Inhibition of RhoA-Dependent pathway and Contraction by 
Endogenous Hydrogen Sulfide in Rabbit Gastric Smooth Muscle Cells

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Inhibitory neurotransmitters, chiefly nitric oxide and vasoactive intestinal peptide, increase cyclic nucleotide levels and inhibit muscle contraction via inhibition of MLC kinase and activation of MLC phosphatase. H2S produced as an endogenous signalling molecule synthesized mainly from L-cysteine via cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) regulate muscle contraction. The aim of this study was to analyse the expression of CSE and H2S function in the regulation of MLCP activity, MLC20 phosphorylation and contraction in isolated gastric smooth muscle cells. Both mRNA and protein expression of CSE, but not CBS was 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 and PKC-sensitive phosphorylation of CPI-17, MLC20 phosphorylation and sustained muscle contraction. 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 and a known target of H2S, 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 MLC phosphatase activity leading to MLC20 dephosphorylation and inhibition of muscle contraction.
INTRODUCTION

In gastrointestinal smooth muscle phosphorylation of Ser^{19} on the 20-kDa regulatory light chain of myosin II (MLC$_{20}$) by Ca$^{2+}$/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 Ca$^{2+}$, or [Ca$^{2+}$]$_i$ and activation of Ca$^{2+}$/calmodulin-dependent MLCK. The initial increase in [Ca$^{2+}$]$_i$ and MLCK activity are transient. MLC$_{20}$ 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 phosphorylation of the endogenous MLCP inhibitor CPI-17 by 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 cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG) results in muscle relaxation (21, 23, 31, 34, 35, 37, 38, 39, 53).

Recent studies have demonstrated that hydrogen sulfide (H$_2$S), 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, 37-30, 32, 41, 45, 47, 50, 56). H$_2$S is synthesized endogenously from L-cysteine via the pyridoxal-5'-phosphate-dependent enzymes, cystathionine-$\gamma$-lyase (CSE) and
cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) (4, 28, 29, 51). Reduction in the levels of H$_2$S accompanied by hypertension and reduced endothelium-dependent relaxation of vascular muscle in mouse lacking H$_2$S generating enzyme cystathionine-γ-lyase (CSE) and downregulation of CSE/L-cysteine pathway in spontaneously hypertensive rats underscores the importance of endogenous H$_2$S in the regulation of smooth muscle function (3, 51, 52, 54). H$_2$S exerts its function by acting on various targets, but, unlike NO and CO, it does not affect soluble guanylyl cyclase (sGC) activity and cGMP formation. One of the most studied mechanisms for vascular muscle relaxation by H$_2$S is activation of K$_{ATP}$ channels (10, 12, 13, 49, 57).

Regulation of gastrointestinal motility in vivo is complex and reflects interplay of autonomic nervous system, enteric nervous system, interstitial cells of Cajal (ICC) and smooth muscle cells. In the gastrointestinal tract, H$_2$S-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 H$_2$S (50). Although, it is well-established that H$_2$S inhibits gastrointestinal motility in vivo and both agonist- and electrically-induced contractions in vitro, it is not known for certain the site of H$_2$S biosynthesis and the mechanism of action. In guinea pig ileum and mouse stomach and colon, the inhibitory effect of H$_2$S was not affected by the K$_{ATP}$ channel inhibitor glibenclamide (7, 50), whereas in the isolated segments of human, mouse and rat colon the inhibitory effect of H$_2$S was dependent on both glibenclamide-sensitive and apamin-sensitive K$^+$ channels (14). Our aims in the present study is to determine the effects of endogenous 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 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 cystathionine β synthase (CBS) and cystathionine γ
lyase (CSE) were obtained from Proteintech (Chicago, IL); antibody to 3-
mercaptopyruvate sulfurtransferase (3-MST) was obtained from Sigma-Aldrich (St.
Louis, MO); antibodies to phosphor-MYPT1 (Thr696), phospho-CPI (Thr38) and
phosphor-MLC20 (Ser19) were obtained from Santa Cruz Biotechnology, Inc. (Dallas,
TX); [32P]ATP, [125I]cAMP and [125I]cGMP were obtained from PerkinElmer (Cambridge,
MA). Western blotting and chromatography material were from Bio-Rad Laboratories
(Hercules, CA), Y27632, RNAqueous™ kit was obtained from Ambion (Austin, TX); 1H-
[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and L-N⁵-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 Sciences, (Maryland); PCR reagents were from Applied Biosystems, Roche;
SuperScript™ II Reverse Transcriptase and TOPO TA Cloning® Kit Dual Promoter
were from Invitrogen, (CA); EcoR I was from New England Bio Labs; Dulbecco’s
modified Eagle’s medium (DMEM) was from Fisher Scientific. DL-propargylglycine (PPG), L-cysteine and other reagents were from Sigma (St. Louis, MO).

New Zealand white rabbits were purchased from RSI Biotechnology, (Clemmons, NC). C57BL/6 mice purchased from Jackson laboratories (Bar Harb or, ME). The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. All procedures were performed in accordance with the recommendations of the Institutional Animal Care and Use Committee of Virginia Commonwealth University (VCU). Normal human colon and gastric tissues were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), a non-profit 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 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% (v/v) 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 allowed to disperse spontaneously. The cells were harvested by filtration through 500µm Nitex and centrifuged twice at 350g for 10 min. In some experiments dispersed
smooth muscle cells were cultured in Dulbecco’s modified Eagle’s medium 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, Austin, TX) 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, Foster, CA) with random hexanucleotide primers. Reversibly transcribed cDNA (5 µl) was amplified by PCR under standard conditions using the HotMaster Taq DNA polymerase kit (Epicentere 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 GGG 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 CCA AAG 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).

**Mouse CBS:** forward, 5’ ACT ACG 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 co-transfected with 2 μg pcDNA3 vector and 1 μg of pGreen Lantern-1 DNA to monitor transfection efficiency (36, 37).

**Western blot analysis for 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 (Hercules, CA). 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 (1:1,000) to CBS and CSE and then for 1 h with secondary antibody conjugated with horseradish peroxidase. The protein bands were visualized by enhanced chemiluminescence.

**Assay for Rho kinase activity.** Rho kinase activity was determined in cell extracts by immunokinase assay as previously described (36). Freshly dispersed smooth muscle cells were treated with the contractile agonist carbachol (1 μM) in the presence or absence of different concentrations of NaHS or L-cysteine for 10 min and solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. Equal amounts of protein extracts were incubated with Rho kinase-2 antibody plus protein A/G agarose overnight at 4°C. Immunoprecipitates were washed twice with a phosphorylation buffer containing 10 mM MgCl₂ and 40 mM HEPES (pH 7.4) and then incubated for 5 min on ice with myelin basic protein (MBP) (1 mg/ml) as a substrate for Rho kinase activity. The kinase
reaction was initiated by the addition of 10 µCi of $[^{32}\text{P}]$ATP (3,000 Ci/mmol) and 20 µM ATP, followed by an incubation for 10 min at 37°C. $^{32}\text{P}$-labeled myelin basic protein was absorbed onto phosphocellulose disks, and repeated washings with 75 mM phosphoric acid removed free radioactivity. The extent of phosphorylation was determined from the radioactivity on phosphocellulose discs by liquid scintillation.

**Assay for protein kinase C (PKC) activity.** One ml of cells (2x10$^6$ cells/ml) were incubated with carbachol (1 µM) in the presence or absence of different concentrations of NaHS or L-cysteine for 10 min and the reaction 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, 1 mM phenylmethylsulfonylfluoride (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 Ca$^{2+}$/phospholipid-dependent incorporation of $^{32}\text{P}$ from $[^{\gamma}\text{32}\text{P}]$ATP into histone as described before (33) and expressed as cpm/mg protein.

**Measurement of phosphorylated CPI-17 and MYPT1.** One ml of cells (2-3 x 10$^6$ cell/ml) was treated with carbachol (1 µM) in the presence or absence of different concentrations of NaHS or L-cysteine for 10 min and solubilized on ice for one hour in medium containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 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 MYPT1 (Thr$^{696}$) or CPI-17 (Thr$^{38}$) and then for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2000). The protein bands were identified by enhanced chemiluminescence reagent (36).

**Assay for cAMP and cGMP.** Cyclic AMP and cGMP production was measured by radioimmunoassay using $[^{125}\text{I}]$cAMP or $[^{125}\text{I}]$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-dihydro-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/mg 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 MgSO$_4$, 1.15 mM NaH$_2$PO$_4$, 15 mM NaHCO$_3$, 10.5 mM glucose and 2.5 mM CaCl$_2$. The stomach was emptied of its contents and the proximal part of the stomach 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 5 ml tissue bath containing oxygenated (95% O$_2$/5% CO$_2$) Kreb’s solution at a pH of 7.4 at 37°C. The tissues were mounted between a glass rod and an isometric transducers (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 minutes during equilibration. To measure NaHS or L-cysteine induced effect on
contraction, the strips were precontracted with 10 µM CCh, and after obtaining stable sustained contraction different concentrations of L-cysteine or NaHS were cumulatively added. In a separate study 10 µM glibenclamide, a K<sub>ATP</sub> 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 tension above basal levels were used to test the effect of L-cysteine or NaHS. Time control studies demonstrated that response to 10 µM was reproducible following 2 h incubation in Krebs buffer. Muscle strips were used within two hours after isolation.

**Measurement of contraction freshly isolated muscle cells.** Contraction in freshly dispersed colonic smooth muscle cells was determined by scanning micrometry as previously described (33-38). All cell suspensions were studied within one hour after dispersion. Freshly isolated muscle cells (0.4 ml containing 10⁶/cell ml) from circular muscle layer of stomach were preincubated for 10 min with different concentration 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<sub>ATP</sub> 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 100X magnification and the length of first 50 cells encountered randomly was measured using an image-splitting eyepiece connected to a micrometre. 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 mean±SEM of n, where n represents one sample from one animal for single experimental replicate. Differences were analysed by Student’s t-test and considered significant with a probability of p<0.05. Regression analysis 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 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. 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 H$_2$S. Muscle strips from stomach were allowed to equilibrate to a passive tension of 1 g. Carbachol (10 µM) induced a sustained contraction of 1.9±0.21 grams (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 EC$_{50}$ 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 pre-incubation of tissues for 15 min with the nitric oxide synthase inhibitor L-NAME (100 µM) or soluble guanylyl cyclase inhibitor ODQ (10 µM) (inhibition of carbachol-induced contraction: 85±5% to 91±6% with NaHS and 77±7% to 80±6% with ODQ). The concentrations used
in the present study are not toxic because after washout the contractile activity to carbachol was rapid and complete (data not shown).

To further understand the loci and mechanism of action of H$_2$S 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 CCh-induced contraction in a concentration-dependent fashion (Fig. 3B). The EC$_{50}$ 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$_2$S is independent of K$_{ATP}$ 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 carbachol-induced contraction: 88±6% to 93±5% with NaHS and 86±7% to 91±5% with ODQ)

L-cysteine or NaHS also caused inhibition of CCh-induced contraction in gastric muscle cells isolated from mouse stomach. CCh induced 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 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 H$_2$S on cAMP and cGMP. Previous studies in gastrointestinal muscle have shown that inhibition of contraction in response to inhibitory transmitters is mediated via 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 (Figs. 4A and 4B) or absence (Figs. 4C and 4D) 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 in the presence and absence of IBMX, respectively). Similarly, SNP (1 µM), NO donor, caused significant increase in cGMP (542±25% and 421±31% in the presence and absence of IBMX, respectively).

Pre-treatment of cells with NaHS (100 µM) or L-cysteine (100 µM) caused significant augmentation of 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, pre-treatment of cells with NaHS (100 µM) or L-cysteine (100 µM) caused significant augmentation of 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$_2$S can modulate cyclic nucleotide levels upon concurrent
stimulation of adenylyl cyclase and soluble guanylyl cyclase activities and generation of cAMP and cGMP.

**Inhibition of Rho kinase and PKC activity by H$_2$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 (bisindolmaleimide) 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$_2$S on muscle contraction is mediated via inhibition of Rho kinase and PKC activities. Treatment of muscle cells for 10 min with CCh (10 µM) caused a significant increase in Rho kinase activity (18915±2312 cpm/mg protein, p<0.001; n=5) above basal levels (3625±562 cpm/mg protein). Addition of L-cysteine or NaHS caused inhibition of CCh-induced Rho kinase activity in a concentration-dependent manner (Fig. 6A). The EC$_{50}$ 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 less than 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 (10 µM) caused significant increase in Rho kinase activity (16472±2145 cpm/mg protein, p<0.001; n=4) above basal levels (2635±398 cpm/mg protein). 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 (4352±506 cpm/mg protein, p<0.001; n=5) above basal levels (762±106 cpm/mg protein). Addition of L-cysteine or NaHS also caused inhibition of CCh-induced PKC activity in a concentration-dependent manner (Fig. 6B). The EC$_{50}$ 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 less than 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 (10 µM) caused significant increase in PKC activity (3965±452 cpm/mg protein, p<0.001; n=4) above basal levels (653±102 cpm/mg protein). Addition of L-cysteine (100 µM) and NaHS (100 µM) significantly inhibited CCh-induced Rho kinase 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 MLC phosphatase activity via phosphorylation of MYPT1 at Thr$^{696}$ and CPI-17 at Thr$^{38}$, respectively (17, 36, 46). Consistent with inhibition of Rho kinase and PKC activities, both NaHS (1 mM) and L-cysteine (10 mM) caused inhibition of CCh-induced phosphorylation of MYPT1 at Thr$^{696}$ and CPI-17 at Thr$^{38}$ (Fig. 6C). These studies suggest that exogenous H$_2$S in response to NaHS and endogenous generation of H$_2$S via activation of CSE by L-cysteine caused inhibition of sustained contraction via inhibition of Rho kinase mediated phosphorylation of MYPT1 and PKC-mediated phosphorylation of CPI-17.
Inhibition of Rho kinase and PKC activities by L-cysteine via CSE. The possible involvement of CSE in the inhibition of Rho kinase and PKC activities in response to L-cysteine was examined by 2 approaches. In the first approach cultured muscle 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 versus 39±5% inhibition with control) (Fig. 7A). In contrast, inhibitory effect of NaHS (100 µM) was not affected by CSE siRNA (56±5% inhibition with siRNA versus 60±6% inhibition in control cells). Similarly, transfection of CSE siRNA blocked the inhibitory effect of L-cysteine (100 µM) on CCh-induced PKC activity (6±4% inhibition with siRNA versus 41±4% inhibition with control). In contrast, inhibitory effect of NaHS (100 µM) was not affected by CSE siRNA (59±5% inhibition with siRNA versus 57±7% inhibition with control) (Fig. 7B).

In the second approach a CSE selective inhibitor DL-propargylglycine (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 versus 44±% inhibition in control cells) and PKC activities (3±5% inhibition versus 41±3% inhibition in control cells). The inhibitory effect of NaHS (100 µM) was not affected by PPG (Figs. 8A and 8B). Consistent with the reversal of inhibition of Rho and 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 versus 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**

H$_2$S, regarded as a third gasotransmitter, is receiving increasing interest, as much as NO and CO have received previously, to understand its physiological functions (22, 27-29, 51, 52). The role of H$_2$S 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 H$_2$S synthesizing enzyme (50). However, expression of CSE and function of H$_2$S in gastrointestinal smooth muscle cells is 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 H$_2$S inhibit muscle contraction, and the inhibition of contraction was associated with inhibition of Rho kinase and PKC activities and stimulation of MLC phosphatase activity leading to MLC$_{20}$ dephosphorylation. These findings may further explain the loci and mechanism of action of H$_2$S in the regulation of gastrointestinal motility. The important findings of this study are summarized as follows: i) transcripts of the H$_2$S-synthesizing enzymes CSE and 3-MST, but not CBS have been detected in isolated muscle cells, where it is responsible for H$_2$S production; ii) endogenous activation of CSE by L-cysteine or H$_2$S donor NaHS inhibited contractile agonist-induced contraction in muscle strips and isolated muscle cells in a concentration-dependent manner; iii) the inhibitory effect of L-cysteine or NaHS on muscle contraction was not affected by glibenclamide; iv) L-cysteine and NaHS inhibited agonist-induced
Rho kinase and PKC activity, Rho kinase-sensitive MYPT1 phosphorylation at Thr^{696}, PKC-sensitive CPI-17 phosphorylation at Thr^{38} and stimulated MLC^{20} dephosphorylation at Ser^{19}; v) inhibition of CSE, to reduce H_{2}S generation, by PPG, reversed the effect of L-cysteine, on Rho kinase and PKC activity and sustained contraction; vi) suppression of CSE expression also blocked the effect of L-cysteine on Rho kinase and PKC activity providing strong evidence that H_{2}S 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 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 H_{2}S to the muscle layers and efficient metabolism of H_{2}S to thiosulfate and sulphate by colonic mucosa (48). In humans and mice, H_{2}S can be produced up to mM range in the gastrointestinal tract and efficient oxidation process by sulfide quinine 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 H_{2}S to the inner muscle layers of GI tract.

In our studies in gastric smooth muscle, both L-cysteine and NaHS are effective at µM and mM concentrations, consistent with concentrations that caused effects in other systems (3, 6, 50). In several tissues (e.g., brain, liver, kidney) free H_{2}S concentrations are low in µM range, except in aorta where concentrations are 20-100
times greater than in other tissue (26). It is predicted that tissue produces micro molar concentration of H$_2$S in short time to elicit a response (55). It is estimated that only ~30 µM H$_2$S released within seconds upon addition of 100 µM NaHS (55). The actual concentration of H$_2$S in the gastrointestinal smooth muscle and the physiological and pathophysiological relevance of H$_2$S at low and high micro molar level are yet to be determined.

Recent studies in vascular and visceral smooth muscle have clearly established H$_2$S as a mediator of smooth muscle relaxation. Expression of CSE is down regulated in hypertensive animal models and CSE$^{-/-}$ KO mice are hypertensive and exhibit reduced endothelium-dependent vasorelaxation; these effects are associated with decrease in H$_2$S generation in these mice (54). It is established that the effect of H$_2$S on vasodilation is mainly brought about by activation of $K_{ATP}$ channels and this is consistent with the blockade of H$_2$S effect by the $K_{ATP}$ channel inhibitor glibenclamide (57). Additional targets such as voltage-dependent Ca$^{2+}$ channels, Ca$^{2+}$-dependent K$^+$ channels and an ill-defined endothelium-dependent mechanism appear to play minor role in H$_2$S induced vasodilation (50-52). Generalization of these inhibitory mechanisms to other smooth muscle is problematic. The mechanism of inhibition of contraction by H$_2$S 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 TRPV1 receptors (42). A glibenclamide insensitive effect of NaHS was also observed mouse aorta (25), mouse bronchial rings (24) and mouse fundus and distal colon (8, 9) and guinea pig ileum (50). However, inhibition of spontaneous contractions by NaHS in human and rat colon was affected by
glibenclamide and apamin (14). In these nerve-muscle preparation H$_2$S 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 nitric oxide synthase or soluble guanylyl cyclase in both muscle strips and isolated muscle cells. Similarly, inhibition of contraction by NaHS in mouse distal colon strips was not affected by NO synthase inhibitor, L-NAME, sGC inhibitor, ODQ, or adenylyl cyclase inhibitor SQ22536, (9). These results are consistent previous studies in mouse distal colon and guinea pig ileum (8, 50) and suggest that H$_2$S can induce muscle relaxation independent of NO signalling. However, L-cysteine and NaHS augmented SNP-induced cGMP formation and forskolin-induced cAMP formation. These results suggest that H$_2$S can modulate cyclic nucleotide levels upon concurrent activation of adenylyl cyclase and soluble guanylyl cyclase and generation of cAMP and cGMP. H$_2$S, in vivo, can regulate generation of NO (1, 45) and also augment the effect of NO (3, 5). Recent studies have shown that H$_2$S reacts with oxidized thiol species to generate persulfides with significant nucleophilic character (19). The significance of this pathway and the interaction of persulfides with 8-nitro-cGMP in smooth muscle function are not known. Treatment of vascular muscle cells for 24 h with H$_2$S was shown to affect smooth muscle cell adhesion by decreasing the expression of α5-β1 integrin expression (55). Inhibition of contraction by H$_2$S in our studies could not be due to their effect on integrin expression.
and adhesion as gastric muscle strips or cells were pre-treated for 10 min to examine
the effect of H$_2$S on agonist-induced muscle contraction.

In our studies in gastric muscle cells, inhibition of agonist-induced muscle
contraction by H$_2$S was accompanied by decrease in Rho kinase and PKC activities
suggesting activities suggesting that inhibition of contraction could be due to inhibition of
agonist-stimulated Rho kinase and PKA activities. The precise molecular targets of H$_2$S
in the pathways that lead to inhibition of Rho kinase and PKC activity are not clear. S-
sulfhydration provides a possible mechanism by which H$_2$S alters the function of several
proteins and this process appears common to posttranslational 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 H$_2$S 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 sulfhydration is
a well characterized mechanism, the physiological mechanism in the reversal of
sulfhydration is not clear. Sulfhydration is reversed by reducing agents such as
dithiothreitol (40).

In summary, our studies show that inhibition of Rho kinase activity and MYPT1
phosphorylation, and ensuing disinhibition of MLCP activity, as well as inhibition of PKC
activity and CPI-17 phosphorylation, and ensuing blockade of CPI-17 inhibitory effect on
MLC phosphatase activity in response to H$_2$S lead to inhibition of contraction.
Acknowledgements

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and m3-mediated MLC20 (20 kDa regulatory light chain of myosin II) phosphorylation
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Table 1. EC$_{50}$ (in μM) values for the inhibitory effect of NaHS and L-cysteine. The inhibitory effect of NaHS and L-cysteine on carbachol-induced Rho kinase and PKC activities and contraction was examined in rabbit gastric muscle.

<table>
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<th>PKC</th>
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<tr>
<td>NaHS</td>
<td>75±13</td>
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<td>L-Cysteine</td>
<td>485±32</td>
<td>132±25</td>
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**FIGURE LEGENDS**

**Figure 1. Selective Expression of CSE 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 (-) of reverse transcriptase (RT). (B) Both CBS (337 bp) and CSE (384 bp) expression was identified in mouse brain by RT-PCR using primers specific for CBS and CSE. RT-PCR was performed in the presence (+) or absence of (-) of reverse transcriptase. (C) Protein expression was analysed 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 CSE and 35 kDa corresponding to 3-MST was detected by chemiluminescence using antibody to CSE (1:1000 dilution) or 3-MST (1:1000) in both brain and muscle cells of the stomach. A band of 63 kDa corresponding to CBS in the brain but not in smooth muscle cells of the stomach was detected. Numbers indicate densitometry ratio values to the loading control.

**Figure 2. Inhibition of carbachol-induced contraction by L-cysteine and NaHS in muscle strips.** (A) Gastric muscle strips from rabbit stomach were allowed to equilibrate at resting tension (1g) for 1 h before initiation of experiments. Muscle strips were treated with carbachol (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. Contraction in response to CCh was considered 100 percent and the results are expressed as percent of CCh-induced contraction. (B) The effect of L-cysteine (100 µM) and NaHS (100 µM) on CCh (10 µM)-induced contraction was examined in the presence of K_\text{ATP} channel inhibitor, glibenclamide (10 µM). Contraction in response to CCh was considered 100 percent and the results are expressed as percent of CCh-induced contraction. Values are means±SEM of 5-7 experiments.

Figure 3. Effect of L-cysteine and NaHS on carbachol-induced contraction in isolated muscle cells. (A) Dispersed muscle cells from rabbit stomach were treated with different concentrations of carbachol (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 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 10 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 10 µM CCh was considered 100 percent and the results are expressed as percent of CCh-induced contraction. (C) The effect of L-cysteine (100 µM) and NaHS (100 µM) on 10 µM CCh-induced contraction was examined in the presence of K_\text{ATP} channel inhibitor, glibenclamide (10 µM). Contraction (30±3% decrease from the control
cell length of 107±5 µm) in response to 10 µM CCh was considered 100 percent and the results are expressed as percent of CCh-induced contraction. Values are means±SEM of 4-6 experiments.

**Figure 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 IBMX (A) or absence of IBMX (C) as described in the 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 the methods. Results are expressed as pmol/mg protein. Values are means±SEM of 4-6 experiments. ** p<0.01 significant stimulation compared to basal levels. Please note the differences in the Y-axis scale for cyclic nucleotide levels in the presence or absence of IBMX.

**Figure 5. Effect of L-cysteine and NaHS on cAMP and cGMP levels in response to forskolin or SNP in isolated muscle cells.** (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 the methods. (B) Dispersed muscle cells from rabbit stomach were treated with SNP (10 nM) in the presence or
absence of L-cysteine (100 μM) or NaHS (100 μM) for 5 min and cGMP levels were measured by radioimmunoassay in the absence of IBMX as described in the methods. Results are expressed as pmol/mg protein. Values are means±SEM of 6 experiments.

** p<0.01 significant stimulation compared to basal levels.  # p<0.05 significant augmentation compared to forskolin- or SNP-induced increase in cyclic nucleotide levels.

Figure 6. Effect of L-cysteine and NaHS on carbachol-induced Rho kinase and PKC activities and phosphorylation of MYPT1, CPI-17 and MLC_{20} in isolated muscle cells. (A) Muscle cells isolated from rabbit stomach were treated with 10 μM carbachol (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 the methods. Results are expressed as percent of CCh-induced increase in Rho kinase activity (18915±2312 cpm/mg protein above basal levels of 3625±562 cpm/mg protein). Values are means±SEM of 4 experiments. (B) Muscle cells isolated from rabbit stomach were treated with 10 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 the methods. Results are expressed as percent of CCh-induced increase in PKC activity (4352±506 cpm/mg protein above basal levels of 762±106 cpm/mg protein). Values are means±SEM of 4 experiments. (C) Muscle cells isolated from rabbit stomach were treated with 10 μM CCh for 10 min in the presence or absence of L-cysteine (100 μM) or NaHS (100 μM). Phosphorylation of MYPT1 at Thr^{696}, CPI-17 at...
Thr$^{38}$ and MLC$_{20}$ at Ser$^{19}$ 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.

**Figure 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 hours. Cell were stimulated with 10 µM carbachol (CCh) for 10 min 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 cpm/mg protein. Values are means±SEM 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.

**Figure 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 10 µM carbachol (CCh) for 10 min 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 PPG (10 µM). Rho kinase (A) or PKC (B) activity was measured by immune kinase assay as described in the methods. Results are expressed as cpm/mg protein. Values are means±SEM of 4 experiments. ** p<0.01 significant inhibition of CCh-induced Rho kinase or PKC activity.
Figure 9. Inhibition of CSE activity blocks the inhibitory effect of L-cysteine on CCh-induced contraction. Dispersed muscle cells from rabbit stomach were stimulated with 10 µM carbachol (CCh) for 10 min 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 DL-propargylglycine PPG (10 µM). Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in cell length from the control cell length of 110±5 µm. Results are expressed as percent decrease in cell length. Values are means±SEM of 4 experiments. ** p<0.01 significant inhibition of CCh-induced contraction.
Figure 1

A) CSE in muscle cells

Mouse | Rabbit | Human
MW | + | - | - | + | - | RT

B) Mouse brain

CBS | CSE
MW | + | - | - | RT

C) CSE

RG | HG | MG | MB
0.42 | 0.56 | 0.63 | 0.36

CBS

RG | HG | MG | MB
0.92

β-actin

RG | HG | MG | MB
0.18 | 0.20 | 0.31 | 0.35

3-MTS

β-actin

RG | HG | MG | MB
0.18 | 0.20 | 0.31 | 0.35

Ratio
Figure 3

A) Contraction (% decrease in length) vs. CCh (log M)

- L-cysteine
- NaHS

B) % of CCh-induced contraction vs. Log (M)

- L-cysteine
- NaHS

C) % of CCh-induced contraction vs. Glibenclamide

- NaHS
- L-cysteine
Figure 5

A) cAMP (pmol/mg protein)

B) cGMP (pmol/mg protein)
Figure 6

(A) % of CCh-induced Rho kinase activity

(B) % of CCh-induced PKC activity

Log (M)

NaHS
L-cysteine

C)

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Figure 8

A) Rho kinase activity (cpm/mg protein)

B) PKC activity (cpm/mg protein)