Novel mechanism of hydrogen sulfide-induced guinea pig urinary bladder smooth muscle contraction: The role of BK channels and cholinergic neurotransmission

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Abstract

Hydrogen sulfide (H₂S) is a key signalling molecule regulating important physiological processes, including smooth muscle function. However, the mechanisms underlying H₂S-induced detrusor smooth muscle (DSM) contractions are not well understood. This study investigates the cellular and tissue mechanisms by which H₂S regulates DSM contractility, excitatory neurotransmission, and large-conductance voltage- and Ca²⁺-activated K⁺ (BK) channels in freshly-isolated guinea pig DSM. We used a multidisciplinary experimental approach including isometric DSM tension recordings, colorimetric ACh measurement, Ca²⁺ imaging, and patch-clamp electrophysiology. In isolated DSM strips, the novel slow release H₂S donor, P-(4-methoxyphenyl)-p-4-morpholinylphosphinodithioic acid morpholine salt (GYY4137), significantly increased the spontaneous phasic and nerve-evoked DSM contractions. The blockade of neuronal voltage-gated Na⁺ channels or muscarinic ACh receptors with tetrodotoxin or atropine, respectively, reduced the stimulatory effect of GYY4137 on DSM contractility. GYY4137 increased ACh release from bladder nerves, which was inhibited upon blockade of L-type voltage-gated Ca²⁺ channels with nifedipine. Furthermore, GYY4137 increased the amplitude of the Ca²⁺ transients and basal Ca²⁺ levels in isolated DSM strips. GYY4137 reduced the DSM relaxation induced by the BK channel opener, NS11021. In freshly-isolated DSM cells, GYY4137 decreased the amplitude and frequency of transient BK currents recorded in a perforated whole cell configuration and reduced the single BK channel open probability measured in excised inside-out patches. GYY4137 inhibited spontaneous transient hyperpolarizations and depolarized the DSM cell membrane potential. Our results reveal the novel findings that H₂S increases spontaneous phasic and nerve-evoked DSM contractions by activating ACh release from bladder nerves in combination with a direct inhibition of DSM BK channels.

Key Words: Detrusor smooth muscle, GYY4137, acetylcholine, Ca²⁺ transients
Introduction

Detrusor smooth muscle (DSM) is the major component of the urinary bladder wall. Coordinated complex mechanisms involving the contraction and relaxation of DSM facilitate bladder voiding and filling phases (1). DSM contractions in rodents are induced by two main neurotransmitters, ACh and ATP, which are released from parasympathetic nerve endings (1). However, in humans, evidence points to ACh as the major neurotransmitter triggering DSM voiding contractions (7, 34). In experimental animals and humans, activation of muscarinic ACh receptors (mAChRs) depolarizes the DSM cell membrane potential, enhances action potential generation, promotes influx of Ca\(^{2+}\) via L-type voltage-gated Ca\(^{2+}\) (Ca\(_{\text{V}}\)) channels, and leads to increased DSM contractility (6, 14, 41). Recently, it was demonstrated that mAChR activation leads to an inhibition of large conductance voltage- and Ca\(^{2+}\)-activated K\(^+\) (BK) channels in rat and human DSM cells via a Ca\(^{2+}\)-dependent mechanism. This suggests the existence of a functional link between the mAChRs and BK channels in the DSM of the urinary bladder (35, 36).

The BK channels are highly expressed in DSM and have been recognized as key regulators of DSM excitability and contractility (38, 39). These K\(^+\) channels contribute to maintenance of the resting membrane potential and modulation of the repolarization phase of spontaneous action potentials that determine the DSM spontaneous phasic contractions (38, 39). Pharmacological inhibition of the BK channels with iberotoxin, charybdotoxin or paxilline enhances the DSM excitability and contractility (14, 16, 21, 50, 51) whereas BK channel pharmacological activators, such as NS11021 and NS1619, reduce the generation of spontaneous action potentials and related DSM phasic contractions (21, 24, 28, 32), confirming the important regulatory role of the BK channels (38, 39).

Hydrogen sulfide (H\(_2\)S) is an important signalling molecule, exerting a wide range of biological effects in mammalian tissues (47), and is proposed to function as a neuromodulator
It has been suggested that \( \text{H}_2\text{S} \) regulates \( \text{Ca}^{2+} \) homeostasis in neuronal cells via activation of L-type \( \text{Ca}_\text{V} \) channels, and thereby regulates the neurotransmitter release (12, 27, 52). In the central nervous system, \( \text{H}_2\text{S} \) promotes synaptic release of glutamate (3). Furthermore, it has been proposed that \( \text{H}_2\text{S} \) enhances \( \text{ACh} \) released in central preganglionic terminals, and thus regulates gastrointestinal function (42).

In the lower urinary tract, \( \text{H}_2\text{S} \) is primarily synthetized via cystathionine \( \gamma \)-lyase, although cystathionine \( \beta \)-synthase has also been found to be expressed (9-11). Since \( \text{H}_2\text{S} \) and its synthases are present in the lower urinary tract tissues of various species, it is believed that endogenous \( \text{H}_2\text{S} \) might play a role in the physiological function of the bladder and/or in pathological conditions such as overactive bladder (11). It has been suggested that in DSM, \( \text{H}_2\text{S} \) induces concentration-dependent contraction by stimulating tachykinins release and activation of \( \text{NK}_1 \) and \( \text{NK}_2 \) receptors (37). In the ureter and bladder neck, it is believed that \( \text{H}_2\text{S} \) induces smooth muscle relaxation by stimulating capsaicin sensitive primary afferents which release inhibitory neuropeptides (8, 10). Besides these few recent reports, there is no information about the role of \( \text{H}_2\text{S} \) in the cholinergic neurotransmission or BK channel activity in DSM.

In the current study, for the first time we investigated \( \text{H}_2\text{S} \) regulatory mechanisms controlling \( \text{ACh} \) release and BK channel activity in freshly-isolated guinea pig DSM strips and cells. We employed the novel slow-release \( \text{H}_2\text{S} \) donor, \( \text{P}-(4\text{-methoxyphenyl})-p-4\text{-morpholinylphosphinodithioic acid morpholine salt} \) (GYY4137). GYY4137 is a water soluble compound that slowly releases \( \text{H}_2\text{S} \) in both aqueous solutions in vitro and after intravenous or intraperitoneal administration in vivo (30, 48). GYY4137 is a novel \( \text{H}_2\text{S} \) donor that better reflects the endogenous physiological release of \( \text{H}_2\text{S} \) and it is much more stable compared to the other known \( \text{H}_2\text{S} \) donors. Thus, GYY4137 represents an improved pharmacological tool for study of the physiological effects of \( \text{H}_2\text{S} \) (30, 48).
Materials and Methods

Ethical approval: Experimental procedures were carried out in accordance with the Animal Use Protocol #1747 reviewed and approved by the University of South Carolina Institutional Animal Care and Use Committee.

Animal housing, euthanasia, and DSM tissue harvesting: A total of 42 adult male Hartley-Albino guinea pigs (Charles River Laboratories, Raleigh, NC), with weight average of 448±11 g were used in this study. The guinea pigs were housed within the Animal Resource Facilities at the University of South Carolina. The animals had free access to food and water, and were exposed to 12 h light/dark cycles. The guinea pigs were euthanized by CO₂ inhalation using an automated CO₂ delivery system (SmartBox™, Euthanex Corp., Palmer, PA). Upon thoracotomy, the bladders were cut open above the bladder neck and transferred to a Petri dish containing dissection solution. The urothelium and lamina propria were carefully removed. DSM strips (5-6 mm long and 2-3 mm wide) were prepared for Ca²⁺ imaging experiments, isometric DSM tension recordings, and DSM single cell isolation.

Isometric DSM tension recordings: The isometric DSM tension recording experiments were conducted as previously described (22, 51). Briefly, DSM strips were attached to an isometric force-displacement transducer and suspended in a 10 ml temperature-controlled (37°C) water bath containing physiological saline solution (PSS) and aerated with 95% O₂-5% CO₂ (pH 7.4). DSM strips were stretched to 10 mN of initial tension and washed with fresh PSS every 15 min during an equilibration period of 45–60 min. Nerve-evoked DSM contractions were generated by applying electrical field stimulation (EFS), using a pair of platinum electrodes mounted in the tissue baths parallel to the DSM strips. The EFS pulses were generated using a PHM-152I stimulator (MED Associates, St. Albans, VT) and delivered rectangular pulses with the following parameters: 0.75 ms pulse width, 20 V pulse...
amplitude, 3 s stimuli duration and, polarity was reversed for alternating pulses.

**Ca^{2+} transients and global Ca^{2+} level measurements:** DSM strips were pinned on a Sylgard base with a window of 3 mm in diameter in a glass bottom Petri dish. DSM preparations were carefully stretched and were allowed to equilibrate for 30 min in PSS at 35°C. Next, preparations were incubated in PSS containing 8 µM of fura-2 AM for 2 h at 35°C in a dark room. The fura-2 AM solution was then removed and strips were washed three times with PSS. The recording chamber was mounted on the stage of an inverted fluorescent microscope (OLYMPUS IX81) equipped with a 40x objective. Images were captured on a Hamamatsu C10600 camera connected to a computer running the MetaFluor 7.7.2.0 software (Molecular Devices, Union City, CA). Next, DSM strips loaded with fura-2 were excited at 340 nm and 380 nm wavelengths light for 20 ms with 0.6 s intervals. Relative changes in [Ca^{2+}]_{i} were expressed as the emission intensity ratio (F_{340}/F_{380}). The Ca^{2+} transients and global Ca^{2+} levels were acquired using ImageJ, and the Ca^{2+} transients were picked up with a threshold of 20% above the basal Ca^{2+} level. All Ca^{2+} imaging experiments were carried out at room temperature (22–23°C).

**ACh measurements:** Total ACh was measured with a choline/ACh assay kit (ab65345, Abcam) using the colorimetric method following the manufacturer’s instructions. Briefly, the samples were collected from the incubation medium containing the DSM strips, before (control) and after GYY4137 stimulation and kept at -20 ºC. Fifty µl sample was then mixed with 50 µl of the reaction mixture in a 96 well plate and allowed to incubate at room temperature and protected from the light for 30 min. ACh is converted to choline by acetylcholinesterase. The absorbance of 570 nm light by the solution was measured using an ELx 808 Ultra Microplate Reader (BioTek Instruments Inc., Winooski, VT). The total choline concentration ([Cho]) was calculated as a product of the amount of choline (A_{Cho}) in the sample well determined from the standard curve and the volume of sample (S_v) used in
the reaction ([ACh]=ACho/Sv) and was expressed as nmol/ml.

**DSM single cell isolation:** DSM cells were isolated as previously described (22, 51). Briefly, DSM strips were placed in 2 ml dissection solution supplemented with 1 mg/ml BSA, 1 mg/ml papain, and 1 mg/ml dithiothreitol and incubated for 12-18 min at 37°C. The DSM strips were then transferred to 2 ml dissection solution supplemented with 1 mg/ml BSA, 0.5 mg/ml type II collagenase, 0.5 mg/ml trypsin inhibitor, and 100 µM CaCl₂ and incubated at 37°C for 12–15 min. After the incubation, DSM strips were washed with fresh dissection solution containing BSA 1 mg/ml. Individual cells were released from the tissue by passing the enzyme-treated DSM strips through a Pasteur pipette.

**Patch-clamp electrophysiological recordings:** Patch-clamp electrophysiological experiments were performed as previously described (22, 32, 51). Briefly, several drops of the dissection solution containing freshly-isolated DSM cells were placed into a recording chamber and were allowed to adhere to the glass bottom for at least 20 min. Next, the cells were washed several times with extracellular solution. The amphotericin-B perforated whole cell recording method was employed to measure transient BK currents (TBKCs) and resting membrane potential. Whole cell currents were recorded using an Axopatch 200B amplifier, Digidata 1440A, and pCLAMP version 10.2 software (Molecular Devices). An eight-pole Bessel filter 900CT/9L8L (Frequency Devices, Ottawa, IL) was used to filter the recorded currents. The patch-clamp pipettes were prepared from borosilicate glass (Sutter Instruments, Novato, CA) and pulled using a Narishige PP-830 vertical puller (Narishige Group, Tokyo, Japan). Then they were fire-polished with a Microforge MF-830 (Narishige Group) to give a final tip resistance of 3-6 MΩ. TBKCs were recorded at -20 mV. Resting membrane potential was recorded using the current-clamp mode of the patch-clamp technique (Ih=0). Single BK channel recordings were conducted on excised patches using the inside-out configuration at +60 mV. All single channel experiments were carried out with a symmetrical solution.
containing 140 mM KCl and ~300 nM free [Ca^{2+}] for pipette and bath compartments, as previously described (32). The final tip resistance of the electrodes for single channel recording was 6-15 MΩ. All patch-clamp experiments were carried out at room temperature (22–23°C).

**Chemicals and solutions:** Dissection solution contained (in mM): 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 glucose, 10 HEPES, and 2 MgCl₂, pH 7.3, adjusted with NaOH. The Ca^{2+}-containing PSS was prepared daily and had (in mM): 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, 1.2 MgSO₄, and 11 glucose, and was aerated with 95% O₂-5% CO₂ to get pH 7.4. The extracellular solution for whole cell patch-clamp experiments had (in mM): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. The pipette solution for whole cell patch-clamp experiments had (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, pH adjusted to 7.2 with NaOH and supplemented with freshly dissolved (every 1–2 h) 200 µg/ml amphotericin-B. The symmetrical K⁺ solution for single channel recording had (in mM): 140 KCl, 1.08 MgCl₂, 5 EGTA, and 3.16 CaCl₂, pH adjusted to 7.2 with NaOH. The free Ca^{2+} concentration of ~300 nM was calculated using the WEBMAXC Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm, Chris Patton). Papain was purchased from Worthington (Lakewood, NJ); dithiothreitol, collagenase type II, tetrodotoxin (TTX) and N’-[3,5-Bis (trifluoromethyl)phenyl]-N-[4-bromo-2- (2H-tetrazol-5-yl-phenyl)thiourea (NS11021) from Sigma-Aldrich (St. Louis, MO); BSA, amphotericin-B and atropine from Thermo Fisher Scientific (Fair Lawn, NJ); and P-(4-methoxyphenyl)-p-4-morpholinylyphosphinodithioic acid morpholine salt (GYY4137) from Tocris (Bristol, UK).

Stock solution of GYY4137 was dissolved in DMSO and the final concentration of DMSO in the bath did not exceed 0.06%.

**Data analysis and statistics:** MiniAnalysis software (Synaptosoft, Inc., Decatur, GA) was
used to analyse DSM phasic contraction amplitude and muscle force integral (determined by integrating the area under the curve of the phasic contractions). Data were normalized to the spontaneous contractions prior the addition of the first concentration of GYY4137 (taken as 100%). The 5 min recordings prior to the addition of each concentration of GYY4137 were analysed. For the EFS-induced contractions, the contraction amplitude and muscle force were normalized to the values at frequency of 50 Hz under control conditions (taken as 100%). The TBKC amplitude and frequency, and the spontaneous transient hyperpolarizations were also analysed using MiniAnalysis software. Data were normalized to the TBKCs prior the addition of GYY4137 (taken as 100%). The whole cell current-clamp (Ih=0) recordings were analysed using version 10.2 of the Clampfit software (Molecular Devices) and the last 5 min of the stable recordings prior to the application of GYY4137 were used as a control. The BK channel open probability (NPo) were obtained using the build-in algorithm in Clampfit, which calculates open probability as (To)/(To + Tc), where To is the total open time and Tc is the total closed time during the recording interval. Single-channel events were analyzed over 10 min recording prior to and after addition of GYY4137. The NPo was normalized to the control values and taken as 100%. GraphPad Prism 4.03 software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. Sensitivity to GYY4137 was expressed as pD2, and calculated using a computerized nonlinear regression analysis (GraphPad Software). The data are summarized as mean±SEM of n=number of DSM cells or strips and N=number of guinea pigs, and compared using unpaired or paired Student's t-test, as indicated. Differences in the rate of ACh release were analysed using one-way ANOVA analysis of variance following by Bonferroni post hoc test. A P value of <0.05 was considered statistically significant.
Results

GYY4137 increases spontaneous phasic contractions in freshly-isolated DSM strips: To examine the effects of the H₂S donor, GYY4137, on DSM contractility we applied cumulative concentrations of GYY4137 (0.1 nM-10 µM) on freshly-isolated DSM strips exhibiting spontaneous phasic contractions. GYY4137 significantly increased the spontaneous phasic contraction amplitude (pD₂=7.4±0.2 and Eₘₐₓ=477.5±94.4%) and muscle force integral (pD₂=7.0±0.3 and Eₘₐₓ=625.1±164%) in a concentration-dependent manner (n=12, N=8, P<0.05; Figs. 1 & 2). Because H₂S increases the cholinergic neurotransmission in type I glomus cells (31), we sought to investigate the effects of GYY4137 on DSM contractility in the presence of TTX (1 µM), a blocker of neuronal voltage-gated Na⁺ channels, which blocks the propagation of the nerve impulse. TTX (1 µM) significantly decreased the GYY4137 stimulatory effects on DSM phasic contraction amplitude (Eₘₐₓ=189.8±13.2%; n=7, N=4, P<0.05; Fig. 1A and B) and muscle force integral (Eₘₐₓ=242.4±39.9%; n=7, N=4, P<0.05; Fig. 1A and C). These results suggest that H₂S has a pre-junctional effect and may function as a neuromodulator in the urinary bladder.

GYY4137 increases the contractility of freshly-isolated DSM strips in an mAChR-dependent manner: Activation of mAChRs by ACh plays a key role in triggering bladder voiding contractions (7, 34). Therefore, we next investigated whether mAChRs are involved in the GYY4137-evoked DSM contractions. DSM strips were pretreated with atropine, a mAChR antagonist. In the presence of atropine (1 µM), GYY4137 increased the phasic contraction amplitude and muscle force integral to Eₘₐₓ=184.2±12.9% and 240.3±35.5%, respectively (n=7, N=4, P<0.05; Fig. 2).

We further investigated the effects of GYY4137 on the nerved-evoked DSM contractions. GYY4137 (3 µM) caused a small but statistically significant increase of the EFS-induced DSM contractions. The contraction amplitude and force at 50 Hz were increased to...
111.5±1.4% and 114.0±1.1%, respectively (n=8, N=5, P<0.05; Fig. 3). Atropine (1 µM) inhibited the EFS-induced DSM contraction amplitude and force to 45.4±4.2% and 26.4±4.1% of the control values at 50 Hz, respectively. In the presence of 1 µM atropine, GYY4137 (3 µM) did not have any significant effects on the EFS-induced DSM contractions (n=6, N=4, P>0.05; Fig. 3D-E). Collectively, these results suggest that H2S enhances DSM contractility in a mAChR-dependent manner.

**GYY4137 increases neuronal ACh release in freshly-isolated DSM strips through a mechanism involving influx of Ca2+ via L-type Cav channels:** To further investigate the role of H2S on the ACh release from bladder nerves, we measured the amount of ACh released from DSM strips using the colorimetric method. GYY4137 increased the ACh release in a concentration-dependent manner (1 nM-3 µM). GYY4137 (1 µM) increased the ACh release from 0.0028±0.0006 nmol/ml, under control conditions, to 0.0187±0.0025 nmol/ml (n=5, N=5, P<0.05; Fig. 4). Nifedipine (1 µM), an L-type Cav channel blocker, significantly decreased the GYY4137 (1 µM)-induced ACh release to 0.0069±0.0007 nmol/ml (n=5, N=5, P<0.05; Fig. 4). These results suggest that in the bladder H2S increases the ACh release through a mechanism involving Ca2+ influx via L-type Cav channels.

**GYY4137 increases spontaneous Ca2+ transients and basal Ca2+ levels in freshly-isolated DSM strips:** We further investigated the effects of GYY4137 on spontaneous Ca2+ transients and basal Ca2+ levels of fura-2 loaded DSM isolated strips. DSM strips generated spontaneous Ca2+ transients with a mean frequency of 0.76±0.06 min⁻¹, amplitude (F340/F380) of 0.18±0.04, and a basal F340/F380 of 0.66±0.09 under control conditions. GYY4137 (3 µM) increased spontaneous Ca2+ transient amplitude and basal Ca2+ levels to 0.29±0.06 and 0.79±0.11 (12 traces; n=6, N=6, P<0.05; Fig. 5), respectively, but it did not have any significant effect on the Ca2+ transient frequency (12 traces; n=6, N=6, P>0.05; Fig. 5).

**GYY4137 attenuates DSM relaxation induced by BK channel pharmacological
**Activation with NS11021:** BK channels are considered the most important physiologically-relevant K\(^+\) channels that regulate DSM function (38, 39). Thus, we sought to investigate the role of BK channels in the H\(_2\)S-induced DSM contractions. We constructed concentration-response curves for NS11021, a selective BK channel opener, in the absence or presence of GYY4137 (3 \(\mu\)M). NS11021 (10 \(\mu\)M) decreased the spontaneous phasic contraction amplitude and muscle force integral to 21.9±3.5% and 15.9±4.1%, respectively (n=8, N=5; Fig. 6). GYY4137 (3 \(\mu\)M), significantly reduced the DSM relaxation effect of NS11021 and caused a rightward shift of the concentration-response curves. In the presence of GYY4137 (3 \(\mu\)M), NS11021 (10 \(\mu\)M) reduced the DSM spontaneous phasic contraction amplitude and muscle force integral to 42.0±5.8% and 29.9±3.7%, respectively (n=8, N=5, P<0.05; Fig. 6). These results suggest that GYY4137-induced DSM contractions involve interactions with the BK channels.

**GYY4137 reduces the amplitude and frequency of TBKCs in freshly-isolated DSM cells:** To further investigate the effects of H\(_2\)S on BK channels, TBKCs were recorded using the amphotericin-B perforated whole cell patch-clamp technique at a holding potential of -20 mV. DSM cells exhibited TBKCs with a mean frequency and amplitude of 0.66±0.1 Hz and 39.6±10.9 pA, respectively, and an average cell capacitance of 31.8±2.3 pF (n=7, N=7). GYY4137 (3 \(\mu\)M) significantly decreased the frequency and amplitude of TBKCs to 52.3±8.8% and 84.4±5.2% of the control values, respectively (n=7, N=7, P<0.05; Fig. 7). These results suggest that H\(_2\)S inhibits TBKC activity in DSM cells.

**GYY4137 decreases the single BK channel open probability in excised patches from freshly-isolated DSM cells:** To determine whether H\(_2\)S directly modulates the open probability of BK channels, single BK channel activity was recorded in excised membrane patches using the inside-out configuration of the patch-clamp technique at +60 mV. GYY4137 (3 \(\mu\)M) decreased the BK channel open probability to 44.8±12.6% of the control
values (n=7, N=7; \textit{P}<0.05; \textbf{Fig. 8}). Under control conditions, the single BK channel current amplitude was 10.8±3.2 pA, and GYY4137 (3 µM), did not change the amplitude of the BK channel currents, 10.5±2.6 pA (n=7, N=7; \textbf{Fig. 8A and C}). The single channel recordings suggest that H$_2$S directly inhibits BK channel activity.

GYY4137 depolarizes the resting membrane potential in freshly-isolated DSM cells: BK channels have a key role in maintaining DSM cell excitability (38, 39). For this reason, we sought to determine the effects of GYY4137 on the DSM cell resting membrane potential. DSM cell membrane potential was recorded using the amphotericin-B perforated whole cell patch-clamp technique in current-clamp mode. Under control conditions, DSM cells exhibited a membrane potential average of -25.1±3.6 mV with mean cell capacitance of 35.4±2.9 pF (n=9, N=9). GYY4137 (3 µM) caused a small but statistically significant depolarization of DSM cell membrane potential to -22.3±3.6 mV (n=9, N=9, \textit{P}<0.05; \textbf{Fig. 9}). Five of the 9 cells tested exhibited spontaneous transient hyperpolarizations. GYY4137 (3 µM) decreased the amplitude and the frequency of the spontaneous transient hyperpolarizations to 68.4±9.3% and 71.3±9.7% (n=5, N=5, \textit{P}<0.05; \textbf{Fig. 9}) of the control values, respectively. These results indicate that GYY4137 depolarizes DSM cell resting membrane potential, and thereby increases DSM cell excitability.

\section*{Discussion}

The present study used an innovative multi-level experimental designed to investigate the role of H$_2$S in guinea pig DSM function. Our results provide compelling evidence for a novel regulatory mechanism by which H$_2$S induces DSM excitability and contractility. H$_2$S: (1) promotes neuronal ACh release through a mechanism involving influx of Ca$^{2+}$ via L-type Ca$_{V}$ channels; (2) inhibits BK channels in DSM cells and reduces single BK channel open probability in excised patches from DSM cells; (3) inhibits the BK channel-mediated
spontaneous transient hyperpolarizations and depolarizes the DSM cell membrane potential; (4) increases the amplitude of Ca$^{2+}$ transients and the basal Ca$^{2+}$ levels; and (5) increases DSM phasic contractions.

In DSM, the myogenic nature of the spontaneous phasic contractions is determined by the spontaneous action potentials (14, 15). The influx of Ca$^{2+}$ via L-type Cav channels initiates the depolarization phase of DSM action potentials thereby increasing the global intracellular Ca$^{2+}$ concentration which triggers DSM phasic contractions (14, 15). The repolarization phase of DSM action potentials is initiated upon activation of the BK channels, which is associated with a reduction in intracellular Ca$^{2+}$ and DSM relaxation (38). Thus, the amplitude of DSM phasic contractions is directly related to Ca$^{2+}$ transients and cell membrane depolarization (4, 15, 18). Our results obtained from isometric DSM tension recordings showed a concentration-dependent increase in DSM spontaneous phasic contraction amplitude and muscle force integral induced by the H$_2$S donor, GYY4137 (Figs. 1 & 2). In agreement with our data, others have shown that low concentrations of H$_2$S significantly increase the smooth muscle contractions in the gastrointestinal tract (13, 53) and coronary vessels (40).

In the present study, we used a multi-level approach spanning the molecular, cellular, and tissue levels to reveal the precise mechanism of H$_2$S-induced DSM contractions. The various techniques and approaches have different advantages and disadvantages. For example, the isometric DSM tension recordings and ACh release measurements were conducted at physiological temperature (~37ºC) whereas the patch-clamp electrophysiology and Ca$^{2+}$-imaging experiments were done at room temperature (~21ºC). At physiological pH (~7.4) and physiological temperature (~37ºC), 1 mM GYY4137 releases ~4 µM of H$_2$S in vitro (30).

Increasing evidence for the role of H$_2$S as a neuromodulator has emerged in literature
In our study, the neuronal voltage-gated Na$^+$ channel blocker, TTX, reduced the ability of GYY4137 to stimulate DSM spontaneous phasic contractions (Fig. 1). Our results are in line with previous observations showing that TTX partially reduces the contractile effect of NaHS in the rat urinary bladder (37). This suggests a possible presynaptic effect of H$_2$S in the regulation of the neurotransmitter release, which contributes to the increase in DSM spontaneous phasic contractions.

The relative contributions of cholinergic and purinergic pathways to DSM voiding-contractions appear to vary depending on the species and disease states under pathophysiological conditions (2). ACh is considered to play the primary excitatory role for physiological bladder contraction (7, 34). In our study, the blockade of mAChRs with atropine significantly reduced the stimulatory effect of GYY4137 on DSM spontaneous phasic contractions suggesting that H$_2$S increases DSM contractility in a mAChR-dependent manner (Fig. 2). Furthermore, our data demonstrate that GYY4137 increases the amplitude and muscle force of the nerve-evoked DSM contractions (Fig. 3). Consistently, in the presence of atropine, GYY4137 did not produce any significant change on the nerve-evoked purinergic DSM contractions. We further provided direct evidence that GYY4137 facilitated the neuronal ACh release in a concentration-dependent manner (Fig. 4). Collectively, our findings suggest that H$_2$S increases the ACh release from bladder nerve terminals, leading to enhancement of DSM contractions. In agreement with our data, others have reported that H$_2$S increases ACh release in frog neuromuscular junctions (43), and in the central preganglionic nerve terminals of gastrointestinal smooth muscle from mouse (42).

In the synaptic junctions of the urinary bladder nerves, neuronal L-type Cav channel activation play a key role facilitating cholinergic neurotransmission (45). In the current study, we used nifedipine, which by blocking the extracellular Ca$^{2+}$ influx in the bladder nerve terminals, decreased the ability of GYY4137 to induce neuronal ACh release (Fig. 4). This
indicates that \( \text{H}_2\text{S} \) promotes neuronal ACh release in a \( \text{Ca}^{2+} \)-dependent manner. Similarly, previous studies have demonstrated that \( \text{H}_2\text{S} \) raises ACh release in a \( \text{Ca}^{2+} \)-dependent manner in type I glomus cells (31), in neurons (12, 52), astrocytes (33) and microglia (29).

In the urinary bladder, the activation of mAChRs by ACh depolarizes the DSM cell membrane potential, and so promotes the generation of \( \text{Ca}^{2+} \) transients leading to overall enhancement of DSM contractility (17, 18, 54). Recently, it has been shown that in DSM cells type-3 mAChRs are functionally coupled to the BK channels, which mediate the mAChR-induced membrane depolarization (35, 36). Our results obtained from \( \text{Ca}^{2+} \) imaging demonstrate that GYY4137 increased the basal intracellular \( \text{Ca}^{2+} \) concentrations and the amplitude of \( \text{Ca}^{2+} \) transient in freshly-isolated DSM strips (Fig. 5).

Since part of the GYY4137-induced DSM contractions is TTX- and atropine-insensitive, we further investigated the possible post-synaptic effects of GYY4137 on DSM contractility. \( \text{H}_2\text{S} \) has been reported to inhibit whole cell BK channel currents in type I glomus cells of the carotid body of mice, rats, and humans (31, 46). BK channels that are highly expressed in DSM cells, but not in the bladder innervating neurons (49), are critical regulators of the DSM function (38, 39). NS11021 is a potent and specific BK channel opener that is very selective for the channel at concentrations <10 µM. Pharmacological activation of BK channels with NS11021 has been previously shown to decrease DSM spontaneous phasic contractions (28). Our results demonstrated that GYY4137 significantly attenuates the inhibitory effects of the BK channel opener NS11021 on DSM contractility (Fig. 6). As the effects of NS11021 were mitigated by GYY4137 within the range of concentrations selective for the BK channel, this supports a modulatory role for these channels in the response induced by \( \text{H}_2\text{S} \).

Recent evidence suggests that mAChRs are linked to BK channels to enhance DSM excitability (35, 36). Specifically, activation of mAChRs with carbachol leads to an inhibition
of the spontaneous transient hyperpolarizations and depolarizes DSM cell membrane potential via a Ca\(^{2+}\)-dependent mechanism (35, 36). To further examine the mechanism involved in the effects of H\(_2\)S on BK channels activity, we performed patch-clamp electrophysiology on freshly-isolated DSM cells. This approach helped us to separate the H\(_2\)S stimulatory effects caused by neuronal ACh release from the direct DSM effects involving BK channel inhibition.

Our patch-clamp data on single DSM cells revealed that GYY4137 decreases the TBKCs amplitude and frequency (Fig. 7). This further suggests a modulatory role for the BK channels in the cellular mechanisms of GYY4137-induced DSM contractions. TBKCs are mediated solely by the BK channels and are generated by “Ca\(^{2+}\) sparks”, rapid and localized Ca\(^{2+}\) releases from the ryanodine receptors of the sarcoplasmic reticulum (19, 20, 39). The inhibition of TBKCs leads to cell membrane potential depolarization and subsequent activation of L-type Ca\(_V\) channels in DSM (38, 39). In comparison, BK channel pharmacological inhibition with iberiotoxin or paxilline suppresses the TBKCs, depolarizes the DSM resting membrane potential in humans (21, 50), guinea pigs (19), and rats (23), thus causing activation of L-type Ca\(_V\) channels and DSM contractions.

To assess the potential direct interactions between H\(_2\)S and BK channels, we further studied the effects of GYY4137 on single BK channel activity in excised patches from DSM cells. Our single BK channel recordings demonstrated that GYY4137 reduced the BK channel open probability, suggesting a direct modulation of the BK channel by H\(_2\)S (Fig. 8). These results are in agreement with those reported in glomus cells of rats and humans, where BK channel activity is acutely inhibited by H\(_2\)S in single channel recordings from excised membrane patches using the inside-out configuration (46). We further examined the effects of GYY4137 on the membrane potential (Fig. 9). Our results demonstrated that GYY4137 depolarizes the DSM cell membrane potential and decreased the amplitude and frequency of
the spontaneous transient hyperpolarizations, which are known to be mediated by the BK channels (38, 39). Because the BK channel is considered to be the most important and physiologically-relevant channel controlling DSM function (38, 39), small changes in BK channel activity are linked to significant changes in DSM contractility as observed in the present study. This further supports a direct inhibitory effect of H2S on BK channel activity. Indeed, our results agree with previous reports showing that H2S directly inhibits BK channels in various types of cells treated with NaHS or gas-bubbled solutions containing dissolved H2S gas (31, 46). Contrary to our findings, other study showed that H2S augments whole cell currents in mouse tracheal smooth muscle (25) and increases BK channels open probability in rat pituitary tumor cells (44). The H2S effects have been demonstrated to be cell/tissue specific (47). Thus, potential differential regulatory H2S effects on BK channel activity can be explained by differences in BK channel splice variants, differential BK channel regulatory mechanisms, or BK channel-indirect mechanisms (38, 47).

Since in the cytosol H2S is able to dissociate to HS−, it is not clear if H2S and/or HS− are the effective agents. However, it has been shown that the negatively charged C-terminal site of the BK channel could prevent the binding of HS− (5), thus supporting H2S as a direct acting molecule on the BK channel activity.

In summary, our results provide evidence for a novel regulatory pathway in DSM whereby H2S promotes neuronal ACh release via mechanisms dependent on neuronal Ca2+ influx, and a direct inhibition of DSM BK channels. Both mechanisms work in concert to enhance DSM excitability and contractility (Fig. 10).
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Figure Legend

Figure 1. GYY4137 increased spontaneous phasic contractions in freshly-isolated DSM strips, an effect that was significantly inhibited by TTX. (A) Original isometric DSM tension recordings showing that the neuronal voltage-gated Na⁺ channel inhibitor, TTX (1 µM), decreased the stimulatory effects of GYY4137 on DSM spontaneous phasic contractions. (B & C) Summary data indicating that TTX (1 µM) decreased the GYY4137-induced DSM spontaneous phasic contraction amplitude (B) and muscle force integral (C); (n=12, N=8, in the absence of TTX; n=7, N=4 in the presence of TTX; asterisks indicate unpaired Student's t-test: *P<0.05, **P<0.01, GYY4137 vs. GYY4137 + TTX).

Figure 2. Pharmacological inhibition of mAChR with atropine led to a decrease in the stimulatory effect of GYY4137 on DSM spontaneous phasic contractions. (A) Original isometric DSM tension recordings showing that the mAChR inhibitor, atropine (1 µM), reduced the ability of GYY4137 to enhance DSM spontaneous phasic contractions. (B & C) Summary data indicating that atropine (1 µM) decreased the stimulatory effect of GYY4137 on DSM spontaneous phasic contraction amplitude (B), and muscle force integral (C); (n=12, N=8, in the absence of atropine; n=7, N=4, in the presence of atropine; asterisks indicate unpaired Student's t-test: *P<0.05, **P<0.01; GYY4137 vs. GYY4137 + atropine).

Figure 3. GYY4137 increased the nerve-evoked DSM contractions. (A) Original isometric DSM tension recordings showing that GYY4137 (3 µM) increased the EFS-induced contractions. (B & C) Summary data indicating that GYY4137 (3 µM) increased the nerve-evoked DSM contraction amplitude (B) and muscle force integral (C); (n=8, N=5; asterisks indicate paired Student's t-test: *P<0.05, **P<0.01, ***P<0.001; EFS vs. EFS + GYY4137).
Summary data showing a lack of GYY4137 effect on the purinergic component of the nerve-evoked DSM contractions amplitude (D), and muscle force integral (E); (n=6, N=4; asterisks indicate paired Student's t-test: *P<0.05, **P<0.01, ***P<0.001, control vs. atropine).

Figure 4. GYY4137 promoted neuronal ACh release. Summary data showing that in the absence of stimulation by GYY4137, the rate of ACh release did not change during the experimental time frame (time controls) (n=5, N=5). GYY4137 increased the ACh release in DSM strips in a concentration-dependent manner (n=5, N=5; asterisks indicate one-way ANOVA: **P<0.01, ***P<0.001; time control vs GYY4137). Nifedipine (1 µM), an L-type CaV channel blocker, reduced the GYY4137-induced ACh release; (n=5, N=5; the number sign indicate one-way ANOVA: #P<0.05, ##P<0.01, ###P<0.001; GYY4137 vs. GYY4137 + nifedipine).

Figure 5. GYY4137 increased spontaneous Ca^{2+} transients and basal Ca^{2+} levels in freshly-isolated DSM strips. (A) Sequence of Ca^{2+} images at intervals of 0.6 s showing Ca^{2+} transients generated within a DSM strip before and after application of GYY4137 (3 µM). (B) An original recording of spontaneous Ca^{2+} transients from freshly-isolated DSM strips illustrating that GYY4137 (3 µM) increased the amplitude of the Ca^{2+} transients and basal intracellular Ca^{2+} levels. (a & b) Insets depicting Ca^{2+} transients on an expanded time scale before (a), and after application of 3 µM GYY4137 (b). (C & D) Summary data indicating that GYY4137 (3 µM) increased the spontaneous Ca^{2+} transient amplitude (C), and basal intracellular Ca^{2+} levels (D); (12 traces; n=6, N=6; asterisks indicate paired Student's t-test: **P<0.01, control vs. GYY4137). GYY = GYY4137; ns = non-significant.
**Figure 6.** GYY4137 reduced the relaxation effects of NS11021, a BK channel selective opener, on the DSM spontaneous phasic contraction. (A) Original isometric DSM tension recordings showing the relaxation effects of NS11021 (100 nM-30 µM) on the spontaneous phasic contractions of DSM isolated strips in the absence or presence of 3 µM GYY4137. (B & C) Summary data indicating that GYY4137 (3 µM) reduced the relaxation effects of NS11021 on spontaneous phasic contraction amplitude (B) and muscle force integral (C) of DSM strips; (n=8, N=5 in the absence of GYY4137; n=8, N=5 in the presence of GYY4137; asterisks indicate unpaired Student's t-test: *P<0.05, **P<0.01; NS11021 vs. NS11021 + GYY4137).

**Figure 7.** GYY4137 decreased TBKC activity in freshly-isolated DSM cells. (A) An original recording showing that GYY4137 (3 µM) decreased the amplitude and frequency of TBKCs in a single freshly-isolated DSM cell. Insets depicting TBKCs on an expanded time scale before and after GYY4137 (3 µM) application. (B) Summary data showing that GYY4137 (3 µM) significantly decreased the amplitude and frequency of TBKCs; (n=7, N=7; asterisks indicate paired Student's t-test: *P<0.05, **P<0.01, control vs. GYY4137).

**Figure 8.** GYY4137 decreased single BK channel open probability in excised patches from freshly-isolated DSM cells. (A) An original single BK channel recording in an excised patch using inside-out configuration at +60 mV. (B & C) Summary data illustrating a significant decrease in the channel open probability following application of 3 µM GYY4137 (B), and the lack of effect on single BK channel current amplitude (C); (n=7, N=7; asterisks indicate paired Student's t-test: **P<0.01, control vs. GYY4137). GYY = GYY4137.

**Figure 9.** GYY4137 depolarized DSM cells membrane potential. (A) An original current-
clamp recording illustrating that GYY4137 (3 μM) decreases the spontaneous transient
hyperpolarizations and depolarizes DSM cell membrane potential. Portions of the recording
are shown on an expanded time scale showing the control and the decrease in the amplitude
and frequency of the spontaneous transient hyperpolarizations following GYY4137
application. (B) Summary data showing that GYY4137 (3 μM) significantly depolarizes
DSM cell membrane potential; (n=9, N=9; asterisks indicate paired Student’s t-test:
**P<0.01, control vs. GYY4137). GYY = GYY4137; ns = non-significant.

Figure 10. Proposed novel signalling pathway by which H₂S induces DSM phasic
contractions. H₂S promotes neuronal ACh release from bladder nerve endings, in a Ca²⁺-
dependent manner. In parallel, H₂S inhibits BK channels directly, and thus depolarizes the
DSM cell membrane potential causing an increase in the basal Ca²⁺ levels and the amplitude
of the Ca²⁺ transients. Collectively, this leads to an increase in DSM phasic contractions.
Figure 1

A

B

C

GYY4137 (log[M])

TTX (1 μM)

GYY4137 (log[M])

Muscle Force (%)

Amplitude (%)

GY4137

TTX + GYY4137

TTX + GYY4137

TTX + GYY4137

GY4137

GY4137

0 150 300 450 600

0 150 300 450 600

0 150 300 450 600

-10 -9 -8 -7 -6 -5

-10 -9 -8 -7 -6 -5

-10 -9 -8 -7 -6 -5

5 mN

10 min

5 mN

10 min

5 mN

10 min

* ********

* ** **

* * * ** *

GYY4137 log[M]

GYY4137 log[M]

GYY4137 log[M]
Figure 2

**A**

Atropine (1 μM)

GYY4137 (log[M])

5 mN

10 min

**B**

GYY4137

Atropine + GYY4137

Amplitude (%)

GYY4137 log[M]

**C**

GYY4137

Atropine + GYY4137

Muscle Force (%)

GYY4137 log[M]
Figure 3

A

GYY4137 (3 μM)

B

Control

GYY4137

Amplitude (%)

EFS (Hz)

C

Control

GYY4137

Muscle Force (%)

EFS (Hz)

D

Control

Atropine

Atropine + GYY4137

Amplitude (%)

EFS (Hz)

E

Control

Atropine

Atropine + GYY4137

Muscle Force (%)

EFS (Hz)
Figure 4

The graph shows the concentration of [ACh] nmol/ml over time for different concentrations of [GY4137] and [Nifedipine (1 μM) + GYY4137]. The x-axis represents time in minutes (0, 20, 40, 60, 80) and the y-axis represents [ACh] nmol/ml. The bars indicate significant differences compared to the Time Control group, with *** for P < 0.001, ** for P < 0.01, # for P < 0.05, and ns for not significant.
Figure 5

A

Control

GYY4137 (3 μM)

B

GYY4137 (3 μM)

C

Ca^{2+} Trans. Amp. (F_{340}/F_{380})

Control GYY (3 μM)

D

Basal Ca^{2+} Level (F_{340}/F_{380})

Control GYY (3 μM)
Figure 6

A

NS11021 (log[M])

GYY4137 (3 μM)

NS11021 (log[M])

Muscle Force (%)

B

NS11021

GYY4137 + NS11021

Amplitude (%)

C

NS11021

GYY4137 + NS11021

Muscle Force (%)

A

B

C
Figure 7

A

GYY4137 (3 μM)

B

TBKCs (%)

0 20 40 60 80 100

Amplitude Frequency

* **
Figure 8

A

Control

GYY4137 (3 μM)

B

C

NPO (%)

GYY (3 μM)

Single channel current amplitude (pA)

Control GYY (3 μM)

0 3 6 9 12 15

0 5 pA

4 s

ns

**
Figure 9