Involvement of Potassium Channels and Calcium-Independent Mechanisms in Hydrogen Sulfide–Induced Relaxation of Rat Mesenteric Small Arteries

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ABSTRACT

Endogenous hydrogen sulfide (H2S) is involved in the regulation of vascular tone. We hypothesized that the lowering of calcium and opening of potassium (K) channels as well as calcium-independent mechanisms are involved in H2S-induced relaxation in rat mesenteric small arteries. Amperometric recordings revealed that free [H2S] after addition to closed tubes of sodium hydrosulfide (NaHS), Na2S, and GYY4137 [P-(4-methoxyphenyl)-P-4-morpholiny-l-phosphinothioic acid] were, respectively, 14%, 17%, and 1% of added amount. The compounds caused equipotent relaxations in isometric myographs, but based on the measured free [H2S], GYY4137 caused more relaxation in relation to released free H2S than NaHS and Na2S in rat mesenteric small arteries. Simultaneous measurements of [H2S] and tension showed that 15 μM of free H2S caused 61% relaxation in superior mesenteric arteries. Simultaneous measurements of smooth muscle calcium and tension revealed that NaHS lowered calcium and caused relaxation of NE-contracted arteries, while high extracellular potassium reduced NaHS relaxation without corresponding calcium changes. In NE-contracted arteries, NaHS (1 mM) lowered the phosphorylation of myosin light chain, while phosphorylation of myosin phosphatase target subunit 1 remained unchanged. Protein kinase A and G, inhibitors of guanylate cyclase, failed to reduce NaHS relaxation, whereas blockers of voltage-gated Kv7 channels inhibited NaHS relaxation, and blockers of mitochondrial complex I and III abolished NaHS relaxation. Our findings suggest that low micromolar concentrations of free H2S open K channels followed by lowering of smooth muscle calcium, and by another mechanism involving mitochondrial complex I and III leads to uncoupling of force, and hence vasodilation.

Introduction

The three gasses hydrogen sulfide (H2S), nitric oxide (NO), and carbon monoxide have biologic effects at low concentrations and are toxic at high concentrations (Szabo, 2007; Sun et al., 2011). The reported physiologic levels of H2S vary greatly; in both humans and rodents, plasma levels ranging from below 1 μM and up to 300 μM have been reported (Olson, 2011). Part of this variance can probably be ascribed to the techniques used to measure H2S because it is often the total sulfur pool rather than the free H2S that is measured (Olson et al., 2014). Although it is not well understood which of the forms H2S, HS–, or S2– are relevant for the biologic effects, free H2S is thought to mediate most of the effect on vascular tone (Kimura, 2014).

The precise role of H2S in regulating blood pressure remains unclarified as knockout of cystathionine gamma-lyase (CSE), which is considered the most important H2S producing enzyme in the cardiovascular system, has yielded disparate

ABBREVIATIONS: ACh, acetylcholine; ANOVA, analysis of variance; BKCa, large conductance calcium-activated potassium channels; [Ca2+]i, intracellular calcium concentration; CRC, concentration-response curve; CSE, cystathionine gamma-lyase; DIDS, 4,4′-disothiocyanato-2,2′-stilbenedisulfonic acid; GYY4137, P-(4-methoxyphenyl)-P-4-morpholiny-l-phosphinothioic acid; H2S, hydrogen sulfide; KATP, ATP-sensitive potassium channel; KPSS, high-potassium physiologic saline solution; KT5720, (9-[Ca2+]i), 1, 3, 10, 12-hexahydroxy-10-hydroxy-9-methyl-1-oxo-9, 12-epoxy-1H-dinol[1,2,3-fg:3′,2′]-1′,6-dipyridyl[9,8-i][1,6]-benzo-diazepine-10-carboxylic acid hexyl ester; L-NOARG, N G-nitro-L-arginine; MITO-TEMPO, (2-(2,6,6-tetramethylpiperidin-1-oxyl)-4-hydroxy-2-oxoethyl)triphenylphosphonium chloride monohydrate; MLC, myosin light chain; MLCP, myosin light chain phosphatase; MYPT-1, myosin phosphatase target subunit 1; NE, norepinephrine; NaHS, sodium hydrosulfide; OX, nitric oxide; ODH, 1H-[1,2]oxadiazolo[4,3-a]quinolin-1-one; PSS, physiologic saline solution; ROS, radical oxygen species; Rp-8-PCPT-cGMPS, (Rp)-8-[para-chlorophenylthio]guanosine-3′,5′-cyclic monophosphorothioate; SNP, sodium nitroprusside; TEA, tetraethylammonium; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; U46619, 9, 11-dideoxy-11α, 9α-epoxymethano-prostaglandin F2α; XE991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride; Y27632, 4-[1R]-l-aminoethyll-N-pyriridin-4-ylcyclohexane-1-carboxamide.

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results. One CSE knockout mouse was found to be normoten-
sive (Ishii et al., 2010); another CSE knockout mouse was
reported to have substantially lower levels of plasma H2S as
well as age-dependent hypertension (Yang et al., 2008).
Although injection of a H2S salt transiently reduces mean
arterial pressure (Ali et al., 2006; Yang et al., 2008), the effect
on the vascular tone is complex. In non-mammalian species,
H2S induces dose-dependent constriction and/or constriction
followed by relaxation (Dombkowski et al., 2005). In arteries
from rats, it has been suggested that low H2S concentrations
cause contractions whereas high H2S concentrations induce
relaxation of mesenteric, aorta, and gastric arteries as well as
of human mammary arteries (Ali et al., 2006; Kubo et al., 2007;
Webb et al., 2008; d’Emmanuele di Villa Bianca et al., 2011).
These studies were based on the application of the H2S
calctions NaHS and Na2S, thought to yield high transient
concentrations of H2S; therefore, the use of slow-release
H2S donors such as P-(-4-methoxyphenyl)-P-4-morpholinyl-
phosphinodithioic acid (GY4137) has been advocated
(Papapetropoulos et al., 2015). However, GYY4137 also causes
both vasodilation and vasoconstriction (Li et al., 2008; Salomone
et al., 2014). This suggests that the actual concentrations
reaching the vascular smooth muscle are of major importance
for the observed effect and mechanisms underlying H2S-
induced vasodilation.
A number of other mechanisms underlying H2S-induced
vasorelaxation have been suggested, including involvement
of NO (Zhong et al., 2003; Cheang et al., 2010), release of
calcitonin gene-related peptide from nerve-endings due to
induction of Ca2+ sparks (Liang et al., 2012; Jackson-Weaver et al., 2013)
and also that they reduce global [Ca2+]i (Jackson-Weaver et al., 2011, 2013; Li et al., 2012). It has been shown that the H2S donors NaHS and Na2S activate
calcium sparks (Liang et al., 2012; Jackson-Weaver et al., 2013)
and also that they reduce global [Ca2+]i in cerebral arterioles
(Liang et al., 2012). However, so far there have been no attempts to directly correlate vascular smooth muscle cell
calcium to changes in vascular tone by simultaneous measure-
ments of [Ca2+]i and relaxation.
We hypothesized that lowering of calcium and opening of
K channels as well as calcium-independent mechanisms are
involved in H2S-induced relaxation. To investigate this hy-
thesis, we performed the following measurements: 1) the
release of H2S from NaHS, Na2S, and GYY4137 was examined
by the use of a H2S microsensor; 2) changes in [Ca2+]i were
measured simultaneously with relaxation to determine
whether lowering of [Ca2+]i contributes to H2S relaxation; 3)
the phosphorylation of myosin light chain (MLC) and myosin
phosphatase target subunit 1 (MYPT-1) was measured; and 4)
the involvement of different K channels and the mitochondrial
electron chain complexes was investigated using selective
blockers in rat small mesenteric arteries.

Materials and Methods

Solutions and Chemicals. The following drugs were used:
epinephrine (NE), acetylcholine (ACh), 4-aminopyridine, antymicin A,
NaHS, Na2S, tetraethylammonium (TEA), 4,4-diisothiocyanato-2,2-

dibenzene disulfonic acid (DIDS), linopirdine, MIOTEMPO [2-(2,6,6-

tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl] triphenylphosphonium
chloride monohydrate, L-NOARG (N-NOarginine), retigabine,
Rp-8-pCPT-cGMPS (Rp-8-para-chlorophenylglycamina-3,5-cyclic
monophosphorothioate), KTS720 ([8S,10S,12R]-2,3,9,10-dihydroxy-
10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-f:3,4,1-

kl]pyrrolo[3,4-i][1,6]-benzo-diazocine-10-carboxylic acid hexyl ester),
XE991 (10,10-bis(4-pyridinylmethyl)-9(10H)-anthracene dihydrochloride,
potassium polysulfide (K2Sx), retone, sodium nitroprusside
(SNP), and glibenclamide from Sigma-Aldrich (St. Louis, MO).
ODq (1H-1,2,4-oxadiazol-4,3-aquinocinal-1-one) was obtained from
Tocris (Bristol, United Kingdom), and Fura-2 AM and pluronier P127
were purchased from Invitrogen (Taasstrup, Denmark). Iberiotoxin
was purchased from Latoxan, Valence, France. GYY4137 was synthe-
sized as previously described elsewhere (Li et al., 2008).

The NaHS and Na2S solution was made fresh every day.
To neutralize pH of the solution, hydrochloric acid was added until
a pH of 7.35–7.45 was obtained. The composition of the physiologic salt
solution (PSS) was NaCl 119 mM, NaHCO3 25 mM, glucose 5.5 mM,
CaCl2 1.6 mM, KH2PO4 1.18 mM, MgSO4 1.17 mM, and EDTA
0.027 mM. The composition of the lysis buffer was 20 mM tris-HEC,
5 mM EGTA, 150 mM NaCl, 20 mM glycophosphate, 10 mM NaF,
1% Triton X-100, 0.1% Tween-20, and 1x Halt Protease and Phospha-
tase Inhibitor Cocktail. The sample buffer composition was dithio-

treitol 6 mM, Tris-HCl 350 mM, dithiothreitol and sodium laureyl
sulfate 10%, glycerol 30%, and bromophenol blue 0.12%

Hydrogen Sulfide Measurements. For measurement of the H2S
concentration, a hydrogen sulfide microsensor (Unisense A/S, Aarhus,
Denmark) was used. The microsensor is a miniaturized amperometric
sensor consisting of an internal reference and a sensing and guard
anode. H2S from the environment is driven by the external partial
pressure and will penetrate through the sensor tip membrane into the
alkaline electrolyte. Because the sensor is sensitive to temperature,
we performed all calibrations and measurements at 37°C, as this
temperature is physiologically appropriate. The sensor was calibrated
below pH 4.0 because all the added Na2S would be in the H2S form; at
pH 7.4 it a larger part of the H2S is in the HS− form and thus is not
measured by the sensor. Calibration was made by dilution of a stock
solution of 1 M NaH2S, which was dissolved in N2-flushed PSS in a
closed tube. Measurements were performed in PSS (pH 7.4, 37°C) in
closed tubes; after addition of the donors, H2S concentration was
measured for 30 minutes. However, GYY4137 has been reported to
release more H2S at low pH (Li et al., 2008), we also measured the
release of H2S from GYY4137 at pH 3.0.

Microvascular Myograph Studies. This study followed the
recommendations in the Guide for the Care and Use of Laboratory
Animals of the U.S. National Institutes of Health and the ARRIVE
Guidelines. Wistar rats (10–12 weeks) were euthanized by cervical
dislocation followed by exsanguination, and the mesentery bed was
removed and placed in ice-cold PSS. Third branch mesenteric arteries
were dissected and mounted on wire myographs (as previously
described elsewhere) for recordings of isometric tension (Mulvany
The 1.5–2.0 mm long arterial rings, with diameters of approximately 200–300 μm, were mounted on 40-μm stainless steel wires and kept in PSS at 37°C and bubbled with 5% CO₂/21% O₂/74% N₂. After 30 minutes of equilibration, the arteries were normalized. Experiments were performed on arteries stretched to 90% of \(L_{100}\), where \(L_{100}\) is defined as the circumference of the relaxed artery exposed to a transmural pressure of 100 mmHg. Before conducting experiments, the viability of the arterial segment was tested. The mesenteric arteries were contracted twice by NE (10 μM). To test the presence of functional endothelium, the arteries were contracted with NE (3 μM) before ACh (10 μM) was added. Arteries were only included if they developed an active force corresponding to a transmural pressure of 100 mmHg and relaxed a minimum of 60% to ACh (10 μM).

Simultaneous Measurements of H₂S and Relaxation. For simultaneous measurements of force and H₂S concentration, a segment of the mesenteric superior artery was mounted on two 100-μm thick steel wires in a single chamber myograph as previously described elsewhere for simultaneous measurements of force and NO concentration (Simonsen et al., 1999). A H₂S-sensitive microelectrode with tip diameter of 50–80 μm (Unisense A/S, Aarhus, Denmark) was first calibrated as described earlier; by use of a micromanipulator, it was introduced into the lumen of the artery while another sensor was placed in the organ bath. Mesenteric superior arteries were normalized and tested as described for small mesenteric arteries, only difference being that 1 μM of NE was used to contract the arteries.

Experimental Protocol. To investigate the mechanism of H₂S-induced vasodilation, arteries with endothelium were incubated with either vehicle or inhibitors before concentration–response curves (CRCs) were constructed for NaHS, Na₂S, or GYY4137 in preparations contracted with NE. The control and examination of drugs were run in parallel, and only one CRC was constructed for each vasodilator per animal.

To investigate the involvement of K channels in H₂S-induced relaxation, CRCs for NaHS were performed on NE-contracted arteries incubated with blockers of different K channels: glibenclamide (10 μM), TEA (1 mM and 3 mM), iberiotoxin (100 nM), 4-AP (0.5 mM), linopirdine (10 μM), and XE991 (10 μM) were incubated with arteries for 30 minutes before dilation was induced by NaHS.

To investigate other pathways suggested to be involved in NaHS relaxation, CRCs for NaHS were performed on NE-contracted arteries incubated with L-NOARG (100 μM), ODQ (3 μM), KT5720 (200 nM), and Rp-8-pCPT-cGMPS (20 μM) (incubated with arteries for 30 minutes), and rotenone (1 μM), antimycin A (1 μM), Tempol [4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl] (300 μM), MITO-TEMPO (10 μM), and DIDS (100 μM) (incubated with arteries for 15 minutes). Rotenone (1 μM), antimycin A (1 μM), Tempol (300 μM), and MITO-TEMPO (10 μM) were also investigated in arteries contracted with 60 mM K (K₆₀PSS). To obtain comparable levels of contraction in arteries incubated with rotenone or antimycin A, NE was added on top of high-potassium physiologic saline solution (KPS).

Simultaneous Measurements of Intracellular Calcium Levels and Tension. Measurements of intracellular calcium levels were performed in mesenteric small arteries as previously described elsewhere (Rodriguez-Rodriguez et al., 2008). After mounting, equilibration, and normalization, the arteries were loaded with Fura-2 AM (8 μM) and loading mix (Pluronic F127) in the dark for 2 hours. The myograph was placed on a Zeiss inverted microscope (Leica Microsystems...
For detection of MLC, phosphorylated MLC (pMLC), MYPT, and phosphorylated MYPT (pMYPT), mesenteric small arteries were frozen in ice-cold acetone with trichloroacetic acid (pMLC), MYPT, and phosphorylated MYPT (pMYPT), mesenteric small arteries were frozen in ice-cold acetone with trichloroacetic acid (10%) and dithiothreitol (10 mM) and then placed in a −80 °C freezer for 24 hours before being washed 3 times with acetone with dithiothreitol (10 mM). Before homogenization with pellet pestles (Sigma, St. Louis, MO), the samples were heated to 50°C for 10 minutes in 15 μl of 50% lysis buffer and 50% sample buffer. After homogenization they were sonicated for 45 seconds and centrifuged for 10 minutes at 10,000 rpm at 4°C. The sampling in acetone and direct dissolving in sample buffer is incompatible with our protein measurements. The same volume was added to the gels for the blotting, and the samples were heated to 50°C for 10 minutes in 15 μl of 50% lysis buffer and 50% sample buffer. After homogenization they were sonicated for 45 seconds and centrifuged for 10 minutes at 10,000 rpm at 4°C. The sampling in acetone and direct dissolving in sample buffer is incompatible with our protein measurements. The same volume was added to the gels for the blotting, and the results expressed as a ratio of phosphorylated to unphosphorylated protein.

A linear relation is required for quantitative protein expression (Eaton et al., 2013). We have previously observed that the densitometric measurements of MYPT-1 correlated linearly with pMYPT and MLC with pMLC. Therefore, pMYPT and pMLC were expressed as ratios of their respective unphosphorylated proteins.

All samples were run simultaneously to minimize any differences in transfer. Samples and a prestain marker (Bio-Rad Laboratories, Hercules, CA) were loaded onto the gel. Immunoblotting was performed as described previously elsewhere (Hedegaard et al., 2014). The following antibodies were applied: MYPT 1:6000 (Sc-25618; Santa Cruz Biotechnology), pMLC 1:1000 (3672S; Cell Signaling Technology, Beverly), pMLC 1:2000 (3671S; Cell Signaling Technology), MLC 1:1000 (3672S; Cell Signaling Technology, Beverly), and secondary anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) 1:4000.

Data Calculation and Analysis. All recordings and calculations were performed by PowerLab data system and Chart 5.5 (ADInstruments, Oxfordshire, United Kingdom) or Labscribe (World Precision Instruments, Hitchin, United Kingdom). The mechanical responses of the vessel segments were measured as active wall tension (ΔT), which is the change in force (ΔF) divided by twice the segment length (2L). The CRCs were compared with controls by a two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Differences between means were analyzed by unpaired two-tailed t test. One-way ANOVA followed by Bonferroni post-test or a Student t test was used to analyze difference between mean relaxation to one dose of NaHS or SNP. P < 0.05 was considered statistically significant for all tests. All graphs and statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). The results are given as mean ± S.E.M.

Results

Release of H₂S from NaHS, Na₂S, and GYY4137. The release of free H₂S from the donors was examined by use of a microsensor in closed containers containing PSS. The H₂S microsensor responded with changes in current to micromolar concentrations of Na₂S, and the output current of the probes correlated linearly with the concentrations of Na₂S (Fig. 1A). The amount of H₂S released from 300 μM of NaHS in PSS at pH 7.4 was 43 μM (14%); during the 30 minutes of measurements the concentration slowly decreased to 33 μM (11%) (Fig. 1B). The corresponding curve for 300 μM Na₂S in PSS at pH 7.4 had a similar appearance, with a fast release of H₂S reaching a maximum of 50 μM (17%) after 2 minutes, and a slow decrease resulting in a final concentration of 39 μM (13%) after 30 minutes (Fig. 1C). Addition of 300 μM GYY4137 to PSS at pH 7.4 failed to change the electrode current, whereas 300 μM GYY4137 added to PSS at pH 3.0 gave a slower release of H₂S than NaHS and Na₂S, reaching a maximum after
6 minutes, after which a stable level of H₂S at 3 μM (1%) was obtained that remained stable through the next 25 minutes (see Fig. 1D).

**Effect of Exogenous H₂S.** To test the response of mesenteric arteries to different H₂S donors, CRCs for NaHS, Na₂S, and GYY4137 were obtained in arteries contracted to NE. NE induced stable contractions; however, in some cases they were oscillatory around a stable mean tension as previously described for rat mesenteric small arteries (see Fig. 2, A and B) (Peng et al., 2001). NaHS and Na₂S induced contraction starting from 10 μM (Fig. 2A), and only at higher concentrations (100 μM) was the contraction followed by relaxation; at 3 mM the arteries relaxed 100% (see Fig. 2, A and C). NaHS induced comparable relaxations in NE- and U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin F₂₀)‑contracted mesenteric arteries (n = 6, results not shown). GYY4137 induced relaxations starting at a concentration of 30 μM, and maximum relaxation was reached at 300 μM; no constriction was observed with GYY4137 (see Fig. 2, B and C).

Based on the added amounts, the compounds caused equipotent relaxations; NaHS gave EC₅₀ values of 188 ± 33 μM (n = 13), Na₂S of 187 ± 52 μM (n = 5), and GYY4137 of 107 ± 21 μM (n = 6). Impurities in NaHS have been suggested as an explanation for low sensitivity, but our results show that NaHS and Na₂S induced equipotent and also reproducible relaxations in mesenteric arteries. Based on the estimated concentration of free H₂S showing that a maximum of, respectively, 14% of NaHS, 17% of Na₂S, and 1% of GYY4137 (at pH = 3.0) is present on the H₂S form, the curves depicted in Fig. 2D are obtained. Based on the measured free H₂S, GYY4137 caused more relaxation in response to released free H₂S with EC₅₀ values of 1.3 ± 0.2 μM (n = 6), whereas NaHS and Na₂S were equipotent, yielding EC₅₀ values of 21 ± 4 μM for NaHS (n = 13) and 34 ± 12 μM for Na₂S (n = 5) in rat mesenteric small arteries.

To correlate the actual H₂S concentrations to the relaxations, simultaneous measurements of tension and H₂S concentrations were obtained for NaHS and GYY4137. NaHS (1–1000 μM) induced concentration-dependent increases in the current of the H₂S microsensors and simultaneously relaxed the mesenteric artery (Fig. 3B). At 300 μM NaHS, the H₂S concentration measured with the intraluminal and extraluminal H₂S-sensitive microsensors was, respectively, 15 ± 1.6 μM and 6.5 ± 0.5 μM, and the artery relaxed 61 ± 1.9 μM (n = 3). GYY4137 failed to induce any increase in current of the H₂S microsensors; at 300 μM GYY4137, the H₂S concentration measured with the intraluminal and extraluminal H₂S-sensitive microsensors was, respectively, 0.7 ± 0.8 μM and 0.04 ± 0.1 μM (Fig. 3C). GYY4137 did not yield detectable H₂S at pH 7.4 in closed containers or in the myograph; therefore, NaHS was used for the further investigation of the mechanisms underlying H₂S-induced relaxation in rat mesenteric arteries.

**Effect on Smooth Muscle Calcium Levels of NaHS.** Simultaneous measurements of tension and intracellular calcium levels were conducted. Norepinephrine induced stable increases in intracellular calcium and contraction (Fig. 4A). Increasing the concentration of NaHS resulted in slight increases in calcium, while the artery relaxed at concentrations from 100 μM to 1 mM. At concentrations equal to or above 1 mM, NaHS simultaneously reduced the intracellular calcium concentration and tension (Fig. 4, A and B). In arteries contracted with high extracellular potassium, the fall in calcium was abolished, and the maximum relaxation was reduced compared with the NaHS relaxation in NE-contracted preparations (Fig. 4, B and C).

The lowering of calcium in NE-contracted arteries shows that H₂S-induced vasodilation involves mechanisms dependent on changes in intracellular calcium levels. The calcium-dependent mechanisms likely involve the K channels because

![Fig. 3.](image-url)
relaxation was attenuated in potassium-contracted arteries. Another part of the relaxation is independent of changes in calcium, as we still observed some relaxation in the potassium-contracted arteries where no change in calcium was observed.

Effect of K Channels Blockers on NaHS Relaxation.
To investigate the involvement of K channels in H$_2$S-induced relaxation, CRCs for NaHS were performed on arteries incubated with blockers of different K channels. None of the blockers changed contraction levels to NE (Supplemental Table 1). Glibenclamide (10 μM) failed to inhibit NaHS-induced relaxation (Fig. 5A), but it significantly reduced relaxations induced by pinacidil, an opener of K$_{ATP}$ channels (Supplemental Fig. 1). This suggests that K channels other than K$_{ATP}$ channels are involved in NaHS relaxation. Inhibition with TEA (1 mM and 3 mM), which has been suggested to inhibit BK$_{Ca}$ (Nelson and Brayden, 1993) and K$_V$7 channels expressed in Chinese hamster ovary cells with IC$_{50}$ values of 3–5 mM (Hadley et al., 2000), yielded a significant reduction of NaHS-induced relaxation (Fig. 5B); the higher concentration of TEA (3 mM) had a larger effect on NaHS relaxation than 1 mM. TEA (1 mM) failed to reduce SNP relaxation ($n = 4$, results not shown) or GYY4137-induced relaxation ($n = 5–6$, results not shown). Iberiotoxin, a selective blocker of BK$_{Ca}$, failed to inhibit NaHS relaxation (Fig. 5C) and the same was observed when inhibiting voltage-dependent K channels by 4-aminopyridine (Fig. 5D).

XE991 (10 μM), an inhibitor of K$_V$7 channels, reduced the relaxations induced by retigabine, an opener of K$_V$7 channels ($n = 5$ $P = 0.004$, results not shown). XE991 failed to change the CRCs for NaHS, but it significantly reduced the relaxation to 300 μM NaHS (Fig. 5E). Another blocker of K$_V$7 channels, linopirdine, inhibited the CRCs for NaHS, giving further support for the involvement of K$_V$7 channels in NaHS relaxation (Fig. 5F).

Investigation of Protein Kinase Inhibitors and Rotenone on NaHS Relaxation. Following previously published results, we investigated a series of pathways potentially involved in NaHS relaxation. The effect of the Cl$^-$/HCO$_3$-exchanger inhibitor DIDS was found to inhibit the CRC not only for NaHS, but also for SNP and ACh ($n = 6$, results not shown), suggesting that the effect is either unspecific or dependent on the presence of NO. However, the NaHS relaxations were unaltered in the presence of 100 μM L-NOARG, a NO synthase inhibitor ($n = 6$, results not shown).
To investigate the role of the cyclic nucleotide pathways in H$_2$S relaxation, the cGMP and cAMP pathways were blocked. ODQ (3 x 10$^{-6}$ M), an inhibitor of guanylyl cyclase, markedly reduced relaxations induced by the NO donor SNP but failed to change NaHS relaxation (Fig. 7, A and B). RP-8-cPTP-cGMPS (20 μM), an inhibitor of PKG, also inhibited SNP-induced relaxation but failed to change NaHS relaxation (Fig. 7, A and B). RP-8-cPTP-cGMPS and ODQ were also found to inhibit relaxation by polysulfides (K$_2$Sn) (Supplemental Fig. 2). KT5720 (200 nM), an inhibitor of protein kinase A, inhibited forskolin-induced relaxation but failed to reduce NaHS relaxation (Fig. 7, C and D).

NaHS has been proposed to be converted by sulfide quinone oxoreductase, leading to sulfation of the mitochondrial complexes. Therefore, rotenone and antimycin A were added, which are inhibitors of, respectively, complex I and complex III. In vessels constricted with high extracellular potassium, NaHS 3 x 10$^{-4}$ M induced 57% ± 3% relaxation (n = 12), which was inhibited in the presence of rotenone (1 μM) and antimycin A (1 μM) to 7.8% ± 3.8% and 0.4% ± 0.4%.

Fig. 5. Effect of K channels blockers in NE (3 mM)-contracted mesenteric arteries on the responses to cumulative concentrations of NaHS (A) Effect of glibenclamide (10 mM) (n = 6–28). (B) Effect of TEA (1 mM and 3 mM) (n = 7–28). (C) Effect of iberiotoxin (100 nM) (n = 7–9). (D) Effect of 4-aminopyridine (0.5 mM) (n = 7–9). (E) Effect of XE991 (10 mM) #P, 0.05 by t test (n = 9–28). (F) Effect of linopirdine (10 mM) (n = 9–28). All data are presented as mean 6 S.E.M., *P, 0.05 by two-way ANOVA or Bonferroni posttest.
respectively; these relaxations were not inhibited by the superoxide mimic Tempol (300 μM) or the mitochondria-specific superoxide mimic MITO-TEMPO (10 μM) (Fig. 8A). In NE-contracted arteries, rotenone and antimycin A also inhibited relaxation from NaHS (Fig. 8B), but the superoxide mimetics failed to affect relaxation. SNP (10⁻² M), an NO donor, relaxed the NE-contracted arteries to the same level in the absence or presence of the inhibitors (Fig. 8C).

Discussion

The main findings of our study are that low micromolar concentrations of free H₂S lead to relaxations involving the lowering of smooth muscle [Ca²⁺]. This is supported by the observation that high extracellular K and inhibition of Kᵥ7 channels caused inhibition of NaHS-induced vasodilation, suggesting that low micromolar concentrations of free H₂S lead to relaxations involving K channels followed by lowering of [Ca²⁺], and of MLC phosphorylation in NE-contracted preparations. Moreover, in preparations activated with high extracellular K, NaHS induced relaxations independent of changes in [Ca²⁺], and MLC phosphorylation, probably via direct inhibition of the mitochondrial electron transport leading to force suppression. The latter observation was supported by the observation that NaHS relaxations were sensitive to inhibitors of mitochondrial complex I and III.

Evaluation of H₂S Concentration and Relaxation.

The H₂S concentration measured with microelectrodes in our study reflects the free [H₂S], which is the amount added to the bath where the electrode is placed minus clearance by degradation and diffusion. The addition of 300 μM NaHS to a closed container resulted in sustained free H₂S concentrations of 14%–17%, but taking pH 7.4 into account the measured levels correspond to the expected levels of free H₂S. In the myograph bath, the free H₂S concentrations were also lower compared with those in a closed container. In pressurized rat mesenteric arteries, 10–300 μM of added NaHS induced relaxation (White et al., 2013; in our study, 100–1000 μM NaHS induced relaxations. Although there are differences in pressure versus isometric mounted small arteries (Buus et al., 1994; Wesselman et al., 1997), our electrode measurements...
Plasma concentrations of H$_2$S have been reported to vary from 0.1 to 100 μM in patients (Goslar et al., 2011). Taking into account that a maximum of 14%–17% of the added NaHS or Na$_2$S was observed to be in the free H$_2$S form, this significantly lowers the concentration of free H$_2$S needed to induce vasodilation, giving us an estimated EC$_{50}$ of 34 and 21 μM. Furthermore, the simultaneous measurements showed that 15 μM of H$_2$S at the luminal side caused 61% relaxation of the superior mesenteric artery. These findings suggest that the concentration range of free H$_2$S generated by NaHS relaxation in our study. However, we found that TEA and iberiotoxin (a blocker of BK Ca channels) failed to affect any corresponding changes in [Ca$^{2+}$]$_i$ in rat mesenteric arteries. These findings suggest K channels are involved in NaHS-induced relaxation.

**Involvement of Calcium-Independent Mechanisms in H$_2$S-Induced Vasodilation.** Vascular tone is dependent on the relative activities of MLCK and MLCP, where activation of MLCK kinase or inhibition of MLCP can increase phosphorylation of MLCK and thereby initiate vascular smooth muscle contraction (Somlyo and Somlyo, 2003). Inhibition of MLCP, but not Rho-kinase, reduced the H$_2$S-induced relaxation. (A) Effect of KT5720 (200 nM) in NE (3 μM)-contracted mesenteric arteries on the responses to cumulative concentrations of SNP (two-way ANOVA, *P < 0.05, n = 3–7). (B) Effect of KT5720 (200 nM) in NE (3 μM)-contracted mesenteric arteries on the responses to cumulative concentrations of forskolin (two-way ANOVA, *P < 0.05, n = 3). Data are presented as mean ± S.E.M.

In the present study, simultaneous measurements of [Ca$^{2+}$]$_i$ in NE-contracted preparations revealed that NaHS lowered [Ca$^{2+}$]$_i$ and induced relaxation and, as expected based on the calcium measurements, phosphorylation of MLC decreased compared with controls. However, in preparations contracted with high extracellular potassium, NaHS induced less relaxation without any corresponding changes in [Ca$^{2+}$]$_i$ in rat mesenteric arteries. These findings suggest K channels are involved in NaHS-induced relaxation.

**K$_{ATP}$ channels (Zhao and Wang, 2002; Tang et al., 2005; Webb et al., 2008), K$_V$7 channels (Schleifenbaum et al., 2010; Martelli et al., 2013; Hedegaard et al., 2014), and BK Ca$_{Ca}$ (Liang et al., 2012; Jackson-Weaver et al., 2013) have been suggested to be involved in H$_2$S vasodilatation. Glibenclamide, 4-aminopyridine (a blocker of voltage-gated K channels), and iberiotoxin (a blocker of BK Ca$_{Ca}$ channels) failed to affect NaHS relaxation in our study. However, we found that TEA (1–3 mM) reduced NaHS relaxation; this concentration range was previously found to inhibit K$_V$7 channels (Hadley et al., 2000). In agreement with these findings, XE991 and linopirdine, blockers of K$_V$7 channels, inhibited NaHS relaxation, suggesting that K$_V$7 channels are involved in NaHS relaxation. However, the reductions in NaHS relaxation by XE991, linopirdine, and TEA were markedly less than observed in KPSS versus NE-contracted preparations, suggesting that other K channel subtypes may also contribute to NaHS relaxation.

**Involvement of K Channels and Calcium Lowering in H$_2$S-Induced Vasodilation.** Opening of K channels leads to reduced [Ca$^{2+}$]$_i$ and vasodilation. NaHS and Na$_2$S were found to activate calcium sparks and reduce global [Ca$^{2+}$]$_i$ (Liang et al., 2012; Jackson-Weaver et al., 2013). In the present study, simultaneous measurements of [Ca$^{2+}$]$_i$ in NE-contracted preparations revealed that NaHS lowered [Ca$^{2+}$]$_i$ and induced relaxation and, as expected based on the calcium measurements, phosphorylation of MLC decreased compared with controls. However, in preparations contracted with high extracellular potassium, NaHS induced less relaxation without any corresponding changes in [Ca$^{2+}$]$_i$ in rat mesenteric arteries. These findings suggest K channels are involved in NaHS-induced relaxation.

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**Involvement of Calcium-Independent Mechanisms in H$_2$S-Induced Vasodilation.** Vascular tone is dependent on the relative activities of MLCK and MLCP, where activation of MLCK kinase or inhibition of MLCP can increase phosphorylation of MLCK and thereby initiate vascular smooth muscle contraction (Somlyo and Somlyo, 2003). Inhibition of MLCP, but not Rho-kinase, reduced the H$_2$S-induced relaxation of mouse gastrointestinal smooth muscle (Dhaese and Lefebvre, 2009); in the pig bladder, H$_2$S lowered tension without changing the calcium concentration (Fernandes et al., 2013). In our study, H$_2$S initially evoked vasodilation
while the intracellular calcium levels increased or were maintained at the same level. These results suggest that besides a lowering of \([\text{Ca}^{2+}]_i\) by high NaHS concentrations, calcium desensitization is involved in NaHS relaxation of rat mesenteric small arteries. However, the phosphorylated MYPT-1/MYPT-1 ratio did not change after exposure to NaHS in the mesenteric arteries, suggesting that other mechanisms than MLCP are involved in the calcium desensitization of the vascular smooth muscle contractile apparatus.

In large arteries, inhibition of phosphodiesterase type 5 was found to be involved in NaHS relaxation (Bucci et al., 2012), and NaHS was also recently suggested to contain polysulfides and by oxidation of protein kinase G to cause relaxation in mouse mesenteric arteries (Stubbert et al., 2014). However, in our study ODQ and RP-8-cPTP-cGMPS, an inhibitor of protein kinase G, inhibited SNP relaxation and relaxation induced by the polysulfide K2Sn, while NaHS relaxation was unaltered, suggesting that polysulfides and NaHS cause relaxation through different pathways. Nor did we observe any effect on NaHS relaxation when protein kinase A was inhibited by KT5720. These findings do not exclude an interaction of H2S with phosphodiesterases or protein kinase A and G, but they suggest that other mechanisms are involved in NaHS-induced uncoupling of calcium from the contractile apparatus in mesenteric small arteries.

In perfused trout gills, it has been suggested that sulfide induces vasoconstriction by a mechanism involving mitochondrial complexes I, II, and IV, and by enhancing the formation of radical oxygen species (ROS) (Skovgaard and Olson, 2012). The interaction of H2S with the mitochondria is complex, as lower concentrations may stimulate the mitochondrial electron transfer through a mechanism involving metabolism by sulfide quinone oxoreductase (Szabo et al., 2014), whereas higher concentrations of H2S may inhibit mitochondrial electron transfer by interaction with cytochrome C (Goubert et al., 2007; Módis et al., 2013). In preparations contracted with high extracellular potassium to exclude the contribution of K channels, the mitochondrial complex I and III inhibitors rotenone and antimycin A both abolished NaHS relaxation in rat mesenteric arteries, but rotenone and antimycin A did not change the relaxation induced by exogenously added NO and hence the bioavailability. Moreover, NaHS relaxations were not inhibited in the presence of the superoxide mimetic Tempol or the mitochondrial superoxide scavenger MITO-TEMPO. Although ROS may lead to relaxation in mesenteric arteries, these findings do not suggest that ROS contribute to NaHS relaxation. Inhibition of cytochrome C by NaHS would lead to decreased ATP synthesis (Módis et al., 2013). Therefore, one may speculate that the lower ATP levels resulting from NaHS inhibition of the mitochondria lead to reduced actin-MLC cross-bridge turnover or to an increased smooth muscle AMP to ATP ratio followed by activation of AMP kinase and vascular relaxation (Rubin et al., 2005). That NaHS relaxation was also reduced by rotenone and antimycin A in NE-contracted preparations suggests that both K channels and a mitochondrial pathway contribute to NaHS relaxation in mesenteric small arteries.

Our findings suggest that low micromolar concentrations of free H2S lead to relaxations involving K channels followed by lowering of smooth muscle calcium. Moreover, in preparations activated with high extracellular potassium, NaHS induced relaxations independent of the changes in \([\text{Ca}^{2+}]_i\) and MLC phosphorylation, probably by direct inhibition of the mitochondrial electron transport, leading to force suppression. The effect of H2S on both K7 channels and the mitochondrial pathways may contribute to a cardioprotective effect of low concentrations of H2S.

**Fig. 8.** Effect of rotenone and antimycin A on NaHS and SNP relaxation. (A) Effect of rotenone, antimycin A, Tempol, and MITO-TEMPO in KPSS-contracted mesenteric arteries on the response to 300 μM NaHS (one-way ANOVA, \(P < 0.05\) by Bonferroni post-test, \(n = 5\)). (B) Effect of rotenone, antimycin A, Tempol, and MITO-TEMPO in NE (3 μM)-contracted mesenteric arteries on the response to 300 μM NaHS (one-way ANOVA, \(P < 0.05\) by Bonferroni post-test, \(n = 5\)). (C) Effect of rotenone, antimycin A, Tempol, and MITO-TEMPO in NE (3 μM)-contracted mesenteric arteries on the response to 100 μM SNP (one-way ANOVA, \(P < 0.05\) by Bonferroni post-test, \(n = 5–11\)). Data are presented as mean ± S.E.M.
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References


H₂S Induces Vasodilation in Small Arteries 63