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Ph.D. Thesis

Thrombin and Trypsin Modulate Pacemaker Currents of Interstitial Cells of Cajal from Small Intestine through Proteinase Activated Receptors 1 and 2

Graduate School of Chosun University

Department of Physiology

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Advisor: Prof. Jae Yeoul Jun

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ABBREVIATIONS

FFA	flufenamic acid
FITC	fluorescein isothiocyanate
GI	gastrointestinal
GIT	gastrointestinal tract
ICC	interstitial cells of Cajal
JNK	c-jun N-terminal kinase
MAPK	mitogen activated protein kinase
NSCCs	non-selective cation channels
PARs	proteinase activated receptors
PCR	polymerase chain reaction
PGP	protein gene product
RT	reverse transcriptase
SMC	smooth muscle cell

국문초록

Thrombin and Trypsin Modulate Pacemaker Currents of Interstitial Cells of Cajal of Small Intestine through Proteinase Activated Receptors 1 and 2

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카할사이질 세포는 위장관 운동을 조절하는 향도잡이 세포로 평활근의 자발적 수축의 근간인 서파을 발생시키고 전파하며 위장관 신경계의 신호를 전달하고 또한 신장 수용체로서의 역할을 담당한다. 단백질 분해효소인 트롬빈과 트립신은 G-단백질과 연결된 protease-activated receptor (PARS)를 통해 여러 부위에서 다양한 생리학적 기능을 하고 있다. 위장관내 존재하는 많은 세포들에서도 발현되며 특히 소장내강에도 높은 농도로 존재하고 있다. 단백질 분해효소는 조직손상, 염증 및 복구과정 등 병태생리학적 현상과 관련되어있다. PARS 의 활성화는 위장관 운동에도 영향을 보여 동물의 종이나 조직의 부위에 따라 평활근을 수축 또는 이완하고 있다. 그러나 현재까지 카할사이질 세포에 대한 PARS 의 존재와 생리학적 역할에 대한 보고는 없다. 본 연구는 카할사이질 세포에서 PARS 활성 물질인 트롬빈과 트립신의

향도잡이 활동도에 대한 효과와 작용기전을 규명하고자 마우스 소장에서 배양된 세포에 전기생리학적 기법(patch-clamp technique), RT-PCR, 및 세포내 칼슘농도를 측정하였다.

배양된 카할사이질 세포에서 RT-PCR 결과 PARS 1 과 PARS 2 과 발현되었다. 트롬빈 (PARS 1 활성제) 과 트립신 (PARS 2 활성제)는 모두 막전압의 저분극을 유발하였으며, 향도잡이 전류에 대해 긴장성 내향성 전류를 발생과 더불어 전류의 발생빈도와 크기를 감소하였다. 비선택성 양이온 통로 차단제 (flufenamic acid), 세포외 나트륨 또는 칼슘 제거 용액 및 세포내 내형질 세망으로부터 칼슘 분비 억제제 (tharpsigargin)의 존재하에서는 트롬빈과 트립신의 작용이 차단되었다. 그러나 COX-2 억제제(naproxen) 또는 mitogen-activated protein kinases 억제제들에 의해서는 차단되지 않았다. 트롬빈과 트립신은 세포내 칼슘농도를 증가시켰다. 이러한 결과들은 카할사이질 세포에 PARS 1 과 PARS 2 가 존재하며 비선택성 양이온 통로를 활성화 시켜 향도잡이 활동도를 조절하며 이 과정에 세포내 칼슘 분비가 관여하고 있음을 보여준다.

결론적으로 PARS 의 위장관 운동성에 대한 효과는 카할사이질 세포에 영향을 주어 나타낼 수 있음을 시사하며 이는 위장관 운동성 조절 기전을 규명하는 데 있어 중요한 기초 자료를 제공할 것으로 생각된다.

1. Introduction

1.1 Interstitial Cells of Cajal and Gastrointestinal Motility

1.1.1 History of Interstitial Cells of Cajal

The term "interstitial cells of Cajal" (ICC) refers to several types of cells located in the musculature of the gastrointestinal (GI) tract and, morphologically and functionally, intercalated between the segments of the enteric nervous system and smooth muscle cells. As early as 1893, Santiago Ramón y Cajal, a Spanish neuroanatomist used the Golgi technique and methylene blue staining to describe spindle-shaped or stellate cells associated with autonomic nerve endings of the intestine; he termed them "cellules interstitielles". Because they had long ramified cell processes and oval nuclei with sparse perinuclear cytoplasm, Cajal believed them to belong to a distinct type of neurons (Ramón, 1892; Ramón, 1893; Ramón, 1904). In the century to follow, a heated debate was led concerning the origin, function, distribution, and identification of ICC. ICC reached its historical renaissance with the analyses of Thuneberg, who hypothesized that ICC act as intestinal pacemakers and participate in the transmission of impulses to the intestinal musculature, a transmission analogous to that in the heart muscle (Thuneberg, 1982). Some researchers have provided abundant evidence that ICC are implicated in the generation of slow waves that act as an electrical basis for pacemaking functions (Faussone, 1987). Today, ICC is given a central place in research studies aimed at understanding gastrointestinal contractions and elucidating thepathogenesis of various motility disorder.

1.1.2 Morphology and Localization of ICC in the Gastrointestinal Tract

ICC is spindle-shaped or stellate, has a different number of ramified cell processes. The cell located between smooth muscle cells is spindle-shaped with several long cell processes parallel to the axis of the muscle bundles. The cell surrounding the nerve plexus is more stellate in shape, containing numerous ramified cell processes (Costa, 2000; Goyal, 1996). The ultrastructure of ICC is mainly myoid, most similar to that of smooth muscle cells, with several fibroblastic features. Cytological properties identified by transmission electron microscopy include: ramified profile, high electronic density of the cytoplasm, a well-developed perinuclear Golgi complex and centrioles, an independent basal lamina, numerous, irregularly distributed caveolae of the plasma membrane, membrane-associated dense bands, cytoplasmic dense bodies, abundant subsurface smooth endoplasmic cisternae, numerous mitochondrias, and the presence of bundles of thin and intermediate filaments (Fig. 1) (Burns et al., 1996).

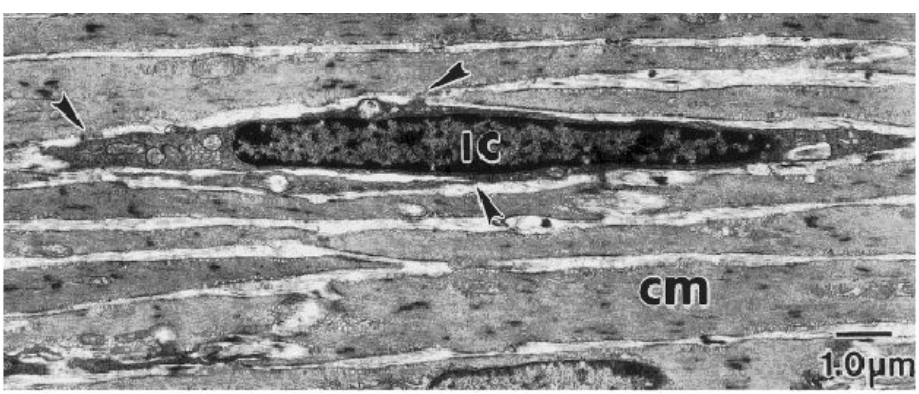

Fig.1. Ultrastructure of ICC in the murine gastric fundus (Burns et al., 1996).

In the human digestive tract, the presence of ICC has been confirmed within and around the muscle layer of the, stomach, small and large intestine (Fig. 2 and 3). ICC is arranged in two- or three-dimensional networks and bundles of mutually

associated cells that form close contacts with the nerve plexus and smooth muscle cells (Drndarevic, 2001; Goyal, 2000).

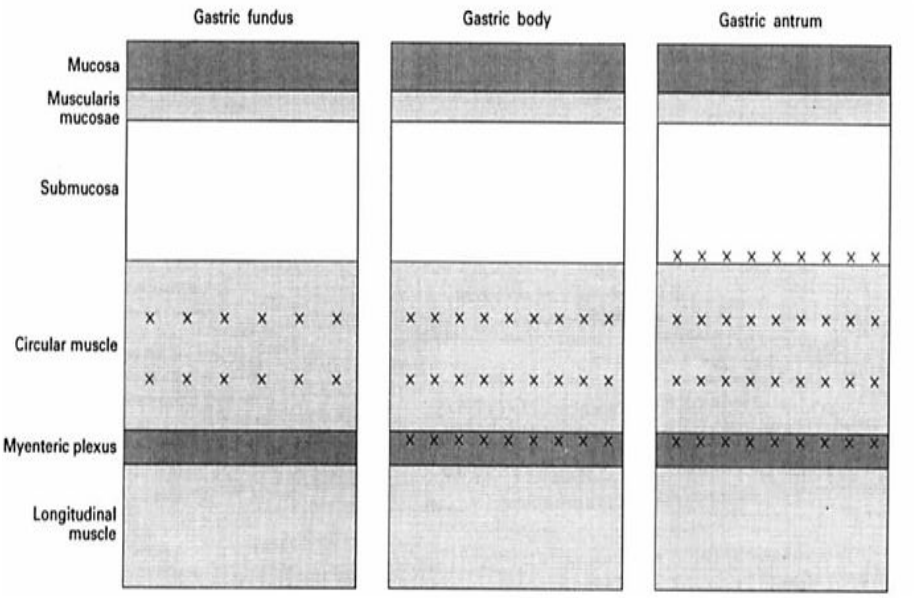

Fig.2. Location of ICC in the human stomach (Drndarevic, 2001; Goyal, 2000). The location of human ICC has been mapped in human stomach. The ICC of the human stomach show regional variation. There are few ICC in the fundus, found only in the circular muscle layer. In the corpus and antrum many such cells are seen, one network within the circular muscle layer and another in the intramuscular plane; an additional network of ICC is present in the submucosal layer of the antrum.

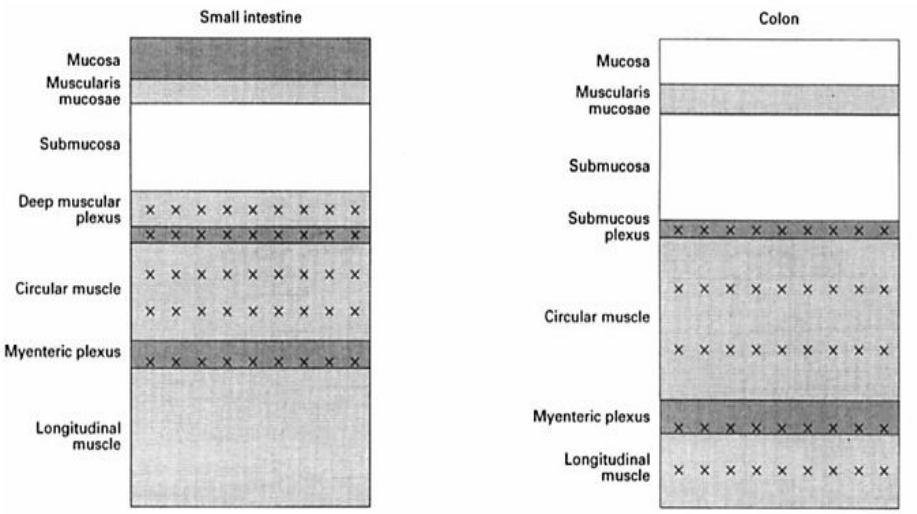

Fig.3. Location of ICC in the human small intestine and colon (Drndarevic, 2001). The location of human ICC has been mapped in small intestine and colon. In the small intestine, ICC associated with myenteric plexus are arranged in networks of bundles; they are innervated by nerve elements of myenteric plexus, which extend into both the layers of smooth muscle. The ICC of the deep muscular plexus ramify in the inner zone of the outer division of circular muscle, penetrate the innermost circular layer and are also found at the submucosal border. In the human colon, ICCs are found at the submucosal border of the circular muscle, ICCs were also seen in the main bulk of the circular muscle layer and in the longitudinal muscle layer.

1.1.3 Identification of ICC

The kit receptor is encoded by the proto-oncogene c-kit located on the W (white spot) locus of chromosome 5 of mouse; it is a tyrosine kinase similar to other receptors for growth factors whose interactions with the ligands initiate signaling via kinase cascade to the cell nucleus (Yarden, 1987; Chabot, 1988). The discovery that ICC expresses the kit receptor has represented a fundamental advance in the study of these cells. By using PCR and immune-histochemistry techniques, studies

to characterize the distribution and the function of ICC in the gastrointestinal tract were greatly advanced.

ANO1 acts as a calcium-activated chloride channel, belongs to the anoctamin family. Calcium activated chloride channels are membrane proteins that are responsible for the passive flow of Cl^- into and out of the cell. Recently, the Ca^{2+}-activated Cl^- channels are attracting a lot of attention in the study of ICC, it is hypothesized that Ca^{2+}-activated Cl^- channels are involved in the generation of pacemaker activity in cultured ICC (Zhu et al., 2009), and the lack of any slow wave activity or rhythmic contraction of smooth muscles in the digestive tract of Ano1 knockout mice. Furthermore, it has been shown that Ano1 labels all classes of ICC and represents a highly specific marker for studying the distribution of ICC in mouse and human tissues with an advantage over Kit (Hwang et al., 2009).

1.1.4 Functions and Pacemaker Activity of ICC

There are two main roles proposed for the ICC: First, they act as the pacemakers for most of the Gl tract (Thuneberg, 1982). GI motility depends on the spontaneous mechanical contractions of the smooth muscles. GI smooth muscle cells show cyclic depolarizations of the membrane potential, called slow waves which determine the frequency of smooth muscles contractions. ICC are pacemaker cells which generate rhythmic pacemaker potentials by producing spontaneous inward pacemaker currents. ICC are connected to smooth muscle cells by gap junctions, and the pacemaker currents induced by ICC are directly transmitted to smooth muscle cells through gap junctions and induce the slow waves which cause smooth muscles contractions. The electrical properties of ICC have been studied using several different techniques. Isolated ICC has been examined using conventional patch-clamp recording techniques. This approach, which has been applied to ICC, allows a description of the specific populations of ion channels present in their membranes (Langton et al., 1989; Tokutomi et al., 1995) and an analysis of the cellular mechanisms which regulate the channels (Ward et al., 2000; Koh et al.,

2002). Second, they act as intermediaries in the control of muscle activity by the enteric nervous system (Berezin, 1988). ICC make close contacts with both excitatory and inhibitory enteric motor neurons by synaptic-like structures (Hirst et al., 2003), and It has been shown that cultured ICC possess receptors and second messenger systems responsible for their role in excitatory (such as tachykinin receptors, muscarinic receptors) and inhibitory (NO) neural transmission; and also their ability to respond to hormones (Jun et al., 2004; So et al., 2009; Park et al., 2007). In the enteric nervous system, numerous neurotransmitters and hormones are released and regulate GI motility. Therefore, ICC are physiological and therapeutic targets for numerous neurotransmitters and hormones.

1.2 Proteinase and Proteinase Activated Receptors

1.2.1 A Brief Introduction of Proteinase and Proteinase Activated Receptors

The coagulation cascade proteinase, thrombin, is well recognized for its role in generating fibrin clots from the cleavage of fibrinogen. In addition, thrombin is known as a key factor for triggering signaling pathways in platelets and endothelial cells. Other serine proteinases, like trypsin, have also been observed to activate signal transduction events. The receptors responsible for the actions of these proteinases are called PARs, a family of G protein-coupled receptors have recently been discovered (Coughlin, 2005; Ramachand, 2008), it currently consists of four family members (Kawabata, 2001). PAR 1, PAR 3 and PAR 4 are thrombin receptors (Vu et al., 1991; Xu et al., 1998), while PAR 2 is a receptor for trypsin. Given the broad spectrum of roles that PARs have in normal and pathological tissue function, these receptors are emerging as potential therapeutic targets for several diseases including arthritis, colitis, asthma, neurodegenerative conditions, tumour invasion and cardiovascular diseases.

1.2.2 Structure and Activation of Proteinase Activated Receptors

PARs are seven trans-membrane-spanning, G-protein-coupled receptors. They are activated through the proteolytic cleavage of the N-terminal domain of the receptor (Vu et al., 1991; Coughlin et al., 1992), Four members of this receptor family have been cloned till far: PAR 1, PAR 3 and PAR 4 which are activated by thrombin, and PAR 2 that is activated by trypsin or humanmast cell tryptase (Rasmussen et al., 1991; Bohm et al., 1996). Activation of PARs is achieved by proteolytic unmasking of the N-terminal cryptic receptor-activating sequence, which subsequently binds to the body of the receptor as a tethered ligand (Fig. 4). Short synthetic peptides based on the proteolytically revealed receptor sequences (PAR-activating peptides) can selectively activate PAR 1, PAR 2 or PAR 4, so far, activating peptides for PAR 3 have not been identified (Table 1). Analogues of the tethered ligand are of great interest in the case of PAR 1, PAR 2 and PAR 4, since they constitute specific receptor agonists that can be used to understand the function of PARs.

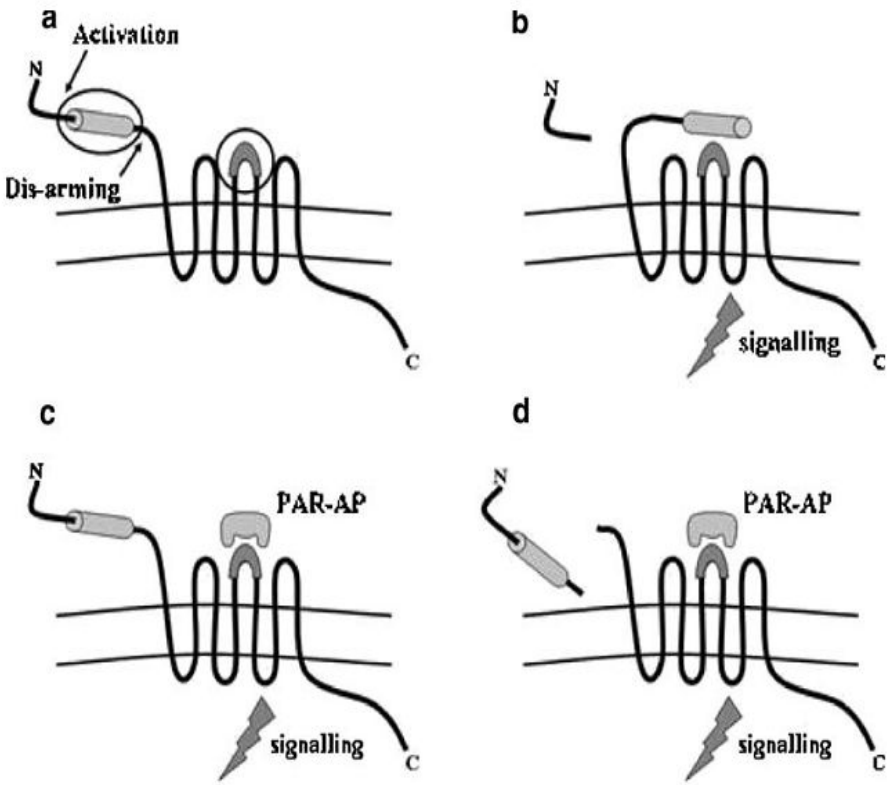

Fig.4. Mechanism of activation and disarming of PARs (Vu et al., 1991).

The scheme shows the potential sites of receptor activation or disarming (a). Proteolytic activation (reaction b) results from the exposure of the tethered ligand sequence that interacts with the extracellular domain of the receptor to initiate signaling. Peptide-mediated activation (reaction c) can occur in the absence of receptor proteolysis via the interaction of a PAR-activating peptide with the extracellular receptor domains. Disarming of the receptor (reaction d) prevents proteolytic activation of the receptor because of the removal of the tethered ligand domain, but still allows for activation by an activating peptide.

	Species	Tethered ligand sequence	Selective agonist peptides	Major activating proteases
PAR-1	human	SFLLRN	TFLLRN/Cit-NH_2	thrombin
PAR-2	human	SLIGKV	SLIGKV/SLIGRL	trypsin, tryptase
	mouse/rat	SLIGRL	SLIGRL/Tc-NH_2	trypsin, tryptase
PAR-3	human	TFRGAP		thrombin
PAR-4	human	GYPGQV	GYPGQV	thrombin, trypsin

Table1. Tethered ligand sequences and agonists of PAR (Coughlin et al., 1992).

1.2.3 Localization of the Proteinase Activated Receptors in the Gastrointestinal Tract

PAR 1 is expressed in many cell types and tissues. In the GI tract, PAR 1 has been localized in endothelial cells, in the lamina propria and in the submucosa (Bohm et al., 1996). In the human, PAR 3 has been detected in the stomach and small intestine, although the cell type that expresses PAR 3 remains to be identified (Ishihara et al., 1997). PAR 4 expression has been identified in many tissues and cells (lung, pancreas, thyroid, testis, platelets, placenta, skeletalmuscle, etc.) with especially high levels observed in the small intestine. Moderate expression of PAR 4 was also detected in the human colon (Xu et al., 1998). The presence of PAR 2 has been revealed in a variety of tissues, both in rodents and in humans, with particularly prominent expression observed in the GI tract (Bohm et al., 1996; Nystedt et al., 1995). Northern blot analysis of human and mouse tissues has revealed that PAR 2 is strongly expressed in the small intestine, colon, liver, pancreas and more weakly detected in the stomach. PAR 2 is also highly expressed by human colon adenocarcinoma cells (Bohm et al., 1996). More recent studies have shown that PAR 2 can be prominently localized to enterocytes of the villi and crypts of LieberkuÈ hn of the rat small intestine, where it is present at the plasma membrane and in the Golgi apparatus (Kong et al., 1997), PAR 2 was detected both at the apical and basolateral membrane of enterocytes and has also been detected at

the plasma membrane of myocytes in muscularis externa and muscularis mucosa of rat jejunum (Kong et al., 1997).

1.2.4 Roles for PARs in Physiology and Disease

PARs are expressed in a wide variety of tissues and they have important physiological and pathophysiological roles in a wide range of biological systems, including the cardiovascular system, GI system, respiratory system, and peripheral nerves. In cardiovascular system, PARs regulate platelet function, endothelial permeability, leukocyte adhesion and nitric oxide release, thereby affecting vascular smooth muscle tension. Under pathological conditions, such as endothelial dysfunction occurred, PARs activation could directly cause vasoconstriction (Damiano, 1999). In respiratory system, PARs activation having profound effects on bronchomotor tone, airway inflammatory responses and in the development of lung fibrosis (Cocks, 1999). In nerves system, PARs activation in neurons modulates cell morphology and process extension as well as cell survival and also play very important roles in the pathogenesis of neurotrauma, intracerebral haemorrhage and ischaemia, as well as neuroinflammatory and neurodegenerative Conditions (Vergnolle, 2001). In GI system, all PARs are expressed in the GI tract: in the mucosal epithelial cells, the smooth muscle elements and the enteric nervous system cells. Activation of PARs in the GI tract modulates a variety of alimentary functions such as glandular exocrine secretion, epithelial ion transport, and smooth muscle motility (Kawabata, 2002). PARs play critical roles in modulation of motility of the GI mooth muscle. Modulation of gastrointestinal smooth muscle motility by PAR1, PAR2 and PAR4 has been described in vitro (Cocks et al., 1999a; Kawabata et al., 2000b; Mule et al., 2002; Saifeddine et al., 1996; Sekiguchi et al., 2007) as well as in vivo (Kawabata et al., 2001). In vitro study, it has been shown that activation of PAR 1 and PAR 2 cause contraction of rat and mouse gastric longitudinal muscle (Saifeddine et al., 1996). In vivo study, activation of PAR 1 and PAR 2 facilitate GI transit in mice (Kawabata et al., 2001). The underlying mechanisms for the motility modulation by PARs vary with species and the parts of the gastrointestinal tract. Involvement of contractile PGs has been

shown in PAR2-mediated contraction in the gastric longitudinal smooth muscle of rats (Saifeddine et al., 1996) and PAR1- and PAR2-mediated contraction in the gastric and ileal longitudinal smooth muscle of mice (Sekiguchi et al., 2007).

1.3 Rationale and Objectives

Cultured ICC isolated from the small intestine retains the ability to generate the electrical rhythmicity, and they are a powerful model for studying the pacemaker mechanism and the modulation mechanism by varies neurotransmitters and hormones as well as proteases. Under both physiological and pathophysiological conditions, the GI tract is exposed to a large array of proteases. Since it has already reported that activation of PARs by trypsin and thrombin modulates GI motility, and ICC provides important regulatory functions in the motor activity of the GI tract, however, there is no report as to the activation of PARs on cultured ICC. The aim of this project is to study the action of PARs on the pacemaker potential generated by cultured ICC of the small intestine, and try to explore the signaling pathways and molecular interactions.

2 MATERIAL AND METHODS

2.1 Experimental Animals

Balb/C mice were used in the present experiment and were treated as per the guiding principles for the care and use of animals approved by the ethics committee in Chosun University and the National Institutes of Health Guide, South Korea for the care and use of laboratory animals. And every effort was made to minimize the number as well as their suffering.

2.2 Tissue Isolation and ICC Culture

Balb/C mice (3-5 days old) of either sex were anesthetized with diethyl ether and sacrificed by cervical dislocation. Abdominal cavities were opened from the ventral surface and small intestines (from 1 cm below the pyloric ring to the cecum) and

colon (just below from cecum to rectum) were removed. Intestines were opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution, tissues were pinned to the base of a Sylgard dish, and mucosa was removed by sharp dissection. Small stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated in Ca^{2+} free Hank's solution for 15 minutes. The muscle strips were enzymetically digested by incubating at 37°C for 12 minutes in the enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1 mg/ml, bovine serum albumin (Sigma) 1 mg/ml, trypsin inhibitor (Sigma) 0.5 mg/ml and triturated using fire blunted glass tubes with a range of holes. Cells were seeded onto sterile glass cover slips (20 mm) coated with poly - L-lysine (200 µl, Sigma) in a 35 mm culture dish, and cultured at 37°C in a 95 % O2 and 5 % CO2 in moisturized incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and stem cell factor (SCF, 5 ng/ml, Sigma). On the second day of culture, the growth media were replaced with growth media without stem cell factor and incubated further in the same condition till used.

2.3 Whole Cell Patch Clamp Recording

The whole-cell configuration of the patch-clamp technique was used to record membrane potentials (current clamp) from cultured ICC. Membrane potentials were amplified by Axopatch 200B (Axon Instruments, Foster, CA, USA). Data were digitized with a 16-bit analog to digital converter (Digidata 1322A, Axon instrucments) and stored directly on-line using pClamp software (Version9.2; Axon instrucments). Command pulse was applied using an personal computer and pClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz and were analyzed using pClamp and Graph Pad Prism (version 5) software.

2.4 Measurement of Intracellular Ca^{2+} Concentration

The 24-30 hrs cultured and well grown ICC on cover slips (25 mm) was rinsed twice and incubated for 10 minutes with physiological bathing solution. The cells were then, incubated in 1 μM fluo-4/AM with 5% CO_2 at 37°C for 10 minutes and washed twice with the physiological bath solution. Cover slips with interstitial cells of Cajal mounted on a perfusion chamber. The cells were scanned every 0.4 seconds with Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer 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. During the Ca^{2+} imaging, the temperature of the perfusion chamber containing the cultured ICC was maintained at 30°C. The variations in intracellular Ca^{2+} fluorescence emission intensity were expressed as peak fluorescence intensity ratio (F1/Fo) with respect to time of peak fluorescence, where Fo is the minimum fluorescence intensity during image capturing.

2.5 RNA Isolation and RT-PCR

Single Cell Isolation

The path pipette with relatively little bigger pore size was used to pick the single cultured ICC, the pipette is moved closer to the single ICC and collected using applied suction to the pipette that resulted in aspiration of the cell into the patch pipette. The pipette contents were subsequently ejected into a sterile 1 ml eppendorf tube containing sterile and chilled PBS. Cells were rapidly placed in ice and the total RNA was isolated using the Trizol reagent according to the manufacturer's instruction before performing PCR.

Whole Dish Total RNA Isolation

The tissue of small intestine were cultured on 100mm culture dish separately. After 48 hours of the culture, total RNA isolation was performed. The culture dish was washed 2 times with ice-cold PBS and was placed on the ice. Then the cells were scraped in 1 ml of PBS solution and transferred to the eppendorf tube. The tubes

are then centrifuged at 8000 rpm for 5 minutes. The cell pellet was then used for the total RNA isolation by Trizol reagent according to the manufacturer's instruction before performing PCR.

The First-strand cDNA was synthesized and polymerase chain reaction (PCR) was performed with specific primers (Table 2) using one-step RT-PCR Premix kit (iNtRON Biotechnology Inc., Korea), and secondary PCR was performed using 1 μl of the first PCR product. The PCR products were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

Table2: Primers for PCR

Genes	reference	Nucleotides (5' →3')	Size (bp)
c-kit	AY536430	GCACAGAAGGAGGCACTTATACCT TGAGACAGGAGTGGTACACCTTTG	215
myosin	NM_013607	AGCAGGAGGTGGAACACAAGAAGA GGAAGCCACATCTTTGGCCAGTTT	197
PGP 9.5	NM_011670	GCCAACAACCAAGACAAGCTGGAA GCCGTCCACGTTGTTGAACAGAAT	213
PAR-1	NM_010169	CCTATGAGCCAGCCAGAATC ACGTTCAGAGGAAGGCTGAC	305
PAR-2	NM_007974	TGGGAGGTATCACCCTTCTG CCAGGTTGGCCATGTAAATC	330
PAR-3	NM_010170	TGGGTATTTGGCGAGGTCATGTGCCG TCGACGACATCGTGGCAGGTGGTGA	287
PAR-4	NM_007975	TGCTGTATCCTTTGGTGCTG CATGAGCAGAATGGTGGATG	371

RT-PCR was performed using the following profile: 45°C for 30 min for reverse transcription followed by 95°C for 5 min to active the Taq polymerase (i-star Taq, iNtRON Biotechnology Inc., Korea), and then 35 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, followed by an extension step at 72 °C for 10 min.

2.6 Solutions

Extracellular Solution (mM)

5 KCl, 135 NaCl, 2 CaCl2, 10 glucose, 1.2 MgCl2, and 10 HEPES adjusted to PH 7.4 with Tris.

Intracellular Solution (mM)

135 KCl, 2.5 MgCl2, 3 MgATP, 0.1 NaGTP, Creatine phosphate disodium, 0.1 EGTA, and 10 HEPES adjusted to PH 7.2 with Tris.

2.7 Statistical Analysis

Data was expressed as mean ± standard errors. Coefficient correlation was evaluated using the Student's t test. P values of <0.05 were taken to be statistically significant. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

3 Drugs and Chemicals

Thrombin, TFLLR, Trypsin, Flufenamic acid, PD98059, SB203580, JNK inhibitor, genistein.

4 Results

4.1 Effects of Thrombin on Pacemaker Potentials in Cultured ICC

For investigating the effects of Thrombin (an agonist activating PAR 1, PAR 3 and PAR 4), I performed whole cell patch clamp recording from cultured ICC under current clamp mode (I=0). Under control conditions, ICC showed spontaneous pacemaker potentials. The resting membrane potential was -61 ± 3 mV, the amplitude and frequency of pacemaker membrane potential were 31 ± 3 mV and 14 ± 2 cycles/min, respectively. Exposure to thrombin (0.1 unit/ml) produced depolarization of the resting membrane potential with a decrease of the amplitude and frequency of the pacemaker potential (Fig. 5A). In the presence of thrombin, the value of the resting membrane potential, amplitude and frequency of pacemaker

potentials under current clamp mode by thrombin (0.1 unit/ml) was significantly different when compared with the control value, the resting membrane potential was -30 ± 4 mV, the amplitude and frequency of pacemaker potential were 3 ± 1 mV and 9 ± 2 cycles/min, respectively (n = 5, Fig. 5B - D).

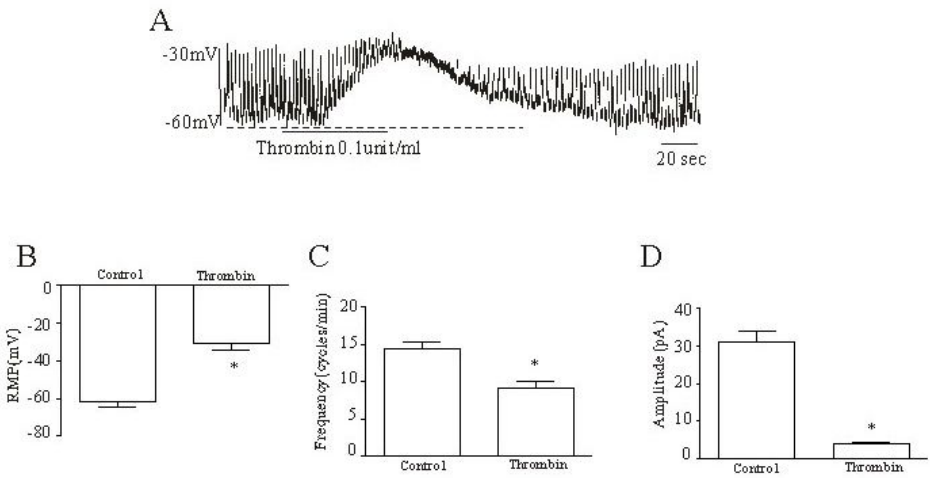

Fig.5. Effect of thrombin (an agonist activating PAR 1, PAR 3 and PAR 4) on pacemaker potentials recorded in cultured ICC. (A) Pacemaker potentials of ICC exposed to Thrombin (0.1 unit/ml) in the current clamping mode (I = 0). Thrombin caused membrane depolarization and decreased frequency and amplitude of pacemaker potentials. Responses to Thrombin are summarized in (B), (C) and (D). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

4.2 The Expression of PAR 1 and PAR 2 mRNA in Cultured ICC and the Effects of TFLLR-NH_2 and Trypsin on Pacemaker Potentials in Cultured ICC

To examine the expression of PARs mRNA in the cultured ICC, both whole dish and single ICC RT-PCR were performed using PARs subtype gene specific primers. In both whole dish and single cultured ICC, RT-PCR detected transcripts for PAR 1 and PAR 2 (Fig. 6C and D). But the specific primers (PAR 3 and PAR 4) did not produce cDNA fragments of appropriate size. After identifying the subtypes of PARs mRNA in the cultured ICC, I tested the effects of TFLLR-NH_2 (a PAR 1 specific agonist) and trypsin (a PAR 2 specific agonist) by whole cell patch clamp recording from cultured ICC under current clamp mode (I=0). Under control conditions, the resting potential was -66 ± 4 mV, the amplitude and frequency of pacemaker membrane potential were 30 ± 4 mV and 16 ± 2 cycles/min. Exposure to TFLLR-NH_2 (5 μM) produced depolarization of the resting membrane potential with a decrease of the amplitude and frequency of the pacemaker potential (Fig. 6A). In the presence of TFLLR-NH_2, the resting membrane potentials were -43 ± 2 mV, the amplitude and frequency of pacemaker potential were 8 ± 2 mV and 11 ± 2 cycles/min, respectively (n = 4, Fig. 6E - G). Exposure to trypsin (100 nM) also produced depolarization of the resting membrane potential with a decrease of the amplitude and frequency of the pacemaker potential (Fig. 6B). Under control conditions, the resting potential was -62 ± 4 mV, the amplitude and frequency of pacemaker membrane potential were 30 ± 2mV and 13 ± 2 cycles/min. In the presence of trypsin, the resting membrane potentials were -34 ± 4 mV, the amplitude and frequency of pacemaker potential were 5 ± 2 mV and 11 ± 2 cycles/min, respectively (n = 5, Fig. 6E - G).

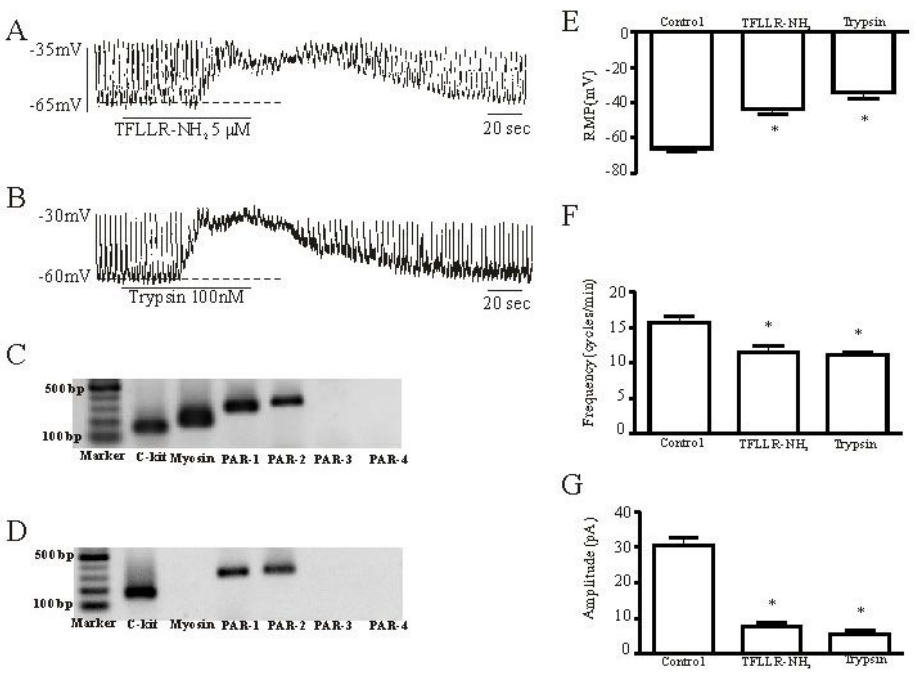

Fig.6. The depolarization of pacemaker potential by TFLLR-NH_2 and Trypsin in cultured ICC from the mouse small intestine, and the expression of mRNA in different muscle cells by RT-PCR using gene specific marker. (A) and (B), Pacemaker potentials of ICC exposed to TFLLR-NH_2 (5μM) and Trypsin (100 nM) in the current-clamping mode (I = 0). Responses to TFLLR-NH_2 and Trypsin are summarized in (E), (F) and (G). (C) Amplified cDNA from intestinal muscle cells cultured in whole dish and visualized in 2% gel. (D) Amplified cDNA prepared from pure ICC and visualized in 2% gel.

4.3 Effects of TFLLR-NH_2 and Trypsin on Pacemaker Currents in Cultured ICC

I examined whether TFLLR-NH_2 and trypsin exposed exogenously to ICC would trigger dose dependent effects on pacemaker currents in voltage clamp mode. Under a voltage clamp at a holding potential of -70 mV, the ICC generates spontaneous inward currents. Exposure to TFLLR-NH_2 (5μM) and trypsin (100 nM) induced tonic inward currents (resting currents) and reduced the frequency and amplitude of pacemaker currents in a dose dependent manner (Fig. 7A - C) and (Fig. 8A - C). The summarized values and a bar graph of the effects of TFLLR-NH_2 and trypsin on pacemaker currents are shown in Fig. 7D - F and Fig. 8D - F (n = 5).

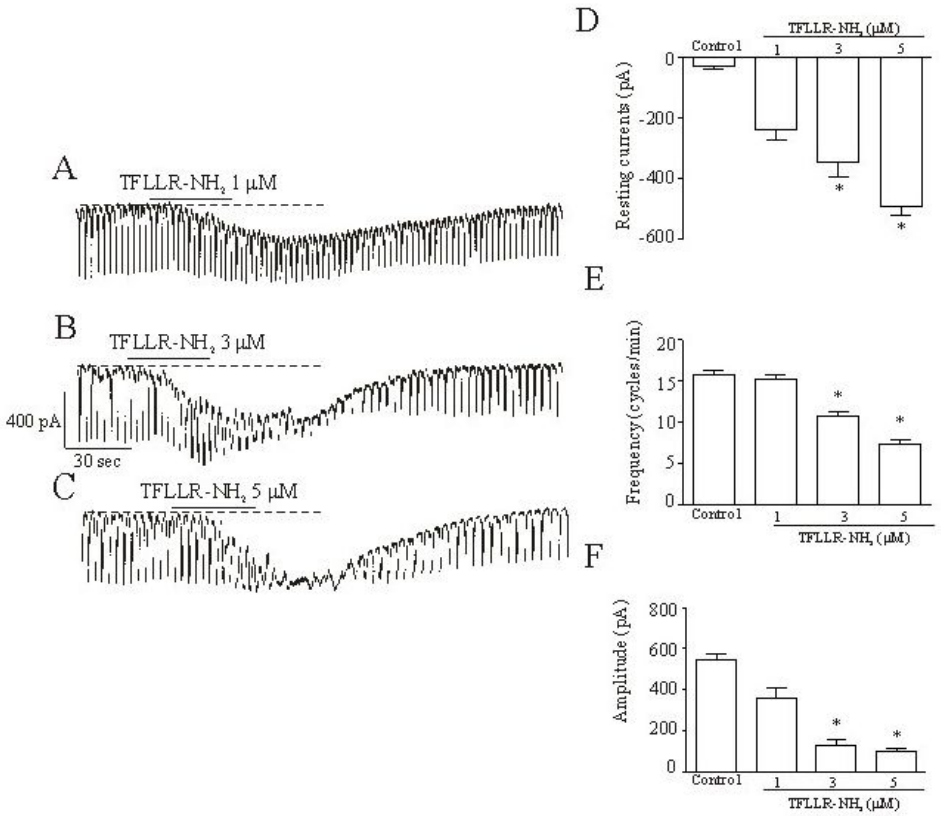

Fig.7. The tonic inward pacemaker currents induced by TFLLR-NH_2 in cultured ICC.

Pacemaker currents of ICC recorded at a holding potential of -70 mV and exposed to various concentrations of TFLLR-NH_2 (from 1 to 5 μM). (A-C) TFLLR-NH_2 caused a concentration-dependent increase in tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. Responses to TFLLR-NH_2 are summarized in (D-F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

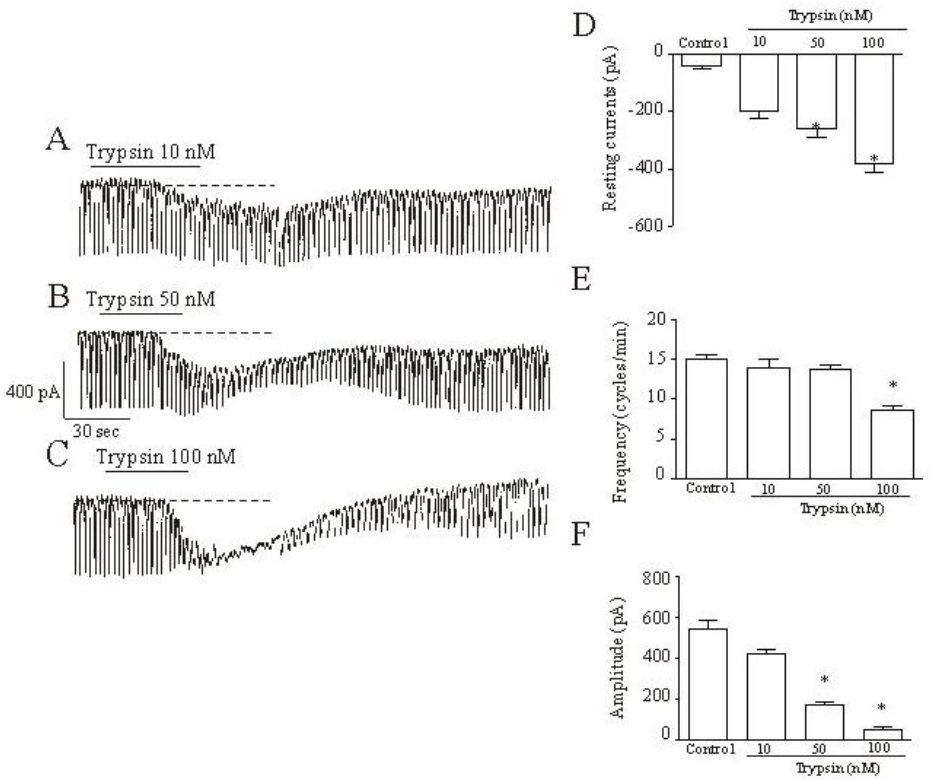

Fig.8. The tonic inward pacemaker currents induced by trypsin in cultured ICC. Pacemaker currents of ICC recorded at a holding potential of -70 mV and exposed to various concentrations of trypsin (from 10 to 100 nM). (A-C) trypsin caused a concentration-dependent increase in tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. Responses to trypsin are summarized in (D-F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

4.4 Effects of an External Na^+-free Solution and Non-Selective Cation Channel Blocker on the TFLLR-NH_2 and Trypsin Induced Effects in Cultured ICC

To determine the characteristic of the tonic inward currents induced by activation of PAR 1 and PAR 2, I tested the effects of TFLLR-NH_2 and trypsin in the presence of external Na^+-free solution and flufenamic acid (a non-selective cationic channels blocker, NSCCs). Under this condition, both external Na^+-free solution and flufenamic acid (10 μM) abolished the generation of pacemaker currents and also blocked the TFLLR-NH_2 and trypsin induced tonic inward currents (Fig. 9 - 12). Under normal conditions, the resting currents of the tonic inward currents induced by TFLLR-NH_2 and trypsin was -422 ± 34 pA and -386 ± 64 pA, respectively, and in presence of external Na^+-free solution, the resting currents was -15 ± 6 pA and -11 ± 6 pA, respectively. (n = 4, Fig. 9B and Fig. 10B). In the presence of flufenamic acid, the resting currents of tonic inward currents induced by TFLLR-NH_2, trypsin was -18 ± 2 pA and -23 ± 5 pA, respectively. (n= 4, Fig. 11B and Fig. 12B). These results suggest that the TFLLR-NH_2 and trypsin induced tonic inward currents are mediated by NSCCs.

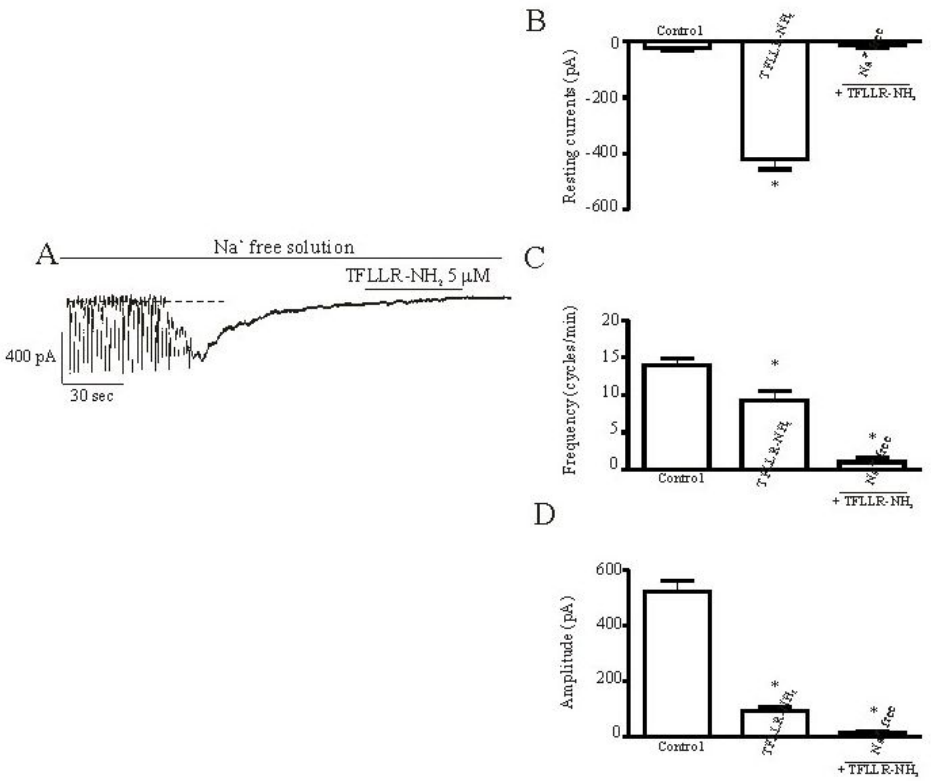

Fig.9. The effect of an external Na^+-free solution on the TFLLR-NH_2 induced effects on pacemaker currents in cultured ICC. (A) The use of an external Na^+-free solution abolished the generation of pacemaker currents. In the presence of Na^+-free solution, TFLLR-NH_2 (5 μM) did not produce tonic inward currents. Responses to TFLLR-NH_2 in the external Na^+-free solution are summarized in (B), (C) and (D). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

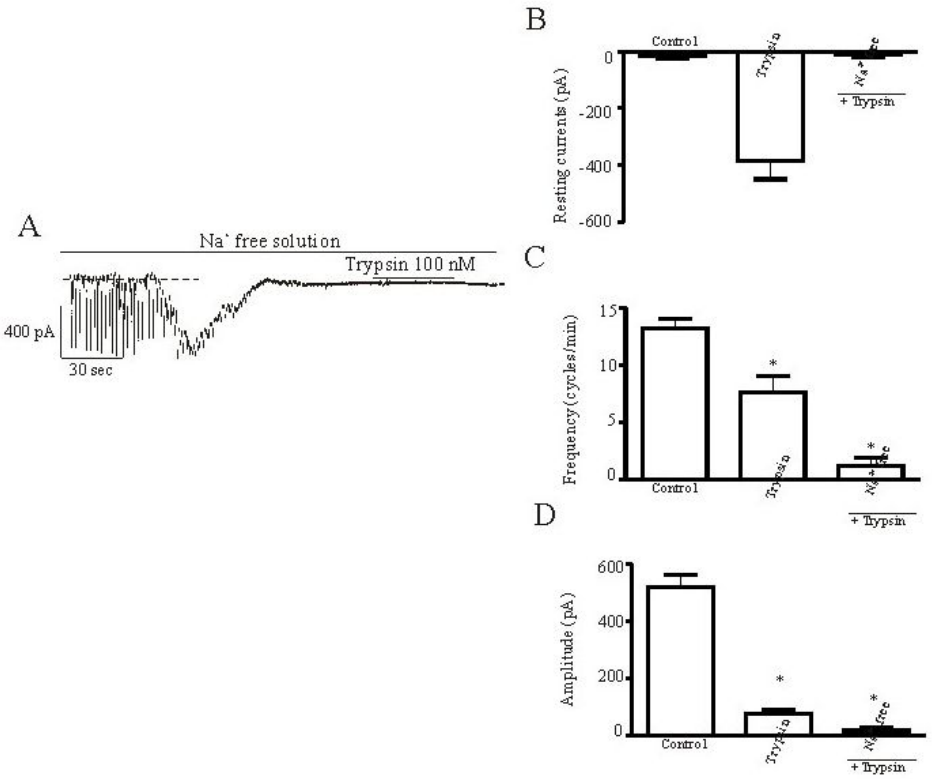

Fig.10. The effect of an external Na^+-free solution on the trypsin induced responses on pacemaker currents in cultured ICC. (A) The use of an external Na^+-free solution abolished the generation of pacemaker currents. In the presence of Na^+-free solution, trypsin (100 nM) did not produce tonic inward currents. Responses to trypsin in the external Na^+-free solution are summarized in (B), (C) and (D). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

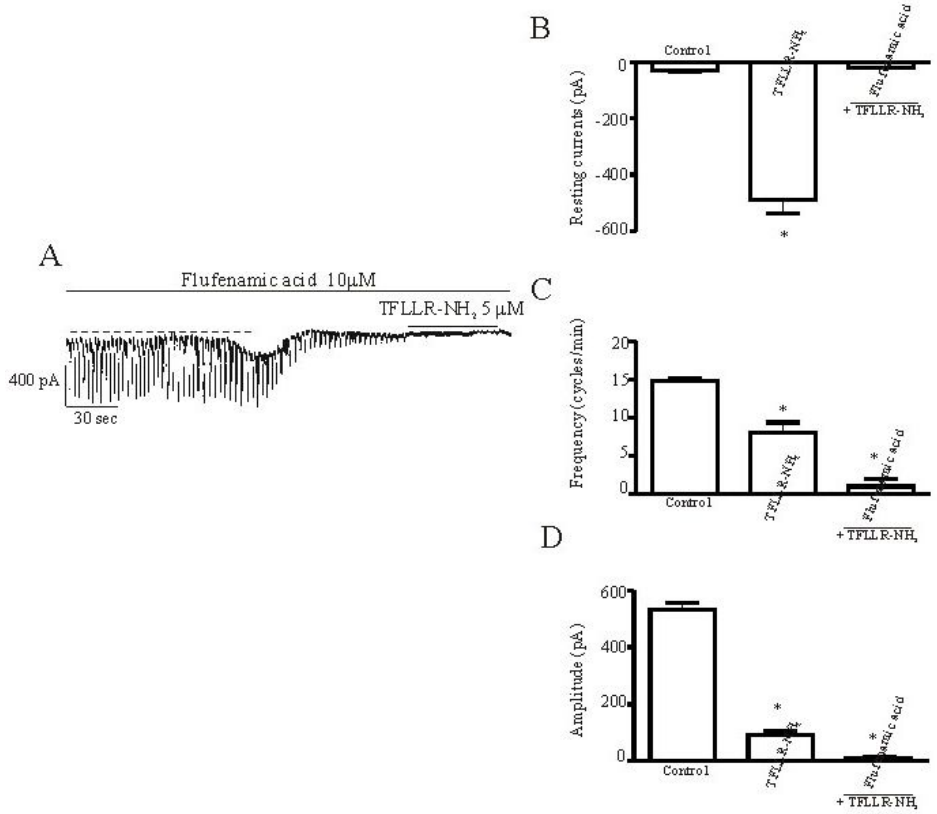

Fig.11. The effect of flufenamic acid on the TFLLR-NH_2 induced effects on pacemaker currents in cultured ICC. (A) flufenamic acid abolished the generation of pacemaker currents and also blocked the TFLLR-NH_2 (5 μM) induced tonic inward currents. Responses to TFLLR-NH_2 in presence of flufenamic acid are summarized in (B), (C) and (D). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

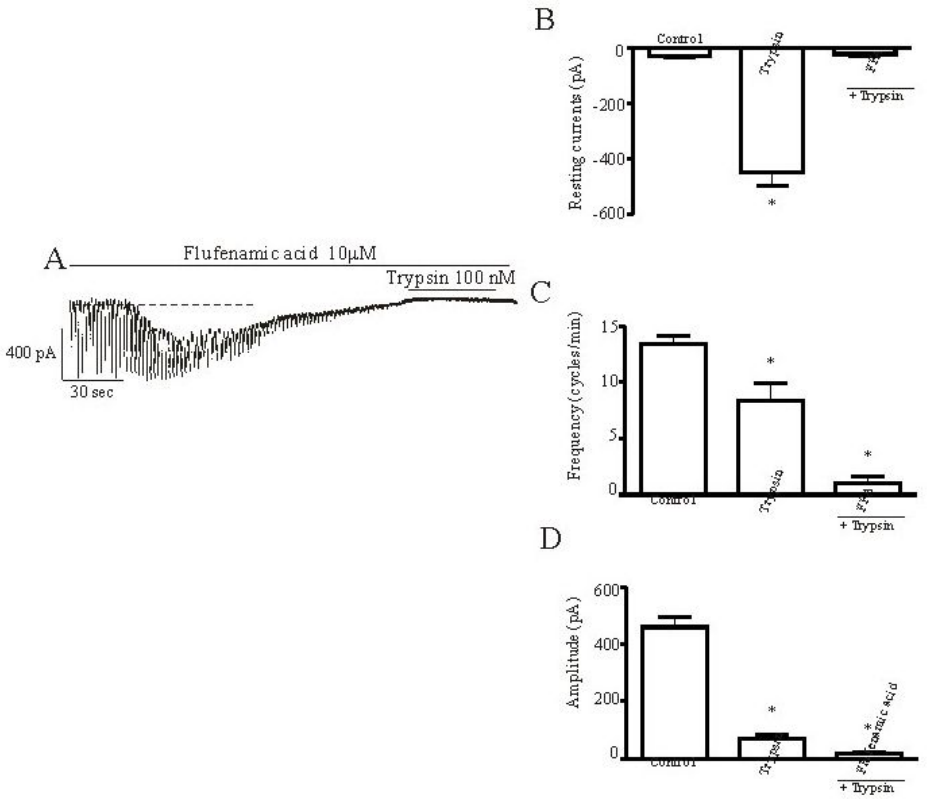

Fig.12. The effect of flufenamic acid on the trypsin induced effects on pacemaker currents in cultured ICC. (A) flufenamic acid abolished the generation of pacemaker currents and also blocked the trypsin (100 nM) induced tonic inward currents. Responses to trypsin in presence of flufenamic acid are summarized in (B), (C) and (D). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

4.5 Effects of an External Ca^{2+}-free Solution and Thapsigargin on the TFLLR-NH_2 and Trypsin Induced Effects in Cultured ICC

To investigate the role of external Ca^{2+} or internal Ca^{2+}, TFLLR-NH_2 and trypsin was tested under external Ca^{2+} free conditions or in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum. Pacemaker currents recorded at a holding potential of -70 mV were completely abolished by external Ca^{2+} free solution, but, the TFLLR-NH_2 and trypsin induced tonic currents were not blocked (Fig. 13A and Fig. 14A). Under external Ca^{2+} free conditions, the value of the resting currents with TFLLR-NH_2 (5 μM) and trypsin (100 nM) was not significantly different when compared with TFLLR-NH_2 (5 μM) and trypsin (100 nM) in the normal conditions (n = 4, Fig. 13C and Fig. 14C). However, thapsigargin (5 μM) inhibited the pacemaker currents of ICC and blocked the TFLLR-NH_2 and trypsin induced tonic currents (Fig. 13B and Fig. 14B). In the presence of thapsigargin, the value of the resting currents with TFLLR-NH_2 (5 μM) and trypsin (100 nM) was significantly different when compared with TFLLR-NH_2 (5 μM) and trypsin (100 nM) in the normal conditions (n = 3, Fig. 13C and Fig. 14C).

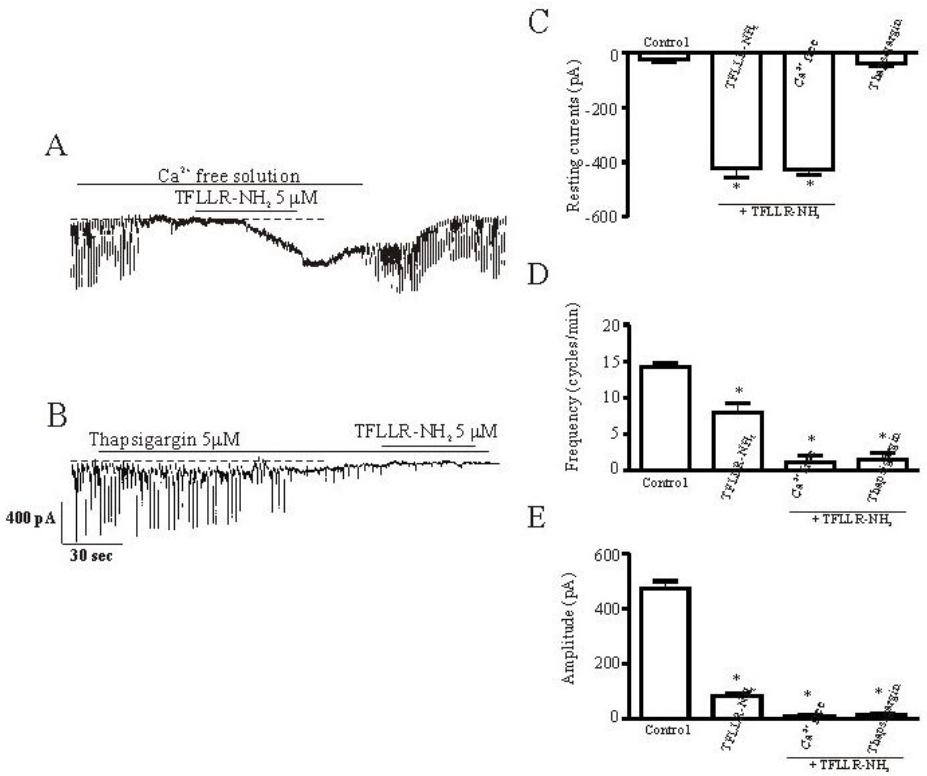

Fig.13. The effect of an external Ca^{2+}-free solution and thapsigargin on the TFLLR-NH_2 induced effects on pacemaker currents in cultured ICC. (A) The use of an external Ca^{2+}-free solution abolished the generation of pacemaker currents. In the presence of Ca^{2+} free solution, TFLLR-NH_2 (5 μM) still produce tonic inward currents. (B) Thapsigargin (5 μM) inhibited the pacemaker currents of ICC and blocked the TFLLR-NH_2 induced tonic currents. Responses to TFLLR-NH_2 in the presence of external Ca^{2+}-free solution and thapsigargin are summarized in (C), (D) and (E). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

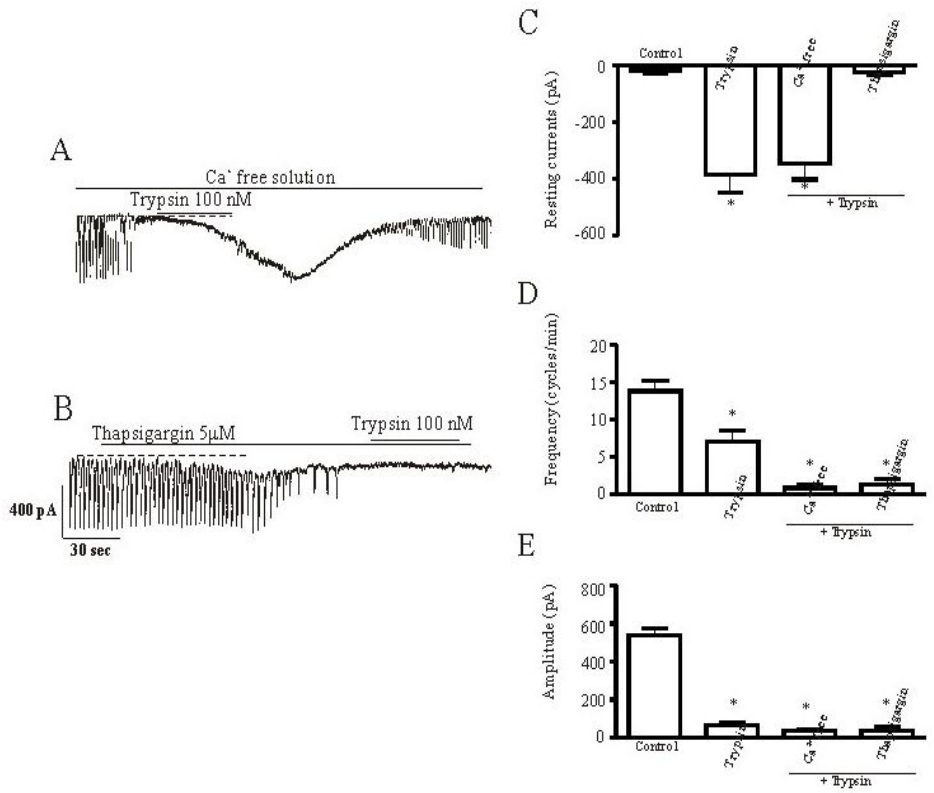

Fig.14. The effect of an external Ca^{2+}-free solution and thapsigargin on the trypsin induced effects on pacemaker currents in cultured ICC. (A) The use of an external Ca^{2+}-free solution abolished the generation of pacemaker currents. In the presence of Ca^{2+} free solution, trypsin (100 nM) still produce tonic inward currents. (B) Thapsigargin (5 μM) inhibited the pacemaker currents of ICC and blocked the trypsin induced tonic currents. Responses to trypsin in the presence of external Ca^{2+}-free solution and thapsigargin are summarized in (C), (D) and (E). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

4.6 Effects of Intracellular Ca^{2+} Intensity by TFLLR-NH_2 and Trypsin in Cultured ICC

Intracellular Ca^{2+} oscillations are considered to be the primary mechanism for the pacemaker activity in cultured ICC. Therefore, I examined the effect of TFLLR-NH_2 and trypsin on intracellular Ca^{2+} oscillations in cultured ICC. In this study, I measured the spontaneous intracellular Ca^{2+} oscillations of ICC which are connected with cell clusters. Under control conditions, Spontaneous intracellular Ca^{2+} oscillations were observed in many ICC which were loaded with 1μM fluo-4AM (Fig. 15A and Fig. 16A), and spontaneous regular intracellular Ca^{2+} oscillations were observed in a time series (Fig. 15C and Fig. 16C). Fig. 15A and Fig. 16A show images of basal (F0) and peak point (F1) of Ca^{2+} oscillations in normal circumstances, and, Fig. 15B and Fig. 16B show images of basal (F0) and peak point (F1) of Ca^{2+} oscillations in the presence of TFLLR-NH_2 (5 μM) and trypsin (100 nM). My result found that TFLLR-NH_2 (5 μM) or trypsin (100 nM) rapidly increased the intracellular Ca^{2+} concentrations in ICC (Fig. 15C and Fig. 16C).

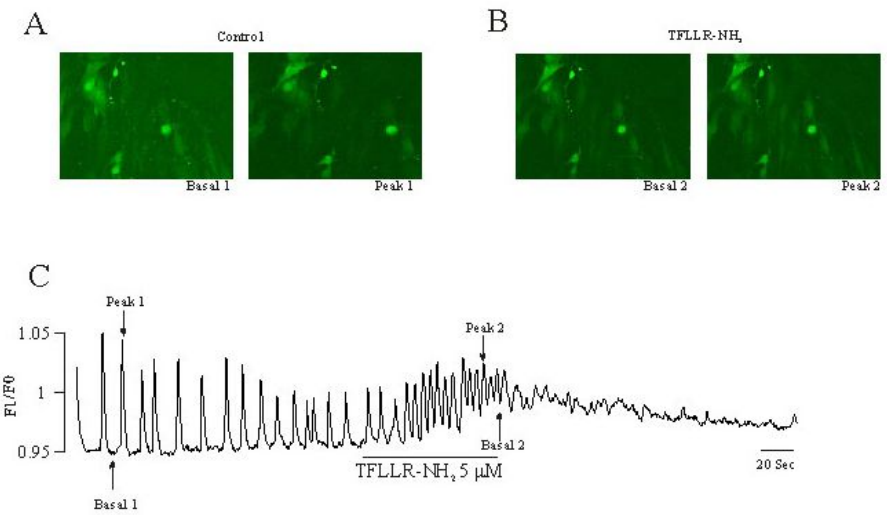

Fig.15. Effects of TFLLR-NH_2 on intracellular Ca^{2+} in cultured ICC. (A) shows the basal and peak point of ICC image in the normal condition. (B) shows the basal and peak point of ICC image in the presence of TFLLR-NH_2 (5 μM). (C) shows the Ca^{2+} oscillations in the presence of TFLLR-NH_2. The interval of representative frame was 1 second and the exposure time of each frame was 500 ms.

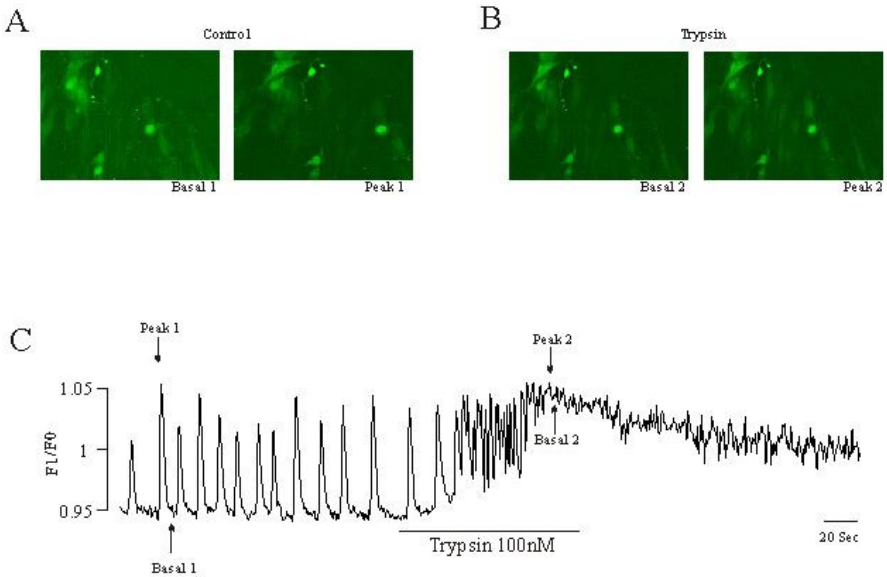

Fig.16. Effects of trypsin on intracellular Ca^{2+} oscillations in cultured ICC. (A) shows the basal and peak point of ICC image in the normal condition. (B) shows the basal and peak point of ICC image in the presence of trypsin (100 nM). (C) shows the Ca^{2+} oscillations in the presence of trypsin (100 nM). The interval of representative frame was 1 second and the exposure time of each frame was 500 ms.

4.7 Effects of Mitogen Activated Protein Kinase inhibitors in the TFLLR-NH_2 and Trypsin Induced Effects in Cultured ICC

MAPKs activation by PAR 1 and 2 agonists has been shown to modulate various cellular responses in various cell types (Lin et al., 2002; Hsieh et al., 2008; Zheng et al., 1998; Shapiro et al., 1996; Sekiguchi et al., 2007). To test the involvement of MAPKs in the TFLLR-NH_2 and trypsin mediated effects in ICC, I examined the effects of MAPKs inhibitors in the TFLLR-NH_2 and trypsin induced effects in ICC. Under this condition, the tonic inward currents induced by TFLLR-NH_2 (5 μM) and trypsin (100 nM) were not blocked by PD 98059 (10 μM), SB 203580 (10 μM), or JNK inhibitor (10 μM), (Fig. 17A - C, Fig. 18A - C). Responses to TFLLR-NH_2 or trypsin in presence of PD 98059, SB 203580, or JNK inhibitor are summarized in (n = 5, Fig. 17D - F, Fig. 18D - F.).

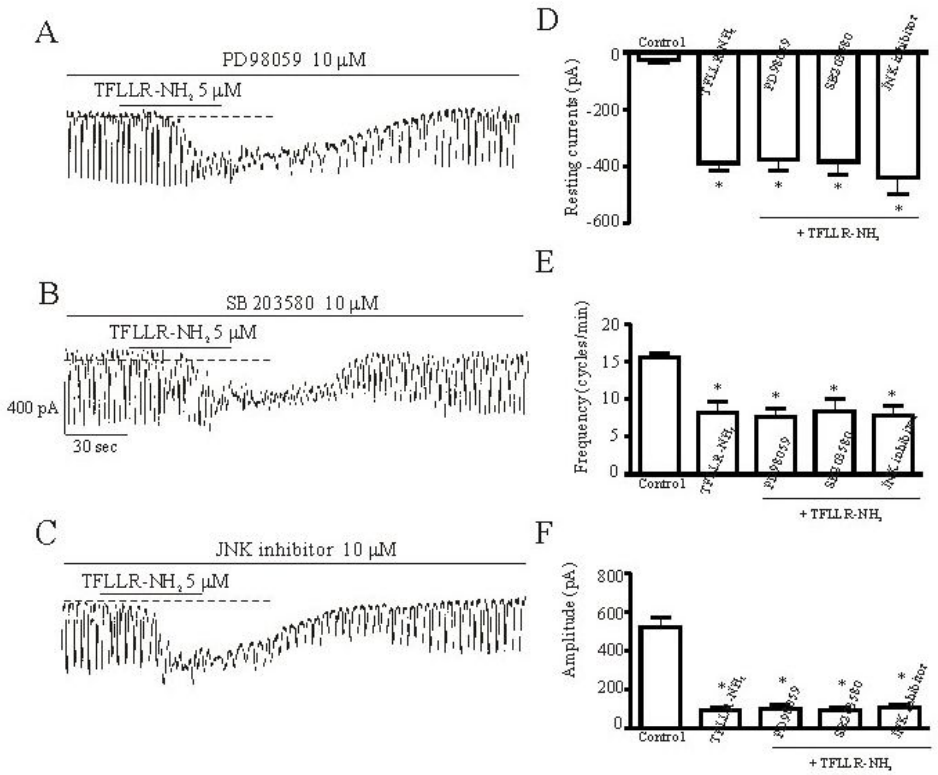

Fig.17. Effects of MAPKs inhibitors on TFLLR-NH_2 induced effects in cultured ICC. (A-C) Pacemaker currents of ICC exposed to TFLLR-NH_2 (5 μM) in the presence of PD 98059, SB 203580 or JNK inhibitor. Responses to TFLLR-NH_2 and in presence of different MAPK inhibitors are summarized in (D), (E) and (F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

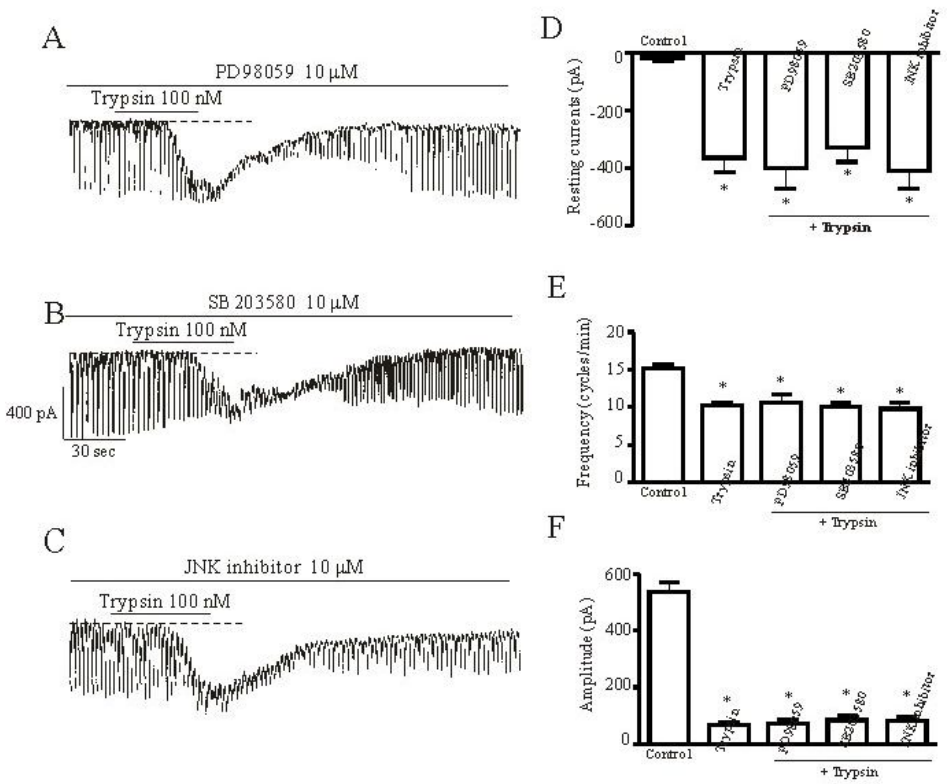

Fig.18. Effects of MAPKs inhibitors on trypsin induced effects in cultured ICC. (A-C) Pacemaker currents of ICC exposed to trypsin (100 nM) in the presence of PD 98059, SB 203580 or JNK inhibitor. Responses to trypsin in presence of different MAPKs inhibitors are summarized in (D), (E) and (F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

4.8 Effects of Tyrosine Kinase, Cyclooxygenase 2 and NF-κB inhibitors in the TFLLR-NH_2 and Trypsin Induced Effects in Cultured ICC

Tyrosine kinase, Cyclooxygenase 2 and NF-κB have been shown to play very important roles in the Thrombin and trypsin induced signaling pathways (Chen et al., 2011; Hirota et al., 2012; Saifeddine et al., 1996; Hsieh et al., 2008; Mule et al., 2002), To assess the role of tyrosine kinase, Cyclooxygenase 2 and NF-κB in TFLLR-NH_2 and trypsin induced effects in cultured ICC, I examined genistein (a tyrosine kinase inhibitor), NF-κB inhibitor or Naproxen (a cyclooxygenase 2 inhibitor), as shown in Figs. 19A – C and Figs. 20A – C. Under this condition, the tonic inward currents induced by TFLLR-NH_2 (5 μM) and trypsin (100 nM) were not blocked by genistein (10 μM), NF-κB inhibitor (10 μM) or Naproxen (10 μM). Responses to TFLLR-NH_2 and trypsin in presence of genistein, NF-κB inhibitor or naproxen are summarized in (n = 4, Figs. 19D - F) and (n = 4, Figs. 20D - F).

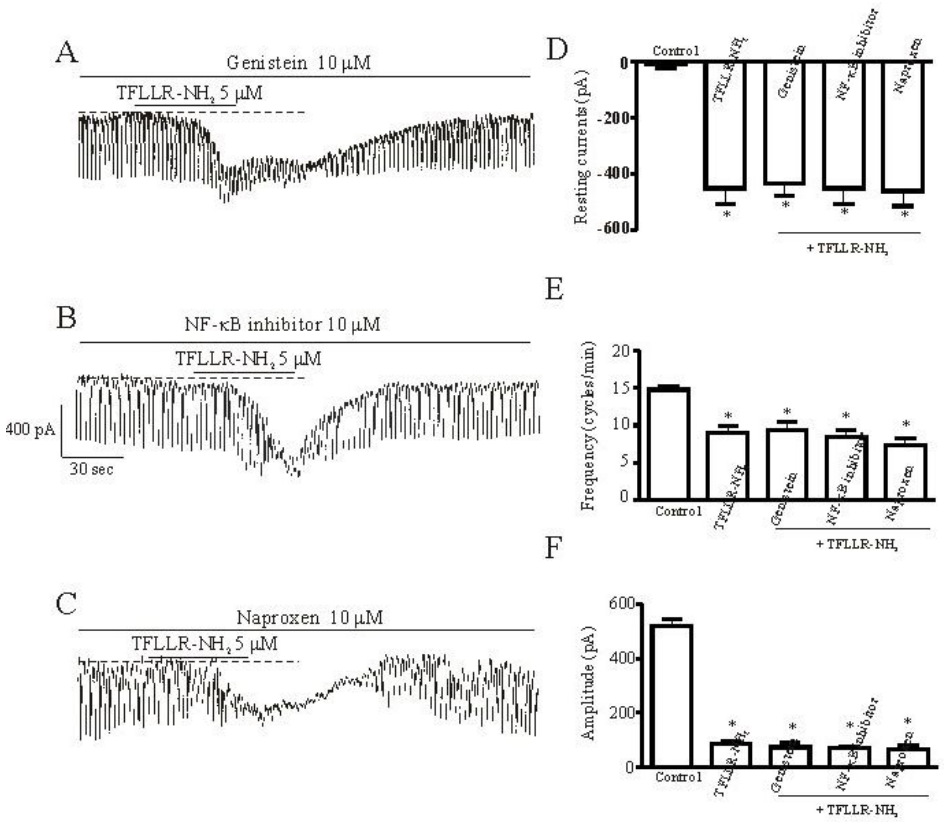

Fig.19. Effects of tyrosine kinase, NF-κB and Cyclooxygenase 2 inhibitors on TFLLR-NH_2 induced effects in cultured ICC. (A - C) Pacemaker currents of ICC exposed to TFLLR-NH_2 (5 μM) in the presence of genistein, NF-κB inhibitor and naproxen. Responses to TFLLR-NH_2 in presence of genistein, NF-κB inhibitor and naproxen are summarized in (D - F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

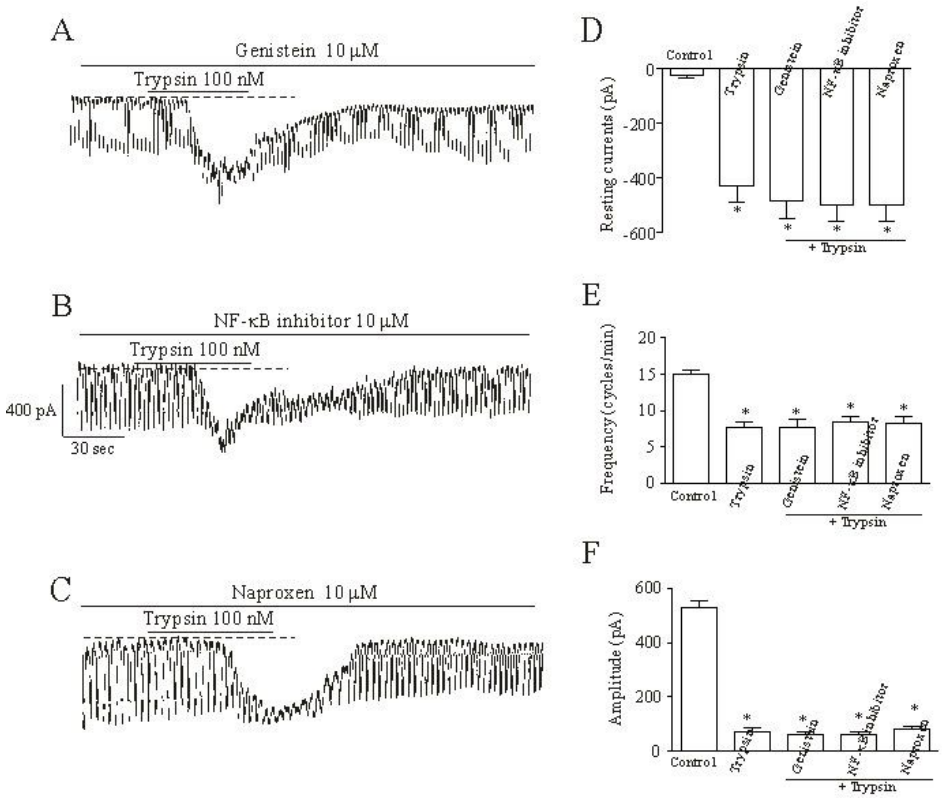

Fig.20. Effects of tyrosine kinase, NF-κB and Cyclooxygenase 2 inhibitors on trypsin induced effects in cultured ICC. (A - C) Pacemaker currents of ICC exposed to trypsin (100 nM) in the presence of genistein, NF-κB inhibitor and naproxen. Responses to trypsin in presence of genistein, NF-κB inhibitor and naproxen are summarized in (D - F). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p < 0.05$). The dotted lines indicate zero current levels.

5 Discussion

The present study demonstrates that activation of either PAR 1 or PAR 2 depolarizes the membrane potential and produces a tonic inward pacemaker current in cultured ICC of the murine small intestine. These effects are mediated through the activation of NSCCs by intracellular Ca^{2+} dependent mechanism.

PARs are composed of four subtypes from PAR 1 to PAR 4, three of which are activated by thrombin (PAR 1, PAR 3 and PAR 4) while the fourth (PAR 2) is activated by trypsin. PARs are widely distributed in the GI tract (Kawabata, 2003; Vergnolle, 2004), in particular, PAR 1 and 2 are abundant in GI tract (Hollenberg, 1999; Kawabata et al., 2000). To date, modulation of gastrointestinal smooth muscle motility by activation of PARs has been well described in vitro throughout the GI tract (Hollenberg et al., 1999; Corvera et al., 1997; Zheng et al., 1998; Cocks et al., 1999a; Kawabata et al., 2000a). It has been suggested that the activation of PAR 1 and 2 produces contraction of the gastric longitudinal smooth muscle from rats or guinea pigs (Hollenberg et al., 1993; Saifeddine et al., 1996). Since ICC are connected to smooth muscle cells by gap junctions, and the pacemaker currents induced by ICC are directly transmitted to smooth muscle cells through gap junctions and induce the slow waves which cause smooth muscles contractions. I think that the thrombin and trypsin induced contractions of the GI smooth muscles may result from the action of PARs on the pacemaker currents of ICC. In the present study, I examined the expression of PARs mRNA in the cultured ICC of small intestine by using the RT-PCR technique, RT-PCR results showed that PAR 1 and PAR 2 are expressed in cultured ICC of the murine small intestine, but, PAR 3 and PAR 4 are not expressed. In addition, with the use of whole patch clamp recording from the cultured ICC, I found that thrombin, an agonist activating PAR 1, PAR 3 and PAR 4 and trypsin, an enzyme activating PAR 2 depolarizes the membrane potential and produces tonic inward pacemaker currents in cultured ICC. In addition, TFLLR-NH_2, a PAR 1 specific agonist highly mimics the effects of thrombin. Therefore, I confirmed that the PAR 1 and PAR 2 are expressed in

cultured ICC of the murine small intestine, and the activation of PARs induced inward currents is mediated by PAR 1 and PAR 2.

NSCCs are a heterogeneous family of channels, widely expressed in non-excitable and excitable cells. NSCCs were found to be predominantly permeable to Na^+, and, to a small degree, Ca^{2+}, the removal of external Na^+ decreased the amplitude of currents carried by NSCCs, Pharmacologically, flufenamate inhibited NSCCs (Kuriyama et al., 1998). As a result of the wide expression of NSCCs, as well as to the fact that the activation of such currents produce a persistent membrane depolarization, NSCCs have been involved in the control of cell excitability and the generation of depolarizing after potentials, plateau potentials, or bursting (Kramer et al., 1985; Swandulla et al., 1985; Partridge et al., 1988; Partridge et al., 1994; Zhang et al., 1995; Congar et al., 1997; Haj-Dahmane et al., 1997; Rekling et al., 1997; Morisset et al., 1999; Egorov et al., 2002). Over the last years, several investigations have suggested that the NSCCs are also responsible for the generation of spontaneous inward pacemaker currents in cultured ICC of murine small intestine (Koh et al., 1998; Thomsen et al., 1998). Reducing the concentration of external Na^+ abolished the generation of pacemaker currents and the external application of NSCCs blocker flufenamic acid also inhibited the generation of pacemaker currents in cultured ICC of murine small intestine (Koh et al., 1998). In addition, it has been reported that NSCCs play a very important role in the excitatory neurotransmitters induced actions on the cultured ICC of the murine small intestine, Cultured ICC possess receptors and second messenger systems responsible for their role in excitatory (such as tachykinin receptors, muscarinic receptors) and inhibitory (NO) neural transmission (Jun et al., 2004; So et al., 2009; Park et al., 2007). It suggested that Carbachol, a muscarinic receptor agonist, depolarized the membrane potential and produced tonic inward pacemaker currents on cultured ICC, and this inward currents induced by Carbachol is carried by NSCCs (So et al., 2009). In another study, Substance P depolarized the membrane potential and produced tonic inward currents on cultured ICC, and this effects is also mediated by NSCCs (Jun et al., 2004). Furthermore, NSCCs has been reported

to play a very important role in the activation of PARs induced responses (Shirakawa et al., 2010; Bair et al., 2008). In the present study, with the use of whole cell patch clamp recording, I found that activation of PARs depolarized the pacemaker potentials and produced tonic inward currents with reduced frequency and amplitude of pacemaker currents in the cultured ICC of the murine small intestine. In the presence of Na^+ free solution and flufenamic acid, the spontaneous pacemaker currents of cultured ICC was abolished, and the PARs mediated tonic inward currents were also blocked. These results strongly suggest that NSCCs are involved in generating the pacemaker currents of cultured ICC and also mediate the activation of PARs on the cultured ICC of the murine small intestine.

The external and intracellular Ca^{2+} are essential for cultured ICC to generate the pacemaker currents, and intracellular Ca^{2+} oscillations are considered to be the primary initiator to generate pacemaker currents. Either removal of extracellular Ca^{2+} or block the Ca^{2+} release from the endoplasmic reticulum abolishes the pacemaker activity in ICC (Torihashi et al., 2002). In addition, the activation of NSCCs also depends on the Ca^{2+} (Lupinsky et al., 2006). More importantly, it has been reported that the activation of PARs activates the NSCCs (Shirakawa et al., 2010; Bair et al., 2008). In the present study, with the use of Ca^{2+} confocal imaging, I observed that the treatment with TFLLR-NH_2 and trypsin initially increased the intracellular Ca^{2+} concentrations and also increased the oscillating Ca^{2+} wave frequency and were followed by a decrease of the intracellular Ca^{2+} concentrations and oscillating Ca^{2+} wave frequency in cultured ICC. I think that this rise of intracellular Ca^{2+} concentrations may either from the external Ca^{2+} influx or from the Ca^{2+} release from the intracellular Ca^{2+} stores. In this study, the removal of extracellular Ca^{2+} abolishes the pacemaker activity in cultured ICC, but, did not block the tonic inward currents induced by activation of PAR1and 2. However, thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum blocked the tonic inward currents induced by activation of PAR1and 2, Therefore, it suggests that the increased concentrations of intracellular Ca^{2+} induced by activation of PAR

1 and 2 is due to the Ca^{2+} release from the endoplasmic reticulum rather than Ca^{2+} influx from the extracellular space.

Cyclooxygenase (COX) is a rate-limiting key enzyme in the synthesis of prostaglandins (PGs). Two COX isoforms have been demonstrated: COX-1, which is constitutively expressed in most tissues, mediates regulating normal physiological responses; COX-2, another COX isoform, which is expressed in several inflammatory diseases and displays a wide range of biological activities in different tissues (Williams et al., 1999; Smith et al., 2000), COX-2 is not detectable in most normal tissues or resting cells, but its expression can be induced by various stimuli to produce proinflammatory PGs during inflammatory responses in several cell types including cultured ICC (Choi et al., 2010; Ohnaka et al., 2000; Yang et al., 2002). It has already shown that COX-2 enzyme is detectable in cultured ICC after exposure of deoxycholic acid and produce PGE_2 which in turn modulates the pacemaker currents (Jun et al., 2005). In another study, high levels of PGE_2 synthesized by COX-2 are involved in mediating the action of H_2O_2 on cultured ICC (Choi et al., 2010). In adition, Thrombin and trypsin have been shown to regulate the activities of COX-2 through MAPKs and NF-κB activation in various cell types (Hsieh et al., 2008; Syeda et al., 2006; Sekiguchi et al., 2007). However, little is known about the signaling mechanisms underlying thrombin and trypsin induced effects on cultured ICC. In this study, I have applied naproxen, a COX-2 inhibitor and found that naproxen didn't block the tonic inward currents induced by activation of PAR 1 and 2, this result indicate that the COX-2 enzyme is not involved in PAR 1 and 2 mediated effects in cultured ICC. In addition, to explore the signaling pathway of the PARs activation, I also tried a NF-κB inhibitor and various MAPKs inhibitors, I found that these inhibitors didn't block the tonic inward currents induced by activation of PAR 1 and 2. These results indicate that the activation of NF-κB and MAPKs is also not involved in PAR 1 and 2 mediated effects in cultured ICC.

Several studies have reported that thrombin and trypsin induced diverse biological effects are due to the activation of tyrosine kinase (Zheng et al., 1998; Flavia et al., 2002; Kawabata et al., 2000b; Darmoul et al., 2004), so, in this study, I also tested the effect of genistein, a tyrosine kinase inhibitor, I found that in the presence of genistein, activation of PAR 1 and 2 still produce a tonic inward current in ICC, this result indicates that the tyrosine kinase is also not involved in PAR 1 and 2 mediated effects.

The signal transduction mechanism of the PARs activation has been well established in many cell types. However, in this study, I found that most of the signaling pathways involved in thrombin and trypsin induced activation in other cell types don't occur in ICC. This indicate that the cultured ICC responses and the signal transduction mechanisms coupled with PARs activation do not appear to be the same in the other cell types and depend on the tissues and the animal species. Moreover, the signal transduction pathways triggered by PAR 1 and PAR 2 are complex and the "cascade" processes are not completely understood.

In conclusion, this study suggests that activation of PAR 1 and PAR 2 modulate the pacemaker activity of ICC through the activation of NSCCs via an intracellular Ca^{2+} release mechanism. My study thus provides novel evidence for the physiological roles of PARs 1 and 2 in modulating the pacemaker activity of cultured ICC from the small intestine.

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Abstract

Interstitial cells of Cajal (ICC) provide important regulatory functions in the motor activity of the gastrointestinal tract. It has already reported that activation of proteinase-activated receptors (PARs) by thrombin and trypsin modulates gastrointestinal motility. However, there is no report as to the activation of PARs on ICC. In the present study, the existence of PAR 1 and PAR 2 mRNA was identified by RT-PCR in ICC. In whole cell patch clamp, thrombin (an enzyme activating PAR 1, PAR 3 and PAR 4), TFLLR-NH_2 (a specific peptide agonist for PAR 1), and trypsin (an enzyme activating PAR 2) produced a large inward current and decreased the frequency and the amplitude of the pacemaker current significantly. The normal pacemaker currents of ICC was abolished in the presence of flufenamic acid (a non-selective cation channels blocker, NSCCs), external Na^{+}-free solution, external Ca^{2+}-free solution and thapsigargin (a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum), and the TFLLR-NH_2 and trypsin induced tonic inward currents was blocked by flufenamic acid, external Na^{+}-free solution and thapsigargin, but not blocked by external Ca^{2+}-free solution. In Ca^{2+} confocal imaging, both TFLLR-NH_2 and trypsin increased the concentration of intracellular Ca^{2+}. These findings demonstrate that activation of either PAR 1 or PAR 2 depolarizes the membrane potential and produces a tonic inward pacemaker current in ICC. These effects are mediated through the activation of NSCCs by intracellular Ca^{2+} dependent mechanism.

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