ORIGINAL ARTICLE

Basic Study

Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice

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Abstract

AIM: To investigate the effect of hydrogen sulfide (H_2S) on smooth muscle motility in the gastric fundus.

METHODS: The expression of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in cultured smooth muscle cells from the gastric fundus was examined by the immunocytochemistry technique. The tension of the gastric fundus smooth muscle was recorded by an isometric force transducer under the condition of isometric contraction with each end of the smooth muscle strip tied with a silk thread. Intracellular recording was used to identify whether hydrogen sulfide affects the resting membrane potential of the gastric fundus *in vitro*. Cells were freshly separated from the gastric fundus of mice using a variety of enzyme digestion methods and whole-cell patch-clamp technique was used to find the effects of hydrogen sulfide on voltage-dependent potassium channel and calcium channel. Calcium imaging with fura-3AM loading was used to investigate the mechanism by which hydrogen sulfide regulates gastric fundus motility in cultured smooth muscle cells.

RESULTS: We found that both CBS and CSE were expressed in the cultured smooth muscle cells from the gastric fundus and that H_2S increased the smooth muscle tension of the gastric fundus in mice at low concentrations. In addition, nicardipine and aminooxyacetic acid (AOAA), a CBS inhibitor, reduced the tension, whereas Nω-nitro-L-arginine methyl ester, a nonspecific nitric oxide synthase, increased the tension. The AOAA-induced relaxation was significantly recovered by H_2S, and the NaHS-induced increase in tonic contraction was blocked by 5 mmol/L 4-aminopyridine and 1 μmol/L nicardipine. NaHS significantly depolarized the membrane potential and inhibited the voltage-dependent potassium currents. Moreover, NaHS increased L-type Ca^{2+} currents and caused an elevation in intracellular calcium ($[Ca^{2+}]_i$).

CONCLUSION: These findings suggest that H2S may be an excitatory modulator in the gastric fundus in mice. The excitatory effect is mediated by voltage-dependent potassium and L-type calcium channels.

Key words: Gastric fundus smooth muscle; Hydrogen sulfide; Tension; Voltage-dependent potassium channel; L-type calcium channel

Core tip: The results demonstrated that the cystathionine β-synthase and cystathionine γ-lyase were both expressed in cultured smooth muscle of the gastric fundus. Hydrogen sulfide (H2S) increased the tension of the gastric fundus and depolarized the resting membrane potential. H2S decreased the current of voltage dependent potassium channel and calcium channel and then increased the intracellular calcium.

INTRODUCTION

Hydrogen sulfide (H2S) has been proved to be a novel gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO) in recent years[1,2]. The endogenous production of H2S in the gastrointestinal tract has been demonstrated in tissue homogenates[3,4]. Two pyridoxal-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine g-lyase (CSE), are mainly responsible for H2S synthesis. CBS and CSE have been found throughout the entire gastrointestinal tract[4] and are detected in several cell types, including smooth muscle cells, enteric neurons, interstitial cells of Cajal (ICC) and epithelial cells, varying between species and regions of the gastrointestinal tract[4-8].

Recently, many reports have demonstrated the role of endogenous and exogenous H2S in gastrointestinal motility. The first work on the role of H2S in gastrointestinal smooth muscle involved the guinea pig ileum smooth muscle, in which cyanide and nitroprusside augmented its relaxation but H2S reversed the relaxation caused by nitric oxide[9]. The ATP-sensitive potassium (K_{ATP}) channel has been demonstrated to contribute to intestinal smooth muscle relaxation in the rat jejunum[10], the human, rat and mouse jejunum and colon[11]. Nevertheless, in the urinary bladder, H2S increased the bladder contraction mediated by capsaicin-sensitive nerves[12].

The gastric fundus is mainly responsible for gastric receptive relaxation; for example, after swallowing, gastric accommodation of the meal involves fundic relaxation *via* activation of the vagal inhibitory pathway. Then, the stored fundic contents are gradually delivered to the caudad stomach *via* peristaltic contractions, modified by the vagal excitatory pathway[13]. This basic theory suggests that the basic tone of the gastric fundus smooth muscle is very important to gastric receptive relaxation. However, few studies have investigated the effect of H2S on gastric fundus motility. The present study aimed to investigate the effect of H2S on gastric fundus motility and its ion channel-based mechanism.

MATERIALS AND METHODS

Ethics

This study was carried out in strict accordance to the recommendation in the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988). The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Jiaotong University School of Medicine (Permit Number: Hu 686-2009).

Animals

Adult male ICR mice aged 5 wk (20-35 g) were provided by the Experimental Animal Center of the Chinese Academy of Sciences, Shanghai, China. The mice were housed at a constant temperature (20-25 ℃) under a 12 h light/dark cycle with free access to water and food.

Tissue preparation and isometric measurements

The mice were killed by cervical dislocation, and the stomach was removed quickly, usually in 2 min, and placed in aerated (95% O2 and 5% CO2) Krebs solution containing the following (in mmol/L): NaCl 121.9, NaHCO3 15.5, KCl 5.9, MgSO4 1.2, KH2PO4 1.2, glucose 11.5, and CaCl2 2.5. The stomach was cut along the lesser curvature, washed with iced Krebs, pinned to the base of a Sylgard dish with the mucosa facing upward, and the mucosa and submucosa were removed. Full-thickness muscle strips (2 mm × 8 mm) of the fundus were obtained along the circular axis. A silk thread (USP 5/0) was attached to both ends of the strips, and the strips were hung along the circular axis in 8-mL organ baths perfused with warm (37 ℃) oxygenated Krebs solution. Mechanical activity was recorded by an isometric force transducer (RM6240C, Chengdu Instrument Factory, China) connected to an amplifier. The strip was equilibrated for 30 min with 0.3-0.5 g of the basal tension before addition of the experimental drugs.

Preparation of cultured smooth muscle cells and immunocytochemistry

Mouse gastric smooth muscle was isolated as described

above, with a few modifications. After washing three times in phosphate-buffered saline (PBS) with 1% antibiotic/antimycotic (Gibco, Grand Island, NY, United States), the muscle was planted in six-well plates immersed in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, United States), supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Gibco, Grand, NY, United States). The culture medium was changed every 48 h, and the cells were subcultured for 4-6 d.

A double-labeling immunocytochemical study was used to examine the expression of CBS and CSE. Cells grown on polylysine-coated sterile glass cover-slips were washed three times with 0.1 mol/L PBS and fixed with 4% paraformaldehyde for 20 min at 4 ℃. The cells were washed in PBS for 10 min and incubated in PBS containing 10% normal goat serum for 30 min on ice, followed closely by being incubated with either rabbit anti-CBS (1:100, Abcam Ltd., Hong Kong) or rabbit anti-CSE polyclonal antibody (1:100, Proteintech Group, Ltd., United States) mixed with mouse monoclonal anti-smooth muscle α-actin (1:100, Santa Cruz Ltd., United States) at 4 ℃ overnight. After washing, the cells were incubated at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100, Jackson Immuno Research, West Grove, PA, United States) mixed with Dylight 594-conjugated goat anti-rabbit IgG (1:100, ImmunoReagents Inc, Raleigh, NC, United States) for 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole for 5 min. The controls used the same procedure but omitted the primary antibodies. The cells were observed under a fluorescence microscope (BX3, Olympus, Tokyo, Japan).

Intracellular microelectrode recordings

The strips (8 mm × 10 mm) were pinned in a chamber with a piece of Sylgard in the bottom with the circular muscle side up and perfused with Krebs solution. A 2-h equilibration is necessary before performing the recording. Nicardipine is present to lessen the movement of the strips. We used a glass microelectrode filled with 3 mol/L KCl (30-60 MΩ of resistance) to impale the cells. Membrane potentials were recorded with a standard electrometer (Duo 773, WPI Inc., Sarasota, FL, United States). The 3% KCl-agar bridge between the bath solution and the Ag-AgCl reference electrode was used to stabilize the electrode potentials.

Cell preparation and voltage patch-clamp experiment

Smooth muscle cells were prepared from the fundus as described above. The strip was incubated in a Ca^{2+}-free solution containing the following (in mmol/L): NaCl 135, KCl 5, glucose 10, Hepes 10, and $MgCl_2$ 1.2, adjusted to pH 7.4 with Tris. The strip was cut into pieces and incubated in 1 mL of digestive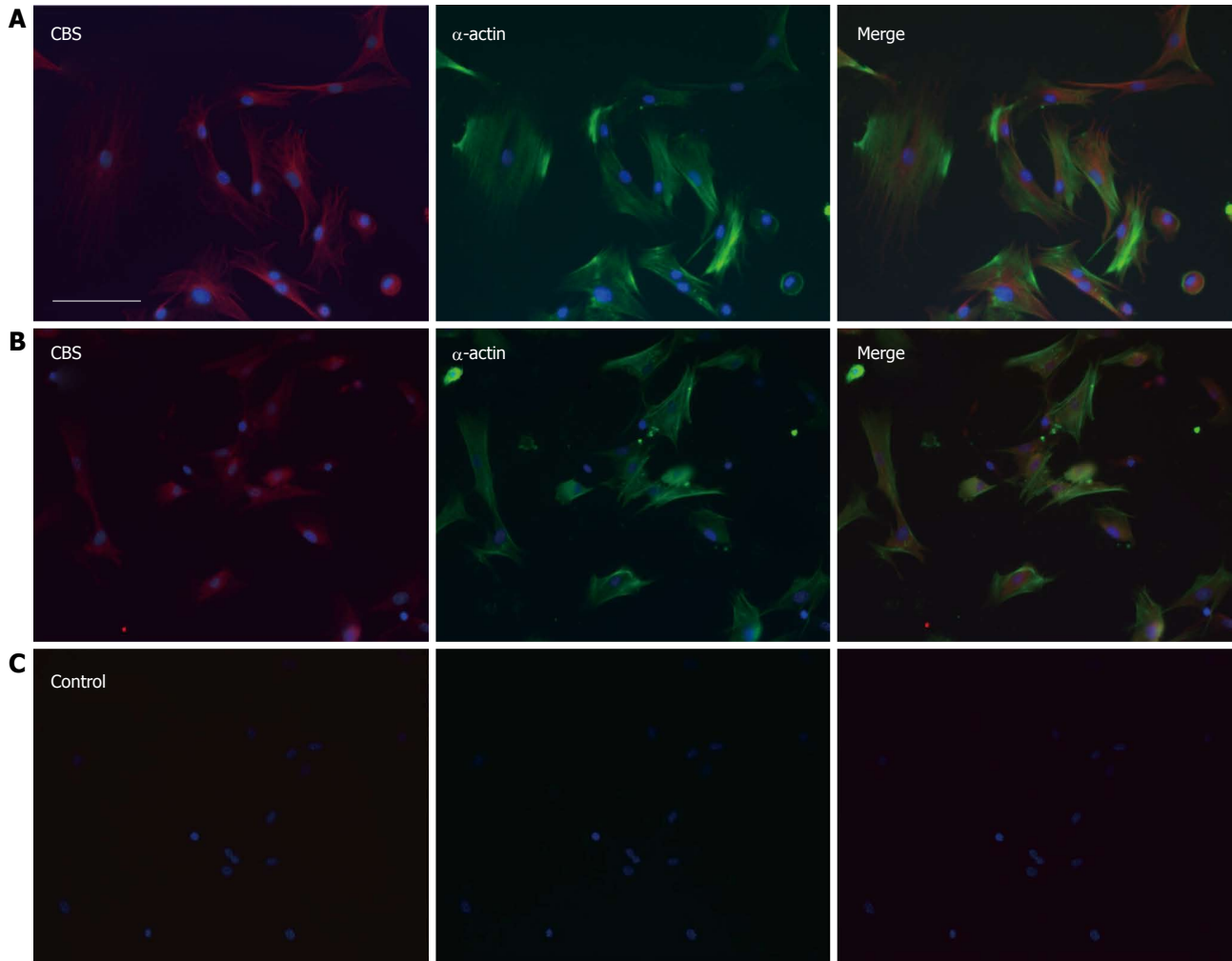 medium (Ca^{2+}-free solution) containing 2 mg of collagenase I (Sigma-Aldrich, St. Louis, MO, United States), 500 μg of papain (Sigma-Aldrich, St. Louis, MO, United States), 2 mg of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, United States) and 1.5 mg of DTT at 37 ℃ for 10 min. After digestion, the tissue fragment was reserved and washed with modified Kraft-Bruhe (KB) solution containing the following (in mmol/L): glutamic acid 50, taurine 20, EGTA 0.5, Hepes 10, $MgCl_2$ 3, KCl 50, KH_2PO_4 20, and glucose 10, adjusted to pH 7.4 with KOH. Then, the solution was triturated with a glass pipette and kept in modified Kraft-Bruhe (KB) solution. The suspension was transferred to a perfusion chamber on the stage of an inverted microscope, and the cells were recorded after being allowed to settle for 30 min. The cells were perfused in physiologic saline solution (PSS) containing the following (in mmol/L): NaCl 135, KCl 5, $CaCl_2$ 2.5, glucose 10, Hepes 10, and $MgCl_2$ 1.2, adjusted to pH 7.4 with Tris. A single 4-channel perfusion system (BPS-4, ALA Inc., Westbury, NY, United States) was used to change the perfusate. The whole-cell patch-clamp technique was used to record the transient outward potassium current and L-type Ca^{2+} current with an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). The pipette resistance was 2-4 MΩ. For recording the transient outward potassium current, the pipette was filled with a solution comprising the following (in mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, Mg-ATP 5, Hepes 5, $MgCl_2$ 1.0 and EGTA 10, adjusted to pH 7.3 with Tris. For recording the L-type Ca^{2+} channel current, the pipette was filled was a solution comprising the following (in mmol/L): CsCl 130, $MgCl_2$ 1, Na_2ATP 5, Na_2GTP 0.5, EGTA 11 and HEPES 10, adjusted to pH 7.3 with CsOH.

$[Ca^{2+}]_i$ measurement

The cells were obtained as previously described and placed on polylysine-coated slides. They were cultivated in a carbon dioxide incubator at 37 ℃. $[Ca^{2+}]_i$ was measured in cells loaded with 1 μmol/L fura-3 acetoxymethyl ester (fura-3AM) (Sigma-Aldrich, St. Louis, MO, United States) dissolved in PSS containing 1 μmol/L F127 in a carbon dioxide incubator for 1 h. After fura-3AM loading, the cells were washed three times in PSS and placed under a fluorescence microscope (BX3, Olympus, Tokyo, Japan). The cells were perfused in a flowing PSS perfusion solution at room temperature.

Drugs

Sodium hydrogen sulfide (NaHS), 4-aminopyridine (4-AP), nicardipine, aminooxyacetic acid (AOAA), DL-propargylglycine (PAG), Nω-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States). All were dissolved in distilled water except nicardipine, which was distilled in DMSO (dimethyl sulfoxide).

Figure 1 Double immunofluorescence labeling of cystathionine β-synthase and cystathionine γ-lyase proteins in cultured mouse gastric smooth muscle cells. A: CBS-IR is present in α-actin IR smooth muscle cells; B: CSE-IR is present in α-actin IR smooth muscle cells; C: Negative control without primary antibodies (scale bar =100 μm). CBS: Cystathionine β-synthase; CSE: Cystathionine γ-lyase; IR: Immunoreactivity.

Statistical analysis

The data were analyzed using Origin 7.5 software and are expressed as mean ± SE. Data from multiple groups were evaluated using one-way analysis of variance followed by a post-hoc Bonferroni test, whereas Student's paired *t*-test was used to evaluate paired data sets. A *P* value < 0.05 was considered statistically significant.
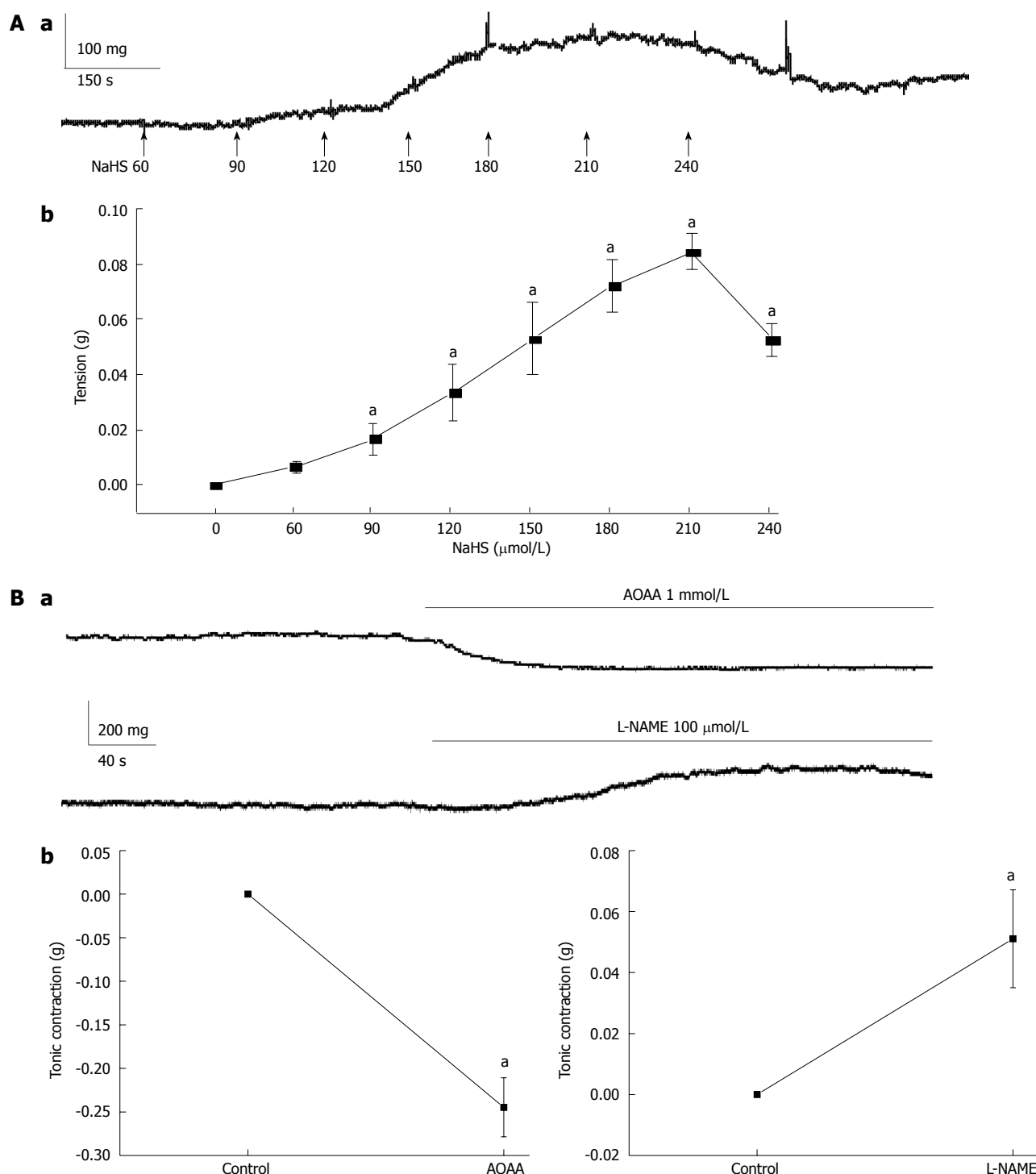
RESULTS

Expression of CBS and CSE in gastric fundus smooth muscles

To determine whether H_2S can be generated from the gastric fundus smooth muscle, CBS and CSE protein immunoreactivity (IR) was examined by double immunofluorescence labeling of cultured fundus smooth muscle cells. We found that both CBS and CSE were expressed in α-actin-positive cells (Figure 1), which suggests that H_2S can be endogenously generated in gastric fundus smooth muscle cells.

Effect of exogenous and endogenous H_2S on gastric fundus smooth muscle contraction

We observed the effect of H_2S on gastric fundus smooth muscle tonic contraction. We observed that NaHS, an H_2S donor, significantly enhanced the tension of the fundus smooth muscle at lower concentrations. The basal tension was increased from 0 mg in the control to 6.71 ± 2.11, 16.86 ± 5.67, 33.57 ± 10.32, 52.86 ± 13.06, 72.00 ± 9.62, 84.43 ± 6.56, and 52.57 ± 5.99 mg in force in cells treated with NaHS at 60, 90, 120, 150, 180, 210, and 240 μmol/L, respectively ($P < 0.05$, $n = 10$; Figure 2Aa, Ab). L-NAME (100 μmol/L), a non-specific inhibitor of NOS, increased the tension of fundus smooth muscle strips from 0 mg in the control to 52.40 ± 16.47 mg in force ($P < 0.05$, $n = 10$), whereas the CBS inhibitor AOAA (1 mmol/L) decreased the tension from 0 mg in force in the control to -241.30 ± 28.57 mg in force ($P < 0.05$, $n = 10$, Figure 2Ba, Bb). The AOAA-induced decrease in the tension was reversed by NaHS (Figure 3Aa, Ab, Ad; $P < 0.05$, $n = 10$). Interestingly, the CSE inhibitor,
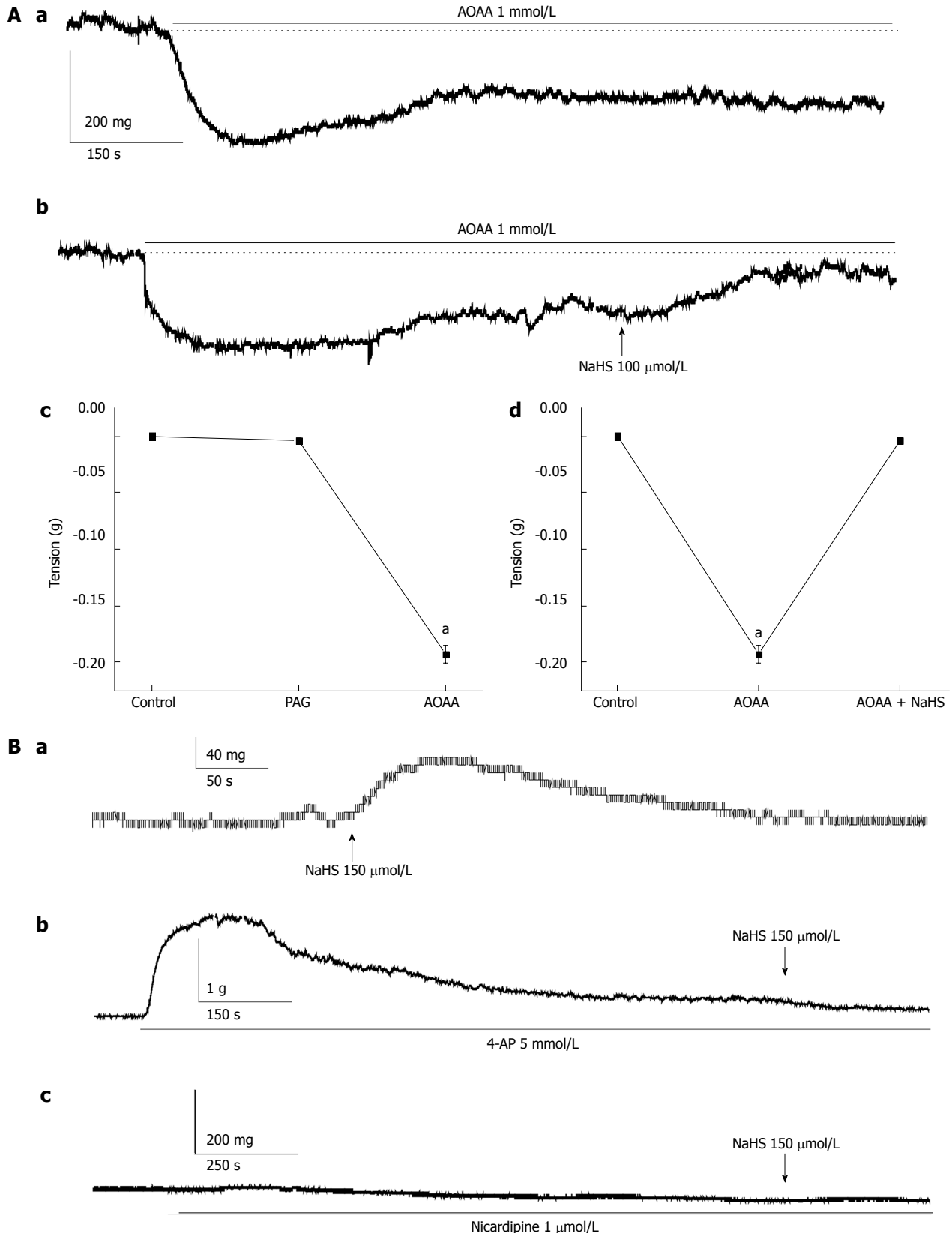
Figure 2 Effect of H₂S on the tension of gastric smooth muscle. A: Different doses of NaHS significantly enhanced the gastric fundus smooth muscle tension (a: Representative trace of changes in gastric fundus smooth muscle tension induced by different concentrations of NaHS; b: Summarized graph showing the effects of different concentrations of NaHS in inducing basal tension); B: Effects of endogenous H₂S on the gastric fundus smooth muscle tension (a: Representative traces of AOAA- and L-NAME-induced basal tension; b: Summarized graph showing the changes in AOAA and L-NAME-induced tonic contraction). Data are expressed as mean ± SE, $n = 10$, [a]$P < 0.05$, *vs* the control. AOAA: Aminooxyacetic acid; L-NAME: Nω-nitro-L-arginine methyl.

PAG (1 mmol/L), did not significantly affect the tension (from 0 mg in force by the control to -4.25 ± 2.81 mg in force, Figure 3Ac, $P > 0.05$, $n = 10$), which indicates that CBS may predominate over the gastric fundus in modulating smooth muscle contraction. Consequently, we investigated whether potassium and calcium channels are involved in the NaHS-induced excitatory effect on fundus smooth muscle tonic contraction. 4-AP (5 mmol/L), a voltage-dependent potassium channel blocker, elicited strong tonic contraction and completely blocked the NaHS-induced enhancement of fundus smooth muscle tone (Figure 3Ba, Bb; $n = 8$). We then tested the effect of nicardipine, an L-type calcium channel blocker, on NaHS-induced fundus

Figure 3 Effects of aminooxyacetic acid, DL-propargylglycine and ion channel blockers on NaHS-induced gastric fundus smooth muscle tonic contraction.
A: Effects of AOAA and PAG on gastric fundus smooth muscle basal tension (a, b: H$_2$S significantly recovered the AOAA-induced decrease in basal tension; c: Summarized graph showing the changes in AOAA- and PAG-induced tonic contractions; d: The recovery effect of NaHS on the AOAA-induced decrease in gastric fundus smooth muscle tension); B: Effects of a potassium channel blocker and L-type calcium channel blocker on NaHS-induced tonic contraction (a: Representative traces of NaHS-induced tonic contraction; b,c: Effect of 4-AP (5 mmol/L) on NaHS-induced tonic contraction, and effect of nicardipine (1μmol/L) on NaHS-induced tonic contraction. Data are expressed as mean ± SE, *n* = 10, [a]*P* < 0.05, *vs* the control. AOAA: Aminooxyacetic acid; PAG: DL-propargylglycine.

A

B

Figure 4 Effect of NaHS on the membrane potential of the gastric fundus smooth muscle. A: Raw trace of the NaHS-induced change in membrane potential; B: Summarized graph showing the change in the NaHS-induced increase in the membrane potential. Data are expressed as mean ± SE, $n = 6$, [a]$P < 0.05$ *vs* the control.

smooth muscle tonic contraction. As shown in Figure 3Ba and Bc ($n = 8$), nicardipine (1 μmol/L) completely blocked the excitatory effect of NaHS (150 μmol/L) on fundus smooth muscle tonic contraction. These results suggest that the excitatory effect of NaHS may be mediated *via* the voltage-dependent potassium channels and L-type calcium channels, resulting in the depolarization of membrane potential and Ca^{2+} influx.

Effect of NaHS on the membrane potential

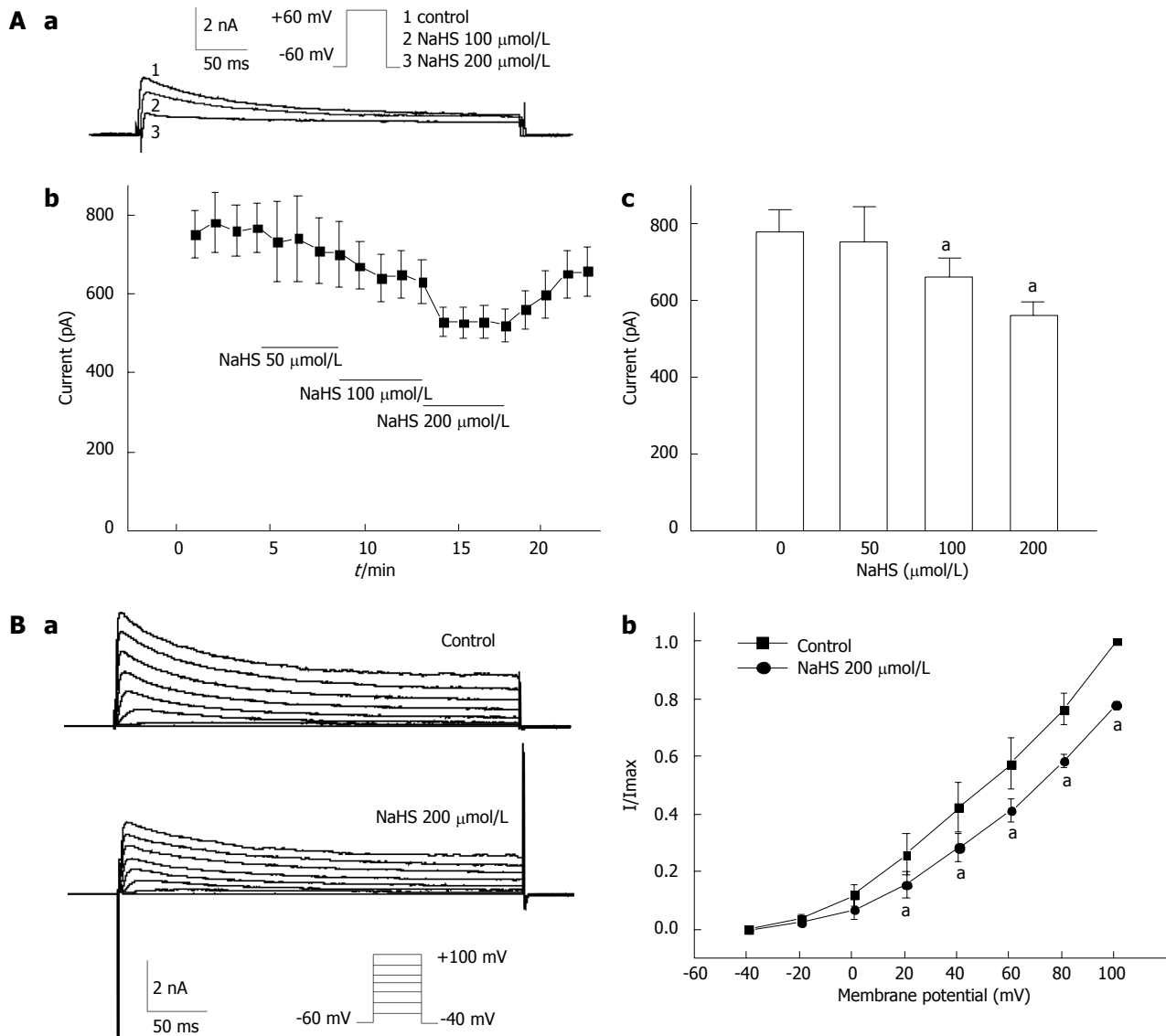To further understand the above results, we observed the effect of NaHS (100 μmol/L) on membrane potential by intracellular recording. We found that NaHS depolarized the membrane potential from -31.82 ± 1.36 mV in the control to -25.44 ± 1.13 mV (Figure 4A, B; $P < 0.05$, $n = 6$). The result indicates that the NaHS-induced excitatory effect may be related to depolarization of the membrane potential.

Effect of NaHS on voltage-dependent potassium current and L-type calcium current

To determine the mechanism of NaHS-induced membrane potential depolarization, we further examined the effect of NaHS on the voltage-dependent potassium current (IKv). Initially, IKv was elicited by a single depolarizing step pulse (in which the membrane potential was held at -60 mV and depolarized to +60 mV in 10-s intervals) for 440 ms using the whole-cell patch-clamp technique in freshly dispersed fundus smooth muscle cells. The mean peak current was increased from 777.26 ± 59.78 pA in the control to 753.89 ± 89.70 pA, 659.86 ± 48.04 pA, and 559.06 ±

36.02 pA by 50 μmol/L, 100 μmol/L, and 200 μmol/L NaHS, respectively (Figure 5Aa, Ab, Ac; $P < 0.05$, $n = 6$). To further determine the effect of NaHS on the current-voltage (I-V) relationship of IKv, IKv was elicited by a step voltage command pulse from -40 mV to +100 mV for 400 ms with a 20-mV increment in 10-s intervals. NaHS significantly decreased IKv at every membrane potential level from +20 mV to +100 mV in the I-V curve (Figure 5Ba). The IKv at +60 mV was decreased by 16.18% ± 4.96% (Figure 5Bb, $P < 0.05$, $n = 6$) with the application of 200 μmol/L NaHS. These results suggest that IKv may contribute to NaHS-induced membrane potential depolarization in mouse gastric fundus smooth muscle.

The L-type calcium current (ICa) was activated by a single depolarizing step pulse (in which the membrane potential was held at -80 mV and depolarized to 0 mV in 10-s intervals) for 440 ms first by using the whole-cell patch-clamp technique. The inward calcium current was increased with the application of a succession of NaHS. The peak relative currents were increased from 1 in the control to 1.13 ± 0.13, 1.26 ± 0.05, and 1.34 ± 0.08, by 50 μmol/L, 100 μmol/L, and 200 μmol/L NaHS, respectively (Figure 6Aa, Ab, Ac; $P < 0.05$, $n = 6$). The effect of NaHS on the I-V relationship of ICa is shown in Figure 6. The bath application of 200 μmol/L NaHS showed augmentation of the peak current on the I-V curve. NaHS significantly increased ICa at membrane potentials from -10 mV to +10 mV in the I-V curve (Figure 6Bb; $P < 0.05$, $n = 6$). The ICa at 0 mV was increased by 22.10% ± 3.90% (Figure 6Bb; $P < 0.05$, $n = 6$) with the application of 200 μmol/L NaHS.

Figure 5 Effects of NaHS on IKv in gastric fundus smooth muscle cells. Aa: Representative traces elicited by a single depolarized step pulse; Ab: The time-dependent effect of different concentrations of NaHS on IKv; Ac: Summarized graph showing the changes in the NaHS-induced inhibition of IKv elicited by a single depolarized step pulse; Ba: Representative traces of the NaHS-induced decrease in IKv in gastric smooth muscle cells; Bb: The I-V relation curve of the NaHS-induced change in IKv. Data are expressed as mean ± SE, n = 6, [a]P < 0.05 *vs* the control.

These results suggest that the L-type calcium channel is involved in the excitatory effect of NaHS on fundus smooth muscle contraction.
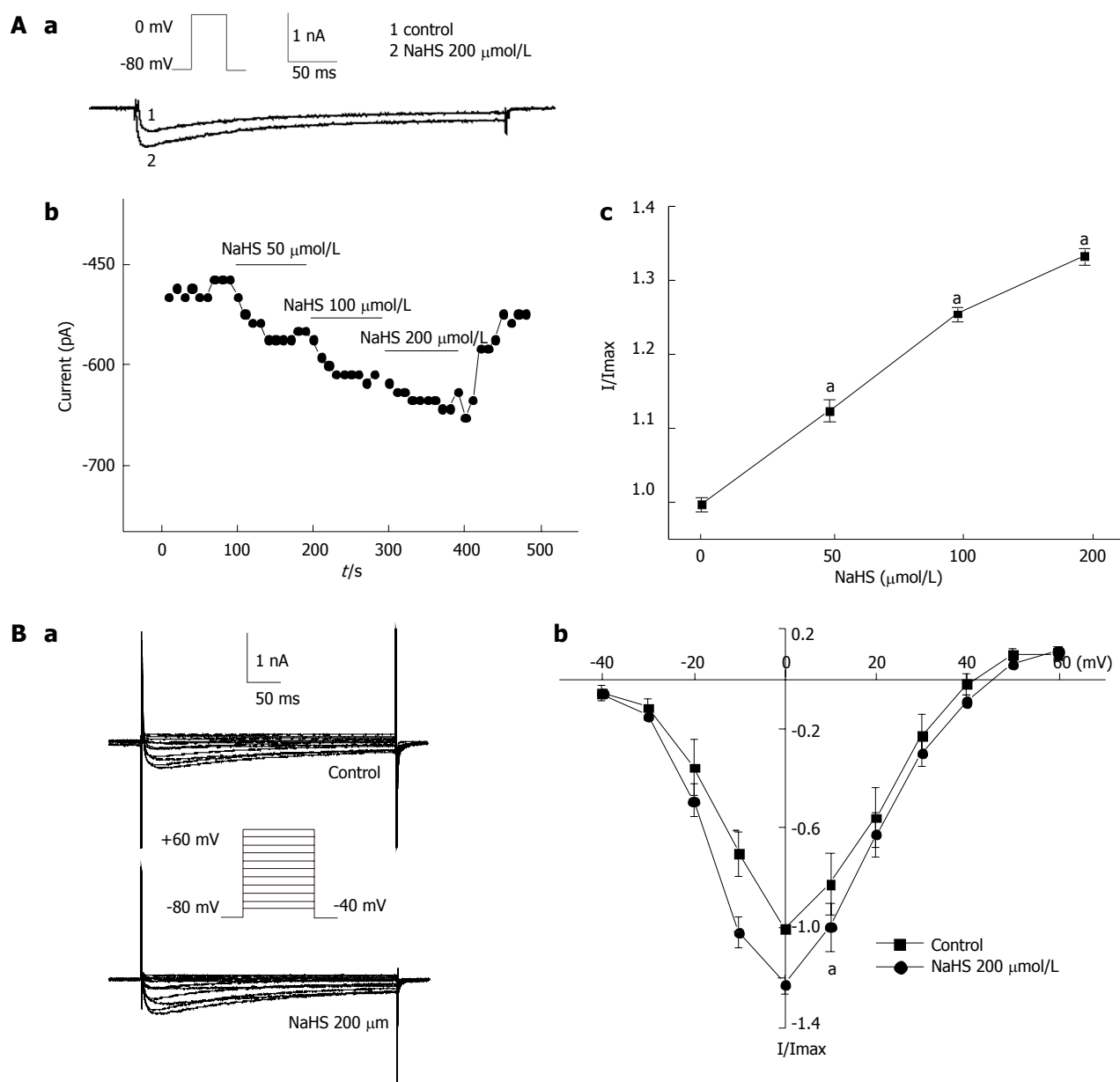
Effect of NaHS on [Ca²⁺]i

The above results suggest that NaHS-induced depolarization activates the L-type calcium channel *via* the inhibition of voltage-dependent potassium currents. Thus, we directly observed the effect of NaHS on changes in intracellular calcium levels. NaHS has been shown to elicit an increase in intracellular Ca^{2+} in cultured fundus smooth muscles cells (Figure 7Aa and Ab). The F/Fo response to NaHS varied in magnitude from 1.02 ± 0.004 in the control to 1.06 ± 0.015, 1.18 ± 0.037, 1.33 ± 0.047, and 1.27 ± 0.023 when treated with 50 μmol/L, 100 μmol/L, 200 μmol/L, and

250 μmol/L NaHS, respectively, with the maximum at 200 μmol/L (Figure 7Ab; P < 0.05, n = 6).

Because extracellular Ca^{2+} entry can result from the opening of L-type calcium channels, we observed whether the change in intracellular Ca^{2+} is associated with L-type calcium channels. When perfused with PSS containing 200 μmol/L NaHS, the F/Fo was markedly increased (Figure 7Ba), but pretreatment with 1 μmol/L nicardipine caused the NaHS-induced increase in F/Fo to be almost completely abolished (Figure 7Ba). Then, we perfused the sample with PSS containing 1 μmol/L nicardipine and 200 μmol/L NaHS, which attenuated the increase in intracellular Ca^{2+} induced by 200 μmol/L NaHS (Figure 7Bb). These results indicate that NaHS increases the intracellular Ca^{2+} levels *via* L-type calcium channels.

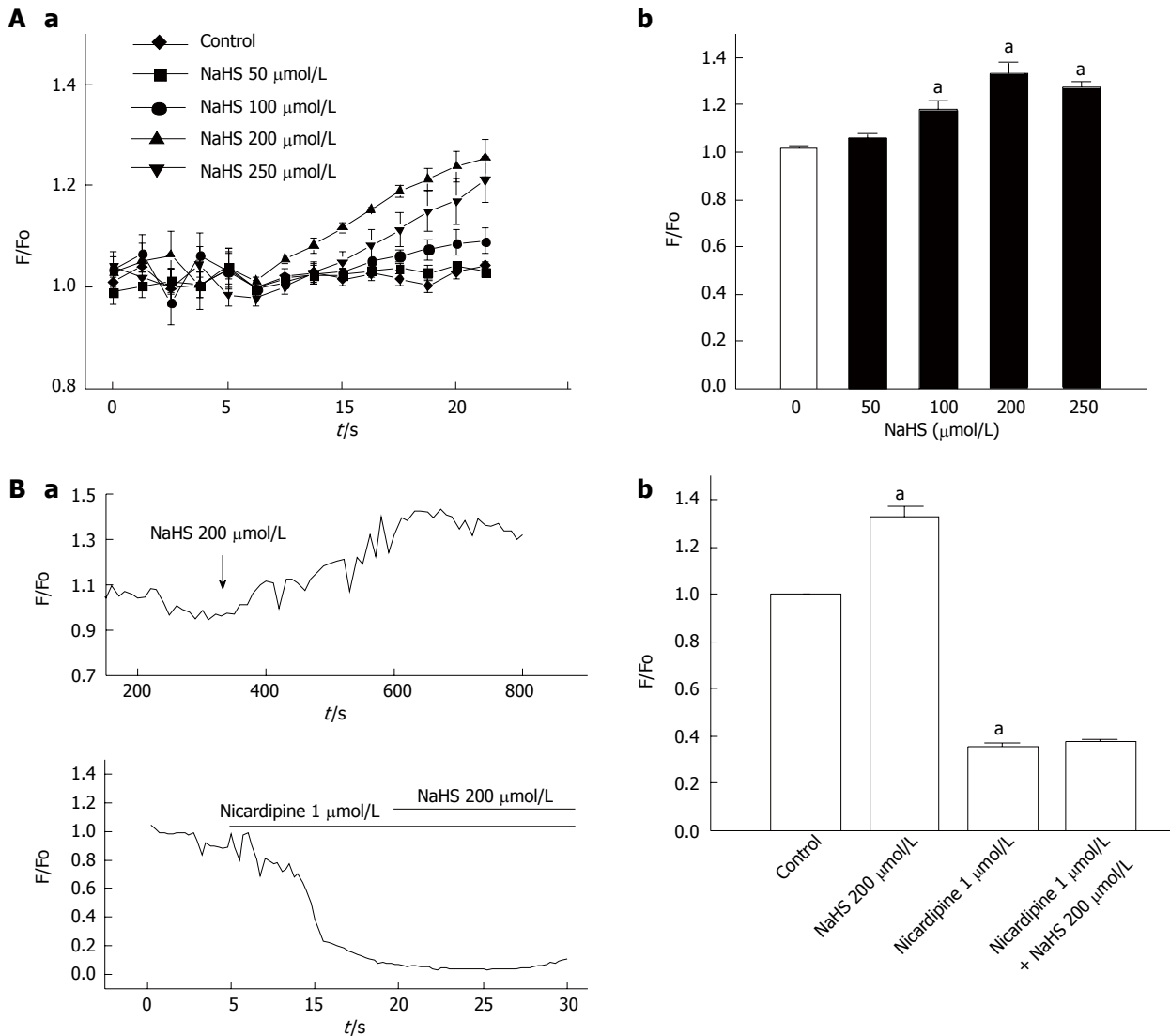**Figure 6 Effects of NaHS on ICa in gastric fundus smooth muscle cells.** Aa: Representative traces elicited by a single depolarized step pulse; Ab: The time-dependent effect of different concentrations of NaHS on ICa; Ac: Summarized graph showing the change in the NaHS-induced inhibition of ICa elicited by a single depolarized step pulse; Ba: Representative traces of the NaHS-induced increase in ICa in gastric smooth muscle cells; Bb: The I-V relation curve of the NaHS-induced change in ICa. Data are expressed as mean ± SE, *n* = 6, $^aP < 0.05$ *vs* the control.

DISCUSSION

Gasotransmitters are gas molecules endogenously synthesized in a regulated manner, causing well-defined physiological and/or pathophysiological effects, acting at specific cellular and molecular targets and employing specific mechanisms of inactivation[2,14,15]. H_2S is of particular interest in the gastrointestinal tract as it is both produced both by gastrointestinal tissues and generated in large quantities by the bacterial flora in the lumen of the gut[3,4,15-18]. In the gastrointestinal tract, both excitatory and inhibitory effects on smooth muscle have been reported. For example, NaHS concentration-dependently relaxed prostaglandin F2a-contracted circular muscle strips of mouse fundus and distal colon[19,20]. NaHS also exerted relaxant effects on guinea pig, rabbit and rat ileum and jejunum preparations[3,10,19,21,22]. Furthermore, NaHS inhibits peristaltic activity in the mouse small intestine and colon[11]. Our previous studies indicated dual effects of H_2S on the spontaneous contraction of gastric antral smooth muscle; for example, a low concentration of NaHS increased tonic contraction, whereas a high concentration reduced the amplitude and tone of gastric smooth muscle spontaneous contraction in guinea pigs[23]. We have explored that NO and H_2S play opposite roles in regulating the tension of gastric antrum before, and they share no common pathways[24]. These studies suggest that the role of H_2S in the regulation of gastrointestinal motility

Figure 7 Effect of NaHS on [Ca2+]i in cultured gastric fundus smooth muscles. Aa: The time-dependent effects of different concentrations of NaHS on [Ca2+]i.
Ab: Summary graph showing the effects of the NaHS-induced increase in intracellular calcium; Ba: Raw traces of the NaHS-induced increase in intracellular calcium.
Representative traces showing the effect of nicardipine on the H2S-induced increase in intracellular calcium; Bb: Summary graph showing the effects of nicardipine on
the H2S-induced increase in intracellular calcium in the presence of nicardipine. Data are expressed as mean ± SE, *n* = 6, [a]*P* < 0.05 *vs* the control.

displays a regional variation. The stomach gastric fundus is involved in receptive contraction concerned with gastric accommodation, and the gastric antrum is involved in gastric emptying concerned with the pyloric pump; in contrast, the jejunum and colon are involved in migrating the motility complex aimed at absorption. Therefore, the effects of H2S in the stomach might differ from those in the jejunum and colon.

In the present study, we found that both CBS and CSE, which catalyze the generation of H2S, were expressed in primary cultured smooth muscle cells (Figure 1). We deduce that H2S can be generated endogenously and continuously in gastric smooth muscle cells and influences physiological processes. Meanwhile, we observed that NaHS at lower concentrations increased the basal tension of smooth muscles in the gastric fundus (Figure 2). AOAA, an inhibitor of CBS, decreased the basal tension, and

PAG, an inhibitor of CSE, did not affect the tension significantly (Figure 3). All these results demonstrate that CBS may be the predominant enzyme in the gastric fundus. Although AOAA is widely used as an inhibitor of several pyridoxal phosphate-dependent enzymes, including aspartate transaminase, 4-aminobutyrate and dopa-decarboxylase[25], it may also inhibit NADH shuttles[26]. In contrast, Martin *et al*[4] have demonstrated that AOAA reduces H2S generation. In our study, we also observed that the decrease in the tension induced by AOAA was significantly reversed by NaHS (Figure 3), suggesting that the AOAA-induced inhibitory effect on gastric fundus smooth muscles was partially mediated by inhibition of CBS to generate endogenous H2S. NO is a well-known relaxation agent for smooth muscle as a contrary experiment control, L-NAME, a nonspecific inhibitor of NOS, significantly enhanced the tension of fundus smooth muscle. As

shown in our results, the effect of AOAA on gastric fundus smooth muscle is the opposite to that of L-NAME, which indicates that H_2S may be an excitatory gaseous transmitter in the gastric fundus under physiological conditions.

It is undeniable that membrane potential is important for electric-contraction coupling. Furthermore, distinct from other parts of the gastrointestinal tract, gastric fundus smooth muscle cells are electrically quiescent or, in some occasions, generate the discharge of membrane noises[27]. We observed the membrane potential of gastric fundus smooth muscles using the intracellular recording technique and found that 100 μmol/L NaHS significantly depolarized the membrane potential (Figure 4). Because voltage-dependent potassium channels are the most important regulators of maintaining the resting membrane potential, we observed the effect of 4-AP, an inhibitor of IK_V, on the NaHS-induced excitatory effect in succession and found that the NaHS-induced tonic contraction of fundus smooth muscle was completely blocked by 4-AP. Gastrointestinal smooth muscle cells express voltage-dependent calcium channels[28,29], and these channels are the backbone of electric-contraction coupling in the gut[30]. Therefore, we also used nicardipine to block the L-type calcium channels and found that the NaHS-induced tonic contraction was completely abolished (Figure 3). These results suggest that H_2S may be activated by L-type calcium channels through the inhibition of IK_V and depolarization of the membrane potential.

However, H_2S inhibited L-type calcium channels, resulting in the inhibition of intracellular calcium concentrations in rat cardiomyocytes[31]. Moreover, H_2S raised the intracellular calcium concentrations in endothelial cells *via* K_{ATP} channels and the Na^+-Ca^{2+} exchanger[32]. These studies indicated that the function of H_2S may be complicated in different tissues. In the present study, to further explore the ion channel mechanism involved in the NaHS-induced excitatory effect on fundus smooth muscles, we observed the effect of H_2S on IK_V, L-type calcium current and intracellular calcium concentration using the whole-cell patch-clamp and calcium imaging techniques. We found that H_2S inhibited IK_V (Figure 5) but increased the L-type calcium current (Figure 6) and intracellular calcium levels (Figure 7). The H_2S-induced increase in intracellular calcium was significantly blocked by nicardipine (Figure 7). We conclude that the excitatory effect of NaHS on the fundus smooth muscle was mediated by intracellular calcium due to the activation of L-type calcium channels *via* inhibition of the IK_V-induced depolarization of the membrane potential. The present study demonstrates that H_2S is an excitatory gaseous molecule in the gastric fundus in mice, but its exact mechanism still needs further investigation.

In summary, we showed that the H_2S-producing enzymes CBS and CSE are expressed in the gastric fundus in mice. H_2S at physiological concentrations may excite the fundus smooth muscles and induce

tonic contraction. CBS may be more important for the excitatory effect of endogenous H_2S on gastric motility. Endogenous H_2S induces the depolarization of membrane potential *via* the inhibition of voltage-dependent potassium channels. In succession, NaHS-induced depolarization activates L-type calcium channels, raises the intracellular calcium level and finally induces fundus smooth muscle tonic contraction. Under physiological conditions, endogenous H_2S and NO levels might maintain a relative balance to ensure the basic physiological tone of fundus smooth muscle.

COMMENTS

Background
Hydrogen sulfide is considered a gaseous signal molecular for its wide effects in pathophysiology process. Numerous studies have shown that hydrogen sulfide serves as a vasodilator. The gastric fundus is responsible for receptive relaxation and little is known on the effect of hydrogen sulfide on the fundus.

Research frontiers
Hydrogen sulfide is mainly catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). The authors previous studies have shown that hydrogen sulfide enhanced the tension of gastric antrum *via* voltage dependent potassium channels and ATP sensitive potassium channels. In this study, the authors demonstrate that hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice.

Innovations and breakthroughs
Recent reports have highlighted the effects of hydrogen sulfide on gastrointestinal muscle and enteric nervous system. This is the first study to report that hydrogen sulfide enhances the tension of gastric fundus smooth muscle *via* raising intracellular calcium.

Applications
This study may represent a future strategy for therapeutic intervention in disorders of gastrointestinal motility by understanding the mechanism of action of hydrogen sulfide on gastric fundus tension.

Terminology
Hydrogen sulfide is mainly catalyzed by CBS and CSE. In the gastrointestinal tract, hydrogen sulfide is involved in gastrointestinal motility, absorption and secretion.

Peer-review
The authors of the manuscript entitled "Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone mediated by voltage-dependent potassium and calcium channels in mice" present a very nice work of basic science physiology on gastric smith muscles.

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