Korean J Physiol Pharmacol
Vol 14: 83−89, April, 2010
DOI: 10.4196/kjpp.2010.14.2.83

The Inhibitory Effects of Hydrogen Sulfide on Pacemaker Activity of Interstitial Cells of Cajal from Mouse Small Intestine

Shankar Prasad Parajuli[1,*], Seok Choi[1,*], Jun Lee[2], Young Dae Kim[2], Chan Guk Park[2], Man Yoo Kim[2], Hyun Il Kim[1], Cheol Ho Yeum[1], and Jae Yeoul Jun[1]

Departments of [1]Physiology and [2]Internal Medicine, College of Medicine, Chosun University, Gwangju 501-759, Korea

In this study, we studied whether hydrogen sulfide (H_2S) has an effect on the pacemaker activity of interstitial cells of Cajal (ICC), in the small intestine of mice. The actions of H_2S on pacemaker activity were investigated using whole-cell patch-clamp technique, intracellular Ca^{2+} analysis at 30°C and RT-PCR in cultured mouse intestinal ICC. Exogenously applied sodium hydrogen sulfide (NaHS), a donor of hydrogen sulfide, caused a slight tonic inward current on pacemaker activity in ICC at low concentrations (50 and 100 μM), but at high concentration (500 μM and 1 mM) it seemed to cause light tonic inward currents and then inhibited pacemaker amplitude and pacemaker frequency, and also an increase in the resting currents in the outward direction. Glibenclamide or other potassium channel blockers (TEA, $BaCl_2$, apamin or 4-aminopydirine) did not have an effect on NaHS-induced action in ICC. The exogenous application of carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) and thapsigargin also inhibited the pacemaker activity of ICC as NaHS. Also, we found NaHS inhibited the spontaneous intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in cultured ICC. In doing an RT-PCR experiment, we found that ICC enriched population lacked mRNA for both CSE and CBS, but was prominently detected in unsorted muscle. In conclusion, H_2S inhibited the pacemaker activity of ICC by modulating intracellular Ca^{2+}. These results can serve as evidence of the physiological action of H_2S as acting on the ICC in gastrointestinal (GI) motility.

Key Words: Interstitial cells of Cajal (ICC), Intestinal motility, Pacemaker currents, Sodium hydrogen sufide (NaHS)

INTRODUCTION

Hydrogen sulfide (H_2S) though traditionally considered as a colorless 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. H_2S is produced in substantial amounts by mammalian tissues and exerts many physiological effects suggesting its potential role as a regulatory mediator. Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets. Abnormal metabolism and functions of H_2S contribute to the pathogenesis of many diseases, and play major roles in physiological and pathological processes such as blood pressure regulation, neurotransmission and inflammatory processes etc [1-3].

Recently, it was reported that H_2S exerts effects on the motility of various tissues. In the urinary bladder, H_2S activated bladder contraction [4]. Interestingly, H_2S had an opposite effect on the isolated ileal muscle strips, relaxing them [5] and also inhibited the motor patterns in human, rat and mouse colon and jejunum [6]. These results mean that H_2S can have dual action, causing either contraction or relaxation in muscle motility.

GI activity is governed by periodic generation of electrical activity called slow waves which are supposed to be generated from interstitial cells of Cajal (ICC) and then propagated to nearby muscle cells via gap junctions [7]. Because ICC play an important role in the regulation of GI motility, it can be the target for many hormones, neurotransmitters, and various endogenous compounds in modulating GI motility.

The effects of H_2S on gastrointestinal motility have been examined, however its role on ICC that is still not well-understood. The present study intends to investigate the role that could be played of exogenously applied H_2S, and subsequently to characterize the effects and mechanism of H_2S-induced action on the pacemaker activity generated from small intestinal ICC.

Received March 15, 2010, Revised March 29, 2010,
Accepted March 30, 2010

Corresponding to: Jae Yeoul Jun, Department of Physiology, College of Medicine, Chosun University, 375, Seosuk-dong, Dong-gu, Gwangju 501-759, Korea. (Tel) 82-62-230-6412, (Fax) 82-62-232-4943, (E-mail) jyjun@chosun.ac.kr
*These authors contributed equally to this work.

ABBREVIATIONS: H_2S, hydrogen sulfide; ICC, interstitial cells of Cajal; NaHS, sodium hydrogen sulfide; GI, gastrointestinal; CSE, cystathionine gama lyase; CBS, cystathionine beta-synthase.

METHODS

Preparation of cells and tissues

Balb/C mice were used in this experiment and were treated as per the guiding principles 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. Every effort was made to minimize both the number of mice and their suffering.

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 the small intestines from 1 cm below the pyloric ring to the cecum were removed. Intestines were opened along the mesenteric border and 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 were equilibrated in Ca^{2+} free Hank's solution for 30 minutes. The muscle strips were enzymatically digested by incubating at 37°C for 12 minutes in an 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 was triturated using fire blunted glass tubes with a range of holes. Cells were seeded onto sterile glass cover slips (20 mm) and 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 was replaced with that without stem cell factor, and was incubated further in the same condition till used.

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 9.2; Axon Instruments). The data were filtered at 5 kHz and were displayed on an oscilloscope and a computer monitor. Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments pertaining to patch clamp were performed at 30°C.

Cell preparation and RNA isolation

Muscle tissue was isolated from mouse small intestine and enzymatically digested, as mentioned previously. Well triturated muscles were seeded onto 100 ml culture dish and were cultured for 2 days in smooth muscle growth medium to get at least 80% confluence. Similarly in another preparation fully automated magnetic cell separation technique (ROBOSEP, Stem Cell Technologies Inc) was used to separate pure population of ICC from the above prepared cell suspension (cells $>10\times10^6/\mu$l) by incubating in magnetic nanoparticles, mouse CD117 antibody and PE selection followed by extensive washing. Total RNA was isolated separately from both preparations using TRIzol reagent according to manufacturer specifications (Invitrogen, Milan, Italy).

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration

$[Ca^{2+}]_i$ mobilization induced by NaHS was examined by Ca^{2+} sensitive indicator fluo-4/AM. The well grown ICC on cover slips (20 mm) was rinsed twice for 15 minutes with a 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 were mounted on a perfusion chamber, and images were acquired with a live cell imaging confocal microscope (600×; Ultraview, Nikon). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During the Ca^{2+} imaging, the temperature of the perfusion chamber containing the cultured ICC was maintained at 30°C. The variations in intracellular Ca^{2+} fluorescence emission intensity were expressed as peak fluorescence intensity ratio (F1/F0) with respect to time of peak fluorescence, where F0 is the minimum fluorescence intensity during image capturing.

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 programmed so that cDNA synthesis was followed immediately with PCR amplification by using gene specific primers of c-kit (Y00864): 5'-cgcctgccgaaatgtatg-3', 3'-ggttctctgggttggggt-5, 161 bp; myosin (NM_013607) 5'-gagaaaggaaacaccaag-3', 3'-aacaaatgaagcctcgtt-5', 233bp; β-actin (NM_007393): 5'-tctagacttcgagcaggaga-3', 3'-aatgtagtttcatggatgcc-5', 176 bp; PGP9.5 (AF172334): 5'-cgatggagattaaccccgagatg-3', 3'-ttttcatgctgggccgtgag-5', 168 bp; CSE (NM_145953): 5'-atcctgggctaccctctcaccctc-3', 3'-tgactcgaacttttaagggtgcgctg-5', 370 bp; CBS (NM_178224): 5' acaccatctgctgcttgctggaca-3', 3'-gagaagggttttgaccaggcacctg-5', 250 bp. The PCR reactions for c-kit, myosin, β-actin, protein gene product (PGP) −9.5 were amplified for 38 cycles in the thermal condition of 55°C for 40 minutes, 94°C for 3 seconds, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 10 minutes. But, the PCR reactions for CSE and CBS were amplified for 40 cycles at the annealing temperature of 62°C for 30 seconds. The PCR products were visualized by 2% agarose gel electrophoresis followed by ethidium bromide staining.

Solutions and drugs

The cells were bathed in a solution containing: 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.2 with Tris. The pipette solution contained 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM Na_2GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, 0.1 mM EGTA, pH adjusted to 7.2 with Tris.

All drugs in this study were purchased from Sigma Chemical Co. and were dissolved in appropriate solvent as mentioned in the product information. NaHS was dissolved in water and used fresh in every experiment.

Statistical analysis

Data are expressed as mean±standard error. Differences in the data were evaluated by Student's t-test. A p values less than 0.05 were taken as a statistically significant difference.

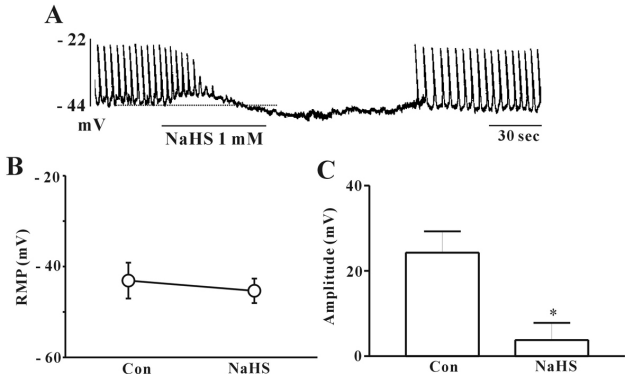

Fig. 1. Effects of NaHS on pacemaker potentials recorded in cultured ICC from mouse small intestine. (A) shows the pacemaker potentials of ICC exposed to NaHS (1 mM) in the current clamping mode (I=0). NaHS induced membrane hyperpolarization and inhibited the amplitude and frequency of pacemaker potential in ICC. The dot lines indicate the control resting membrane potentials levels. Responses to NaHS are summarized in (B) and (C). The bars represent means±SE. *Asterisks mean significantly different from the controls ($p<0.05$).

RESULTS

Effect of NaHS on pacemaker potentials in ICC

For investigating the effects of H_2S, we used a donor of hydrogen sulfide, NaHS in this study. We performed the electrophysiological recording from cultured ICC under current clamp mode (I=0). In control condition, ICC showed pacemaker potentials. The resting membrane potential and amplitude recorded from ICC were measured as −46.2±7.9 mV and 24.25±5.85 mV (n=6) respectively in normal conditions. NaHS (1 mM) when administered to the bathing solution slightly depolarized the membrane and then hyperpolarized the membrane potential and the pacemaker potentials disappeared (Fig. 1A). The value of the amplitude of pacemaker potentials under current clamp mode by NaHS (1 mM) was significantly different when compared with the control value, but not resting membrane potential (n=5, Fig. 1B and C).

Effects of NaHS on pacemaker currents in ICC

We examined whether NaHS exposed exogenously to ICC in a range of physiological relevant concentration (50 μM to 1 mM) would trigger dose dependent effects on pacemaker currents in voltage clamp mode. Under a voltage clamp at a holding potential of −70 mV, the ICC generated spontaneous inward currents called pacemaker currents. The application of 50 and 100 μM NaHS induced the slight inward currents without any change in amplitudes or frequency (Fig. 2A and B). However, in the presence of 500 μM and 1 mM NaHS, the pacemaker currents were largely in-

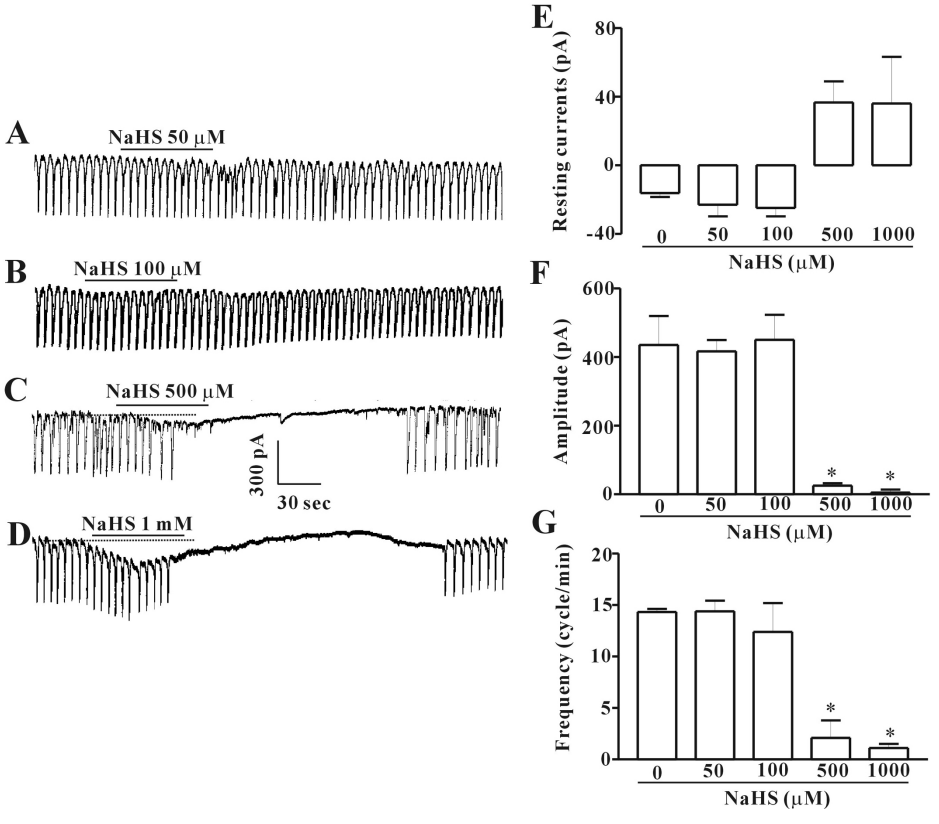

Fig. 2. Effects of NaHS on pacemaker currents recorded in cultured ICC from mouse small intestine. (A∼C), and (D) show pacemaker currents of ICC exposed to NaHS (50, 100, 500 μM or 1 mM respectively) at a holding potential of −70 mV. Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. The dot lines indicate the control resting current levels. (E, F), and (G) summarize the inhibitory effects of NaHS on pacemaker currents in ICC. Bars represent means±SE. *Asterisks mean significantly different from the controls ($p<0.05$).

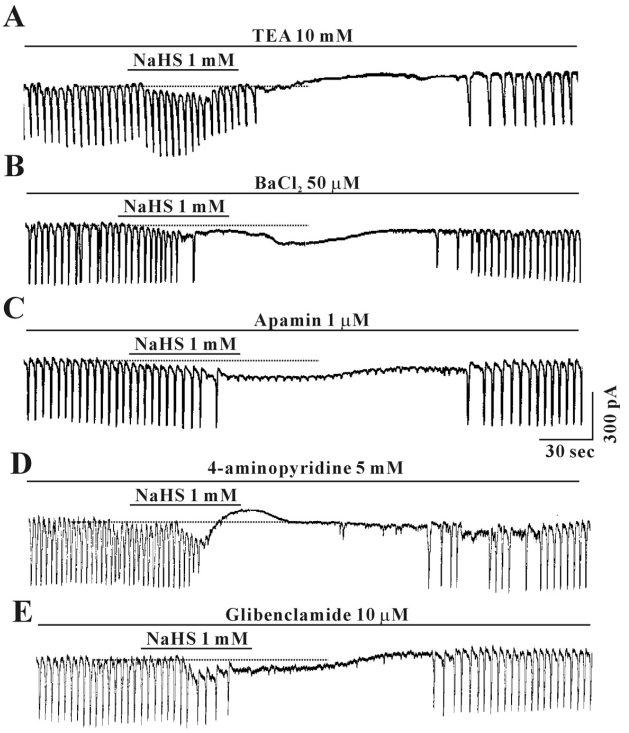

Fig. 3. Effects of TEA, $BaCl_2$, apamin, 4-aminopyridine and glibenclamide on NaHS-induced response in cultured ICC from mouse small intestine. Pretreatment with (A) TEA (20 mM), (B) $BaCl_2$ (50 μM), (C) apamin (100 μM), (D) 4-aminopyridine (5 mM) or (E) glibenclamide (10 μM) did not affect the inhibitory effects of NaHS (1 mM). Vertical solid line scales amplitude of pacemaker current and horizontal solid line scales duration of recording (s) pacemaker currents. The dot lines indicate the control resting current levels. TEA: tetraethylammonium chloride.

hibited and also the slight inward currents appeared before the inhibition of pacemaker currents by NaHS (Fig. 2C and D). Under control conditions, the resting currents, amplitudes and frequency generated from the ICC were recorded as −19.19±2.84 pA, 415.33±84.61 pA and 14.33±0.30 cycles/min respectively. When the ICC were incubated with high concentrations of NaHS, the resting currents were measured as 39.64±8.51 at 500 μM and 38.11±6.08 at 1 mM respectively. The mean control values of pacemaker currents amplitude and frequency were changed respectively to 26.4±9.6 mV and 2.01±1.04 cycles/min at 500 μM and 18.9±5.7 mV and 1.10±0.23 at 1 mM, by addition of NaHS. The value of frequency and amplitude by NaHS (500 μM and 1 mM) was significantly different when compared with control value (n=6, Fig. 2E∼G).

Effects of potassium channels blockers on NaHS-induced responses in cultured ICC

Various kinds of potassium channel blockers were tested in order to investigate which potassium channels mediate NaHS action on pacemaker currents. The application on ICC itself of tetraethylammonium chloride (TEA) (10 mM), a Ca^{2+}-activated K^+ channel blocker, and $BaCl_2$ (50 μM), a inward rectifying K^+ channel blocker, had no effect on the pacemaker currents. In the presence of TEA or $BaCl_2$, NaHS (1 mM) still inhibited the pacemaker currents in ICC (n=6; Fig. 3A and B). Furtherrmore, the treatment of apamin (1 μM), a Ca^{2+}-activated K^+ channel blocker, 4-aminopyridine (5 mM), a transient voltage-dependent K^+ channel blocker and glibenclamide (10 μM), an ATP-sensitive K^+ channel blocker, on ICC itself had no effect on the pacemaker currents. As well NaHS (1 mM) still inhibited the pacemaker currents in ICC when in conjunction with apamin, 4-aminopyridine or glibenclamide (n=5; Fig. 3C∼E).

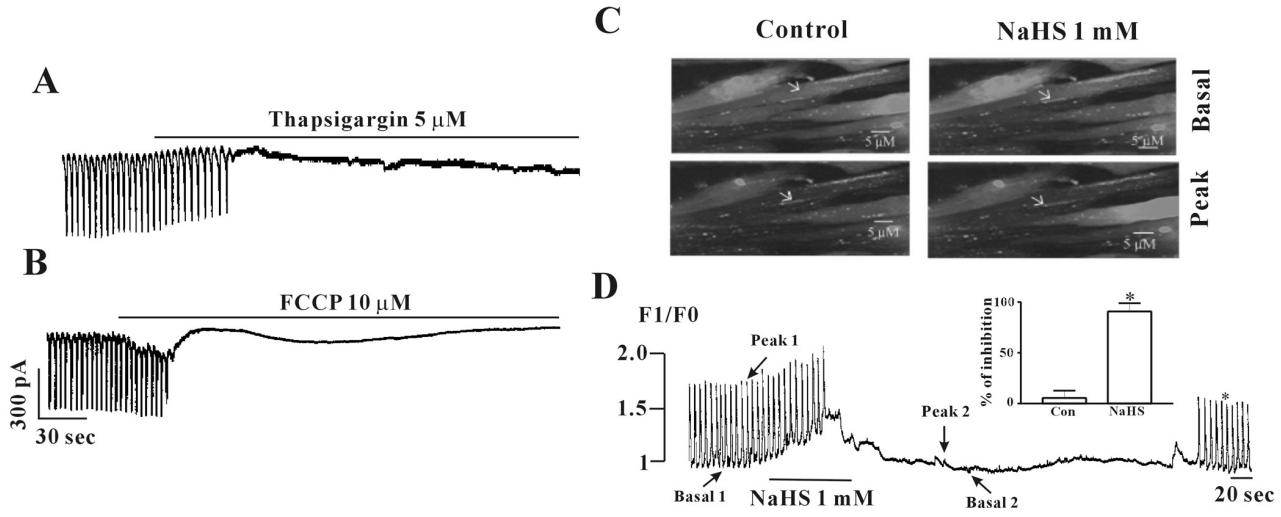

Fig. 4. Effects of thapsigargin or FCCP on pacemaker currents and effects of NaHS on $[Ca^{2+}]_i$ oscillation in cultured ICC from mouse small intestine. (A) Treatment with thapsigargin (5 μM) inhibited the pacemaker currents of ICC. (B) Treatment with FCCP (10 μM) in ICC also inhibited the pacemaker currents. (C) Sequential fluorescence intensity images of fluo-4-loaded cultured ICC in normal condition or in presence of NaHS (1 mM). The interval of representative frame was 1 second and the exposure time of each frame was 500 ms. (D) Fluorescence intensity changes plotted in (C) white marker. (Inset) Graphic representation of the inhibition of intensity at peak point of Ca^{2+} oscillations by NaHS. *($p<0.05$) Significantly different from the untreated control. Con, control.

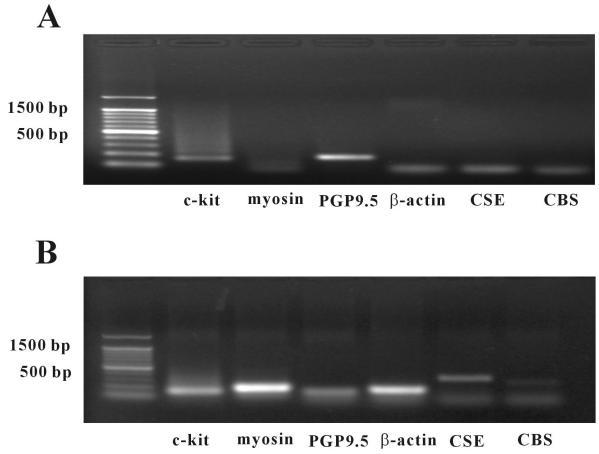

Fig. 5. Agarose gels of the RT-PCR products of CSE and CBS enzymes using cultured cells and separated ICC. (A) Amplified cDNA prepared from pure ICC separated by magnetic cell separation and visualized in 2% gel. (B) Amplified cDNA from unsorted muscle cells and visualized in 2% gel (myosin-smooth muscle cells marker, PGP9.5-neuronal cell marker, CSE-cystathionine gama lyase, CBS-cystathionine beta synthase).

Involvement of intracellular Ca^{2+} on NaHS-induced action in ICC

To rule out the possible involvement of $[Ca^{2+}]_i$ in the NaHS-induced effect on pacemaker currents, thapsigargin, a Ca^{2+}-ATPase inhibitor of endoplasmic reticulum and FCCP, an inhibitor of mitochondrial Ca^{2+} uptake were tested. An exposure of thapsigargin (5 μM) or FCCP (10 μM) to ICC resulted in an inhibition of the pacemaker activity of ICC (Fig. 4A). As with NaHS, thapsigargin and FCCP significantly suppressed both the amplitude and frequency of the pacemaker (n=4; bar graph not shown). We next examined the effect of NaHS on ICC $[Ca^{2+}]_i$ oscillations, because they are considered the primary mechanism for pacemaker activity in gastrointestinal motility. Specifically, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC using cell cluster preparations. Under control conditions, spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICC loaded with fluo4-AM (Fig. 4C), and spontaneous regular $[Ca^{2+}]_i$ oscillations were observed in a time series (Fig. 4D). Fig. 4C shows images of basal (F0) and peak point (F1/F0) of Ca^{2+} oscillations in both normal circumstances and in the presence of 1 mM NaHS. Our results found that in the presence of NaHS, intracellular Ca^{2+} oscillations in ICC rapidly declined (Fig. 4D). The summarized data about inhibitory NaHS-induced action at peak points of Ca^{2+} oscillations are shown in the inset figure (n=6).

No expression of mRNA transcript for CBS and CSE enzymes ICC

A fragment of mRNA for cystathionine- gama-lyase (CSE) (370 bp) and cystathionine beta-synthase (CBS) (250 bp) isolated from sorted ICC or cultured whole tissues were amplified using gene specific primers CBS and CSE, to examine the availability of CBS and CSE to produce H_2S. Cells separated by magnetic cell separation technique from freshly prepared cultured cells using c-kit antibody were enriched with c-kit positives ICC. ICC enriched population expressed c-kit gene (161 bp) but did not express myosin (233 bp) or PGP 9.5 (168 bp). mRNA isolated from magnetic cell separation was also amplified with primer β-actin as a positive control. mRNA neither for CSE nor CBS were amplified in sorted ICC (Fig. 5A). However, 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.

DISCUSSION

In this study, we found that, in high concentration, NaHS inhibited the pacemaker activity of ICC. The higher concentrations (500 μM~1 mM) of NaHS produced slight inward currents and then outward currents with significant suppression of the amplitude and frequency of pacemaker activity in ICC.

Because of its lipid solubility, H_2S can easily penetrate the plasma membranes of cells in the undissociated form. H_2S is produced in substantial amounts by mammalian tissues and exerts many physiological effects, suggesting its potential role as a regulatory mediator. Endogenously produced H_2S in mammals is found to be effective in various ways, depending on what tissue. 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 [5,8,9]. Actually, the maximum inhibitory effects on pacemaker currents from ICC was gained using 1 mM NaHS, which is a relatively higher amount than that of endogenously expressed physiological concentration in mammalian tissues. But it was suggested that the maximum yield of NaHS, a donor of H_2S, is about 33% of applied concentration [10]. Therefore, the exogenously applied NaHS concentration was less than the physiological concentration in this study.

ATP-sensitive K^+ channels regulate the electrical membrane potential, thus they determined cell excitability. Activation of ATP-sensitive K^+ channels produces hyperpolarization of the cell membrane [11]. Previously, we reported that ATP-sensitive K^+ channels existed in mouse intestinal ICC [12]. Related to this, there are some reports that the activation of ATP-sensitive channels by NaHS was proposed as the consequence of ATP depletion due to the inhibition by sulfide of the oxidative phosphorylation [10], with partial involvement of ATP-sensitive K^+ channels on NaHS-induced contraction and relaxation in rat and mouse aorta [13]. These reports show the possibility that NaHS-induced action on pacemaker activity in ICC can be mediated by regulation of ATP-sensitive K^+ channels. Therefore, to study whether NaHS-induced effect was mediated by ATP-sensitive K^+ channels, the action of glibenclamide on NaHS-induced effect was tested. From this, we could not see any influence on NaHS-induced effect in presence of glibenclamide and furthermore, various kinds of K^+ channels blockers could not show any action on NaHS-induced effect in this study. These results mean potassium channels, especially ATP-sensitive K^+ channels may not be involved in NaHS-induced action on pacemaker activity in ICC. Interestingly, it was suggested that two-pore K^+ channels encode stretch-dependent K^+ channels in mouse GI

smooth muscle cell [14] and that sulfur-containing amino acids can enhance spontaneous contraction by blocking stretch-dependent K^+ channels and nitrergic responses in colonic myocytes [15]. Though it is well known that ICC in the GI tract can serve as a stretch receptor, there were no reports of the existence of stretch-dependent K^+ channels in ICC. These results suggest the possibility that NaHS has dual action either contraction by acting on muscle cells or relaxation via ICC in GI motility.

In order to find out how NaHS inhibits the pacemaker activity in ICC, we focused on $[Ca^{2+}]_i$. It is well known that the periodic pacemaker activity of ICC is dependent on $[Ca^{2+}]_i$ oscillation and that this pacemaker mechanism is initiated by the release of Ca^{2+} from the endoplasmic reticulum through the inositol triphosphate (IP_3) receptor followed by re-uptake of Ca^{2+} into the mitochondria [16,17]. In previous reports, we also showed the pacemaker currents were abolished in Ca^{2+}-free solution [18]. Moreover, we could see that thapsigargin, a Ca^{2+}-ATPase inhibitor of endoplasmic reticulum and FCCP, an inhibitor of mitochondrial Ca^{2+} uptake, blocked the pacemaker activity of ICC, as did the NaHS-induced action in this study. These results showed the important role of $[Ca^{2+}]_i$ for pacemaker activity in ICC. Interestingly, the application of FCCP or NaHS showed slight inward currents firstly and then inhibitory action on pacemaker currents from ICC. These can be the conjecturable mechanisms that the blocking of Ca^{2+} re-uptake into the mitochondria from cytoplasm increases the cytoplasmic Ca^{2+} levels in ICC. Then these will inhibit the spontaneous periodic pacemaker activity of ICC. Furthermore, we could see, by looking at Ca^{2+} imaging tests, that NaHS inhibited the $[Ca^{2+}]_i$ oscillation in ICC.

Two classes of pyridoxal-5'-phosphate-dependent enzymes, CBS and CSE are responsible for the majority of the endogenous production of H_2S in mammalian tissues that use L-cysteine as the main substrate [19,20]. 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 and, CBS is mainly expressed in the liver and central nervous system [8,21]. Florucci et al. (2006) have 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 [22]. In the present study, mRNA for both CSE and CBS were not expressed in sorted ICC but prominently expressed in unsorted muscles. It seems that CSE is 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 unsorted muscles may suggest that H_2S is endogenously produced, either in smooth muscle cells or neuronal cells or other populations of cells than ICC.

In conclusion, this study describes the effects of H_2S on ICC in the small intestine of the mouse. H_2S inhibited the pacemaker activity of ICC, which was via internal Ca^{2+} mobilization. Expression of CSE in unsorted muscles but not in sorted ICC suggests that ICC is not the target of H_2S production, but the response of H_2S may be transmitted either from smooth muscle cells or neuronal cells. Thus, the action of H_2S on ICC may explain the inhibitory action of H_2S in GI motility.

ACKNOWLEDGEMENTS

This work was supported by the Korean Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-521-E00006).

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