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The Regulatory Mechanism of Pacemaker Activity from Colonic Interstitial Cells of Cajal

Graduate School of Chosun University

Department of Physiology

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ABBREVIATIONS

GI - gastrointestinal tract

SMP – submuscular plexus

ENS - Enteric nervous system

NO - nitric oxide

ICC - interstitial cells of Cajal

RMP - resting membrane potential

TEM - Transmission electron microscopy

DMP - deep muscular plexus

IM – Intramuscular

SMC – Smooth muscle cell

ER- Endoplasmic reticulum

SCF- Stem cell factor

IP_3 - Inositol trisphosphate

HCN - Hyperpolarization-activated cyclic nucleotide-gated

cAMP - 3'-5'-cyclic adenosine monophosphate

cGMP - 3',5'-cyclic guanosine monophosphate

CNBD - cyclic nucleotide binding domain

KRB - Krebs-Ringer bicarbonate solution

PCR - polymerase chain reaction

RT-PCR – reverse transcription PCR

CsCl - Cesium Chloride

Epac Agonist - 8-(4-Chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate monosodium hydrate

TTX – Tetrodotoxin

K_{ATP}– ATP sensitive potassium channel

CNG - Cyclic nucleotide-gated

국문초록

The Regulatory Mechanism of Pacemaker Activity from Colonic Interstitial Cells of Cajal

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위 장관의 가장 중요한 작용은 음식물을 분해하여 체내 세포에 영양분과 에너지를 공급해 주는 것이다. 이는 위장관내 근육 세포의 수축을 통해 소화운동, 소화액 분비 및 소화 흡수 기능을 발휘하게 된다. 카할세포는 위장관내 평활근 수축의 조절 기능을 담당한다고 알려져 있다. 하지만 아직까지 카할세포에 대한 연구가 미비한 실정이다.

따라서 카할세포의 운동 조절에 관한 기능을 구체적으로 밝히고 자본 연구에서는 cyclic nucleotides의 카할세포에 대한 기능을 확인하고자 하였다. 전기 생리학적 기법을 통해 카할세포에서 향도잡이전류를 확인할 수 있었으며, cAMP의 투여는 향도잡이전류의 빈도수를 증가시키는 것을 알 수 있었다. 더불어 증가되는 빈도수의 결과는 cAMP에 작용하는 phosphodiesterase효소 에서도 관찰할 수 있었다. 또한 adenylyl cyclase의

처치는 향도잡이 전류를 억제 하는 것을 확인할 수 있는 것으로 보아 cAMP가 향도잡이 전류의 조절에 큰 역할을 담당하는 것으로 여겨진다.

이러한 결과를 토대로 hyperpolarization-activated cyclic nucleotide-gated (HCN) 채널이 카할세포에 존재하는지 RT-PCR기법을 통해 확인한 결과 HCN1과 3 아형 채널이 카할세포에 존재한 것을 알 수 있었다. 더불어 전기 생리학적 기법을 통해 다양한 HCN 채널 길항제가 향도잡이 전류를 억제하는 것을 볼 수 있었다. 많은 연구결과에서 카할세포의 향도잡이 전류에 대한 세포내 칼슘의 역할에 대한 보고를 토대로 본 연구에서는 세포내 칼슘에 대한 연구를 시행한 결과 역시 HCN 채널과 세포내 칼슘은 밀접한 관련이 있는 것으로 보여졌다.

이러한 결과는 위장관 중대장의 운동성이 cAMP 의조절을 받는 것으로 여겨지며, 구체적인 부착부위는 세포막에 존재하는 HCN 채널로 생각된다.

본 연구는 기존 소장에서 보여지는 향도잡이 전류에 대한 기전과 서로 다른 조절 기전을 가지고 있으며 이는 소장과 대장운동성에 대한 약물학적 처치에 대한 기초적인 중요 자료를 제공할 것으로 사료된다.

1. INTRODUCTION

1.1 The Gastrointestinal Tract

The gastrointestinal tract (GI) is the long muscular tube stretching from the mouth to the anus which is divided into many functional compartments. It is generally divided into the oesophagus, the stomach, the intestine and the rectum. It plays a crucial role in the absorption and digestion and develops contractile activity to facilitate the movement and mixing of the luminal contents.

1.1.1 Histology of Colon:

The entire length of GI tract is comprised of four distinct layers of cells (Fig.1)

1. The mucosa→ it provides absorptive surface and is a barrier to noxious substances via epithelial cells
2. The submucosa→ it is the supportive connective tissue layer containing the submucosal neural plexus, blood vessel, lymphatics and a thin smooth muscle
3. The muscularis externa→ it provides muscular contraction and is comprised of the myentric neural plexus sandwiched between the circular and longitudinal smooth muscle layers
4. The serosa→ it provides a protective layer of mesothelial cells covering the outside of the tube

In the colon, the wall is divided into two muscle layers, two neural plexuses, a serosa and a mucosa. Longitudinal muscle separated from the inner circular muscle by the Auerbach's or myenteric plexus (MY). The submuscular plexus (SMP) or Meissner's plexus lies between the mucosa and the circular muscle layer. The mucosa consists of an epithelial layer, fibers of the muscularis mucosa and connective tissue (Christensen J. 1994).

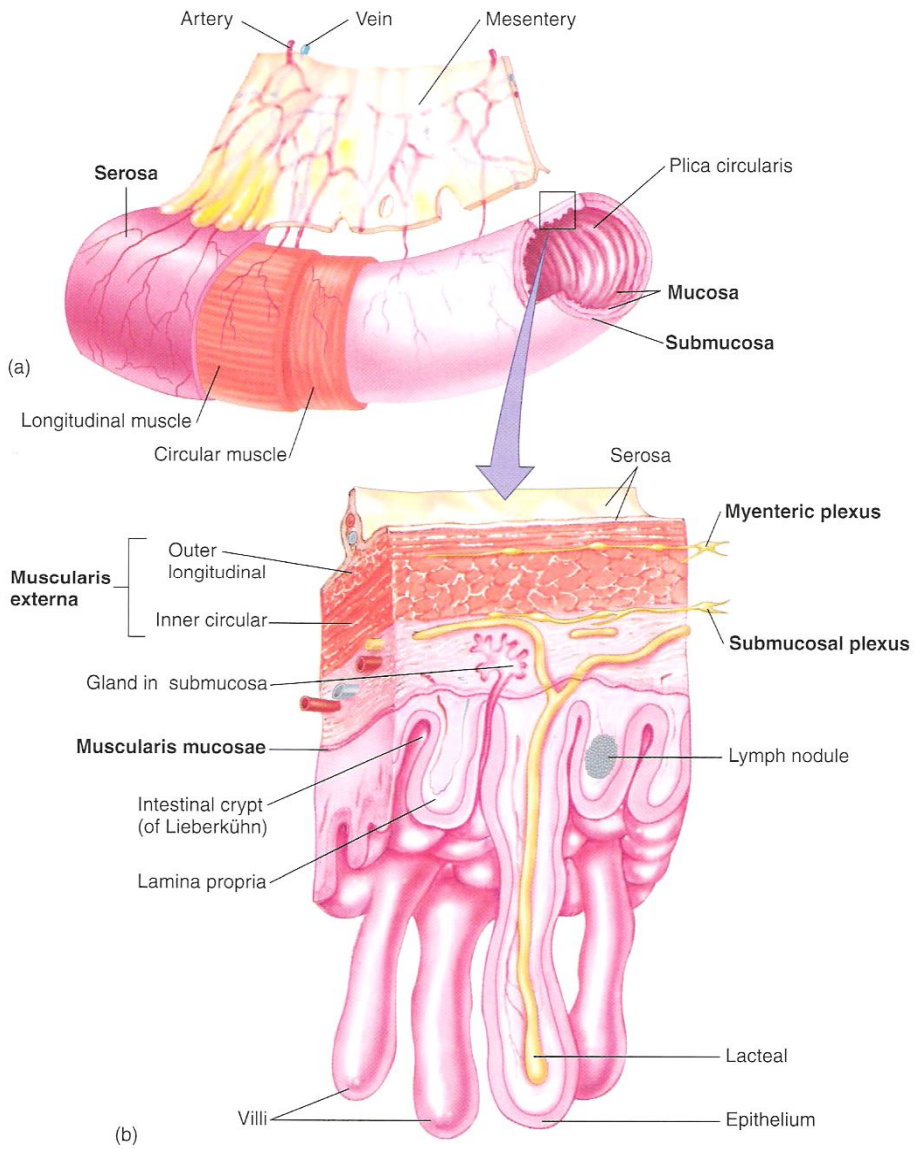


Fig. 1. Figure showing the different layers of cells in the GI tract. (Figure adapted from Human Physiology by Amanda Rausch, Chelsea Kortleever)

1.1.2 Function of Colon

The main functions of the colon motility are

- Extraction of water and the electrolytes from the contents of the intestinal lumen through mixing movements
- Keeping the contents temporally before defecation and also allow the bacterial growth
- Moving the content to the rectum and let defecation under voluntary control through coordinated contractions.

1.2 Regulation of GI Motility

Functional integration of multiple tissues and cell types is necessary to generate the various motility patterns characteristic of each organ in the digestive tract (Wood, 2003). GI motility is regulated in 3 ways,

1. Reflexes that originate outside the digestive system (**long reflexes**)
2. Reflexes that originate inside the digestive system (**enteric nervous system** or **short reflexes**) and
3. GI peptides.

The smooth muscle cells (SMC) receive the regulatory input from various control systems, including motor neurons, ICC, hormones, paracrine substances and inflammatory mediators thereby generating the force (Perrino, 2011).

1.3 Pacemaker Activity

SMCs of the intestines show cyclic depolarizations of the membrane potentials which can be recorded both in vivo as well as in vitro and termed as slow waves. These slow waves are still being generated by the smooth muscles from the isolated

intestine signifying that the pacemaker mechanism is within the intestinal wall. When these cyclic depolarizations reach the calcium channel open threshold, calcium enters inside smooth muscle cell and triggers a muscle to contract. For this process calcium enters the cell through L-type Ca^{2+}channel.

A typical slow wave consists of a rapid depolarization, a partial repolarization and a sustained plateau followed by a progressive and complete repolarization to the resting membrane potential (RMP). However, the smooth muscle itself cannot generate slow waves because it lacks the ionic mechanisms responsible for the pacemaker activity. So, where do these cyclic depolarizations come from? Where is the origin of the rhythm of the intestine? Which are the pacemaker cells?

1.3.1 Interstitial Cells of Cajal

More than one hundred years ago, the Spanish anatomist Santiago Ramon Y Cajal described a cell type that appeared fusiform or stellate, with prominent nuclei and several processes that formed networks in gastrointestinal tissue (Sanders, 1996). These cells constitute a unique class of cells that are commonly referred to as interstitial cells of Cajal (ICC). ICC develop from mesenchymal cells that are also the common precursors for smooth muscle cells (Sanders et al., 1999).

1.3.2Morphology of ICC

The presence of ICC in different regions of the GI wall led anatomists to speculate about a physiological role. It was suggested that ICC may serve as a pacemaker because they are found more prominently in rhythmically contracting tissues (Thuneberg, 1982 & Pellegrini et al., 1997). Using phase contrast microscopy, murine ICC in culture or freshly isolated have been described as triangular (multi-polar) or stellate in shape, with a large nucleus and little surrounding cytoplasm, and possessing 3-5 branches with triangular bifurcations at the end of each process.

It was also reported that ICC in culture demonstrate spontaneous contractile activity, quite often with the processes contracting independent of the cell body and of smooth muscle (Hughes et al., 1998). The electron microscopic analysis of the ultrastructural characteristics of ICC revealed that it contains an elaborate system of sarcoplasm in reticulum, thin and intermediate filaments, and an abundance of mitochondria. ICC can be easily distinguished from the smooth muscle and the glial cell by the lack of myosin filaments and numerous caveolae respectively

1.3.3Classification of ICC

Electron microscopy has been an invaluable tool for examining the ultrastructure of cells within the GI tract. Using this technique, ICC have been noted to have a number of distinct morphological features including the presence of gap junctions between ICC and ICC and ICC and SMC, numerous cavaeoloe, abundant sER and numerous mitochondria (Kumoro et al., 1999). Cells in different locations were also noted to be distinct from one another in that those cells within the muscularis (ICC-IM) and deep muscular plexus region (ICC-DMP) of the small intestine were thin, unbranched cells while those within the myenteric region (ICC-MY), along the submucosal edge (ICC-SM) of the circular muscle (CM) were highly branched cells with numerous processes (Kumoro, 1999; Kumoro et al., 1999; Thuneberg, 1982). The differences in morphology between regions led to the proposal that these cells constituted different cell subpopulations with differing functions. In addition to the ultrastructural features described above, ICC-IM were described as having post-synaptic densities along their cell surface (Beckett et al., 2005). A representation of different types of ICC and their location on GI tract is shown in Fig. 2

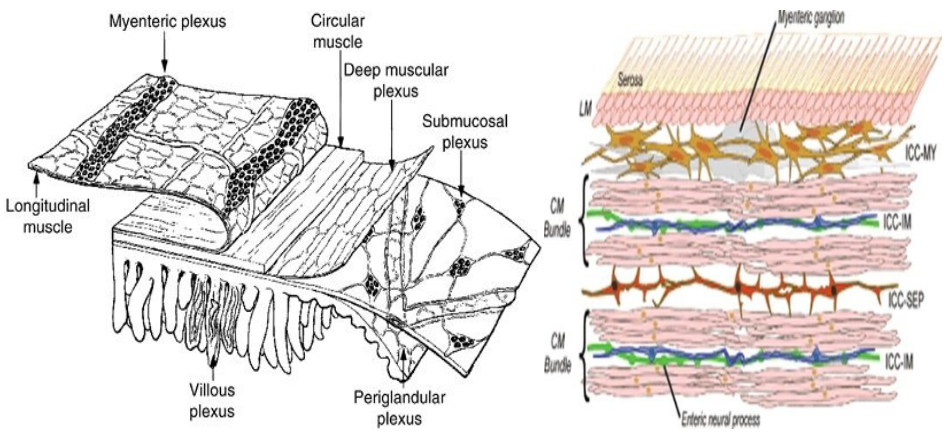


Fig. 2. Location of different types of ICCs in different layers of GI tract (Furness & Costa1978; Hirst, 2001,

1.3.4 ICC Distribution

ICC are differently distributed along the GI tract. Transmission electron microscopy allowed to characterize the ICC ultrastructure and consequently, identify ICC (Faussone-Pellegrini & Thuneberg, 1999).

Gastrointestinal region		ICC-MY	ICC-DMP	ICC -SMP	ICC-IM
Stomach	Antrum	Yes	-	-	Yes
	Corpus	Yes	-	-	Yes
	Fundus	_	-	-	Yes
Small Intestine		Yes	Yes	-	-
Colon		Yes	-	Yes	Yes

Table 1. Distribution of ICC in different regions of the gastrointestinal tract.

The distribution in Table 1 is a summary of what it has been found along the GI tract in different animal species: dog, rat, mouse, rabbit, cat, pig, guinea-pig (Smith et al., 1987a; Smith et al., 1987b; Du & Conklin 1989; Christensen et al., 1992; Sanders1996; Ward et al., 1997; Jimenez et al., 1999; Vanderwinden et al., 2000; Pluja et al., 2001) and humans (Rumessen et al., 1993; Rae et al., 1998; Torihashi et al., 1999).

1.3.5 The KitReceptor and its Ligand Stem Cell Factor

The discovery that ICC expressed the receptor tyrosine kinase c-Kit (Ward et al., 1994; Torihashi et al., 1995) was an important one as it provided a means by which they could be distinguished from other cell types. The c-Kit receptor is a type III receptor protein tyrosine kinase, which also includes PDGFR-α and –β and CSF-1, consisting of an extracellular domain, a single transmembrane domain and a cytoplasmic domain. The c-Kit receptor has been identified in and plays a major role on the development of blood cells, gametocytes, interstitial cells of Cajal, mast cells and melanocytes. The ligand for the Kit receptor is called stem cell factor (SCF; also known as mast cell growth factor; MGF) which binds to c-Kit receptor and thereby activates it. Immunohistochemical labeling of ICC using antibodies raised against c-Kit has confirmed studies from electron micrographs in that two

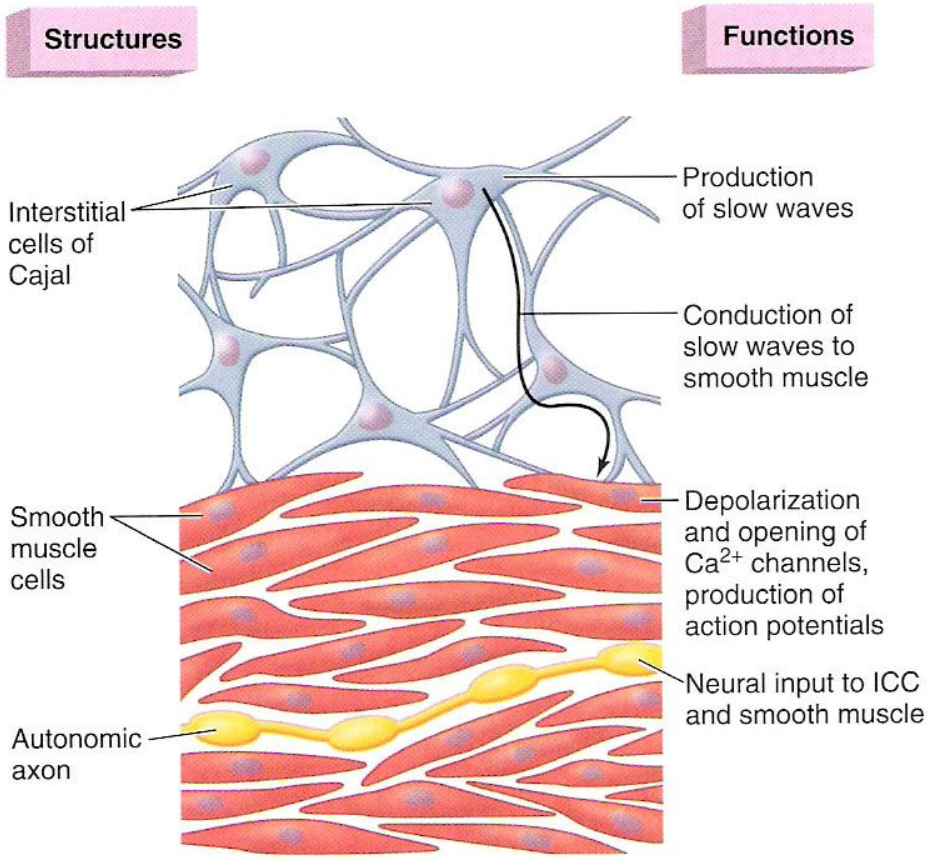
morphological classes of ICC have been determined, i.e. long spindle-shaped ICC-IM and stellate, highly branched cells in plexus regions. Additionally, studies using c-Kit neutralizing antibodies revealed that neutralizing c-Kit led to the disappearance of slow wave activity, electrical events that give rise to phasic smooth muscle contractions and therefore loss of motility.

1.3.6 Physiological Roles of ICC

Specific ICC functions include the generation of electrical pacemaker activity that manifests in smooth muscles as electrical slow waves and contributes to segmenting and propagating (peristaltic) contractile activity (Huizinga, 1999; Sanders, 1996; Sanders et al., 1999). ICC are now regarded as being important players in the control of GI motility due to their implication in pacing of SMC as well as playing important roles in different types of neuromuscular transmission. ICC also serve as an interface between the enteric nervous systems and the smooth musculature by mediating efferent inputs to smooth muscle cells (Hirst & Ward, 2003; Ward & Sanders, 2001), and indirectly, to the pacemaker apparatus (Hirst & Ward, 2003).

The two main and established functions of ICC are:

- ICC generate spontaneous rhythmic electrical oscillations, or slow waves, which cause smooth muscle contractions (Torihashi et al., 1995; Sanders et al., 2000; Daniel, 2001; Huizinga, 2001).
- ICC serve as a connection between enteric nerves and SMC, participating in neurotransmissionand regulating GI motility (Sanders 1996; Huizinga 1999; Ward 2000; Daniel 2001; Huizinga 2001).



Structures and Functions of ICC, SMC and autonomic axon

1.4 Pacemaker Mechanism

The electrical activity of the GI tract is not similar to the electrical activity in the cardiac cells which were thought to be analogous long ago when extensive works were not done in ICC. These currents generally referred as "slow waves" are neither produced by SMCs as the tissue lacking ICC could not able to elicit this current.

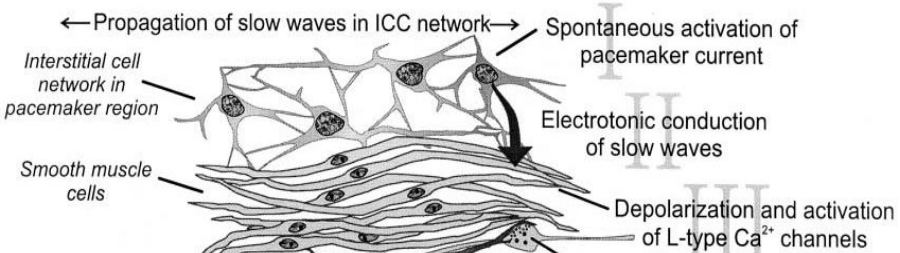


Fig.3. The rhythmoneuromuscular apparatus of GI motility. (Sanders et al, 2000)

This slow waves produced are propagated through the networks of other electrically coupled ICC. ICC-MY and ICC-SM are electrically coupled to neighboring SMCs, facilitating conduction of slow waves into the smooth muscle syncytium. SMCs respond to the slow wave depolarization with the activation of voltage-dependent ion channels (most importantly Ca^{2+} channels). The smooth muscle response is regulated by neural inputs. Both excitatory and inhibitory enteric motor neurons are closely associated with ICC-IM (or ICC-DMP in the small intestine). Neurotransmission occurs at specialized junctions that are within 20 nm of varicose nerve terminals. ICC-IM and ICC-DMP are electrically coupled to neighboring SMCs. Neural modification of the smooth muscle response, via ICC, modulates excitation-contraction coupling between slow waves and contractions.

Studies on electrical rhythmicity in ICC have suggested that inositol trisphosphate (IP_3) receptor-dependent calcium release from endoplasmic reticulum (ER) is crucial for the generation of slow waves. It has been reported that inhibitors of IP_3 receptor blocked pacemaker activity and injection of heparin inhibited pacemaker current and slow waves. Thus, Ca^{2+} release from ER through IP_3 receptor is necessary for $[Ca^{2+}]_i$ oscillation in ICC. It is also well known that ICC have many mitochondria and that mitochondrial Ca^{2+} uptake is thought to be required for

electrical pacemaking in ICC.

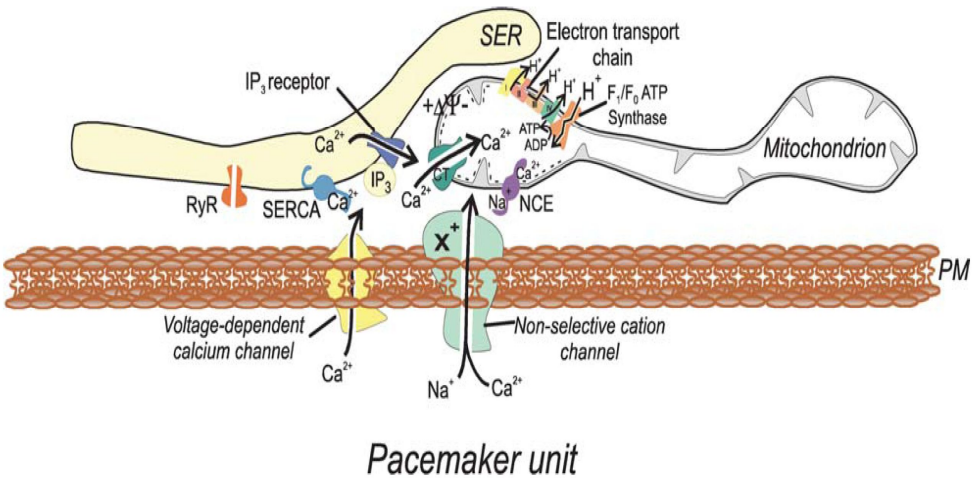


Fig. 4. Figure illustrating the mechanism of pacemaker current generation. **(**Sanders et al, 2006**)**

Isolated ICC-SM are spontaneously active at resting potential values (measured by means of the patch clamp technique), while muscle cells remain at the same potential values. In experimental conditions the influx ion channels in ICC are activated with lower potential values (more negative) than the channels in muscle cells in the same region. Such a relation between the current and potential is typical of low threshold Ca^{2+} channels (T-type of Ca^{2+} channels). This is the ideal type of channels for pacemaker activity, and it can also be found in other pacemaker cells in the organism. The low-threshold Ca^{2+} channels are not inactivated by low depolarization. Close to the resting potential of pacemaker ICC these channels are able to create small influx currents, which very probably are responsible for ICC-SM depolarization towards the threshold potential.

Activation of pacemaker currents depends upon the periodic release of Ca^{2+} from IP_3 receptor-operated stores. Mitochondrial Ca^{2+} uptake is linked in a hitherto unknown way to the activation of pacemaker currents. The uptake and periodic

release of Ca^{2+} from IP_3 receptor-operated stores appears to be the main oscillatory process responsible for GI auto rhythmicity (Ward et al., 2000). Because of the high total resistance of ICC-SM (at least 1 G Ω) a very feeble current is needed to induce significant polarization. With progressing depolarization, the L-type Ca^{2+}channels (the second source of Ca^{2+} influx) are progressively activated. These L-type Ca^{2+} channels are important for electrical activity transmission and a threshold potential increase in smooth muscle cells (Sanders, 1996). Some authors suggested the occurrence of some ionic flow direction rectifier in ICC, important for pacemaker activity, as in the heart. Non-specific Na^+ or K^+ channels activated by hyperpolarization perform the function of a rectifier in myocytes. Ca^{2+} channels in ICC-SM, initiate the pacemaker activity and the voltage-gated K^+ channel terminates the cycle by depolarization. It is therefore also necessary to investigate the voltage-gated channels in other types of ICC (Langton et al., 1989; Lee & Sanders, 1993)

1.5 Pacemaking Activity in ICC and its Transmission to Smooth Muscle Cells

Many reports after the extensive work have shown that ICC provide the pacemaker activity associated with rhythmic contractions of the stomach and small intestine. ICC-MY are responsible for producing rhythmic inward currents when recording in voltage clamp mode or rhythmic oscillations in membrane potential when recorded in current clamp mode. The slow waves generated by the smooth muscle were transmitted from ICC-MY and that the ICC-MY are necessary for this electrical activity. This has been demonstrated by the use of c-Kit neutralizing antibody where irregular intestinal contractility, loss of ICC-MY and absence of intestinal slow waves were shown. Use of mutant mouse (Sl/SLd) which has a mutated Steel locus which produces the ligand steel factor for the c-Kit receptor, ICC-MY failed to develop in the small intestine and slow wave activity is lost.

Intact ICC networks are critical for generation and propagation of slow waves. ICC

are coupled to SMCs through low resistance electrical junctions. SMCs respond to the depolarization produced by slow waves with activation of voltage-dependent Ca^{2+} channels. For contractile responses, the most important smooth muscle channel is the dihydropyridine-sensitive (L-type) Ca^{2+} channel. Other voltage-dependent channels, such as K^+ channels, tune the response of the muscle cells and facilitate or prohibit the development of Ca^{2+} action potentials (spikes) or sustained Ca^{2+} entry during the plateau phase of the slow wave.

Also, ICC also play an important role in mediating motor inputs from the enteric nervous system; muscles lacking ICC have significantly attenuated responses to cholinergic and nitrergic enteric nerve stimulation. Excitability responses of SMCs to slow wave depolarization are regulated by excitatory and inhibitory motor inputs from the enteric nervous system and by the effects of hormones and paracrine substances.

It is likely that cells throughout the ICC network are spontaneously active but cycle-to-cycle differences in excitability or region-specific differences in pacemaker frequency can generate either temporary or long-term pacemaker dominance. Within a small region of the ICC network, most of the cells are spontaneously active at the same general frequency. Thus, the primary pacemaker (the cell or sub-cellular compartment responsible for initiating each cycle) can vary between each slow wave. Inward current and depolarization from the primary pacemaker 'entrains' the activity of coupled pacemakers, creating a wave of activity with specific and regular propagation velocity properties. A faster pacemaker will dominate other coupled pacemaker cells running at slower frequencies; however, entrainment will fail if a propagating slow wave runs into a refractory region. Thus, the extent of the ICC network over which a primary pacemaker can maintain dominance depends upon the propagation velocity and the spontaneous frequency of other pacemaker cells in the network.

1.6 Ion Channels

Ion permeation is crucial for a variety of biological functions such as nervous signal transmission and osmotic regulation (Hille, 2001). Many human and animal diseases are also associated to defects in ionic channels function, the majority of them arising from mutations in the genes encoding the channel proteins. Ionic channels are proteins inserted in the membrane lipid bilayer by forming aqueous pores through the cell membrane. Different kinds of ionic channels are present in nature, differing in functional, gating and selectivity properties. The open state of the channel pore may be regulated by different factors that open and close the channel mouth allowing and preventing the ionic flux. The gating mechanisms can be based on physical factors like the transmembrane potential difference such as happens in voltage gated channels (Catterall, 2000), or pressure-induced structural changes as the case of mechanosensitive channel(Chang et al., 1998). In other classes of channels, chemical factors are also determinant in triggering the channel gate, as the presence of ligand molecules (i.e. in cyclic nucleotide activated channels) or pH variations (i.e. in KcsA potassium channel (Cuello et al., 1998; Schrempf et al., 1995). An important property of the open pore is the selective permeability, which is the ability to allowing only to a restricted class of ions to flow through the channel pore in large amount.

1.6.1 Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels

Hyperpolarization- activated cyclic nucleotide (HCN) currents were discovered during the late 1970s in sino-atrial node cells where they determine the rate of the heart beating, and were termed "pacemaker" currents (Brown et al., 1979; Brown & DiFrancesco, 1980; DiFrancesco, 1981a & 1981b; Yanagihara & Irisawa, 1980). The same currents were found in hippocampal pyramidal cells (Halliwell & Adams, 1982; Maccaferri et al., 1993) and in photoreceptors cells (Fain et al., 1978; Baylor

et al., 1984; Hestrin, 1987). At the time of discovery, the properties of the channels seemed to be unique, especially the activation upon hyperpolarization; currents were, therefore, termed I_h (h for hyperpolarization-activated), I_f (f for funny) or I_q (q for queer).

Recently, in both vertebrates and invertebrates, genes coding for HCN channels have been identified. This HCN channels belong to the family of voltage-gated potassium channels. In vertebrates, 4 different types of HCN (1-4) channels are found having high sequence homology between each other. HCN channels contain six membrane-spanning helices (S1-S6), including a positively charged voltage-sensing S4 segment, and an ion conducting pore between S5 and S6. In the C terminus, the channels carry a cyclic nucleotide-binding domain (CNBD) capable of binding 3'-5'-cyclic adenosine monophosphate (cAMP) and 3',5'-cyclic guanosine monophosphate (cGMP) (the latter with less affinity), that act as modulators on channel properties.

1.6.2 Cyclic Adenosine Monophosphate Modulatory Mechanism

Several aspects of the HCN channel behaviors are modulated when cAMP binds to the intracellular CNBD of HCN channels. It speeds up the opening of the channel, shifts the V1/2 value to the positive, increases maximal open probability and also the closing of the channel is slowed. It is thought that cAMP modulation and voltage sensing machinery in HCN channels work independently of each other.

1.7 Electrophysiology: An Overview

Ion channels are responsible for the electrical behavior of cells. Cells are simple electrical circuits containing the capacitor and the conductor in series. The lipid bilayer acts as a capacitor as it separates the changes and also maintains a voltage deference i.e. membrane potential across its boundary. Ion channels provide the flow of the ions by acting as a conductor. Electrophysiology is the electrical recording technique that enables the measurement of the ionic flow and the potential difference related to them.

1.7.1 Voltage Clamp

The voltage clamp technique was first developed by Cole, Marmont and Hodgkin, Huxley and Katz in 1949. This techniques allow to clamp i.e. hold the membrane at different potential making it possible to measure how much *ionic current* crosses a cell's membrane at any given voltage

1.7.2 Current Clamp

It is another technique where the membrane potential of the cell is recorded by injecting the current into a cell through the recording electrode. Unlike in voltage clamp, the membrane potential is not held constant but is free to vary. This technique is used to study how a cell responds when electric current enters a cell; this is important for instance for understanding how neurons respond to neurotransmitters that act by opening membrane ion channels.

1.7.3 Patch Clamp Technique

The patch clamp technique was first developed by Neher and Sakmann in 1976. The principle of this technique is to isolate a patch of membrane electrically from the external solution and to record current flowing into the patch. It uses a glass micropipette, also referred as an electrode, having an open tip which fuses with an intact cell membrane to form a stable high resistance "giga seal". Now the current can be measured in the "cell attached" configuration. "Whole cell" configuration, the current from an entire population of ion channels, can also be recorded by rupturing the seal then allowing the pipette solution and the intracellular solution to equilibrate. Additionally, the membrane patch can be pulled away from the rest of the membrane to form an "inside-out" or "outside-out" excised parch. In the inside –out configuration, the cytoplasmic side of the membrane faces into the bath solution, whereas in the outside-out configuration the extracellular side faces into the bath.

1.8 Thesis Rationale and Objectives

Undisturbed GI motility or peristalsis is critical for effective digestion and absorption of our food and its ingredients. The ICC-MY are of major importance for motor control of the GI tract. In recent years, there is growing evidence that ICC serve as electrical pacemakers and generate spontaneous electrical slow waves which constitute the basic electrical rhythm in the gastrointestinal tract. Many gastrointestinal motility disorders have been associated with abnormal numbers or disorders of ICC so far.

Many studies have been done on the ICC of the small intestine but very less information is reported on the current generated by ICC of the colon. I hypothesize that the current generated by the ICC of colon is different to the current generated by the ICC of the small intestine. As the function of both colon and small intestine are different, I also hypothesize that the expression of the receptors and the action of the neurotransmitters on ICC of both the small intestine and colon are also different.

The goal of this project is to compare the current generated by the ICC of both small intestine and colon. Specifically, I have tried to study the action of cyclic nucleotides on the pacemaker potential generated by ICC of the colon and the receptors responsible for the action. Comparison of the cyclic nucleotides effect is also done on the pacemaker current generated by the ICC of small intestine.

2. MATERIALS AND METHODS

2.1 Ethical Approval

BALB/c mice aged 3-5 days old of either sex used for these studies. Mice were anesthetized with diethyl ether and sacrificed by cervical dislocation. All animals were treated ethically according to the guiding principles for the care and use of animals in the field of physiological sciences approved by the Institutional Animal Use and Care Committee at Chosun University College of Medicine.

2.2 Tissue Isolation and ICC Culture

Immediately after cervical dislocation, an abdominal incision was made and the entire small intestine and colon were removed and placed in Krebs-Ringer bicarbonate solution. Microdissection was performed to uncoil the intestine. The small intestine was excised 1 cm below the pyloric ring to the cecum and the colon just below from cecum to the rectum. They were opened along the mesenteric border and the luminal contents were washed away with ice-cold KRB solution.

The isolated tissue was pinned to the base of a Slygard dish, the mucosa and submucosa were removed by peeling and only the tunica muscularis of small intestine and colon were used after sharp dissection. The tissue isolated was then equilibrated with ice-cold Ca^{2+}-free Hanks solution. The tissues were transferred into collagenase-containing enzyme solution and incubated, without stirring, at 37°C for 15 min. Following incubation in the enzyme solution, tissues were washed repeatedly (3-5 times) with Ca^{2+}-free solution the tissues were triturated through a series of three blunt pipettes of decreasing tip diameter to create a cell suspension and placed onto sterile glass coverslips coated with poly-L lysine in 35-mm culture dishes and incubated at 37℃ in a 95% O_2 and 5% CO_2 incubator in smooth muscle growth medium (Lonza, Walkersville, MD, USA) supplemented with 2%

antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF) (5 ng/ml; Sigma). After 24 hrs, the media is replaced with the one without containing SCF and allowed to grow for another 24hrs before performing the patch clamp.

2.3 Electrophysiological Recordings

To conduct the voltage clamp and current clamp technique in single cells on required essential pieces of equipment including: a vibration isolation table to prevent mechanical interference, a Faraday cage to prevent electrical interference, an inverted microscope to view both the preparation and the glass electrode, a perfusion system to perfuse the preparation, a macro and micromanipulators to position the glass electrode and electronic equipments like headstage, amplifier, analogue to digital converter (Digitizer), software and the computer to apply the protocol, record and analyze data. Before experimentation, the proper assembly of the above equipment and configuration of the pClamp software was confirmed.

Extracellular solution and intracellular solutions are prepared carefully and were titrated to a pH of 7.38 - 7.42 and 7.2-7.25 respectively. Osmolarity of both the solutions are maintained same or near equal for the easy sealing and better survival of the cell after patch. An extreme difference in osmolarity is detrimental to cellular health as it may cause cell death via shrinkage or rupture.

2.3.1 Pipettes

The glass microelectrode is prepared from the fire polished borosilicate glass capillaries (Harvard Apparatus) with the help of puller (Narishige PP-83, Japan) which gives the blunt tipped electrode. The heater of the puller is well adjusted such that the electrode prepared produces resistance between 3-5 MΩ.

2.3.2 Cell Recording and Identification

After 48hrs of the culture, the culture plates were taken out from the incubator and placed in the recording chamber equipped on the inverted microscope. The cells were then perfused with the extracellular solution and left for at least 15 minutes to equilibrate. The cells were maintained at 30^{o}C. The cells were then visualized under the microscope and ICC to be patch were identified.

Glass microelectrode were filled with intracellular solution and mounted on the proper pipette holder and tightly sealed such that there would not be any leakage. The headstage was angled around 45^{o} and lowered into the extracellular solution. Once the electrode dips into the extracellular solution, pipette resistance and the resultant current were displayed in the Clampex program (pClamp software). The current pulse was then zeroed by turning the pipette offset knob on the Axon 200B amplifier. Cell was approached by the use of both macro and micro manipulators. As the electrode touches the cell membrane, the pipette resistance starts increasing and almost doubles. At this point, negative pressure was applied quickly but gently which helped to form the giga ohm (GΩ) seal. Furthermore, application of the negative command potentials rapids up the formation of GΩ seal.

Once the GΩ seal was formed, the pipette capacitance was reduced and then the cell membrane was ruptured. It was done either by the continuous application of the negative pressure to the cell membrane or by pressing the Zap switch on the amplifier which injects the sudden burst of current rupturing the cell membrane. Large capacitive transients and a low access resistance as viewed in the clampex interface, signaled rupture of the small patch of cell membrane within the mouth of the pipette.

The current and the membrane potential were then recorded using the gap-free protocol saved in the clampex software. Currents or potentials were amplified using

Axopatch 200B (Axon Instruments, Foster City, CA, USA). Data were digitized with a 16-bit analog to digital converter (Digidata 1322A, Axon instruments) and stored directly on-line using pClamp software (version 9.2, Axon instruments). Command pulse was applied using a personal computer and pClamp software (version 9.2, Axon Instruments). All data were analyzed using pClamp (version 9.2; Axon Instruments), Graphpad Prism (version 5.0, Graphpad Software Inc., San Diego, CA). The data were filtered at 5 KHz.

2.4 Measurement of Intracellular Calcium Oscillation

24-30 hrs cultured ICC were used for the measurement of intracellular Ca^{2+}. The media from the culture dish was removed and the cells were washed with the extracellular solution 2 times and incubated at 37^{o}C for 10 minutes. Fluo-4/AM was then loaded to the cells at the final concentration of 1μM and incubated for further 15 minutes at 37^{o}C in dark. Fluo-4/AM dye was removed by washing the cell twice with the extracellular solution and placed on the 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 (×200; Hamamatsu Instrument, Hamamatsu, Shizuoka, Japan). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of the Ca^{2+}imaging, the temperature of the perfusion chamber containing the cultured ICC was kept at 30°C. The variations of $[Ca^{2+}]_i$ fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging.

2.5 Solutions

Ca^{2+}-free Solution (mM)

125 NaCl, 5.36 KCl, 15.5 NaOH, 0.336 Na_2HPO4, 0.44 KH_2PO_4, 10 glucose, 2.9 sucrose, and 11 HEPES, adjusted to pH 7.4 with Tris buffer.

Enzymatic Solution

1.3 mg collagenase (Worthington, Lakewood, NJ, USA), 2 mg fatty acid-free bovine serum albumin, and 2 mg trypsin inhibitor in 1 ml of Ca^{2+} solution.

Extracellular Solution (mM)

5 KCl, 135 NaCl, 2 $CaCl_2$, 10 glucose, 1.2 $MgCl_2$, and 10 HEPES adjusted to pH 7.4 with Tris.

Intracellular Solution (mM)

135 KCl, 2.5 $MgCl_2$, 3 MgATP, 0.1 NaGTP, 2.5 Creatine phosphate disodium, 0.1 EGTA, 10 HEPES adjusted to pH 7.2 with Tris.

2.6 Analysis of Electrophysiological Data

Data are expressed as means ± standard errors of the mean. The student's *t* test was used, where appropriate, to evaluate differences in the data. *P* values < 0.05 were taken as statistically significant. The n values reported in the text refer to the number of cells used in the patch-clamp experiments. For patch clamp experiments, peak currents were analyzed before and during application of drugs.

2.7 RNA Isolation and RT-PCR

Single Cell Isolation

The field in the culture dish was selected where there are lots of single cell isolated from each other. The patch pipette with relatively little bigger pore size was used to pick the 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 1ml 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 both small intestine and the colon, after enzymatic digestion, were cultured on 100mm culture dish separately. After 48hrs 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. 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**)**. For the single cell gene amplification, secondary PCR was performed using 1μl of first PCR product. All PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide fluorescence.

Table 2 : Primers for PCR

Official symbol for mRNA	GenBank Accession No.	Primer Sequence	Binding Position	Product Size
c-Kit	AY536430	S-GCACAGAAGGAGGCACTTATACCT A-TGAGACAGGAGTGGTACACCTTTG	S-1184 A-1398	215
Myosin	NM_013607	S-AGCAGGAGGTGGAACACAAGAAGA A-GGAAGCCACATCTTTGGCCAGTTT	S-3888 A-4084	197
PGP 9.5 (Uch/1)	NM_011670	S-GCCAACAACCAAGACAAGCTGGAA A-GCCGTCCACGTTGTTGAACAGAAT	S-467 A-679	213
HCN 1	NM_010408	S-GCCATGCTGAGCAAGCTGAGATTT A-TCCGATCGAGTCGGTCAATAGCAA	S-1807 A-2131	325
HCN 2	NM_008226	S-CACAGCCATGCTGACAAAGCTCAA A-ATCTAGCCGGTCAATAGCCACAGT	S-1613 A-1937	325
HCN3	NM_008227	S-TCTGCAGTTTCTGGTCCCTATGCT A-ACTGCTCCACCTGCTTGTACTTCT	S-896 A-1203	308
HCN 4	NM_001081192	S-ATCGTGGTGGAGGACAACACAGAAT A-GACACAGCAGAAGCATCATGCCAA	S-952 A-1288	337

RT-PCR was performed using the following amplification profile: 45°C for 30 min for reverse transcription followed by 95°C for 5 min to activate the Taq polymerase (*i-star*Taq, iNtRON bio 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.

2.8 siRNA Transfection

siRNA oligodeoxynucleotides were chemically synthesized by Bioneer (Daejeon., Korea). The following siRNAs were used:

sense, GUCAUCAUACCAGUUGGAA(dTdT);

antisense, UUCCAACUGGUAUGAUGAC(dTdT) and

sense, CUCACUGAUGGAUCCUACU(dTdT);

antisense, AGUAGGAUCCAUCAGUGAG (dTdT) targeting HCN1 and HCN3 respectively.

The above siRNAs were dissolved to make a stock solution of 50 μm/ml. A day before the transfection, the ICC cells were cultured on 25mm poly-L-Lysine coated coverslip placed in 35 mm culture plates with the growth media supplemented with FBS but without antibiotics to get the 50-60% confluent at the transfection. siRNA were mixed with Lipofectamine™ RNAiMAX (Invitrogen) according to manufacturer's instruction to make the final concentration of 20 nM. This siRNA – RNAiMax mixture is then added to the cells having the media without serum and antibiotic followed by incubating them for 6 hrs at 37^{o}C in CO_2 incubator. The media was then changed with the fresh one containing serum and further incubated. These cells were then patch clamped after 24 hrs, 30 hrs and 40 hrs.

3. Drugs and Chemicals

8-Bromoadenosine 3′,5′-cyclic monophosphate, Rolipram, SQ 22536, 2′,5′-Dideoxyadenosine, Zatebradine hydrochloride, ZD7288 hydrate, Cesium Chloride, (-)-*cis*-Diltiazem hydrochloride, TTX, Nifedipine, Nicardipine, 8-(4-Chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate monosodium hydrate, KT 5720.

4.RESULTS

4.1 Pacemaker Potential and Current Generated by ICC Present in Colon

There have been lots of studies done on the slow waves of the colon tissue and different patterns of the current have been identified from the circular muscle and the longitudinal muscle. Even though ICC are present in the colon tissue, not much work have been done compared to the ICC of small intestine. The possible reason could be difficulty in culturing the cells and also could be possible that the size of the colon from the 3-5 days old mice are too small to differentiated into proximal colon, mid colon and the distal colon.

In our cultured cells from the colon, we also had the mixture of the ICC from all the different locations of the colon as the explant from the colon was hardly 1cm in length so it was impossible to distinguish and culture them separately.

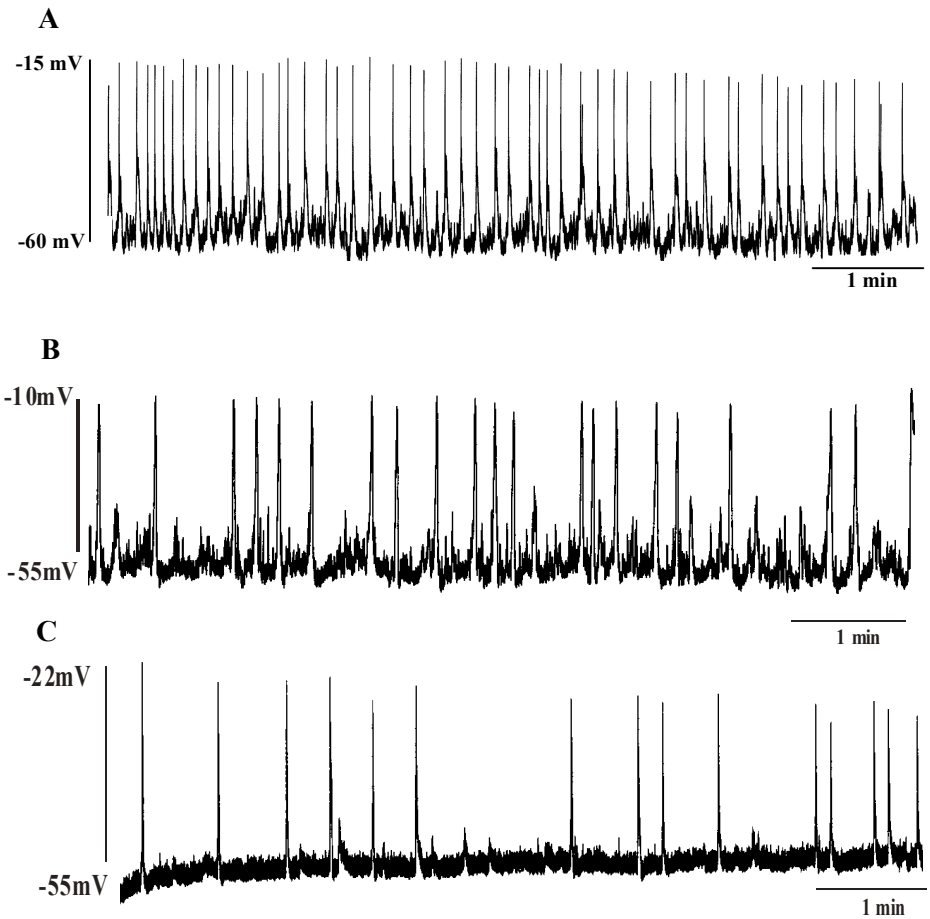


Fig.5. Different patterns of membrane potentials recorded from the ICC of Murine colon. (A) Membrane potential with high frequency (B) Membrane potential showing the moderate frequency and (C) the frequency with the least no of cycles min^{-1}.

After identifying the ICC under the microscope and making the whole cell patch, three different pacemaker potential patterns were observed. The amplitude of all the patterns were almost same ranging from 35 - 45 mV but differed in the frequency cycles. Some patterns have very high frequency with 6 ± 2 cycles min^{-1}, some with frequency 4 ± 2 cycles min^{-1} and others with 2 ± 1 cycles min^{-1}. We believe the ICC showing high frequency may belong to the proximal colon, with moderate cycles to the mid colon and the ICC's showing least frequency from distal colon. The resting membrane potentials were ranged between -60 ± 5 mV.

They differed with the resting membrane potential and the frequency generated by the ICC of the small intestine where the resting membrane potential were found to be -70 ± 3 mV and the frequency 14 ± 2 cycles min^{-1} but there was no significant difference in the amplitude being 30 ± 7.59 mV.

Under voltage clamp (holding potential -70 mV), ICC from the colon generated the spontaneous inward current alike small intestine which is also termed as "pacemaker current". The resting current level of the colon is almost same like of small intestine with 122 ± 20 pA but differed with the amplitude and the frequency.

Fig.6. Pacemaker current generated by the murine colonic ICC under the voltage clamp of -70 mV. The current generated has the amplitude of above 1200 pA with frequency 2 cycles min^{-1}.

The amplitude of the pacemaker current from the colon ICC were found to be twice or thrice fold bigger than that of small intestine with the values 1200 ± 300 pA compared to 400 ± 50 pA of small intestine. The frequency of colon pacemaker current was 2.9 ± 2.62 cycles min^{-1} compared to 14 ± 2 cycles min^{-1} of small intestine.

4.2 Elimination of Neural and Smooth Muscle Cells Electrical Activity

The culture dish used to record the membrane potential by patching contains both smooth muscle cells and the neural cells in it. These cells are interconnected to each other and also the currents are propagated to smooth muscle via gap junction. The pacemaker potential generated by the single cell is not robust so the recordings are done from the ICC which are a part of network and connected to the small clump of cells.

While patching some time the neural cells or the smooth muscle cells may get patched and as they also generate the spontaneous current, it is important to eliminate them.

The neuron cells were determined by the use of tetrodotoxin (TTX). Neurons on the culture dish are rich of TTX sensitive sodium channels. By treatment of the TTX to the dish, active conductance of the enteric neurons was blocked. After the treatment of the TTX 1 μM in the culture dish, the pacemaker potential was not affected and still generated the pacemaker potential. Under control conditions, the frequency of pacemaker currents was 7.89 ± 2.5 cycles $5min^{-1}$, and membrane potential and pacemaker potential amplitudes were -58.68 ± 2.47 mV and 41.4 ± 7.98 mV, respectively. After treatment of the TTX, the value of resting membrane potential, frequency and amplitude remained close to control conditions at -56.74 ± 4.74 mV, 8.12 ± 3.56 cycles $5min^{-1}$ and 44.37 ± 7.9 mV respectively (Fig.7).

It is important to eliminate the slow waves produced by the smooth muscle because after the elimination of neural activity, the slow waves from smooth muscle cells may be present. This can be differentiated either by viewing the plateau phase of the pacemaker potential or the treatment of the L-type Ca^{2+} channel blocker.

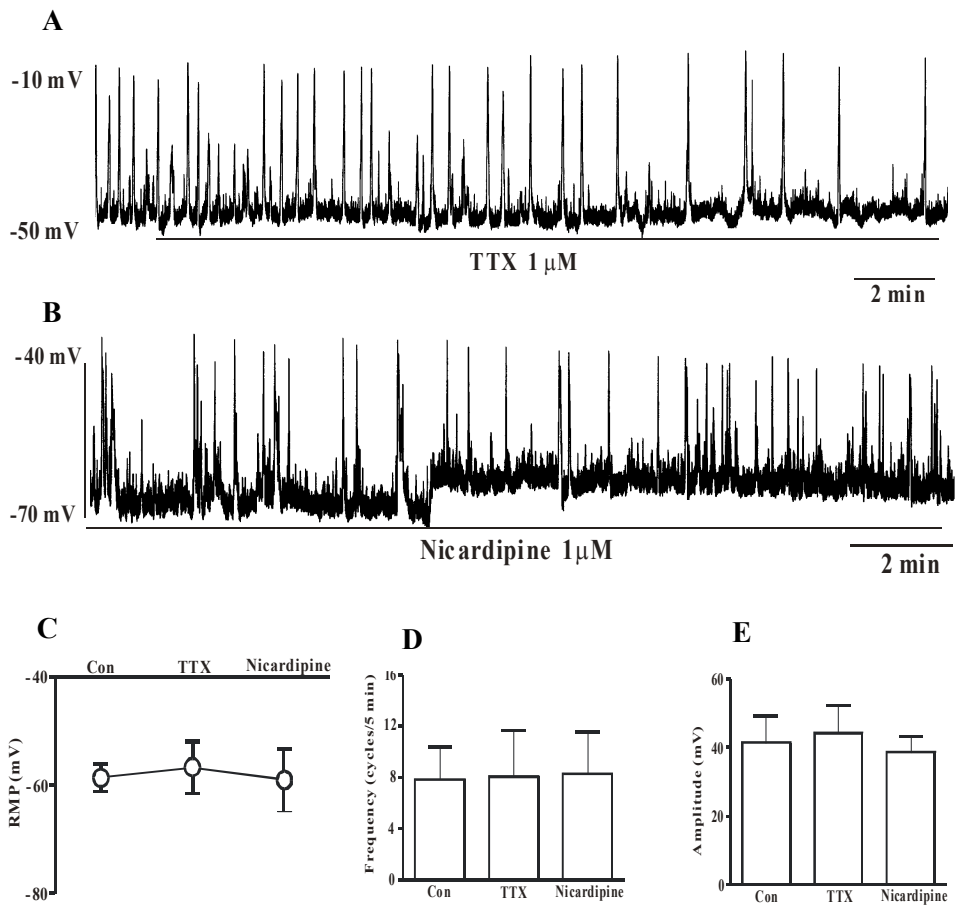


Fig. 7. Spontaneous membrane potential oscillations were recorded from colon ICC in the presence of (A) TTX and (B) nicardipine (1 μM). (C) Graphical representation of the change in resting membrane potential after their treatment. The histogram summarization shows the change in (D) frequency and (E) in amplitude after the treatment of TTX and nicardipine.

During the slow depolarization, voltage dependent L-type Ca^{2+} channels are opened in the smooth muscle cells, which lead to generation of calcium action potentials if threshold is achieved. So to eliminate the slow waves from smooth muscle cells, 1 μM nicardipine, blocker of L-type Ca^{2+} channel, was used. There was no blockage of the pacemaker potential even after treatment of nicardipine for the longer time (Fig.7B). This proved that the patching was done on the ICC and the pacemaker potential is solely generated by them. The resting membrane potential, frequency and amplitude values obtained are -59.08 ± 5.75 mV, 8.35 ± 3.25 cycles $5min^{-1}$and 38.66 ± 4.84 mV respectively (Fig. 7 C, D and E).

4.3 Effects of Cyclic Nucleotide on Pacemaker Potential

4.3.1 Action of Cyclic AMP on Pacemaker Potential.

The action of cyclic nucleotides had been accessed in the pacemaker potential generated by colonic ICC. In our previous investigations, we have reported that in small intestine, 8-bromo cAMP had no role in the pacemaker current under voltage clamp at -70 mV.

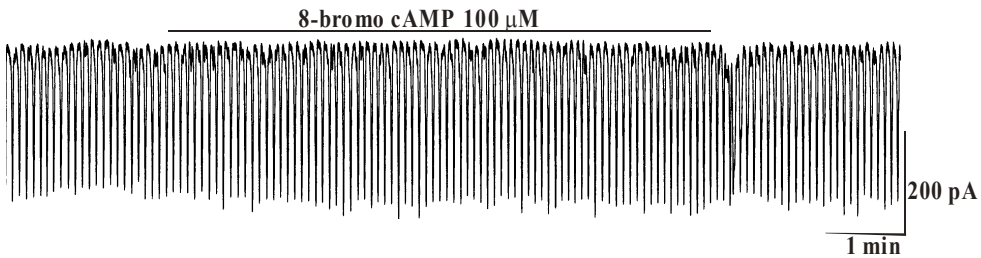


Fig. 8. Spontaneous inward currents were more robust and regular when recorded from ICC in networks from the small intestine. Cell was held at −70 mV. 8-bromo-cAMP was treated to the cells at the concentration of 100 μM.

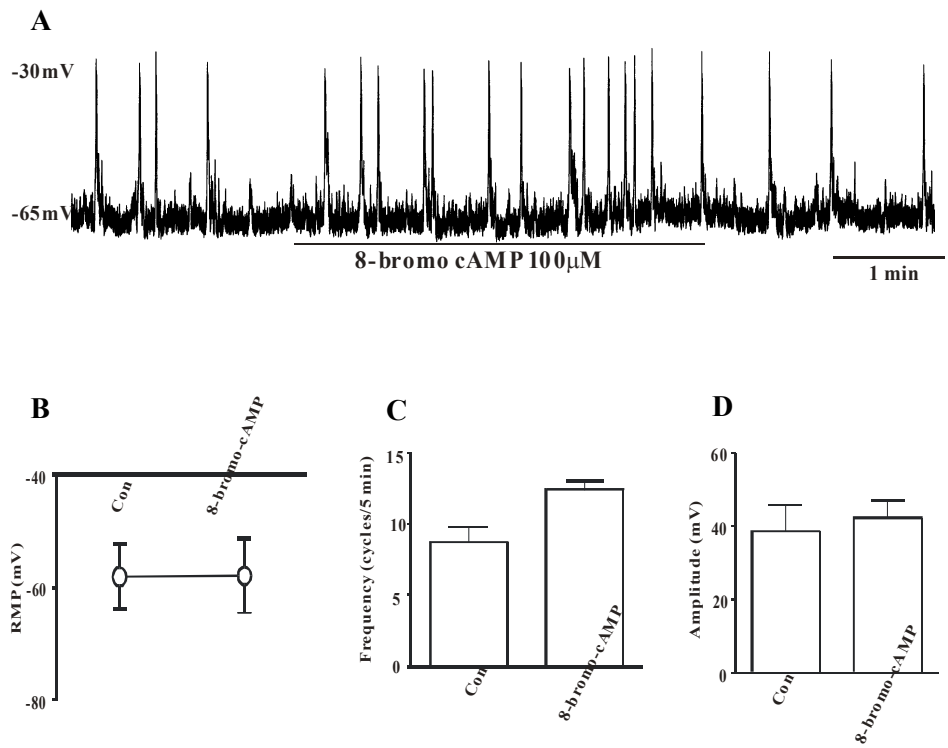


Fig. 9. Effect of cAMP on the spontaneous pacemaker potential. Under current clamp mode (*I=0*) (A) Addition of 100 μM 8-bromo-cAMP increased the frequency of the pacemaker potential. Changes in the resting membrane potentials were represented in (B), (C) and (D) are the bar graphic representation of changes in frequency and amplitude respectively after the treatment of 8-bromo-cAMP.

We found the pacemaker potential generated by colon ICC is completely different from the pacemaker potential generated by the small intestine in terms of current pattern and the frequency. So here we checked if the cyclic nucleotide modulated the pacemaker potential generated by colonic ICC.

First, we assessed the effect of 8-bromo cAMP at the concentration of 100 μM to the pacemaker potential of colonic ICC. There was no significant difference in the resting membrane potential compared to the control condition but had the significant change in the number of frequency generated. After the treatment of 100 μM 8-bromo-cAMP, the frequency changed from 8.68 ± 1.12 cycles $5min^{-1}$under control condition to 12.39 ± 0.57 cycles $5min^{-1}$. Under wash out, the frequency was returned to the normal state. The resting membrane potential (-58.68 ± 2.47 mV) and amplitude (36.61 ± 7.16 mV) did not changed from control and had the value of -57.9 ± 6.66 mV and 42.38 ± 4.61 mV respectively.

This proves that cAMP has action in the pacemaker frequency which was absent in small intestine.

4.3.2 Role of Phosphodiesterase Enzyme Inhibitors.

The phosphodiesterase (PDE) is an enzyme which breaks the phosphodiester bond. The cyclic nucleotide phosphodiesterases comprise a group of enzymes that degrade the phosphodiester bond in the second messenger molecules cAMP and cGMP. Inhibitors of PDE can prolong or enhance the effects of physiological processes mediated by cAMP by inhibition of their degradation by PDE.

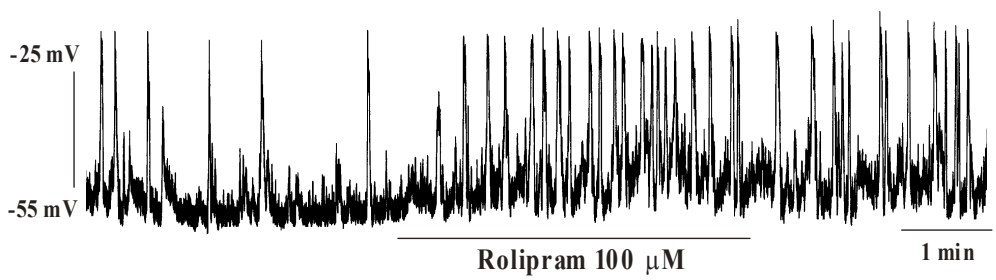


Fig. 10.1. Effect of the drugs to block the phosphodiesterase IV enzyme activity. Selective cAMP-specific phosphodiesterase (PDE4) inhibitor, Rolipram 100 μM, was treated on pacemaker potential under current clamp.

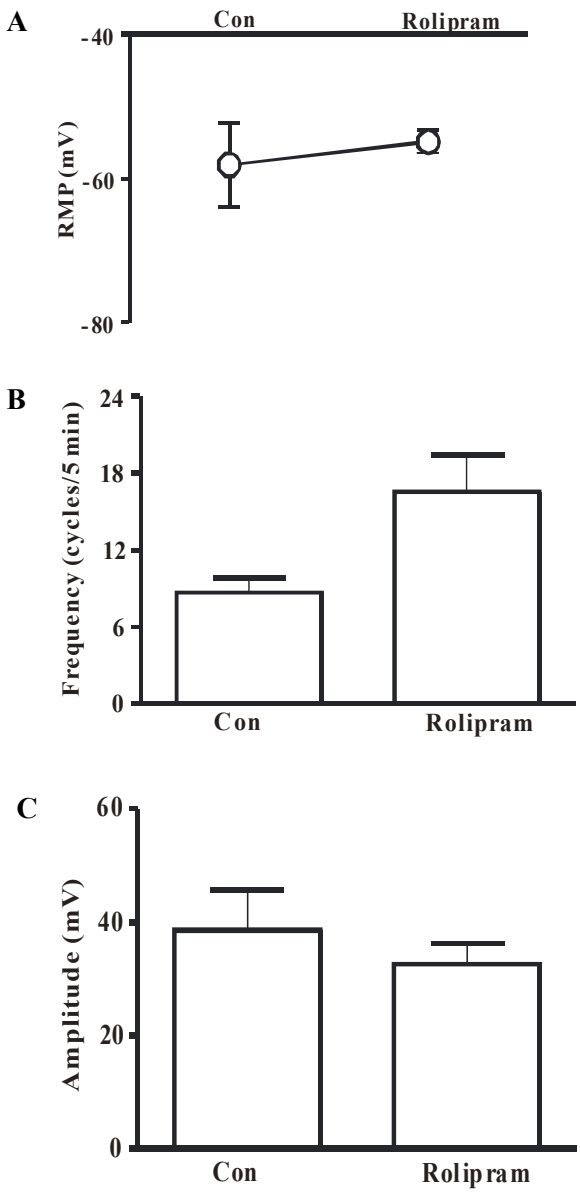


Fig. 10.2. Summarized data of the phosphodiesterase IV inhibitors. Changes in the resting membrane potential are summarized in (A). (B) and (C) are the summarized data of frequency and amplitude respectively in response to rolipram.

Rolipram, selective cAMP-specific phosphodiesterase (PDE4) inhibitor was used at the concentration of 100 μM to the pacemaker potential of the colon ICC. It has been reported that upon treatment of the PDE inhibitors, the intracellular concentration of cyclic nucleotide increases up to 7 folds.

After prolong treatment of Rolipram 100 μM, the frequency of the pacemaker potential increased from 8.68 ± 1.12 cycles $5min^{-1}$under control condition to 16.5 ± 2.94 cycles $5min^{-1}$ (Fig. 10.1and 10.2 B) but has the minimal effect on the resting membrane potential and amplitude which were changed to -54.85 ± 1.67 mV and 32.56 ± 3.65 mV from -58.08 ± 5.78 mV and 38.61 ± 7.16 mV (Fig. 10.2A and C) in control.

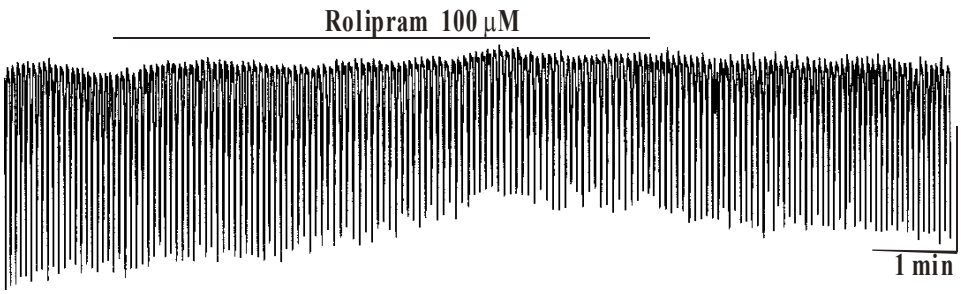


Fig. 11. Effect of the phosphodiesterase IV enzyme inhibitors on pacemaker current generated by small intestine ICC holding at -70 mV. Effect of Rolipram100 μM on pacemaker current.

Rolipram was also treated to the small intestine pacemaker current generated at the cells voltage clamped at -70 mV. Rolipram 100 μM had not adverse effect on the pacemaker current generated by small intestine ICC. There was not any increase in the frequency cycle but little decrement of amplitude size was observed.

4.3.3 Effect of cAMP on Spontaneous Ca^{2+} Puffs Generated by Colonic ICC.

Colonic ICC in the culture dish loaded with fluo-4 AM generated spontaneous intracellular Ca^{2+} transients that occurred either as highly localized events or Ca^{2+} waves. Spontaneous Ca^{2+} transient in colonic ICC are due to Ca^{2+} release from IP_3 receptors and therefore are termed Ca^{2+} puffs.

In order to investigate whether cyclic nucleotides has any role on $[Ca^{2+}]_i$, 8-bromo-cAMP was treated to the culture dish at the concentration of 100 μM.

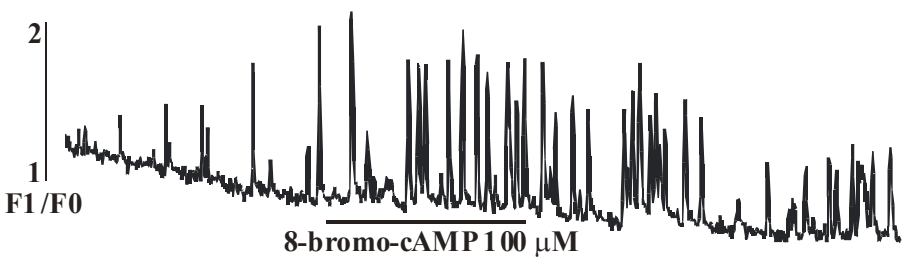


Fig. 12.1. Effect of the 8-bromo-cAMP on the intracellular calcium oscillation.

The Ca^{2+} wave frequency significantly increased after the treatment of 100 μM of cell permeable analogue of cAMP.

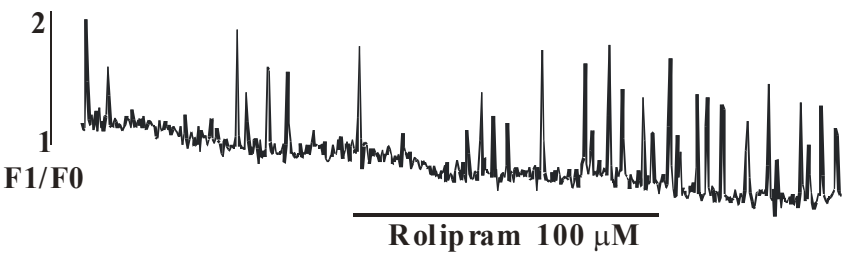


Fig. 13. Effect of phophodiesterase IV enzyme inhibitors in the $[Ca^{2+}]_i$ of the colon ICC. Upon treatment of Rolipram 100 μM, the calcium oscillation frequency increased.

After obtaining the similar effect of cAMP like the electrical recordings, I then checked the effect of the phosphodiesterase IV enzyme inhibitor on the spontaneous Ca^{2+} wave.

Rolipram 100 μM increased the frequency of the $[Ca^{2+}]_i$ oscillation alike the patch clamp recording data indicating that the intracellular cAMP have role in the Ca^{2+} oscillation in the cell which is linked to the generation of the pacemaker potential in ICC.

4.4 Modulation of the Pacemaker Potential Generated in Colonic ICC by cAMP

4.4.1 Action of adenylyl cyclase inhibitors in colonic pacemaker potential

Adenylyl cyclase also known as adenylatecyclase (AC) is a part of G protein signaling cascade which helps to transmit the message from outside of the cell to inside. On activation with the signal, it activates and converts adenosine triphosphate to cAMP. cAMP is known as a second messenger.

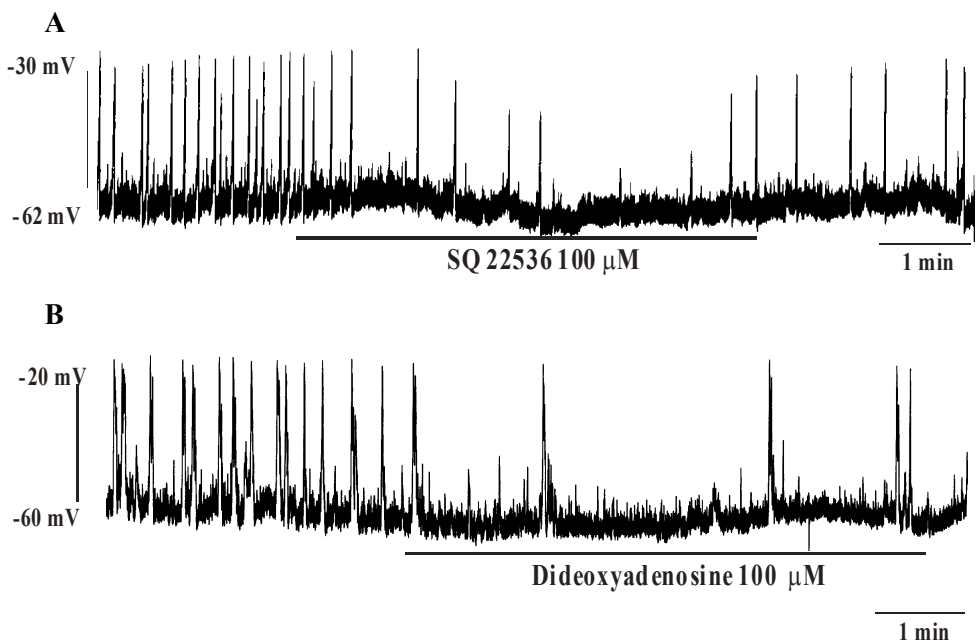


Fig. 14.1a. Membrane currents recorded after the treatment of adenylyl cyclase inhibitors. (A) Upon treatment of SQ 22536 on the spontaneously generated membrane potential, the frequency of the current generation is nearly abolished. (B) Decrement in the frequency of pacemaker potential was observed after the treatment of Dideoxyadenosine 100 μM

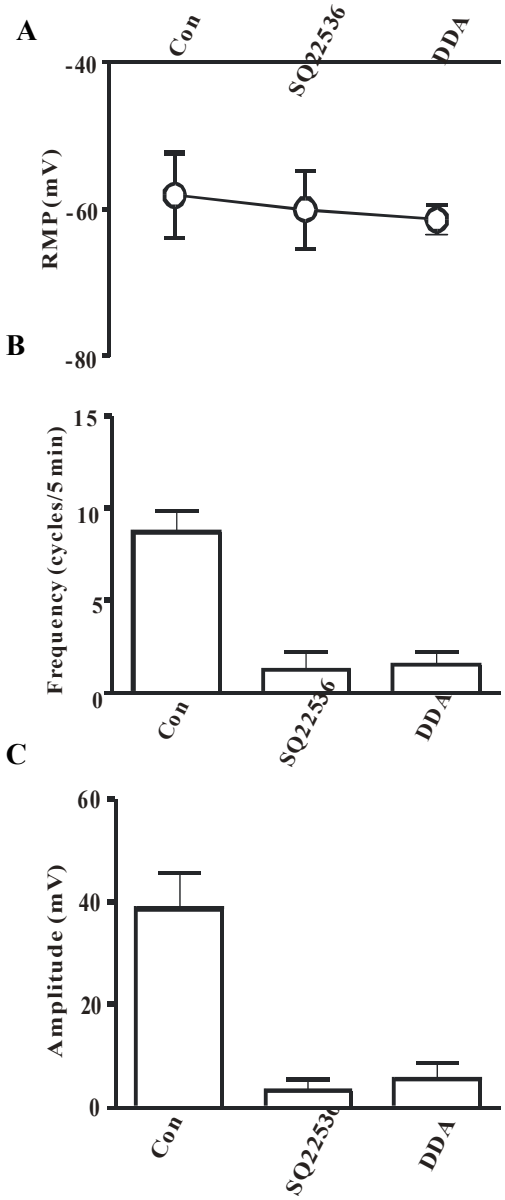


Fig. 14.1b. Statistical analysis of the adenylylcyclase inhibitors. Graph (A) shows the change in the resting membrane potential. Graph (B) and graph (C) are the statistical representation of changes in frequency and amplitude respectively. Statistical significance of the effect of SQ 22536 and dideoxyadenosine are compared with control

In the previous result we have shown that after the treatment of cell permeable cAMP, the frequency of the pacemaker potential was increased and similar effect was seen after the degradation of cAMP was blocked by inhibiting the PDE enzyme. These results proved that cAMP modulates the pacemaker potential. But these results do not signify if cAMP has any role in the generation of the pacemaker potential.

To assess the role of the cAMP in the generation of the pacemaker potential, we the cells with cell permeable adenylyl cyclase inhibitor SQ 22536. After the inhibition of the enzyme the intracellular cAMP is decreased as no further ATP is converted to cAMP.

Upon treatment of SQ 22536 100 μM (Fig. 14.1aA, it significantly decreased the frequency and the amplitude of the pacemaker potential and almost inhibited it completely. The frequency decreased to the value of 1.25 ± 0.95 cycles $5min^{-1}$and amplitude to 3.22 ± 2.20 mV compared to the control value of 8.68 ± 1.12 cycles $5min^{-1}$ and 36.61 ± 7.16 mV respectively (Fig. 14.1b Band C)

This was further confirmed by the use of 2′,5′-Dideoxyadenosine (DDA), another type of cell-permeable adenylyl cyclase inhibitor. Identical results were obtained after the treatment of 100 μM dideoxyadenosine (Fig. 14.1a B) which decreased the pacemaker potential frequency significantly. Compared to the control frequency and amplitude of 8.68 ± 1.12 cycles $5min^{-1}$ and 36.61 ± 7.16 mV, they decreased to 1.5 ± 0.7 cycles $5min^{-1}$ and amplitude to 5.43 ± 3 mV respectively after the treatment of DDA (Fig. 14.1b B and C).

There was no significant difference in the resting membrane potential after the treatment of SQ 22536 (-59.4 ± 5.2 mV) and DDA (-63.65 ± 3.35 mV) compared to control value of -58.08 ± 5.75 mV (Fig. 14.1b A).

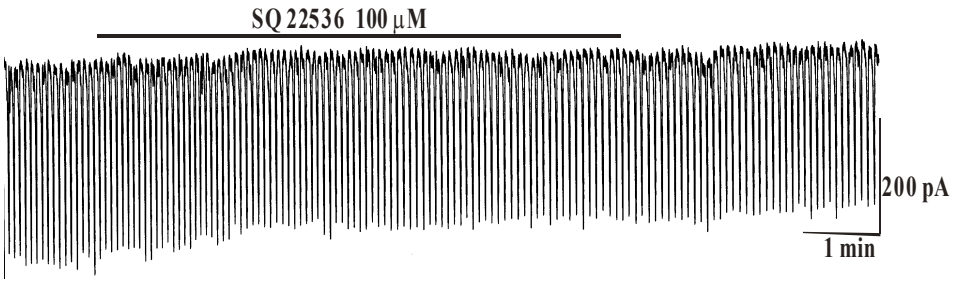


Fig. 14.2. Effect of the adenylylcyclase inhibitor in pacemaker current generated by small intestine ICC. Adenylylcyclase inhibitor, SQ 22536 100 μM, has no effect on the ICC of small intestine generating the spontaneous current holding at -70 mV.

When the same concentration of SQ 22536 was treated to the pacemaker current generated by the small intestine, it showed no effect signifying that cAMP has no role in the generation of pacemaker current in the small intestine but in case of colon, it plays an important role during the generation of pacemaker potential.

4.4.2 Inhibition of [Ca^{2+}]$_i$ by Adenylyl Cyclase Inhibitor

It has been observed that after inhibition of the adenylyl cyclase enzyme, the pacemaker potential generated by colonic ICC is abolished indication the role of cAMP for their generation. Similarly the external application of the cAMP also increased the frequency in both pacemaker potential as well as Ca^{2+} wave. For further verification of the result, we applied the adenylylcyclase inhibitor, SQ 22356 100 μM, to the Ca^{2+} wave generating cell clusters of colonic ICC.

Upon prolonged treatment of the inhibitor, it inhibited the generation of spontaneous calcium wave.

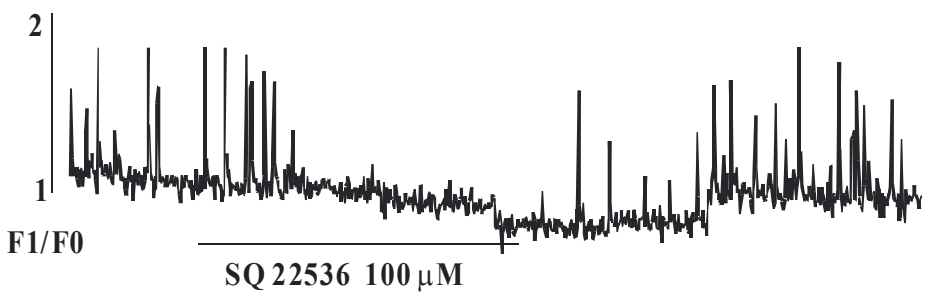


Fig. 14.3. Measurement of [Ca^{2+}]$_i$ in the presence of adenylyl cyclase inhibitor. Typical intracellular calcium oscillation producing the periodic Ca^{2+} spikes are abolished in the presence of SQ 22536 (100 μM)

4.4.3 Effect of the Protein Kinase A (PKA) inhibitor and Epac agonist.

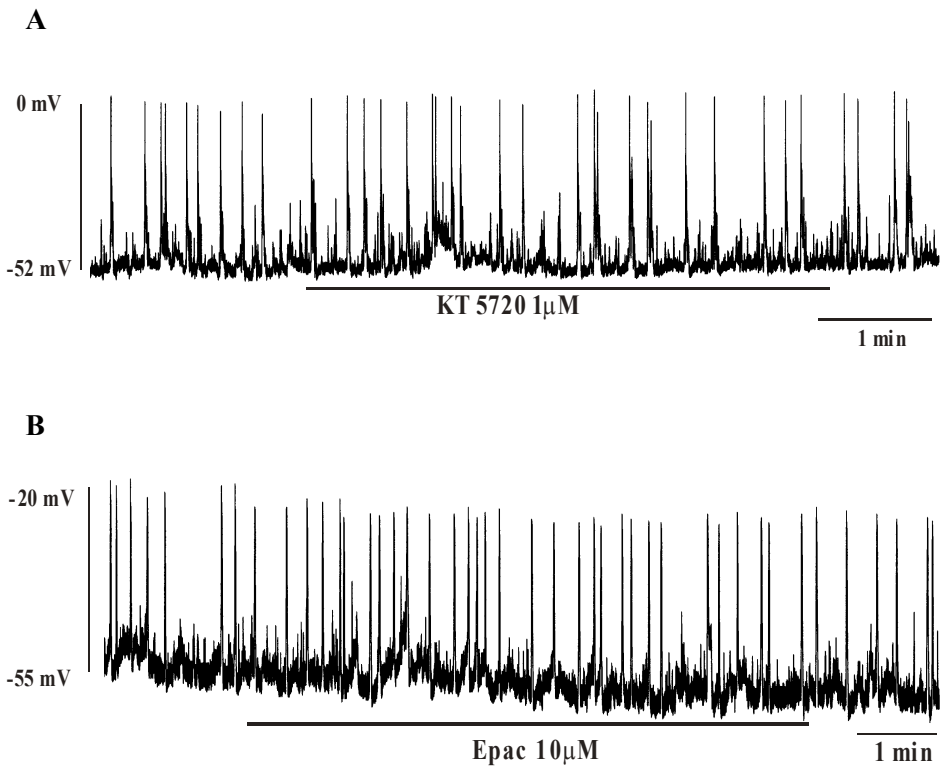


Fig. 14.4a. Effect of Protein Kinase A (PKA) inhibitor and Epac on pacemaker potential generated by colonic ICC in current clamp (*I=0*) mode. (A) KT5720, a specific PKA inhibitor, when treated to colonic pacemaker potential generating cell at the concentration of 1 μM had no effect on both frequency and the resting membrane potential. (B) Similarly treatment of Epac agonist 10 μM showed no effect.

A

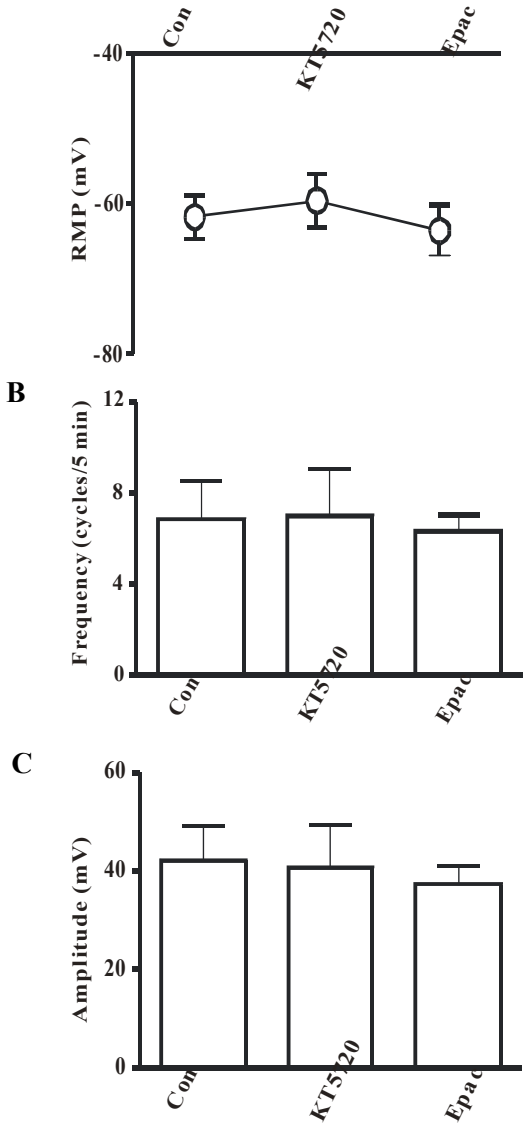


Fig 14.4b. Graphs (A), (B) and (C) show the statistical analysis of the PKA inhibitor and the Epac agonist on the resting membrane potential, frequency and amplitude respectively.

Protein Kinases are key regulators of cell function that constitute one of the largest and most functionally diverse gene families. Protein kinase A (also known as the cyclic AMP-dependent protein kinase or A kinase) is an enzyme that covalently decorates proteins with phosphate groups. The unique characteristic of protein kinase A is that its activity is regulated by fluctuating levels of cAMP within cells. Intracellular concentration of cAMP provides the most fundamental control over activity of protein kinase A. The exchange factor directly activated by cAMP (Epac) is a newly discovered direct target for cAMP.

In this step, we tried to check if the action of cAMP in the pacemaker potential is through the activation of the PKA and also investigated if the modulation of cAMP is through Epac or not. To verify this we treated KT 5720, specific, cell-permeable inhibitor of protein kinase A (PKA) and Epac agonist. After the treatment of both KT 5720 and Epac, the pacemaker potential was analyzed. There was not any change in the pacemaker potential even after the prolong treatment of the both PKA inhibitor and Epac agonist. The resting membrane potential, frequency and the amplitude -61.81 ± 2.92 mV, 8.58 ± 2.1 cycles $5min^{-1}$ and 42.13 ± 6.91 mV in control condition remained nearly constant after the treatment of KT 5720 at -59.65 ± 3.46 mV, 8.76 ± 2.6 cycles $5min^{-1}$ and 40.65 ± 8.69 mV and Epac agonist -63.65 ± 3.37 mV, 7.92 ± 0.89 cycles $5min^{-1}$ and 37.25 ± 3.82 mV.

Had there been the activation of PKA by cAMP, the pacemaker potential frequency should have been decreased just like after the treatment of the adenylyl cyclase inhibitor. These results signify that the modulation of pacemaker potential by cAMP is independent of PKA and Epac.

4.5 No Involvement of Cyclic Nucleotide Gated (CNG) Channel

Cyclic nucleotide-gated (CNG) channels are nonselective cation channels first identified in retinal photoreceptors and olfactory sensory neurons. They are opened by the direct binding of cyclic nucleotides, cAMP and cGMP. Cyclic nucleotides directly activate CNG channels by binding to a site on the channel protein.

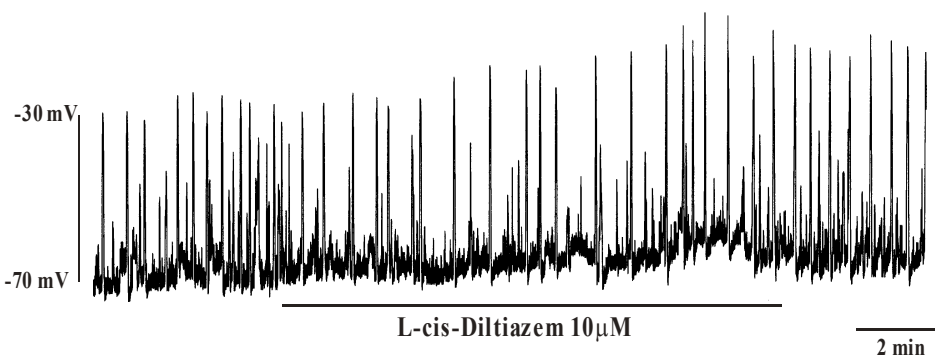


Fig. 15.1a. Effect of inhibitor of cyclic nucleotide gated (CNG) channel. The trace shows that the treatment of L-cis-Diltiazem has no effect on the pacemaker potential of the colonic ICC.

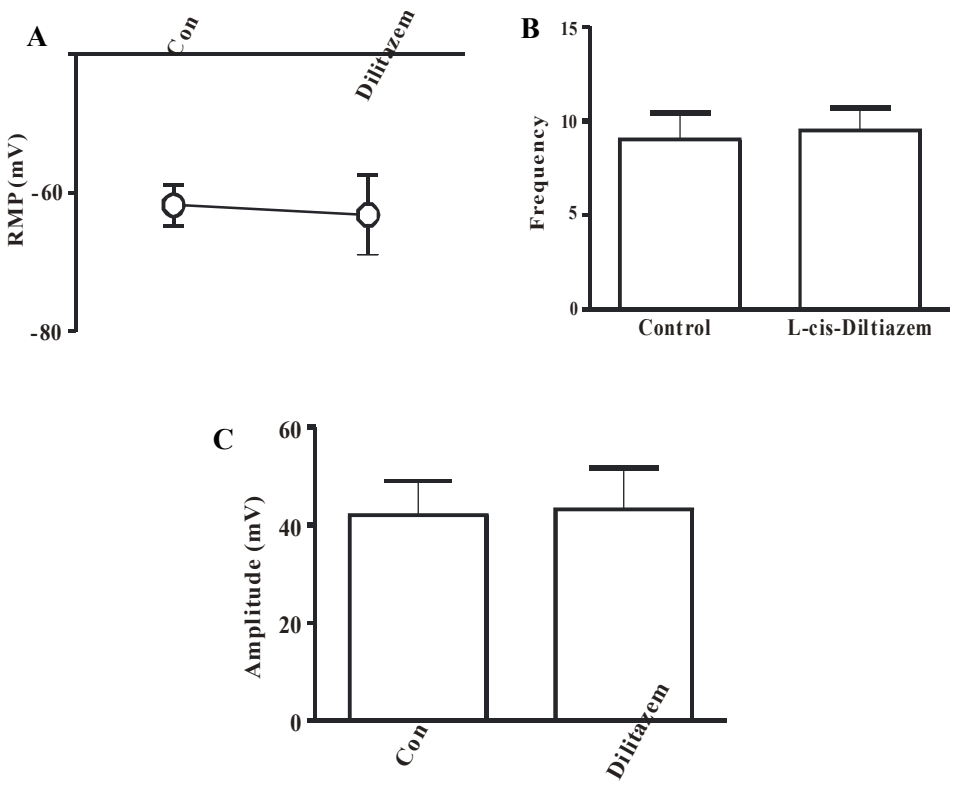


Fig. 15.1b. The changes in the resting membrane potential, frequency and the amplitude after the treatment of L-cis-Diltiazem are graphically represented in (A), (B) and (C) respectively.

As our previous studies clarifies that that action of cAMP on the pacemaker potential was not mediated through the activation of PKA. So we hypothesized that ICC of the colon may possess CNG channel where these intracellular cAMP bind to them and mediate the action. To verify this, the cells in the culture were exposed to the L-cis-Diltiazem 10 μM, a potent CNG channel blocker, to the pacemaker potential recorded in current clamp (*I=0*). L-cis–Diltiazem failed to show any action on the pacemaker potential generated by colonic ICC. There was no

observation of any change in the resting membrane potential and the frequency. The value of the resting membrane potential, frequency and the amplitude in the control were -61.81 ± 2.92 mV, 8.58 ± 2.1 cycles $5min^{-1}$ and 42.13 ± 6.91 mV which were observed -63.22 ± 5.82 mV, 8.35 ± 0.95 cycles $5min^{-1}$ and 43.22 ± 8.43 mV respectively after the treatment of L-cis-diltiazem (Fig. 15.1a and 15.1b A, B, C).This result proved that the cAMP mediated action was not mediated through CNG channel.

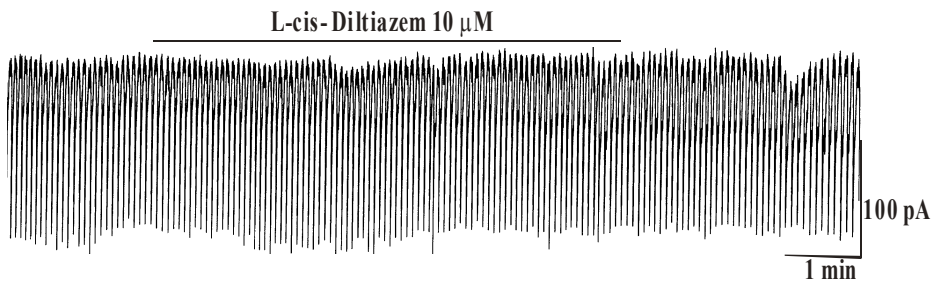


Fig. 15.2. Effect of L-cis-diltiazem, a CNG channel blocker, on the pacemaker current generated by small intestine ICC at a holding potential of -70 mV.

Even in the pacemaker current recorded in voltage clamp at -70mV from the small intestine, there was no effect of L-cis-diltiazem. This shows the possibility that ICC do not express the CNG channel both in small intestine ICC and the colonic ICC.

4.6 Identification of Hyperpolarization-activated Cation (HCN) Channels in Colonic ICC

The above results showed that there is the role of cAMP in the pacemaking mechanism of the colonic ICC. But our results showed that cAMP failed to activate PKA for its action and also ruled out the possibility of the HCN channel expression on them as the CNG channel blocker has no role in the pacemaker potential.

Many experiments, after thorough investigations, reported that HCN channels contribute to the spontaneous rhythmic activity on both the heart and the brain. It has also been proved that cAMP modulated the HCN channels by binding specifically to the channel protein. We hypothesized that there could be the possibility of cAMP binding to the HCN channels thereby modulating the channel and helping for the rhythmic generation of the pacemaker potential in the colonic ICC. cAMP is well known to modulate the HCN channel facilitating the channel opening.

To test this hypothesis we exposed the cells generating the membrane potential to different non specific HCN channel blockers

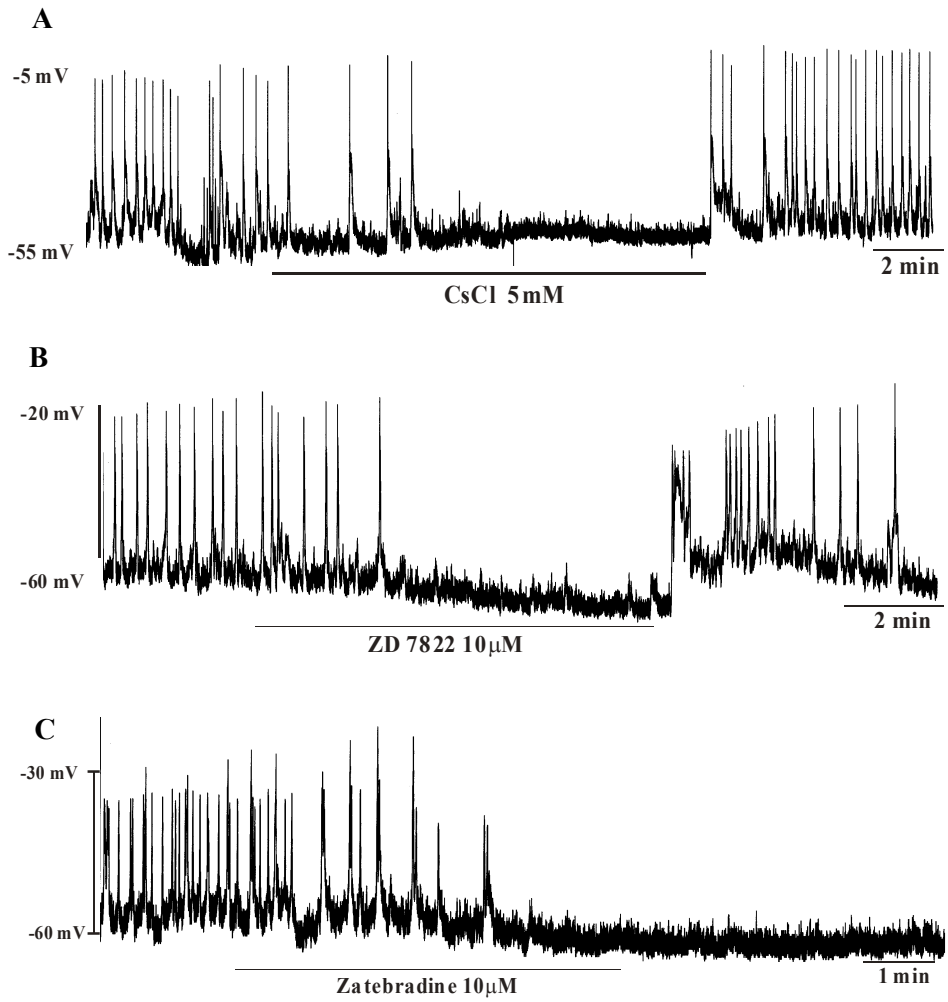


Fig. 16.1a. Effect of different types of blockers knows to block the HCN channels. (A) Cesium Chloride, a well known for blocking the HCN channels, when treated at 5 mM blocked the membrane potential generation. (B) ZD 7822 (10 μM), potent HCN channel blocker, also inhibited the generation of spontaneous membrane potential generation. (C) Abolition of membrane potential was also observed upon treatment of Zatebradine 10 μM.

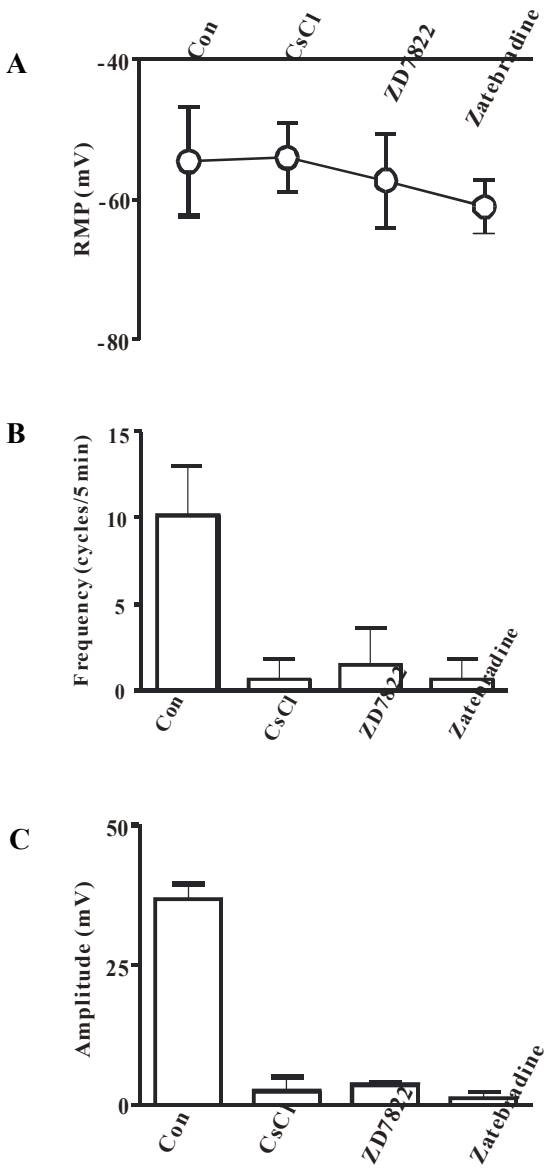


Fig. 16.1b. Statistical analysis of the HCN blockers on spontaneous membrane potential. Graphical representation on change of resting membrane potential (A), changes in the number of frequency (B) and changes in the amplitude (C) after the treatment of the HCN blockers.

4.6.1 Effect of HCN Channel Blockers on Pacemaker Potential Generated by Colonic ICC

It is well documented that Cesium chloride (CsCl) at higher concentration is known to block the HCN channel. The pacemaker potential was recorded from the cultured ICC from the colon in current clamp (*I=0*). To this potential, CsCl at the concentration was treated. 5 mM CsCl attenuated the spontaneously generated pacemaker potential. The frequency and amplitude decreased from 10.13 ± 2.87 cycles $5min^{-1}$ and 36.65 ± 2.75 mV to 0.66 ± 1.15 cycles $5min^{-1}$ and 2.43 ± 2.55 mV respectively. There was no change in the value resting membrane potential (-53.96 ± 4.96 mV) from control (-54.51 ± 7.76 mV) (Fig. 16.1aA and 16.1b A, B, C)

To confirm the CsCl induced inhibition of the pacemaker potential generated by the ICC is due to the blockade of the HCN channels, we observed the effects of ZD 7288 and Zatebradine, a more selective HCN channel blocker, on the pacemaker potential. Indeed, both the blockers mimicked the effect of CsCl on pacemaker potential (Fig. 16.1a B and C). Upon treatment of the ZD 7288, the frequency and amplitude of 10.13 ± 2.87 cycles $5min^{-1}$ and 36.65 ± 2.75 mV at control condition decreased to 1.5 ± 2.12 cycles $5min^{-1}$ and 3.55 ± 0.63 mV and similarly the pacemaker frequency rate and amplitude decreased to 0.66 ± 1.15 cycles $5min^{-1}$ and 1.2 ± 10.5 mV when treated with Zatebradine (Fig. 16.1b A, B, C).

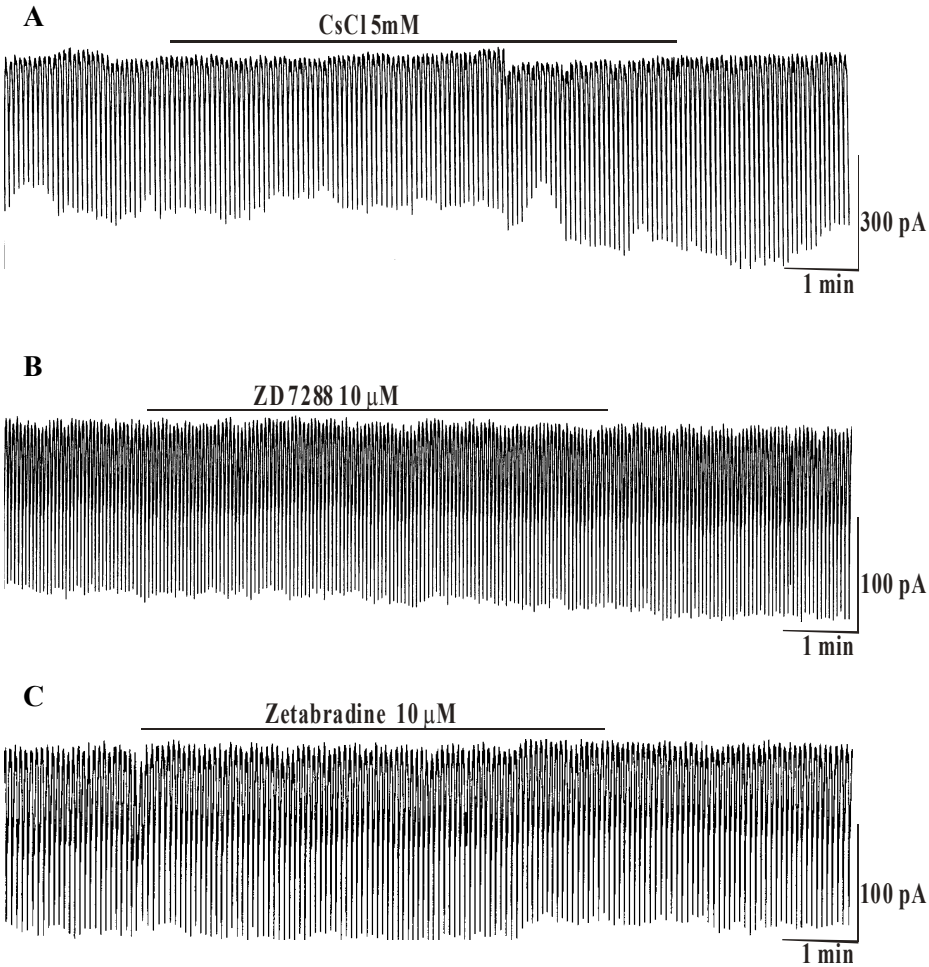


Fig. 16.2. Treatment of the HCN channel blockers on the spontaneous pacemaker currents generated by ICC from small intestine at a holding potential of -70 mV. All HCN channel blockers (A) CsCl 5 mM, (B) ZD 7288 10 μM and (C) Zatebradine 10 μM failed to show any action on the pacemaker current from the small intestine ICC.

These HCN channel blockers were also used to the pacemaker currents generated by ICC from small intestine after patching at the holding potential of -70 mV. All three HCN channel blockers namely 5 mM CsCl, 10 μM ZD7288 and 10 μM zatebradine had no effect on the pacemaker current.

This signifies that that the generations of pacemaker potential in colon is regulated by HCN channels and are regulated by cAMP which is completely different in small intestine.

4.6.2 Effect of HCN Channel Blocker in Intracellular Ca^{2+}Oscillation

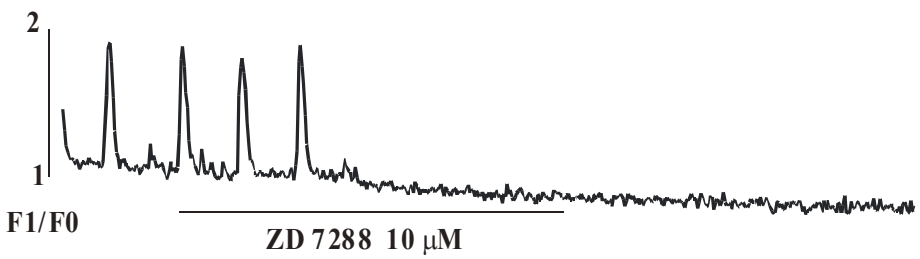


Fig 16.3. Effects of ZD 7288 10 μM, specific HCN channel blocker, on intracellular Ca^{2+} oscillation in cultured ICC from mouse colon.

As many reports suggested that $[Ca^{2+}]_i$ oscillations in ICC were considered to be the primary mechanism for the pacemaker activity in gastrointestinal activity, I examined the effects of ZD 7288 on $[Ca^{2+}]_i$ oscillations in ICC. In this study, I measured the spontaneous $[Ca^{2+}]_i$ oscillations of ICC which were connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICC loaded with fluo-4. Fig.16.3 shows images of basal (F0) and peak point (F1/F0) of Ca^{2+} oscillations. In the presence of 10 μM ZD 7288, $[Ca^{2+}]_i$ oscillations in ICC declined rapidly.

4.6.3 Expression of the HCN Subtypes in Colon ICC.

The results we got till now showed that HCN channel plays the role in the modulation of pacemaker potential mediated by cAMP. The HCN channel family comprises four members (HCN1–HCN4) that share approximately 60% sequence identity to each other. There is evidence that HCN subunits can co-assemble to form heteromers. HCN channels are found in neurons and heart cells.

We examined the expression of the HCN subtypes in the total mRNA extracted from the cells cultured from the whole tissue and from the total mRNA isolated from the single ICC cells by using RT-PCR.

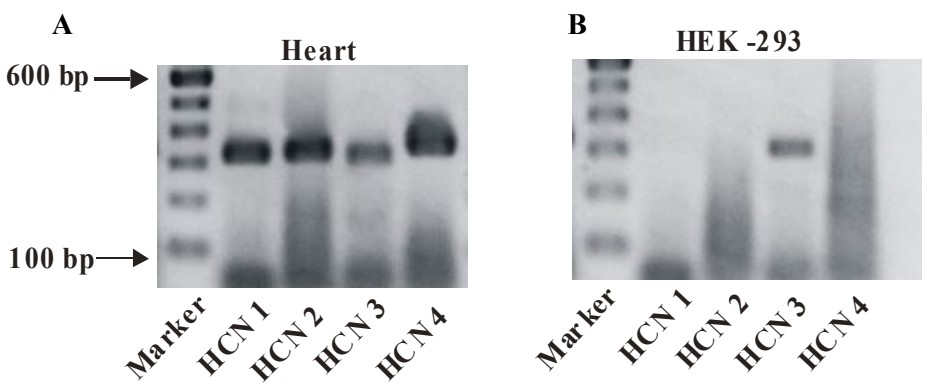


Fig.17.1. Detection by RT-PCR of mRNA encoding for HCN channels in (A) heart as the positive control and (B) in HEK-293 cell line as the negative control.

The RNA extracted from the heart of the mouse was used as the positive control and the RNA extracted from the HEK-293 cell line was used as the negative control.

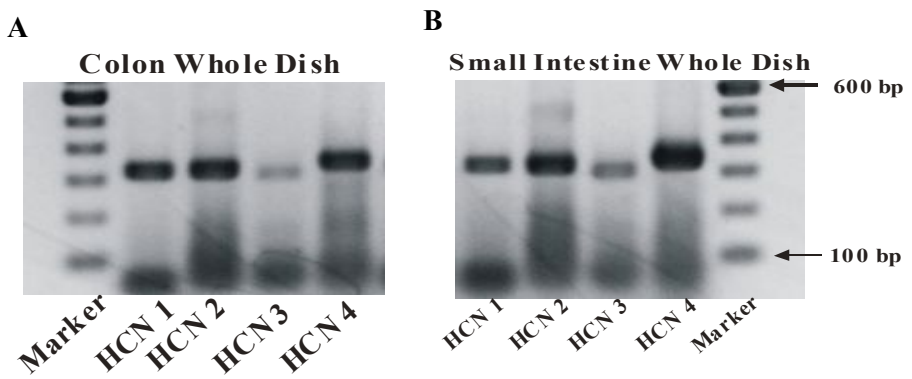


Fig 17.2. Gel electrophoresis of the RT-PCR products using primers for 4 types of HCN. (A) and (B) shows the product of RT-PCR for the HCN channels from the whole dish of cultured colon and small intestine respectively.

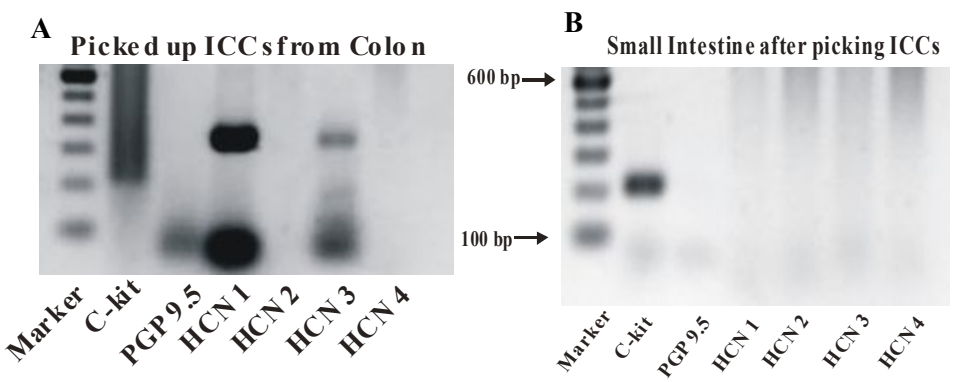


Fig 17.3. Gel electrophoresis of the single cell RT-PCR products using primers for 4 types of HCN channels and the control for c-Kit and PGP 9.5 (A) and (B) shows the product of RT-PCR for the HCN channels after picking up the single cell from the cultured colon and small intestine dish respectively. Lane 2 and 3 of both the gel are used for c-kit and PGP 9.5 and the remaining lanes from 4 -7 are used for HCN1 -4.

Of the 4 subtypes of HCN channel examined, mRNA for all 4 subtypes were expressed in the heart which is used as the positive control. Only HCN3 subtype was used in the HEK -293 cell line which was used as the negative control.

When RT-PCR performed to the total RNA extracted from the cells cultured from the whole tissue, all 4 subtype of HCN channel were detected. Similar result was observed in the PCR product from the whole cell of the small intestine. This could possibly because of the enteric neurons present in the whole tissue culture dish.

Total RNA extracted from the single ICC from the colon was then used for reverse transcription followed by the amplification step using the primers for 4 different subtypes of HCN channel. Primers for the neuron marker, PGP 9.5, and the c-kit marker were also used in the RNA isolated from the single cell. The ICC from the colon expressed the mRNA encoding for HCN1 and HCN3. The neuron marker PGP 9.5 did not get expressed in the gel running image implying that there were not enteric neurons present in the sample. Furthermore the ICC market, c-kit mRNA was expressed.

When performed the single cell RT-PCR in the small intestine ICC, the purified mRNA expressed mRNA encoding for c-kit but I found no evidence of mRNA encoding for the HCN channel subtypes.

4.6.4 RNA Interference Confirms Role of HCN Channels for the Generation of Pacemaker Potential

We next evaluated whether HCN1 and HCN3 is the target for cAMP binding and modulating the generation of pacemaker potential. Custom synthesized BioRP purified siRNA directed against HCN1 and HCN3 mRNA sequences were used. Mock transfection is performed for the control.

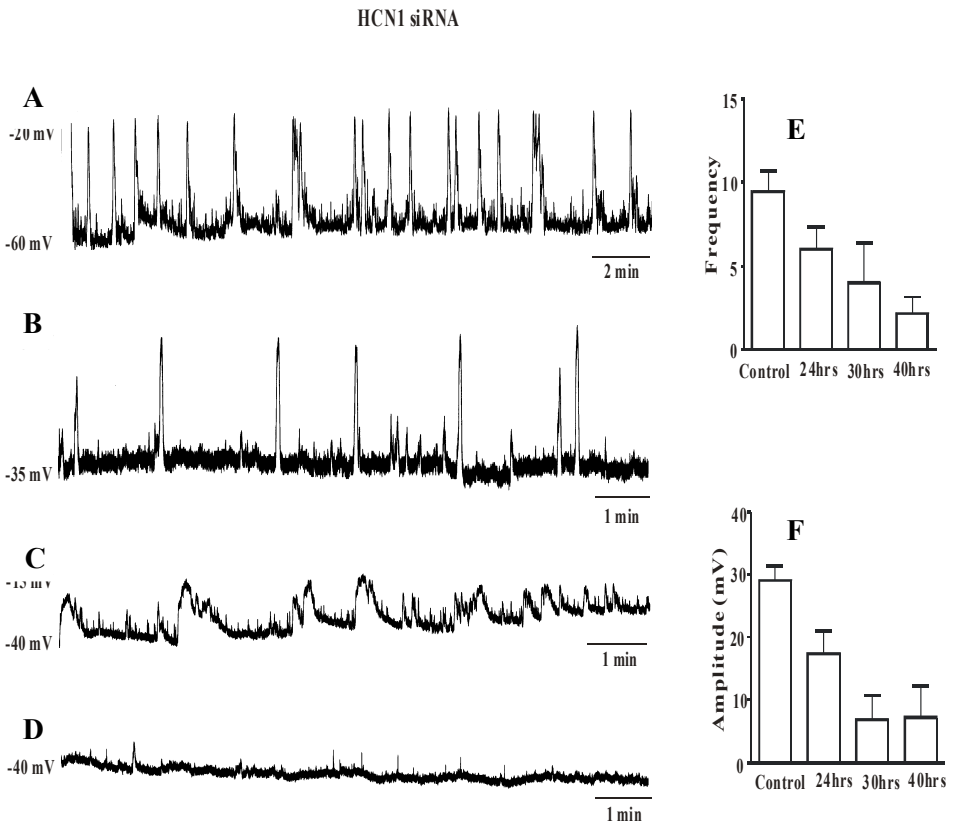


Fig 18.1. Recordings of colon ICC transfected with siRNA targeted to HCN1 gene in different intervals. Control (A) cell generated the regular membrane potential while (B), C) and (D) recorded in 24, 30 and 40hrs respectively showed the gradual decrement in frequency and amplitude. (E) and (F) are the summation of the data acquired.

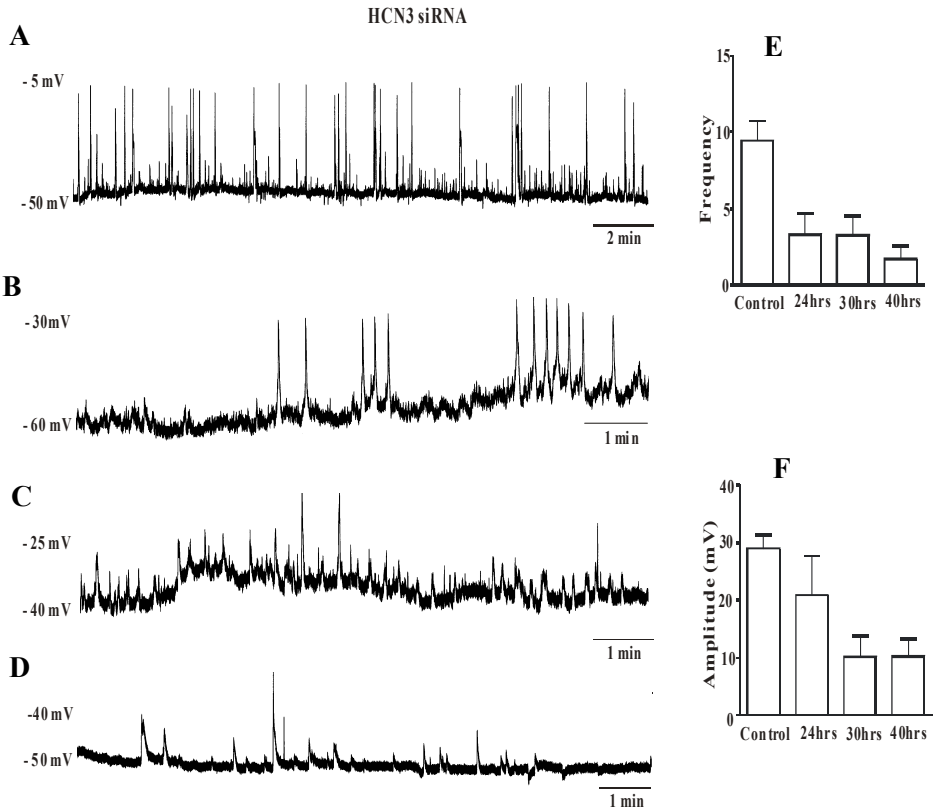


Fig. 18.2. Recordings of colon ICC transfected with siRNA targeted to HCN3 gene in different intervals. Control (A) cell generated the regular membrane potential while (B), C) and (D) recorded in 24, 30 and 40 hrs respectively showed the gradual decrement in frequency and amplitude. (E) and (F) are the summation of the data acquired.

The culture dish were transfected for different intervals before patch clamping at current clamp (*I=0*) were performed. Mock transfection was done for every siRNA transfection interval. The pacemaker potential generated by the control dishes for siRNA HCN 1 were found to be normal with frequency of 9 ± 2 cycles $5min^{-1}$ and 29 ± 2 mV. When the pacemaker potential was recorded from siRNA targeted for HCN1 gene after 24 hrs, there was slight decrease in the frequency as well as in the amplitude values being 7 ± 2 cycles $5min^{-1}$ and 18 ± 4 mV. Frequency and the amplitude further decreased when the pacemaker potential was recorded from the cell transfected for 30 hrs and complete abolishment of the pacemaker potential was observed after transfecting the cultured cells with siRNA targeted for HCN 1 for 40 hrs.

Similar effects were seen with the culture cells transfected with siRNA targeting the HCN3 mRNA. The frequency of the control dish was found to be 9 ± 3 cycles $5min^{-1}$ which decreased to 3 ± 2 cycles $5min^{-1}$. Not much difference was found after transfecting the cells for 30 hrs but at 40 hrs, pacemaker potential almost got abolished having the frequency of 2 ± 1 cycle $5min^{-1}$. Amplitude of the pacemaker potential also decreased in context to the increased transfection time. The amplitude of 29 ±2 mV was observed in the control which decreased to 20 ± 4 mV, 10 ± 4 and 9 ± 3 mV at 24, 30 and 40 hrs respectively.

5. DISCUSSION

The experiments described within this dissertation have examined the spontaneous pacemaker activity of murine colon. In the present studies, I have cultured the ICC cells from the colon tissue of the mouse. I evaluated the different kind of pacemaker potentials generated by the cultured ICC by utilizing the patch clamp technique. The main findings are discussed in more details in this section.

As the current evaluation from the colon ICC for the first time, I evaluated the different membrane potentials generated by them. During the studies I found three different patterns of the cells mainly differing in the frequency of the generation of the membrane potential. The first pattern had high frequency of 6 frequencies min^{-1}, the second with 3 frequencies $min^{-1}$and last kind of cell produced hardly 1 cycle $2min^{-1}$. As the colon tissue isolated from the 3-5 days old mice is too small to distinguish the region and culture the cells from different regions separately, our culture system possessed all cells from all regions of the colon. So I believed the difference in the frequency number must have been from the ICC cells from different regions. The high frequency of the membrane potential generation must have been produced by the ICC present in the proximal region of the colon, the membrane potential with 3 cycles min^{-1} might be from the ICC of the mid colon region and the last one with least frequency might be from the distal colon.

For these studies, the choice of the cell is the one producing the frequency of around 2~3 cycles min^{-1}. When patching is done under voltage clamp at a holding potential of -70 mV, the current generated by the cell is approx.1200 pA. In these studies, I also performed the patching on the ICC from the small intestine and the comparison is done between the two such that it would be easy to elucidate the results and find out the difference. In comparison to small intestine, the frequency of the membrane potential produced is less which is normally found 14 – 16 cycles

min^{-1} but the pacemaker current generated by the colon ICC is large when compare to small intestine which usually have the amplitude of 400 pA.

Our culture cells also have the neuron and the smooth muscle cells. Neurons also has the capability to produce the current spontaneously and when recorded from the smooth muscle cells, we can see the spontaneous current generation from them transferred from the pacemaker cells. It is important to remove the chance of recording the current from the both of these cells. This has been achieved by the treatment of tetrodotoxin which is the blocker of sodium channels. As neurons highly express the TTX sensitive channels, upon treatment of TTX, the current generation is inhibited. Similarly, depolarization of slow waves in smooth muscle is due to the activation of L-type Ca^{2+} channel. When L-type Ca^{2+} channels blockers are used the slow wave from smooth muscle is not observed. Hence nifedipine or nicardipine, a selective blocker of L-type Ca^{2+} channel, are used to eliminate the chance of recording the current from smooth muscle.

The current and the membrane potential generated by the ICC are not affected by the treatment of sodium channel or the L-type Ca^{2+} channel blockers. The cells showing no any change on the membrane potential after treating them were further evaluated for other drug treatments. At the start of the experiments, TTX was used in the bath solution and was continuously flown to the cells while performing the whole cell patch.

In our previous studies, we have shown in the small intestine ICC that cAMP has no role in the pacemaker current but cGMP showed the inhibitory effect. The main purpose of this study is to evaluate the action of the cyclic nucleotides in the membrane potential generated by the colonic ICC and also find out if the current generated by both small intestine and colonic ICC are same or has any difference in them.

Upon treatment of the cAMP exogenously to the cells that showed the spontaneous membrane potential, the rate of the frequency increased suggesting that the cAMP has a role to play in the generation of the membrane potential. The action of both the cAMP was confirmed by the treatment of the phosphodiesterase enzyme inhibitors. Phosphodiesterase is a ubiquitous enzyme that catalyses the hydrolysis of phosphodiester bonds. It is responsible for the hydrolysis of cAMP. The breakdown of cAMP is under the control of the enzyme phosphodiesterase, so when the inhibitor is used, the intracellular concentration of the cAMP is increased. In this experiment, rolipram, selective cAMP-specific phosphodiesterase (PDE4) inhibitor is used. After its treatment cAMP concentration of the ICC are increased. When rolipram was treated, it showed the similar effect as shown by the treatment of the cAMP externally. This suggests that the cAMP has the role in the generation of the spontaneous membrane potential.

Activation of pacemaker currents depends upon periodic release of Ca^{2+} from IP_3 receptors. Ca^{2+} release from IP_3 receptors triggers Ca^{2+} uptake by mitochondria, possibly by gating the rapid uptake mode of the uniporter (Sparagna et al., 1995; Litsky & Pfeiffer, 1997). Uptake and periodic release of Ca^{2+} from IP_3 receptor-operated stores appears to be the main oscillatory process responsible for GI autorhythmicity. Thus the pacemaker mechanism is a highly integrated process requiring physical proximity and coordination between ion channels and transport proteins in the ER, mitochondria and plasma membrane (Ward et al., 2000). So all the drugs which are treated exogenously to the cells generating the spontaneous membrane potentials are also treated to the cells while measuring their Ca^{2+} oscillation by live imaging confocal microscopy. The similar effect of increment in Ca^{2+} oscillation was recorded after the treatment of 8-bromo-cAMP and the rolipram. So I tried finding out the action of the cAMP in details.

The mechanism by which the intracellular cAMP generated is by the action of the enzyme adenylyl cyclase that synthesizes cAMP from adenosine triphosphate (ATP). The role of cAMP on the membrane potential is further analysed by

inhibiting the enzyme adenylyl cyclase. Upon treatment of the inhibitor, the level of cAMP in the cell is decrease. To this I treated the drug SQ22536 which decreased the intracellular production of the cAMP. On this doing, the membrane potential generated by ICC was completely abolished. This phenomenon was verified by using another kind of adenylyl cyclase inhibitor named dideoxyadenosine. The similar potential abolishment was observed confirming the role of cAMP in the spontaneous generation of the membrane potential by the ICC of the colon tissue which is not obtained from the small intestine ICC whose pacemaker current with these drugs were carried our simultaneously.

The cAMP–PKA signal transduction pathway is a ubiquitous cascade that modulates numerous cellular events within neurons (Abel & Kandel, 1998; Self et al., 1998). As the cAMP showed its action on the colonic ICC, it may possibly by the activation of PKA. Our result showed that the action of cAMP is not via PKA signaling pathway as the PKA inhibitor, KT5720, has no action on the membrane potential of the colonic ICC.

A family of novel cAMP sensor proteins, named Epac (exchange protein directly activated by cAMP) or cAMP-GEF (cAMP-regulated guanine exchange factor), has been identified (de Rooij et al 1998, Kawasaki et al., 1998). These proteins contain a CBD that is homologous to that of PKA R subunits and the prokaryotic transcription regulator, cAMP receptor protein (CRP). Epac proteins bind to cAMP with high affinity and activate the Ras superfamily small GTPases Rap1 and Rap2.
To test if the cAMP mediated action is through the Epac, I treated the colonic ICC with 8-pCPT-2′-O-Me-cAMP**,** potent and specific membrane-permeant activator of exchange factors directly activated by cAMP, but it showed no action on the spontaneous membrane potential suggesting that the action of cAMP is also not through Epac, a new receptor for cAMP.

Cyclic nucleotide-gated (CNG) channels play a fundamental role in signal transduction of sensory neurons. Upon binding of cyclic nucleotides, CNG

channels open and thereby cause changes in membrane potential. As cAMP did not activated both PKA and Epac, I thought it may be binding to CNG channel for its action. I treated the colonic ICC to L-cis-diltiazem which is known to block the CNG channel selectively. Our result shows that the CNG channels are not expressed in the ICC and that the action of cAMP on the colonic ICC is not via binding to the CNG channels as the blocker of CNG failed to show any action on the membrane potential generated by the colonic ICC.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels comprise a small subfamily of proteins within the superfamily of pore-loop cation channels. In mammals, the HCN channel family comprises four members (HCN1-4) that are expressed in heart and nervous system. The current produced by HCN channels has been known as I_h (or I_f or I_q). I_h has also been designated as pacemaker current, because it plays a key role in controlling rhythmic activity of cardiac pacemaker cells and spontaneously firing neurons. Regulation of HCN channels by cAMP is mediated by the proximal portion of the cytosolic COOH terminus (Zagotta et al., 2003).Binding of cAMP increases channel activity by removing tonic channel inhibition that is conferred by C-linker-CNBD (Weinger et al., 2001; Wang et al., 2001). As the ICC are also pacemaking cells and observed to have the modulation of its activity by cAMP in colon tissue, I thought it could be by binding with HCN channels. Non specific HCN channels isoform blockers were treated to the colonic ICC which generated the spontaneous membrane potential. It is well recorded that high concentration of CsCl blocks the HCN channel. The inhibitory action of CsCl is seen in our colonic ICC. The result was further verified with some drugs known to block the HCN channels specifically. ZD 7822 and Zatebradine, well known HCN channel blockers, also blocked the rhythmic generation of the membrane potential suggesting the presence of HCN channels and its role in the pacemaker activity in colon ICC.

The expression of HCN channels in the colon ICC is further investigated by the expression of the mRNA. In brain, all four HCN subunits have been detected

(Notomi & Shigemoto, 2004). HCN are also defined to express in the enteric neurons (Xiao et al., 2004). mRNA extracted from the brain was used as the positive control and all types of HCN channel isoforms were detected. Similarly, negative control was also used and for this purpose total RNA was extracted from HEK -293 cells. When performed the RT-PCR on the total RNA extracted from the whole dish containing all smooth muscle and enteric neurons, all types of the HCN channels were expressed. Single cell RT-PCR was performed such that there is no chance of amplification of the mRNA from other cells. Our results show that after performing the RT-PCR from single cell, there is no expression of the smooth muscle marker as well as the marker of enteric neurons. In this condition mRNA expressed for HCN1 and HCN3 were detected. This result showed that the modulation of the cAMP on pacemaker mechanism of colon ICC is through binding of the HCN channels.

This result is further verified by silencing the genes that are responsible for the HCN1 and HCN3 channels. After siRNA transfection, the membrane potential generation by the colonic ICC was hampered. All the drugs treated for the verification of the HCN channels were also treated to the pacemaker currents generated by the ICC of the small intestine and showed no effect on them. This suggests that the mechanism of the current generation on both small intestine and colon ICC are different.

Increased intracellular Ca^{2+} may also stimulate adenylyl cyclase (Litvin et al., 2003), resulting in increased cAMP-hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channel signaling (for review, see Dash et al., 2007; Wang et al., 2007).Although initially identified in sinoatrial node cells (McCormick & Pape, 1990), the current generated by HCN channels has now been found in a variety of cell types with spontaneous activity such as thalamic relay neurons (Solomon &Nerbonne,1993) and superior colliculus-projecting neurons (Pape, 1996). It has been reported that upon activation, HCN channels permeate the Ca^{2+} (Yu et al., 2003). I suggest through these experiments and results that the Ca^{2+} influx through

the HCN channels plays an important role in the generation of spontaneous membrane potential in the colonic ICC. This also explains that intracellular cAMP binds to the HCN channel thereby activating making the path for the Ca^{2+} influx inside the cell.

Abnormalities, or absence of ICC has been well documented in a variety of clinical disorders (Streutker et al., 2007). Hirchsprung's disease, a colonic disorder, which was believed due to neuron is now proved due to the loss of ICC (Wang et al., 2009). Elsewhere, the contributions of ICC dysfunction to diabetic gastroenteropathy (Ordo¨g, 2008) are being explored and the first case of chronic intestinal pseudo-obstruction in a child related to the regional loss of ICCs in the distal ileum and colon has been described (Struijs et al., 2008). Serotonin has long been a target of new drugs for IBS. Spiller (Spiller, 2008), in a review of the role of serotonin in IBS, provides a biological basis for the use of different approaches to diarrhea and constipation predominant IBS, given that the former is associated with enhanced postprandial release of serotonin, whereas the latter demonstrates an impaired response. HCN channel presence in ICC, in this study, and its role on the pacemaker activity of the colon can be made target for the treatment of colonic disorders.

In conclusion, ICC in colon generate the membrane potentials in slower rate compared to the small intestine, thus the difference in the motility between the two. HCN channels are found to have the pivotal role in the generation of the membrane potential in colon. Furthermore, the basal intracellular cAMP is required for the activation of the HCN channels and to maintain the pacemaker activity.

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ABSTRACT

The gastrointestinal tract is all about the digestion, absorption and the movement of the luminal contents by the co-ordinated muscular contractions. Interstitial cell of Cajal (ICC), a network of specialized cells, has been already reported as the participant in the regulation of smooth muscle contractility within the gastrointestinal tract. Colon is the part of this GI tract whose motility supports the important role in absorption, storage and defecation. In the colon, a number of studies have demonstrated that ICCs are associated with myentric and submuscular plexuses. The major focus of this dissertation is to record the current from the ICC of colon in network and find out its regulation by cyclic nucleotides.

In the present thesis, it has been demonstrated, using the patch clamp technique, the membrane potential generated by the cultured ICC from the colon tissue and the effect of the cyclic nucleotides. The comparisons are made between the pacemaker current generated by the small intestine ICC which has been widely studied till date.

3'-5'-cyclic adenosine monophosphate (cAMP) is an important second messenger. It forms when the membrane enzyme adenylyl cyclase is activated. The treatment of external cAMP increased the frequency of the pacemaker potential. Similar activity has been observed after inhibiting the phosphodiesterase enzyme acting on cAMP. The membrane potential generated by the colon ICC is completely abolished when the activity of the adenylyl cyclase is blocked. This suggests that cAMP has the role in the generation of the membrane potential. This result was verified further by inhibiting the action of phosphodiesterase enzyme acting on cAMP which also resulted in the increment of the frequency of the membrane potential generation. This action of cAMPis neither through the activation of protein kinase A nor through the binding to the Epac channel. This action is found out to be through the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Out of 4 different subtypes, HCN1 and HCN3 are found in the ICC of the colon. Blocking of the HCN channels also blocked the pacemaker potential of the ICC and moreover these 2 channels act as heteromers as silencing the gene of one channel also affects the action of another one.

In conclusion, cAMP binds to the HCN channel thereby activating it which then allows calcium influx inside the cell or may help release the calcium from the intracellular stores. Calcium oscillation in ICC is well studies and been suggested for the generation of pacemaker potential. Thus cAMP plays an important role in the generation of the pacemaker potential in colon ICC which is completely different from the pacemaker current generation in small intestine ICC.

Dedicated

To

Animals used for Research

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