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석사학위논문

Receptor Tyrosine and MAP Kinase are involved in effects of 5-HT on interstitial Cells of Cajal in Murine Intestine

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Pawan Kumar Shahi의 석사학위 논문을 認准함

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지도교수: 전 제 열

조선대학교 대학원 의학과

Serotonin (5-hydroxytruptamine or 5-HT)는 위장관에 널리 분포하면 위장관의 생리학적 기능 수행에 있어 5-HT의 기능은 매우 중요한 것으로 알려져 있다. 따라서, 위장관내 존재하는 Cajal 사이질 세포에서 발생하는 전기적 현상에 대한 5-HT의 효과와 작용기전을 규명하고자 생쥐 작은 창자에서 배양된 Cajal 세포에서 세포막 전압 고정법 및 세포 내 Ca^{2+} 분석을 시행하였다. 정상상태에서의 세포막 전압 고정법 시행시 Cajal세포는 서파와 자발적인 내향성 전류(향도잡이 전류)를 발생하였고 5-HT의 투여는 일시적인 내향성 전류를 발생시키는 것을 알 수 있었다. 또한 5-HT_4와 5-HT_7 수용체 길항제인 SDZ 205557과 SB 269970은 5-HT의 효과를 부분적으로 억제하였다. 더불어 5-HT3 수용체 길항제인 3-TCM는 완벽하게 억제하는 것을 보여주었다. 하지만, GDPβS를 이용한 G 단백질 관여 실험에서는 5-HT효과에 영향을 주지 못하였다.

Ca2+ -free 용액을 전 처리는 ICC에서 발생하는 자발적 향도잡이 전류를 억제하였으며, 5-HT에 대한 효과도 억제하였다. 이와 더불어, 다양한 MAP kinases 길항제를 이용한 실험에서 JNK II 길항제만이 5-HT의 효과를

감소시켰으며, 수용체 tyrosine kinase 길항제인 genistein과 herbimycin A 또한 억제 효과를 보여주었다.

이와 같은 결과는 ICC에서 발생되는 향도잡이 전류에 대한 5-HT의 역할을 확인시켜주는 것으로 그 작용은 5-$HT_{3,4,7}$을 통하여 이루어 지는 것을 알 수 있었다. 또한 그 작용은 수용체 typrosine kinase와 MAP kinase 활성화를 통해 이루어 지는 것을 시사한다.

INTRODUCTION

Cellular Physiology of gastrointestinal Smooth Muscle

Gastrointestinal smooth muscle exhibits variable tone, on which are superimposed rhythmic contractions driven by electrical slow waves. The latter are cycles of membrane depolarization and repolarization that originate in a network of pacemaker cells, the interstitial cells of Cajal, located at the boundaries and within the substance of the circular muscle layer. Ca^{2+} influx during the depolarization phase of each slow wave triggers a transient contraction. Release of excitatory neurotransmitters, chiefly acetylcholine(Ach), causes further depolarization and Ca^{2+} influx and activated signaling cascades that result in Ca^{2+} release and greater contraction which is discussed in details later.

Interstitial Cells of Cajal (ICC)

In 1911, Santiago Ramon Y. Cajal described a network of cells in gastrointestinal tissues and called them the primitive neurons. Cajal thought that these stellate cells with long branched processes are a special type of neurons, the interstitial neurons. He characterized them as accessory components that modify the smooth muscle contraction and are themselves the subject of nervous system regulation (Cajal 1893, 1911). Thuneberg (1982) stated that only a subset of cells identified by Cajal were associated with smooth muscle. These cells were qualified as interstitial cells of Cajal. (ICC). With the development of transmission electron microscopy an increased number of publications dealing with morphology and physiology of Cajal's cells appeared. There

are some structural variations between species and differences due to their different location in the gut, but the ultra-structure of ICC can be characterized by the following features:

1) Numerous large mitochondria present in processes,
2) Large bundles of intermediate filaments (vimentin),
3) Absence of thick myosin filaments,
4) Presence of surface caveolae,
5) Differently developed basement membrane,
6) synapse like contacts between the ICC and tertiary nerve bundles,
7) Well developed smooth and rough endoplasmic reticulum, and
8) Close apposition of gap junctions with smooth muscle cells (Huizinga et al. 1997).

Although the interstitial cells of Cajal were identified at the end of the 19th century, their developmental origin and functions remain uncertain. The discovery that ICC express the protooncogene c-kit, and that its gene product is the tyrosine kinase receptor Kit, opened new possibilities for studying ICC (Maeda et al. 1992, Ward et al. 199). The interstitial cells of Cajal form a three-dimensional network of cells placed between and in the smooth muscle layers. Isolated ICC are electrically active and create ion currents for pacemaker function. They also have electrical slow-wave activity, which determines the phasic contraction frequency in the stomach, small intestine and colon. Sanders (1996, 1999) uses in his reviews following classification of ICC: IC-MY are cells in the myenteric region of the stomach, small intestine and colon; IC-SM (submucosal ICC) are cells located along the submucosal surface of the circular smooth muscle layer in the colon; IC-DMP are Cajal's cells along the deep muscular plexus in

the small intestine; IC-IM are the intramuscular Cajal's cells in esophagus, stomach and colon (Fig. 1).

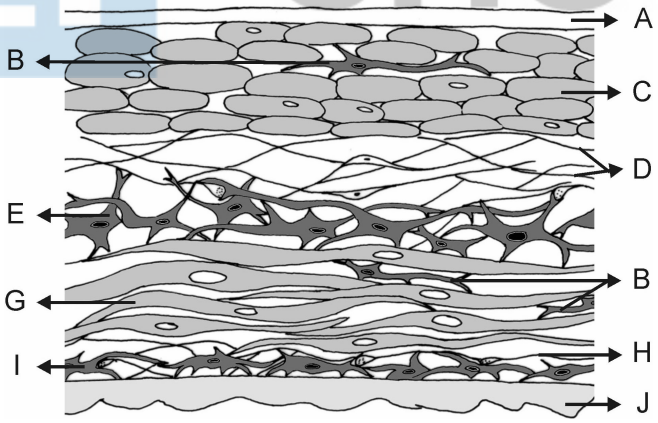


Fig. 1. *Scheme of the gut wall anatomical configuration. Detailed organization and the presence of ICC subclasses varies along the gut. A) serosa; B) intramuscular ICC (IC-IM) present in the esophagus, stomach and colon; C) longitudinal muscle layer; D) plexus yentericus Auerbachi; E) myenteric ICC (IC-MY) mainly in the stomach, small intestine and colon; G) circular muscle layer; H) plexus submucosus Meissneri; I) submucosal ICC (IC-SM) in the colon; J) submucosa.*

Table 1. A Classification Scheme for ICC

IC-MY_{ST} , IC-MY_{SB}, and IC-MY_C	ICC within the intermuscular space between the circular and longitudinal muscle layers (myenteric region) of stomach, small bowel, and colon, respectively
IC-SM ICC	along the submucosal surface of the circular muscle bundles of the colon
IC-DMP[a]	ICC within the deep muscularis plexus region of the small intestine
IC-IM_{ES} , IC-IM_{ST} , and IC-IM_C	Intramuscular ICC of the esophagus, stomach, and colon, respectively

[a]IC-DMP may be a specialized class of IC-IM, but the fact that these cells are only distributed within the

plane of the DMP, have a unique and well-described morphology, and have a unique expression of myofilament proteins, whereas IC-IM are often distributed at variable depths within the muscle layers and have fibroblast-like features, suggests that a special classification is reasonable for IC-DMP.

The results of many studies and detailed observations in the GI tract have served as the basis for this classification. The individual subpopulations play important, but varying roles in the motility of GI tract. Assuming that the ICC are unique for the gut and have properties which are not present in neurons and smooth muscle cells suggests that they could be an ideal target for pharmacological intervention in motility disorders (Huizinga et al. 1997).

Organization and Electrophysiology of Interstitial Cells of Cajal; and Smooth Muscle Cells in Gastrointestianl Tract

The functional role of the gastrointestinal (GI) tract is to digest and absorb nutrients. These processes are facilitated by the arches rated movement of the luminal contents(i.e, food, enzymatic and fluid secretions, digestion products, waste) from the mouth to the anus. The main elements of the motor apparatus consist of three critical cell types (enteric neurons, interstitial cells of Cajal [ICC], and smooth muscle cells).

Smooth muscle cells are clearly the force-producing element through most of the GI tract, but like many motor systems in the body, these cells alone are incapable of producing the coordinated movements of GI motility. The first level of motor coordination comes from intrinsic electrical activity, known as slow waves, which can propagate over many centimeters and organize contractile events. ICC are the active

element within pacemaker regions with unique intracellular timing mechanisms and ionic conductances that generate the pacemaker currents that underlie slow waves. ICC are coupled electrically to each other and to neighboring smooth muscle cells via gap junctions. Slow waves are generated in ICC, actively propagate in ICC networks, and conduct passively to smooth muscle cells. Smooth muscles cells do not have the apparatus to generate or propagate slow waves, but these cells express a number of voltage-dependent ion channels, most importantly voltage dependent ”L-type” Ca^{2+} channels that respond to slow wave depolarizations. Depolarization of smooth muscle cells increases the opening of Ca^{2+} channels, resulting in Ca^{2+} entry and contraction. Oscillations of the smooth muscle cell membrane potential in response to slow waves produce periods of low and increased Ca^{2+} channel probability. Thus, the contractile behavior of smooth muscle cells in regions with slow wave activity is naturally periodic, leading to motility patterns such as peristalsis and segmentation.

The amplitude of slow waves and the force of contractions in response to each slow waves are regulated to a significant extent by the enteric nervous system. Both inhibitory and excitatory neural inputs occur and can change the smooth muscle contractile response to slow waves from weak to powerful. For example, excitatory neural input can increase the amplitudes of slow waves, increase Ca^{2+} entry, and enhance the force of contraction. Inhibitory neural inputs, via activation of K^{+} channels or suppression of inward current conductances in smooth muscle cells, reduce the amplitude of slow waves and reduce contractile force.

Major excitatory and inhibitory neural control is mediated through a class of ICC that is intermingled within bundles of smooth muscle cells (intramuscular ICC [ICC-IM]). ICC-IM form synaptic structures with the varicose nerve terminals of enteric neurons

and gap junctions with neighboring smooth muscle cells. Other substances that condition the response of smooth muscle to slow wave depolarizations include hormones, paracrine substances, and inflammatory mediators. The site(s) of action of some these bioactive agents may also be at ICC, but the importance of ICC as targets for these substances currently is poorly understood. Several motility disorders have now been associated with loss of ICC, thus, the study of these cells has produced exciting new hypothesis about normal and abnormal GI functions.

ICC and the peristalsis in GI tract.

In the 1970s, information about the pacemaker regions in the GI tract began to appear gradually. Electrophysiological studies with isolated portions of intestinal wall confirmed that the circular muscle layer, if separated from the longitudinal muscle layer, ceases to generate slow waves, and that the longitudinal layer remains electrically active only at some sites. Suzuki et al. (1986) confirmed that the slow waves can be observed only at the sites where the presence of Cajal's cells has been demonstrated by ex post staining; thus, ICC can be considered as the initiators of slow waves. The slow waves represent the pacemaker activity of GI tract contractility and are ready to transform the excitatory neural stimulus into coordinated peristaltic movements for 2h daily. Measurements performed on the cat, dog, and rabbit small intestine, even on human jejunum (Hara et al. 1986) have demonstrated that the myenteric pacemaker region is the dominant source of slow waves in the small intestine. The dominant pacemaker in the stomach is localized in the proximal portion of its body, in the myenteric region. Each part of the stomach, with the exception of fundus, includes the pacemaker mechanism, a dense network of ICC-MY, with an increasing number of these cells

towards the antrum (Faussone-Pelegrini et al. 1989). The pacemaker in the colon resides at the submucosal surface of the circular muscle layer (Smith et al. 1987). There is, however, an additional pacemaker in the colon, which is located along the septa separating the individual circular muscle bundles. ICC-SM cells are present in proximal colon only whereas they are completely absent in its distal portion. The myenteric pacemaker generates small membrane potential oscillations, spreading into both circular and longitudinal muscle layers. It is difficult to determine what is the role of ICC in the spreading of the slow waves. As the ICC network becomes impaired, slow waves are abolished and their spreading is not measurable. To compare the propagation of slow waves with that of fast action potentials is not convenient, because these processes have different threshold potentials. The time needed for spreading of slow and fast activation differs in its dependence on muscle syncytium resistance (Publicover and Sanders 1989). Intracellular measurements have revealed that the slow waves spread along the long axis and around the circumference of the colon so that the ICC represent a basal pathway of activation in the colon (Sanders et al. 1990). Electric activity of the circular and longitudinal muscle layers of gastrointestinal tract organs is synchronized. Slow waves of circular and longitudinal muscle cells are in phase, indicating that a link must exist between these two layers. This connection is mediated by IC-MY, forming gap junctions with muscular cells in the small intestine and colon.

The pacemaker apparatus

ICC in pacemaker regions of the *tunica muscularis* are highly branching cells arranged into an electrically coupled network that extends between the circular and longitudinal muscle layers of GI organs, between bundles of smooth muscle cells, and along the submucosal surface of the circular muscle layer in the colon. Individual ICC, although intrinsically active, can do little in isolation to provide and distribute pacemaker activity. Intact ICC networks are critical for generation and propagation of slow waves. ICC are coupled to smooth muscle cells through low resistance electrical junctions. Slow waves are conducted to smooth muscle cells, but these cells lack the conductances necessary to actively propagate or regenerate slow waves. Smooth muscle cells 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.

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 smooth muscle cells 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

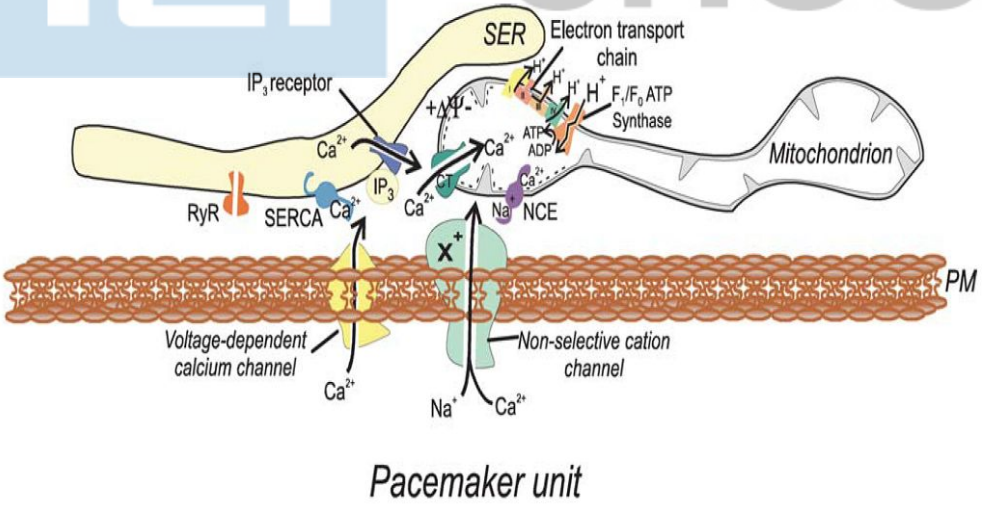
region of the ICC network, most of the cells are spontaneously active at the same general frequency. Thus, the primary pacemaker (the cell or sub-cellular compartment responsible for initiating each cycle) can vary between each slow wave. Inward current and depolarization from the primary pacemaker 'entrains' 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.

The pacemaker mechanism

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 interstitial Cajal's cells 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 IC-SM depolarization towards the

threshold potential.

FIG. 2



Pacemaker unit

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 calcium channels (the second source of Ca^{2+} influx) are progressively activated. These L-type calcium 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 and Sanders 1993)

Serotonin and Interstitial Cells of Cajal

The coordinated movement of food along the GI tract is dependent on 5-HT-mediated regulation of smooth muscle tone, peristalsis, mucosal secretion, and visceral perception via an interaction with intrinsic enteric and extrinsic afferent neurons, the interstitial cells of Cajal, smooth muscle cells and enterocytes. The majority of the body's serotonin (5-HT) is produced by the gastrointestinal tract. Ninety per cent of 5-HT is produced by enterochromaffin cells (EC), found in the gastrointestinal epithelium. However, the source of 5-HT that can activate the different receptors found is still not clear for all the cell types that express 5-HT receptors, including ICC. The other major source of 5-HT in the gastrointestinal tract are enteric neurons, in particular interneurons. In non-diseased tissue, it is unlikely that ICC and myenteric neurons respond to 5-HT released by the EC in the mucosa because the 5-HT transporters present in serotonergic neurons, mucosal and submucosal cells will rapidly take up 5-HT. However, a subset of intrinsic primary afferent neurons located in the submucosal plexus project to the myenteric plexus and are activated by mucosal 5-HT. These submucosal to myenteric projections may result in mucosal 5-HT activating myenteric neurons and, indirectly, ICC. In inflammation, 5-HT transporters have been shown to be down regulated and 5-HT production increased making it more likely that mucosally generated 5-HT can reach ICC and activates the expressed receptors. Another possible

source of 5-HT are serotonergic interneurons. Serotonergic interneurons represent about 2% of the total number of enteric neurons. However, while in the myenteric plexus they may represent a possible source of 5-HT for other myenteric neurons, interneurons do not make direct contact with ICC and therefore would be unlikely to provide sufficient 5-HT to activate ICC 5-HT receptors. Mast cells can represent a source for 5-HT. Mast cells are known to be in close apposition to ICC and therefore may be one of the sources of 5-HT. Finally, platelets are a rich source of 5-HT and may serve as a significant 5-HT source. It is not currently known if platelet derived 5-HT can activate ICC 5-HT receptors.

5-HT has several distinct functions in the gastrointestinal tract. 5-HT acts as a paracrine factor transducing information from EC to intrinsic primary afferent neurons and to adjacent cells in the mucosa and submucosa. Furthermore, 5-HT is a neurotransmitter and is also increasingly recognized as a survival factor. Control of gastrointestinal motility requires the coordinated activity of several cell types including nerves, smooth muscle cells and interstitial cells of Cajal (ICC). ICC are now known to be essential for normal motility. Several receptors are expressed on ICC including 5-HT receptors. An understanding of the potential role that 5-HT receptors expressed on ICC may play in the control of ICC function and survival requires an understanding of the different roles that ICC play in the control of gastrointestinal motility.

5-HT and its Receptors

5-HT was discovered in the 1930s and named “enteramine” as it was first extracted from the intestine. Since its discovery, it has become clear that 5-HT serves many diverse physiological functions, such as the regulation of sleep, appetite, mood,

neuroendocrine secretion, sexual behavior, cognition, and GI function via an interaction with multiple 5-HT receptors. To date 5-HT receptors belonging to seven families (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7), have been identified. With Exception of the 5-HT_3 receptor, which is ligand- gated ion channels, each of the 5-HT receptors identified is a seven- transmembrane domain, G- protein-coupled receptor. The 5-HT_1 and 5-HT_5 receptor families are negatively and the 5-HT, 5-HT_6, and 5-HT_7 receptors are positively coupled to adenylyl cyclase. Binding of 5-HT to the Gq-coupled 5-HT_2 receptor activates phospholipase C resulting in the release of inositol triphosphate and an elevation of cytosolic calcium. With regard to GI disorders, various 5-HT receptor ligands have been evaluated clinically to treat conditions such as IBS-D, IBS-C, chronic constipation, functional dyspepsia, and gastroparesis.

5-HT Receptors in the GI tract

5-HT_1 Receptor family

The 5-HT_1 receptor family consists of the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} subtypes. Radio ligand binding and mRNA expression studies in the rat have provided evidence that 5-HT_{1A} receptors are expressed on submucosal and myenteric neurons throughout the GI tract, with a particularly high density in the stomach. 5-HT_{1A} receptor activation produces presynaptic inhibition of fast and slow excitatory neurotransmission in the ENS and hyperpolarization of myenteric IPANs resulting in a reduction in the amplitude of excitatory postsynaptic 5-HT_{1A} receptor activation is associated with inhibition of electrically evoked contractions of guinea pig ileum and stomach circular smooth muscle (Buchheit and Buhl 1999; Mir et al. 1988), and relaxation of the dog proximal stomach and mouse fundus potentials. Expression of 5-HT_{1B} and to a lesser

extent 5-HT_{1D} mRNA has been described in the bovine ileum and colon (Engel et al. 2006). mRNA for both receptor subtypes is present in human and rat sensory neurons consistent with an involvement in sensory neurotransmission. The 5-HT_{1E} receptor (McAllister et al. 1992) appears to be localized entirely within the CNS. The 5-HT_{1F} receptor is closely related to the 5-HT_{1E} subtype, possessing greater than 70% sequence homology across the seven transmembrane domains and like the latter appears to be largely restricted to the CNS.

A number of studies have indicated an important physiological role in GI function for a peripheral 5-HT receptor characterized by high affinity for [3H] 5-HT, and termed the 5-HT_{1P} subtype (Mawe et al. 1986). The 5-HT_{1P} receptor has not been cloned, and its molecular identity therefore remains elusive. It has been suggested that the 5-HT_{1P} receptor is either the 5-HT_7 receptor or a heterodimer of the dopamine D2 receptor with either the 5- HT_{1B} or 5-HT_{1D} receptor (Liu and Gershon 2005; Monro et al. 2005; Tonini 2005). 5-HT_{1P} receptors are localized on IPANs in the submucosal and myenteric plexuses and in the intestinal mucosa. 5-HT, released from enterochromaffin cells, following mucosal stimulation, initiates reflexes via activation of 5-HT_{1P} receptors on the submucosal IPAN terminals (Pan and Gershon 2000). 5- HT1P receptor activation and subsequent stimulation of submucosal VIP- and CGRP-containing afferent neurons are considered to have a critical role in initiation and maintenance of the peristaltic reflex.

5-HT_2 Receptor family

The 5-HT_2 receptor class comprises the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} subtypes, which possess 6–50% sequence identity. The 5-HT_{2A} and 5-HT_{2B} receptor subtypes are present

in the CNS and periphery, while 5-HT_{2C} receptors appear to be restricted to the CNS (Hoyer et al. 2002; Leysen 2004). 5-HT_{2C} receptors are considered to lack a role in GI physiology and pathophysiology, in contrast to the 5-HT_{2A} and 5-HT_{2B} receptor subtypes. In the rat and guinea pig stomach antrum or corpus, 5-HT_{2A} receptor activation results in contraction (Komada and Yano 2007; Tamura et al. 1996), while in the rat fundus, a relaxation is observed (Komada and Yano 2007). Activation of 5-HT_{2A} receptors in canine and guinea pig isolated colonic longitudinal muscle results in contraction (Briejer et al. 1995a; Prins et al. 1997), and in vivo, motility is stimulated in the middle and distal colon (Nagakura et al. 1996a). 5-HT_{2A} receptor activation also produces contraction of human isolated jejunal smooth muscle cells

The 5-HT_{2B} receptor was originally cloned from the rat stomach fundus (Foguet et al. 1992. mRNA for the 5-HT_{2B} receptor is widely expressed in the human and rodent GI tracts (Fiorica-Howells et al. 2000; Borman et al. 2002). In human colon, 5-HT_{2B} receptor mRNA and protein are present in the longitudinal and, to a lesser extent, circular muscle layers and in myenteric neurons (Borman et al. 2002). The 5-HT_{2B} receptor is also expressed on interstitial cells of Cajal in the human and mouse intestine and is postulated to have a proliferative role.

5-HT_3 Receptor

5-HT_3 receptors are ligand-gated cation channels. Evidence for expression of 5-HT_3 receptors in ICC comes from work carried out in the rat small intestine. In rat intestine, 5-HT_3 receptors are expressed by different cell types including functionally distinct classes of neurons, ICC and endocrine cells. 5-HT_3 receptors are found in both ICC-MP and ICC-DMP. However not all ICC appeared to express 5-HT_3 receptors, that is some

ICC at both the ICC-MP and ICC-DMP did not appear to express 5-HT_3 by immunohistochemistry. The role of the 5-HT_3 receptor on ICC is currently unknown. 5-HT_3 receptors are ligand-gated cation channels and allow entry of several cations including Ca^{2+} in some but not all types of 5-HT_3 receptors. 5-HT_3 receptor activation results in neurotransmitter release, including acetylcholine, from presynaptic terminals and neuronal excitation of postsynaptic cells. Given the known function of 5-HT_3 receptors it can be hypothesized that activation of 5-HT_3 receptors on ICC would result in rapid depolarization and therefore alteration or initiation of the slow wave.

THE 5-HT_4 Receptor

5-HT_4 receptors are G protein-coupled metabotropic receptors. The 5-HT_4 receptor was first cloned in 1995 (Gerald et al. 1995), and in recent years, many splice variants of the receptor, which differ only in the sequence of their intracellular COOH-terminal domain, have been identified i.e., 5-HT 4(a), 5-$HT_{4\ (b)}$, 5-$HT_{4(c)}$, 5-$HT_{4(d)}$, 5-$HT_{4(e)}$, 5-$HT_{4(f)}$, 5-$HT_{4(g)}$, 5-$HT_{4(i)}$, and 5-$HT_{4(n)}$. In the mouse, mRNA for the 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, 5-$HT_{4(e)}$, and 5-$HT_{4(f)}$ splice variants is expressed, albeit to different extents, in the submucosal plexus (Liu et al. 2005a). The 5-$HT_{4(a)}$ and 5-$HT_{4(b)}$ but not 5-$HT_{4(e)}$ and 5-$HT_{4(f)}$ isoforms are also expressed in the myenteric plexus of the small and large intestine Evidence for expression of 5-HT_4 receptors in ICC comes from work carried out in the mouse and guinea pig small intestine. Studies using an antibody to 5-HT_4 showed that together with the presence of 5-HT_4 receptors in subsets of enteric neurons and smooth muscle cells, the 5-HT_4 receptor was also expressed in ICC-MP. The expression of 5-HT_4 in ICC-DMP appeared to be much less robust. Similar to the 5-HT_3 receptor, the role of 5-HT_4 receptors in ICC is not known as no functional studies have been carried

out. 5-HT_4 receptor agonists acting presynaptically enhance neurotransmitter release at neuron-neuronal synapses while 5-HT_4 receptor agonists acting on smooth muscle cause relaxation. The 5-HT_4 receptor is also thought to be coupled by Gs to the stimulation of adenylyl cyclase, resulting in an increase in cyclicAMP (cAMP) and activation of protein kinase A. cAMP has been shown to mediate slow wave amplitude and frequency in murine ICC suggesting a potential role for the 5-HT receptor in modulating slow waves. The 5-HT_4 receptor also appears to be involved in neuronal development and survival. Whether activation of the 5-HT_4 receptor does in fact play role in the survival of ICC or is involved in the generation and propagation of the electric slow wave by increasing cAMP levels remains to be elucidated.

5-HT_5 and 5-HT_6 Receptor families

The 5-HT_5 receptor family consists of two members, designated 5-HT_{5A} and 5-HT_{5B}; only the former member is expressed in the human as the coding sequence of the 5-HT_{5B} receptor is interrupted by stop codons (Nelson 2004). Both receptor subtypes are essentially limited in distribution to the CNS, although the 5-HT_{5A} receptor has also been found in the carotid body. There are no data suggesting a role for the 5-HT_5 receptor in GI function.

The 5-HT_6 receptor has been cloned from several species including humans (Kohen et al. 1996; Ruat et al. 1993). Within the CNS, there is considerable interest in the role played by the 5-HT_6 receptor in the regulation of feeding, cognition, affective states, and seizures (Woolley et al. 2004), although there appears to be extremely limited 5-HT_6 receptor expression in the periphery. Despite the interaction of the 5-HT_6 receptor with many neurotransmitter systems known to influence GI function (e.g., cholinergic,

dopaminergic, and GABAergic) and detection of weak receptor expression in the rat stomach (Ruat et al. 1993), there are no definitive data demonstrating a functional role for the 5-HT_6 receptor in the GI tract.

5-HT_7 Receptors

The 5-HT_7 receptor has been detected in the CNS and periphery (Vanhoenacker et al. 2000), although the majority of physiological effects attributed to 5-HT_7 receptor activation occur in the CNS (e.g., increased excitability of hippocampal neurons, regulation of prefrontal cortex development, and thermoregulation; Bacon and Beck 2000; Beique et al. 200; Hedlund et al. 2003). However, the 5- HT_7 receptor is present in the ENS and is postulated to play a role in GI physiology. Each of the 5-HT_7 receptor splice variants is present in the human stomach, small intestine, and colon (Irving et al. 2007; Jasper et al. 1997; Krobert et al. 2001), while 5-HT_7 receptor immunoreactivity is present in myenteric and submucosal IPANs, NO synthase- and VIPimmunoreactive descending neurons, and in smooth muscle cells of the ileum (Tonini et al. 2005). Activation of 5-HT_7 receptors on IPANs results in a slow excitatory postsynaptic potential, while stimulation of 5-HT_7 receptors in the smooth muscle of the ileum and colon produces relaxation

Table 2: Summary of 5-HT receptor characteristics in GI tract.

Receptor Type	Receptor Sub-type	Signal Transduction Mechanism(s)	Primary localization	Functional Role
5-HT$_1$	**5-HT$_{1A}$**	**Gi/o**	Intrinsic sensory neurons, interneurons, excitatory motor neurons, enterocytes	Relaxation, modulation of visceral sensitivity
	5-HT$_{1B}$	**Gi/o**	Intrinsic and extrinsic sensory neurons, smooth muscle cells	Contraction/ relaxation
	5-HT$_{1D}$	**Gi/o**	Intrinsic and extrinsic sensory neurons, smooth muscle cells	Contraction/ relaxation
	5-HT$_{1E}$	**Gi/o**	Not present	Not Applicable
	5-HT$_{1F}$	**Gi/o**	Cellular localization not described	Relaxation
5-HT$_2$	**5-HT $_{2A}$**	**Gq/11**	Enteric neurons, enterocytes, smooth muscle cells	Contraction/ relaxation
	5-HT$_{2B}$	**Gq/11**	Enteric neurons, smooth muscle cells	Contraction
	5-HT$_{2C}$	**Gq/11**	Not present	Not Applicable
5-HT$_3$	**5-HT $_{3A/B}$**	**Cation Channels (Na^+, Ca^{2+},K^+)**	Intrinsic and extrinsic sensory neurons, ICC, secretomotor neurons, enterocytes	Modulation of visceral sensitivity and motility, secretion
5-HT$_4$	**5-HT$_4$ splice Variants**	**Gs; increase $[Ca^{2+}]_i$ 5-HT$_{4(a)}$; Gi/o 5-HT$_{4(b)}$**	Intrinsic sensory neurons, interneurons, ICC, excitatory and inhibitory motor neurons, smooth muscle cells, enterocytes	Contraction/relaxation, stimulation of motility, secretion
5-HT$_5$	**5-HT $_{5A}$**	**Gi/o**	Not Present	Nor Applicable
	5-HT$_{5B}$	**None identified**	Not Present	Nor Applicable
5-HT$_6$		**Gs**	Limited expressions mRNA in stomach, cellular localization not described	No definite role
5-HT$_7$		**Gs**	Intrinsic sensory neurons, inhibitory motor neurons, smooth muscle cells	Relaxation, modulation of Visceral sensitivity

MATERIAL AND METHODS

Animal: -

Balb/C mice (3～7 days old) of either sex were anesthetized with diethylether 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 physiology sciences approved by the institutional animal use and care committee at Chosun University, College of Medicine. The small intestine was excised 1 cm below the pyloric ring to the cecum and opened along the mesenteric border. Luminal Contents were washed away with Krebs-Ringer bicarbonate solution.

Cell Culture

The isolated tissue was pinned to the base of Slygard dish, and the mucosa was removed by the sharp dissection. Small Strips of intestinal muscle were equilibrated in Calcium Free Hank's Solution containing (in mM) KCL-5.36, NaCl- 125, NaOH – 0.336, Na_2HCO_3-0., Glucose- 10, Sucrose – 2.9 and HEPES – 11 and the pH is adjusted to 7. 4 with Tris for 30 minutes. Cells were dispersed by incubating for 15 minutes at 37^{o}C in an enzymatic solution containing Collagenase (Wortington Biochemicals, Lakewood, NJ, USA 1.3mg/ml, bovine serum albumin (BSA) (Sigma, St.Louis, MO, USA) 2mg/ml, Trypsin Inhibitor (Sigma) 2mg/ml, and ATP 0.27mg/ml. The cells were then finely chopped and placed onto sterile glass coverslips coated with Poly-L Lysine in 35mm culture dishes and incubated at 37 ^{o}C in a 95% O_2 – 5% CO_2 incubator in SMGM (smooth muscle Growth Medium, Cambrex Bio Science, Walkersville, MD, USA)

supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5ng/ml, Sigma). Interstitial Cells of Cajal (ICC) were identified immunologically using a monoclonal antibody for kit protein.

Patch – Clamp Experiments

Patch clamp technique is a technique in electrophysiology that allows the study of individual ion channels in cells**.** All patch-clamp methods rely on a very high-resistance seal between a micropipette and a membrane; the seal is usually attained by gentle suction. The four most common variants include

- Cell-attached patch,
- Inside-out patch,
- Outside-out patch, and
- Whole-cell clamp.

Patch-clamp methods are commonly used to voltage clamp, that is control the voltage across the membrane and measure current flow, but current-clamp methods, in which the current is controlled and the voltage is measured, are also used.

The whole – cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from the cultured ICC. Currents or potentials were amplified using an Axopatch 1-D (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 9.2; Axon instruments). The data were filtered at 5 KHz. All experiments were carried out at 30 ^{o}C.

Solutions: -

Bathing Solution (External Solution) For ICC recording		
	Concentration	Mol. Wt.
$NaCl_2$	135mM	58.
KCl	5mM	7.55
$MgCl_2$	1mM	203.3
D-Glucose	10mM	180.2
HEPES	10mM	238.3
$CaCl_2$	1.8mM	17.0
Adjust pH 7. with Tris base		

solution used for ICC recordings

Table 3. Bathing solution and Internal

Internal Solution For ICC Recording		
	Concentration	Mol. Wt.
KCl	10mM	7.55
$MgCl_2$	2.5mM	203.3
MgATP	2.7mM	507.2
Na_2GTP	0.1mM	523.2
Phospho-creatine	2.5mM	255.1
HEPES	5mM	238.3
EGTA	0.1mM	380.
Adjust pH 7. with Tris base		

Measurement of the Intracellular Ca^{2+} Concentration

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by using fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at −20 °C. The cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution. The coverslips were then incubated in the bath solution containing 5 M fluo-3 with 5% CO_2 at 37°C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and 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). Fluorescence was excited at a wavelength of 88 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^{o}C. The variations of intracellular Ca^{2+} fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging.

Drugs and Chemicals

Drugs used for the experiments, 5-Hydrogytryptamine (5-HT), SB 269970, 3-Tropanylindole – 3 – carboxylate methiodide, SDZ 205557 hydrochloride, PD 98059, SB 203580, Guanosin – 5 – [B-thio] diphosphate- trilithium salt, Herbimycin A, Geinstein were purchased from Sigma-Aldrich, St. Louis, MO, USA. JNK inhibitor II is purchased from Calbiochem. Appropriate solvents either DMSO or distilled water were used to dissolve the drugs and stock solutions (10mM or 100mM) were prepared and stored in aliquots at temperatures designated. All the light sensitive drugs were protected from the light by wrapping the tubes containing drugs with the aluminium foil. The required concentrations of the drugs were prepared at the time of experiment and were added to the bath solution. All drugs were applied to the whole cell preparation by superfusion. The final concentration of DMSO in all the drug preparations was less than 0.5%.

Statistical analysis

Data is expressed as the means ± standard errors (S.E.). Differences in the data were evaluated by the student's t test. A P values less than 0.05 were taken as a statistically significant difference. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

RESULTS

Spontaneous membrane potential and pacemaker current generated by ICC at resting state.

Under a current clamp mode, the spontaneous depolarization generated my ICC. The mean resting potential was -63 ± 2mV and ICC produced electrical pacemaker potentials.

Under a voltage clamp at a holding potential of -70mV, ICC showed an inward currents oscillations, so called pacemaker currents. The frequency of the pacemaker currents was 14 ± 2 cycles /min and the amplitude was -600 ± 50pA.

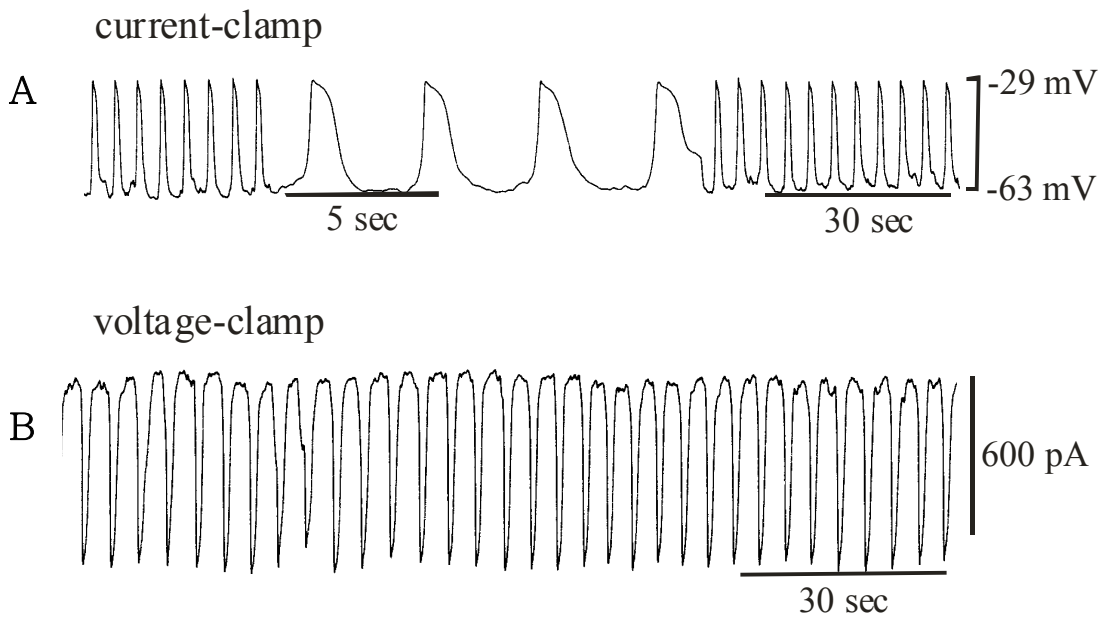


FIG 3. (A) Spontaneous depolarizations and (B) Inward currents in cultured ICC of the murine small intestine.

Effect of 5-HT on membrane potential generated by ICC

Recordings from the cultured ICC under the current clamp mode (*I*=0) showed spontaneous pacemaker potentials. The resting membrane potential was -63 ± 2 mV and the amplitude was 40 ± 5 mV. In the presence of 5-HT (10 μmol /ml), the membrane potentials were depolarized to -32 ± 16mV, and the amplitude of the pacemaker potentials was decreased to -28 ± 14 mV.

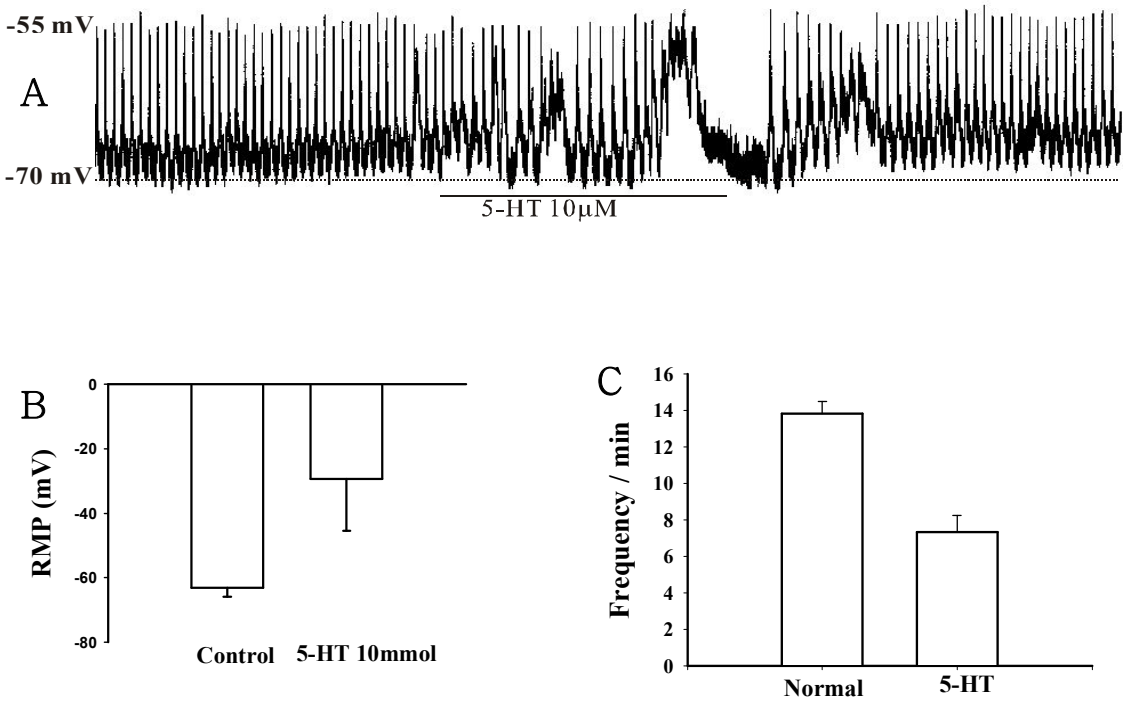


Fig. 4 Effect of 5-HT on the pacemaker potential in the cultured ICC of murine small intestine. **(A)**. Pacemaker potentials of ICC exposed to 10μmol 5-HT in the current clamping mode (I=0). A summarized bar graph showing the 5-HT induced effects on pacemaker potential of ICC. **(B)** Pacemaker potential at normal condition is -63 ± 2 mV and in presence of 5-HT is 32 ± 16mV. **(C)** Frequency in absence and presence of 5-HT and the dotted lines indicate zero current levels.

Effect of 5-HT at different concentration on ICC

Under a voltage clamp at a holding potential of -70mV, ICC generated spontaneous inward currents, which is referred to as 'pacemaker currents'. The frequency of the pacemaker current was 14 ± 2 cycles per min. When the 5-HT was treated to ICC, it showed a transient depolarization of the pacemaker current. At the concentration of 1 μmol the effect of 5-HT on the pacemaker current is not significant (Fig. 5A) (n=5). Then the concentration was increased to 10 μmol. At this concentration there was a significant transient effect. The resting current was -65 ± 2 pA (Fig. 5B) and the corresponding frequency was found to be 9 ± 2 cycles min-1 (n=15). Further the effect of 5-HT was observed at higher concentration. For that 100 μmol of 5-HT was treated on the pacemaker current. It showed the similar effect to the 10 μmol concentration (Fig.5C).

The value what obtained after the treatment of 5-HT have significantly different from the control values and the 5-HT at the concentration of 10 μmol showed the maximum effect so the further experiments were carried out at this concentration.

5-HT also has the desensitization effect on the pacemaker current so after the treatment of the drug, the cells were washed for 15 minutes to washout all the drug completely before treating drugs again.

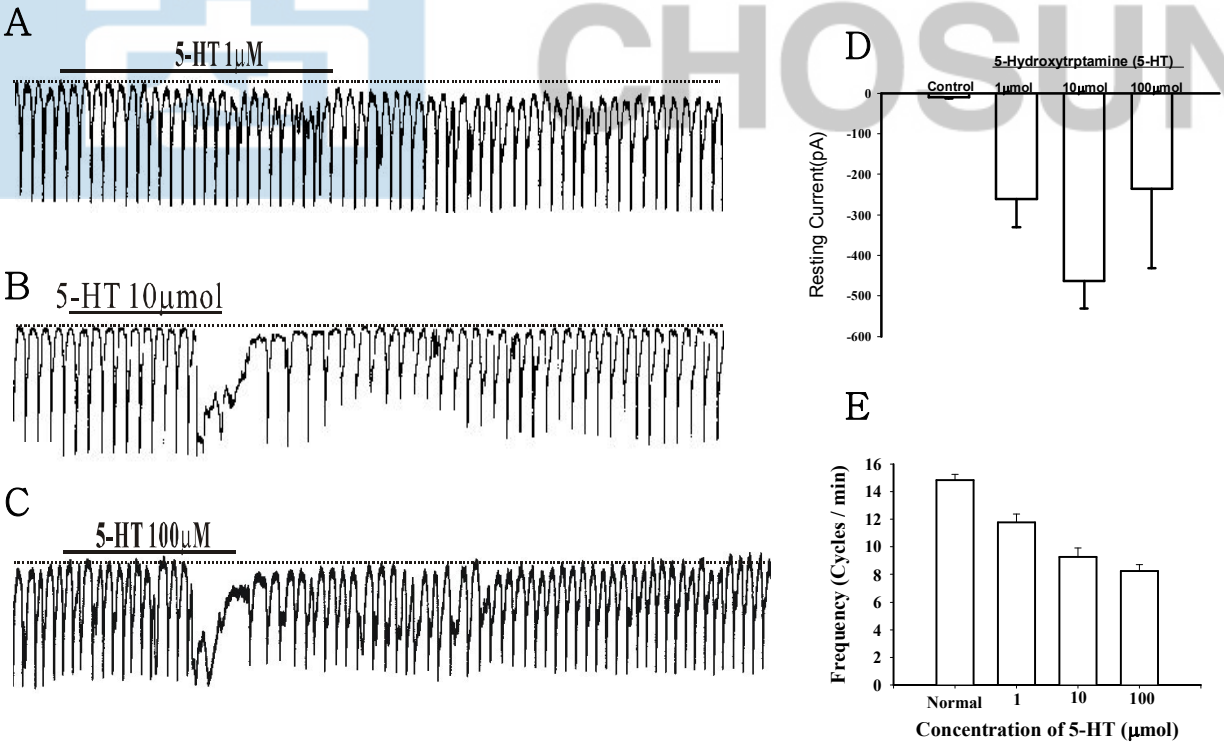


Fig.5 The dose-dependent effects of 5-HT on pacemaker currents in cultured ICC of murine small intestine. **(A), (B), (C)** show the pacemaker currents of ICC exposed to 5-HT (1, 10 and 100 μmol) at a holding potential of -70 mV. **(D), (E)** summarize the inward current generated by 5-HT on pacemaker currents in ICC. Bars represent means ± S.E.

No involvement of G-protein in the action of 5-HT in cultured ICC

To explore whether the G-protein is involved in the action of 5-HT, the effect of GDPβS, a nonhydrolyzable guanosine 5-diphosphate (GDP) analogue which permanently inactivates GTP-binding proteins was tested. Under the control conditions at a holding potential of -70 mV, the resting current of pacemaker currents is -10 ± 2 pA and the inward current produced by the 5-HT 10μmol was -623 ± 42 pA. In the presence of GDPβS in the pipette, the effect of the 5-HT was not blocked but surprisingly the tonic inward current produced was more compared to the single treatment of 5-HT. The inward current produced in presence of GDPβS 1mM was found to be -970 ± 152 pA. Why the inward current produced is more needs to be studied more but the data obtained suggests that the G-proteins may not be involved in 5-HT induced effects on cultured ICC.

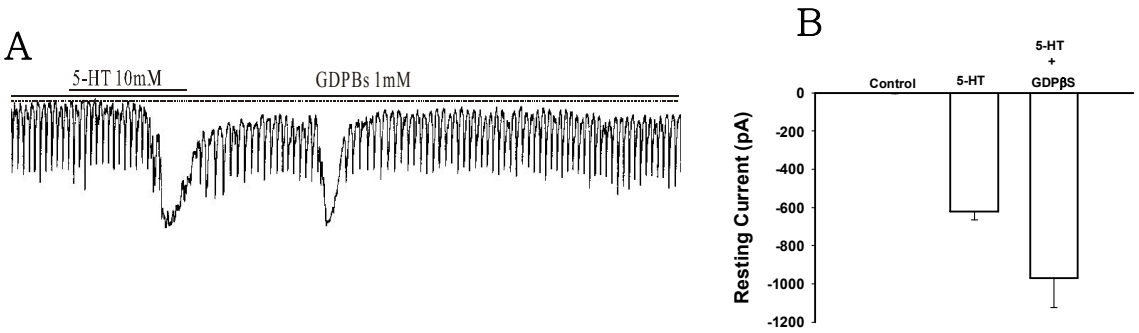


Fig. 6 Effect of 5-HT (10 μmol) in the presence of drug inactivating GTP-binding proteins. **(A)** under the controlled condition at the holding potential of -70 mV, whole cell patch clamp performed with the internal solution filled with 1mM GDPβS and 5-HT's effect on it. **(B).** The bars represent mean ± SE values and the dotted lines indicate zero current levels

Effect of external Ca^{2+} in 5-HT induced inward current on ICC

To investigate the role of external Ca^{2+}, 5-HT was tested under external Ca^{2+} -free conditions. Pacemaker currents recorded at a holding potential of -70mV were completely abolished by external Ca^{2+} -free solution. In this condition, 5-HT induced inward current was suppressed in absence of external Calcium. In the external Ca^{2+} -free solution, the inward currents produced by 5-HT was -235 ± 39 pA. These values were significantly different when compared with 5-HT in normal Ca^{2+} containing solution.

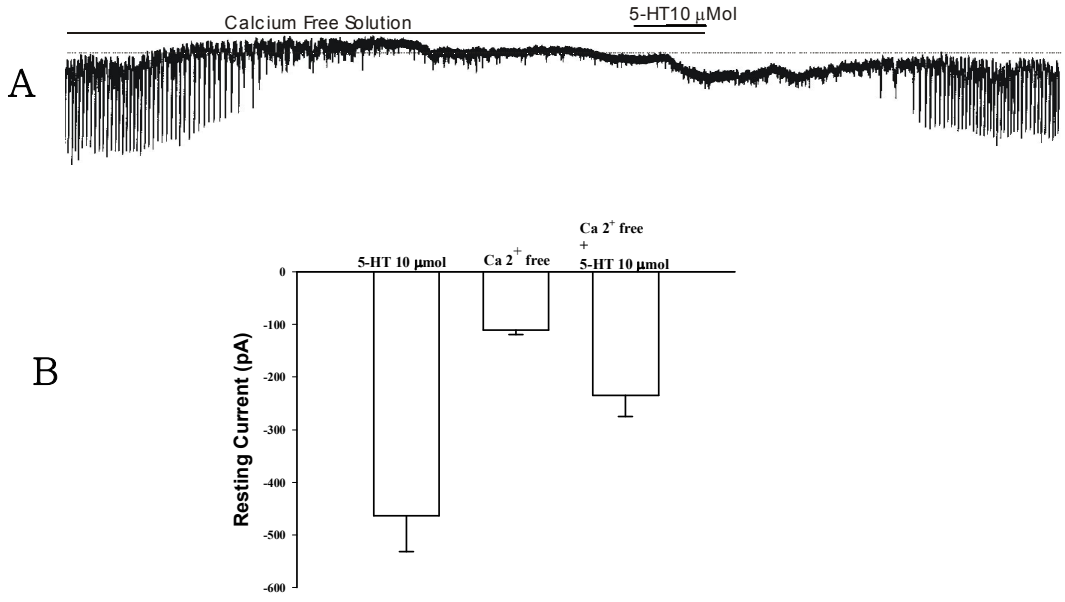


Fig. 7 Effects of an external Ca^{2+} free solution, upon 5-HT - induced responses on pacemaker currents in cultured ICC of the murine small intestine. **(A)** The external Ca^{2+} -free solution abolished the generation of pacemaker currents. Under this condition, 5-HT -induced inward currents were suppressed. Responses to 5-HT in the external Ca^{2+} -free solution is summarized in **(B).** Bars represent mean values ± S.E. The dotted lines indicate the zero current levels.

Excitation of $[Ca^{2+}]_i$ oscillation by 5-HT

Because many reports suggested that $[Ca^{2+}]_i$ oscillations in ICC are considered to be the primary mechanism for the pacemaker activity in gastrointestinal activity, we examined the effect of5-HT on $[Ca^{2+}]_i$ oscillations in ICC. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC which are connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICC which were loaded with fluo-4 (Fig-). Figure a, c, e show images of basal (F0) and peak point (F1/F0) of Ca^{2+} oscillations. Further-more, in the presence of 5-HT (10μmol), $[Ca^{2+}]_i$ concentration in ICC was increased i.e. excited. $[Ca^{2+}]_i$ oscillations was studied with all the drugs used in the patch clamp recordings

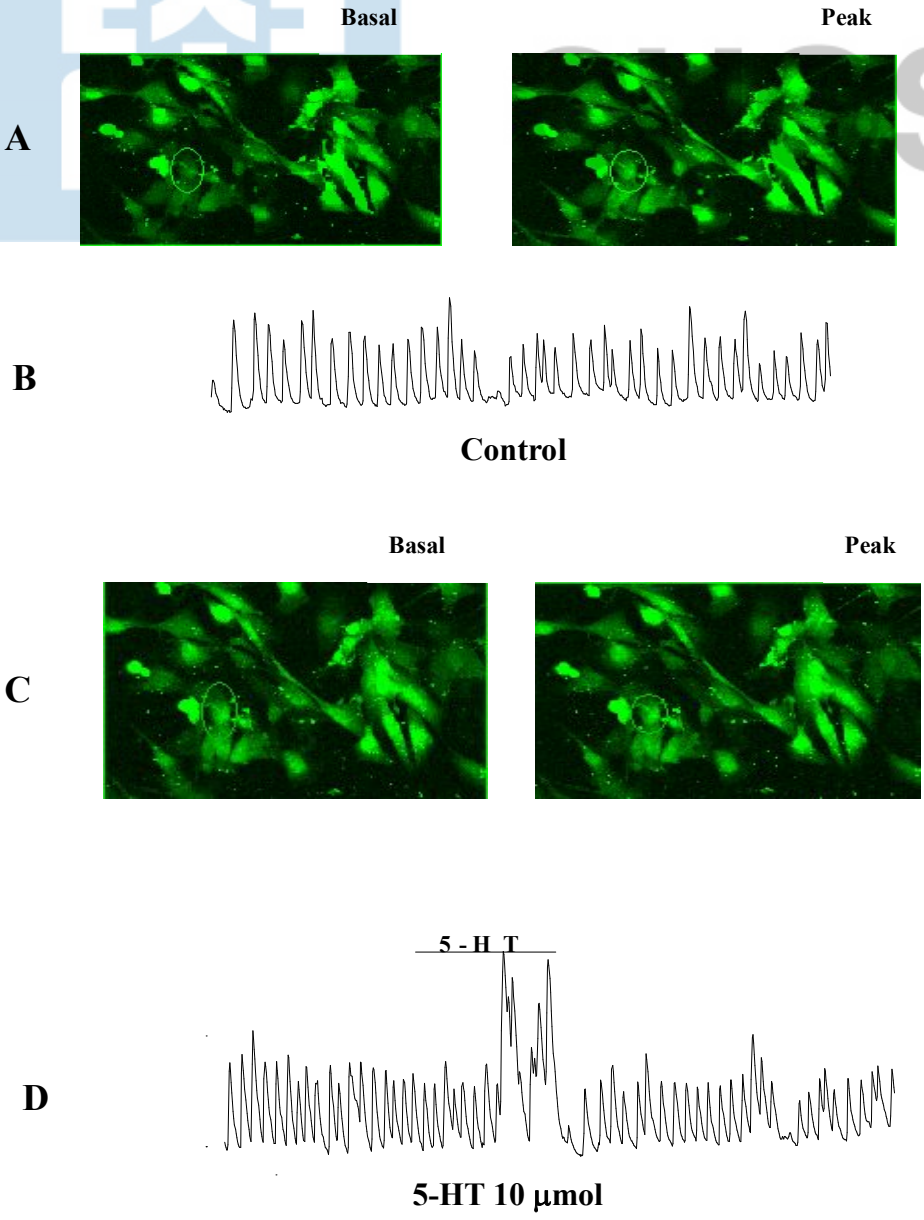


Fig.8 Effects of 5-HT on intracellular Ca^{2+} oscillation in cultured ICC from mouse small intestine. Sequential fluorescence intensity images of fluo-4-loaded cultured ICC in normal condition. **(A), (C)** shows the basal and peak point of ICC in absence and presence of 5-HT (10μmol)**. (B), (C)** Calcium oscillation recorded in normal condition and in presence of 5-HT respectively.

Identification of 5-HT receptors in ICC

To identify the receptor subtypes of serotonin, different serotonin receptor antagonists were pretreated and then co- treated with 5-HT. SDZ 205557, 5-HT_4 receptor antagonist, SB 269970, 5-HT_7 receptor antagonist, 3- Tropanylindole – 3 – carboxylate methiodide (3-TCM), 5-HT_3 antagonist and SB 204741, 5-HT_{2B} receptor antagonist, all were pretreated to cultured ICC at the concentration of 10μmol for 12 min and then co-treated with 5-HT 10μmol.

When the 5-HT 10μmol was alone treated to the cultured ICC it produced the inward current -55±80 pA. After the pretreatment of the 5-HT_4 receptor antagonist SDZ 205557, it partially blocked the inward current produced by 5-HT. the resting current produced in the presence of SDZ 205557 was -206±100 pA. 5-HT_7 receptor antagonist SB 269970 also partially blocked the inward current produced by 5-HT with the inward current -319± 29 pA.

Observing the above data, there was possibility that both these receptors play role on the 5-HT induced inward current. As there was no specific drug which antagonizes 5-HT_4 and 5-HT_7 receptors, the separate antagonists were mixed and treated to cultured ICC and then the 5-HT was applied on the pretreated ICC. The effect of the 5-HT 10μmol induced inward current was totally blocked with the resting current -26 ± 3 pA.

5-HT induced inward current was also blocked by the 5-HT_3 receptor antagonist 3-TCM. These results suggest that 5-HT 3, 4 and 7 receptors are responsible for the 5-HT induced inward current on ICC.

It has been reported that 5-HT_{2B} receptor is also present on ICC and is responsible for the proliferation of ICC. 5-HT_{2B} receptor antagonist SB 204741 10 μmol was used to check if it has any role on the 5-HT induced inward current. The pretreated ICC with SB

204741 did not block the effect of 5-HT suggesting that it has no role on the inward current produced by 5-HT. (Data not shown).

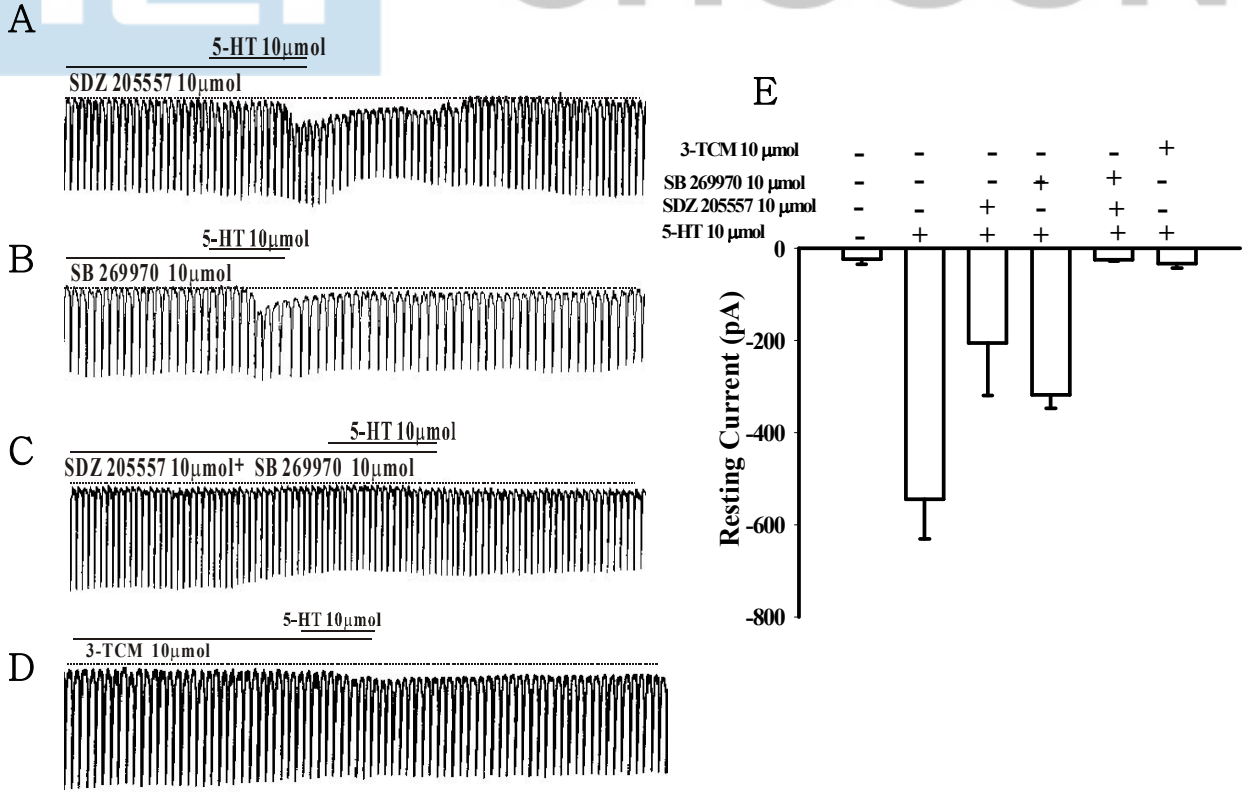


Fig.9 Effects of 5-HT antagonists on 5-HT induced responses on pacemaker currents in cultured ICC of the murine small intestine. **(A)** Pacemaker currents of ICC exposed to 5-HT (10 μmol) in the presence of 5-HT_4 receptor antagonist SDZ 205557 (10 μmol). In the presence of 5-HT_4 receptor antagonist, 5-HT still caused partial inward currents. **(B)** Pacemaker currents of ICC exposed to 5-HT (10 μmol) in the presence of 5-HT_7 receptor antagonist SB 269970 (10 μmol). Similar 5-HT effect was observed alike in presence of 5-HT_4 receptor antagonist. **(C)** Mixture of 5-HT_4 and 5-HT_7 receptor antagonists was treated to pacemaker current generating ICC and 5-HT (10 μmol) is exposed to it. There was a total blockage of the 5-HT effect. **(D)** Pacemaker currents of ICC exposed to the selective 5-HT_3 receptor antagonist (10 μmol). 5-HT_3 receptor antagonist in turn completely blocked the effect of 5-HT. Responses to 5-HT in presence of different receptor antagonists are summarized in **(E).** Bars represent mean values ± S.E. The dotted lines indicate the zero current levels.

Involvement of tyrosine kinase in the 5-HT induced inward current of pacemaker ICC.

The effects of the tyrosine kinase inhibitors on 5-HT induced inward current were studied on whole cell patches at a holding potential of -70mV. Genistein 10 μmol and Herbimycin A 5μmol were used to find out if the 5-HT induced inward current has any involvement of tyrosine kinase or not. In the experiments carried out, the 5-HT 10 μmol only produced the inward current of –521 ± 71 pA. When Genistein and Herbimycin were used, they both blocked the effect of 5-HT significantly with the inward current of -84 ± 15 pA and -112 ± 2 pA respectively. Furthermore, Diadzein (10 μM), an inactive analogue of Genistein, was used as a control for Genistein. No significant changes in inward current produced by 5-HT was observed with Diadzein (10 μM, n = 3, Fig.) compared with the 5-HT single treatment with inward current of -382 ± 110 pA. This finding suggests that the 5-HT induced effect is dependent on tyrosine kinase activation.

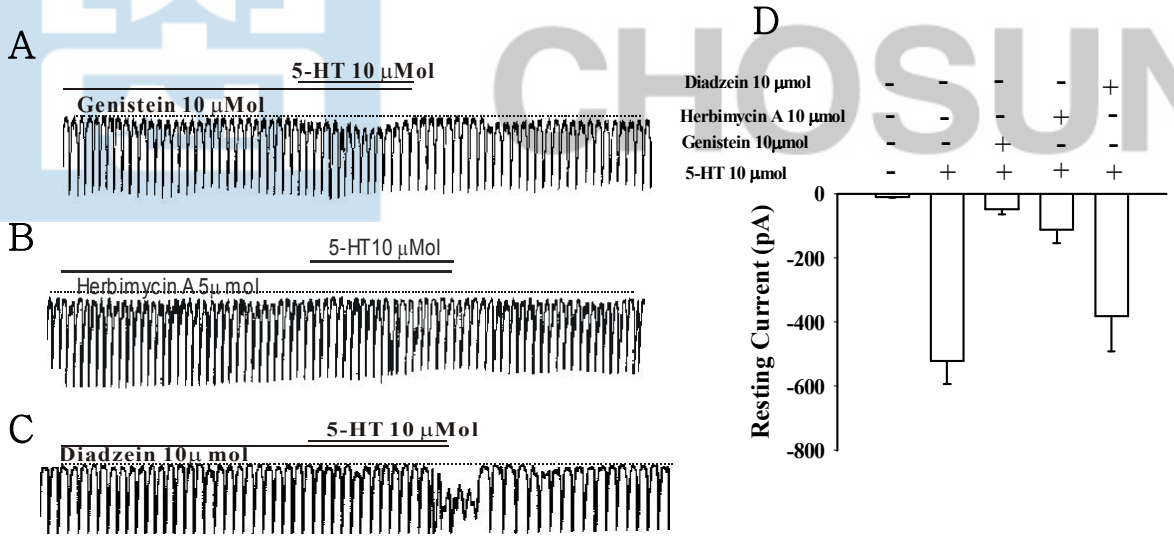


Fig. 10 Action of 5-HT in the presence of receptor tyrosine kinase inhibitors. **(A)** In presence of receptor tyrosine kinase inhibitor Genistein (10 μmol) and **(B)** Herbimycin A (10 μmol), 5-HT (10 μmol) did not show any effect in the pacemaker current generating ICC. **(C)** 5-HT showed the transient inward current in presence of Diadzein (10 μmol), an inactive analogue of Genistein. **(D)** Graphical representation of the effects by 5-HT in presence of different receptor tyrosine kinase blockers. The dotted lines indicate zero current levels.

Involvement of mitogen-activated protein kinases (MAPKs) in the 5-HT induced depolarization of pacemaker currents

Since many reports suggested 5-HT activate MAPKs in many cell types, investigation was done to find out whether MAPKs are involved in the 5-HT induced effects using PD98059, a p44/42 MAPK inhibitor, or SB203580, a p38 MAPK inhibitor, or a JNK (c-jun NH_2-terminal kinase) II inhibitor. In the presence of PD 98059, 5-HT showed the inward current but not so inward in absence of PD 98059. This means p44/42 has some role in the 5-HT induced inward current as PD 98059 partially blocked the effect. In presence of SB203580, there was no change in the effect of 5-HT on pacemaker current but JNK II inhibitor completely blocked the effect of the 5-HT in pacemaker current. The data thus obtained signifies that p38 MAPK has no action but c-jun NH_2-terminal kinase is involved in the 5-HT induced inward current.

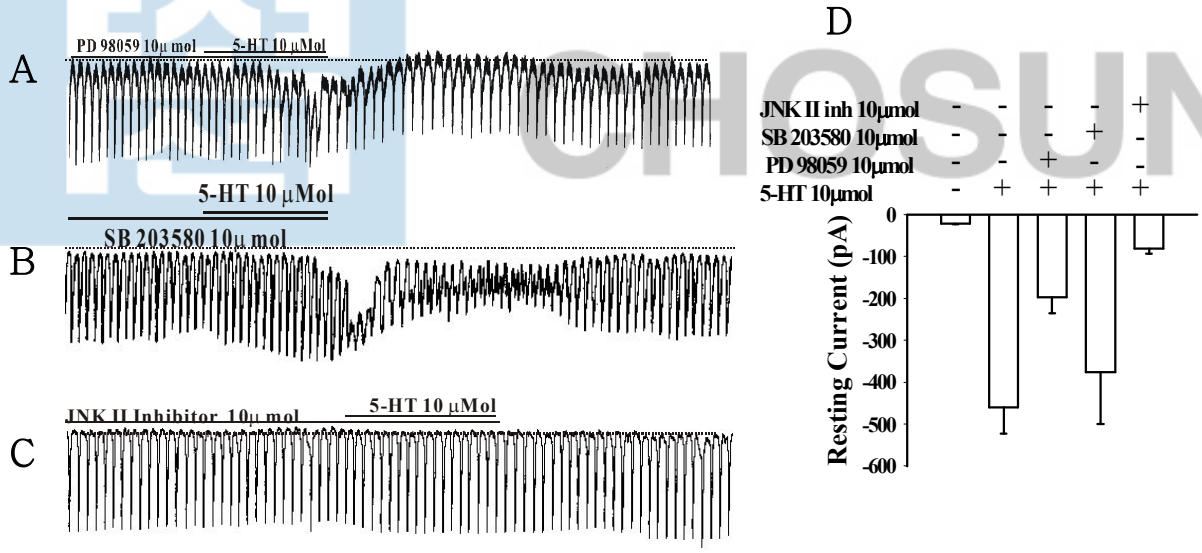


Fig.11 The effect of PD98059, SB203580, and the JNK inhibitor on the 5-HT induced effects on pacemaker currents of ICC from the murine intestine. **(A)**. The effect of 10μmol 5-HT on pacemaker currents after pretreating cells with 10 μM PD98059, a p44/42 MAPK inhibitor, for 15 minutes. **(B).** The effect of 10μmol 5-HT on pacemaker currents after pretreating cells with 10 μmol SB203580, a p38 MAPK inhibitor, for 15 minutes. **(C)**. The effect of 10μmol 5-HT on pacemaker currents after pretreating cells with 10 μmol JNK inhibitor, c-jun NH_2-terminal kinase inhibitor, for 15 minutes. **(D)**. Summarized data of 5-HT effects in presence of different MAP kinase inhibitors. The bars represents mean ± SE values (n=6/group) and the dotted lines indicate zero current levels.

DISCUSSION

In the present study, it was found out that 5-HT depolarizes the membrane potential and the pacemaker current in intestinal ICC. The inward current produced by 5-HT is not persistent but was found to be transient. The effect produced by 5-HT is mediated by the 5-HT_3, 5-HT_4 and 5-HT_7 receptors without the involvement of G- protein and receptor tyrosine and MAP kinase are involved in the 5-HT induced process.

The majority of serotonin (5-hydroxyruptamine, 5-HT) in the body is produced in the gastrointestinal tract, where it functions as a neurotransmitter, a neuromodulator and a paracrine factor. 5-hydroxytryptamine is produced and released in the gastrointestinal tract by enterochromaffin cells in the mucosa. 5- hydroxytryptamine acts on multiple distinct 5-HT receptors. Of the 7 classes of 5-HT receptors 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptors are known to be expressed in the gastrointestinal tract. ICC generate spontaneous pacemaker inward currents that depolarize the membrane; these spread to smooth muscle *via* gap junctions resulting in the depolarization of the membrane in smooth muscle and lead to contractions by generating an action potential through voltage dependent Ca^{2+} channel activation. Therefore ICC determines the frequency of contractions.

To date, many experiments were carried out and the 5-HT receptors in ICC were found but its physiological action on ICC and which receptor types are involved have not been carried out. There are many subtypes of 5-HT receptors, and their distribution in gastric tissues is heterogeneous, some distribute on nerve terminals and others distribute on smooth muscle (Gershon, 2004) The main objective of the present study was to determine the action of 5-HT on the spontaneous pacemaker current generated by ICC

and the receptor types involved and its signaling mechanism.

In the present study, when 5-HT (10 μmol) was exogenously applied to the ICC generating pacemaker current, it depolarized the inward current. The inward current thus produced was not persistent but transient. Depolarization was also observed in the pacemaker potential produced by the ICC. The next goal of this study was to find out which receptor subtypes of 5-HT were involved in the effect produced by 5-Hydroxytryptamine when applied exogenously. Molecular studies were done to find out which 5-HT receptors genes were expressed in ICC. From the molecular studies 5-HT_3, 5-HT_4 and 5-HT_7 were found to be expressed in ICC. Later in the patch clamp studies, ICC showing spontaneous inward current was treated with potent 5-HT_4 receptor antagonist SDZ 205557. This could not able to block the effect of the 5-HT completely suggesting that there may be another receptor responsible for the 5-HT action. 5-HT_7 receptor antagonist SB 269970 was pretreated to the spontaneously pacemaker current generating ICC and the action of 5-HT on the same cell was observed. Similar effect like 5-HT_4 antagonist was observed. This suggested that both the receptors might be responsible and acting for the 5-HT action on ICC. As commercial antagonist specific for both 5-HT_4 and 5-HT_7 receptors was not available, the mixture of both antagonist was pretreated and action of 5-HT was observed. This completely blocked the effect of 5-HT action suggesting that 5-HT_4 and 5-HT_7 receptors were present in ICC. Similar result was obtained in the calcium imaging experiments as well.

In the ENS, 5-HT_3 receptor immunoreactivity is expressed on neurons of the myenteric and submucosal plexuses, interstitial cells of Cajal and fibers in the circular and longitudinal muscle layers, submucosa and mucosa (Glatzle et al. 2002). 5-HT_3 receptor activation is associated with increased electrically evoked contractions of Guinea pig

and mouse stomach corpus and fundus circular smooth muscle (Bucggeit and Buhl 1994; Xue et al. 2006). This is supported by our results as well because in the presence of 5-HT 3 antagonist 3-TCM, 5-HT showed not inward current in the ICC. 5-HT_{2B} receptor was reported to be present in ICC which regulates the proliferation of ICC in mouse Jejunum (Gianrico et al. 2007). SB 204741, 5-HT_{2B} receptor antagonist, was used and it did not block the inward current produced by the exogenously applied 5-HT suggesting that 5-HT_{2B} receptor has no role in the 5-HT mediated action on ICC. This results thus obtained suggests 5-HT_3, 5-HT_4 and 5-HT_7 receptors are responsible for the 5-HT action in ICC.

In the next step, whether G-protein is involved in the 5-HT mediated action on ICC was carried out. For this experiment, GDPβS, a nonhydrolyzable guanosine 5-diphosphate (GDP) analogue which permanently inactivates GTP-binding proteins was used in the pipette solution. Even in the absence of GTP-binding protein, 5-HT showed the inward current and suggests that there is no involvement of G-protein in the action. Similar result was suggested in 5-HT_4 receptor by Aline Dunuis et al 2007. In presence of GDPβS, the effect of 5-HT on ICC was found more and detailed study is required.

When the extracellular calcium was omitted from the bath solution, the pacemaker current produced by ICC was completely inhibited. Even in absence of extracellular calcium, 5-HT showed its effect but not as normal in presence of extracellular calcium indicating that the extracellular calcium is required for the action of 5-HT.

The MAPKs signaling pathway plays an important role in the mediation of cellular responses including visceral smooth muscle contraction. Three principal MAPKs are expressed in various tissues: p44/42 MAPK, JNK, and p38 MAPK. Reports are there demonstrating activation of MAP kinase by 5-HT_7 receptors in hippocampal neurons

(D.S. Cowen et al, 2001). In the present study, PD98059, an inhibitor of p44/42 MAPK, partially inhibited 5-HT induced inhibition of pacemaker currents suggesting p44/42 MAPK may be involved in the modulation of pacemaker currents by 5-HT. SB203580, a p38 MAPK inhibitor, did not inhibited the action of 5-HT but JNK (c-jun NH_2-terminal kinase) II inhibitor completely inhibited the effect of 5-HT. the results obtained suggests that JNK is responsible for the action of the 5-HT and this is the first time reported.

Receptor tyrosine kinase also has a role in the 5-HT effect on ICC as the Genistein and Herbimycin A, receptor tyrosine kinase blocker, both inhibited the effect of 5-HT. Diadzein, an inactive analogue of Genistein failed to block the effect of 5-HT. These data proves that receptor tyrosine kinase has role on the action.

In the presence of exogenous 5-HT, intracellular Ca^{2+} level is increased. The periodic pacemaker activity of ICC is dependent on intracellular Ca^{2+} oscillations. The pacemaker mechanism is initiated by release of Ca^{2+} from the endoplasmic reticulum and is followed by reuptake of Ca^{2+} into the mitochondria (Sanders et al., 2006). In the studies done on 5-HT, the intracellular calcium was increased and returned back to normal condition showing similar effect as electrophysiological studies.

Experiments were carried out to see changes in intracellular calcium by 5-HT in presence of different 5-HT receptors antagonists as well as MAP kinase inhibitors used for the electrophysiology experiments. Similar results were obtained and the electrophysiological data were verified to be correct.

SUMMARY

Serotonin (5-hydroxytruptamine or 5-HT) is present in abundance within the gut, most stored in enterochromaffin cell granules. It plays a critical physiological role in regulation of Gastrointestinal (GI) function which is related to the expression of multiple 5-HT receptor types and subtypes. The effects of 5-HT on electrical responses of the membrane were investigated in Interstitial Cells of Cajal (ICC) isolated from the murine small intestine. For the analysis, whole cell patch clamp technique, Confocal microscopy for change in intracellular Calcium oscillation and molecular studies were performed. On application of 5-HT (10□mol) on the spontaneous pacemaker current generating ICC, it showed the transient inward current. In presence of 5-HT_4 and 5-HT_7 receptor antagonists, SDZ 205557 and SB 269970, the effect was partially blocked but completely antagonized in the presence of mixture of both the antagonists. 5-HT_3 receptor antagonist 3-TCM also abolished the effect of 5-HT. There is has no involvement of G protein as GDP□S, a nonhydrolyzable guanosine 5-diphosphate (GDP) analogue which permanently inactivates GTP-binding proteins, did not affect the 5-HT induced effect. The pretreatment with Ca^{2+} -free solution abolished the generation of pacemaker currents and suppressed the 5-HT -induced action. Moreover, MAP kinase is involved in 5-HT activity as JNK II Inhibitor, c-jun NH2-terminal kinase inhibitor, completely abolished the 5-HT effect where as PD 98059, a p44/42 MAPK inhibitor, and SB 203580, a p38 MAPK inhibitor, partially blocked and has no effect on 5-HT action respectively. In addition, Genistein and Herbimycin A, receptor tyrosine kinase inhibitors, also inhibited the 5-HT effect on ICC but Diadzein, an inactive analogue of Genistein failed to do so. These results suggest 5-HT when exogenously

applied to pacemaker current generating ICC produces transient inward current through 5-HT_3, 5-HT_4 and 5-HT_7 and this occur by the activation of receptor tyrosine kinase and MAP kinase activation.

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