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The comparison of Pacemaker Activity from Interstitial Cells of Cajal among Stomach, Small intestine and Colon

Graduate School of Chosun University

Department of Medicine

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위, 소장, 대장의 카할 간질세포에서 발생되는 향도잡이 활동도에 대한 비교 연구

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ABBREVIATIONS

GI - Gastrointestinal

SMC - Smooth Muscle Cells

ICCs - Interstitial Cells of Cajal

Ca^{2+} - Calcium

ER - Endoplasmic Reticulum

RyR – Ryanodine Receptors

IP_3R - Inositol 1,4,5-trisphosphate Receptors

ANO1 Channel - Anoctamin1 Channel

CACC - Ca^{2+} Activated Cl^- Channel

NSCC - Non-selective Cation Channels

HCN - Hyperpolarization-activated Cyclic Nucleotide-gated Channels

TRP - Transient Receptor Potential Channels

CICR - Calcium-induced Calcium Release

cAMP - 3',5'-cyclic Adenosine Monophosphate

ODQ - Oxadiazolo Quinoxalin

cGMP - 3′,5′-cyclic Monophosphate

SNAP - S-Nitroso-N-acetylpenicillamine

TPH - Tryptophan Hydroxylase

EC - Enterochromaffin Cells

CNS - Central Nervous System

STICs - Spontaneous Transient Inward Currents

STDs - Spontaneous Membrane Potentials

DDA - 2', 3'-dideoxyadesnoine

ACh - Acetylcholine

초 록

위, 소장, 대장의 카할 간질세포에서 발생되는 향도잡이 활동도에 대한 비교 연구

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서론 : 카할 사이질 세포는 자발적인 향도잡이 활동도를 생산하며 이는 위장관 평활근 세포로부터 생산되는 서파에 영향을 주어 수축을 조절하는 기능을 가지고 있다. 기존 많은 보고에서 서파는 위, 소장 그리고 대장에서 서로 다른 양상이 관찰된다. 하지만 이에 대한 정확한 연구를 밝혀지지 않아 본 연구를 통해 확인해 보았다.

연구방법 : 연구 결과 도출하기 위해 본 연구자는 마우스의 위, 소장 그리고 대장에서 카할 사이질 세포를 배양하였고 배양된 세포를 이용하여 whole cell patch clamp 방법을 실시하였다.

결과 : ANO1 이온통로 억제제와 T-type 칼슘 이온통로 억제제는 위와 대장의 카할 사이질 세포의 향도잡이 활동도를 억제하였지만 소장에는 큰 역할을 보여주지 못하였다. 더불어 HCN 이온통로 억제제와 ATP 민감성 K^+ 이온통로 억제제는 대장 카할 사이질 세포의 향도잡이 활동도에만 유의성 있게 효과를 나타내었다. 또한 세포막 투과가 가능한 cAMP와 cGMP을 투여하였을 때 역시 대장 카할 사이질 세포에서만 향도잡이 활동도에 영향을 나타내었다. cAMP와 cGMP 관련 억제제 역시 대장 카할 사이질 세포의 향도잡이 활동도에 효과를 나타내었다. 더불어 신경전달물질은 위장관 운동성에 매우 중요한 역할을 하기에 본 연구에서 위, 소장 그리고 대장 카할 사이질 세포의 향도잡이 활동도에 대한 효과를 확인해 보았다. 흥분성 신경전달물질은 모든 부위의 향도잡이 활동도에 비슷한 양상의 효과를 확인할 수 있었다.

결론 : 카할 사이질 세포에서 발생되는 향도잡이 활동도에 중요한 역할을 담당하는 것으로 알려진 ANO1과 T-type 칼슘이온 이온통로는 위 그리고 대장의 카할 사이질 세포에서만 기능적 역할을 담당하는 것으로 확인되었다. 또한 HCN 그리고 ATP 민감성 K^+ 이온통로는 대장 카할 사이질 세포에 영향을 주는 것으로 확

인되었다. 더불어 대장 카할 사이질 세포에만 cAMP가 기능적 역할을 가지는 것으로 확인되었고 cGMP는 모든 부위에 그 효과를 관찰할 수 있었다. 위장관 운동성에 중요한 역할을 담당하는 신경전달물질은 모든 부위에서 비슷한 효과가 관찰되었다.

1. INTRODUCTION

1.1 The gastrointestinal tract and its motility

The GI tract is an important visceral organ which is consist of pharynx, esophagus, stomach, small intestine, colon and rectum, has a four-layers structure including mucosa, submucosa, muscular layers and serosa. It plays a vital role on taking in nutrients and eliminating waste products fromits motility, which is elicited by phasic contractions in both circular and longitude muscles and contains peristalsis and segmentation. The motility in the GI tract which exists from stomach to colon dependson the response of GI smooth muscles to enteric neural, hormonal, and paracrine factors.

1.2 Slow waves

The existing studies found that GI smooth muscle has spontaneous, periodic and phasic contractions. The quiescent and tonic activities of smooth muscles origin from the regular depolarization and repolarization of membrane potential in SMC called slow waves.

1.2.1 Slow waves mechanism

Slow waves are essential property of smooth muscle but do not evoke smooth muscle contraction by themselves. When the membrane potential of slow waves reach to threshold and release of Ca^{2+} relying on the opening action of voltage-dependent Ca^{2+} channels and lead to the periodic entry of Ca^{2+}. The electrical activity results in the persistent excitation-contraction process of smooth muscles (Huizinga et al. ,1995).

1.2.2 The different pattern of slow waves in stomach, small intestine and colon

Those contractile activities vary from stomach to colon in the forms of different slow waves. In corpus and antrum of stomach, slow waves depolarization are adequate to cause enough Ca^{2+} entry and contractions of SMC. In small intestine and colon only some small contractions can be elicited by depolarization of slow waves, and most of the contractions are caused by generation of Ca^{2+} action potentials.

Slow waves which elicit smooth muscles contractions vary from the region. In corpus

and antrum of stomach, slow waves depolarization are adequate to cause enough Ca^{2+} entry and contractions of SMC. Slow waves have 3 cycles per minute frequency and long-lasting plateau potential part. In small intestine and colon only some small contractions can be elicited by depolarization of slow waves, and most of the contractions are caused by generation of Ca^{2+} action potentials. Slow waves in small intestine have 12-15 cycles per minute and the frequency has a gradient to 6 cycles per minute along to the colon. As for the region, in small intestine, slow waves have the same amplitude and frequency in circular and longitude layer. In colon, the plateau potentials were in cells with the contractions of SMC in small amplitude and higher frequency. The action potentials in serosal surface in LM or CM has a frequency of 3-4 cycles per minute and large amplitude (Kito et al., 2011).

Former researcher shows that slow waves result from intrinsic pacemaker activity. Some recordings made by implements of SMC and ICCs indicate that pacemaker activity origins from ICCs.

1.3 Interstitial cells of Cajal

The intrinsic rhythmicity origins from the pacemaker activities of interstitial cells of Cajal in the pacemaker regions (Sanders et al., 2012). The spontaneous pacemaker potentials generated by ICCs consists of various shapes, frequencies, amplitudes and durations. The former researchers have some recognitions on ICCs. In the late period of 19th, a Spanish neuroanatomist named Santiago Ramón y Cajal found a type of spindle-shaped and stellate cells with prominent nuclei and varicose processes that formed networks in GI tissues by using Golgi technique and staining with methylene blue, he named them as “cellues interstitilles”. Later on, other researchers termed these cells as a new class of cells referred to ICCs rather than neurons (Sanders et al., 1996). Those cells were considered as the pacemaker cells in the GI tract due to the adjacent structure between ICCs and SMC and were suggested to drive the contraction through the smooth muscle (Thuneberg et al., 1982).

1.3.1 The morphology and identification of ICCs

In the different organs of individual species (like rat, guinea-pig, mice and dogs), the

shape, distribution, and ultrastructural features of ICCs vary from the anatomical locations throughout the digestive tract. Most of all, ICCs have small body size and several lengthened processes. ICCs were characterized by researchers through ultrastructural features (Komuro T, 1999), which helps to identify them. The presence of ICCs includes:(a) plenty of mitochondrials and caveolaes; (b) a discontinuous basal lamina;(c) plenty intermediate filaments; (d) well-developed Golgi apparatus, few ribosomes, rough and smooth ER; (e) close connections and gap junctions with nerves and SMC, can form a network through the intestinal wall, and with SMC(Al-Shboul OA, 2013).

For a long time, to investigate ICCs further is extremely hard because there is no specific labels of the ICCs until the discovery of the expression of c-kit, a protooncogene that encodes a receptor tyrosine kinase (Huizinga, 1995). c-kit modulates hematopoiesis, gametogenesis, melanogenesis as well as the proliferation in several progenitor cells such as mast cells, melanocytes and ICCs (Duttlinger et al., 1995). Physiological analysis demonstrated that the mechanisms for the spontaneous pacing of contractions in isolated gut segments are defective in the anti-c-kit mAb-treated mice, W/Wv mice and even W/+ mice. These findings suggest that c-kit plays a crucial role in the development of the pacemaker mechanism of ICCs that is responsible for the generation of autonomic gut motility (Maeda et al., 1992). However, c-kit also expressed in the mast cells and recent years, another specific marker of ICCs was extensively accepted and studied by the researchers, called TMEM16A, anoctamin-1 or CACC channel, which was considered as an important channel in the generation of the pacemaker activity of ICCs (Gomez-Pinilla et al., 2009).

1.3.2 Distribution of ICCs

ICCs have been found in many tissues both inside of GI tract and outside of the GI tract such as urethra (Sergeant et al., 2006), the upper urinary tract , myocardium, uterus, fallopian tube, myometrium, human placenta and the ciliary muscle in monkeys. Most attention of ICCs was attracted by researchers in GI tract because of the wide-spread and the important function in the GI tract. ICCs are found throughout the whole tract containing esophagus, stomach, small intestine, large intestine and pancreas.

Small number of ICCs are more densely in the antrum and corpus including ICC-MY and ICC-IM. In the fundus, only ICC-IM were detected (Hirst et al., 2002). In the small intestine, there are ICC-MY, ICC-IM, and ICC-DMP. ICC-MY have various receptors for kinds of neurotransmitters and hormones and close associations with myenteric nerves. ICC-DMP forms synapse like contacts with nitrergic and cholinergic nerves and specialized connections with both nerves and SMC. Gap junctions with SMC are wide-existed, prompting them to transmit information from nerves to muscles (Al-Shboul et al., 2013). ICC-IM were found remarkably in the inner layer of muscularis propria (Wang et al., 2003). ICCs in the colon include subtypes of ICC-MY, ICC-IM, and ICC-SMP. ICC-SMP refers to the class of ICCs which is located in the submucosal nerve plexus (SMP), just beneath the innermost circular layer. ICC-IM are distributed throughout the musculature and range with SMC in both muscle layers (Mazzia et al., 2000). ICCs located in the submucosal layer is less dense than other position. Compared with small intestine, total number of ICCs is less in the colon.

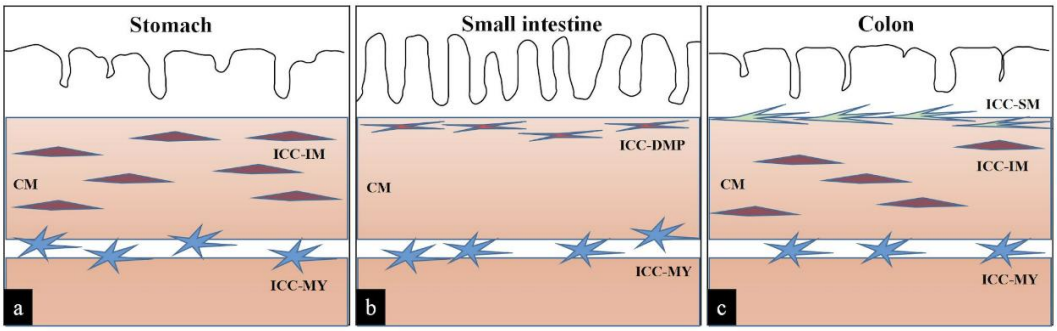

Fig. 1. Distribution of ICCs in stomach, small intestine and colon.

1.3.3 Physiological function of ICCs

Recently, ICCs in the GI tract has aroused people's concern. Early studies found that slow waves were abolished when ICCs were obliterated by some experimental ways in intact GI tissues (Ördög et al., 1999). Further electrical physiological techniques (whole-cell patch clamp) confirmed that a kind of rhythmical, electrical activity was obtained in isolated and cultured ICCs, the waveforms and properties of which were

similar to slow waves in intact tissues (Koh et al., 1998).

(a) ICCs act as pacemaker cells in the GI tract. Current studies suggested that ICCs generate the rhythmic, spontaneous, active inward current and then propagate to the neighboring SMC through the close connections called gap junctions. SMC induce the cyclic depolarization in the membrane which is termed as slow wave potential. Slow wave can determine the frequency of smooth muscle contraction.

(b) ICCs act as an intermediate to modulate the enteric neurotransmission. ICCs express lots of receptors for neurotransmitters and hormones, and have the close synaptic contacts (< 20nm) with excitatory and inhibitory neurons. Based upon the structural relationship between enteric nerve and smooth muscle, many studies have strongly suggested the role of ICCs to transduce the neurotransmission signaling in GI networks (Daniel et al., 1984)

(c) ICCs in stretching sensing. Some researchers found the response of stretch is disappeared in W/Wv mice (which lack of ICC-IM), which suggested that ICC-IM might play a significant role in the stretch sensitivity in gastric muscles (Won et al., 2005). Some studies showed that a mechanosensitive Na^+ channel current exists in intestinal ICCs in human body and seems to have an action on the control of motility in the digestive tracts (Strege et al., 2003). In spite of previous studies confirmed this phenomenon, further experiments in this field need to be done in the future.

1.3.4 Pacemaker mechanism

ICCs attract considerable attentions as pacemaker cells which generate pacemaker activities and propagate them to neighboring cells such as SMC within tunica muscularis according to present studies. However, the mechanism of pacemaker activity of ICCs is still under controversial.

A majority of researches are performed in this field and proposed that a chloride channel line behind the rhythmic depolarization that lead to the upstroke of the slow waves, rhythmic activation of chloride channels is believed to be activated by Ca^{2+} released from the ER through IP_3R and RyR (Huizinga et al., 2002). Some researchers show that blockers of the IP3 receptor and calcium pump in the membrane of ER inhibit slow-wave activity and the whole-cell inward current produced by the murine

ICCs c-kit^{+} cells developed in cell culture (Ward et al., 2000). This result proposed that Ca^{2+} oscillation is likely to initiate the pacemaker activity from ICCs.

Moreover, some researchers confirmed that Ca^{2+}-dependent inward current was involved in pacemaker activity in intact muscle strips and bundles because pacemaker activity of cells was reduced when treated with membrane-permeable Ca^{2+} buffers (Kito et al., 2003). The Ca^{2+}-dependent conductance has been taken to be a Cl^{-} conductance, because pacemaker activity were blocked by a variety of Cl^{-} channel blockers in guinea-pig (Kito et al., 2002) and murine muscles (Hirst et al., 2002) even in the ICC-MY in small intestine (Kito et al., 2003). Furthermore, studies of isolated and cultured ICCs have suggested that spontaneous activity is generated by activation of a non-selective cation conductance (Sanders et al., 2007).

This oscillation of Ca^{2+} inside of the cytoplasm probably results in the activation of the Ca^{2+}-dependent ion channels located cross the membrane, it was proved to be CACC channel, ANO1, encoded by Tmem16a. Several conductance, such as CACC and NSCC such as HCN channels and TRP channels have been suggested to be denoted to the generation of pacemaker activity from ICCs (Choi et al., 2022)

Recently, some studies mentioned a kind of theory that the underlying mechanism of generation and propagation of pacemaker activity in ICC-MY in modulating the contraction of smooth muscle.

(a) First of all, Ca^{2+} transients occurring during the plateau phase of propagating slow waves are largely caused by the release of Ca^{2+} from stores via RyR receptors and IP3R receptors in the membrane of ER.

(b) This Ca^{2+} influx into excluded volume or microdomain trigger activation of CACC and lead to massive Cl^{-} influx, producing STICs. STICs result in STDs (Drumm et al., 2017).

(c) STDs activate voltage-dependent Ca^{2+} channel (T-type Ca^{2+} channel) which leads masses of Ca^{2+} flow into cytoplasm and triggers activation of IP_3R. Summation of Ca^{2+} release sustains activation of ANO1 and generate pacemaker activity.

(d)Because pacemaker activity depolarizes the membrane potential of adjacent cells such as SMC and activates the voltage-dependent Ca^{2+} channel (L-type), which results in the excitation-contraction coupling, slow waves are generated and make the contraction in

the smooth muscle (Drumm et al., 2017).

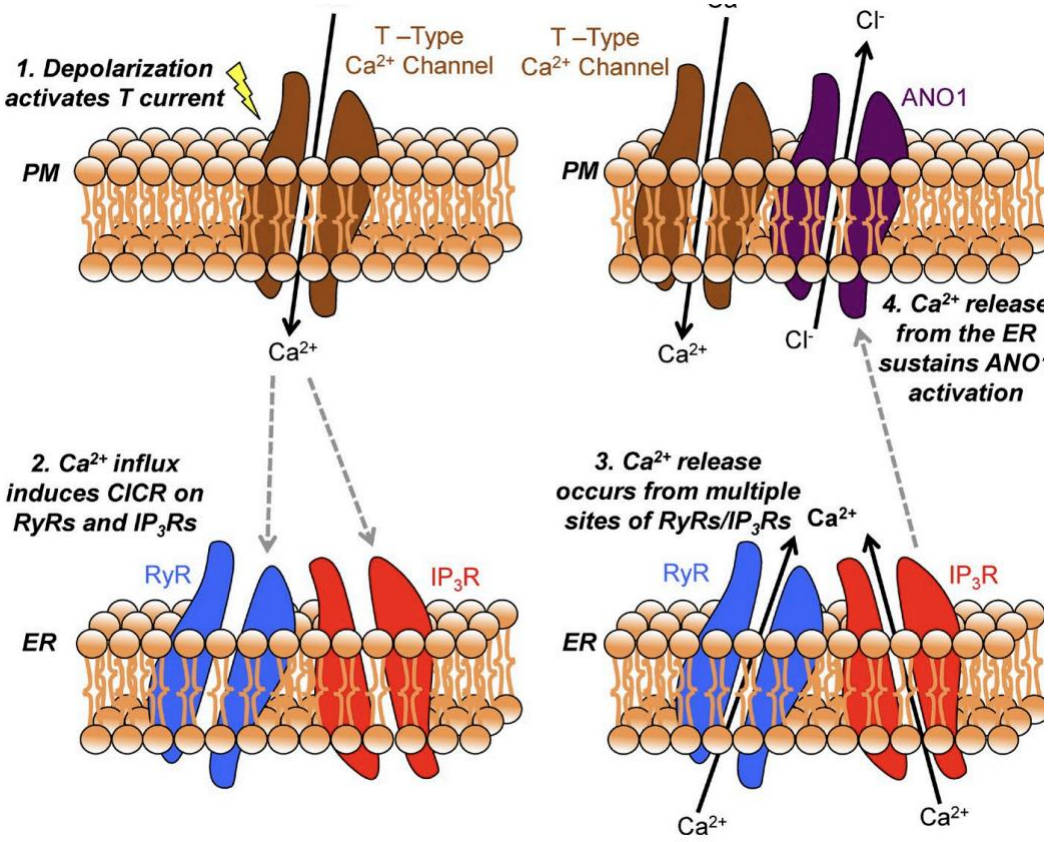

Fig. 2. Schematic showing the mechanism by which pacemaker activity in the mousepropagate via asynchronous ER Ca^{2+} release. RyR, ryanodine receptor; PM, plasma membrane; CICR, calcium-induced calcium release. (Drumm et al., 2017)

HCN channels are voltage-gated channels that are encoded by the HCN 1-4 gene family. They are primarily found in the heart system and in the central and peripheral nervous systems (Notomi et al., 2004), and allow for the passage of Na^+ and K^+ ions through these channels. HCN channels are known as pacemaker cells in the heart and are activated by both voltage changes and cellular cAMP. HCN channels are crucial for regulating neuronal excitability, rhythmic neuronal activity, and synaptic transmission, and thus play a role in numerous physiological functions. In the past, there have been suggestions that HCN channels may be involved in pacemaker activity in ICCs in the

GI tract (Shahi et al., 2014).

Until now, there was no report indicates that pacemaker activities shows difference in each pattern of the organ. Since that I make the comparison of pacemaker activity in stomach, small intestine and colon via studying the related ion channels by performing current clamp from mice.

2. MATERIALS AND METHODS

2.1 Experimental Animals and Ethical Approval

Following the guidelines set out in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, all experiments were carried out and approved by the ethics committee of Chosun University. The mice were given a standard diet and had unrestricted access to water. Male and female ICR mice aged between 5 and 8 days were anesthetized with diethyl ether and humanely killed through cervical dislocation.

2.2 Preparation of Cells and Tissue Isolation

The colon was removed from below the cecum to the rectum, and the middle portion of the colon was used. Small intestine was from 1cm below the pyloric ring to the cecum. The colon and small intestine were opened along the mesenteric border. Opened tissues were pinned to the bottom of a Sylgard dish filled with ice-cold Ca^{2+} free Hank's solution. After removing the luminal contents, the mucosa and submucosa was removed by dissection. The isolated tissue was equilibrated in Ca^{2+}-free Hank's solution for 30 min. Cells were scattered with an enzyme solution containing 1.3mg/mL collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2mg/mL bovine serum albumin (Sigma, St. Louis, MO, USA), 2mg/mL trypsin inhibitor (Sigma), and 0.27 mg/mL ATP, then incubated in 37℃ water bath for 14 minutes. After 3 washes with Ca^{2+}-free Hank's solution to remove enzyme, the tissues were triturated by a series of blunt pipettes of decreasing tip diameter. Small pieces of tissue were put onto sterile glass coverslips coated with 200μl poly-L-lysine (Sigma-Aldrich) in 35mm culture dishes. Cells were cultured in smooth muscle growth medium (Medium 231; Gibco, Grand Island, NY, USA) supplemented with 1% antibiotics/antimycotics (Gibco, Grand Island, NY, USA), and 5ng/mL urine stem cell factor (SCF; Sigma) at 37°C/5% CO_2.

2.3 Electrical Activity Recording

Current clamp mode was used to record pacemaker potentials in stomach, small intestine and colon ICCs when a network-like structure appeared after culturing (2–3 days). Pacemaker potentials were amplified using an Axopatch 200B (Axon Instruments,

Foster, CA, USA). Data were filtered at 5kHz 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 operations were performed at 30℃.

2.4 Solutions

2.4.1 Bath solution

135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 10mM Glucose, 10mM HEPES and 1.8mM $CaCl_2$; adjust the pH to 7.4 with Trisma-base.

2.4.2 Ca^{2+}-free Hank's Solution (mM):

135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 10mM Glucose, 10mM HEPES; adjust the pH to 7.4 with Trisma-base.

2.4.3 Enzymatic Solution:

2μg/ml collagenase (Worthington Biochemical Co., Lakewood, NJ, USA), 2μg/ml bovine serum albumin (Sigma, St. Louis, MO, USA), 1μg/mL trypsin inhibitor (Sigma, St. Louis, MO, USA), mixed and dissolved in the 2ml Ca^{2+}-free Hank's Solution.

2.4.4 Pipette solution

Pipette solution were loaded into the micropipette to manipulate patch clamp which contain 140mM KCl, 2.5mM $MgCl_2$, 0.1mM EGTA, 2.7mM MgATP, 0.1mM Na_2GTP, 2.5mM Creatine Phosphate Disodium, and 5mM HEPES, pH was adjusted to 7.2 with Trisma-base.

2.5 Reagents

The chemicals used in this study is as shown as followed table.

Drug names	Companies	Catalog No.
T16Ainh-A01	Calbiochem	613551
MONNA	TOCRIS	5770
CACC	sigma	SML0916
ML 218	sigma	SML0385
ZD 7288	sigma	Z3777
Cscl	sigma	C3309
glibenclamide	sigma	G0639
8-bromo-cAMP	sigma	B7880
2',3'Dideoxyadenosine	MedChemExpress	HY-W013441
8-bromo-cGMP	sigma	B1381
ODQ	sigma	O3636
cch	sigma	C-4382
serotonin (5-HT)	sigma	H7752
SNAP	Calbiochem	487910

Table 1. Drugs and Chemicals

2.6 Statistical analysis

Data are expressed as the means ± standard errors. Student's t-test for analysis paired data to evaluate the differences. P value less than 0.05 was considered statistically significant. The n-values reported in the text refer to the number of cells used in the experiments.

3. RESULTS

3.1 Pacemaker Activity Generated by ICCs Present in Stomach, Small intestine and Colon

Previous studies have described the slow waves in stomach, small intestine and colon tissue, but not much comparison among them. After culturing the cells from mouse and distinguishing the ICCs under the microscope, three types of pacemaker potential can be observed during the whole cell patching.

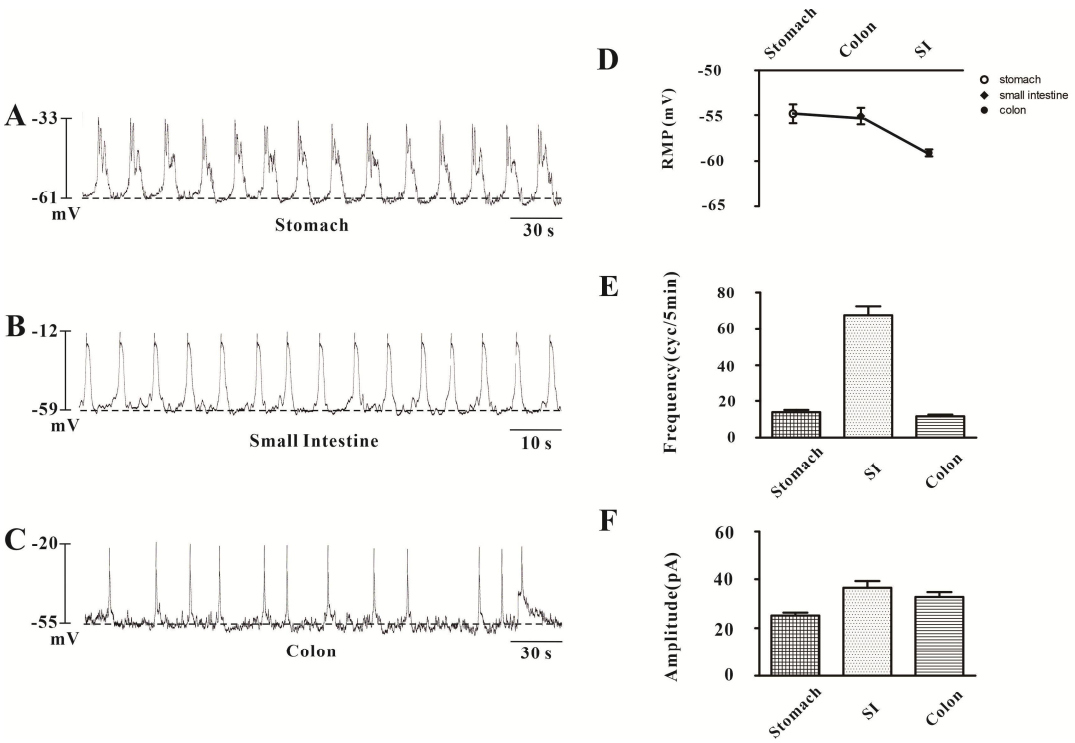

Fig. 3. Three typical pacemaker activities recorded from cultured ICCs in stomach, small intestine and colon from mice. (A) Pacemaker activities in cultured ICCs from murine stomach (B) Pacemaker activities in small intestinal ICCs (C) Pacemaker activities in colonic ICCs (D-F). Changes in the resting membrane potentials, summarized data of frequency and amplitude.

The typical resting membrane potential in stomach is around -61mV, with an amplitude of 25mV. The frequency is 16cycles/5min and the duration is bigger than small intestine and colon with long plateau potential part (n>20). In the small intestine part, pacemaker activities are more stable, which means the occurance of upstroke shows more rhythmicity. The frequency is around 70cycles/5min and amplitude is 36mV. The resting membrane potential is nearly the same as stomach, which is almost -59mV. The duration of potential is much shorter than stomach because of the high frequency (n >20). The colon part has smaller amplitude than small intestine part, nearly 33mV, and the frequency is about 12cycles/5min. The duration is similar to small intestine and the resting membrane potential is near -55mV (n>20).

3.2 The Role of ANO1 Channel in ICCs in stomach, small intestine and colon.

The activation of ANO1 channel causes peridically release of Ca^{2+} from ER, which is a primary mechanism of pacemaker activities of ICCs. The TMEM16A protein has been taken as a calcium-activated chloride channel (Caputo et al., 2008) in vascular SMC.

When treated with 5μM T16Ainh-A01, the frequency of membrane potentials in stomach decrease impressively from 18 cycles/5min to 0 cycles/5min, while resting membrane potential from -54mV to -61mV. No considerable changes of the frequency, resting membrane potentials and amplitude show in small intestine. Resting membrane potential raised from -61.5mV to -58mV (Fig. 4D) while frequency decreases from 17cycles/5min to 1cycle/5min (Fig. 4E). The result shows that T16Ainh-A01 inhibits stomach and colon pacemaker activities significantly, but has no prominent effect on small intestine.

MONNA is a potent ANO1 channel blocker. The application of MONNA hyperpolarized the membrane and the hyperpolarization is suggestive of a Cl^- conductance being activated (Boedtkjer et al., 2015).

I tested 5μM MONNA solution on ICCs to find out the possible effect on pacemaker activities. After treating, the resting membrane potential decreased from -63mV to -69mV in stomach and frequency from 15cycles/5min to 0, which means 5μM MONNA inhibited the pacemaker activities in stomach. The inhibition was also shown in colon, which frequency decreased from 14cycles/5min and resting membrane potential

from -52mV to -56mV. For small intestine, no inhibition was found with 5μM MONNA (Fig. 5).

In small intestine, both CACC and T16Ainh-16A. Studies of TMEM16A/CACC have utilized both T16Ainh and CACC. After treatment, frequency of stomach ICCs diminished from 12cycles/5min to 0 and resting membrane potential from -59mV to -64mV. Almost no change in the small intestine was observed, from frequency to amplitude. In colon, the frequency show similarity to stomach, decreasing from 12 cycles/5min to 0. The resting membrane potential changed from -58mV to -60mV(Fig. 6). The pacemaker activities were inhibited by CACC in stomach and colon. CACC showed no effect on small intestine.

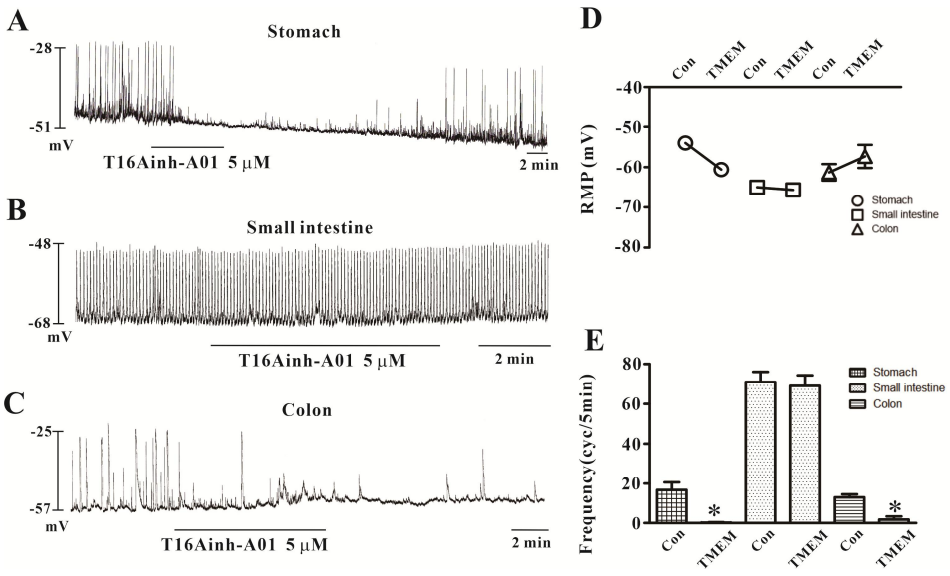

Fig. 4. (A-C) Effects of T16Ainh-A01 (5μM) on pacemaker activities of stomach, small intestine and colon. (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

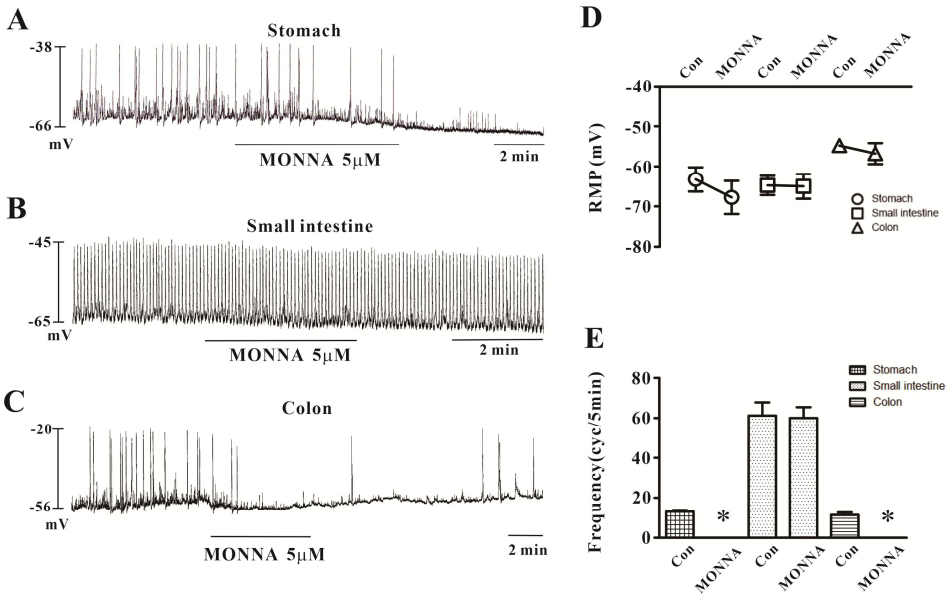

Fig. 5. (A-C) Effects of 5μM MONNA on pacemaker activities induced by cultured ICCs from stomach, small intestine and colon of murine. (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

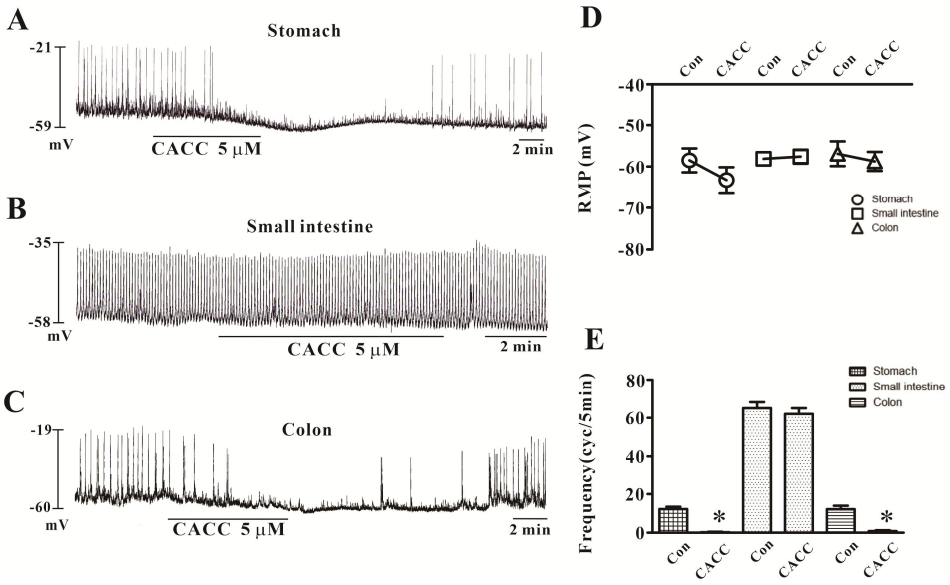

Fig. 6. (A-C) Effects of 5μM CACC on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.3 Role of T-type Ca^{2+} channel in ICCs in stomach, small intestine and colon

T-type Ca^{2+} channels have a prominent place on the basis of known mechanism of pacemaker activity of ICCs, which is in cooperated with ANO1 channels to take actions. A robust and specific blocker of T-type Ca^{2+} channels - ML 218 (10μM) were used to explore the difference of mechanism from stomach, small intestine and colon. After the treatment of ML 218, the frequency were dramatically inhibited in stomach and colon, but had no effect on small intestine (Fig. 7). This result suggested that T-type Ca^{2+} channels play a prominent role on generating pacemaker activity in stomach and colonic ICCs.

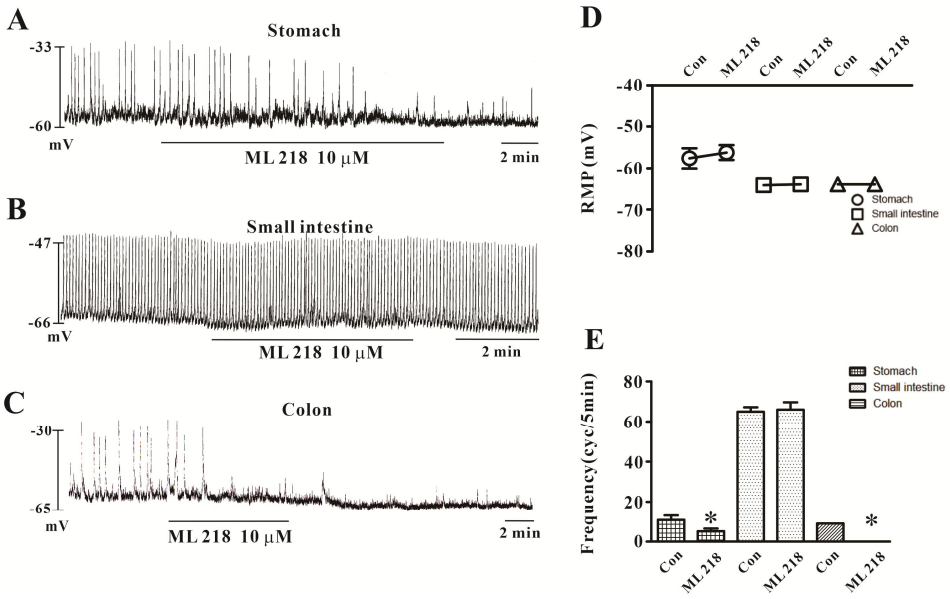

Fig. 7. (A-C) Effects of ML 218(10μM) on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.4 Mechanism of each tissue on involvement of HCN channels in cultured ICCs.

It is cleared that HCN channels can be activated by hyperpolarization of membrane, and the current through these channels work as an important pacemaker protein in the heart and brain. We believed that HCN channels are present in colonic ICCs and related to generation of spontaneous pacemaker activity (Shahi et al., 2014). Based on these studies, I also tested the inhibitor of HCN channel (ZD 7288 10μM and CsCl 5mM) in order to compare the possible difference among stomach, small intestine and colon. The results displayed that only colonic pacemaker activities were inhibited by both blockers, while the pacemaker activities induced by ICCs from stomach and small intestine had no any change (Fig. 8). It suggested that only colonic ICCs generating pacemaker activity require the HCN channels.

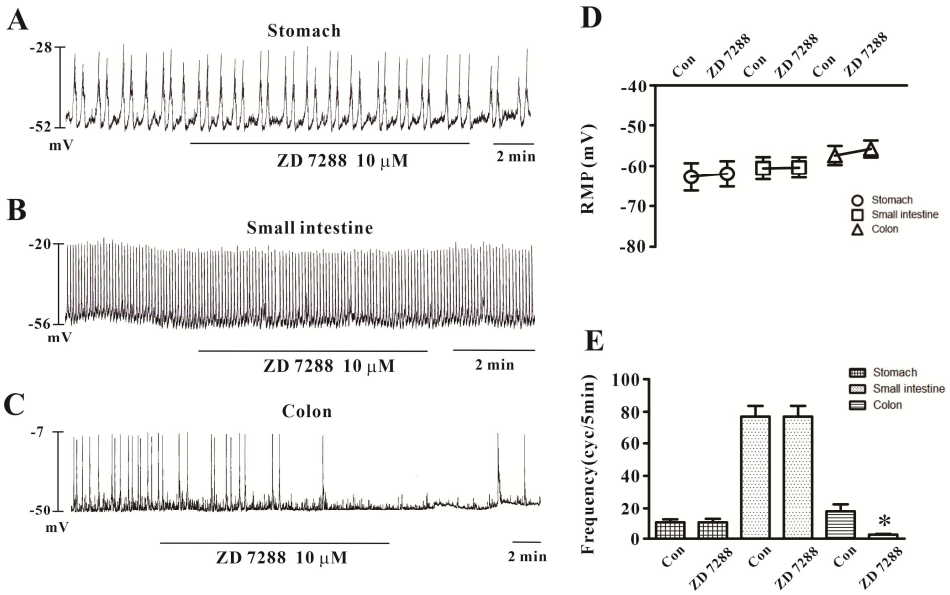

Fig. 8. (A-C) Effects of ZD 7288 10μM on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

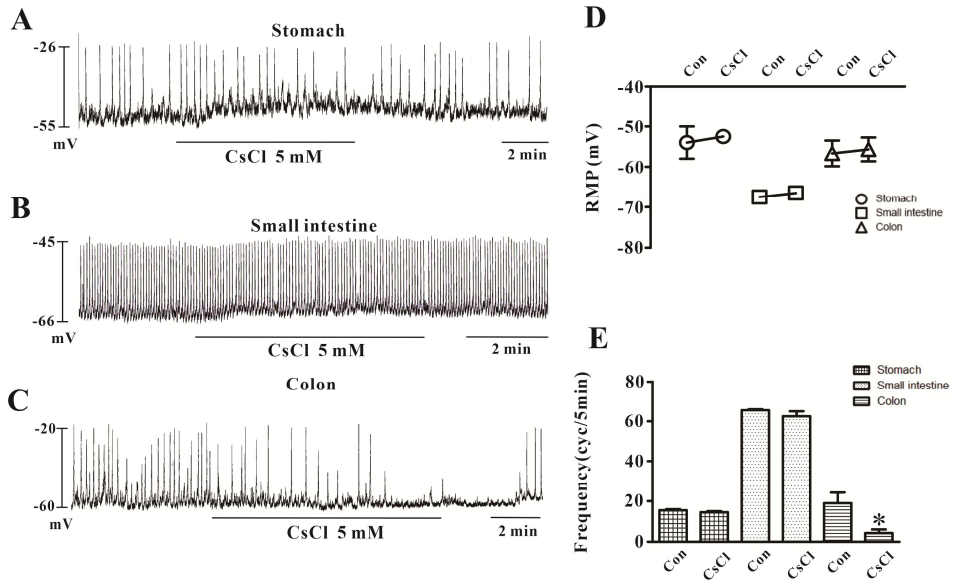

Fig. 9. (A-C) Effects of CsCl 5mM on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.5 The different function of K_{ATP} channels in three parts of GI tract

ATP-sensitive K^+(K_{ATP}) channels have been reported as a maintainer of resting membrane potentials and regulate cell excitability in various of cells. Previous study found that K_{ATP} channels are present in colonic ICCs and are comprised of Kir 6.1 and SUR 2B subunits(Na et al., 2017). These channels may have important role in maintaining resting membrane potential through basal activation. These K_{ATP} channels are modulated by protein kinase C. In contrast, in small intestinal ICCs, K_{ATP} channels are comprised of Kir 6.2 and SUR 2B subunits and are not activated under basal conditions. I tested the inhibitor of K_{ATP} channel blocker (glibenclamide 10μM) in stomach, small intestine and colon, respectively. It showed significant depolarization and rise of frequency in colonic ICCs and had no effect on small intestinal and stomach ICCs (Fig. 10).

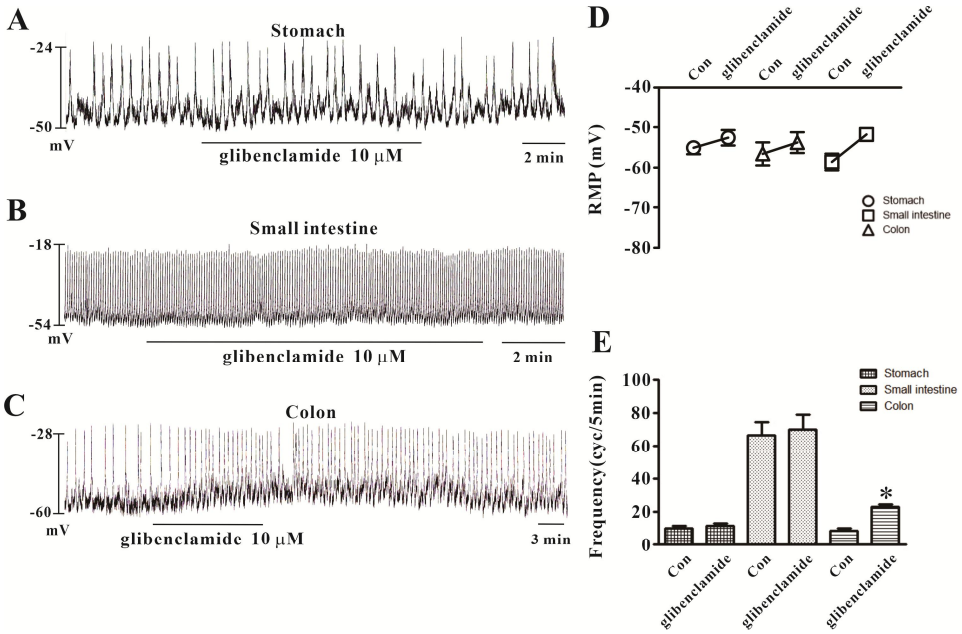

Fig. 10. (A-C) Effects of glibenclamide 100μM on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.6 Effect of cAMP on Pacemaker activity in stomach, small intestine and colon.

Basal cAMP involved in generating pacemaker potentials in colonic ICCs. It is well known that intracellular cAMP directly activates HCN channels (Choi et al., 2022). And HCN channels play an important role of generating pacemaker potential in colonic ICCs. cAMP mediates excitatory responses which can increase heart rates and neuronal firing rates.

Some of the research found that slow waves were sensitive to cAMP in colon. The cell-permeable-cAMP (8-bromo-cAMP) and adenylyl cyclase inhibitor (DDA) were performed in this experiment. The result show that the frequency in colon was increase by 8-bromo-cAMP 100μM, but had no effect in stomach and small intestine (Fig. 11). Meanwhile after treating DDA 100μM, Pacemaker activity was completely eliminated in

the stomach and colon But did not show any effect in small intestine (Fig. 12).

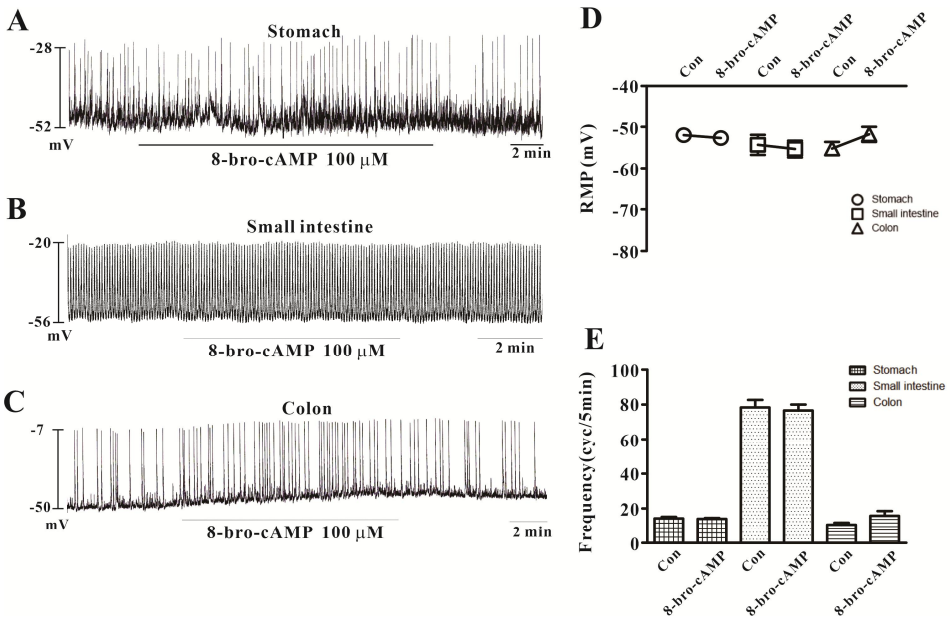

Fig. 11. (A-C) Effects of 8-bromo-cAMP (100μM) on pacemaker activities of stomach, small intestine and colonic ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

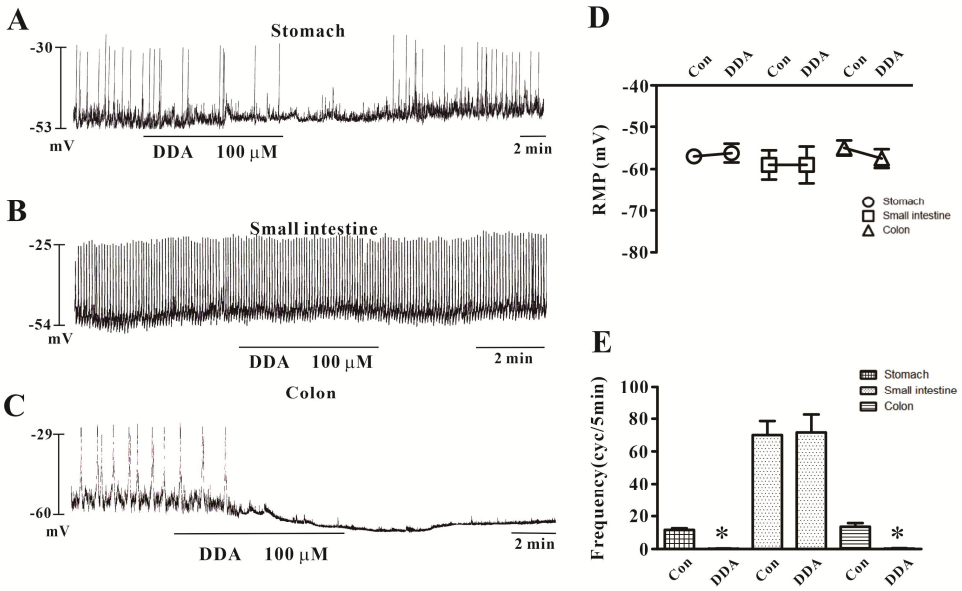

Fig. 12. (A-C) Effects of dideoxyadenosine (100μM) on pacemaker activities of stomach, small intestine and colonic ICCs which were cultured from murine and recorded with whole-cell patch clamp; (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.7 The role of cGMP in GI tract.

cGMP serves as a secondary signaling molecule. The cGMP signaling pathway is involved in the regulation of GI smooth muscle contractility (Sanders et al., 1998). The actions of cGMP at the cellular level are mediated by cGMP-dependent protein kinase, cGMP-degrading phosphodiesterase, and cyclic nucleotide-gated ion channels.

Cell-permeable 8-bromo-cGMP has the ability to block the pacemaker potential of small intestinal ICCs (Koh et al., 2000). The data from this study indicate that basal intracellular cGMP levels maintain the resting pacemaker potential frequency of colonic ICCs (Shahi et al., 2013). As such, I conducted experiments using 8-bromo-cGMP and selective soluble guanylyl cyclase inhibitor (ODQ) in the stomach, small intestine, and colon. Under control conditions in current clamp mode, 8-bromo-cGMP 100μM completely inhibited pacemaker activity frequency in the stomach and colon, but only

partially decreased it in the small intestine (Fig. 13). Conversely, ODQ only increased the pacemaker activity frequency in the colon but show no obvious effect on stomach and colon.

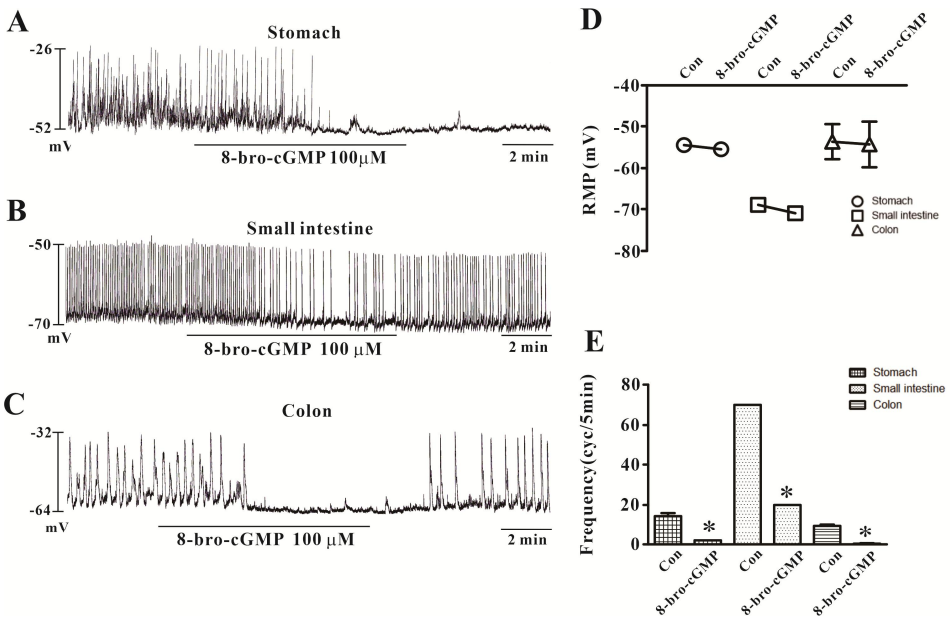

Fig. 13. (A-C) Effects of 8-bromo-cGMP(100μM) on pacemaker activities of stomach, small intestine and colonic ICCs which were cultured from murine and recorded with whole-cell patch clamp; (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

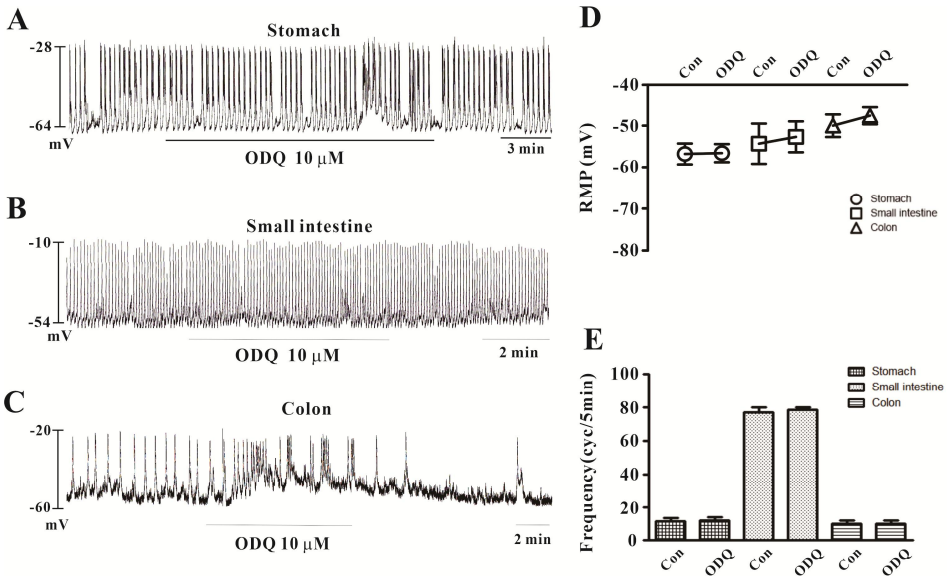

Fig. 14. (A-C) Effects of ODQ (10μM) on pacemaker activities of stomach, small intestine and colonic ICCs which were cultured from murine and recorded with whole-cell patch clamp; (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.8 Effect of Carbachol on Pacemaker activity in stomach, small intestine and colon.

Carbachol (CCh) is classified as acholinergic agonist. Recently study found that CCh is a powerful activator of ICCs activity, which is mediated through the ANO1 channel (Sanders et al., 2011). Hence, I tested effects of CCh in stomach, small intestine and colon. Under the current clamp mode (I=0), ICCs generated spontaneous pacemaker potentials from stomach, small intestine and colon. After a period of stability, Exposure to CCh (100nM) induce a huge depolarization of the resting membrane potential through 3 regions. And only increase of the frequency of the pacemaker potential in colon (Fig. 15).

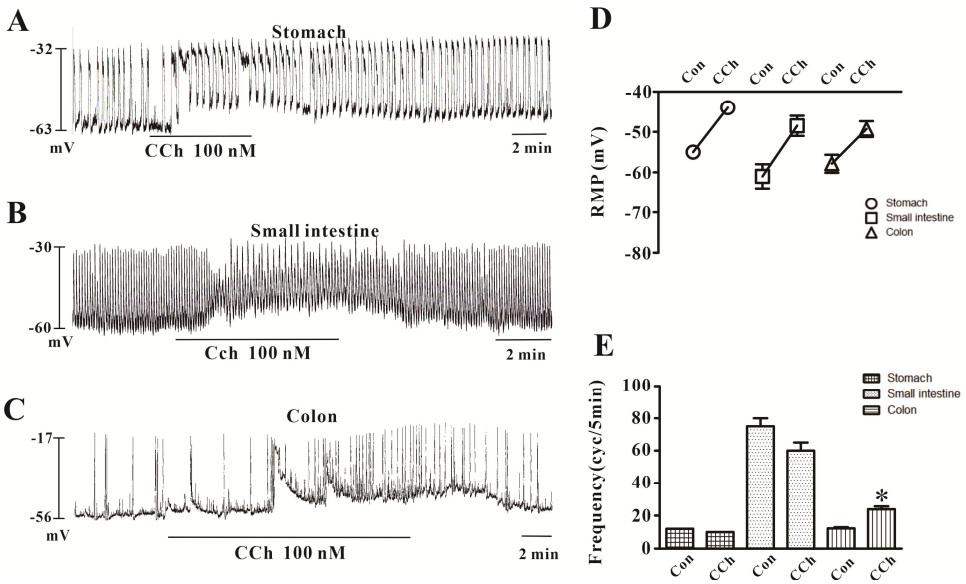

Fig. 15. (A-C) Effects of CCh 100nM on pacemaker activities of stomach, small intestine and colon ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

3.9 Effect of 5-HT on Pacemaker activity in stomach, small intestine and colon.

Serotonin (5-HT) plays a crucial role in the regulation of GI motility, enteric neurogenesis, mucosal growth/maintenance, intestinal inflammation, osteogenesis, and hepatic regeneration. Early studies on the role of 5-HT showed that 5-HT release increases from the gut when motor activity occurs.Therefore, I tested 5-HT in stomach, small intestine and colon. Data illustrate 5-HT induce huge depolarization in 3 parts. But the duration time of depolarization is much shorter than others. The tonic current were dramatically induced and frequency were increased in colon after treatment of serotonin. In small intestine, a short depolarization was produced by 5-HT, while frequency were reduced and depolarization was generated in by 5-HT on stomach. (Fig. 16)

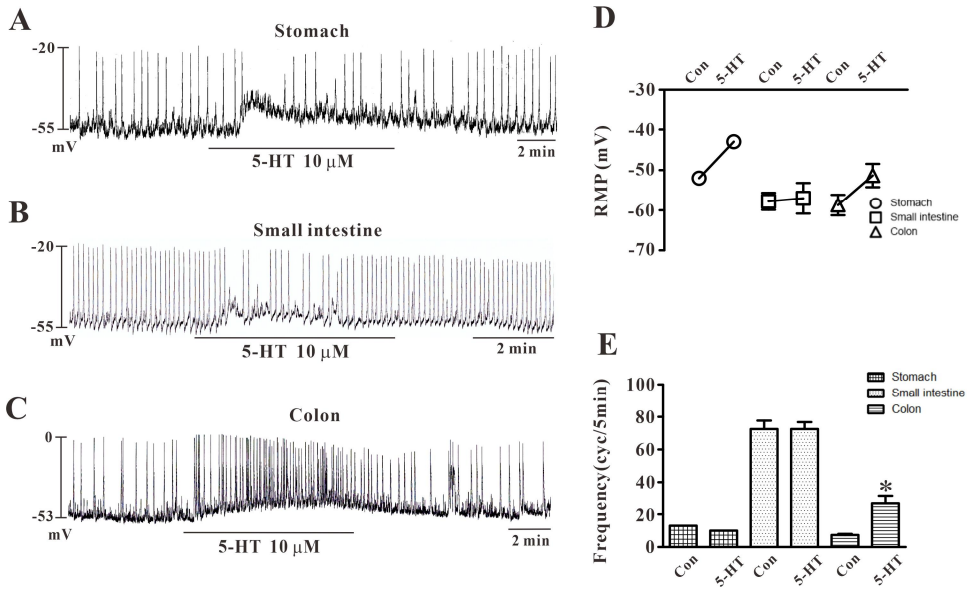

Fig. 16. (A-C) Effects of 5-HT (10μM) on pacemaker activities of stomach, small intestine and colonic ICCs which were cultured from murine and recorded with whole-cell patch clamp (D-E) Bar graphic represent resting membrane potential and frequency comparison. Bars represent mean values ± standard error (SE). *Asterisks mean significantly different from the control ($p < 0.05$).

4. DISCUSSION

This study showed that the pacemaker activity induced by ICCs are different in pattern, frequency, resting membrane potential, and amplitude in stomach, small intestine and colon. As for the mechanism of pacemaker activity, the possible involved mechanism might be different in each part. In order to know the difference of each tissue, I cultured the ICCs from stomach, small intestine and colon to perform patch clamp and investigate mechanism of pacemaker activity in each part of GI tract and function of various of neurotransmitters.

This study showed that the amplitude and frequency is different in stomach, small intestine and colon, the difference of each area might be determined by different pattern of slow wave in SMC.

Current studies suggested that Ca^{2+}-activated Cl^- channel (ANO1) is a specific protein which is encoded by ANO1 and involved in generating the pacemaker activity in colonic and small intestinal ICCs in mouse (Gomez-Pinilla et al., 2009). In the present study, I tested three types of ANO1 channel blockers in pacemaker activity to compare the effect and explore the function of ANO1 channels in each part. In stomach and colon, these three blockers displayed inhibition of frequency on pacemaker activity. However, all of them did not show any effect in small intestinal ICCs. It suggests that ANO1 channel is of considerable importance on provoking pacemaker activity in cultured ICCs in stomach and colon.

Previous research has found that T-type Ca^{2+} channels are expressed and functional in ICCs in the small intestine of mice (Zheng et al, 2014). Whole-cell patching membrane potential recordings show that T-type currents are crucial for the generation and spread of pacemaker activity in ICCs networks. Localized Ca^{2+} release events from the ER activate adjacent ANO1 channels, leading to the production of STICs. These STICs produce STDs of the cell membrane(zhu et al., 2015). When a threshold potential is reached for the regenerative activation of T-type channels, Ca^{2+} entry enhances the activation of ANO1 and further depolarizes the cell. This leads to the generation of pacemaker activity which spreads to coupled cells and depolarizes adjacent cells. Depolarization triggers T-type currents in adjacent cells, and the resulting Ca^{2+} entry

and release activates ANO1 channels in these cells. This sequence explains cell-to-cell propagation in ICCs networks. Therefore, the T-type Ca^{2+} channel blocker was used in the cultured ICCs in the stomach, small intestine, and colon in this study. As expected, the results were consistent with the effects of the ANO1 drug, with pacemaker activity being blocked by ML 218 in the stomach and colon but not in the small intestine. This suggests that T-type Ca^{2+} channels are crucial for the generation of pacemaker activity in the stomach and colonic ICCs of mice.

HCN channels have been reported that are entailed in production of pacemaker channels and the current flow through it was known as If current which is treated to launch the rhythmic activity in the sinoatrial node and permeable to the Na^{+} and K^{+}. As reported in our previous study, HCN were described as being involved in the generation of pacemaker potential in ICCs (Shahi et al., 2014). Additionally, cytosolic cAMP were supposed to be the second messenger in the downstream signaling pathway, intracellular cAMP levels are maintained by the production of adenylyl cyclase and breakdown of phosphodiesterase (Gomez-Pinilla et al., 2009). The cAMP was believed as the activator of HCN channels, and maintain the activation for HCN, which was reported as a candidate for generation of pacemaker activity in ICCs. In the past, we have already published HCN-related articles what experiments were performed in cultured ICCs in colon of murine. I tested HCN channel blockers (ZD 7288 and CsCl) in cultured ICCs, only colonic ICCs showed inhibition by these blockers and it proves that HCN channels may take part in regulation of pacemaker activity and may be an effective therapeutic target for abnormal colonic motility disorders in colon.

K_{ATP} channels have been reported for maintaining resting membrane potential and regulating cell excitability in various tissues. Our previous study has reported that K_{ATP} channels are expressed and comprised of Kir 6.1 and SUR 2B subunits and activated in basal state and take a leading role in sustaining resting membrane potentials in colonic ICCs. Whereas in small intestinal ICCs, K_{ATP} channels are comprised of Kir 6.2 and SUR 2B subunits and are not activated under resting state. 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). Glibenclamide (a K_{ATP} channel blocker) did not block the frequency in stomach and small intestine. Same with

previous results, it depolarized the resting membrane potential and increase the frequency in colonic ICCs. These results implies that closing of K_{ATP} channels can make a significant depolarization in colonic ICCs and it means that is an important channels in murine colon but stomach and small intestine.

The function of intracellular cAMP and cGMP on pacemaker activity in ICCs have been reported in previous studies. Intracellular cAMP was believed as an important activator for generating pacemaker activity in ICCs, by contrast, cGMP can inhibit the pacemaker activity. Therefore, I tested 8-bromo-cAMP and a specific adenylyl cyclase inhibitor in each part in cultured ICCs. Frequency were increased by cAMP in colonic ICCs while it did not show any effect in another two tissue. However, as for impact of DDA, it exhibited inhibition in each part. These results indicate that cAMP is involved in generating of pacemaker activity in colonic ICCs and it might be related to stomach, while it has no function on small intestine.

Many neurotransmitters, such as excitatory neurotransmitter including ACh, 5-HT have recently been a hot topic because of their roles in the gut physiology and their potential roles in GI pathophysiology. Many physiological process like blood flow or gut motility were regulated and controlled by these neurotransmitters (Zhu et al., 2011). Pacemaker activity induced by ICCs are determined by excitatory and inhibitory enteric motoneurons that innervate the tunica muscularis. In the case of excitatory motoneurons, CCh is a cholinomimetic drug that binds and activates acetylcholine receptors were tested in cultured cells of each part and displayed the huge depolarization in pacemaker activity. The result indicates that CCh take an important role on individual part in stomach, small intestine and colon. Furthermore, another kind of excitatory neurotransmitter, serotonin was discussed in this study. Serotonin is a critical neurotransmitter in GI tract which was found to modulate enteric nervous system (ENS) development and neurogenesis, motility, secretion, inflammation, sensation, and epithelial development (Margolis et al., 2014). Therefore, I treated 5-HT in each part and I found that it showed dramatic depolarization in each part, however, compared with other two, the effect in small intestine of serotonin is much short, the frequency in stomach and small intestine were not changed as same as colon, which is tonic and quite faster than control. This suggests that 5-HT induced motility in GI tract may function in ICCs for

each.

In conclusion, pacemaker activity induced by small intestinal ICCs is much faster than colon and stomach, but the resting membrane in three parts is similar. Among that, ANO1, T-type Ca^{2+} channel play an important role in both stomach and colon to pace the electrical activity, however, HCN channel and K_{ATP} channel was found to generate and maintain the membrane potential in colon, but not activated in small intestine and stomach. cAMP only acts on colon without other two, while cGMP take function on all. In addition, the mentioned neurotransmitters above take effect on stomach, small intestine and colon.

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6. ABSTRACT

Background: ICCs are known to produce spontaneous pacemaker activity which is thought to have an effect on GI motility produced by the contraction of smooth muscles in GI tract. In many previous reports, motility has been observed to be different in the stomach, small intestine, and colon. However, this has not been clearly studied, so this study aimed to investigate it.

Method: In order to draw results from this research, we cultured ICCs from stomach, small intestine, and colon of ICR mice and performed whole cell patch clamp to to record the membrane potential and compare the difference between them.

Results: ANO1 or T-type Ca^{2+} channel inhibitor suppressed the spontaneous pacemaker activity of ICCs in the stomach and colon, but did not have a significant effect in the small intestine. In addition, HCN or ATP-sensitive K^{+} channel inhibitor only significantly affected the spontaneous pacemaker activity of ICCs in the colon. Also, when the cell-permeable cAMP and cGMP were applied, they only affected the spontaneous pacemaker activity of ICCs in the colon. The inhibitors that are related to cAMP and cGMP also affected the spontaneous pacemaker activity of ICCs in the colon. Furthermore, neurotransmitters play a very important role in GI motility, so this study investigated the effect of spontaneous pacemaker activity of ICCs in the stomach, small intestine and colon. The effect of excitatory neurotransmitters was similar in all parts.

Conclusion: ANO1 and T-type Ca^{2+} channels, which are known to play an important role in the spontaneous pacemaker activity of ICCs, were found to only function in the stomach and colon. In addition, HCN and ATP-sensitive K^{+} channels were found to only affect ICCs in the colon. Furthermore, cAMP was found to only function in the colon, while the effect of cGMP could be observed in all parts. Neurotransmitters, which play an important role in GI motility, had similar effects in all parts.

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