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 activity and sustained muscle contraction. The inhibitory effects of l-cysteine, but not NaHS, were blocked upon suppression of CSE expression by siRNA or inhibition of its activity by DL-propargylglycine (PPG) suggesting that the effect of l-cysteine is mediated via activation of CSE. Glibenclamide, an inhibitor of KATP channels, had no effect on the inhibition of contraction by H2S. Both l-cysteine and NaHS had no effect on basal cAMP and cGMP levels but augmented forskolin-induced cAMP and SNP-induced cGMP formation. We conclude that both endogenous and exogenous H2S inhibit muscle contraction, and the mechanism involves inhibition of Rho kinase and PKC activities and stimulation of MLCP activity leading to MLC20 dephosphorylation and inhibition of muscle contraction.

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+ 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).

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), cystathione-β-synthase (CBS), and 3-mercaptoppyruvate sulfurtransferase (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 underscores 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 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...
smooth muscle function and identify the targets involved in mediating the effects of H2S using muscle strips and isolated muscle cells from the stomach of rabbit and mouse. Our results demonstrate that both endogenous and exogenous H2S induce muscle relaxation and the mechanism involves inhibition of Rho kinase and PKC activities leading to stimulation of MLCP activity, MLC20 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-MLC20 (Ser19) were obtained from Santa Cruz Biotechnology (Dallas, TX); [32P]ATP, [32P]cAMP, and [32P]GMP were obtained from PerkinElmer (Cambridge, MA). Western blotting and chromotography 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 from Aldrich (St. Louis, MO); antibodies to phospho-MYPT1 (Thr696), phospho-MYPT1 (Thr696), and [125I]cGMP were obtained from PerkinElmer (Cambridge, MA).

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 KH2PO4, 0.6 mM MgCl2, 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 μm 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 passaged once for use in various studies.

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 μg) was used to synthesize cDNA using Superscript II reverse transcriptase (Applied Biosystems) with random hexanucleotide primers. Reversibly transcribed cDNA (5 μl) was amplified by PCR under standard conditions with the HotMaster Taq DNA Polymerase Kit (Epicentre Biotechnologies, Madison, WI) in a final volume of 50 μl 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 GCC GC-3′ (560 bp); human CSE: forward, 5′-TGG ATG GGG CTA AGT ACT GTT TGG 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 (EcoRI) of the eukaryotic expression vector pDNA3. Recombinant plasmid cDNAs were transiently transfected into smooth muscle cultures for 48 h. The cells were cotransfected with 2 μg pDNA3 vector and 1 μg 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...


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

<table>
<thead>
<tr>
<th>NaHS</th>
<th>75 ± 13</th>
<th>62 ± 8</th>
<th>96 ± 12</th>
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<tr>
<td>L-cysteine</td>
<td>485 ± 32</td>
<td>132 ± 25</td>
<td>706 ± 42</td>
<td>956 ± 48</td>
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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 containing 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 computer recording system (Polyview). Preparations were allowed to equilibrate for 1 h at resting tension (1 g) before initiation of experiments, 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 concentrations of L-cysteine or NaHS were cumulatively added. In a separate study, 10 μM glibenclamide, a K₁ATP 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 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. Contraction in freshly dispersed gastric smooth muscle cells was determined 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 K₁ATP 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 splitting 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 experimental replicate. Differences were analyzed by Student’s t-test and considered significant with a probability of P < 0.05. Regression analysis

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 treated 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 K₁ATP channel inhibitor (10 μM glibenclamide). Muscle cell contraction (30 ± 3% 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.
was performed using GraphPad Prism 5. A statistical software program was used (GraphPad Software, San Diego, CA).

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. 1C). No detectable CBS protein was obtained with CBS-specific antibody. Control studies detected the presence of CBS in homogenates obtained from mouse brain (Fig. 1C). These results suggest that muscle cells express CSE and 3-MST, and this is consistent with the tissue-specific expression of CSE and CBS and with more preferential expression of CBS in brain and nervous system (28, 51).

Inhibition of contraction by H₂S. Muscle strips from stomach were allowed to equilibrate to a passive tension of 1 g. CCh (100 μM) induced a sustained contraction of 1.9 ± 0.21 g (n = 5) above basal tension (the amplitude of the tonic contraction was measured). Addition of l-cysteine (1 μM-10 mM) or NaHS (1 μM-10 mM) induced inhibition of CCh-induced contraction in a concentration-dependent fashion (Fig. 2A). The EC₅₀ was 75 ± 13 μM for NaHS and 485 ± 32 μM for l-cysteine (Table 1). Maximal inhibition of 92 ± 7% and 82 ± 8% was obtained with NaHS (10 mM) and l-cysteine (10 mM), respectively (Fig. 2A). 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 K_ATP 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 for 10 min with CCh 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-dihydroxy-l-methyl-1-(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 EC50 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 H2S is independent of KATP channel activation. The inhibitory effect of NaHS (1 mM) or l-cysteine (10 mM) was unaffected by preincubation of cells for 15 min with t-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).

Effect of H2S 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 adenyl 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 H2S can modulate cyclic nucleotide levels upon concurrent stimulation of adenyl cyclase and sGC activities and generation of cAMP and cGMP.

Inhibition of Rho kinase and PKC activity by H2S: 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 H2S 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⁻¹-mg protein⁻¹; P < 0.001; n = 5) above basal levels (3,625 ± 562 counts-min⁻¹-mg protein⁻¹). Addition of l-cysteine or NaHS caused inhibition of CCh-induced Rho kinase activity in a concentration-dependent manner (Fig. 6A). The EC50 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

![Graph](http://ajpcell.physiology.org/)
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 mM) and l-cysteine (10 mM), 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 (3,965 ± 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. 6C). 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.

\[ \text{Fig. 6. Effect of l-cysteine and NaHS on CCh-induced PKC activity and phosphorylation of MYPT1, CPI-17, and 20-kDa regulatory light chain of myosin II (MLC20) in isolated muscle cells.} \]

A. Muscle cells isolated from rabbit stomach were treated with 1 μM CCh for 10 min to activate Rho kinase in the presence or absence of different concentrations of l-cysteine or NaHS. Rho kinase activity was measured by immunokinase assay as described in MATERIALS AND METHODS. Results are expressed as percentage of CCh-induced increase in Rho kinase activity (18,915 ± 2,312 counts·min⁻¹·mg protein⁻¹) above basal levels of 3,625 ± 562 counts·min⁻¹·mg protein⁻¹. Values are means ± SE of 4 experiments.

B. Muscle cells isolated from rabbit stomach were treated with 1 μM CCh for 10 min to activate PKC activity in the presence or absence of different concentrations of l-cysteine or NaHS. PKC activity was measured by immunokinase assay as described in MATERIALS AND METHODS. Results are expressed as percentage of CCh-induced increase in PKC activity (4,352 ± 506 counts·min⁻¹·mg protein⁻¹) above basal levels of 762 ± 106 counts·min⁻¹·mg protein⁻¹. Values are means ± SE of 4 experiments.

C. Muscle cells isolated from rabbit stomach were treated with 1 μM CCh for 10 min in the presence or absence of l-cysteine (100 μM) or NaHS (100 μM). 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 ± 4% inhibition in control cells) and PKC activities (3 ± 5% inhibition vs. 41 ± 3% inhibition in control cells). The inhibitory effect of NaHS (100 μM) was not affected by PPG (Fig. 8, A and B). Consistent with the reversal of inhibition of Rho and PKC activities by 10.220.33.6 on August 28, 2017 http://ajpcell.physiology.org/ Downloaded from
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 MLCP activity leading to MLC20 dephosphorylation. These activations, 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 vasodilation 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 vasodilation (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...
The formation 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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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
1. Altauzy Z, Ju YJ, Yang G, Wang R. The concentration of S-sulphydra- 
ination by contraction and specific antagonists. Eur J Pharmacol
2. Bitar KN, Makhlouf GM. Receptors on smooth muscle cell: character-
7: ra87, 2014.
7. Denizalti M, Durlu-Kandilci NT, Bozkurt TE, Sahin-Erdemli I. Hydro-


