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博士學位論文

Pharmacological Investigation of Hydrogen sulfide on Interstitial Cells of Cajal from Murine Small Intestine

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Abbreviations

ATP	adenosine triphosphate
$[Ca^{2+}]_c$	cytosolic calcium
cAMP	cyclic adenosine monophosphate
CBS	cystathionine-B- synthesis
cGMP	cyclic guanosine monophosphate
CSE	cystathionine-y layase
DIDS	[4,4' - Diisothiocyanatostilbene - 2,2' - disulfonic acid, disodium salt]
FFA	flufenamic acid
FITC	fluorescein isothiocyanate
GI	gastrointestinal
H_2S	hydrogen sulfide
ICC	interstitial cells of Cajal
IC-MY	myonteric interstitial cells
IP_3	inositol 1,3,5 triphosphate
JNK	C-jun N-terminal kinase
K_{ATP}	ATP sensitive potassium channel
LTP	long term potential
MAPK	mitogen activated protein kinase
NaHS	sodium hydrogen sulfide
NMDA	N- Methyl -D- aspartate
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phospholypase C
PGP	protein gene product
RT	reverse transcriptase

SMC	smooth muscle cells
SQ	9-(tetrahydro-2-furanyl)-9H-purin-6-amine
STICs	spontaneous transient inward currents
STOCs	spontaneous transient outward currents

Abstract

Pharmacological Investigation of Hydrogen sulfide on Interstitial Cells of Cajal from Murine Small Intestine

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Pharmacological investigation on the role of hydrogen sulfide in pacemaker activity and signal mechanisms in cultured interstitial cells of Cajal (ICC) from mouse was carried out using whole cell patch-clamp techniques at 30°C. Spontaneously active ICC generated pacemaker potential in the current clamp mode and pacemaker currents at a holding potential of −70 mV. Exogenously applied sodium hydrogen sulfide, a donor of hydrogen sulfide responded dose dependently to ICC. Hydrogen sulfide triggered effects on pacemaker activity by membrane polarization in lower concentration (100-300 μM) without changing pacemaker amplitudes and pacemaker frequency, and produced membrane hyperpolarization with inhibited pacemaker amplitude and pacemaker frequency of the pacemaker currents generated from ICC, and also increased resting currents in the outward direction in higher concentration (1 mM). NaHS induced inward excitatory pacemaker currents were abolished with pre-treatment of flufenamic acid, an inhibitor of voltage-independent non-selective cation channels. Sodium and calcium ion depleted bathing solution also abolished pacemaker currents as well as NaHS induced inward pacemaker currents but DIDS, an inhibitor of calcium sensitive chloride channels itself suppressed the pacemaker currents but did not response to NaHS induced

pacemaker currents. Similarly, thapsigargin, an inhibitor of Ca^{2+}-ATPase suppressed the NaHS induced effect and not responded to Calphostin C, an inhibitor of protein kinase C. NaHS induced inhibitory outward current was suppressed by pre-treatment of glibenclamide, an inhibitor of ATP-sensitive potassium channels without altering amplitudes and frequency suggest the partial involvement of ATP sensitive potassium channel but inhibitors of other potassium channels not responded to NaHS induced effect on pacemaker currents. Pre-treatment with ODQ (a guanylate cyclase inhibitor) did not change amplitudes and frequency of NaHS induced pacemaker current from ICC, but partially enhanced the inhibitory outward current. Similarly, pre-treatment of L-NAME, an inhibitor of nitric oxide also not affected the action of NaHS. But SQ 22536, an inhibitor of cAMP partly blocked the NaHS induced inhibitory outward currents. Calcium indicator (fluo-4) loaded ICC produced spontaneous calcium waves and NaHS significantly enhanced Spontaneous $[Ca^{2+}]_i$ oscillations. These results suggest that activation of non-selective cation channels, and influx of sodium and efflux of calcium ion from internal store is responsible for inward pacemaker currents while inhibitory mechanism on pacemaker activity might be due to partly activation of ATP-sensitive K^+ channels via partially dependent cAMP. NaHS regulated pacemaker activity was found independent to protein kinase C. Furthermore, RT-PCR results revealed that ICCs enriched population lack mRNA for neither CSE nor CBS, but a prominent expression of mRNA for CSE in whole dish cells could be the strong evidence to elucidate that ICC are not the target of hydrogen sulfide but the response of hydrogen sulfide may be transmitted either from smooth muscle cells or neuronal cells.

국 문 초 록

생쥐 소장에서 분리된 Interstitial Cells of Cajal에 대한 Hydrogen sulfide의 작용 기전

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위장관 운동성에 대한 hydrogen sulfide (H_2S)의 기능은 지금까지 평활근 수준으로 많이 연구가 이루어져 있지만, 위장관의 향도잡이 세포의 역할을 담당하는 Interstitial cells of Cajal (ICC)에 대한 H_2S의 연구는 아직까지 이루어지지 않고 있다. 따라서 본 연구는 세포막 고정법을 이용하여 ICC의 향도잡이 활동도에 대한 H_2S의 영향에 대하여 연구하고자 하였다. 전압고정 상태에서 낮은 농도 (100 - 300 μM) 의 H_2S는 긴장성 내향성 전류를 발생시키고, 높은 농도 (1 mM)에서는 ICC에서 발생되는 향도잡이 전류를 억제하였다. 낮은 농도의 H_2S에 의해 나타나는 내향성 전류는 비선택성 양이온 통로 억제제인 flufenamic acid와 Na^+ 또는 Ca^{2+}-free 용액에 의해 억제되었다. 이와 마찬가지로, Ca^{2+}-ATPase 억제제인 thapsigargin도 H_2S의 효과를 억제하였지만 protein kinase C 억제제인 calphostin C는 영향을 주지 못하였다.
높은 농도의 H_2S에 의한 억제효과는 ATP-sensitive K^+ 이온통로 억제제인 glibenclamide에 의해 약한 억제효과를 보인 것으로 보아, 부분적으로 ATP-sensitive K^+ 이온통로가 그 역할을 담당하는 것으로 생각되어 진다. 이와 더불어 다른 종류의 K^+ 이온통로 억제제는 H_2S의 효과에 큰 영향을 주지 못하였다. 또한 guanylate과 adenylate cyclase 억제제인 ODQ와 SQ-22536 그리고

nitric oxide 억제제인 NAME는 H_2S의 향도잡이 전류에 대한 역할에 영향을 주지 못하였다.

Ca^{2+} 표시자인 fluo-4로 처리된 ICC는 자발적인 Ca^{2+} 진동을 보여주었고 H_2S는 이러한 자발적인 Ca^{2+}의 진동을 활성화시키는 역할을 담당하였다.

이러한 결과는 H_2S가 비선택성 양이온과 세포 내 그리고 세포외 Ca^{2+}을 이용하여 긴장성 내향전류를 발생시키는 것으로 사료되며, H_2S에 의한 억제효과는 부분적이긴 하지만 ATP-sensitive K^+ 이온통로를 통하여 그 역할을 담당하는 것으로 생각된다.

I. Introduction

Hydrogen sulfide (H_2S), though traditionally considered as a toxic gas, recent reports suggest that it has a crucial physiological role as a third gasotransmitter after nitric oxide (NO) and carbon monoxide (CO) in the mammalian body. Rui Wang (2002) has recommended designating these gaseous transmitters as gasotransmitters because they are small molecules of gas, and are freely permeable to membrane. These gasotransmitters, mainly NO, CO and H_2S molecules share common chemical features and biological action modes, but differ from classical neurotransmitters and hormonal factors such as acetylcholine. Most of the actions of these "gasotransmitters" center on ion channels – sodium, potassium and calcium – and clearly have potentially major effects on their targets. Their production and metabolism are enzymatically regulated, and their effects are not dependent on specific membrane receptors. Moreover, they have well-defined specific functions at physiologically relevant concentrations. Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets. Being a gas, H_2S directly acts on ATP-sensitive K^+ channels independent of membrane receptors (Zhao et al., 2001). Abnormal metabolism and functions of H_2S contribute in the pathogenesis of many diseases, and plays a major role in physiological and pathological processes such as blood pressure regulation, neurotransmission, inflammatory processes etc.

H_2S is a colorless gas with a strong odor of rotten eggs. Reiffenstein et al (1992) has suggested that in the mammalian body, at a physiological pH of 7.4, approximately one-third of H_2S exists as the undissociated form and two-thirds as the hydrosulfide anion (HS^-). It can easily penetrate the plasma membranes of cells in the undissociated form because of its lipid

solubility. Its solubility in lipophilic solvents is fivefold greater than in water. Hydrogen sulfide is produced in substantial amounts by mammalian tissues and exerts many physiological effects suggesting its potential role as a regulatory mediator. In the context of the digestive system, Florucci et al (2006) have reported emerging roles of hydrogen sulfide in maintenance of mucosal integrity, regulation of blood flow, and modulation of inflammatory reactions.

Endogenously produced H_2S in mammalian is found to be expressed variously from tissues to tissues. According to Zhao et al (2001), the H_2S concentration of rat serum was <46 μM while the physiological concentration of H_2S in rat brain tissue has been reported to be 50-160 μM (Hosoki et al., 1997; Abe & Kimura, 1996). Two classes of pyridoxal-5'-phosphate-dependent enzymes— cystathionine beta-synthase (CBS) and cystathionine- gama-lyase (CSE) are responsible for the majority of the endogenous production of H_2S in mammalian tissues that use L-cysteine as the main substrate (Bukovska et al., 1994; Erickson et al., 1990; Stipanuk et al., 1982). CSE is a key enzyme of the trans-sulfuration pathway, which interconverts L-methionine and L-cysteine. It also uses L-cysteine as an alternative substrate to form H_2S (Erickson et al., 1990). As a pyridoxal phosphate-dependent enzyme, CSE is expressed in a range of mammalian cells and tissues, and it seems to be the main H_2S-forming enzyme in the liver, kidney, and cardiovascular system (Barber et al.,1999; Zhao et al., 2001). Hooski et al (1997) recently found hydrogen sulfide to exert a range of biological effects in gastrointestinal smooth muscle preparations in vitro. Previous report suggest that physiologically relevant concentration of H_2S specifically potentiate the activity of the NMDA

receptor and hippocampal long term potentiation is altered in CBS knockout mice (Eto et al., 2002a; Kimura et al., 2000).

Gastric motility is achieved through the coordinated activity of the enteric nervous system (ENS), interstitial cells of Cajal (ICC), and smooth muscle (SM) cells. It has been noted that ICC express c-kit and proper development of ICC depends upon signaling via the kit receptor pathway (Sanders et al., 1999). ICC and the ENS supply Smooth muscle cells with the necessary stimuli to contract and their coordinated contraction generates motility (Alberto and Martin, 2007). Furthermore, ICC control gastrointestinal motility, some pace slow waves and others act in enteric neurotransmission (Daniel, 2001).

Pacemaker ICC netwoks vary both in different regions of the gut, such as stomach, small intestine and large intestine and in different species such as mice, rats, guinea pigs and dogs. Sanders et al (1999) classified ICC networks as inter-muscular (ICC-MY), submucosal (ICC-SM), intramuscular (ICC-IM, ICC-DMP) based on their distribution pattern. Although ICC variants have been found in several locations within the gut wall, the ICC of the myenteric plexus (ICC-MY), which lies between the circular and longitudinal muscle layers, are considered largely responsible for the generation and maintenance of slow wave activity. Gastrointestinal activity is regulated by periodic generation of electrical activity called slow waves and which is supposed to be generated from interstitial cells of Cajal (Ward et al., 1994). Electrical slow waves generated by interstitial cells of Cajal account for the generation of spontaneous myogenic contractile behavior of the intestinal musculature (Sanders, 1996). The slow waves generated from ICC propagate into the intestinal circular muscle coat and trigger action potentials, in turn initiate contraction.

Previous studies on ICC found that pacemaking by ICC depends on cycling of Ca^{2+} between intracellular compartments (endoplasmic reticulum and mitochondria) mediated by specialized channels and pumps (Lu et al., 1995; Sanders et al., 2000; Suzuki et al., 2000; Ward et al., 2000) and rhythmic electrical activity per second is the consequence of periodic openings of voltage-insensitive, non-selective cation channels driven by the intracellular Ca^{2+} oscillations (Koh et al., 2002; Ward et al., 2000). Other investigators have suggested a role for Ca^{2+}regulated Cl^{-} channels in pacemaker activity (Huizinga et al., 2002; Tokutomi et al., 1995). Initiation of the electrical slow wave appears to be involved both pacemaker channels in ICC and intracellular calcium oscillations (Dickens et al., 1999). Similarly, Nakayami et al (2007) has suggested that intracellular calcium oscillation be the primary mechanism for the generation of pacemaker potentials, which may account for characteristic features of GI pacemaker activity. ICC generates the slow wave and modulates enteric neurotransmission. There is evidence from cultured murine ICC identifying both Ca^{2+} regulated non-selective cation channels and large-conductance Cl^{-} channels as the pacemaker channels. Simultaneous recordings of electric activity from ICC-MY and nearby smooth muscle cells have demonstrated that electric activity occurs first in ICC-MY and then initiates electric responses in the smooth muscle cells (Ward et al., 2000).

Although high concentrations of H_2S inhibit synaptic responses, physiological concentrations of H_2S facilitate the induction of long term potentiation (LTP) in the hippocampus. These observations suggest that endogenous H_2S functions as a neuromodulator in the brain. The pathway (s) through which H_2S exerts its dilatory activity has not been completely established, although it is evident that potassium channels, especially those sensitive to ATP (K_{ATP}), are involved, and, when opened by H_2S, the

resulting hyperpolarization initiates relaxation (Zhao et al., 2001; Abe and Kimura, 1996). Furthermore, it has been reported (Nagai et al., 2004) that exogenously applied NaHS regulates calcium waves in astrocytes and in primary rat microglial cells (Lee et al., 2006) but the role of NaHS in regulation of intracellular calcium in ICC is not yet clear.

Though few reports are available on the role of gasotransmitters in physiological and pathological processes such as blood pressure regulation, neurotransmission, and inflammatory processes including gastrointestinal motility, as far I know functional role of hydrogen sulfide in the modulation of gastrointestinal activity has not yet examined. The present study intends to investigate the role of exogenously applied hydrogen sulfide in regulating the pacemaker currents generated from the iterstitial cells of Cajal with physiological or pharmacological interventions. And the study will also explore the specific molecular targets of hydrogen sulfide on its target proteins. The role of H_2S in gastrointestinal activity will be elucidated by characterization of H_2S modulated pacemaker currents generated from interstitial cells of Cajal and signaling pathway involved to evoke H_2S effect will be addressed by electrophysiological recordings in cultured interstitial cells of Cajal from murine small intestine. In addition to electrophysiological study of NaHS on pacemaker activity, the role of NaHS on intracellular calcium mobilization will be revealed by calcium imaging in interstitial cells of Cajal. Present work also aims to explore the expression of H_2S producing enzymes CSE and CBS in pure ICC population as well as whole cell culture by RT- PCR experiments.

II. Materials and Methods

2.1. Experimental animals :

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

2.2 Preparation of cells :

Balb/C mice (8-13 days old) of either sex were anesthetized with diethyl ether and sacrificed by cervical dislocation. Abdominal cavities were opened from the ventral surface and small intestines from 1 cm below the pyloric ring to the cecum were removed. Intestines were opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution, tissues were pinned to the base of a Sylgard dish, and mucosa was removed by sharp dissection. Small stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated in Ca^{2+} free Hank's solution for 30 minutes. The muscle strips were enzymetically digested by incubating at 37°C for 12 minutes in the enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1 mg/ml, bovine serum albumin (Sigma) 1 mg/ml, trypsin inhibitor (Sigma) 0.5 mg/ml and triturated using fire blunted glass tubes with a range of holes. Cells were seeded onto sterile glass cover slips (20 mm) coated with poly - L-lysine (200 µl, Sigma) in a 35 mm culture dish, and cultured at 37°C in a 95 % O_2 and 5 % CO_2 in moisturized incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San

Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and stem cell factor (SCF, 5 ng/ml, Sigma). On the second day of culture, the growth media were replaced with growth media without stem cell factor and incubated further in the same condition till used.

2.3 Patch clamp experiments :

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Membrane currents or Membrane potentials were amplified by Axopatch 1-D (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, Vally view, OF, USA). Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C.

2.4 Immunohistochemical experiments :

Enzymatic digested intestinal preparation was seeded onto poly-L-lycine coated 20 mm cover slip in 35 mm culture dish and incubated for 24 hours in smooth muscle growth medium supplemented with murine stem cell factor (SCF, 5 ㎍/ml, Sigma) and 2 % antibiotics/antimycotics (Gibco, Grand Island, NY, USA). The humidified incubator was maintained with 5% CO_2 and 95% O_2 at 37°C. Well grown monolayer cells after 24 hours were washed with warm PBS for 30 minutes and treated with 5μl/ml C-kit antibody (FITC-antimouse), 1:200. The preparation was incubated for 15

minutes in room temperature and then washed with warm PBS. The C-kit labeling was observed and images were captured in excitation of 488 ηm under confocal microscope (Ultra VIEW ™ Confocal Imaging System).

2.5 Measurement of intracellular Ca^{2+} concentration :

Intracellular calcium mobilization induced by sodium hydrogen sulfide was examined by calcium sensitive indicater fluo-4/AM. The well grown ICC on cover slips (25 mm) was rinsed twice for 15 minutes with physiological bathing solution. The cells were then, incubated in 5 μM fluo-4/AM with 5% CO_2 at 37°C for 10 minutes and washed for 10 minutes with the physiological bath solution. Cover slips with ICC mounted on a perfusion chamber, and image acquired with a live cell imaging confocal microscope (600x; Ultraview, Nikon). Fluorescence was excited at a wavelength of 488 ηm, and emitted light was observed at 515 ηm. During the Ca^{2+} imaging, the temperature of the perfusion chamber containing the cultured ICCs was maintained at 30°C. The variations in intracellular Ca^{2+} fluorescence emission intensity were expressed as peak fluorescence intensity ratio (F_1/F_o) with respect to time of peak fluorescence, where F_o is the minimum fluorescence intensity during image capturing.

2.6 Molecular biological analysis :

2.6.1 Cell preparation and RNA isolation :

Muscles were isolated from mouse small intestine and enzymatically digested as mentioned previously. Well triturated muscles containing smooth muscle cells, ICC, neuronal cells, mast cells etc were seeded for 100 ml cultured dish and cultured for 2 days in smooth muscle growth

medium to get at least 100% confluation. Therefore, RNA isolated from this culture is referred as whole dish RNA. Fully automated magnetic cell separation technique ($ROBO_{SEP}$, Stem Cell Technologies Inc) was used to separate pure population of ICCs from above prepared cell suspension (cells>10 x 10^6/µl) by incubating in magnetic nanoparticles, mouse CD 117 kit and PE selection with extensive washing. ICC were labeled to C-kit antibody mouse CD 117 and PE selection cocktail and co-incubated with magnetic nanoparticles. When the CD 117 and PE selection labeled cells placed in magnet for 5 minutes and extensively washed with buffer solution C-kit positive cells were obtained as a positive selection. Total RNA were isolated separately from both preparations using TRIzol reagent according to manufacturer specifications (Invitrogen, Milan, Italy).

2.6.2 cDNA synthesis and amplification :

RT-PCR and cDNA amplification was carried by following Superscript™ One-Step RT-PCR with Platinum Taq (invitrogen). Thermal cycler was programed so that cDNA synthesis was followed immediately with PCR amplification automatically. RT-PCR kit contained the component of 2x reation mix, RT/platinum Taq Mix and 5 mM magnessium sulfate. Well vertexed and centrifuged components were added to the microcentrifuge tubes placed in ice as the following composition:- 2x reaction mix 25 µl, magnessium sulfate (5 mM) 12.5 µl, forward and reverse primer of each 2 1µl (10 pM/µl), tempalte RNA 2µl (2 µg/µl), RT/Platinum Taq Mix 1 µl. The final volume was adjusted to 50 µl, and, gently mixed and centrifuged to settle all the components at the bottom of the amplification tube. The PCR reactions for c-kit, myosin, β-actin, protein gene product (PGP) -9.5 amplified for 38 cycles in the thermal condition of 55° for 40 minutes, 94° for 3 seconds, 94° for 30 seconds, 58° for 30 seconds, 72° for 10 minutes.

But, the PCR reactions for CSE and CBS were amplified for 40 cycles at the annealing temperature of 62° for 30 seconds. The PCR products were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining. Gene specific primers used to amplified mRNA for different genes are summarized in table 1.

Table 1: A summarized information on gene specific primers used in amplification of corresponding mRNA from intestinal muscles.

S.N	Genes	reference	nucleotides	size(bp)
1	c-kit	Y00864	forward: CGCCTGCCGAAATGTATG reverse: GGTTCTCTGGGTTGGGGT	161
2	myosin e	NM_013607	forward: GAGAAAGGAAACACCAAG reverse: AACAAATGAAGCCTCGTT	233
3	b-actin	NM_007393	forward: TCTAGACTTCGAGCAGGAGA reverse: AATGTAGTTTCATGGATGCC	176
4	P G P 9.5	AF172334	forward: CGATGGAGATTAACCCCGAGATG reverse: TTTTCATGCTGGGCCGTGAG	168
5	CSE	NM_145953	forward: ATCCTGGGCTACCCTCTCACCCTC reverse: TGACTCGAACTTTTAAGGGTGCGCTG	370
6	CBS	NM_178224	forwar: ACACCATCTGCTGCTTGCTGGACA reverse: GAGAAGGGTTTTGACCAGGCACCTG	250

2.7 Physiological Bathing Solution :

A bathing solution is needed for constant superfusion over the cells coated on cover slips in culture dish. The composition of the bathing solution is very important in the whole-cell recordings. This solution used while experiments were in progress or in the initial stages of the experiment or to treat the drugs. Bath solution is also used in the name of normal or external solution as its chemical components were similar to the intact cells bathed on the physiological solution. Na^+ free and Ca^{2+} free solution were prepared by elimination of $CaCl_2$ and replacing NaCl with

NMDG from the external solution respectively. Well dissolved components of external solution buffered at pH 7.4 using tris base and stored at 4°C till used. The concentration of chemical components in physiological bathing solution were : KCl, 5 mM; NaCl, 135 mM; $CaCl_2$, 2 mM; glucose, 10 mM; $MgCl_2$, 1.2 mM and HEPES, 10 mM.

2.8 Internal Solution :

Patch pipette solution mimics the intracellular composition of interstitial cells of Cajal and its (pipette filling solution) composition is very important in the whole-cell recording. Well dissolved components of internal solution buffered at pH 7.2. To ensure the perfect giga seal, the pipette filing solution was filtered with a 0.22 μm filter because small amounts of dirt and debris can prevent giga seal formation. The aliquots (1 ml) of internal solution were stored frozen. The concentration of chemical components in internal solution were : K-aspartate, 120 mM; KCl, 20 mM; $MgCl_2$, 5 mM; K_2ATP, 2.7 mM; Na_2GTP, 0.1 mM; creatine phosphate disodium, 2.5 mM; HEPES, 5 mM; EGTA, 0.1 mM.

2.9 Drugs and chemicals :

All drugs were purchased either from Calbiochem Co (SQ 22536, flufenamic acid, calphostin C)., or Sigma Chemical Co (NaHS, ODQ, apamin, $BaCl_2$, TEA, thapsagargin, L-NAME, DIDS,) or RBI, Natick, NA, U.S.A (glibenclamide) or Invitrogen, Molecular Probes (Fluo 4/AM), and dissolved in appropriate solvent. Light sensitive drugs were protected from light and theirs appropriate aliquots were stored at -20°C, -4°C or at room temperature as mentioned on product information. Sodium hydrogen sulfide was used fresh in every experiment.

2.10 Statistical analysis :

Data analysis was carried out by using sigma plot software 9th version and was expressed as mean ± standard errors. Coefficient correlation was evaluated using the Student's 't' test. P values of <0.05 were taken to be statistically significant. The 'n' values reported in the text refer to the number of cells used in patch-clamp experiments.

III. Results

3.1. interstitial cell of Cajal expresses c-kit receptors :

Muscles isolated from murine gut wall comprises neuronal cells, smooth muscle cells and different types of ICC. ICC were identified immunohistochemically using c-kit antibody. Cells transfected with c-kit antibody, the proto-oncogene that encodes the receptor tyrosine kinase showed the immuno positive reactivity. Morphologically these c-kit positive cells were characterized with more or less triangular shape with multiple processes and are interconnected with other cells forming a networks as shown in figure 1a and b.

3.2 Spontaneous activity in interstitial cells of Cajal :

ICC for patch clamp were selected based on the typical morphological characteristics identified by immunohistochemical study (figure 1a and b). ICC are the origin of pacemaker activity and spontaneously generate pacemaker currents under voltage clamp at holding potential of -70 mV, and generate pacemaker potentials under current clamp (I=0). In present study, as shown in figure 2a, ICC generated pacemaker currents were recorded with resting currents, amplitudes and pacemaker frequency as -18.65 ± 3.23 pA, 761.67 ± 79.49 pA and 14.08 ± 0.44 cycles/min (n=30) respectively in voltage clamp. Similarly, amplitudes and frequency of membrane potential recorded from ICC under current clamp were measured as 18.2 ± 2.9 mV and 16.25 ± 0.85 cycles/minutes respectively (figure 2b).

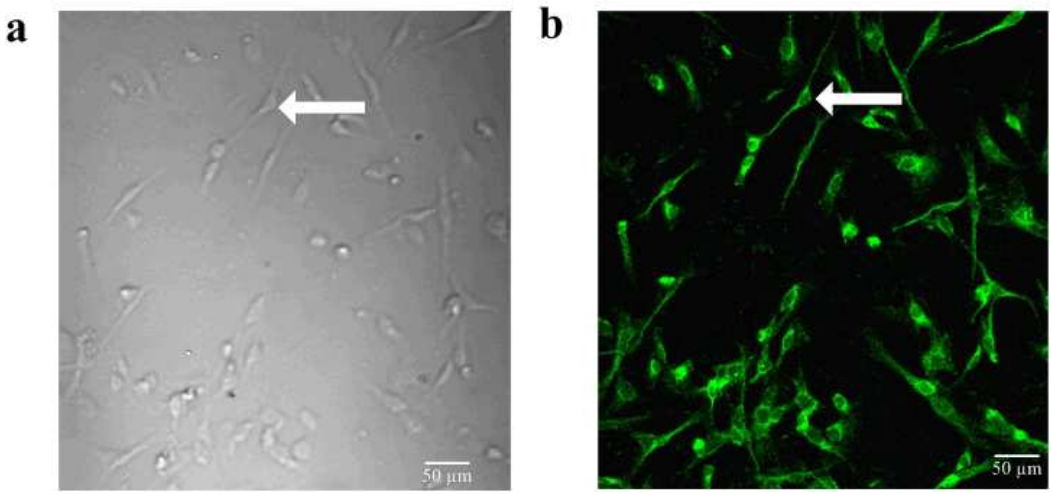

Figure 1: Myonteric interstitial cells of Cajal isolated from the small intestine of mouse cultured for 48 hours in smooth muscle growth medium. (A) Phage contrast image of culture. (B) Same cells immunohistochemicaly labeled with c-kit antibody. Arrow indicates the typical ICC used for electrophysiological recordings.

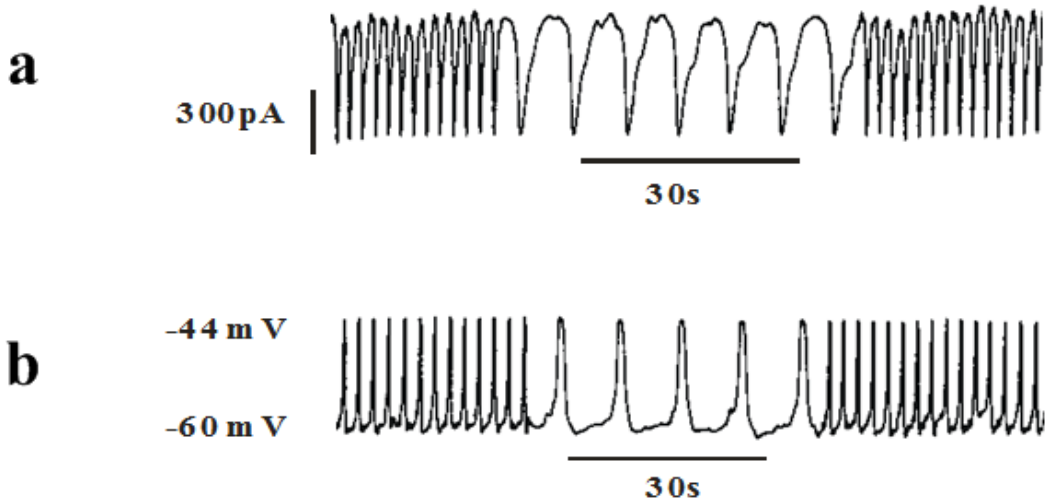

Figure 2: Pacemaker currents and pacemaker potentials generaterd from cultured Interstitial cells of Cajal from murine small intestine. (a) Pacemaker currents recorded under voltage clamp at −70 mV holding potential. (b) ICC produces membrane potentials under current clamp mode (I=0). Horizontal solid lines scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents.

3.3 Sodium hydrogen sulfide regulates the pacemaker current and membrane potentials generated from ICC :

Sodium hydrogen sulfide, a donor of hydrogen sulfide when exposed exogenously to ICC in a range of physiological relevant concentration (50 μM to 1 mM) evoked dose dependent effects on pacemaker currents in voltage clamp mode. It produced tonic inward currents in low concentration (50 μM - 100 μM) without significant change in amplitudes and frequency, and inhibited the frequency and amplitudes deviating the resting currents in outward direction at higher concentrations (300 μM-1 mM) under voltage clamp mode at a holding potential -70 mV. Similarly, NaHS when administered to bathing solution and recorded under current clamp (I=0), depolarized the membrane potential at low concentration and inhibited the resting currents and membrane hyper polarization in higher concentration (1 mM) as shown in Figure 3a and 3b.

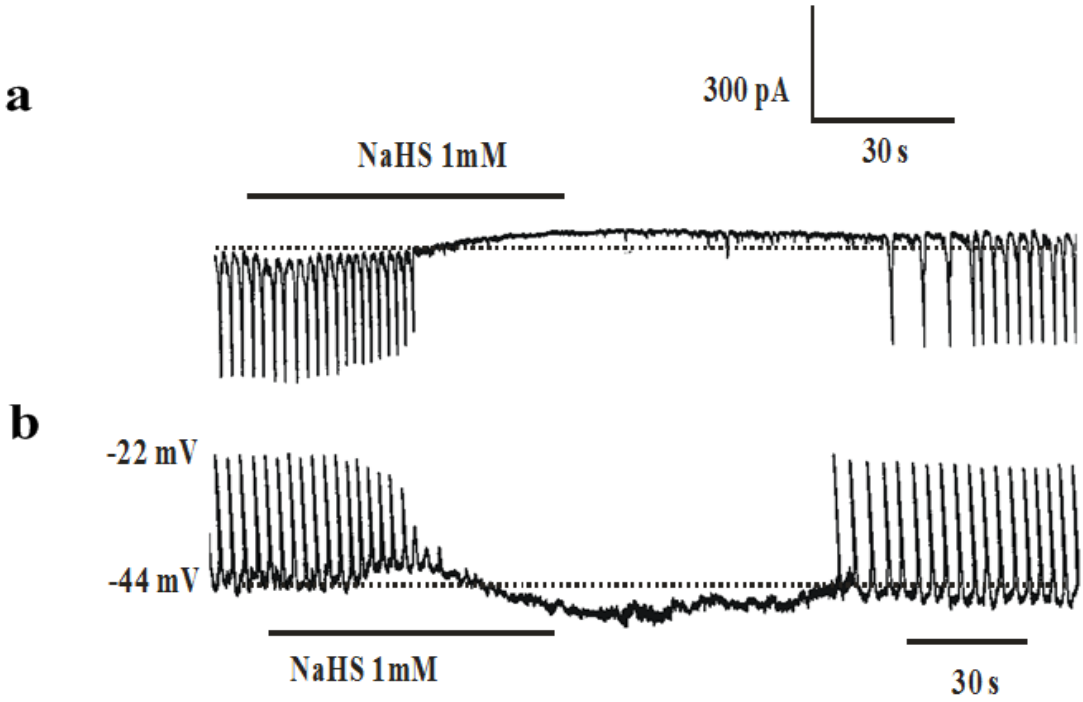

Figure 3: Effects of NaHS in cultured Interstitial cells of Cajal from murine small intestine. (a) ICC exposed to 1 mM NaHS initially depolarized the membrane and produced inward currents followed by inhibitory outward currents under voltage clamp mode at a holding potential of −70mV. (b) ICC exposed to 1 mM NaHS initially depolarized the membrane and then hyperpolarized by inhibiting frequency and amplitudes of pacemaker potentials under current clamp mode (I=0). Horizontal solid lines in figure 3a and 3b scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents. Horizontal dotted line represents the base line currents.

3.4 NaHS regulates the pacemaker activity in ICC in a concentration dependent manner :

ICC cultured from the murine small intestine was exposed to sodium hydrogen sulfide in a range of concentration from 50 μM to 1 mM. NaHS evoked its effect on pacemaker currents under voltage clamp mode at holding potential of -70 mV in a dose dependent way (figure 3a-d). Under control conditions, pacemaker amplitudes and frequency generated from the ICC were recorded as 18.65 ± 3.23 pA , 761.67 ± 79.49 pA and 14.08 ± 0.44 cycles/min respectively. When the ICC were incubated with 50 μM, 100 μM, 300 μM and 1 mM NaHS, the resting currents were measured as -219 ± 99.9, -464.44 ± 110.33, -211.94 ± 25.94, and -195 ± 31.84 pA as sown in figure 3e, respectively. Interestingly, in addition to tonic inward current, 1 mM NaHS produced outward current of 119.166 ± 34.52 pA (figure 4e). Similarly, pacemaker amplitudes were recorded as 616.66 ± 132.93, 650 ± 73.02, 13.75 ± 2.39 and 10.31 ± 3.14 pA respectively in response to ICC incubated in 50, 100, 300 μM and 1 mM NaHS (figure 4f). In the meantime , the mean control values of pacemaker frequency were changed respectively from 14.08 ± 0.44 to 14.66 ± 0.88, 14.33 ± 0.33, 0.5 ± 0.28 and 0.4 ± 0.14 cycles/minute in administration of 50 μM, 100 μM, 300 μM and 1 mM NaHS to bathing solution (figure 4g).

3.5. Role of Calphostin C on NaHS induced effects in pacemaker activity of ICC :

To examine the possible involvement of PKC pathway in H_2S modulated pacemaker currents from ICC, they were exposed to calphostin C (0.1 μM). Pacemaker currents did not response to administration of calphostin C (0.1 μM) for 10 minutes. Pre-incubation of ICC in calphostin C was followed by co-treatment with NaHS (1 mM). The change in resting currents during

pre-treatment and co-treatment were monitored. The mean control values (-195 ± 31.84 pA) of resting currents did not change (-170 ± 15.27 pA) significantly after ICC exposing to Calphostin C (figure 5a and 5b).

3.6. Effects of adenylyl cyclase inhibitor on NaHS induced effects in ICC pacemaker activity :

To examine whether the regulation of NaHS induced effects on pacemaker currents is mediated by cAMP pathways, SQ 22536, a specific inhibitor of adenyl cyclase was used. SQ 22536 (10 μM) applied to bath solution containing ICC did not response itself on the pacemaker activity. In continuous presence of SQ 22536 (10 μM) in bathing solution, NaHS (1 mM) was exposed to ICC, and monitored pacemaker activity. NaHS induced Pacemaker currents produced from ICC did not alter significantly in terms of pacemaker amplitudes (from 3.33 ± 0.76 to 5.66 ± 2.33 pA) and pacemaker frequency (0.77 ± 0.17 to 1.16 ± 0.44 cycles/minute) but a significant inhibition in NaHS inhibited resting currents (119.166 ± 34.52 to -16.66 ± 30.5517.64 pA) was recorded in the present study (figure 6a and 6b).

3.7 Effects of Flufenamic acid, DIDS and depletion of sodium in bathing solution on NaHS-induced pacemaker currents :

To rule out the possible functional mechanism of involvement of voltage independent non-selective cation channel and calcium activated chloride channels for producing tonic inward current by NaHS, effect of flufenamic acid, a voltage independent non-selective cation channel blocker; DIDS, calcium activated chloride channel blocker; and sodium depleted bathing solution were examined. ICC responded individually to FFA (50 μM), DIDS (100 μM) and sodium depleted bathing solution by completely inhibiting

amplitude, pacemaker frequency and deviating resting currents to outward direction except in a case of sodium depleted medium (figure 7a-c). Pacemaker currents were recorded in FFA inhibited condition with co-application of NaHS (1 mM). NaHS induced inward currents were significantly suppressed by 78.6% (-195 ± 31.84 to -42 ± 15.93 pA) in simultaneous administration of FFA and NaHS as shown in figure 7a and 7d (n=5). The inhibition of NaHS induced effects on pacemaker currents by FFA suggested that non-selective cation channels may be involved in NaHS regulated pacemaker currents. Similarly, ICC were pre-incubated in DIDS (100 μM) for 10 minutes and co-incubated with NaHS (1 mM). Unlike flufenamic acid, DIDS (100 μM) partially suppressed by 23.6% (-232.5 ± 48.67 to -177.5 ± 2.5 pA) the NaHS induced inward currents in simultaneous administration of DIDS and NaHS as shown in figure 7b and 6e (n=4). ICC were also incubated in sodium depleted external solution and recorded the pacemaker activity. Amplitudes and pacemaker frequency were completely abolished and resting currents shifted inwards. NaHS (1 mM) was applied simultaneously with sodium depleted normal solution and monitored the pacemaker activity. NaHS induced inward currents were significantly suppressed (67.2 %) from -195 ± 31.84 to -64 ± 16 pA by removal of sodium from bathing solution.

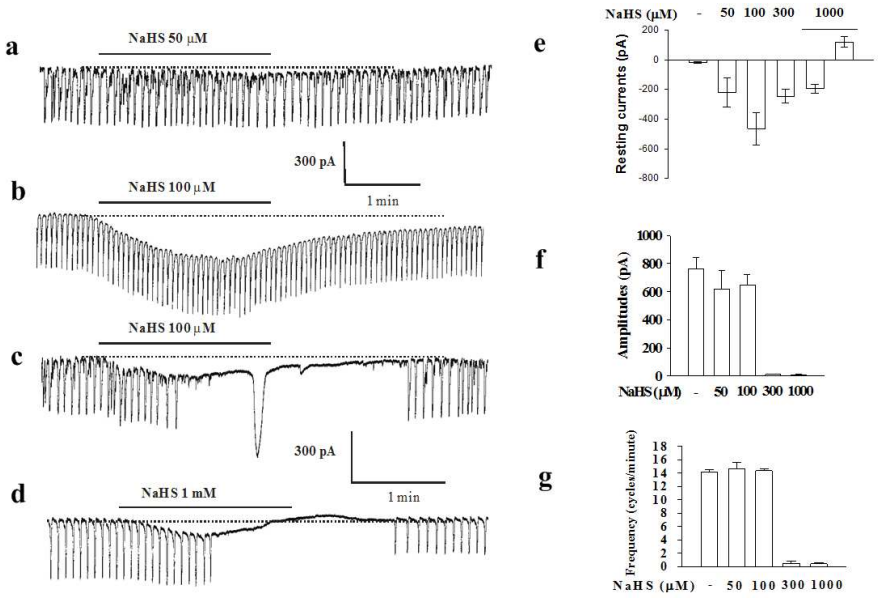

Figure 4: Effects of NaHS in cultured Interstitial cells of Cajal from murine small intestine. (a-d) ICC exposed to NaHS in a range of 50 μM, 100 μM, 300 μM and 1 mM concentration respectively at a holding potential of -70 mV under voltage clamp. (3d-f) Graphical presentation of NaHS induced effects on resting currents, amplitudes and frequency of pacemaker currents produced from ICC, respectively. Horizontal solid lines in figure 4a-4d scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents.

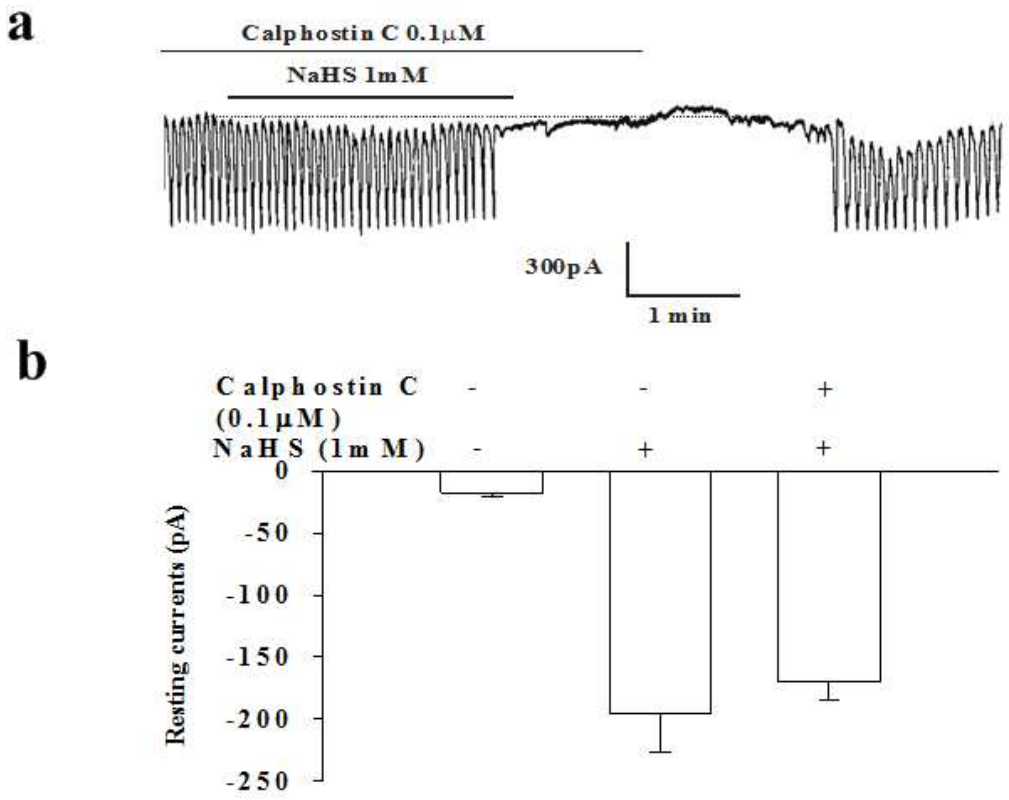

Figure 5: Effect of calphostin C, a potent inhibitor of protein kinase C on NaHS induced pacemaker currents in cultured interstitial cells of Cajal from murine small intestine. (a) Pre-treatment of calphostin C followed by co-treatment of NaHS (1 mM). (b) Graphical presentation on effects of calphostin C on NaHS induced resting currents of pacemaker currents. Horizontal solid line scales the time of patch clamp recording while vertical solid line scales the amplitudes of pacemaker currents in figure 4a. Horizontal dotted line indicates the baseline currents.

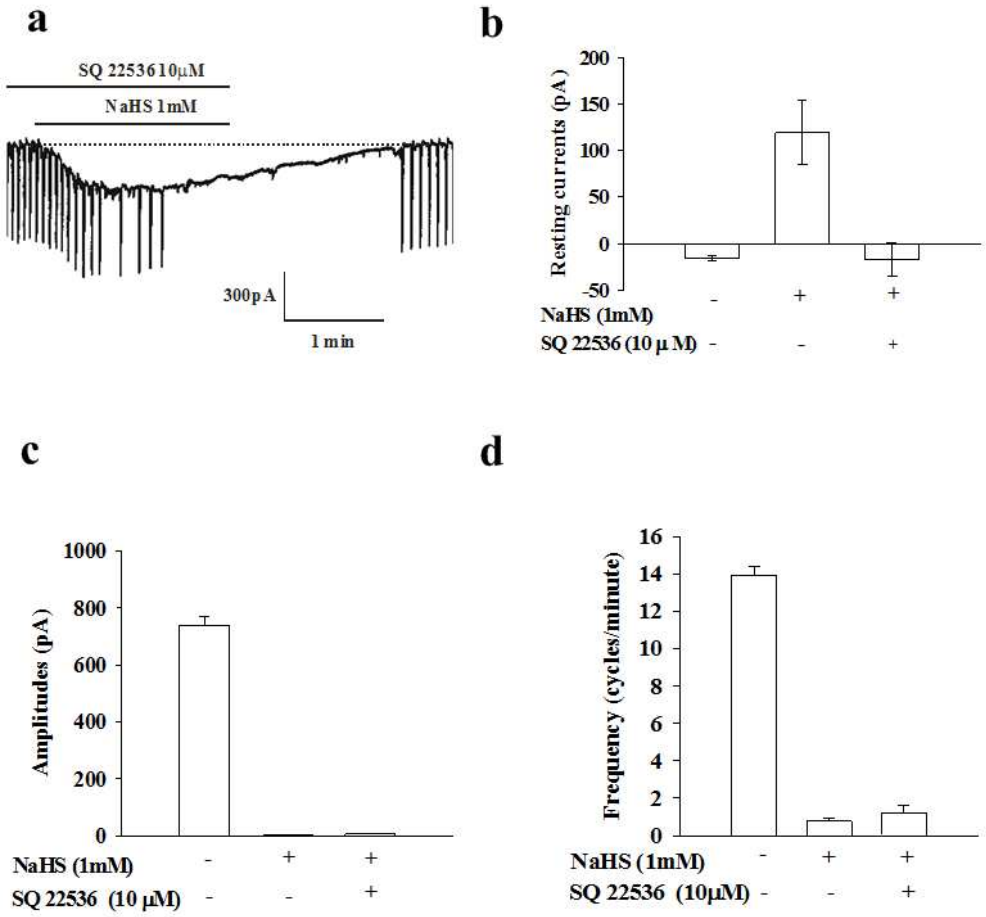

Figure 6: Effect of SQ 22536, an inhibitor of adenyl cyclase on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) pre-treatment of SQ followed by co-treatment of NaHS. (b-d) Graphical presentation of NaHS induced effects on resting currents, amplitudes and frequency respectively. Horizontal solid line in figure 6a scales the time of patch clamp recording while vertical solid line scales the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents.

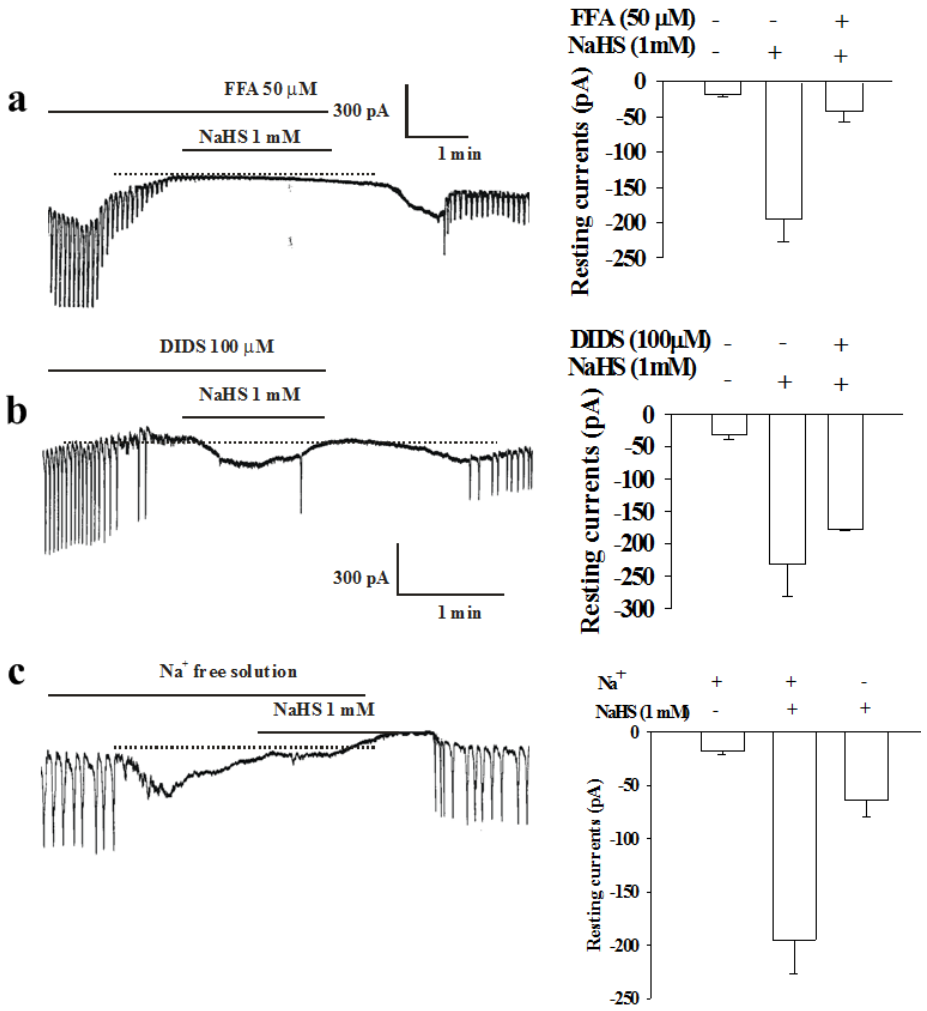

Figure 7: Effects of flufenamic acid, DIDS and Na^+ ion depleted bathing solution on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) pre-treatment of flufenamic acid followed by co-treatment of NaHS. (b) pre-treatment of DIDS followed by co-treatment of NaHS. (c) Depletion of Na^+ in bathing solution and administration of NaHS (1 mM). (d) Graphical presentation on effects of flufenamic acid on NaHS induced pacemaker currents. Horizontal solid lines scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents.

3.8 Effects of Thapsigargin and Calcium ion depletion in NaHS induced tonic inward currents produced from ICC :

To explore the effect of intracellular calcium on NaHS- induced pacemaker activity, ICC were exposed to calcium depleted bathing solution as well as thapsigargin, Ca^{2+} ATPase inhibitor of endoplasmic reticulum. Pacemaker current generated from ICC were completely inhibited in absence of Ca^{2+} and in presence of thapsigargin (5 μM) in bathing solution. ICC were further incubated simultaneously in NaHS (1 mM) and Ca^{2+} depleted medium and pacemaker activity was monitored under voltage clamp at -70 mV. Similarly, ICC were pre-treated with thapsigargin (5 μM) and followed by simultaneous treatment with NaHS (1 mM). Thapsigargin significantly abolished (95.8 %) the NaHS induced resting currents from -195 ± 31.84 to -8 ± 14.04 pA but Ca^{2+} depleted bathing solution did not significantly change the NaHS induced effect on pacemaker activity as shown in figure 8a and c. The NaHS induced resting current is partially suppressed (11.8%) from -195 ± 31.84 to -172 ± 63.15 pA. This result may suggest that NaHS induced effect on pacemaker activity is regulated by Ca^{2+} released from internal store (endoplasmic reticulum) rather than Ca^{2+} influx from extracellular store.

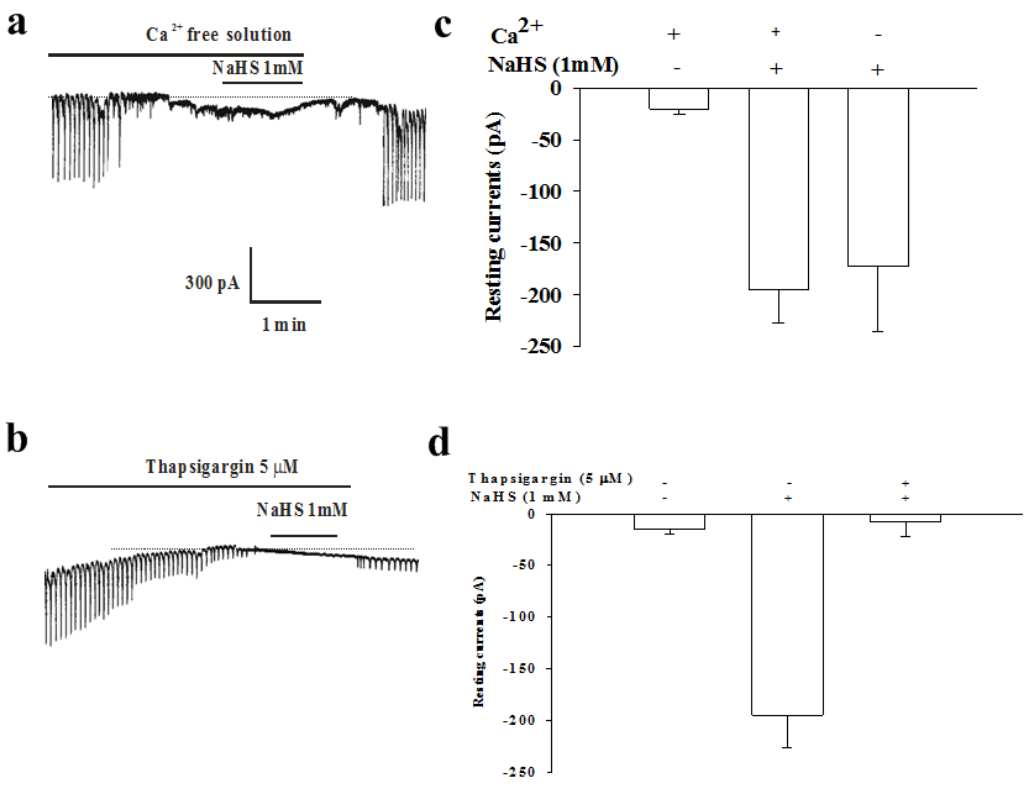

Figure 8: Effects of Ca^{2+} free and thapsigargin on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) Incubation of ICC in Ca^{2+} free solution and followed by co-treatment with NaHS (1 mM). (b) Pre-treatment of thapsigargin (5 μM) followed by co-treatment with NaHS (1 mM). (c-d) Graphical presentation on effects of Ca^{2+} depleted medium and in presence of thapsigargin respectively on NaHS induced pacemaker currents. Horizontal solid lines in figure 8a and 8b) scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents.

3.9 Effects of guanylyl cyclase inhibitor and L-NAME, an inhibitor of NO on NaHS induced effects in ICC pacemaker activity :

To explore whether NaHS induced effects on pacemaker currents is mediated by cGMP pathways, ODQ, a specific inhibitor of soluble guanylyl cyclase was used. ODQ (20 μM) itself when administered to ICC did not alter the pacemaker activity. In continuous presence of ODQ (20 μM) in bathing solution, ICC was exposed to NaHS (1 mM) and pacemaker activity was monitored. NaHS-induced Pacemaker currents produced from ICC was not altered significantly in terms of pacemaker amplitudes (from 3.06 ± 0.63 to 3 ± 1 pA) and pacemaker frequency (0.77 ± 0.17 to 0.83 ± 0.16 cycles/minute) but significant enhancement in NaHS inhibited resting currents by 56.64 % (119.166 ± 34.52 to 186.66 ± 35.27 pA) was measured as shown in figure 9a and 9c. Similarly, to explore whether NaHS induced inhibitory effect is mediated by the production of nitric oxide (NO), ICC were pre-incubated in L-NAME (20 μM), an inhibitor of inducible nitric oxide synthases for 10 minutes followed by co-incubation in NaHS (1 mM). NaHS induced inhibitory effects on pacemaker currents remained unchanged as shown in figure 9b.

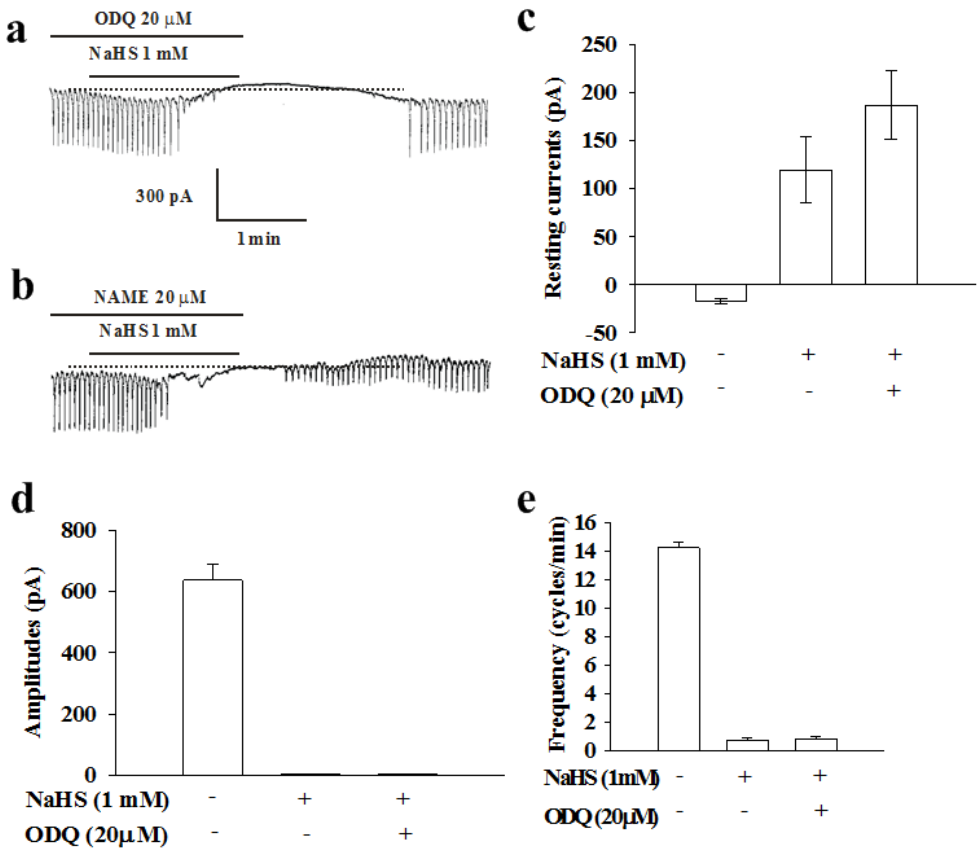

Figure 9: Effects of ODQ and L-NAME on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) Pre-treatment of ODQ (20 μM) for 10 minutes and followed by co-treatment of NaHS (1 mM). (b) Pre-treatment of L-NAME (20 μM) for 10 minutes and followed by co-treatment with NaHS (1 mM). (c, d and e) Graphical presentation of effects of ODQ on NaHS induced pacemaker currents. Horizontal solid lines in figures (9a and 9b) scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents.

3.10 Effect of ATP sensitive potassium channels on NaHS induced pacemaker currents :

To rule out the possible involvement of ATP activated potassium channels, ICC were exposed to glibenclamide (10 μM), an ATP sensitive potassium channels blocker for 10 minutes and NaHS (1 mM) was treated in continuous presence of glibenclamide as shown in figure 10a. Glibenclamide itself did not alter the pacemaker currents but NaHS induced outward currents was significantly suppressed (119.16 ± 34.52 to 0.46 ± 0.16 pA) by 99.6 % in simultaneous treatment of glibenclamide and NaHS (figure 9b), but it did not cause any significant change on inhibitory effects of NaHS on pacemaker amplitudes and pacemaker frequency. Therefore, the partial involvements of ATP sensitive potassium channel might be responsible to regulate NaHS induced pacemaker activity in ICC (n=5).

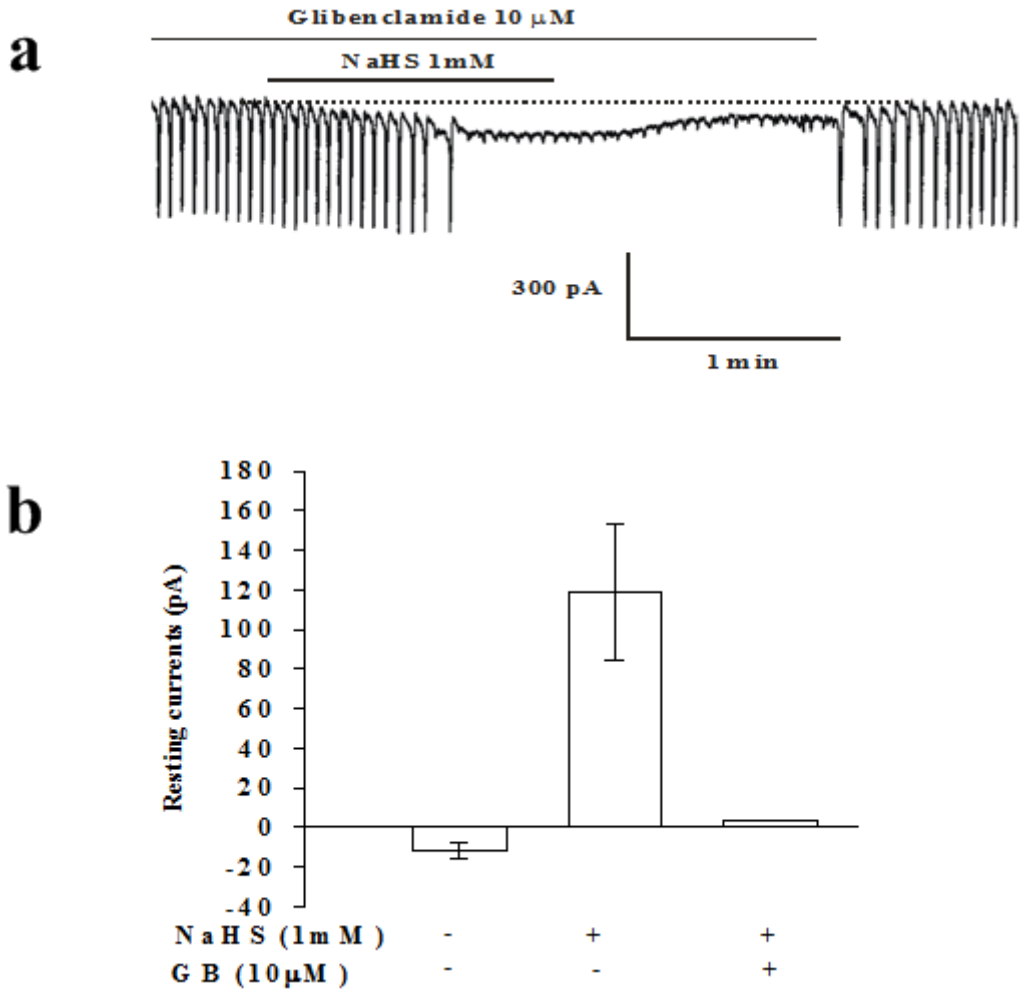

Figure 10: Effects of Glibinclamide on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) Pre-treatment of glibenclamide (10 μM) followed by co-treatment of NaHS (1 mM). (b) Graphical presentation of effects of glibenclamide on NaHS induced pacemaker currents. Horizontal solid line in figure 10a scales the time of patch clamp recording while vertical solid line scales the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents. GB= glibenclamide.

3.11 Effect of Ca^{2+} sensitive potassium channels on NaHS induced pacemaker currents :

To investigate the possibility of involvement of Ca^{2+} sensitive potassium channels for inhibitory action of NaHS on ICC, ICC were first incubated in 20 mM tetraethylammonium chloride (TEA) and co-treated with NaHS (1 mM). TEA itself did not change the pacemaker activity. NaHS induced pacemaker activity was recorded without any significant alteration in inhibitory pacemaker currents as shown in figure 11a. Similarly, apamin, an inhibitor of apamin sensitive and calcium activated potassium channel on NaHS induced effects was tested. ICC were incubated in 0.1 μM apamin and co-treated with NaHS (1 mM). Apamin itself did not change the pacemaker activity. NaHS induced pacemaker activity was recorded without any significant alteration in inhibitory pacemaker currents in presence of inhibitor as shown in figure 11b. It may suggest that Ca^{2+}-activated potassium channels may not be involved in NaHS induced effects on pacemaker currents.

3.12 Effect of Ba^{+} on NaHS induced pacemaker currents :

The role of Ba^{+} in extracellular mediam to influence the potassium current induced by NaHS was examined. ICC were pre-treated with 50 μM $BaCl_2$ for 10 minutes and NaHS (1 mM) was applied simultaneously as shown in figure 11c. The pacemaker current induced by NaHS was not altered significantly but outward current was found suppressed. This result showed that potassium channels may be involved partially in NaHS regulated pacemaker activity in gastrointestinal tract.

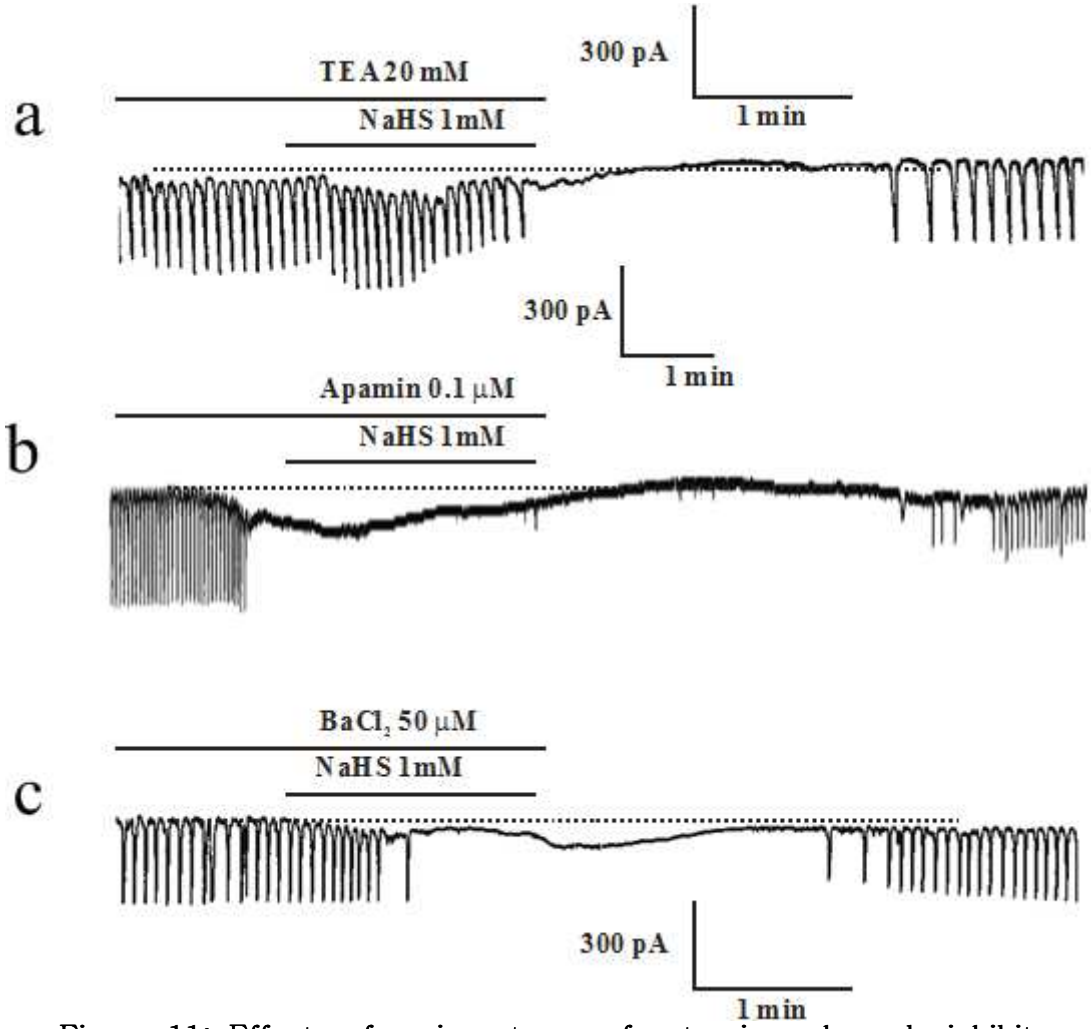

Figure 11: Effects of various types of potassium channels inhibitors on NaHS induced pacemaker currents in cultured Interstitial cells of Cajal from murine small intestine. (a) Pre-treatment of TEA (20 mM) followed by co-treatment with NaHS (1 mM). (b) Pre-treatment of apamin (0.1 μM) followed by co-treatment with NaHS (1 mM). (c) Pre-treatment of $BaCl_2$ (50 μM) followed by co-treatment with NaHS (1 mM). Horizontal solid lines in figure 11a-c scale the time of patch clamp recording while vertical solid lines scale the amplitudes of pacemaker currents. Horizontal dotted lines indicate the baseline currents.

3.13 Experiment on extracellular calcium mobilization in ICC :

Well cultured ICC were incubated in fluo-4/AM, a cell permeable calcium indicator dye and intracellular calcium oscillations were recorded. Spontaneous calcium waves were generated from ICC and clearly observed under live cell imaging confocal microscope as a calcium spark. As shown in figure 12c, frequency of calcium waves recorded from ICC (n=4) was 8.66 ± 1.33 cycles/minutes. Exogenous application of 1 mM NaHS induced duel effects on intracellular calcium mobilization in ICC. Initially intracellular calcium level in ICC was obviously increased by 12.5% from basal level of 0.528 ± 0.196 to 0.59 ± 0.22 (F1/F0) and then declined by 49.1% from control level of 0.528 ± 0.196 to 0.268 ± 0.224 (F1/F0) in presence of 1 mM NaHS which was significantly lower than basal calcium level (0.528 ± 0.196 F1/F0). Interestingly, in addition to elevation in calcium intensity, NaHS also significantly increased the frequency of calcium waves by 38.46% from 8.66 ± 1.33 to 12 ± 1.15 cycles/minutes as shown in figure 12c.

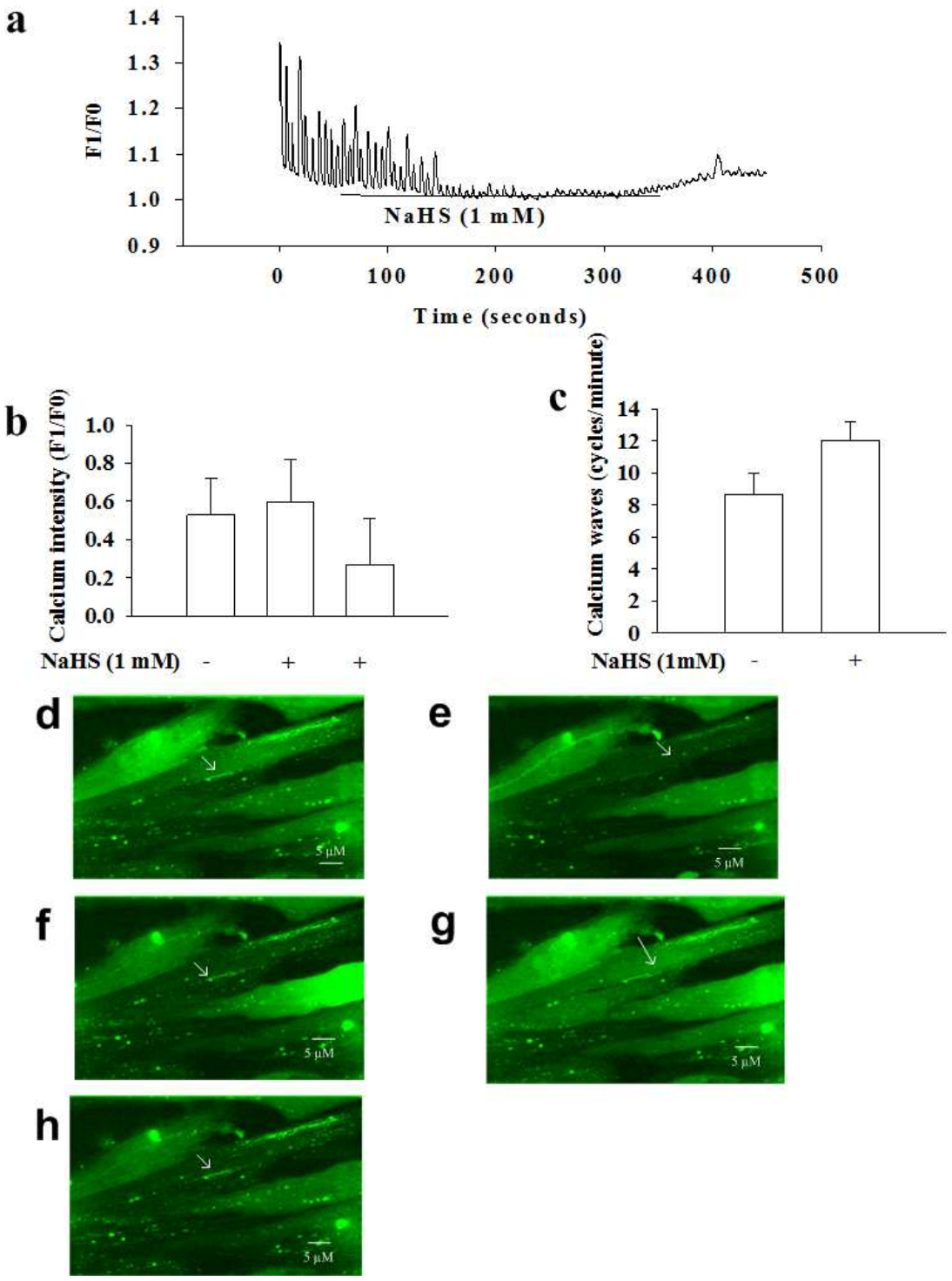
a
F1/F0
1.4
1.3
1.2
1.1
1.0
0.9
NaHS (1 mM)
0
100
200
300
400
500
Time (seconds)
b
Calcium intensity (F1/F0)
1.0
0.8
0.6
0.4
0.2
0.0
NaHS (1 mM) - + +
c
Calcium waves (cycles/minute)
14
12
10
8
6
4
2
0
NaHS (1mM) - +
d
e
f
g
h
5 μM

Figure 12: Effects of NaHS on intracellular calcium oscillation in ICC. (a) Intracellular calcium wave (F1/F0) recorded from ICC loaded with Fluo-4/AM, calcium indicator dye in control condition. (b) NaHS (1 mM) induced change in intracellular calcium intensity. (c) Graphical presentation on effect of NaHS on periodic calcium oscillation (cycles/minutes). (d-e) Images of ICC acquired (5 f/s) in basal and calcium accumulated condition in absence of NaHS. (f-g) Images of ICC acquired (5 f/s) in calcium accumulated condition in presence of NaHS (1 mM). (h) Image acquired during inhibition of calcium waves. The arrows indicated the region of interest to analyze the intracellular calcium level before and after treatment. The horizontal solid line at right bottom corner of the images measures the length of region of interest (ICC).

3.14 Purified population of ICC expresses c-kit genes :

To evaluate the physiological importance of endogenously generated H_2S by the activation of enzymes responsible were determined in gut musularis tissues. Cells separated by magnetic cell separation technique from freshly prepared muscle cells using c-kit antibody were enriched with c-kit positives ICC. To examine whether purified population of ICC were contaminated with smooth muscle cells and neuronal cells, RT-PCR was performed after isolation of mRNA using one step RT-PCR kits and PCR products were visualized. ICC enriched population expressed c-kit gene expression (161 bp) but did not express myosin (233 bp) and PGP 9.5 (168 bp) as shown in figure 13a. mRNA isolated from magnetic cell separation was also amplified with primer β-actin as a positive control. A fragment of mRNA for β-actin (176 bp) was detected in ICC sorted population. The results suggest that ICC enriched population isolated by magnetic cell separation was not contaminated with smooth muscle and neuronal cells.

3.15 Expression of CSE and CBS enzymes in musculature of mouse small intestine :

To examine the availability of CSE and CBS to be responsible for the production of hydrogen sulfide in gut tunica musculature, a fragment of mRNA for CSE (370 bp) and CBS (250 bp) isolated from sorted ICC or cultured whole tissues were amplified using gene specific primers CSE and CBS. As shown in figure 13a and 13b, mRNA neither for CSE nor CBS were amplified in sorted ICC. In a mean time a fragment of mRNA for CSE (370 bp) and CBS (250 bp) isolated from cultured whole tissues were amplified. A prominent amplification of mRNA for CSE (370 bp) and

sparsely amplification of mRNA for CBS (250 bp) was observed in cultured whole tissues.

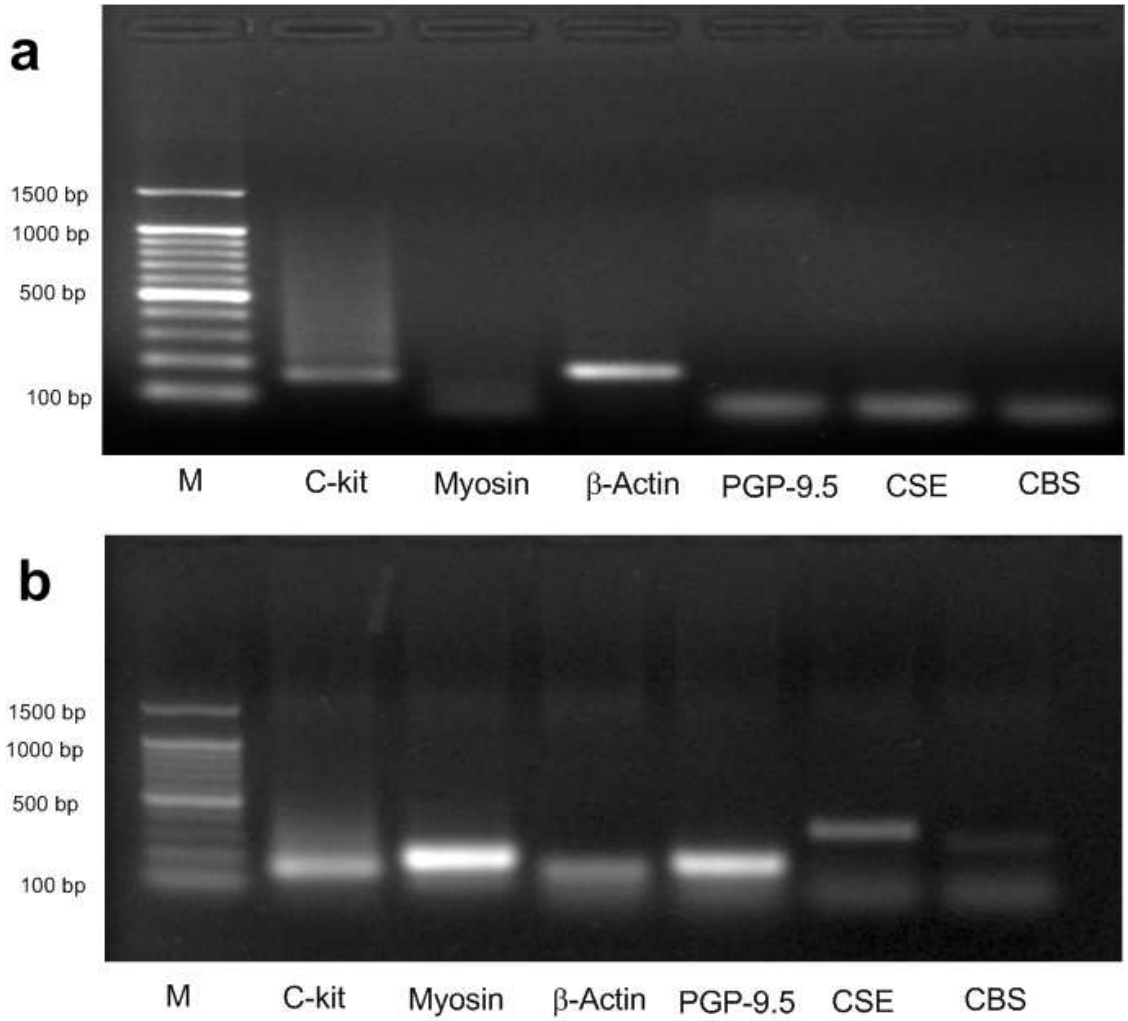

Figure 13: Expression of mRNA in different muscle cells and enzymes detected by RT-PCR using gene specific marker. (a) Amplified cDNA prepared from pure ICC separated by magnetic cell separation and visualized in 2% gel. (b) Amplified cDNA from intestinal muscle cells cultured in whole dishand visualized in 2% gel. M-DNA marker, PGP-neuronal cell marker, CSE-cystathionine gama lyase, CBS-Cystathionine beta synthase.

IV. Discussion

Hydrogen sulfide (H_2S) is among a family of endogenous molecules of gas, defined as gasotransmitters and plays a crucial role in physiological and pathological processes such as blood pressure regulation, neurotransmission, and inflammatory processes including gastrointestinal motility. Several reports have suggested that the mode of action of gasotransmitters is different than that of classical neurotransmitters and centers on ion channels –sodium, potassium and calcium and clearly have potentially major effects on their targets. As far my knowledge, though extensive research has been carried out in the gasotransmitters, the physiological role of hydrogen sulfide especially on gastrointestinal motility is still scant.

In the present study, NaHS concentration-dependently regulated pacemaker activity acting on interstitial cells of Cajal (ICC). A simple and reliable immunohistochemical method revealed that ICC expressed c-kit, the proto-oncogene that encodes the receptor tyrosine kinase (Maeda et al.,1992; Ward et al., 1994,1995; Torihashi et al., 1995; Huizinga et al., 1995). ICC are the origin of pacemaker activity and spontaneously generate the inward pacemaker currents called as slow waves. These electrical slow waves generated by interstitial cells of Cajal account for the generation of spontaneous myogenic contractile behavior of the intestinal musculature (Ward et al., 1994). The slow waves initiated from ICC propagate into the intestinal circular muscle coat via gap junctions and trigger action potentials, in turn initiate contraction.

Pacemaker activity in cultured ICC was studied using patch clamp technique. It revealed that ICC generated spontanious periodic inward

currents under voltage clamp. ICC were exposed exogenously to different concentrations of NaHS and the pacemaker activity was monitored. NaHS evoked dose dependent effects on pacemaker currents in voltage clamp mode. Interestingly, in presence of NaHS in bathing solution ICC generated duel effects. In present study the maximum inhibitory effects on pacemaker currents from ICC was gained in 1 mM NaHS which is relatively higher than that of endogenously expressed physiological concentration in mammalian tissues. As suggested by Reiffenstein et al (1992) the maximum yield of NaHS, a donor of hydrogen sulfide is about 33% of applied concentration, the exogenous application of NaHS in present study was under the physiological concentration. Initially membrane currents depolarized without alteration of amplitudes and frequency and then membrane hyperpolarized with inhibited frequency and amplitudes when ICC was exposed to higher concentration (300-1000 μM) under current clamp mode.

Koh et al (1998) and Thomsen et al (1998) have suggested that pacemaker currents generated from ICC are mediated by the activation of voltage independent non-selective cation channels. It has been considered that the inward pacemaker current is mediated by the activation of non-selective cation channels. Non-selective cation channels can carry an electric charge for ICC pacemaking currents, and many of these channels can permeate Ca^{2+} (Nakyama et al., 2007). As suggested by Kim et al (2007), ICC was exposed to FFA, an inhibitor of non-selective cation channel blocker and co-treated with NaHS to explore the involvement of non-selective cation channels in pacemaker currents induced by NaHS. NaHS induced effect was significantly blocked by FFA. This evidence strongly supports that voltage independent non-selective cation channels is expressed in ICC of murine small intestine and modulates the NaHS

induced inward currents.

Abe and Kimura (1996) have reported that in addition to K_{ATP} channels, NMDA receptors may be the target of H_2S. In the presence of a weak titanic stimulation, NaHS facilitated the induction of hippocampal long-term potentiation (LTP) in rat hippocampal slices by enhancing the NMDA induced inward current. Interaction of H_2S and NMDA receptors was possibly mediated by the activation of a cAMP dependent protein kinase pathway. NaHS increased cAMP production in primarily cultured rat cerebral and cerebellar neurons or in selected rat brain neuronal and glial cell lines. Kimura et al (2000) also reported that NaHS enhanced the production of cAMP, and increased the sensitivity to NMDA stimulation of NMDA receptors expressed in oocytes. Present study is consistent with NMDA stimulation as NaHS enhanced cAMP production. NaHS induced effect on pacemaker currents is partially suppressed by application of SQ 22536, an inhibitor of cAMP. Lee et al (2006) has also reported that cAMP pathway is involved in NaHS regulated calcium homeostasis in primary rat microglial cells. Present results in part suggest that, cAMP pathway might be involved in NaHS regulated pacemaker activity.

It was hypothesized that NaHS induced inward current is closely related with calcium homeostasis and modulated by influx of calcium from external medium or from the store operating calcium release from internal sources. To explore whether NaHS has the role on intracellular calcium mobilization on NaHS induced pacemaker activity, ICC were exposed to calcium depleted bathing solution as well as thapsigargin, Ca^{2+}-ATPase inhibitor of endoplasmic reticulum. Tokutomi et al (1995) has suggested that the pacemaker currents were abolished in Ca^{2+} depleted medium. Moreover, Torihashi et al (2002) in an investigation on murine ICC repoted that thapsigargin, blocker of ER Ca^{2+}-ATPases increased and then

decreased $[Ca^{2}]_i$ and eventually blocked the oscillations. This indicated that the channel involved in oscillation was store-operated and activated by the depletion of Ca^{2+} in ER. The present study is consistent with them as Ca^{2+} depleted medium significantly suppressed the pacemaker currents but NaHS induced effect remained unaffected in calcium depleted medium. Intracellular Ca^{2+} recycling to endoplasmic reticulum was attenuated by the use of thapsigargin. In the mean time the effect of NaHS was tested in calcium depleted condition. Pacemaker currents were remarkedly suppressed by the inhibition of calcium recycling to internal sources. NaHS induced inward pacemaker current was also completely blocked by thapsigargin. This result provides strong evidence for necessity of Ca^{2+} influx from external sources to regulate gastrointestinal smooth muscle contraction and to generate pacemaker currents in ICC. Furthermore, it suggests that NaHS induced effect on pacemaker activity is regulated by Ca^{2+} release from endoplasmic reticulum rather than Ca^{2+} influx from extracellular store.

The activation of non-selective cation channels allows net inward current carried predominately by Na^{+} under physiological condition and consequently leads to excitatory action in GI smooth muscles as suggested by Kuriyama et al (1998). In present study, NaHS induced effect was blocked by Na^{+} depletion in bathing solution but DIDS, an inhibitor of calcium activated chloride channels, did not alter the NaHS induced effects may suggest that NaHS induced effect in pacemaker activity in ICC is mediated by activation of non-selective cation channels rather than involvement of Ca^{2+} sensitive chloride channels. Several reports available to suggest either non-selective cation channels or Ca^{2+} activated chloride channels are principally involved to produce inward currents from ICC (Choi et al., 2006; Kim et al., 2007). Hartzell et al (2005) has also reported

that Cl_{Ca} channels contribute to a range of cellular events including the regulation of smooth muscle tone. Though Cl_{Ca} channels is involved in regulating pacemaker activity by elevation of $[Ca_2+]_c$ to produce inward Cl^- currents, NaHS induced inward current is not mediated by Cl_{Ca} channels in present study. The blocking effects of FFA and Na^+ depleted medium but insignificant effects of DIDS on NaHS induced inward currents, clearly justified the involvement of non-selective cation channels as the principal regulator in NaHS induced inward currents.

It is hypothesized that NaHS induced inhibitory pacemaker activity is regulated either via cGMP pathway or activation of potassium channels. Therefore, their involvement on NaHS induced pacemaker currents were examined by using corresponding inhibitors. To determine whether the H_2S induced effect on pacemaker activity of ICC was mediated by the cGMP pathway, the effect of H_2S on ICC in the presence of the soluble guanylyl cyclase (sGC) inhibitor ODQ was studied. Park et al (2007) recently reported the involvement of cGMP pathway in regulation of gastrointestinal motility via nitric oxide secretion. According to them, nitric oxide (NO), the first gasotransmitter act on ICC and regulates pacemaker activity and drives slow waves via cGMP pathway. NO stimulated cGMP was inhibited by the application of ODQ. Unlike that report, in present study, ODQ, an inhibitor of cGMP did not suppressed the NaHS induced effect despite the partial enhancement of outward currents and has revealed that NaHS regulated pacemaker activity is independent to cGMP pathway. Zhao et al (2001) suggested that unlike NO and CO, H_2S relaxed vascular tissues are independent of the activation of cGMP pathway, while the vasorelaxation induced by NO was virtually abolished by ODQ, a specific inhibitor of soluble guanylyl cyclase, the H_2S induced vasorelaxation was not inhibited by ODQ. In fact, ODQ even potentiated the vasorelaxant effect of H_2S. In

present study, ODQ did not suppress the NaHS induced effects. Instead, interestingly, it enhanced NaHS induced outward resting currents without notable alteration in amplitudes and frequency. The synergistic actions of H_2S and ODQ can not be fully understood yet. However, hypothetically, the interaction between ODQ and H_2S may have generated vasorelaxant free radicals, which further relaxed vascular tissues (Zhao et al., 2001).

It has been reported that, Ileum preparation pre-treated with L-NAME failed to influence the effect of NaHS on contractions due to electrical stimulation showed that nitric oxide does not contribute to the inhibitory effect of NaHS in the field stimulated guinea pig ileum (Teague et al., 2002). But, in another study, Zhao and Wang (2002) found that the endothelium- derived NO is likely released by H_2S because the application of L-NAME to the endothelium intact vascular tissues partially inhibits the H_2S induced vasorelaxation. The present study in ICC from small intestine is consistent with former but differs from later as L-NAME, an inhibitor of NO when exposed to ICC did not alter the NaHS induced inhibitory effect on pacemaker currents. As the ICC are free from endothelium, endothelium NO is not released by the action of NaHS to ICCs and therefore, the application of L-NAME to NaHS induced inhibitory pacemaker currents might not have effects. This result has suggested that release of nitric oxide does not contribute to the inhibitory effect of NaHS on pacemaker currents recorded in myonteric ICC from mouse small intestine.

Jin-Song and Philip (2006) has carried out the signaling mechanism of cardioprotection of hydrogen sulfide preconditioning in rat hearts or cardiac myocytes. They have suggested the involvement of protein kinase C to produce cardioprotective effects by increasing the amplitude of electrically-induced $[Ca^{2+}]_i$ transients. This is not consistent with present

study as the use of calphostin C, an inhibitor of protein kinase C did not response to NaHS induced pacemaker activity and have suggested that calcium activated protein phosphoryalation is not involved in regulating NaHS induced gastrointestinal motility.

Lee et al (2006) found that exogenously applied NaHS has increased $[Ca^{2+}]_i$ in microglial cells in a dose-dependent manner. It has been suggested that exogenous H_2S triggers a calcium influx via plasma membrane and calcium release from intracellular store. This influx is partly dependent on activation of adenylyl cyclase and independent of the phospholipase C, protein kinase C and inositol triphosphate pathway. Present study is consistent with them as increase in intracellular calcium, partially inhibition of adenylyl cyclase and no alteration of NaHS induced effects by calphostin C was measured. NaHS elevated calcium might be released from internal store and may be regulated by cAMP formation in PKC independent manner as calphostin C, a potent inhibitor of protein kinase C did not influence the NaHS induced effects, but did by thapsigargin an inhibitor of ATPase.

Many key biological processes are regulated by cytoplasmic calcium signals. Such signals can arise either from the release of stored calcium or the influx of calcium across the plasma membrane, but more characteristically, from both routes (Putney 2001). A common mechanism by which such cytoplasmic calcium signals are generated involves receptors that are coupled to the activation of phospholipase C (Putney 1986). Phospholipase C generates inositol 1,4,5- trisphosphate (IP3), which in turn mediates the discharge of Ca^{2+} from intracellular stores. The fall in Ca^{2+} concentration within the lumen of the Ca^{2+} storing organelles (most commonly, components of the endoplasmic reticulum) activates plasma membrane Ca^{2+} channels. In present study, NaHS induced calcium

mobilization is closely related to the depletion of sarcoplasmic reticulum/endoplasmic reticulum calcium stores which evokes calcium entry across the plasma membrane in a process termed store operated (SOC) or capacitative calcium entry (CCE). Localized Ca^{2+} release from the ER in smooth muscle, identified as Ca^{2+} sparks, may activate either K_{Ca} or Cl_{Ca} channels to generate spontaneous transient outward (STOCs) and inward (STICs) currents, respectively (Nelson et al., 1995 ; Piper et al 2002). STOCs hyperpolarize the membrane potential and promote closure of voltage-dependent Ca^{2+} channels to facilitate smooth muscle relaxation. STICs depolarize the plasma membrane and activate Ca^{2+} entry via voltage-dependent Ca^{2+} channels to promote contraction. Ca^{2+} released via IP3R is accumulated by mitochondria. Upon stimulation, the free Ca^{2+} concentration in the cytoplasm can be rapidly elevated this increase occurs through the opening of channels in the sarcoplasmic/endoplasmic reticulum.

As suggested by Reiffenstein et al (1992) activation of ATP-sensitive channels by NaHS was proposed in these experiments as the consequence of ATP depletion due to the inhibition by sulfide of the oxidative phosphorylation. Many reports have suggested that H_2S act on ATP sensitive potassium channel. In present study of NaHS induced inhibitory effect was mimicked to the pinacidil, an opener of ATP sensitive potassium channels. Therefore, to study whether NaHS induced outward inhibitory effect was mediated by K_{ATP} channels, action of glibenclamide on NaHS induced effect was tested. As shown in figure 10a, the pre-treatment of glibenclamide followed by co-treatment with NaHS significantly suppressed the NaHS induced outward currents but did not change the NaHS inhibited pacemaker amplitudes and pacemaker frequency. Such a partial contribution of ATP sensitive potassium channels on NaHS induced contraction and relaxation in rat and mouse aorta were reported (Kubo et

al 2007). This result strongly suggests the partial involvement of ATP sensitive potassium channels in NaHS induced pacemaker currents in murine small intestinal interstitial cells of Cajal.

As the intracellular calcium increment was measured in the ICC, to rule out whether calcium activated potassium channels are involved in the inhibitory pacemaker currents produced in ICC, test on calcium sensitive potassium channels were carried out in present study. Calcium sensitive potassium channels are activated by raised levels of intracellular calcium and are consequently called calcium activated potassium channels. Calcium activated channels are sensitive to either TEA or apamin. Neither of calcium activated potassium channels blockers inhibited the NaHS induced inhibitory outward currents in the present study suggest that calcium sensitive potassium channels are not involved in NaHS regulated pacemaker activity. Moreover, it has been suggested that potassium currents are more sensitive to extracellular Ba^{+}. The NaHS induced inhibitory currents was found partially sensitive to extracellular Ba^{+} as NaHS induced outward current is suppressed in presence of $BaCl_2$ without alteration of inhibited pacemaker frequency and amplitudes. This partial sensitivity of Ba^{+} to NaHS induced effect suggest that NaHS partially activate the ATP sensitive potassium channels in murine small intestinal interstitial cells of Cajal to modulate pacemaker activity.

In addition to the patch clamp, the pharmacological study of NaHS induced effect on pacemaker activity in gastrointestinal ICC was carried with measurement of calcium fluctuation using live cell imaging technique. Some investigators reported that the periodic pacemaker activity of ICC is dependent on intracellular Ca^{2+} oscillation and this pacemaker mechanism is initiated by release of Ca^{2+} from the endoplasmic reticulum through inositol triphosphate (IP3) type 1 receptor and is followed by reuptake of Ca^{2+}

into the mitochondria (Suzuki et al., 2000; Ward et al., 2000). It has also been reported that exogenously applied NaHS was able to regulate calcium waves in astrocytes (Nagai et al., 2004) and in primary rat microglial cells (Lee et al., 2006). In present study, ICC produced calcium oscillations and calcium level is significantly elevated with enhancing frequency of calcium waves as shown in figure 12. This elevation in calcium intensity together with increase in frequency of calcium oscillation is mimicked with NaHS induced inward currents recorded in voltage clamp mode at -70 mV. Furthermore, thapsigargin, an inhibitor of store operating calcium release blocker suprresed the intracellular calcium. This provides the strong support that NaHS induced pacemaker activity in ICC is due to periodically release of Ca^{2+} from endoplasmic reticulum of ICC.

Cystathionine -synthase and cystathionine -lyase are responsible for the majority of the endogenous production of H_2S in mammalian tissues. Although CBS is mainly expressed in the liver and central nervous system, CSE is expressed in cardiovascular and gastrointestinal smooth muscle cells. Florucci et al (2005) found that, although both CBS and CSE were expressed by the gastric mucosa, CSE was reported to be the main enzyme involved in the H_2S generation in the stomach. Florucci et al (2006) have also reported expression of CSE mainly in the liver, vascular and nonvascular smooth muscle, but, a low level of expression of the CSE transcript, protein, and enzymatic activity is also detectable in the small intestine and stomach of rodents. In present work , mRNA expression for CSE and CBS in sorted ICC as well as whole gut tissue was examined. The result revealed that mRNA for both CSE and CBS were not expressed in ICC enrich populated cells but a prominent expression of CSE and sparse expression of CBS in cultured gut tunica muscularis was detected. So, present result showed that CSE to be the dominant H_2S producing enzyme in gastrointestinal tract though a sparse mRNA for CBS was also

detected. This expression of mRNA for CSE and CBS in total RNA isolated from gut tissues may suggest that H_2S is endogenously produced either in smooth muscle cells or neuronal cells or other population of cells except ICC.

V. Conclusion

Exogenously applied sodium hydrogen sulfide, a donor of hydrogen sulfide responded dose dependently and resulted duel effects on pacemaker activity of interstitial cells of Cajal identified with c-kit antibody. Separate mechanisms were found to produce excitatory effects in lower concentration and inhibitory effects on higher concentration. Voltage independent non-selective cation channels by allowing Na^+ entry to cells was found responsible to mobilize intracellular calcium and release of calcium from endoplasmic reticulum also took part to regulate intracellular calcium in NaHS induced pacemaker activity. Similarly, ATP sensitive potassium channels partly activating cAMP in a protein kinase C independent manner regulated the inhibitory action of hydrogen sulfide on pacemaker activity produced from ICC. Further more, the inhibitory action of NaHS on ICC was found independent to nitric oxide release but a significant potentiation of soluble gunyal cyclase was found. From the experiment on calcium intensity measurement, and patch-clamp suggest that NaHS act on ICC by elevating intracellular calcium level. So, taking together all the pharmacological experiments on NaHS induced pacemaker activity and intracellular calcium elevation strongly suggest that H_2S could be the principal regulator of gastrointestinal motility.

RT-PCR results carried out for detection of enzymes involved in endogenous production of hydrogen sulfide revealed that ICC enriched population lack mRNA for neither SMCs and neuronal cells. Further more, mRNA expression for neither CSE nor CBS in ICC enriched population, but a prominent expression of mRNA for CSE in tissues isolated from tunica muscularis of mouse small intestine was detected. Thus RT-PCR results strongly suggest that CSE is the major hydrogen sulfide producing enzymes and expressed in either smooth muscle cells or neuronal cells. Therefore,

present work suggest that ICC are not the target of hydrogen sulfide but the response of hydrogen sulfide may be transmitted either from smooth muscle cells or neuronal cells. Present study also concludes that besides the partly involvement of ATP sensitive K^+ and adenyl cyclase in NaHS induced inhibitory effects, other mechanism might have been involved. Therefore, an extensive investigation on signaling pathway involved in NaHS regulated Gastrointestinal motility is recommended.

VI. References

Abe K., & Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.,* 16, 1066-1071 (1996).

Alberto C., & Martin L. B. A Quantitative Model of Gastric Smooth Muscle Cellular Activation. *Annu. Biomedic. Engin.,* (2007).

Barber T., Triguero A., Martinez L. I., Torres L., Garcia C., Miralles V. J., & Vina J. R. Elevated Expression of Liver {gamma}-Cystathionase Is Required for the Maintenance of Lactation in Rats. *J. Nutr.,* 129, 928-933 (1999).

Bukovska G., Kery V., & Kraus J. P. Expression of human cystathionine beta-synthase in *Escherichia coli*: purification and characterization. *Protein Exp. Purif.* 5, 442-448 (1994).

Choi S., Park D. Y., Cheol H. Y., Chang I. Y., You H. J., Park C. G., Kim M. Y., Kong I. D., So I., Kim K. W., & Jun J. Y. Bradykinin modulates pacemaker currents through bradykinin B_2 receptors in cultured interstitial cells of Cajal from the murine small intestine. *Br.J. Pharmaco.,* 148, 918-926 (2006).

Daniel E.E. Physiology and Pahophysiology of the Interstiial Cell of Cajal: From Bench to Beside III. Interaction of Interstiial Cell of Cajal with neuromediators: an interium assessment. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 281, G1329-G1332 (2001).

Dickens E. J., Hirst G. D. S., & Tomita T. Identification of rhythmically active cells in guinea-pig stomach. *J. Physiol., (Lond)* 514, 515-531 (1999).

Erickson P. F., Maxwell I. H., Su L. J., Baumann M., & Glode L. M. Sequence of cDNA for rat cystathionine y- lyase and comparison of deduced amino acid sequence with related *Escherichia coli* enzymes. *Biochem. J.,* 269, 335-340 (1990).

Eto K., Ogasawara M., Umemura K., Nagai Y., & Kimura H. Hydrogen sulfide is produced in response to neuronal excitation. *J. Neurosci.,* 22, 3386-3391 (2002).

Fiorucci S., Antonelli E., Distrutti E., Rizzo G., Mencarelli A., Orlan S., Zanardo R., Renga B., Sante M. D., Morelli A., Cirino G., & Wallace J. L. Inhibition of Hydrogen Sulfide Generation Contributes to Gastric Injury Caused by Anti-Inflammatory Nonsteroidal Drugs. *Gastrointerol.* 129(4), 1210-1244 (2005).

Fiorucci S., Distrutti E., Cirino G., & Wallace J. L. The Emerging Roles of Hydrogen Sulfide in the Gastrointestinal Tract and Liver. Gastrointerol. 131(1), 259 -271 (2006).

Hartzell C., Putzier I., & Arreola J. Calcium-activated chloride channels. *Annu. Rev. Physiol.,* 67, 719-758 (2005).

Hosoki R., Matsiki N., & Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophysic. Res. Commun.,* 237, 527-541 (1997).

Huizinga J. D., Thuneberg L., Kluppel M., Malysz J., Mikkelsen H. B. & Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature,* 373, 347-349 (1995).

Huizinga J. D., Zhu Y., Ye J., & Molleman A. High-conductance chloride

channels generate pacemaker currents in interstitial cells of Cajal. *Gastroenterol.,* 123, 1627-1636 (2002).

Kim H. S., Parajuli S. P., Yeum C. H., Park J.S., Jeong H. S., So I., Kim K. W., Jun J. Y., & Choi S. Effects of Ginseng Total Saponins on Pacemaker Currents of Interstitial Cells of Cajal from the Small Intestine of Mice. *Biol. Pharm. Bull.* 30(11), 2037—2042 (2007).

Kimura H. Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. *Biochem. Biophys. Res. Commun.,* 267, 129-133 (2000).

Bian J. S., & Moore P. K. Signaling mechanism of the cardioprotection induced by hydrogen sulfide preconditioning. *J. Mol. and Cell.Cardiol.,* 41, 1039-1079 (2006).

Koh S. D., Sanders K. M., & Ward S. M. Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the mouse small intestine. *J. Physiol.*, *513*, 203-213 (1998).

Koh S. D., Jun J. Y., Kim T. W., & Sanders K. M. A Ca^2 inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cells of Cajal. *J. Physiol.,* 540, 803-814 (2002).

Kubo S., Doe I., Kurokawa Y., Nishikawa H., & Kawabata A. Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: contribution of dual modulation of vascular tension. *Toxicol..* 232 (1-2), 138-146 (2007).

Kuriyama H., Kitamura K., itoh T., & Inoue R. Physiologiacal features of visceral smooth muscle cells, with special references to receptors and ion channels. *Physiol. Rev.*, 78, 811-820 (1998).

Liu L. W., Thuneberg L., & Huizinga J. D. Cyclopiazonic acid, inhibiting the endoplasmic reticulum calcium pump, reduces the canine colonic pacemaker frequency. *J. Pharmacol. Exp. Ther.,* 275, 1058-1068 (1995).

Lee S. W., Hu Y. S., Hu L. F., Lu Q., Dawe G. S., Moore P. K., Wong P. T. H., & Bian J. S. Hydrogen sulphide regulates calcium homeostasis in microglial cells. *Gila.,* 54, 116-124 (2006).

Maeda H., Yamagata A., Nishikawa S., Yoshinaga K., kobayashi S., Nishi K., & Nihikawa S. Requirement of c-kit for development of intestinal pacemaker system. *Development,* 116, 369-375 (1992).

Nagai Y., Tsugane M., Oka J., & Kimura H. Hydrogen sulfide induces calcium waves in astrocytees. *Faseb J.,* 18, 557-559 (2004).

Nakayama S., Kajioka S., Goto K., Takaki M., & Liu H. N. Calcium associated mechanisms in gut pacemaker activity. J. Cell. mol. Med. 11(5), 958-968 (2007).

Nelson M. T., Cheng H., Rubart M., Santana L. F., Bonev A. D., Knot H. J., & Lederer W. J. Relaxation of arterial smooth muscle by calcium sparks. *Science,* 270, 633-637 (1995).

Park C. G., Kim Y. D., Kim, Y. M., Jun S., Choi S., Yeum C. H., Parajuli S. P., Park S., Seong J. H., So I., Kim W. K., & Jun J. Y. Inhibition of pacemaker currents by nitric oxide via activation of ATP-sensitive K^+ channels in cultured interstitial cells of Cajal from the mouse small intestine. *Naunyn-Schmiedeberg's Arch Pharmacol.* 376, 175-184 (2007)

Piper A. S., Greenwood I. A., & Large W. A. Dual effect of blocking agents on Ca^{2+} activated Cl^- currents in rabbit pulmonary artery smooth

muscle cells. *J. Physiol,.* 539, 119-131 (2002).

Putney, J. W., Jr. A model for receptor regulated calcium entry. *Cell Calcium,* 7, 1-12 (1986).

Putney, J. W., Jr. The Pharmacology of Capacitative Calcium Entry. *Molecular Intervenions.,* 1(2), 84-94 (2001).

Reiffenstein R. J. Hulbert W. C., & Roth S. H. Toxicology of hydrogen sulfide. *Ann. Rev. Pharmacol. Toxicol.,* 32, 109-134 (1992).

Sanders K. M. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroentrol.*, 111, 492-515 (1996).

Sanders K. M., Ordog T., Koh S. D., Torihashi S., & Ward S. M. Developmental and plasticity of interstitial cells of Cajal. Neurogastroenterol. Mot. 11, 311-388, (1999).

Sanders K.M., Ordog T., Koh S. D., & Ward S. M. A novel pacemaker mechanism drives gastrointestinal rhythmicity. *News Physiol. Sci.* 15, 291-298 (2000).

Stipanuk M. H., & Beck P. W. Characterization of the enzymic capacity for cysteine desulphydration in liver and kidney of the rat. *Biochem. J.,* 206, 267-277 (1982).

Suzuki H., Takano H., Yamamoto Y., Komuro T., Saito M., Kato K., & Mikoshiba K. Properties of gastric smooth muscles obtained from mice which lack inositol trisphosphate receptor. *J. Physiol.,* 525, 105-111 (2000).

Teague B., Asiedu S., & Moore P. K. The smooth muscle relaxant effect of hydrogen sulfide in vitro: evidence for a physiological role to control intestinal contractility. *Br. J. Pharmacol.,* 137, 130-145 (2002).

Thomsen L., Robinson T. L., Lee J. C. F., Farraway L., Hughes M. J. H., Andrews D. W., & Huizinga J. D. Interstitial cells of Cajal generates a rhymic pacemaker current. *Nature,* 4, 848-851 (1998).

Tokutomi N., Maeda H., tokutomi Y., Sato D., Sugita M., Nishigawa S., Nakao J., Imamura T., & Nishi K. Rhythmic chloride currents and physiolgical roles of the intestinal *c-kit* positive cell. *Pflugers. Arch.,* 431, 169-177 (1995).

Torihashi S., Ward S. M., Nishikawa S. I., Nishi K., Kobayashi., & Sanders K. M. c-kit development of iterstitial cells and elecrical activity in the murine gastrointestinal tract. *cell tissue res.,* 280, 97-111 (1995).

Torihashi S., Fujimoto T., Claudia Trost C., & Nakayama S. Calcium Oscillation Linked to Pacemaking of Interstitial Cells of Cajal. *Biochem. J.,* 277 (21), 19191-19197 (2002).

Wang R. Two's company there is a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J., 16*, 1792-1798 (2002).

Ward S. M., Burns A. J., Torihashi S., & Sanders K. M. Mutation of proto-oncogene c-kit blocks development of intestinal cells and intestinal electrical rythmicity in murine mutants. *J. Physiol.,* 480*, 91-97* (1994).

Ward S. M., Burns A. J., Torihashi S., Harney S. C., & Sanders K. M. Impaired development of Interstitial cells and intestinal electrical rhythmicity in steel mutants. *Am. J. Physiol.,* C1577-1588 (1995).

Ward S. M., Ordog T., Koh S. D., Baker S. A., Jun J. Y., Amberg G., Monaghan K., & Sanders K. M. Pacemaking in Interstitial Cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J. Physiol.,* 525, 355-361 (2000).

Zhao W., Zhang J., Lu Y., & Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J.,* 20, 6008-6016 (2001).

Zhao W., & Wang R. H_2S-induced vasorelaxation and underlying cellular and molecular mechanisms. *Am. J. Physiol., Heart Circ. Physiol.,* 283, H474-H480 (2002).

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PUBLICATIONS

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sensitive K^+ Channels. *Korean J Physiol Pharmacol.,* **11**, 15-20, February, 2007.

2. Kim H. S., **Parajuli S. P.,** Yeum C. H., Park J. S., Jeong H. S., So I., Kim K. W., Jun J. Y., and Choi S. Effects of Ginseng Total Saponins on Pacemaker Currents of Interstitial Cells of Cajal from the Small Intestine of Mice. *Biol. Pharm. Bull.* 30(11) 2037—2042 (2007).

3. Park C. G., Kim Y. D., Kim M. Y., Kim J. S., Choi S., Yeum C. H., **Parajuli S. P.,** Park S., Seong J. H., So I., Kim W. K., and Jun J. Y. Inhibition of pacemaker currents by nitric oxide via activation of ATP-sensitive K+ channels in cultured interstitial cells of Cajal from the mouse small intestine. *Naunyn-Schmiedeberg's Arch Pharmacol.* **376,** 175-184 (2007).

4. Choi S., **Parajuli S. P.**, Lim G. H., Kim J. H., Yeum C. H., Yoon P. J., and Jun J. Y. Imipramine Inhibits A-type Delayed Rectifier and ATP-Sensitive K^+ Currents Independent of G-Protein and Protein Kinase C in Murine Proximal Colonic Myocytes. *Arch Pharm Res* **29**(11), 998-1005, 2006.

5. **Parajuli S. P.,** and Pradhananga T. M. Limnological Investigation of Some ponds of Kathmandu valley. *ECOPRINT,* **9**(1), (2002).

6. **Parajuli S. P.** Ethanobotanical study at Khanbari Muniipality of Sankhuwasabha Disttrict, Nepal. *Banko Janakari* (Journal of Forestry Information for Nepal) **10**(2) 29-34, 2000.

7. **Parajuli S. P.,** and Jha P. K. Phytoplannkton and Physico-Chemical Parameters of Water in Kamal Pokhari, Bhaktapur, Nepal. *Proceeding, Third National Conference on Science and Technology (RONAST),* (1999).

Seminar and paper presentation

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- **2007 The 15th Federation Meeting of Korean Basic Medical scientists.** Dejan, Korea, April 2007.

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- **Shankar Prasad Parajuli,** Jae Yeoul Jun, Seok Choi, Cheol Ho Yeum, Pyung Jin Yoon. Action of Sphingosine Monophosphate on Pacemaker activity in Interstitial Cells of Cajal from Murine Small intestine. **Bangalore Bio 2006,**Bangalore, India. Jun, 2006.

저작물 이용 허락서

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논문제목	한글 생쥐 소장에서 분리된 Interstitial Cells of Cajal에 대한 Hydrog sulfide의 작용 기전				
	영문 Pharmacological Investigation of Hydrogen sulfide on Interstitial Cells of Cajal from Murine Small Intestine				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(0) 반대( )

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