Inhibition of RhoA-dependent pathway and contraction by endogenous hydrogen sulfide in rabbit gastric smooth muscle cells

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Nalli AD, Rajagopal S, Mahavadi S, Grider JR, Murthy KS. Inhibition of RhoA-dependent pathway and contraction by endogenous hydrogen sulfide in rabbit gastric smooth muscle cells. Am J Physiol Cell Physiol 308: C485–C495, 2015. First published January 7, 2015; doi:10.1152/ajpcell.00280.2014.—Inhibitory neurotransmitters, chiefly nitric oxide and vasoactive intestinal peptide, increase cyclic nucleotide levels and inhibit muscle contraction via inhibition of myosin light chain (MLC) kinase and activation of MLC phosphatase (MLCP). H2S produced as an endogenous signaling molecule synthesized mainly from l-cysteine via cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) regulates muscle contraction. The aim of this study was to analyze the expression of CSE and H2S function in the regulation of MLCP activity, 20-kDa regulatory light chain of myosin II (MLC20) phosphorylation, and contraction in isolated gastric smooth muscle cells. Both mRNA expression and protein expression of CSE, but not CBS, were detected in smooth muscle cells of rabbit, human, and mouse stomach. L-cysteine, an activator of CSE, and NaHS, a donor of H2S, inhibited carbachol-induced Rho kinase and PKC activity, Rho kinase-sensitive phosphorylation of MYPT1, PKC-sensitive phosphorylation of CPI-17, and MLCP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG). Inactivation of specific targets in the signaling pathways mediating contraction upon phosphorylation by PKA or PKG results in muscle relaxation (21, 23, 31, 34, 35, 37, 38, 39, 53).

Recent studies have demonstrated that hydrogen sulfide (H2S), a gaseous transmitter like NO and carbon monoxide (CO), is involved in the regulation of several physiological functions including gastrointestinal motility (7, 9, 15, 16, 17, 20, 22, 27–30, 32, 41, 45, 47, 50, 56). H2S is synthesized endogenously from l-cysteine via the pyridoxal-5'-phosphate-dependent enzymes, cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopropionate sulfurransferase (3-MST) (4, 28, 29, 51). Reduction in the levels of H2S is accompanied by hypertension and reduced endothelium-dependent relaxation of vascular muscle in mouse lacking the H2S-generating enzyme CSE. Downregulation of CSE/MLC20 pathway in spontaneously hypertensive rats underscore the importance of endogenous H2S in the regulation of smooth muscle function (3, 51, 52, 54). H2S exerts its function by acting on various targets, but, unlike NO and CO, it does not affect soluble guanylyl cyclase (sGC) activity. One of the most studied mechanisms for vascular muscle relaxation by H2S is activation of KATP channels (10, 12, 13, 49, 57).

Regulation of gastrointestinal motility in vivo is complex and reflects interplay of the autonomic nervous system, enteric nervous system, interstitial cells of Cajal (ICC), and smooth muscle cells. In the gastrointestinal tract, H2S-synthesizing enzymes have been shown to be expressed by enteric neurons, interstitial cells of Cajal, and epithelial cells (16, 28, 44, 47). Expression of CBS and CSE is tissue specific (28, 47). Inhibition of CSE, but not CBS, caused an increase in contraction of ileum suggesting an inhibitory role of endogenous H2S (50). Although it is well established that H2S inhibits gastrointestinal motility in vivo and both electrically and agonist-induced contractions in vitro, it is not known for certain the site of H2S biosynthesis and the mechanism of action. In guinea pig ileum and mouse stomach and colon, the inhibitory effect of H2S is dependent on both electrically and agonist-induced contractions in vitro, it is not known for certain the site of H2S biosynthesis and the mechanism of action. In guinea pig ileum and mouse stomach and colon, the inhibitory effect of H2S was not affected by the KATP channel inhibitor glibenclamide (7, 50), whereas in the isolated segments of human, mouse, and rat colon the inhibitory effect of H2S was dependent on both glibenclamide-sensitive and apamin-sensitive K+ channels (14). Our aim in the present study is to determine the effects of endogenously released and exogenously applied H2S on muscle contraction; muscle relaxation; protein kinase C; H2S; Rho kinase

IN GASTROINTESTINAL SMOOTH muscle phosphorylation of Ser19 on the 20-kDa regulatory light chain of myosin II (MLC20) by Ca2+/calmodulin-dependent myosin light-chain kinase (MLCK) is essential for muscle contraction (17, 39, 46). Excitatory neurotransmitters such as acetylcholine initiate contraction by increasing cytosolic Ca2+, or [Ca2+]i, and activating Ca2+/calmodulin-dependent MLCK. The initial increases in [Ca2+]i and MLCK activity are transient. MLC20 phosphorylation and contraction, however, are sustained via inhibition of MLC phosphatase (MLCP). Inhibition of MLCP is initiated by two RhoA-dependent pathways: one involves phosphorylation of MYPT1, the regulatory subunit of MLCP by Rho kinase, and the other involves phosphorylation of the endogenous MLCP inhibitor CPI-17 by protein kinase C (PKC) (36, 39).

Inhibitory neurotransmitters such as vasoactive intestinal peptide (VIP) and nitric oxide (NO) induce relaxation through generation of cAMP and cGMP and activation of cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG). Inactivation of specific targets in the signaling pathways mediating contraction upon phosphorylation by PKA or PKG results in muscle relaxation (21, 23, 31, 34, 35, 37, 38, 39, 53).
smooth muscle function and identify the targets involved in mediating the effects of H\(_2\)S using muscle strips and isolated muscle cells from the stomach of rabbit and mouse. Our results demonstrate that both endogenous and exogenous H\(_2\)S induce muscle relaxation and the mechanism involves inhibition of Rho kinase and PKC activities leading to stimulation of MLCP activity, MLC\(_{20}\) dephosphorylation, and inhibition of contraction.

**MATERIALS AND METHODS**

Reagents. Antibodies for CBS and CSE were obtained from Proteintech (Chicago, IL); antibody to 3-MST was obtained from Sigma-Aldrich (St. Louis, MO); antibodies to phospho-MYPT1 (Thr696), phospho-CPI (Thr38), and phospho-MLC\(_{20}\) (Ser19) were obtained from Santa Cruz Biotechnology (Dallas, TX); \(^{[32P]}\)ATP, \(^{[38S]}\)cAMP, and \(^{[35S]}\)gGMP were obtained from PerkinElmer (Cambridge, MA). Western blotting and chromatography materials were from Bio-Rad Laboratories (Hercules, CA); RNaqueous Kit was obtained from Ambion (Austin, TX); 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), Y27632, and L-N\(^{6}\)-nitroarginine methyl ester (L-NAME) were obtained from Cayman Chemical (Ann Arbor, MI); Effectene Transfection Reagent, QIAEX II Gel Extraction Kit, and QIAprep Spin Miniprep Kit were from Qiagen (Hilden, Germany); other reagents were from Sigma.

New Zealand white rabbits were purchased from RSI Biotechnol (Clemmons, NC). C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University (VCU). All procedures were approved by the Institutional Animal Care and Use Committee of VCU. Normal human gastric tissues were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), a nonprofit organization that provides human organs and tissue. The studies using human tissues from NDRI are approved for exempt from VCU Institutional Review Board.

**Preparation of dispersed smooth muscle cells.** Smooth muscle cells were isolated from the circular muscle layer of the stomach of rabbit, human, and mouse by sequential enzymatic digestion, filtration, and centrifugation, as previously described (33–38). Briefly, smooth muscle strips were incubated for 30 min at 31°C in 15 ml of medium containing 120 mM NaCl, 4 mM KCl, 2.6 mM KH\(_2\)PO\(_4\), 0.6 mM MgCl\(_2\), 25 mM HEPES, 14 mM glucose, 2.1% (vol/vol) Eagle’s essential amino acid mixture, 0.1% collagenase (type II), and 0.1% soybean trypsin inhibitor. At the 30-min digestion period, the cells that had spontaneously detached in collagenase containing medium were discarded and partly digested tissues were washed with 50 ml of enzyme-free medium and muscle cells were allowed to disperse spontaneously. The cells were harvested by filtration through 500 \(\mu\)m Nitex and centrifuged twice at 350 \(g\) for 10 min. In some experiments, dispersed smooth muscle cells were cultured in DMEM containing 10% fetal bovine serum until they attained confluence and were then dispersed smooth muscle cells were cultured in DMEM containing 10% fetal bovine serum until they attained confluence and were then

**RT-PCR analysis of CBS and CSE.** Cultured gastric muscle cells were treated with RNaqueous reagent (Ambion) followed by an extraction with phenol:chloroform:isoamylalcohol (25:24:1). RNA (5 \(\mu\)g) was used to synthesize cDNA using Superscript II reverse transcriptase (Applied Biosystems) with random hexanucleotide primers. Reversibly transcribed cDNA (5 \(\mu\)l) was amplified by PCR under standard conditions using the HotMaster Taq DNA Polymerase Kit (Epigenet Biotechnologies, Madison, WI) in a final volume of 50 \(\mu\)l containing 100 ng of each primer. The PCR products were separated by electrophoresis in 1.2% agarose gel in the presence of ethidium bromide, visualized by ultraviolet fluorescence, and recorded by a ChemiImager 4400 Fluorescence system. PCR products were purified by using a QIAquick Gel Extraction Kit (Qiagen) and sequenced. The following primers were used: mouse CSE: forward, 5’-ATG GAT GAA GTG TAT GGA GG-3’; reverse, 5’-ACG AAG CCG ACT ATT GAG GT-3’ (384 bp); rabbit CSE: forward, 5’-ACA TTT CGC CAC GCA GGC CA-3’; reverse, 5’-CTC CCA GAG CAA AGG GC-3’ (560 bp); human CSE: forward, 5’-TGG ATG GGG CTA AGT ACT GTT GGC C-3‘; reverse, 5’-CAG AGC CAA AGG GCG CTG GAA A-3’ (371 bp); and mouse CBS: forward, 5’-ACT AGC ATG ACA CCG CCG AG-3’; reverse, 5’-AGT CCT TCC TGT GCG ATG AG-3’ (337 bp).

**Transfection of CSE siRNA into cultured smooth muscle cells.** CSE siRNA was subcloned into the multiple cloning site (EcoR I) of the eukaryotic expression vector pcDNA3. Recombinant plasmid cDNAs were transiently transfected into smooth muscle cultures for 48 h. The cells were cotransfected with 2 \(\mu\)g pcDNA3 vector and 1 \(\mu\)g of pGreen Lantern-1 DNA to monitor transfection efficiency (36, 37).

Western blot analysis for 3-MST, CBS and CSE. Western blot analysis was performed as previously described (36, 37). Briefly, dispersed or cultured smooth muscle cells were solubilized in Triton X-100-based lysis buffer plus protease and phosphatase inhibitors. After centrifugation of the lysates at 20,000 \(g\) for 10 min at 4°C, protein concentrations of the supernatant were determined with the DC Protein Assay Kit from Bio-Rad. Equal amounts of proteins were fractionated by 15% SDS-PAGE and transferred to PVDF membranes. The blots were incubated for 12 h at 4°C with antibodies

Fig. 1. Expression of cystathionine-\(\gamma\)-lyase (CSE) and 3-MST in smooth muscle cells. A: CSE expression was identified in cultured muscle cells from the stomach by RT-PCR using primers specific for CSE. A band of predicted size was amplified using RNA from the stomach of rabbit (560 bp), human (371 bp), and mouse (384 bp). RT-PCR was performed in the presence (+) or absence (−) of reverse transcriptase (RT). B: both CBS expression (337 bp) and CSE (364 bp) expression were identified in mouse brain by RT-PCR using primers specific for CBS and CSE. RT-PCR was performed in the presence (+) or absence (−) of RT. C: protein expression was analyzed in freshly prepared dispersed muscle cells from the stomach of rabbit (RG), human (HG), and mouse (MG) and homogenates of mouse brain (MB) by Western blot. A band of 66 kDa corresponding to CBS and 35 kDa corresponding to 3-mercaptopropionic acid sulfinate isozyme (3-MST) was detected by chemiluminescence

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Cysteine or NaHS in muscle strips.

A

allowed to equilibrate at resting tension (1 g) for 1 h before initiation of /H9262 NaHS (100 /H9262M) in the presence or absence of different concentrations of NaHS or L-cysteine for 10 min, and the reaction was terminated by rapid freezing. The suspension was thawed, homogenized in medium containing 20 mM Tris-HCl, 250 mM sucrose, 1 mM EGTA, 10 mM mercaptoethanol, and 1 mM PMSF, pH 7.5 and centrifuged at 100,000 rpm at 4°C for 30 min. The pellet was resuspended in buffer containing 0.2% Triton X-100 and centrifuged at 100,000 rpm for 20 min, and the supernatant was used as the particulate fraction. PKC activity was measured by Ca2+/phospholipid-dependent incorporation of 32P from [γ-32P]ATP into histone as described before (33) and expressed as counts per minute per milligram of protein.

Measurement of phosphorylated MLC20, CPI-17 and MYPT1. One milliliter of cells (2–3 × 10^6 cells/ml) was treated with CCh (1 μM) in the presence or absence of NaHS or L-cysteine for 10 min and solubilized on ice for 1 h in medium containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 0.75% deoxycholate, 1 mM PMSF, 10 μg/ml of leupeptin, and 100 μg/ml of aprotinin. The proteins were resolved by SDS-PAGE and electrophoretically transferred on to nitrocellulose membranes. The membranes were incubated for 12 h with phospho-specific antibodies to MLC20 (Ser19), MYPT1 (Thr696) or CPI-17 (Thr38) and then for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2,000). The protein bands were identified by enhanced chemiluminescence reagent (36).

Assay for PKC activity. One milliliter of cells (2–3 × 10^6 cells/ml) was treated with CCh (1 μM) in the presence or absence of NaHS or L-cysteine for 10 min and solubilized on ice for 1 h before initiation of experiments. Muscle strips were treated with CCh (10 μM) to induce contraction in the presence or absence of different concentrations of L-cysteine or NaHS. Contractile activity of muscle strips was calculated as maximum force (1.9 ± 0.21 g) generated in response to CCh and the effect of L-cysteine or NaHS was calculated as percent decrease in maximum contraction. Contractile activity of muscle strips was measured by radioimmunoassay using [125I]cAMP or [125I]cGMP as described previously (34). Cells (3 × 10^6 cells) were treated with L-cysteine or NaHS for 60 s in the presence or absence of 3,7-dihydroxy-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX), and the reaction was terminated with 10% trichloroacetic acid. The samples were acetylated with triethylamine/acetic anhydride (2:1) for 30 min, and cAMP or cGMP was measured in duplicate using 100-μl aliquots. The results were expressed as picomoles per milligrams of protein.

Measurement of contraction in muscle strips. Muscle strips from rabbit stomach were collected and rinsed immediately in Kreb’s solution containing 118 mM NaCl, 4.8 mM KCl, 1 mM MgSO4, 1.15 mM Na2HPO4, 15 mM NaHCO3, 10.5 mM glucose, and 2.5 mM CaCl2. The stomach was emptied of its contents, and the proximal part

**Table 1. EC50 values for the inhibitory effect of NaHS and L-cysteine**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Contraction (Strips)</th>
<th>Contraction (Cells)</th>
<th>Rho Kinase</th>
<th>PKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHS</td>
<td>75 ± 13</td>
<td>62 ± 8</td>
<td>96 ± 12</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>485 ± 32</td>
<td>132 ± 25</td>
<td>706 ± 42</td>
<td>956 ± 48</td>
</tr>
</tbody>
</table>

Values are means ± SE in μM. The inhibitory effect of NaHS and L-cysteine on carbachol-induced Rho kinase and PKC activities and contraction was examined in rabbit gastric muscle.
of the stomach was used to prepare the muscle strips by cutting in the
direction of circular muscle layer. Muscle strips were tied at each end
with silk thread and mounted vertically in a 5-ml tissue bath contain-
ing oxygenated (95% O₂-5% CO₂) Kreb’s solution at a pH of 7.4 at
37°C. The tissues were mounted between a glass rod and an isometric
transducer (Grass Technologies, Quincy MA) connected to a com-
puter recording system (Polyview). Preparations were allowed to equilibrate for 1 h at resting tension (1 g) before initiation of experi-
ments, and bath buffer solution was changed every 15 min
during equilibration. To measure the NaHS- or L-cysteine-induced
effect on contraction, the strips were precontracted with 10 μM CCh,
and after a stable sustained contraction was obtained different con-
centrations of L-cysteine or NaHS were cumulatively added. In a
separate study, 10 μM glibenclamide, a K_ATP channel blocker, was
added to the organ bath 15 min before CCh or NaHS or L-cysteine
treatment. At the end of each experiment, the strips were blotted dry
and weighed (tissue wet weight). Contractile activity of muscle strips
was calculated as maximum force generated in response to CCh, and
the effect of L-cysteine or NaHS was calculated as percent decrease in
maximum contraction. Only muscle strips that developed ~2 g of
tension above basal levels were used to test the effect of L-cysteine or
NaHS. Time control studies demonstrated that response to 10 μM
CCh was reproducible following a 2-h incubation in Krebs buffer.
Muscle strips were used within 2 h after isolation.

Measurement of contraction in freshly isolated muscle cells. Con-
traction in freshly dispersed gastric smooth muscle cells was deter-
mined as previously described (33–38). All cell suspensions were
studied within 1 h after dispersion. Freshly isolated muscle cells (0.4
ml containing 10⁴ cell ml) from circular muscle layer of stomach were
preincubated for 10 min with different concentrations of L-cysteine or
NaHS, and then CCh was added for 10 min. The reaction was
terminated with 1% acrolein. The same experiments were repeated in
cells preincubated for 15 min with a K_ATP channel inhibitor 10 μM
glibenclamide. After termination, an aliquot of cell suspension was
placed on a slide under a coverslip. The slide was scanned at ×100
magnification, and the length of first 50 cells encountered randomly
was measured using an image-splitting eyepiece connected to a
micrometer. The technique, as described and validated earlier using
measurements enlarged photomicrographs of cells, consists of split-
ting prismatically the single image of cells, and the movement of
prism is precalibrated using a stage micrometer (2). The resting cell
length was determined in control experiments in which muscle cells
were incubated with 100 μl of 0.1% bovine serum albumin in the
absence of CCh. The mean cell length of 50 muscle cells was
measured by scanning micrometry. Contraction in response to CCh
was expressed as decrease in mean cell length from control cell
length, and relaxation was measured as percent decrease in contractile
response in the presence of L-cysteine or NaHS.

Statistical analysis. Results are expressed as means ± SE of n,
where n represents one sample from one animal for single experimen-
tal replicate. Differences were analyzed by Student’s t-test and con-
sidered significant with a probability of P < 0.05. Regression analysis

![Image](http://ajpcell.physiology.org/)

Fig. 3. Effect of L-cysteine and NaHS on CCh-induced contraction in isolated
muscle cells. A: dispersed muscle cells from rabbit stomach were treated with
different concentrations of CCh for 10 min to induce contraction in the
presence or absence of L-cysteine (100 μM) or NaHS (100 μM). Muscle cell
length was measured by scanning micrometry. Contraction by CCh was
calculated as a decrease in cell length from the control cell length of 109 ± 4
μm. Inset: photomicrographs of dispersed muscle cells [a: control cell; b: CCh
(1 μM)-treated cell]. B: dispersed muscle cells from rabbit stomach were
preincubated for 10 min with 1 μM CCh for 10 min to induce contraction in the presence or absence of different concentrations of L-cysteine or NaHS. Contraction (32 ± 2%
decrease from the control cell length of 112 ± 4 μm) in response to 1 μM
CCh was considered 100%, and the results are expressed as percentage of
CCh-induced contraction. C: effect of L-cysteine (100 μM) and NaHS (100
μM) on 1 μM CCh-induced contraction was examined in the presence of K_ATP
channel inhibitor (10 μM glibenclamide). Contraction (30 ± 2% decrease from
the control cell length of 107 ± 5 μm) in response to 1 μM CCh was
considered 100%, and the results are expressed as percentage of CCh-induced
contraction. Values are means ± SE of 4–6 experiments.
RESULTS

Expression of CSE enzyme in smooth muscle cells from the stomach. mRNA for CSE, but not CBS, was detected in muscle cells from the stomach of rabbit, human, and mouse. A PCR product of the expected size was obtained with CSE-specific primers using RNA isolated from cultured muscle cells derived from the stomach of rabbit (560 bp), human (371 bp), and mouse (384 bp; Fig. 1A). No PCR product of the expected size was obtained with CBS-specific primers with mRNA from muscle cells. Control studies detected the presence of both CBS (337 bp) and CSE (384 bp) with mRNA from mouse brain (Fig. 1B). Western blot analysis using specific antibody to CSE or 3-MST demonstrated the presence of CSE (66 kDa) and 3-MST (35 kDa) in lysates derived from muscle cells from the stomach of rabbit, human, and mouse (Fig. 1B). The inhibitory effect of both l-cysteine (100 μM) and NaHS (100 μM) was not significantly affected in the presence of glibenclamide (10 μM), a selective inhibitor of KATP channels (Fig. 2B). The inhibitory effect of NaHS (10 mM) or l-cysteine (10 mM) was also unaffected by preincubation of tissues for 15 min with the NO synthase inhibitor L-NAME (100 μM) or sGC inhibitor ODQ (10 μM); inhibition of CCh-induced contraction: 85 ± 5% to 91 ± 6% with NaHS and 77 ± 7% to 80 ± 6% with l-cysteine. The concentrations used in the present study are not toxic because after washout the contractile activity to CCh was rapid and complete (data not shown).

To further understand the loci and mechanism of action of H2S in the regulation of muscle contraction, we examined the effect of l-cysteine and NaHS on muscle function in dispersed muscle cells. As shown previously, treatment of dispersed muscle cells from rabbit stomach caused contraction in a concentration-dependent manner with a maximal contraction of 32 ± 4% decrease in cell length from the basal cell length of 104 ± 5 μm (36). Treatment of cells with NaHS (100 μM) or l-cysteine (100 μM) caused inhibition of CCh-induced contraction shifting the dose-response curve to the right (Fig. 3A). Addition of l-cysteine or NaHS caused inhibition of

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**Fig. 4. Effect of l-cysteine and NaHS on cAMP and cGMP levels in isolated muscle cells. A and C: dispersed muscle cells from rabbit stomach were treated with l-cysteine, NaHS, or forskolin (10 μM) for 5 min and cAMP levels were measured by radioimmunoassay in the presence of 100 μM 3,7-dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX; A) or absence of IBMX (C) as described in MATERIALS AND METHODS. B and D: dispersed muscle cells from rabbit stomach were treated with l-cysteine, NaHS, or sodium nitroprusside (SNP; 1 μM) for 5 min and cGMP levels were measured by radioimmunoassay in the presence of 100 μM IBMX (B) or absence of IBMX (D) as described in MATERIALS AND METHODS. Results are expressed as pmol/mg protein. Values are means ± SE of 4–6 experiments. **P < 0.01, significant stimulation compared with basal levels. Please note the differences in the y-axis scale for cyclic nucleotide levels in the presence or absence of IBMX.**
CCh-induced contraction in a concentration-dependent fashion (Fig. 3B). The EC\textsubscript{50} was 62 ± 8 μM for NaHS and 132 ± 25 μM for L-cysteine (Table 1). Maximal inhibition of 93 ± 6% and 92 ± 10% was obtained with NaHS (1 mM) and L-cysteine (10 mM), respectively (Fig. 3B). The inhibitory effect of both NaHS (100 μM) or L-cysteine (100 μM) was not significantly affected in the presence of 10 μM glibenclamide (Fig. 3C). These results suggest that the inhibitory effect of H\textsubscript{2}S is independent of \textit{K}\textsubscript{ATP} channel activation. The inhibitory effect of NaHS (1 mM) or L-cysteine (10 mM) was unaffected by preincubation of cells for 15 min with l-NAME (100 μM) or ODQ (10 μM); inhibition of CCh-induced contraction: 88 ± 6% to 93 ± 5% with NaHS and 86 ± 7% to 91 ± 5% with L-cysteine.

L-cysteine or NaHS also caused inhibition of CCh-induced contraction in gastric muscle cells isolated from mouse stomach. CCh induced a 31 ± 3% decrease in cell length from a basal cell length of 94 ± 5 μm. Addition of L-cysteine (100 μM) or NaHS (100 μM) significantly inhibited the CCh-induced contraction by 39 ± 4% (\(P < 0.01; n = 4\)) and 58 ± 3% (\(P < 0.001; n = 4\)), respectively. The inhibitory effect of NaHS or L-cysteine was not significantly affected in the presence of glibenclamide (data not shown).

\textbf{Effect of H\textsubscript{2}S on cAMP and cGMP.} Previous studies in gastrointestinal muscle have shown that inhibition of contraction in response to inhibitory transmitters is mediated via an increase in cAMP and/or cGMP levels (34, 37–39). We examined whether inhibition of contraction is mediated by increase in cAMP or cGMP levels in response to NaHS and L-cysteine. As shown in Fig. 4, addition of NaHS (100 μM) or L-cysteine (100 μM) had no significant effect on either cAMP or cGMP levels measured in the presence (Fig. 4, A and B) or absence (Fig. 4, C and D) of IBMX. Control studies showed that forskolin (10 μM), an activator of adenylyl cyclase, caused significant increase in cAMP levels (505 ± 31% and 392 ± 28% in the presence and absence of IBMX, respectively). Similarly, SNP (1 μM), a NO donor, caused significant increase in cGMP (542 ± 25% and 421 ± 31% in the presence and absence of IBMX, respectively).

Pretreatment of cells with NaHS (100 μM) or L-cysteine (100 μM) caused significant augmentation in cAMP levels in response to forskolin (100 nM; 289 ± 35% increase with forskolin alone and 564 ± 28 and 452 ± 26% increase in the presence of NaHS or L-cysteine, respectively; Fig. 5A). Similarly, pretreatment of cells with NaHS (100 μM) or L-cysteine (100 μM) caused significant augmentation in cGMP levels in response to SNP (10 nM; 275 ± 21% increase with SNP alone and 624 ± 43 and 495 ± 36% increase in the presence of NaHS or L-cysteine, respectively; Fig. 5B). These results suggest that H\textsubscript{2}S can modulate cyclic nucleotide levels upon concurrent stimulation of adenylyl cyclase and sGC activities and generation of cAMP and cGMP.

\textbf{Inhibition of Rho kinase and PKC activity by H\textsubscript{2}S.} Previous studies have shown that sustained contraction in response to CCh was blocked in the presence of inhibitors of Rho kinase (Y27632) or PKC (bisindolylmaleimide) suggesting that sustained contraction was mediated by activation of Rho kinase and/or PKC in response to CCh (36). We examined the notion that the inhibitory effect of H\textsubscript{2}S on muscle contraction is mediated via inhibition of Rho kinase and PKC activities. Treatment of muscle cells for 10 min with CCh (1 μM) caused a significant increase in Rho kinase activity (18,915 ± 2,312 counts·min\textsuperscript{-1}·mg protein\textsuperscript{-1}; \(P < 0.001; n = 5\)) above basal levels (3,625 ± 562 counts·min\textsuperscript{-1}·mg protein\textsuperscript{-1}). Addition of L-cysteine or NaHS caused inhibition of CCh-induced Rho kinase activity in a concentration-dependent manner (Fig. 6A). The EC\textsubscript{50} was 96 ± 12 μM for NaHS and 706 ± 42 μM for L-cysteine (Table 1). Maximal inhibition of 88 ± 9% and 63 ± 7% was obtained with NaHS (10 mM) and L-cysteine (10 mM), respectively (Fig. 6A). L-cysteine or NaHS at concentrations <1 μM had no effect on CCh-induced Rho kinase activity.

L-cysteine or NaHS also caused inhibition of CCh-induced Rho kinase activity in gastric muscle cells isolated from mouse stomach. CCh (1 μM) caused a significant increase in Rho

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Effect of L-cysteine and NaHS on cAMP and cGMP levels in response to forskolin or SNP in isolated muscle cells. \textbf{A}: dispersed muscle cells from rabbit stomach were treated with forskolin (100 nM) in the presence or absence of L-cysteine (100 μM) or NaHS (100 μM) for 5 min and cAMP levels were measured by radioimmunoassay in the absence of IBMX as described in MATERIALS AND METHODS. Control studies showed that forskolin (10 μM), an activator of adenylyl cyclase, caused significant increase in cAMP levels (505 ± 31% and 392 ± 28% in the presence and absence of IBMX, respectively). Similarly, SNP (1 μM), a NO donor, caused significant increase in cGMP (542 ± 25% and 421 ± 31% in the presence and absence of IBMX, respectively). Pretreatment of cells with NaHS (100 μM) or L-cysteine (100 μM) caused significant augmentation in cAMP levels in response to forskolin (100 nM; 289 ± 35% increase with forskolin alone and 564 ± 28 and 452 ± 26% increase in the presence of NaHS or L-cysteine, respectively; Fig. 5A). Similarly, pretreatment of cells with NaHS (100 μM) or L-cysteine (100 μM) caused significant augmentation in cGMP levels in response to SNP (10 nM; 275 ± 21% increase with SNP alone and 624 ± 43 and 495 ± 36% increase in the presence of NaHS or L-cysteine, respectively; Fig. 5B). These results suggest that H\textsubscript{2}S can modulate cyclic nucleotide levels upon concurrent stimulation of adenylyl cyclase and sGC activities and generation of cAMP and cGMP.

\textbf{Inhibition of Rho kinase and PKC activity by H\textsubscript{2}S.} Previous studies have shown that sustained contraction in response to CCh was blocked in the presence of inhibitors of Rho kinase (Y27632) or PKC (bisindolylmaleimide) suggesting that sustained contraction was mediated by activation of Rho kinase and/or PKC in response to CCh (36). We examined the notion that the inhibitory effect of H\textsubscript{2}S on muscle contraction is mediated via inhibition of Rho kinase and PKC activities. Treatment of muscle cells for 10 min with CCh (1 μM) caused a significant increase in Rho kinase activity (18,915 ± 2,312 counts·min\textsuperscript{-1}·mg protein\textsuperscript{-1}; \(P < 0.001; n = 5\)) above basal levels (3,625 ± 562 counts·min\textsuperscript{-1}·mg protein\textsuperscript{-1}). Addition of L-cysteine or NaHS caused inhibition of CCh-induced Rho kinase activity in a concentration-dependent manner (Fig. 6A). The EC\textsubscript{50} was 96 ± 12 μM for NaHS and 706 ± 42 μM for L-cysteine (Table 1). Maximal inhibition of 88 ± 9% and 63 ± 7% was obtained with NaHS (10 mM) and L-cysteine (10 mM), respectively (Fig. 6A). L-cysteine or NaHS at concentrations <1 μM had no effect on CCh-induced Rho kinase activity.

L-cysteine or NaHS also caused inhibition of CCh-induced Rho kinase activity in gastric muscle cells isolated from mouse stomach. CCh (1 μM) caused a significant increase in Rho
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kinase activity (16,472 ± 2,145 counts·min⁻¹·mg protein⁻¹; P < 0.001; n = 4) above basal levels (2,635 ± 398 counts·min⁻¹·mg protein⁻¹). Addition of l-cysteine (100 μM) and NaHS (100 μM) significantly inhibited CCh-induced Rho kinase activity by 31 ± 4% (P < 0.01; n = 4) and 53 ± 3% (P < 0.001; n = 4), respectively.

Treatment of muscle cells for 10 min with CCh caused a significant increase in PKC activity (4,352 ± 506 counts·min⁻¹·mg protein⁻¹; P < 0.001; n = 5) above basal levels (762 ± 106 counts·min⁻¹·mg protein⁻¹). Addition of l-cysteine or NaHS also caused inhibition of CCh-induced PKC activity in a concentration-dependent manner (Fig. 6B). The EC₅₀ was 135 ± 15 μM for NaHS and 956 ± 48 μM for l-cysteine (Table 1). Maximal inhibition of 83 ± 9% and 66 ± 8% was obtained with NaHS (10 μM) and l-cysteine (10 μM), respectively (Fig. 6B). l-cysteine or NaHS at concentrations <1 μM had no effect on CCh-induced PKC activity.

l-cysteine or NaHS also caused inhibition of CCh-induced PKC activity in gastric muscle cells isolated from mouse stomach. CCh (1 μM) caused significant increase in PKC activity (3,985 ± 452 counts·min⁻¹·mg protein⁻¹; P < 0.001; n = 4) above basal levels (653 ± 102 counts·min⁻¹·mg protein⁻¹). Addition of l-cysteine (100 μM) and NaHS (100 μM) significantly inhibited CCh-induced PKC activity by 26 ± 3% (P < 0.05; n = 4) and 51 ± 5% (P < 0.01; n = 4), respectively.

Previous studies have shown that activation of Rho kinase and PKC leads to inhibition of MLCP activity via phosphorylation of MYPT1 at Thr696 and CPI-17 at Thr38, respectively (Fig. 6). Phosphorylation of MYPT1 at Thr696, CPI-17 at Thr38, and MLC20 at Ser19 was determined by Western blot analysis using phospho-specific antibody. Figure depicts representative blot of 3 separate experiments. Numbers indicate densitometry ratio values to the loading control.
cells were transfected with CSE-specific siRNA and the effect of L-cysteine on CCh-induced Rho kinase and PKC activities was examined. Transfection of CSE siRNA suppressed the expression of CSE and blocked the inhibitory effect of L-cysteine on CCh-induced Rho kinase activity (7 ± 5% inhibition with siRNA vs. 39 ± 5% inhibition with control; Fig. 7A). In contrast, the inhibitory effect of NaHS (100 μM) was not affected by CSE siRNA (59 ± 5% inhibition with siRNA vs. 57 ± 7% inhibition with control). Similarly, transfection of CSE siRNA blocked the inhibitory effect of L-cysteine (100 μM) on CCh-induced PKC activity (6 ± 4% inhibition with siRNA vs. 41 ± 4% inhibition with control). In contrast, the inhibitory effect of NaHS (100 μM) was not affected by CSE siRNA (59 ± 5% inhibition with siRNA vs. 57 ± 7% inhibition with control; Fig. 7B).

In the second approach, the CSE-selective inhibitor PPG was used in dispersed muscle cells (15, 50). Treatment of cells with 10 μM PPG blocked the inhibitory effect of L-cysteine on CCh-induced Rho kinase (8 ± 4% inhibition vs. 44 ± 6% inhibition in control cells) and PKC activities (3 ± 5% inhibition vs. 41 ± 3% inhibition in control cells). Consistent with the reversal of inhibition of Rho and PKC activity by 10.220.33.5 on September 12, 2016 http://ajpcell.physiology.org/ Downloaded from by CSE siRNA (1) or CSE-specific siRNA (2) was determined by Western blot. **p < 0.01, significant inhibition of CCh-induced Rho kinase or PKC activity.

Fig. 7. Suppression of CSE activity blocks the inhibitory effect of L-cysteine on CCh-induced Rho kinase and PKC activities. Rabbit gastric muscle cells in culture were transfected with control siRNA or CSE-specific siRNA for 48 h. Cells were stimulated with 1 μM CCh for 10 min in the presence or absence of L-cysteine (100 μM) or NaHS (100 μM). Rho kinase (A) or PKC (B) activities were measured by immunokinase assay as described in the methods. Results are expressed as counts·min⁻¹·mg protein⁻¹. Values are means ± SE of 4 experiments. Inset: expression of CSE in cells transfected with control siRNA (1) or CSE-specific siRNA (2) was determined by Western blot. **p < 0.01, significant inhibition of CCh-induced Rho kinase or PKC activity.

Fig. 8. Inhibition of CSE activity blocks the inhibitory effect of L-cysteine on CCh-induced Rho kinase and PKC activities. Dispersed muscle cells from rabbit stomach were stimulated with 1 μM CCh for 10 min in the presence or absence of L-cysteine (100 μM) or NaHS (100 μM). In some experiments, L-cysteine or NaHS were incubated in the presence of CSE inhibitor dl-propargylglycine (PPG; 10 μM). Rho kinase (A) or PKC (B) activity was measured by immunokinase assay as described in MATERIALS AND METHODS. Results are expressed as counts·min⁻¹·mg protein⁻¹. Values are means ± SE of 4 experiments. **p < 0.01, significant inhibition of CCh-induced Rho kinase or PKC activity.
PKC activities, treatment of cells with 10 μM PPG also blocked the inhibitory effect of L-cysteine, but not NaHS, on CCh-induced sustained contraction in freshly dispersed muscle cells (3 ± 2% inhibition vs. 53 ± 4% inhibition in control cells; Fig. 9). These results suggest that inhibitory effect of L-cysteine on Rho kinase and PKC activities and on muscle contraction was mediated by the activation of CSE.

**DISCUSSION**

H2S, regarded as a third gasotransmitter, is receiving increasing interest, as much as NO and CO have received. The role of H2S as an endogenous signaling molecule in the regulation of gastrointestinal motility was demonstrated using innervated muscle strips and whole organ with activators and inhibitors of CSE, a H2S-synthesizing enzyme (50). However, the expression of CSE and function of H2S in gastrointestinal smooth muscle cells are unclear. This study provides evidence for the expression of CSE in smooth muscle cells and using isolated muscle cells demonstrates that both endogenous and exogenous H2S inhibit muscle contraction, and the inhibition of contraction was associated with inhibition of Rho kinase and PKC activities and stimulation of MLCP activity leading to MLC20 dephosphorylation. These findings may further explain the loci and mechanism of action of H2S in the regulation of gastrointestinal motility. The important findings of this study are summarized as follows: 1) transcripts of the H2S-synthesizing enzymes CSE and 3-MST, but not CBS, have been detected in isolated muscle cells, where it is responsible for H2S production; 2) endogenous activation of CSE by L-cysteine or the H2S donor NaHS inhibited contractile agonist-induced contraction in muscle strips and isolated muscle cells in a concentration-dependent manner; 3) the inhibitory effect of L-cysteine or NaHS on muscle contraction was not affected by glibenclamide; 4) L-cysteine and NaHS inhibited agonist-induced Rho kinase and PKC activity, Rho kinase-sensitive MYPT1 phosphorylation at Thr696, and PKC-sensitive CPI-17 phosphorylation at Thr38 and stimulated MLCP activity leading to MLC20 dephosphorylation at Ser19; 5) inhibition of CSE, to reduce H2S generation, by PPG, reversed the effect of L-cysteine on Rho kinase and PKC activity and sustained contraction; and 6) suppression of CSE expression also blocked the effect of L-cysteine on Rho kinase and PKC activity providing strong evidence that H2S generated via CSE may be responsible for L-cysteine-induced inhibitory effects. Evidence for the activation of CSE in mediating the effect of L-cysteine was based on the use of PPG in dispersed muscle cells and CSE siRNA in cultured muscle cells.

Although exogenous NaHS has a potent inhibitory effect on muscle contraction both in muscle strip preparations and isolated muscle cells, it was demonstrated that luminal application of NaHS was less effective in isolated strips (14). This could be due to effective barrier function of mucosa to limit the diffusion of H2S to the muscle layers and efficient metabolism of H2S to thiosulfate and sulphate by colonic mucosa (48). In humans and mice, H2S can be produced up to the millimolar range in the gastrointestinal tract and efficient oxidation process by sulfide quinone oxidoreductase, sulphur dioxygenase, and rhodanese would offer protection against high local concentrations (48). Damage to this protective mucosal barrier function, however, as in the ulcerative colitis, may lead to increased access of H2S to the inner muscle layers of gastrointestinal tract.

In our studies in gastric smooth muscle, both L-cysteine and NaHS are effective at the micromolar and millimolar concentrations, consistent with concentrations that caused effects in other systems (3, 6, 50). In several tissues (e.g., brain, liver, and kidney) free H2S concentrations are low in the micromolar range, except in aorta where concentrations are 20–100 times greater than in other tissue (26). It is predicted that tissue produces a micromolar concentration of H2S in a short time to elicit a response (55). It is estimated that only ~30 μM H2S was released within seconds upon addition of 100 μM NaHS (55). The actual concentration of H2S in the gastrointestinal smooth muscle and the physiological and pathophysiological relevance of H2S at a low and high micromolar level are yet to be determined.

Recent studies in vascular and visceral smooth muscle have clearly established H2S as a mediator of smooth muscle relaxation. Expression of CSE is downregulated in hypertensive animal models, and CSE−/− mice are hypertensive and exhibit reduced endothelium-dependent vasorelaxation; these effects are associated with decrease in H2S generation in these mice (54). It is established that the effect of H2S on vasodilatation is mainly brought about by activation of KATP channels and this is consistent with the blockade of H2S effect by the KATP channel inhibitor glibenclamide (57). Additional targets such as voltage-dependent Ca2+ channels, Ca2+-dependent K+ channels, and an ill-defined endothelium-dependent mechanism appear to play a minor role in H2S induced vasodilatation (50–52). Generalization of these inhibitory mechanisms to other smooth muscle is problematic. The mechanism of inhibition of contraction by H2S appears to be species and tissue specific and varies with activation of the muscle. Inhibition of contraction by NaHS in urinary bladder was abolished by...
capsaicin suggesting the involvement of transient receptor potential channel vanilloid subtype 1 receptors (42). A glibenclamide-insensitive effect of NaHS was also observed in mouse aorta (25), mouse bronchial rings (24) and mouse fundus and distal colon (8, 9) and guinea pig ileum (50). However, the inhibition of spontaneous contractions by NaHS in human and rat colons was affected by glibenclamide and apamin (14). In these nerve-muscle preparations, H2S may also affect enteric neurons to regulate smooth muscle function. A direct effect on muscle was implicated in distal colon of mouse and human since the Na+ channel blocker tetrodotoxin had no effect on the relaxation (14). However, an effect on interstitial cells of Cajal would also influence the function of smooth muscle in these preparations. Our studies demonstrated that the inhibitory effect of l-cysteine and NaHS on gastric muscle contraction was not affected by inhibitors of NO synthase or sGC in both muscle strips and isolated muscle cells. Similarly, inhibition of contraction by NaHS in mouse distal colon strips was not affected by the NO synthase inhibitor l-NAME, the sGC inhibitor ODQ, or the adenylyl cyclase inhibitor SQ22536 (9). These results are consistent with previous studies in mouse distal colon and guinea pig ileum (8, 50) and suggest that H2S can induce muscle relaxation independent of NO signaling. l-cysteine and NaHS augmented SNP-induced cGMP formation and forskolin-induced cAMP formation. These results suggest that H2S can modulate cyclic nucleotide levels upon concurrent activation of adenylyl cyclase and sGC and generation of cAMP and cGMP. H2S, in vivo, can regulate generation of NO (1, 45) and also augment the effect of NO (3, 5). Recent studies have shown that H2S reacts with oxidized thiol species to generate persulfides with significant nucleophilic character (19). The significance of this pathway and the interaction of persulfides with S-nitro-cGMP in smooth muscle function are not known. Treatment of vascular muscle cells for 24 h with H2S was shown to affect smooth muscle cell adhesion by decreasing the expression of α5β1-integrin expression (55). Inhibition of contraction by H2S in our studies could not be due to their effect on integrin expression and adhesion as gastric muscle strips or cells were pretreated for only 10 min to examine the effect of H2S on agonist-induced muscle contraction.

In our studies in gastric muscle cells, inhibition of agonist-induced muscle contraction by H2S was accompanied by a decrease in Rho kinase and PKC, suggesting that inhibition of contraction could be due to inhibition of agonist-stimulated Rho kinase and PKC activities. The precise molecular targets of H2S in the pathways that lead to inhibition of Rho kinase and PKC activity are not clear. S-sulfhydration provides a possible mechanism by which H2S alters the function of several proteins, and this process appears common to post-translational modification and analogous to S-nitrosylation of proteins by NO (40, 43). In this process, a hydropersulfide (−SSH) moiety is generated by the addition of sulphur from H2S to the −SH groups of cysteine residues and this results in altered chemical and biological reactivity of proteins. Whereas the covalent modification of proteins by sulhydration is a well characterized mechanism, the physiological mechanism in the reversal of sulhydration is not clear. Sulhydration is reversed by reducing agents such as DTT (40).

In summary, our studies show that inhibition of Rho kinase activity and MYPT1 phosphorylation, and ensuing dis-inhibition of MLCP activity, as well as inhibition of PKC activity and CPI-17 phosphorylation, and ensuing blockade of the CPI-17 inhibitory effect on MLCP activity in response to H2S, lead to inhibition of contraction.

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AUTHOR CONTRIBUTIONS


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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