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MECHANISMS OF ACTION OF THE GASOTRANSMITTER HYDROGEN SULFIDE IN MODULATING CONTRACTILE ACTIVITY OF LONGITUDINAL MUSCLE OF RAT ILEUM

Munenori Nagao, M.D.¹, David R. Linden, Ph.D.², Judith A. Duenes, BA¹, and Michael G. Sarr, M.D.¹

¹ Gastroenterology Research Unit and Division of Gastroenterologic and General Surgery, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA

² Department of Physiology and Biomedical Engineering, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA

Abstract

AIM—To determine mechanisms of action of the gasotransmitter hydrogen sulfide (H₂S) on contractile activity in longitudinal muscle of rat ileum.

METHODS—Ileal longitudinal muscle strips were prepared to measure isometric contractions. Effects of sodium hydrosulfide (NaHS), a donor of H₂S, were evaluated on spontaneous contractile activity and after enhanced contractile activity with bethanechol. L-cysteine was evaluated as a potential endogenous donor of H₂S. We evaluated involvement of extrinsic nerves, enteric nervous system, visceral afferent nerves, nitric oxide, and K⁺_{ATP} channel and K⁺_{Ca} channel activity on the action of H₂S using non-adrenergic/non-cholinergic conditions, tetrodotoxin, capsaicin, L-N^G-nitro arginine (L-NNA), glibenclamide, and apamin, respectively, as well as electrical field stimulation.

RESULT—NaHS dose-dependently and reversibly inhibited spontaneous and bethanechol-stimulated contractile activity (p<0.05). L-cysteine had no inhibitory effect. Non-adrenergic/non-cholinergic conditions, tetrodotoxin, capsaicin, L-NNA, glibenclamide, or apamin had no major effect on total contractile activity by NaHS, although both tetrodotoxin and apamin decreased the frequency of bethanechol-enhanced contractile activity (p<0.05). We could not demonstrate H₂S release by electrical field stimulation but did show that inhibition of cystathionine β synthase, an endogenous source of H₂S, augmented the inhibitory effect of low-frequency electrical field stimulation.

CONCLUSION—H₂S inhibits contractile activity of ileal longitudinal muscle dose-dependently but not through pathways mediated by the extrinsic or enteric nervous system, visceral afferent nerves, nitric oxide, K⁺_{ATP} channels, or K⁺_{Ca} channels.

Keywords

intestinal motility; gasotransmitter; hydrogen sulfide; longitudinal muscle; physiology; motility; contractile activity; ileum longitudinal smooth muscle

Address correspondence to Michael G. Sarr, M.D., Professor of Surgery, Gastroenterology Research Unit (Guggenheim 10-01), Mayo Clinic, 200 1st Ave SW, Rochester, MN 55905. Tel: 507-255-5713. Fax: 507-255-6318., sarr.michael@mayo.edu and frank.deborah@mayo.edu.

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INTRODUCTION

Contractile activity of the small intestine is regulated or modulated by many factors, such as the central nervous system, the enteric nervous system (ENS), gut hormones, mechanical factors, and the local neurohumoral milieu. Neural modulation by the classic neurotransmitters acetylcholine and norepinephrine, or the non-adrenergic, non-cholinergic neurotransmitters, such as VIP, Substance P, and many other neuropeptides, transmit the neural signal by binding to receptors on the post-synaptic cell membrane, inducing the intracellular release of a second messenger, which then leads to alteration of contractile activity. It has become increasingly clear that endogenously-produced biologic gases called “gasotransmitters” can also play an important role in the signal transduction from nerves in the control of small intestinal motility. The two more commonly appreciated gasotransmitters, nitric oxide (NO) and carbon monoxide (CO), are freely permeable across the cell membrane, diffuse into the target cell, and affect intracellular pathways directly; these gasotransmitters are released following “on demand” enzymatic synthesis {Wang, 2003 #18}. Thus, mechanisms of signal transduction by gasotransmitters are different from that of the classic neurotransmitters.

Hydrogen sulfide (H₂S), which is known more commonly as a toxic pollutant, is the newest member of the gasotransmitter family {Kasperek, 2008 #7}. Endogenous H₂S is produced from the substrate L-cysteine by two enzymes, cystathionine beta (β) synthase (CBS) and cystathionine gamma (γ) lyase (CSE). The mechanism of action of H₂S is well studied in vascular smooth muscle {Lowicka, 2007 #2}, where H₂S opens ATP-sensitive potassium channels (K⁺_{ATP} channels) leading to hyperpolarization of the membrane potential, closing of voltage-gated Ca²⁺ channels, and subsequent vasorelaxation.

Very few studies have explored the role and mechanisms of H₂S in the control of small intestinal motility. Prior work in our laboratory demonstrated that the enzymes that generate endogenous H₂S, CBS, and CSE, are expressed in the enteric nerves of the small intestine (Kasperek et al., under review). Moreover, two prior studies reported that exogenous NaHS, an H₂S donor, caused a dose-dependent inhibition of jejunal and ileal contractile activity {Gallego, 2008 #9; Teague, 2002 #11; Hosoki, 1997 #1}. This inhibitory effect was independent of K⁺_{ATP} channel activity {Teague, 2002 #11}. Moreover, mechanisms of action and functional roles of H₂S in the modulation of small intestinal contractile activity remain poorly understood.

The aim of our study was to determine the effects and mechanisms of action of H₂S applied either exogenously and released endogenously on contractile activity in the longitudinal muscle of rat ileum. By using inhibitors which block specific potential pathways of signal transduction, we studied the involvement of the enteric nervous system, primary afferent nerve fibers, NO, and two different types of K⁺ channels (K⁺_{ATP} channel and K⁺_{Ca} channel) in the response to H₂S. By using exogenously applied L-cysteine, the substrate for endogenous production of H₂S, and electric field stimulation in an attempt to release H₂S from enteric nerves, we sought to investigate the effects of endogenous release of H₂S. Our hypothesis was that H₂S released from enteric nerves acts as an endogenous inhibitor of contractile activity of the longitudinal smooth muscle of rat ileum by a direct effect on smooth muscle contractile activity via opening of K⁺_{ATP} channels.

MATERIALS AND METHODS

Preparation of Animals

Procedure and animal care were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Mayo Foundation in accordance with the

guidelines of the National Institutes of Health and the Public Health Service Policy of the Human Use and Care of Laboratory Animals and was approved by the IACUC of the Mayo Clinic.

Recording of Contractile Activity

Male Lewis rats (Harlan-Sprague-Dawley, Indianapolis, IN) weighing 275–350 g were used in the experiments. Rats were anesthetized initially with inhalation of 2% isoflurane (Abbott Laboratories, North Chicago, IL) and maintained by intraperitoneal sodium pentobarbital (30–50 mg/kg; AmproPharmacy, Arcadia, CA). Via a midline celiotomy, a segment of ileum 10 cm proximal to the ileocecal valve was harvested and kept in chilled, modified Krebs-Ringer's bicarbonate solution (concentrations in mmol/L: NaCl 116.4, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 23.8, calcium disodium edentate 0.26, and glucose 11.1) pre-oxygenated with 95% oxygen/5% carbon dioxide (Praxair, Burr Ridge, IL). After opening the ileal segment along its mesenteric border, full-thickness muscle strips (2x8 mm; width x length) were cut in the direction of the longitudinal muscle layer. Purposely, we did not remove the mucosa and submucosa to maintain the transmural anatomy and all enteric neural connections. Both ends of the muscle strip were tied with 5-0 silk and suspended vertically in 10-ml tissue chambers filled with modified Krebs-Ringer's bicarbonate solution kept at 37.5°C and bubbled continuously with 95% oxygen/5% carbon dioxide. One end of the muscle strip was connected to a fixed hook; the other end was attached to a metal hook connected to a noncompliant force transducer (Kulite Semiconductors Products, Inc., Leonia, NJ) to measure the isometric force generated by the muscle strip. Contractile activity was monitored by an 8-channel recorder (Grass 7D polygraph; Grass Instrument Co, Quincy, MA) in real-time while in parallel being displayed and stored digitally on a personal computer using dedicated software (MP-100A-CE and AcqKnowledge; Biopac Systems, Inc., Goleta, CA) for detailed computer analysis later. Our system has been well described previously {Kasperek, 2009 #25; Kasperek, 2007 #27}. At the end of each experiment, the muscle strips were blotted on filter paper and weighed to standardize the contractile data as per mg tissue weight.

Experimental Design

Muscle strips were equilibrated for 60–90 min with washout of the bath solution every 15 min to allow development of stable, spontaneous contractile activity. Thereafter, the optimal length (L_0) of each muscle strip was achieved by incremental stretching at 5- to 10-min intervals to a length beyond which further stretching no longer increased either amplitude or frequency of spontaneous contractile activity. All subsequent experiments were performed at L_0 . Muscle strips not developing a stable and characteristic spontaneous contractile pattern were excluded from the study. Each experimental condition was carried out in at least two muscle strips per rat in a minimum of 6 rats per condition.

To determine the effect of exogenous H_2S , we chose to use the well-established H_2S donor NaHS. At pH of 7.4 and temperature of 37.5°C, 18.5% of NaHS exists as H_2S in solution {Dombkowski, 2004 #24}. To determine a dose-response curve, four different escalating concentrations of NaHS (10^{-5} , 10^{-4} , 5×10^{-4} , 10^{-3}M) yielding concentrations of H_2S in solutions of about 1.8, 18, 90, and 180 μM , respectively, were added to eight muscle strips per rat in 8 rats with washout of the bath solution between each dose. We used NaHS as an exogenous donor of H_2S purposely, because not only is it easier technically, but use of NaHS is also more reliable in attaining an accurate concentration of H_2S in the bath solution than preparing a solution by bubbling of H_2S through the bath. The effect of NaHS (10^{-4} and 10^{-3}M) on pre-contracted muscle strips was studied by application of NaHS 90 s after exposure of the muscle strips to the muscarinic agonist bethanechol at a dose of $3 \times 10^{-6}\text{M}$; this dose of bethanechol caused an increase in the frequency and amplitude of contractions

rather than a tonic contraction {Ohtani, 2000 #23}. We applied these two doses of NaHS cumulatively at an interval of 5 min without washout between doses to determine a dose response during bethanechol-enhanced contractile activity.

Next, non-adrenergic and non-cholinergic (NANC) conditions were established by adding atropine (10^{-7} M), phentolamine (10^{-5} M), and propranolol (5×10^{-6} M) to the bath of two muscle strips to investigate the role of adrenergic and cholinergic neurons in mediating the effect of NaHS as we have reported before {Kasperek, 2009 #25; Kasperek, 2007 #27}. The effect of NaHS was studied at two doses (10^{-4} and 10^{-3} M) beginning 30 min after establishment of NANC conditions; because the muscarinic receptor antagonist atropine was used to establish NANC conditions, we did not study the effect of NaHS after administration of bethanechol. Thereafter, the effect of the global neural inhibitor tetrodotoxin (TTX; 10^{-6} M) on baseline activity was studied in the same two muscle strips as for the NANC conditions as reported before {Kasperek, 2009 #25; Kasperek, 2007 #27}. TTX inhibits voltage-gated, fast sodium channels in nerve cell membranes to prevent depolarization of the cell membrane and the subsequent release of neurotransmitters from virtually all nerves within the muscle strip. After exposure of the muscle strips to TTX for 30 min, the effect of NaHS was evaluated at two doses (10^{-4} and 10^{-3} M). Thereafter, the effect of NaHS was studied again at two doses (10^{-4} and 10^{-3} M) after bethanechol (3×10^{-6} M) in the ongoing presence of TTX.

In two other muscle strips from 6 rats, we used capsaicin to investigate the role of visceral afferent nerves in mediating the effect of NaHS. Capsaicin is an agonist of transient receptor potential vanilloid receptor 1 (TRPV-1) and is a well-established method of desensitizing primary afferent nerve fibers *in vitro* {Schicho, 2006 #3; Holzer, 1991 #31; Holzer, 1991 #32; Patacchini, 2004 #33; Trevisani, 2005 #30}; we confirmed the effectiveness of capsaicin in preliminary experiments by observing a tachyphylaxis, i.e. a lack of an immediate contractile response (which occurred on first exposure to capsaicin) on subsequent doses of capsaicin. After exposure to capsaicin at two separate doses of 10^{-5} and 10^{-4} M for 30 min, the effect of NaHS was studied at two doses of 10^{-4} and 10^{-3} M. Thereafter, the effect of NaHS at two doses (10^{-4} and 10^{-3} M) was studied again after bethanechol (3×10^{-6} M) in the ongoing presence of capsaicin.

In two other muscle strips from 6 rats, we used the NO synthase inhibitor L-NG-nitro arginine (L-NNA) at two separate doses of 10^{-4} and 10^{-3} M to investigate the effect of the NO pathway and/or any interaction with NO in mediating the effect of NaHS. L-NNA inhibits endogenous production of NO at these doses {Kasperek, 2007 #27}. After exposure to L-NNA at two doses (10^{-4} and 10^{-3} M) for 30 min, the effect of NaHS was studied at two doses (10^{-4} and 10^{-3} M). Thereafter, the effect of NaHS was studied again at two doses (10^{-4} and 10^{-3} M) after bethanechol (3×10^{-6} M) in the ongoing presence of L-NNA.

In the last two muscle strips from 6 rats, we used glibenclamide at two doses of 10^{-4} and 10^{-3} M to investigate the involvement of K^{+}_{ATP} channels in the effect of NaHS. Glibenclamide blocks the K^{+}_{ATP} channel at these doses {Gallego, 2008 #9}. After exposure to glibenclamide at two doses of 10^{-4} and 10^{-3} M for 30 min, the effect of NaHS was studied at two doses (10^{-4} and 10^{-3} M). Thereafter, the effect of NaHS was studied again at two doses (10^{-4} and 10^{-3} M) after bethanechol (3×10^{-6} M) in the ongoing presence of glibenclamide.

In two different muscle strips from 6 rats, the effect of L-cysteine was studied at three doses of 10^{-4} , 10^{-3} , and 10^{-2} M with washout between each dose. Because L-cysteine is a substrate for endogenous enzymatic production of H_2S {Kasperek, 2007 #27}, we hypothesized that L-cysteine would increase endogenous production of H_2S , and the H_2S

released would have some effect on contractile activity. We did not study the effect of L-cysteine after bethanechol. In these same two muscle strips, we used apamin to investigate the involvement of K^+_{Ca} channels in the response to NaHS because apamin blocks the K^+_{Ca} channels [Gallego, 2008 #9]. After exposure to apamin at two doses of 10^{-6} and 5×10^{-6} M for 30 min, the effect of NaHS was studied at two doses (10^{-4} and 10^{-3} M). Thereafter, the effect of NaHS was studied again at two doses (10^{-4} and 10^{-3} M) after bethanechol (3×10^{-6} M) in the ongoing presence of apamin.

In six other muscle strips from 6 rats, the response to electrical field stimulation (EFS) was evaluated at 6 and 50 Hz using a constant voltage (20 V), pulse width (0.5 msec), and duration of stimulation (10 s) similar to our previous work [Kasperek, 2009 #25]. We chose 6 Hz as an inhibitory EFS and 50 Hz as an excitatory EFS based on prior work [Kasperek, 2009 #25]. All EFS studies were performed under NANC conditions with atropine (10^{-7} M), phentolamine (10^{-5} M), and propranolol (5×10^{-6} M) in the bath to exclude adrenergic and cholinergic effects induced by EFS. Between each EFS, 10 min were allowed for spontaneous contractile activity to recover before the next EFS was applied; the bath solution was changed after each series of stimulations. First, we evaluated the response of spontaneous contractile activity to EFS under NANC conditions in all six muscle strips as control conditions. After completing these control conditions, we evaluated EFS separately after inhibition of the endogenous H_2S -producing enzymes CBS and CSE, an inhibitor of NO synthase, and a competitive inhibitor of vasoactive intestinal peptide (VIP). First, we used the CBS inhibitor aminooxyacetic acid (AOAA) at a dose of 10^{-4} M. After exposure to AOAA for 30 min in two muscle strips from 6 rats, the effect of AOAA on baseline contractile activity was evaluated for 15 min. Thereafter, the response to EFS was studied in the presence of AOAA. In two other muscle strips, we used the CSE inhibitor DL-propargylglycine (PPG) at a dose of 2×10^{-3} M [Linden, 2008 #8]. After exposure to PPG for 30 min, the effect of PPG on baseline contractile activity was evaluated for 15 min. Thereafter, the response to EFS was studied in the presence of PPG. After washout of the bath solution, we evaluated the effect on baseline contractile activity of the combination of AOAA (10^{-4} M) and PPG (2×10^{-3} M) for 30 min and, thereafter, the response to EFS was evaluated in the presence of both AOAA and PPG.

In two other muscle strips from 6 rats, we evaluated the effects of inhibiting the dominant NANC inhibitory neurotransmitters NO and VIP in an attempt to reveal any more subtle effects of the release of endogenous H_2S by EFS. We used L-NNA at a dose of 10^{-3} M. After determining the response of baseline contractile activity to L-NNA exposure for 30 min, the response to EFS was studied in the presence of L-NNA. After washout of the bath solution, we evaluated the combination of L-NNA (10^{-3} M) and the VIP antagonist [D-p-Cl-Phe⁶,Leu¹⁷]-VIP (10^{-6} M) for 30 min. Baseline contractile activity and the response to EFS were studied in the presence of L-NNA and the VIP antagonist. After washout of the bath solution, we then used the combination of all four inhibitors/antagonists, L-NNA (10^{-3} M), VIP antagonist (10^{-6} M), AOAA (10^{-4} M), and PPG (2×10^{-3} M). After exposure for 30 min, the effect of these four inhibitors on baseline contractile activity and EFS was evaluated.

Data Analysis

Phasic changes in force (total contractile activity) measured as area under the contractile curve (AUC) were analyzed by a data acquisition system (AcqKnowledge, Biopac Systems, Inc., Goleta, GA). We set the baseline tone before each intervention as zero when we calculated the AUC, which enabled us to analyze the phasic contractile activity. In addition to the measurements of AUC, we also measured and analyzed changes of mean amplitude, baseline tone, and frequency under each condition. Thereafter, the effects of each of the drugs NaHS, L-cysteine, NANC conditions, TTX, capsaicin, L-NNA, glibenclamide and apamin on spontaneous activity were measured for 5 min and compared to the baseline

contractile activity for 5 min measured immediately before each drug was administered. This technique allowed us to control for any effects on baseline contractile activity by any of the drugs tested. In contrast, the effect of administration of AOAA and/or PPG and L-NNA alone or in combination with the VIP antagonist on spontaneous contractile activity was measured for 15 min after exposure of the muscle to these agents for 30 min and was compared to the 5 min immediately before administration of the antagonists. When muscle strip contractile activity was enhanced with bethanechol, the subsequent response to NaHS was measured for 5 min and compared to the 90 s of pre-contraction immediately before administration of NaHS and adjusted for a 5-min interval. For the dose responses to NaHS, the responses after pre-contraction, and the responses to the various inhibitors/antagonists, the mean value of individual muscle strips per rat were meaned, and the mean responses across the 6 rats were calculated. Drug responses are given as % change from baseline contractile activity (defined as 0%), with positive values representing an increase and negative values a decrease in contractile activity.

The response to EFS was studied for the 10 s of EFS in all experiments; the “off contraction” that occurred immediately after termination of EFS was not evaluated. According to the findings from our previous studies, we used 6 Hz as an inhibitory frequency, and 50 Hz as a non-inhibitory EFS frequency {Kasperek, 2009 #25; Ohtani, 2000 #23}. Because our previous work suggested differences in the first 4 s and the last 6 s of the total 10 s of EFS, we analyzed separately the effects of EFS for the whole 10 s, the first 4 s, and the last 6 s {Kasperek, 2007 #27; Kasperek, 2007 #28; Kasperek, 2008 #26; Kasperek, 2008 #29}. Contractile activity was expressed as the percent of baseline contractile activity for an equally long interval (4, 6, or 10 s) measured during the 20 s immediately before EFS.

All data are expressed as mean $\pm$ SEM. Analysis of variance (ANOVA) was used to analyze the effects of a dose-response to NaHS, while paired Student's t-tests were used to compare the effects of different drugs and EFS; when individual comparisons were made, we used the conservative Bonferroni correction to correct for the multiple comparisons. In addition, we also used Wilcoxon rank sums when the values were not distributed normally.

Drugs

Apamin, AOAA, atropine sulfate, bethanechol chloride, capsaicin, L-cysteine, glibenclamide, L-NNA, phentolamine hydrochloride, PPG, DL-propranolol hydrochloride, NaHS, TTX, [D-p-Cl-Phe⁶,Leu¹⁷]-VIP were purchased from Sigma-Aldrich, St. Louis, MO. For the stock solution, capsaicin and glibenclamide were dissolved in dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO). L-NNA and DL-propranolol hydrochloride are dissolved in 0.5 N hydrochloric acid (HCl), and 0.1 N HCl was used for further dilutions to 10⁻⁴M of L-NNA. Preliminary experiments showed that 0.5 N HCl and dimethylsulfoxide had no effect on spontaneous contractile activity or pH of the bath solution. All other drugs were dissolved in purified water.

RESULTS

Response to NaHS (Exogenous donor of H₂S)

NaHS at all doses inhibited spontaneous basal activity in a dose-dependent manner ($p < 0.05$) (Figures 1A and 2A). NaHS at the doses of 10⁻⁴ and 10⁻³M also inhibited the contractile activity enhanced by bethanechol (3 $\times$ 10⁻⁶M) ($p < 0.05$) to the same percentage as for spontaneous activity (Figures 1B and 2B). These effects of NaHS on spontaneous (Table 1A) and enhanced contractile activity (Table 1B) occurred by inhibiting total contractile activity (area under the contractile curve) but also by decreasing amplitude, baseline tone, and frequency of contractions (at greater dose of 5 $\times$ 10⁻⁴M on spontaneous contraction).

To investigate the involvement of neural pathways in the response to NaHS, NaHS at the doses of 10^{-4} and 10^{-3} M was used under NANC conditions and after pretreatment with TTX. There were no significant changes in the inhibitory effects of NaHS on total contractile activity under NANC conditions or after pretreatment with TTX (Table 2). The presence of TTX decreased the inhibitory effect of NaHS on the frequency of contractions in bethanechol-treated tissues, but had no effect on total contractile activity, average amplitude, or baseline tone (Table 3).

To investigate the involvement of primary afferent nerve fibers in the response to NaHS, primary afferent nerve fibers were defunctionalized with capsaicin (10^{-5} and 10^{-4} M) as described previously [Schicho, 2006 #3; Holzer, 1991 #31; Holzer, 1991 #32; Patacchini, 2004 #33; Trevisani, 2005 #30]. The lesser dose of capsaicin caused a short-lasting excitatory response immediately after administration, but when evaluated 30 min later, contractile activity had returned to baseline levels, and capsaicin had no persistent effect on spontaneous contractile activity for the next 5-min baseline interval. In contrast, the greater dose of capsaicin decreased spontaneous contractile activity that persisted for the duration of the NaHS experiments (at least 45 min); thereafter, when the tissue chamber was washed, spontaneous contractile activity returned. There was no change in the inhibitory effect of NaHS on total contractile activity after desensitization of primary afferent nerves with capsaicin on total contractile activity either during spontaneous contractile activity (Table 2) or after bethanechol (3×10^{-6} M) (Table 3).

To investigate any interaction between H_2S and the release of NO, L-NNA was used to block NO production. At the doses of 10^{-4} and 10^{-3} M of NaHS, there was no change in the inhibitory effect of NaHS on total contractile activity after pretreatment with L-NNA on either spontaneous contractile activity (Table 2) or after bethanechol (3×10^{-6} M) (Table 3).

Finally to investigate the involvement of K^+_{ATP} and K^+_{Ca} channels in the response to NaHS, glibenclamide and apamin were used. Glibenclamide alone at both doses inhibited spontaneous contractile activity ($p < 0.05$); however, the inhibitory effects of NaHS on total contractile activity either during spontaneous bethanechol-enhanced contractile activity were unchanged (Tables 2 and 3). Apamin had no effects on baseline spontaneous activity, and pretreatment with apamin also had no effect on the inhibitory effects of NaHS either on total contractile activity, average amplitude, or basal tone either during spontaneous or enhanced activity (Tables 2 and 3). The greater concentration of apamin did, however, significantly decrease the inhibitory effect of NaHS on the frequency of contractions in bethanechol-enhanced tissues (Table 3) but not on baseline spontaneous activity (Table 2).

Effect of endogenous substrate of H_2S

Administration of L-cysteine did not alter spontaneous contractile activity at any of the doses evaluated (10^{-4} , 10^{-3} and 10^{-2} M; data not shown). We did not evaluate the effect of L-cysteine on bethanechol-enhanced activity.

Response to EFS

EFS at 6 Hz did not alter total contractile activity (AUC) for the entire 10 s in NANC conditions but did inhibit total contractile activity during the first 4 s of EFS. In the presence of all of the inhibitors we evaluated (PPG, PPG and AOAA, L-NNA, L-NNA and VIP_{antag}, and all four), there was no significant difference in the effect of EFS compared to control conditions (NANC conditions) for the entire 10 s, first 4 s, or last 6 s (Figure 3A) except for an augmentation of inhibition with AOAA alone at each time duration tested. This effect of AOAA was not seen when AOAA was combined with PPG; when mean amplitude, baseline

tone, and frequency were analyzed, no changes were noted, similar to total contractile activity (data not shown).

EFS at 50 Hz increased total contractile activity for the entire 10 s and for the last 6 s in NANC conditions ($p < 0.05$) and caused an inhibition of total contractile activity during the first 4 s of EFS ($p < 0.05$). In the presence of these same inhibitors we used, there were no significant differences in the effects of EFS compared to control conditions (NANC conditions) for the entire 10 s, first 4 s, or last 6 s (Figure 3B). When mean amplitude, baseline tone, and frequency were analyzed, no changes were noted, similar to the lack of effect on total contractile activity (data not shown).

DISCUSSION

The aim of our study was to determine the effects and mechanisms of action of exogenous and endogenously-released H_2S on contractile activity in the longitudinal muscle of the ileum in rats. We studied ileal longitudinal muscle as part of our ongoing, comprehensive approach to characterizing inhibitory neurotransmitters in the small intestine {Kasperek, 2007 #27; Kasperek, 2007 #28; Kasperek, 2008 #26; Kasperek, 2008 #29}. By using several targeted inhibitors which block different potential pathways of signal transduction, we showed that H_2S inhibited reversibly the spontaneous and cholinergically stimulated contractile activity in rat ileal longitudinal muscle. This effect was not mediated via the enteric nervous system, primary visceral afferent nerve fibers, production of NO, K^+_{ATP} channels, or K^+_{Ca} channels. Our experiments with EFS attempted to uncover an inhibitory effect of endogenously-released H_2S from intrinsic nerves. Although we were unable to show a convincing effect of inhibiting CSE, preventing the release of NO, or antagonizing VIP, we did demonstrate an augmentation of the initial inhibitory effect of EFS at 6 Hz by inhibiting CBS.

To the best of our knowledge, there are few reports that describe the effects of H_2S on contractile activity in the longitudinal smooth muscle of ileum in any species. Several reports have shown that exogenous H_2S manifests an inhibitory effect on spontaneous contractile activity in a dose-dependent manner in the ileum of rabbit {Teague, 2002 #11}, as well as in pre-contracted ileal circular muscle of guinea pig {Hosoki, 1997 #1}. We also demonstrated a dose-dependent, reversible inhibitory effect of NaHS on the presence of spontaneous and stimulated contractile activity as well as on mean amplitude, baseline tone, and frequency in the longitudinal muscle of rat ileum. Our data are in large part compatible with the limited data reported previously, although our experiments more comprehensively evaluate the potential mechanisms of action of H_2S .

We used $10^{-5}M$ to $10^{-3}M$ NaHS as an exogenous donor of H_2S ; 18.5% of NaHS in solution exists as H_2S . This approach led to estimated concentrations of H_2S of 1.85 to 185 μM . The concentrations of NaHS we used are similar to those in other reports {Gallego, 2008 #9; Teague, 2002 #11; Hosoki, 1997 #1} that also cause apparent physiologic effects, which suggests that responses to μM concentrations reproduce physiologic responses. This type of experiment tries to reproduce local concentrations of a "gasotransmitter" near the site of action rather than a general tissue concentration; this approach is similar to the approach used for neurotransmitters. Indeed, the local concentrations of H_2S may be greater than the general tissue or serum concentrations, because H_2S is released locally; local effects on synthesis and metabolism of H_2S may be very different near the site of release and the site of action {Linden, 2010 #41}. There was no evidence that these concentrations caused any tissue toxicity, because the recovery of contractile activity after washout of the bath solution was rapid and complete even after repeated applications of the greatest concentration of NaHS ($10^{-3}M$).

The ENS plays an important role in modulating gastrointestinal motility. Much of our previous work and that of many others have focused on the ENS as the site of release of inhibitory neurotransmitters. We explored whether H₂S might induce the release of inhibitory neurotransmitters from intramural neurons. We showed that blocking NANC nerves selectively or blocking neural depolarization with TTX (and thus release of presynaptic vesicles non-selectively) were unable to prevent the inhibitory effect of H₂S on total contractile activity; however, TTX did block partially the inhibitory effect of NaHS on contractile frequency during bethanechol-enhanced contractile activity. These grouped observations suggest that the inhibitory effect of H₂S is not mediated primarily through modulation of enteric neural activity or by pathways stimulating enteric nerves, although H₂S may alter the increased frequency of contractions in response to a cholinergic agonist via a neural pathway.

A recent report has shown that H₂S acts to increase mucosal secretion via stimulating transient receptor potential vanilloid 1 (TRPV-1) receptors on primary afferent nerve fibers {Schicho, 2006 #3}. In the present study, there was no change of the inhibitory effect of H₂S on contractile activity after pretreatment of capsaicin. This observation suggests that the inhibitory effect of H₂S on contractile activity is not mediated via activation of primary visceral afferent nerve fibers; we cannot, however, exclude the possibility that the decrease in baseline spontaneous contractile activity after exposure to the greater dose (10⁻⁴ M) capsaicin might have impacted some of the inhibitory effect of H₂S.

The interaction of NO and H₂S in vascular smooth muscle has been well investigated {Ali, 2006 #12}. In vascular smooth muscle, exposure to NO increases the expression and activity of CSE {Fiorucci, 2006 #19}, while decreasing the expression of NO synthase {Wang, 2002 #4}. In GI smooth muscle, few reports have explored any interaction between NO and H₂S. In rat colon, the effect of H₂S is dependent on NO production {Distrutti, 2006 #40}, but in guinea pig ileum, inhibition of NO production had no effect on the response to exogenous H₂S {Teague, 2002 #11}. We also demonstrated that the inhibitory effect of NaHS in ileal longitudinal muscle of rat was not itself mediated via an NO pathway by using the inhibitor of NO synthase, L-NNA; these findings are compatible with the previous report {Teague, 2002 #11}. We did not, however, explore any interaction between NO and H₂S using an exogenous NO donor, so we cannot exclude potential interactions of H₂S on NO released independently by a non-H₂S mediated effect.

Most all the primary effects of action of H₂S in vascular smooth muscle are mediated by K⁺_{ATP} channels, the opening of which induces hyperpolarization of the cell, closing of voltage-gated calcium channels, and muscular relaxation {Fiorucci, 2006 #19}. In GI smooth muscle, the importance of K⁺_{ATP} channels is more controversial and may vary with anatomic location. K⁺_{ATP} channels play an important role in the effect of H₂S in rat colon {Gallego, 2008 #9; Distrutti, 2006 #40}; however, in guinea pig ileum, blocking of K⁺_{ATP} channels had no effect on the inhibitory effects of H₂S {Teague, 2002 #11}. In our study, we show clearly using glibenclamide pretreatment that the inhibitory effect of H₂S in rat ileal longitudinal muscle was not mediated via K⁺_{ATP} channels. We did show, however, that baseline spontaneous contractile activity after exposure to glibenclamide was decreased, implicating K⁺_{ATP} channels in the modulation of spontaneous contractile activity.

We also explored the role of K⁺_{Ca} channels. In rat colon, Gallego et al {Gallego, 2008 #9} reported that K⁺_{Ca} channels play an important role in mediating the effect of H₂S {Gallego, 2008 #9; Distrutti, 2006 #40}. In contrast, Dhaese et al {Dhaese, 2009 #37} reported that the inhibitory effect of H₂S on contractile activity in rat colon was independent of K⁺_{Ca} channels {Dhaese, 2009 #37}. Gallego et al. studied transmural segments of rat colon, while Dhaese et al. evaluated muscle strips. Our results studying transmural strips of longitudinal

muscle showed that the inhibitory effect of H₂S in rat ileum was not mediated in great part via small conductance, K⁺_{Ca} channels as blocked by apamin. Apamin did, however, decrease the inhibitory effect of NaHS on contractile frequency during bethanechol-enhanced contractile activity, similar to TTX. Because K_{Ca}⁺² channels are involved in the release of neurotransmitters, it is possible that NaHS may have a minor effect on neurotransmitter release. We cannot exclude the possibility that intermediate or large conductance K⁺_{Ca} channels may play an important role in the inhibitory effect of H₂S in ileal longitudinal muscle of rat.

Our last two experimental conditions were designed to try to release H₂S endogenously. Endogenous H₂S is believed to be produced from L-cysteine by CBS and CSE. Although both enzymes have been demonstrated to exist in rat ileum {Hosoki, 1997 #1}, and we have shown previously that both enzymes can be imaged in rat small intestinal enteric nerves by immunohistochemistry (Kasperek et al., under review), we were not able to demonstrate any inhibitory effect on contractile activity when we exposed the muscle strips to L-cysteine, the presumed substrate for CBS and CSE. Linden et al. reported that H₂S can be produced endogenously by mouse colonic muscle harvested carefully without exposure to the mucosal surface {Linden, 2008 #8}.

We tried to evaluate the role of intrinsic neurons in producing and releasing H₂S endogenously by means of delivering EFS at different frequencies. We used a low frequency (6 Hz) to investigate a relative inhibitory stimulus and a greater frequency (50Hz) known to induce a net contractile effect. Although we used inhibitors or antagonists of known inhibitory neurotransmitters to block potential pathways which may be associated with the action of H₂S, there were no consistent differences in the contractile activity as measured by area under the contractile curve, amplitude, and baseline tone, between control and other conditions with inhibitors under NANC conditions. Although Teague et al {Teague, 2002 #11} reported that PPG caused an increase of contractile activity during EFS in guinea pig ileum, our results in rat ileal longitudinal muscle failed clearly to show that PPG increased significantly the contractile activity of ileal longitudinal muscle. Our different results from the study of Teague et al. may be related to the different species (rat vs guinea pig) or the experimental conditions of EFS, such as frequency, voltage, and/or exposure time to EFS. In contrast, our findings with AOAA were of interest, because the inhibitory effect of the first 4 s of EFS at 6 Hz was potentiated by AOAA. This effect suggests that AOAA, either by its likely inhibition of H₂S release or by its inhibition of another opposing transsulfuration metabolic pathway, may somehow alter the release or inhibitory effects of another inhibitory neurotransmitter. Our experiments cannot further elucidate this question. From these results, we conclude that endogenous production and/or release of H₂S may be regulated or mediated by activation of intrinsic (enteric) neurons under the conditions of our EFS experiments. The amount of H₂S released during EFS, however, may have been too low, such that any major effects of H₂S were not detectable in our experiments.

In conclusion, we have demonstrated that H₂S in physiologic concentrations appears to be an inhibitory gasotransmitter in the ileal longitudinal muscle of rat. The inhibitory effect of H₂S did not appear to be mediated by the extrinsic or enteric nervous systems, primary visceral afferent nerve fibers, NO pathways, or K⁺_{ATP} or K⁺_{Ca} channels. This work suggests that the inhibitory effect of H₂S on smooth muscle contractile activity in the longitudinal muscle of rat ileum appears to involve other undetermined pathways. Possible mechanisms by which H₂S may inhibit small intestinal contractile activity include the possibility of biochemical sulfhydration of cysteine residues of membrane or intracellular proteins or by effects on heme in enzymes such as guanylyl cyclase to augment cGMP {Mustafa, 2009 #35; Gadalla, 2010 #36}.

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Figure 1.

Effects of NaHS on A) spontaneous contractile activity. NaHS at 10^{-3}M inhibited contractile activity by decreasing amplitude, baseline tone, and frequency, and B) after precontraction with bethanechol ($3 \times 10^{-6}\text{M}$). Bethanechol increased amplitude and baseline

tone. NaHS at 10^{-3} M inhibited contractile activity after bethanechol by decreasing amplitude, baseline tone, and frequency.

Fig. 2A **Fig. 2B** **Figure 2.**

Effect of NaHS on spontaneous and bethanechol-stimulated contractile activity. A) Spontaneous contractile activity measured by area under the contractile curve for 5 min was defined as 0%; therefore, negative values represent inhibitory effects on contractile activity. NaHS inhibited spontaneous contractile activity in a dose-dependent manner; *p < 0.05 (after Bonferroni correction) compared to spontaneous contractile activity. B) The area under the contractile curve for 5 min of the bethanechol-stimulated pre-contracted condition was

defined as 0%. NaHS at both doses inhibited contractile activity after precontraction with bethanechol; * $p < 0.05$ (after Bonferroni correction) compared to baseline contractile activity.

Figure 3.

Effect of EFS at A) 6Hz and at B) 50Hz for the entire 10 s, first 4 s, and last 6 s of EFS in the presence of inhibitors. The area under the contractile curve immediately before EFS was defined as 0%; positive values represent a stimulatory effect on contractile activity, while negative values represent an inhibitory effect. In the presence of these inhibitors, there was no significant difference of the effect of EFS compared to baseline control condition for the

entire 10 s, first 4 s, or last 6 s in total contractile activity except for an augmentation of inhibition at 6 Hz by AOAA.

Table 1

A) Effect of NaHS on spontaneous contraction (percent change from baseline contractile activity; mean±SEM; n=8 rats)				
	<u>10⁻⁵M</u>	<u>10⁻⁴M</u>	<u>5x10⁻⁴M</u>	<u>10⁻³M</u>
Total contractile activity (AUC [*])	-7±2 ^{**}	-7±2 ^{**}	-60±13 ^{**}	-108±5 ^{**}
Average amplitude	-3±1 ^{**}	-3±1 ^{**}	-20±5 ^{**}	-35±3 ^{**}
Baseline tone	-6±1 ^{**}	-5±2 ^{**}	-32±6 ^{**}	-46±5 ^{**}
Frequency	1±1	0±0	-17±3 ^{**}	-38±2 ^{**}

B) Effect of NaHS after precontraction with bethanechol (percent change from baseline contractile activity; mean±SEM; n=17 rats)		
	<u>10⁻⁴M</u>	<u>10⁻³M</u>
Total contractile activity (AUC [*])	-10±2 ^{**}	-108±3 ^{**}
Average amplitude	-6±1 ^{**}	-53±2 ^{**}
Baseline tone	-21±3 ^{**}	-65±3 ^{**}
Frequency	-5±1 ^{**}	-37±1 ^{**}

* Area under the contractile curve

** p<0.05 compared to baseline contractile activity defined as 0% (ANOVA)

Table 2

Effect of NaHS (10^{-3} M) on spontaneous contraction in the presence of specific inhibitors (percent change from baseline contractile activity; mean $\pm$ SEM; n=6 rats)

	Without inhibitor	NANC	TTX (10^{-6} M)	Capsaicin		L-NNA		Glibenclamide		Apamin	
				10^{-5} M	10^{-4} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M	10^{-6} M	5×10^{-6} M
Total contractile activity (AUC [*])	-108 $\pm$ 5	-124 $\pm$ 13	-128 $\pm$ 13	-122 $\pm$ 8	-183 $\pm$ 24	-147 $\pm$ 19	-125 $\pm$ 14	-140 $\pm$ 21	-158 $\pm$ 18	-122 $\pm$ 14	-135 $\pm$ 12
Average amplitude	-35 $\pm$ 3	-45 $\pm$ 4	-47 $\pm$ 4	-41 $\pm$ 4	-33 $\pm$ 2	-48 $\pm$ 4	-49 $\pm$ 5	-31 $\pm$ 3	-40 $\pm$ 4	-47 $\pm$ 4	-46 $\pm$ 5
Baseline tone	-46 $\pm$ 5	-51 $\pm$ 7	-53 $\pm$ 8	-40 $\pm$ 5	-28 $\pm$ 4	-52 $\pm$ 8	-52 $\pm$ 7	-32 $\pm$ 6	-36 $\pm$ 10	-49 $\pm$ 6	-51 $\pm$ 9
Frequency	-38 $\pm$ 2	-30 $\pm$ 2	-32 $\pm$ 2	-42 $\pm$ 4	-38 $\pm$ 7	-34 $\pm$ 2	-33 $\pm$ 3	-36 $\pm$ 4	-39 $\pm$ 3	-37 $\pm$ 2	-24 $\pm$ 2

* Area under the curve

Table 3
Effect of NaHS (10^{-3} M) after precontraction with bethanechol in the presence and absence of specific inhibitors

	Without inhibitor	Capsaicin		L-NNA		Glibenclamide		Apamin	
		10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻⁴ M	10 ⁻³ M	10 ⁻⁴ M	10 ⁻³ M	10 ⁻⁶ M	5x10 ⁻⁶ M
Total contractile activity (AUC [*])	-108±3	-109±3	-144±20	-112±9	-100±7	-130±23	-210±48	-121±9	-111±9
Average amplitude	-53±2	-55±3	-40±4	-56±6	-54±5	-46±5	-56±3	-56±4	-53±6
Baseline tone	-65±3	-58±3	-45±7	-65±7	-59±7	-49±4	-53±8	-66±6	-60±10
Frequency	-37±1	-33±2	-35±3	-30±2	-29±2	-27±1	-33±6	-27±1	-20±1 ^{**}

* Area under the contractile curve

** p<0.05 compared to control response (without inhibitor) (ANOVA)