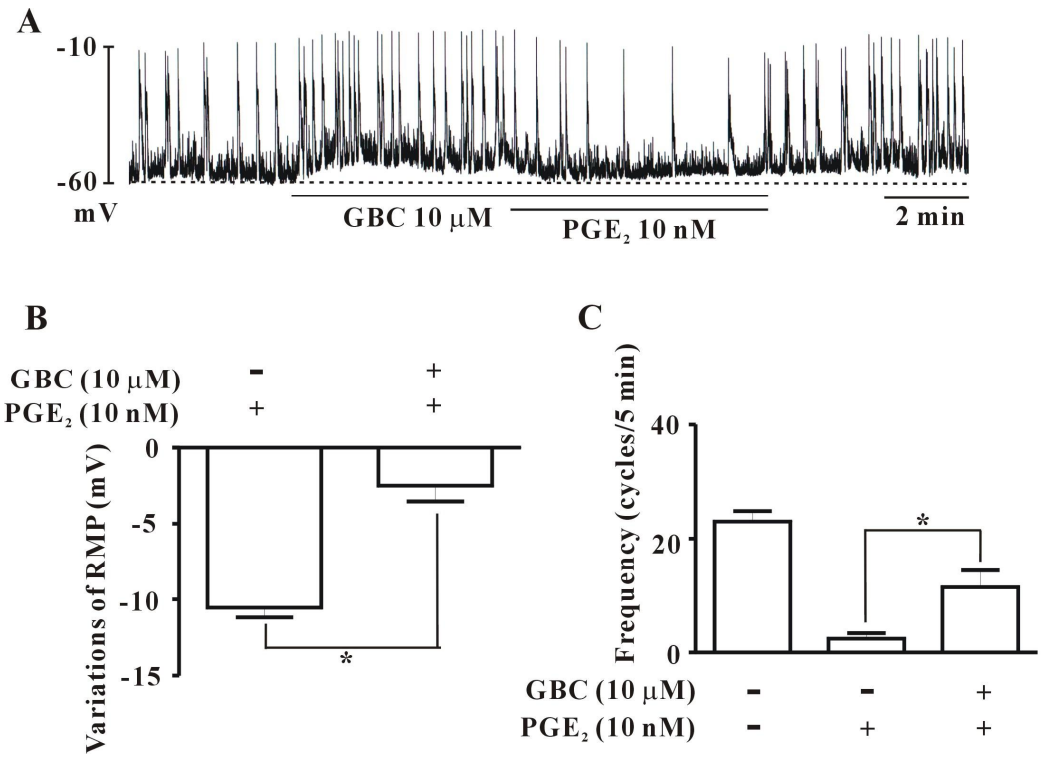

Fig. 17. Effects of PGE_2 (10 nM) on pacemaker potentials in the presence of an ATP-sensitive potassium channel blocker (glibenclamide) in colonic ICC. (A) Pre-treatment of glibenclamide (GBC, 10 μM) blocked the inhibitory effects induced by PGE_2 (10 nM). (B and C) The statistical representation of changes in membrane potentials and frequency. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.9. Voltage-dependent K^+ channel and Ca^{2+}-activated K^+ channels are not involved in high dose PGE_2 induced inhibition of pacemaker potential

To explore whether PGE_2 affects other potassium channels in ICC, I treated colonic ICC with tetraethylammonium (TEA, a voltage-dependent K^+ channel blocker), 4-aminopyridine (4-AP, a voltage-dependent K^+ channel blocker) and apamin (a Ca^{2+}-activated K^+ channel blocker). TEA 5 mM (n = 3), 4-AP 5 mM (n = 3) and Apamin 100 nM (n = 3) did not block the inhibitory effects of PGE_2 10 nM (Fig. 18A ~ 18C). These results suggest that voltage-dependent K^+ channels and Ca^{2+} activated K^+ channels may not be involved in PGE_2 induced inhibition of pacemaker potential in colonic ICC.

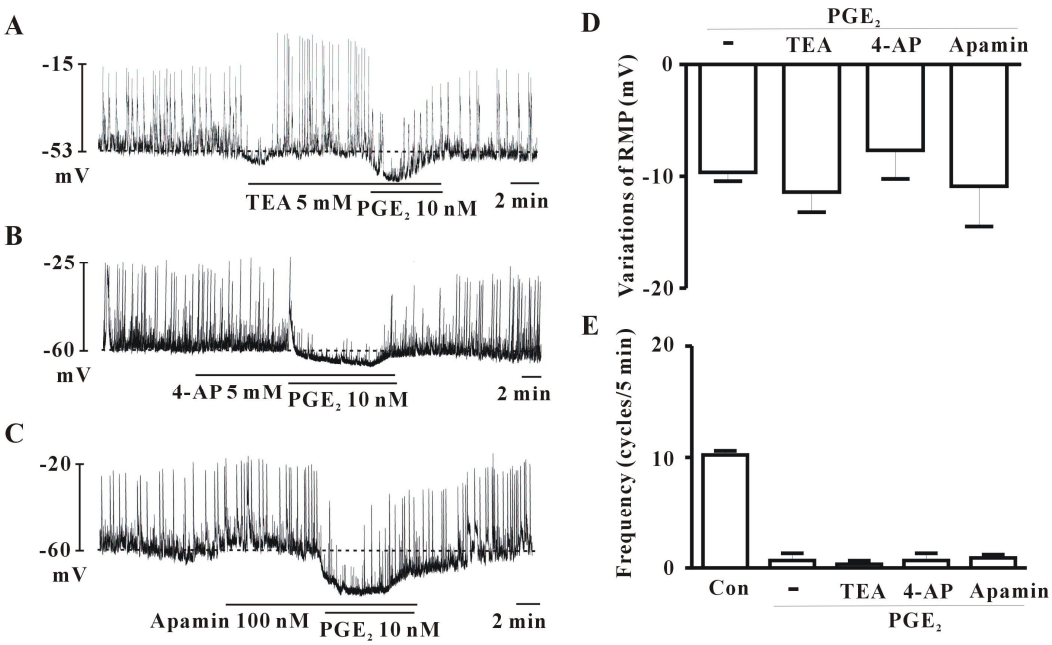

Fig. 18. Effects of high dose PGE_2 (10nM) on pacemaker potentials in the presence of tetraethylammonium (TEA), 4-aminopyridine (4-AP) and Apamin in colonic ICC. (A ~ C) TEA (5 mM), 4-AP (5 mM), Apamin (100 nM) did not block the inhibitory effect induced by PGE_2 (10 nM). The summarize data are shown in E and F. Bar represent mean values ± standard error (SE).

3.10. T-type Ca^{2+} channels may play a role in the inhibition of pacemaker potential induced by high dose PGE_2

T-type current is required for entrainment of pacemaker activity within ICC. To confirm whether the T-type Ca^{2+} channel involved in PGE_2-induced inhibition of pacemaker activity, nickel chloride 50 μM (n = 4) and mibefradil 1 μM (n = 3) (T-type Ca^{2+} channel blockers) were treated. After treating, $NiCl_2$ and mibefradil only inhibited pacemaker potential frequency (from 9.86 ± 1.14 cycles/5min to 0.75 ± 0.48 cycles/5min and 0.67 ± 0.33 cycles/5min), but didn't change the membrane potential (Fig. 19). The data may imply that the activation of T-type Ca^{2+} channels is vital for the generation of pacemaker activity. Hyperpolarization induced by PGE_2 may leading to the closure of T-type Ca^{2+} channel.

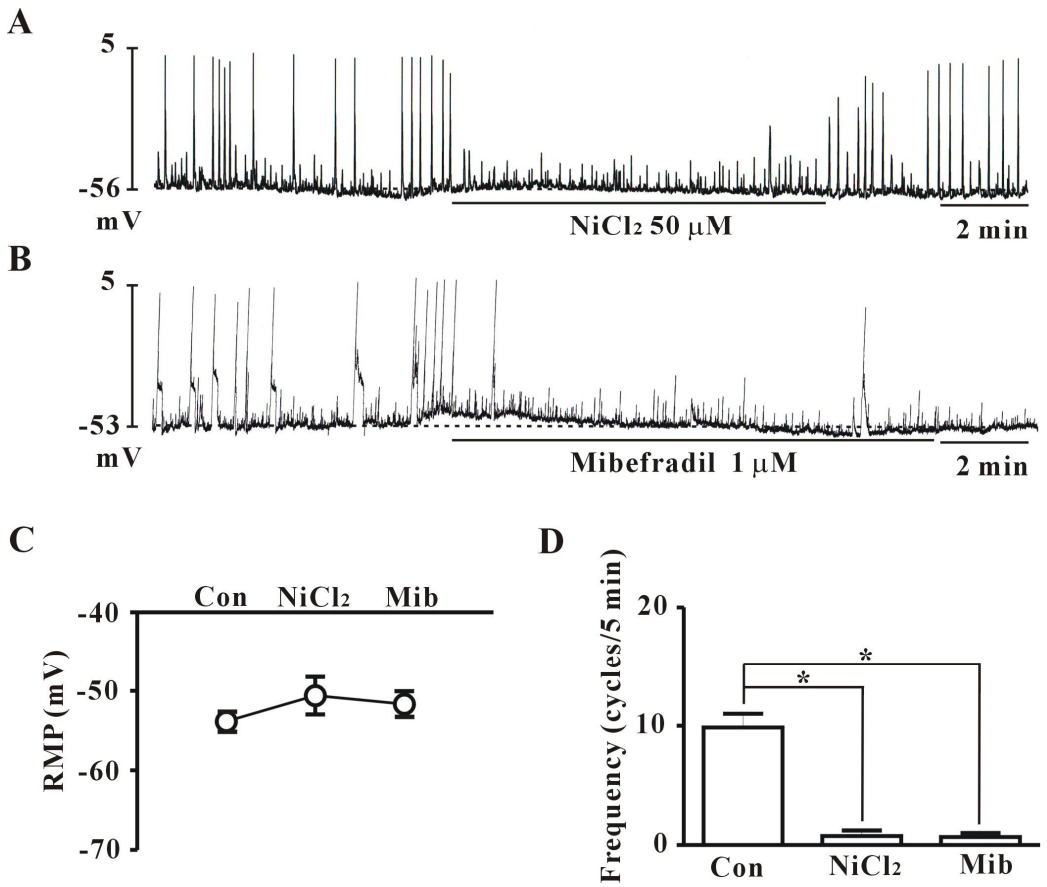

Fig. 19. Effects of Nickel chloride ($NiCl_2$) and mibefradil on pacemaker potentials in colonic ICC. (A and B) $NiCl_2$ and mibefradil inhibited the generation of pacemaker potentials. The responses to $NiCl_2$ are summarized in C and D. Bar represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

4. DISCUSSION

This study has shown that PGE_2 mediated pacemaker activity via EP3 and EP4 receptors in colonic ICC with different concentrations. The depolarization and increase of pacemaker frequency induced by low dose PGE_2 were blocked by SQ22536 (an adenylate cyclase inhibitor). Treatment of sulprostone (an EP3 agonist) mimic the effect induced by low dose PGE_2. Sulprostone induced effect was abolished by pre-treating PLC inhibitor (U-73122), Ca^{2+}-ATPase inhibitor (thapsigargin) and ANO1 channel blockers (TMEM16A inhibitor and MONNA), but not PKC inhibitor (chelerythrine). In recordings of Ca^{2+} imaging, sulprostone increased intracellular Ca^{2+} oscillations. On the contrary, high dose PGE_2 hyperpolarized membrane potential and blocked the generation of pacemaker potentials in a concentration dependent manner. And this inhibitory effect induced by high dose PGE_2 was blocked by a K_{ATP} channel blocker, glibenclamide.

PGE_2 has been reported to be an important regulator of GI motility (Dey *et al*., 2006). Exogenous treatment of PGE_2 leads to both relaxant and contractile response in circular smooth muscle from canine colon (Botella *et al*., 1995). PGE_2 has been also shown to induce relaxation of circular smooth muscle and contraction of the longitudinal muscle in human, guinea-pig and rat small intestine, also in human colon (Bennett *et al*., 1968 and 1981). It is well known that the slow wave of smooth muscle cells (SMC) in GI tract are generated by ICC. Previous study has shown that PGE_2 regulates pacemaker activity through EP2 receptor in small intestinal ICC (Choi *et al*., 2006). Although there are many studies related to the PGE_2 effect on GI tract and small intestinal ICC, the functions of PGE_2 on colonic pacemaker activity has been still unknown. Therefore, I checked the effect of PGE_2 on pacemaker activity of colonic ICC in this study.

In the present study, PGE_2 has shown dual effect depend on variation of concentrations in colonic ICC. Relatively high concentrations of PGE_2 (0.5 nM ~ 10 nM) caused colonic ICC hyperpolarization and cessation of pacemaker potential

frequency. In contrary, low concentration of PGE_2 (0.05 nM ~ 0.3 nM) induced depolarization and enhancement of pacemaker potentials (Fig. 6). PGE_2 exerts its effects through binding to EP receptors, and also signaling through different EP receptor leads to the various effects of PGE_2. It has been reported that PGE_2 enhanced rat colon contractions via EP1 and EP3 receptors (Iizuka *et al*., 2014). Conversely, PGE_2 relaxed mice colon through EP2 and EP4 receptors (Martinez-Cutillas *et al*., 2014). Based on this premise, I performed RT-PCR assays with single ICC cells. EP3 and EP4 receptor was identified in mRNA transcript in colonic ICC (Fig. 7). Furthermore, EP3 agonist (sulprostone) has shown enhancement of pacemaker potential and mimicked the effect induced by low concentration of PGE_2 (Fig. 8A). By the other side, EP4 agonist (CAY10580) inhibited pacemaker potentials and this effects was similar to the high concentration of PGE_2 (Fig. 8B). These results suggested that the dual effects induced by PGE_2 in colonic ICC probably due to activation of EP3 and EP4 receptors. Additionally, one possible explanation for why PGE_2 binding to EP3 and EP4 receptor with different concentration is that PGE_2 has higher binding affinity for EP3 than EP4. The dissociation constants (Kd) of PGE_2 for EP3 and EP4 in mouse is 0.85 nM and 1.9 nM, respectively (Kiriyama *et al*., 1997).

In the present study, PGE_2 with low concentration depolarized the membrane potential and increased frequency by activating EP3 receptor (Fig. 6 ~ 8). Among four EP receptors, EP3 receptor is unique in the sense that it consists of three isoforms in mouse which generated by alternative splicing of the C-terminal tail ($EP3_\alpha$, $EP3_\beta$ and $EP3_\gamma$). These isoforms can coupling to different signaling pathways (Namba *et al*., 1993; Pierce and Regan, 1998). The major signaling pathway of EP3 is involved in the inhibition of adenylate cyclase via $G_{\alpha i}$ activation, which decrease the production of cAMP (Narumiya *et al*., 1999). Along with $G_{\alpha i}$ activation, the EP3 receptor has been shown to augment adenylate cyclase activity by coupling to $G_{\alpha s}$ (Hatae *et al*., 2002; Sugimoto and Narumiya, 2007). In colonic ICC, we previously found that the 8-bromo-cAMP (a cell permeable cAMP analog) increased the pacemaker potential frequency (Shahi *et al*., 2014). It is similar to the effect induced

by low dose PGE_2. In figure 9B, PGE_2 with low concentration increased the membrane potential and frequency, which were blocked by the pre-treatment of SQ-22536 (an adenylate cyclase inhibitor). This result suggested that low dose PGE_2 exert its effect by activating the adenylate cyclase in colonic ICC. In other words, it implied that the variation of cAMP concentration is very important for low dose PGE_2 action.

PGE_2 has been shown to bind to EP3 with a K_d value of 0.9 nM in mouse. However, sulprostone has been identified as a more selective EP3 agonist (Markovič *et al.*, 2017). In order to test the effect of PGE_2 on EP3 receptor, I confirmed the effect of sulprostone and used it to replace PGE_2 0.1 nM in the following study. Sulprostone significantly depolarized the membrane potential and increasing the pacemaker potential frequency with a dose-dependent manner in colonic ICC (Fig. 10). And L798106 (a potent EP3 antagonist) blocked the effect induced by sulprostone (Fig. 11). These results strongly indicated that sulprostone is a selective EP3 agonist and it can mimic the effect induced by PGE_2 with low concentration.

Ca^{2+} is regarded as another second messenger involved in EP3 signaling pathway. It is reported that PGE_2 activates phospholipase C (PLC) and elevates intracellular Ca^{2+} concentration through EP3 receptor in myometrial cells (Asboth *et al.*, 1996). As is commonly known, stimulation of PLC results in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 release Ca^{2+} from endoplasmic reticulum (ER) into the cytoplasm, and DAG stimulates PKC. Meanwhile, periodic Ca^{2+} release and uptake by ER and mitochondria is fundamental pacemaker mechanism of ICC (ward *et al.*, 2000). In this study, PLC inhibitor (U-73122) and Ca^{2+} ATPase inhibitor (thapsigargin) blocked sulprostone induced effect, but not PKC inhibitor (chelerythrine) (Fig. 12 ~ 14). After treating sulprostone, the intracellular concentration of Ca^{2+} was significantly increased in colonic ICC (fig. 15). It suggests that sulprostone depolarized membrane potential and increased pacemaker potential frequency by activating PLC and IP_3 to release Ca^{2+} from ER, which increasing intracellular Ca^{2+} concentration. How did the increased intracellular Ca^{2+} enhance

colonic ICC pacemaker activity after treating sulprostone? Sanders suggested that the Ca^{2+} oscillation is linked to generation of pacemaker activity by periodically activating Ca^{2+}-activated Cl^- channels (ANO1 channels). In the present study, sulprostone induced significant enhancement of pacemaker potential was totally blocked by ANO1 channel blockers (TMEM16A inhibitor and MONNA) (Fig. 16). These suggested that ANO1 channel was involved in the sulprostone induced effect on the pacemaker potential of colonic ICC.

Next I checked the effect of high dose PGE_2 on pacemaker activity of colonic ICC. ATP-sensitive potassium (K_{ATP}) channels have been reported as resting membrane potential maintainer and regulator of cell excitability in various tissues (Clapp and Gurney, 1992). Our previous work has also proved that K_{ATP} channels in colonic ICC were activated in resting state and play an important role in maintaining resting membrane potentials. Activation of K_{ATP} channels leads to hyperpolarization of membrane potentials, whereas inhibition of K_{ATP} channels lead to depolarization of membrane potentials (Na *et al*., 2017). Moreover, the action of PGE_2 in small intestine ICC depends on the activation of K_{ATP} channels (Choi *et al*., 2006). In figure 17B, PGE_2 with high concentration did not shown significant inhibition of pacemaker potential after blocking K_{ATP} channel with glibenclamide (a K_{ATP} channel blocker). In figure 18, TEA, 4-AP and apamin (voltage-dependent K^+ channel and Ca^{2+}-activated K^+ channel blockers) did not blocked the effect induced by high dose PGE_2. These results suggests that the stimulation of high dose PGE_2 may activate K_{ATP} channels thereby hyper-polarizing the membrane potential of colonic ICC.

Voltage-dependent calcium channel were considered as an important portion in the generation of pacemaker activity. Ca^{2+}-induced spontaneous transient depolarizations can trigger the opening of voltage-dependent calcium channels, which in turn serves to further generates slow wave (Blair *et al*., 2014). T-type calcium channels were suggested to be required for generation of pacemaker activity and for active propagation of slow waves (Zheng *et al*., 2014). It was reported that activation of K^+ channels induced hyperpolarization of resting membrane potential, leading to vasodilatation via blocking of voltage-dependent Ca^{2+} channels in vascular smooth

muscle (Jackson, 2000). In the present study, $NiCl_2$ and mibefradil (T-type calcium channel blockers) blocked the generation of pacemaker potentials (Fig. 19). I speculate that high dose PGE_2 opened K_{ATP} channels and hyperpolarized membrane potentials in colonic ICC. T-type channels are closed below their activation threshold. Under these conditions the influx of Ca^{2+} is less, finally inhibited the generation of pacemaker activity and propagation of slow wave. However, it needs further study to confirm this idea.

In summary, PGE_2 shown dual effects on colonic ICC. It activates EP3 receptor with low concentration and activates EP4 receptor with high concentration. Activation of EP3 receptor by low dose PGE_2 or sulprostone depolarizes the membrane potential and increases pacemaker potential frequency. This activation may involves two signaling pathways: 1) enhancement of cAMP concentration; 2) activation of ANO1 channels through PLC dependent intracellular Ca^{2+} release from endoplasmic reticulum. Activation of EP4 receptor by relatively high dose PGE_2 hyperpolarized the membrane potential and abolished pacemaker potential frequency. In this condition, PGE_2 may open ATP-sensitive K^+ channels with an unknown pathway. Thus, considering the distribution of EP receptors in colon, PGE_2 may play multiple role in regulating colonic motility.

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6. ABSTRACT

BACKGROUND: Interstitial cells of Cajal (ICC) generate spontaneous pacemaker acitivity responsible for the production of slow waves in gastrointestinal (GI) smooth muscle. Prostaglandin E_2 (PGE_2) is one of the most important prostanoids found throughout the GI tract and are responsible for multiple important physiological and pathological processes. PGE_2 exert its effects through binding to EP receptors, therefore leading to various signaling pathways.

METHODS: To understand the role of PGE_2 on packmaker activity in colonic ICC, I performed whole cell patch clamp, RT-PCR and Ca^{2+} imaging techniques.

RESULTS: PGE_2 has shown a dual effect on pacemaker potentials in current clamp mode. RT-PCR data suggested the expression of EP3 and EP4 in colonic ICC. Low concentration of PGE_2 depolarized membrane potential and increase the generation of pacemaker activity, and this effect was blocked after pre-treatment of SQ22536 (an adenylate cyclase inhibitor). Meanwhile, the effect induced by low dose PGE_2 was mimic by the sulprostone, an EP3 agonist. Sulprostone induced effect was inhibited by pre-treating PLC inhibitor (U-73122), Ca^{2+}-ATPase inhibitor (thapsigargin) and ANO1 channel blockers (TMEM16A inhibitor and MONNA), but not PKC inhibitor (chelerythrine). In recordings of Ca^{2+} imaging, sulprostone increased intracellular Ca^{2+} oscillations. Conversely, high concentration of PGE_2 hyperpolarized membrane potential and blocked the generation of pacemaker potentials in a concentration dependent manner. However, this inhibitory effect induced by high dose PGE_2 was blocked by a K_{ATP} channel blocker, glibenclamide.

CONCLUSION: Low concentration of PGE_2 activates EP3 may activate adenylate cyclase or excite ANO1 channels through PLC dependent intracellular Ca^{2+} release from endoplasmic reticulum. High concentration of PGE_2 activates EP4 opens ATP-sensitive K^+ channels to regulate pacemaker activity of colonic ICC.

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