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Hydrogen sulfide activates Ca^{2+} sparks to induce cerebral arteriole dilatation

Guo Hua Liang, Qi Xi, Charles W. Leffler and Jonathan H. Jaggar

Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

Key points

- Hydrogen sulfide (H_2S), a gas produced by endothelial cells, relaxes smooth muscle cells within the vascular wall to increase organ blood flow and lower systemic blood pressure.
- Mechanisms by which H_2S produces vasodilatation in the cerebral circulation are unclear.
- We demonstrate that H_2S increases the quantity of calcium ions (Ca^{2+}) contained with the sarcoplasmic reticulum (SR), the intracellular Ca^{2+} store, of cerebral arteriole smooth muscle cells.
- This elevation in SR Ca^{2+} stimulates the generation of local intracellular Ca^{2+} signals called Ca^{2+} sparks, which in turn activate Ca^{2+}-sensitive potassium (K_{Ca}) channels on the cell membrane, leading to membrane hyperpolarization and vasodilatation.
- Elucidating this novel mechanism of H_2S-induced vasodilatation is important to better understand physiological control of blood flow within the brain.

Abstract Hydrogen sulfide (H_2S) is a gaseous vasodilator produced by endothelial cells. Mechanisms by which H_2S induces vasodilatation are unclear. We tested the hypothesis that H_2S dilates cerebral arterioles by modulating local and global intracellular Ca^{2+} signals in smooth muscle cells. High-speed confocal imaging revealed that Na_2S, an H_2S donor, increased Ca^{2+} spark frequency ~1.43-fold and decreased global intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by ~37 nM in smooth muscle cells of intact piglet cerebral arterioles. In contrast, H_2S did not alter Ca^{2+} wave frequency. In voltage-clamped (−40 mV) cells, H_2S increased the frequency of iberiotoxin-sensitive, Ca^{2+} spark-induced transient Ca^{2+}-activated K^+ (K_{Ca}) currents ~1.83-fold, but did not alter the amplitude of these events. H_2S did not alter the activity of single K_{Ca} channels recorded in the absence of Ca^{2+} sparks in arteriole smooth muscle cells. H_2S increased SR Ca^{2+} load ($[Ca^{2+}]_{SR}$), measured as caffeine (10 and 20 mM)-induced $[Ca^{2+}]_i$ transients, ~1.5-fold. H_2S hyperpolarized (by ~18 mV) and dilated pressurized (40 mmHg) cerebral arterioles. Iberiotoxin, a K_{Ca} channel blocker, reduced H_2S-induced hyperpolarization by ~51%. Iberiotoxin and ryanodine, a ryanodine receptor channel inhibitor, reduced H_2S-induced vasodilatation by ~38 and ~37%, respectively. In summary, our data indicate that H_2S elevates $[Ca^{2+}]_{SR}$, leading to Ca^{2+} spark activation in cerebral arteriole smooth muscle cells. The subsequent elevation in transient K_{Ca} current frequency leads to membrane hyperpolarization, a reduction in global $[Ca^{2+}]_i$ and vasodilatation.

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Corresponding author J. H. Jaggar: Department of Physiology, University of Tennessee Health Science Centre, Memphis, TN 38163, USA. Email: jjaggar@uthsc.edu

Abbreviations RyR, ryanodine receptor; SERCA, SR Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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Introduction

Hydrogen sulfide (H_2S), a physiological gasotransmitter, is generated in mammalian cells through the metabolism of L-cysteine by cystathionine β-synthase and cystathionine γ-lyase (Porter *et al*. 1974; Allsop & Watts, 1975; Wang, 2002). H_2S modulates blood flow, blood pressure, synaptic neurotransmission, immune response, hormone secretion and muscle relaxation (Fiorucci *et al.* 2005; Leffler *et al.* 2006; Austgen *et al.* 2011). H_2S induces vasodilatation in many different vascular beds, including rat mesenteric arteries, aorta, tibial arteries and piglet cerebral arterioles (Cheng *et al.* 2004; Liu & Bian, 2010; Schleifenbaum *et al.* 2010; Leffler *et al.* 2011; Liang *et al.* 2011). Several vascular ion channels have been reported to be involved in H_2S-induced vasodilatation, including ATP-sensitive K^+ (K_{ATP}) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, KCNQ channels and L-type Ca^{2+} channels (Cheng *et al.* 2004; Dombkowski *et al.* 2004; Schleifenbaum *et al.* 2010; Leffler *et al.* 2011; Zuidema *et al.* 2010; Liang *et al.* 2011). However, smooth muscle cell ion channels that are specifically targeted by H_2S are unclear as are the mechanisms by which H_2S modulates these proteins.

In smooth muscle cells, ion channels generate and regulate local and global intracellular Ca^{2+} signals, which can control vascular contractility (Jaggar *et al.* 2000). Conversely, local and global intracellular Ca^{2+} signals can regulate the activity of plasma membrane ion channels, which feed back to modify local and global intracellular Ca^{2+} signalling (Jaggar *et al.* 2000). The regulation of local and global Ca^{2+} signals by H_2S in vascular smooth muscle cells is unclear. Establishing such regulation may reveal mechanisms by which this gaseous vasodilator controls vascular contractility.

Three primary Ca^{2+} signals occur in arterial smooth muscle cells, termed Ca^{2+} sparks, Ca^{2+} waves and global $[Ca^{2+}]_i$ (Jaggar *et al.* 2000). Ca^{2+} sparks occur due to the concerted opening of multiple sarcoplasmic reticulum (SR) ryanodine receptor (RyR) channels (Nelson *et al.* 1995; Jaggar *et al.* 2000). Ca^{2+} sparks activate nearby plasma membrane K_{Ca} channels, leading to transient K_{Ca} currents that hyperpolarize the membrane potential. Membrane hyperpolarization reduces voltage-dependent Ca^{2+} channel activity, leading to a reduction in global intracellular Ca^{2+} concentration and vasodilatation (Nelson *et al.* 1995). Ca^{2+} waves are propagating SR Ca^{2+} release events that occur due to the activation of SR inositol trisphosphate-gated Ca^{2+} release channels and RyR channels (Jaggar, 2007). Global $[Ca^{2+}]_i$ is the spatially homogeneous $[Ca^{2+}]_i$ to which plasma membrane Ca^{2+} influx and SR Ca^{2+} release can contribute (Jaggar *et al.* 2000). Voltage-dependent L-type Ca^{2+} ($Ca_V1.2$) channels are a major contributor to global $[Ca^{2+}]_i$ (Jaggar *et al.* 2000). Local Ca^{2+} gradients, termed Ca^{2+} sparklets, are generated by the opening of voltage-dependent Ca^{2+} channels and contribute to global $[Ca^{2+}]_i$ (Santana & Navedo, 2009).

Here, we investigated the regulation of local and global $[Ca^{2+}]_i$ signals by H_2S in cerebral arteriole smooth muscle cells, the underlying mechanisms, and the involvement in H_2S-induced cerebral arteriole dilatation. Data indicate that H_2S elevates $[Ca^{2+}]_{SR}$, which stimulates Ca^{2+} sparks, leading to an increase in transient K_{Ca} current frequency, a reduction in global $[Ca^{2+}]_i$ and vasodilatation. In contrast, H_2S did not alter the activity of Ca^{2+} waves or directly regulate single K_{Ca} channels. These data define a novel mechanism of action of H_2S and indicate that an elevation in SR Ca^{2+} load ($[Ca^{2+}]_{SR}$)is a specific mechanism of vasodilatation induced by this gasotransmitter.

Methods

Tissue preparation

All procedures used were approved by the University of Tennessee Health Science Center Animal Care and Use Committee. The authors have read, and the experiments comply with the policies and regulations of *The Journal of Physiology* given by Drummond (2009). Newborn pigs (1–3 days old, 1–2.5 kg body weight) were anaesthetized with ketamine hydrochloride (33 mg kg^{-1} I.M.) and acepromazine (3.3 mg kg^{-1} I.M.). The brain was removed and maintained in ice-cold (4°C) Hepes-buffered physiological saline solution (PSS) containing (in mM): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, and 10 glucose, with pH adjusted to 7.4 with NaOH. Isolated arterioles (50–200 μm) were dissected from the brain and cleaned to remove basolateral connective tissue. Individual smooth muscle cells were dissociated from cerebral arterioles using a procedure similar to that previously described (Jaggar, 2001). Arterioles were endothelium denuded by intraluminal injection of air for 1 min, as previously described (Adebiyi *et al.* 2008). Unless stated otherwise, all procedures were performed at room temperature (22–25°C).

Confocal Ca^{2+} imaging

Piglet cerebral arterioles (~2 mm in length) were placed in Hepes-buffered PSS containing 10 μM fluo-4 AM and 0.05% Pluronic F-127 for 20 min at room temperature followed by a 30 min wash with Hepes-buffered PSS to allow indicator deesterification. Bath solution that contained (in mM): 110 NaCl, 30 KCl, 10 Hepes, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (adjusted to pH 7.4 with NaOH) was used to depolarize arteries to approximately −40 mV, as previously described (Jaggar, 2001). Fluorescence images were collected using a Noran OZ laser-scanning confocal microscope (Noran Instruments, Middleton, WI,

USA) and a ×60 water immersion objective (1.2 NA) attached to a TE300 microscope (Nikon), as described previously (Jaggar, 2001). Fluo-4 AM was illuminated at 488 nm using a krypton–argon laser, and emitted light >500 nm was captured. Images (56.3 × 52.8 μm) were recorded every 16.67 ms (i.e. 60 images per second). Custom analysis software (kindly provided by Dr M. T. Nelson, University of Vermont) was used to detect Ca^{2+} signals in smooth muscle cells. For detection of Ca^{2+} sparks, an area 1.54 × 1.54 μm (7 × 7 pixels, i.e. 2.37 μm^2) in each image (F) was divided by a baseline (F_0) that was determined by averaging 10 images without Ca^{2+} spark activity. The entire image area was analysed to detect Ca^{2+} sparks. A Ca^{2+} spark was identified as a local increase in F/F_0 that was >1.2. Arterial Ca^{2+} spark frequency (measured in Hz) was calculated by averaging values from at least two different areas of the same arteriole. Ca^{2+} waves were defined as an elevation in F/F_0 >1.2 that propagated for at least 20 μm. Ca^{2+} waves were detected by placing 2.2 × 2.2 μm (10 × 10 pixels, i.e. 4.84 μm^2) in each smooth muscle cell and by using a method similar to that for Ca^{2+} spark detection. Changes in local or global $[Ca^{2+}]_i$ were calculated using the pseudoratio method: $[Ca^{2+}] = KR/(K/([Ca^{2+}]_{rest} + 1 - R))$. K is the apparent affinity of fluo-4 AM for Ca^{2+} (770 nM; Woodruff *et al.* 2002), R is the fractional fluorescence increase (F/F_0), and $[Ca^{2+}]_{rest}$ is $[Ca^{2+}]_i$ at F_0. $[Ca^{2+}]_{rest}$ was 224 nM, as previously determined by ratiometric imaging of fura-2 in newborn cerebral arterioles (Li *et al.* 2006). Global $[Ca^{2+}]_i$ fluorescence was calculated from the same images used for Ca^{2+} spark and wave analysis and was the mean pixel value of 100 different images acquired during a 10 s period (Cheranov & Jaggar, 2004; Xi *et al.* 2005; Cheranov & Jaggar, 2006).

Patch-clamp electrophysiology

Isolated smooth muscle cells were allowed to adhere to a glass coverslip in the bottom of a chamber for 10 min before experimentation. K^+ currents were measured using the amphotericin B perforated-patch configuration with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). The bath solution was Hepes-buffered PSS, and the pipette solution contained (in mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 Hepes, and 0.05 EGTA, with pH adjusted to 7.2 using KOH. Membrane currents were filtered at 1 kHz and digitized at 4 kHz. In each patch under each condition, transient K_{Ca} current frequency and amplitude or K_{Ca} channel activity was calculated from at least 5 min of continuous gap-free data.

Sarcoplasmic reticulum Ca^{2+} load measurements

$[Ca^{2+}]_{SR}$ was estimated by measuring the amplitude of $[Ca^{2+}]_i$ transients induced by caffeine (10 or 20 mM), a RyR channel activator (Cheranov & Jaggar, 2002, 2004; Xi *et al.* 2005). Endothelium-denuded arterioles were incubated in Hepes-buffered PSS containing 5 μM fura-2 AM and 0.1% Pluronic F-127 for 30 min at room temperature. After wash, arteries were allowed to de-esterify the indicator for 15 min. Fura-2 AM was alternately excited with 340 or 380 nm light using a xenon arc lamp and a personal computer-driven hyperswitch (Ionoptix, Milton, MA, USA). Background corrected ratios were collected every 1 s at 510 nm using a Dage MTI integrating CCD camera (IonOptix).

Pressurized artery diameter measurements

A cerebral arteriole segment ∼2 mm in length was cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation, Burlington, VT, USA). The chamber was continuously perfused with PSS of the following composition (mM): 112 NaCl, 4.8 KCl, 24 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 10 glucose, equilibrated with a mixture of 21% O_2, 5% CO_2 and 74% N_2, and maintained at 35°C. Steady-state changes in intravascular pressure were achieved by elevating and lowering an attached reservoir and monitored using a pressure transducer. Intraluminal PSS was static during experiments. Arterioles were observed with a charge-coupled device camera attached to an inverted microscope (Nikon TS 100). Arteriole diameter was measured by using the automatic edge-detection function of IonWizard software (Ionoptix, Milton, MA, USA) and digitized at 1 Hz using a personal computer. Tested compounds were applied via chamber perfusion to maintain constant concentration during experiments.

Pressurized artery membrane potential measurements

Arterioles were maintained at 40 mmHg for 2 h to ensure steady-state myogenic tone had occurred prior to obtaining membrane potential recordings. Membrane potential was measured by impaling arterioles with glass microelectrodes filled with 3 M KCl (50–90 mΩ) from the adventitial side. Membrane potential was recorded using a WPI FD223 amplifier, pCLAMP 9.2 software (Molecular Devices) and a personal computer. Successful intracellular impalements required a rapid negative potential change upon insertion; a stable voltage for at least 25 s; a fast positive voltage deflection upon removal, and a <10% change in tip resistance after impalement.

Statistical analysis

Values are reported as means ± SEM; n refers to the number of events analysed, unless otherwise specified.

Student's *t* test was used for comparison of paired data, except for statistical analysis of Ca^{2+} spark frequency and amplitude and Ca^{2+} wave frequency, where a non-parametric Wilcoxon's matched pairs test was used. ANOVA with the Student–Newman–Keuls *post hoc* test was used for multiple group comparison. $P < 0.05$ was considered significant.

Chemicals

Fluo-4 AM, fura-2 AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). Iberiotoxin was purchased from California Peptide Research Inc. (Napa, CA, USA). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical (St Louis, MO, USA).

Results

H_2S activates Ca^{2+} sparks and reduces global $[Ca^{2+}]_i$ in smooth muscle cells of intact cerebral arterioles

The regulation of local and global Ca^{2+} signals by H_2S was measured in smooth muscle cells of piglet cerebral arteriole segments. For this study, we used Na_2S as an H_2S donor. Na_2S (10 μM) increased mean Ca^{2+} spark frequency from ~2.8 Hz to 4.0 Hz, or 1.43-fold (Fig. 1*A* and *B*). In contrast, H_2S did not change mean Ca^{2+} spark amplitude or Ca^{2+} wave frequency (Fig. 1*C* and *D*). H_2S decreased mean global F/F_0 by ~16%, which translates to a reduction in global intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ from 224 ± 29 nM (Li *et al.* 2006) to 187 ± 12 nM (Fig. 1*E*). These data indicate that H_2S elevates Ca^{2+} spark frequency and reduces global $[Ca^{2+}]_i$, but does not alter Ca^{2+} waves in cerebral arteriole smooth muscle cells.

H_2S activates transient K_{Ca} currents in isolated arteriole smooth muscle cells

Ca^{2+} sparks activate transient K_{Ca} currents in cerebral artery and arteriole smooth muscle cells (Jaggar *et al.* 2000, 2002). H_2S regulation of Ca^{2+} spark-induced transient K_{Ca} currents was measured in isolated cerebral arteriole smooth muscle cells using the perforated patch-clamp configuration. At a membrane potential of −40 mV, Na_2S (10 μM) increased mean transient K_{Ca} current frequency from ~0.23 to 0.42 Hz, or 1.83-fold (Fig. 2*A* and *B*). In contrast, Na_2S did not alter mean transient K_{Ca} current amplitude (Fig. 2*A* and *C*). To further investigate Na_2S regulation of transient K_{Ca} current amplitude, events were divided into small (<25 pA), medium (25–50 pA) and large (>50 pA) amplitude groups, as we have done previously (Li *et al.* 2008). Na_2S did not significantly alter the amplitude distribution of these groups (Fig. 2*A* and *D*). When applied in the presence of Na_2S, iberiotoxin, a selective K_{Ca} channel blocker, essentially abolished transient K_{Ca} currents, reducing mean frequency and amplitude to ~0.5 and 10.2% of that in Na_2S (Fig. 2*B* and *C*). These data indicate that H_2S-induced Ca^{2+} spark activation leads to an elevation in transient K_{Ca} current frequency, but

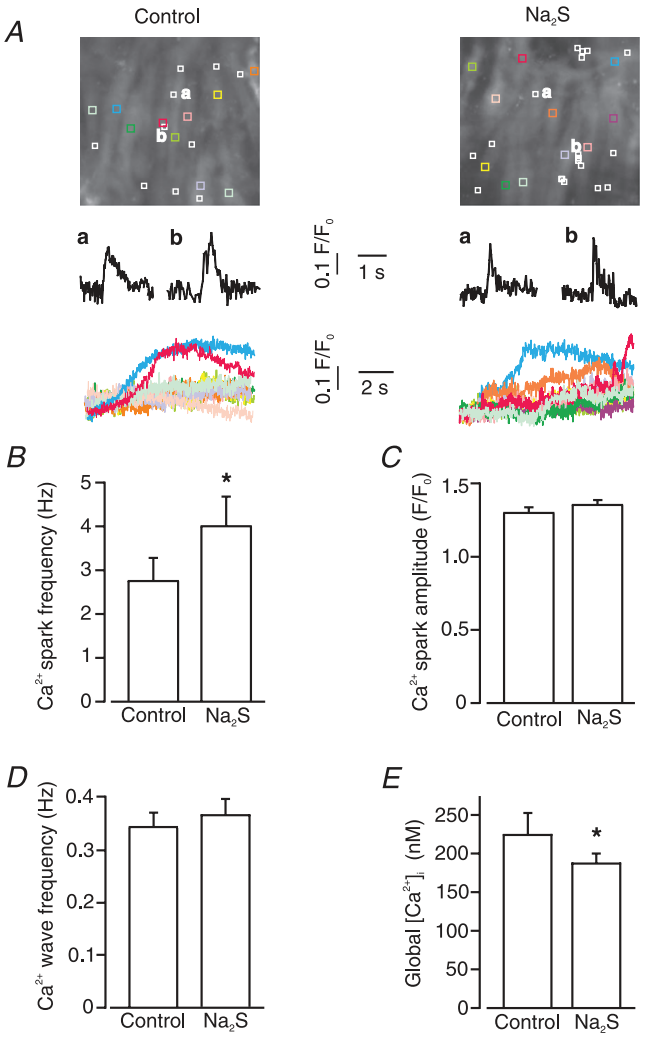

Figure 1. Hydrogen sulfide (H_2S) regulates local and global Ca^{2+} signals in cerebral arteriole smooth muscle cells
A, confocal images in upper panels illustrate average fluo-4 fluorescence in smooth muscle cells in the same arteriole in control and Na_2S (10 μM). White boxes illustrate locations where sparks occurred during 10 s of imaging. Coloured boxes illustrate locations from where corresponding normalized fluorescence (F/F_0) over time traces from each cell shown in the lower panels were determined. Middle panels show two representative Ca^{2+} sparks that occurred at locations labelled as *a* and *b* in each condition. Lower panels indicate intracellular Ca^{2+} changes that occurred in corresponding coloured boxes over time with Ca^{2+} waves evident in a proportion of cells. H_2S increased Ca^{2+} spark frequency (*B*), but did not change Ca^{2+} spark amplitude (*C*) ($n = 8$ for each condition). *D*, H_2S did not alter Ca^{2+} wave frequency ($n = 8$ for each condition). *E*, H_2S decreased global $[Ca^{2+}]_i$ from 224 ± 29 nM to 187 ± 12 nM ($n = 8$). *$P < 0.05$ *vs.* control.

does not alter transient K_{Ca} current amplitude, in cerebral arteriole smooth muscle cells.

H_2S does not directly activate K_{Ca} channels in cerebral arteriole smooth muscle cells

In piglet cerebral arteriole smooth muscle cells, a proportion (~40%) of Ca^{2+} sparks do not activate a transient K_{Ca} current (Jaggar *et al.* 2002). Carbon monoxide, a gasotransmitter that elevates K_{Ca} channel Ca^{2+} sensitivity induces coupling of K_{Ca} channels to these smaller amplitude Ca^{2+} sparks (Jaggar *et al.* 2002; Xi *et al.* 2004; Li *et al.* 2006). To investigate the possibility that H_2S stimulates transient K_{Ca} currents through an effect on K_{Ca} channels, the regulation of single K_{Ca} channels by H_2S was measured in isolated cerebral arteriole smooth muscle cells. Thapsigargin (100 nM), a SR Ca^{2+}-ATPase (SERCA) inhibitor, was used to deplete $[Ca^{2+}]_{SR}$ and abolish transient K_{Ca} currents. From a control activity (NP_o) of 0.35 ± 0.1, H_2S did not alter K_{Ca} channel activity ($95 \pm 17\%$ of control, $P > 0.05$; Fig. 3). These data indicate that H_2S does not activate K_{Ca} channels, further supporting other data here that H_2S activates K_{Ca} channels solely by elevating Ca^{2+} spark frequency.

Na_2S elevates sarcoplasmic reticulum Ca^{2+} load

$[Ca^{2+}]_{SR}$ regulates Ca^{2+} spark frequency (Wellman *et al.* 2001; Cheranov & Jaggar, 2002). Therefore, the regulation of $[Ca^{2+}]_{SR}$ by H_2S was studied by measuring the amplitude of caffeine-induced $[Ca^{2+}]_i$ transients in endothelium-denuded piglet cerebral arterioles. Caffeine (10 mM)-stimulated $[Ca^{2+}]_i$ transients under control conditions (Fig. 4*A* and *B*). Na_2S increased caffeine (10 mM)-induced $[Ca^{2+}]_i$ transients to $161 \pm 13\%$ (1st application) and $153 \pm 11\%$ (2nd application) of control ($n = 5$ arterioles, $P < 0.05$; Fig. 4). Conceivably, Na_2S may elevate caffeine-induced $[Ca^{2+}]_i$ transients by increasing RyR caffeine sensitivity. To investigate this possibility, experiments were repeated using a higher caffeine concentration (20 mM). Na_2S similarly increased caffeine (20 mM)-induced $[Ca^{2+}]_i$ transients to $149 \pm 14\%$ (1st

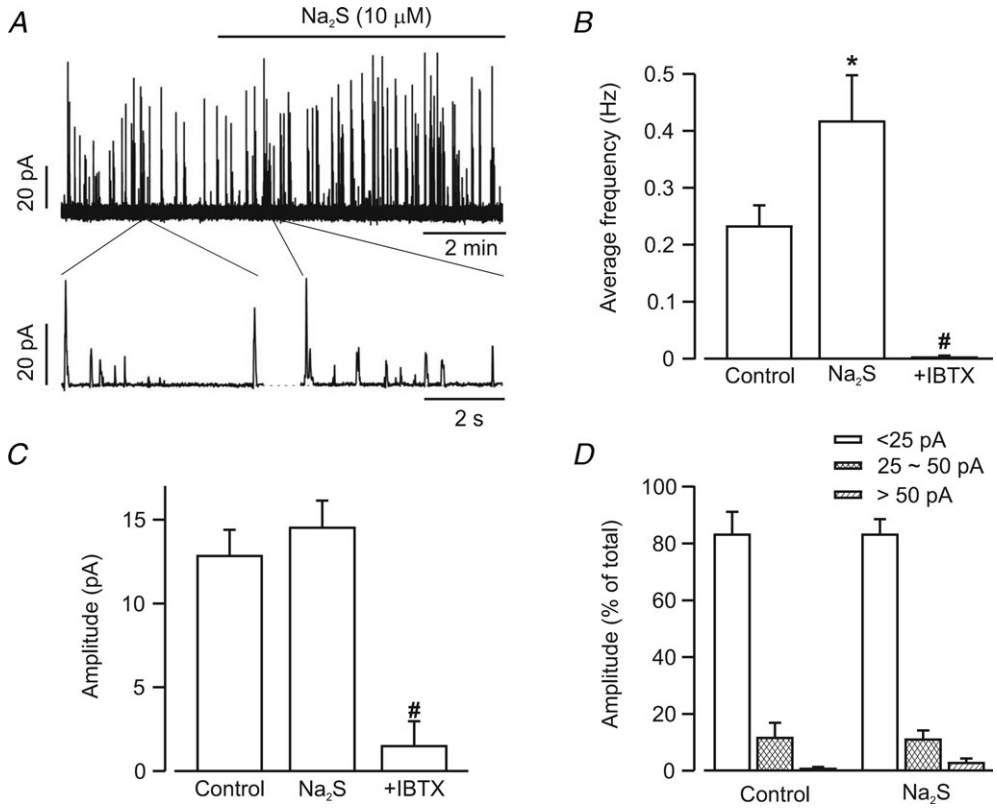

Figure 2. H_2S increases transient K_{Ca} current frequency in cerebral arteriole smooth muscle cells
A, representative recording illustrating Na_2S (10 μM) induced an elevation in transient K_{Ca} current frequency, but not amplitude. *B*, mean transient K_{Ca} current frequency in the same cells in control ($n = 11$ cells) and Na_2S (10 μM, $n = 11$ cells), and in Na_2S+iberiotoxin (IBTX, 100 nM, $n = 5$ cells). *C*, transient K_{Ca} current mean amplitude was not altered by Na_2S ($n = 11$ cells), but was reduced by iberiotoxin (IBTX, $n = 5$ cells). *D*, Na_2S did not change the proportion of transient K_{Ca} currents in each transient K_{Ca} current amplitude category ($n = 11$ cells for control and Na_2S). All currents were recorded at a steady holding potential of −40 mV. $^*P < 0.05$ *vs.* control. #$P < 0.05$ *vs.* Na_2S.

application) and 167 ± 12% (2nd application) of control ($n = 5$ arterioles, $P < 0.05$). Data were not significantly different when comparing the first and second caffeine applications at 10 and 20 mM ($P > 0.05$ for each). These data indicate that H_2S elevates $[Ca^{2+}]_{SR}$.

H_2S induces iberiotoxin-sensitive hyperpolarization in pressurized cerebral arterioles

To investigate the functional significance of H_2S-induced transient K_{Ca} current activation, the membrane potential of pressurized arterioles was measured using glass microelectrodes. At an intravascular pressure of 40 mmHg, mean arteriole membrane potential was ∼−30.3 mV (Fig. 5*A* and *B*). Na_2S (10 μM) hyperpolarized pressurized arterioles to ∼−47.9 mV, or by 17.6 mV (Fig. 5*A* and *B*). Application of iberiotoxin, a selective K_{Ca} channel blocker, in the presence of Na_2S returned mean membrane potential to ∼−39.0 mV, inhibiting the Na_2S-induced hyperpolarization by ∼51% (Fig. 5*A* and *B*).

H_2S dilates cerebral arterioles by activating RyR and K_{Ca} channels

Edge-detection myography was performed to study contractility regulation by H_2S and involvement of Ca^{2+} spark and K_{Ca} channel activation in pressurized arterioles. At 40 mmHg, arterioles constricted from a mean passive diameter of 231 ± 10 μm to a myogenic diameter of 132 ± 8 μm, or by 43% ($n = 11$). Na_2S (10 μM) increased mean arteriole diameter by ∼33 μm (Fig. 6*A* and *C*). Iberiotoxin (100 nM) or ryanodine, a RyR channel blocker (10 μM), did not alter the diameter of pressurized arterioles when applied alone (Fig. 6*B* and *D*). In contrast,

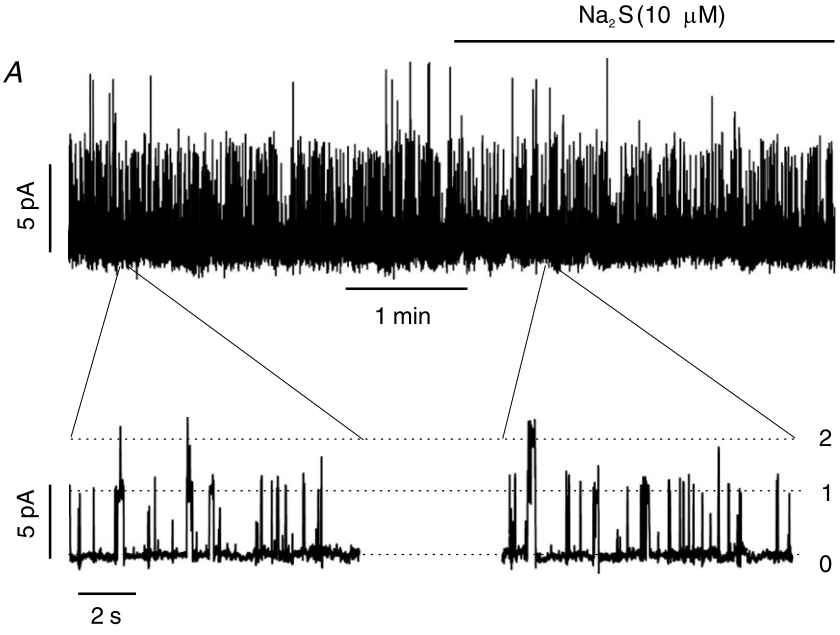

Figure 3. H_2S does not alter single K_{Ca} channel activity in intact arteriole smooth muscle cells
A, representative trace illustrating that H_2S did not change single K_{Ca} channel activity in an arteriole smooth muscle cell. *B*, mean data ($n = 6$ cells). All currents were recorded at a steady holding potential of 0 mV. Cells were pre-treated with thapsigargin (100 nM) to inhibit Ca^{2+} spark-induced transient K_{Ca} currents.

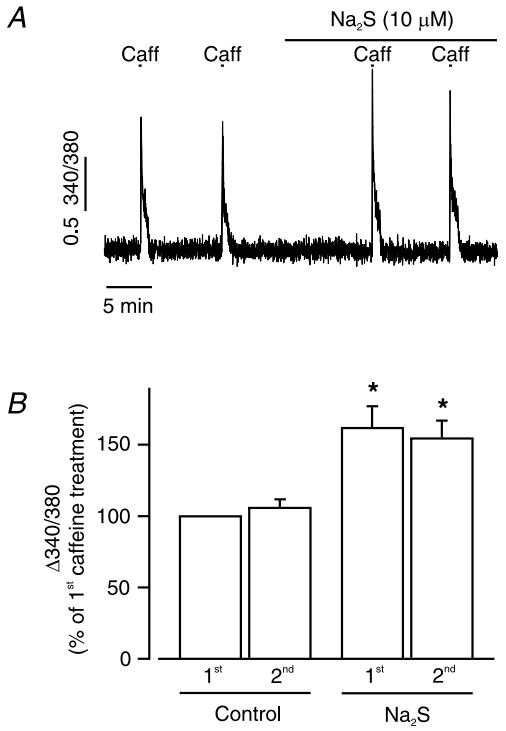

Figure 4. H_2S elevates $[Ca^{2+}]_{SR}$
A, original trace illustrating that Na_2S (10 μM) increased caffeine (Caff, 10 mM)-induced $[Ca^{2+}]_i$ transients in an isolated piglet cerebral arteriole. 340/380, 340 to 380 nm ratio. *B*, mean change in fura-2 AM ratio in two sequential control conditions and Na_2S ($n = 5$ arterioles). $^*P < 0.05$ *vs.* 1st caffeine-induced $[Ca^{2+}]_i$ transient.

iberiotoxin partially reversed Na_2S-induced vasodilatation from ~32 to 20 μm, or by 38% (Fig. 6*A* and *B*). Ryanodine also partially reversed Na_2S-induced vasodilatation from ~35 to 22 μm, or by 37% (Fig. 6*C* and *D*). These data indicate that H_2S-induced cerebral arteriole vasodilatation occurs in part via Ca^{2+} spark-induced K_{Ca} channel activation in piglet cerebral arteriole smooth muscle cells.

Discussion

Here, we investigated the regulation of local and global intracellular Ca^{2+} signals by H_2S in smooth muscle cells of piglet cerebral arterioles. Major findings of this study are that (1) H_2S activates Ca^{2+} sparks but not Ca^{2+} waves, and reduces global intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), (2) H_2S activation of Ca^{2+} sparks leads to an increase in transient K_{Ca} current frequency, (3) H_2S increases $[Ca^{2+}]_{SR}$, (4) H_2S does not directly activate K_{Ca} channels, (5) H_2S hyperpolarizes pressurized cerebral arterioles via K_{Ca} channel activation, and (6) H_2S dilates pressurized cerebral arterioles and this dilatation is partially reversed by RyR and K_{Ca} channel blockers. Collectively, these data indicate that H_2S elevates $[Ca^{2+}]_{SR}$, which stimulates Ca^{2+} sparks that increase transient K_{Ca} currents, leading to membrane hyperpolarization, a reduction in global $[Ca^{2+}]_i$ and vasodilatation.

H_2S is generated endogenously in a wide variety of mammalian tissues, including brain, liver, heart, aorta and kidney (Ubuka, 2002). Physiological concentrations of H_2S in rat and human blood are between 5 and 50 μM (Zhao *et al.* 2001; Ubuka, 2002; Li *et al.* 2005; Elsey *et al.* 2010). In piglet cerebrospinal fluid, we measured a mean H_2S concentration during stimulation with hypercapnia of approximately 4 μM (Leffler *et al.* 2011). Here, we used Na_2S, a commonly used H_2S donor, to elevate extracellular H_2S. Recent phase 1 human clinical trials have also used Na_2S as a potential therapeutic treatment for myocardial infarction (Li *et al.* 2009). Na_2S at 10 μM generates ~5 μM H_2S gas in physiological saline solution at room temperature within 1 min (Liang *et al.* 2011). Therefore, the Na_2S concentration studied here produces

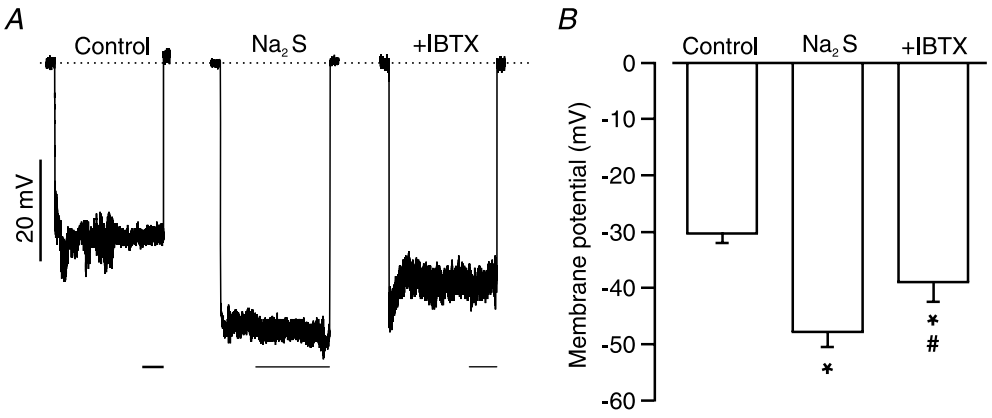

Figure 5. H_2S induces iberiotoxin-sensitive hyperpolarization in pressurized arterioles
A, original recordings of membrane potential upon microelectrode impalement in pressurized (40 mmHg) arterioles in control, Na_2S (10 μM) and Na_2S (10 μM) + iberiotoxin (100 nM). Dotted line indicates 0 mV. Scale bars are 20 s. *B*, mean data (control, $n = 6$; Na_2S, $n = 5$; Na_2S + iberiotoxin, $n = 5$). $^*P < 0.05$ when compared with control and # when compared with Na_2S.

H_2S concentrations within the physiological range. In donating H_2S, Na_2S contributes only micromolar Na^+ as an additional atom. Bath solutions used for our experiments contain 140 mM Na^+. A 10 μM Na^+ elevation will have insignificant effects on Ca^{2+} sparks, transient K_{Ca} currents and arteriole contractility. We have previously shown that Na_2S and NaHS, another H_2S donor, produce quantitatively similar K_{ATP} current activation in cerebral arteriole smooth muscle cells, indicating that these donors act through H_2S donation (Liang *et al.* 2011).

In arterial smooth muscle cells, a single Ca^{2+} spark activates multiple K_{Ca} channels, leading to a transient K_{Ca} current (Nelson *et al.* 1995). Asynchronous transient K_{Ca} currents hyperpolarize arterial membrane potential, thereby reducing voltage-dependent Ca^{2+} channel activity and $[Ca^{2+}]_i$, leading to vasodilatation (Jaggar *et al.* 2000). Several vasodilators, including those that activate adenylyl cyclase or soluble guanylyl cyclase, activate Ca^{2+} sparks (Porter *et al.* 1998; Cheranov & Jaggar, 2006; Mandala *et al.* 2007; Li *et al.* 2008). Such Ca^{2+} spark stimulation elevates transient K_{Ca} current frequency, leading to membrane hyperpolarization, a reduction in voltage-dependent Ca^{2+} channel activity, a decrease in $[Ca^{2+}]_i$, and vasodilatation (Jaggar & Nelson, 2000; Cheng & Lederer, 2008). Here, we provide the first direct evidence that H_2S activates Ca^{2+} sparks in arteriole smooth muscle cells. H_2S increased Ca^{2+} spark frequency, but did not alter Ca^{2+} spark amplitude. Consistent with this result, H_2S increased mean transient K_{Ca} current frequency, but did not alter mean amplitude. We have previously demonstrated that transient K_{Ca} current amplitude is not normally distributed in piglet cerebral arteriole smooth muscle cells (Li *et al.* 2008). Therefore, H_2S-induced changes in transient K_{Ca} current amplitude may not be revealed by comparing mean data. Previous studies from our laboratory indicated that although carbon monoxide (CO) elevates the effective coupling of K_{Ca} channels to Ca^{2+} sparks, astrocyte-derived CO did not alter transient K_{Ca} current mean amplitude (Jaggar *et al.* 2002; Xi *et al.* 2004; Li *et al.* 2008). Categorizing transient K_{Ca} currents into three amplitude groups indicated that CO increased the frequency of smaller amplitude transient K_{Ca} currents more than larger events, thereby depressing the mean amplitude (Li *et al.* 2008). The emergence of a new population of transient K_{Ca} currents occurs due to CO-induced coupling of previously uncoupled smaller amplitude Ca^{2+} sparks to K_{Ca} channels (Jaggar *et al.* 2002). Similar to our previous study, data here indicate that the largest proportion of transient K_{Ca} currents are smaller events (<25 pA) in cerebral arteriole smooth muscle cells (Li *et al.* 2008). In contrast to CO, which elevates K_{Ca} channel sensitivity to Ca^{2+} sparks (Jaggar *et al.* 2002; Li *et al.* 2008), H_2S did not alter transient K_{Ca} current amplitude distribution. These data indicate that H_2S does not elevate effective coupling of K_{Ca} channels to Ca^{2+} sparks. Consistent with a lack of effect of H_2S on transient K_{Ca} current mean amplitude or amplitude distribution, H_2S also did not alter single K_{Ca} channel activity in intact arteriole smooth muscle

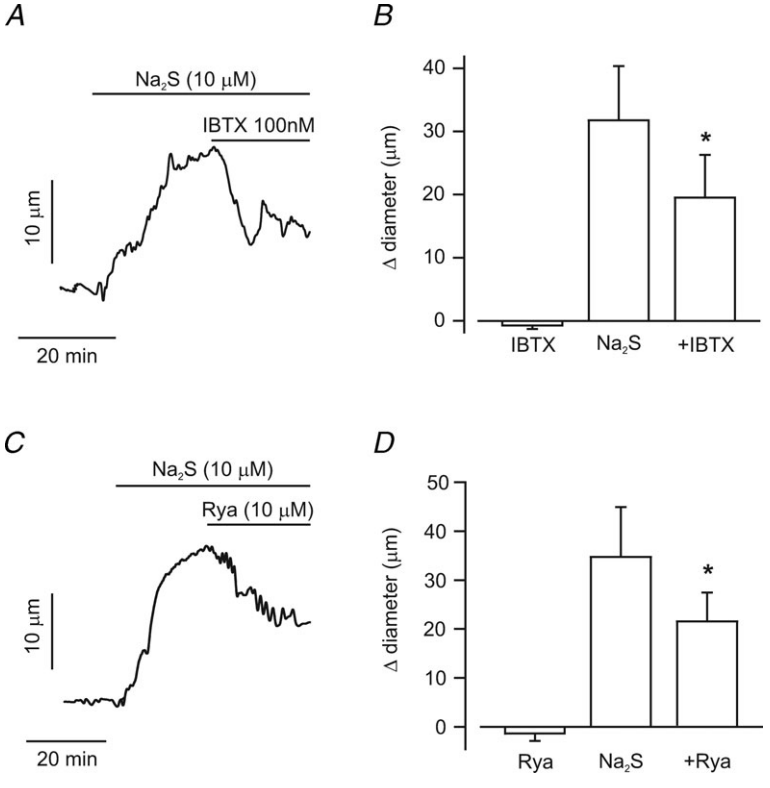

Figure 6. H_2S dilates pressurized (40 mmHg) piglet cerebral arterioles via RyR and K_{Ca} channel activation
A, representative trace illustrating that iberiotoxin (IBTX, 100 nM), partially reverses vasodilatation induced by Na_2S (10 μM). *B*, mean data (IBTX, $n = 5$; Na_2S and Na_2S + IBTX, $n = 9$ for each). *C*, representative trace illustrating that ryanodine (Rya, 10 μM) partially reverses vasodilatation induced by Na_2S (10 μM). *D*, mean data (Rya, Na_2S, and Na_2S + Rya, $n = 6$ for each). $^*P < 0.05$ *vs.* Na_2S.

cells. These data provide further support for our finding that H_2S activates transient K_{Ca} currents specifically by elevating Ca^{2+} spark frequency. In contrast to our findings, NaHS, another H_2S donor, at 0.1–1 mM, had no effect on transient K_{Ca} currents, but inhibited transient K_{Ca} currents at a very high concentration (4 mM) in guinea-pig gastric myocytes (Zhao *et al.* 2009).

H_2S has been reported to both activate and inhibit K_{Ca} channels. Previous studies indicated that H_2S activated K_{Ca} channels in different tissues (Sitdikova *et al.* 2010; Zuidema *et al.* 2010; Jackson-Weaver *et al.* 2011). Iberiotoxin blocked H_2S-induced vasodilatation in rat mesenteric arteries, decreased H_2S-induced K_{Ca} channel activation in rat pituitary tumour cell outside-out patches, and reversed an H_2S-induced elevation in whole-cell K_{Ca} current in cultured human microvascular endothelial cells (Sitdikova *et al.* 2010; Zuidema *et al.* 2010; Jackson-Weaver *et al.* 2011). In contrast, H_2S inhibited carotid body glomus cell K_{Ca} channels and recombinant K_{Ca} channels expressed in human embryonic kidney cells (Telezhkin *et al.* 2009; Li *et al.* 2010). Explanations for these different observations may include channel subunit composition, including whether and which β subunit isoforms are present and the α:β ratio, cell type and species studied, H_2S concentrations used, and whether experiments were performed in intact cells or inside-out membrane patches, which may modify pH and oxidation state, and thus the proportion of H_2S to HS^-. It is also important to note that we have shown 300 μM and 1 mM Na_2S induce an alkaline shift in pH from 7.4 to 7.54 and 7.75, respectively, in physiological saline solution (Liang *et al.* 2011). In contrast, lower concentrations of H_2S donors, similar to those used here, do not alter pH. Therefore, pH alterations may underlie results in studies where high concentrations of H_2S donor or H_2S gas have been used. Of note, alkaline pH shifts Ca^{2+} sparks to waves in arterial smooth muscle cells and has been demonstrated to induce vasoconstriction (Austin & Wray, 1993; Heppner *et al.* 2002). Intracellular acidification also inhibits K_{Ca} channels by reducing Ca^{2+} sensitivity and open time in rabbit tracheal smooth muscle cells (Kume *et al.* 1990).

An elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark frequency in cardiac myocytes and smooth muscle cells (Satoh *et al.* 1997; Cheranov & Jaggar, 2002). To examine mechanisms by which H_2S increased Ca^{2+} spark frequency, we measured H_2S regulation of $[Ca^{2+}]_{SR}$. Na_2S increased the amplitude of $[Ca^{2+}]_i$ transients produced by two different millimolar caffeine concentrations similarly. These data indicate that Na_2S elevates $[Ca^{2+}]_{SR}$. In contrast, Na_2S did not alter global $[Ca^{2+}]_i$ in these experiments. This is expected, because for these experiments the bath solution contained 6 mM K^+, in contrast to the experiments shown in Fig. 1 where the bath solution contained 30 mM K^+ to depolarize smooth muscle cells to physiological voltage. In 6 mM K^+, unpressurized arterial potential is $\sim$−60 mV, and global $[Ca^{2+}]_i$, Ca^{2+} spark frequency, and K_{Ca} channel activity is low. Under this condition, an Na_2S-induced elevation in Ca^{2+} sparks and thus, K_{Ca} channel activity would not be expected to further reduce global $[Ca^{2+}]_i$. These data indicate that an Na_2S-induced elevation in $[Ca^{2+}]_{SR}$ stimulates an increase in Ca^{2+} spark frequency, and thus transient K_{Ca} current frequency. Mechanisms by which H_2S elevates $[Ca^{2+}]_{SR}$ are likely to involve SERCA activation. Phospholamban is an endogenous inhibitor of SERCA (Hutter *et al.* 2002; Toyoshima *et al.* 2003). Protein kinase C, cAMP/cGMP-dependent protein kinases and Ca^{2+}–calmodulin-dependent protein kinase II phosphorylate phospholamban, leading to an increase in SERCA activity (Wegener *et al.* 1986; Simmerman *et al.* 1986; Karczewski *et al.* 1997). H_2S can also S-sulfhydrate cysteine residues, including in K_{ATP} channels, which elevates channel activity (Mustafa *et al.* 2011). Conceivably, H_2S may activate SERCA through direct sulfhyrdration or via regulation of an upstream SERCA regulator, including phospholamban. It is possible that H_2S also activates RyR channels and thus, Ca^{2+} sparks, by a SR Ca^{2+} load-independent mechanism. Future studies will investigate further the mechanisms by which H_2S activates RyR channels to elevate Ca^{2+} spark frequency.

Consistent with H_2S-induced Ca^{2+} spark and transient K_{Ca} current activation, our data indicate that H_2S hyperpolarizes pressurized arterioles and reduces global $[Ca^{2+}]_i$ in arteriole smooth muscle cells. These data are consistent with the established concept that transient K_{Ca} current activation leads to membrane hyperpolarization, which reduces voltage-dependent Ca^{2+} channel activity, and therefore, global $[Ca^{2+}]_i$ (Jaggar & Nelson, 2000; Cheng & Lederer, 2008). Recent evidence indicated that NaHS, an H_2S donor, produced iberiotoxin-sensitive membrane hyperpolarization in mesenteric arteries (Jackson-Weaver *et al.* 2011). In contrast, NaHS hyperpolarized coronary arteries via a 4-aminopyridine-sensitive mechanism (Cheang *et al.* 2010). Here, H_2S activated Ca^{2+} sparks and reduced global $[Ca^{2+}]_i$, but did not alter Ca^{2+} waves. RyR channels can contribute to Ca^{2+} waves in arterial smooth muscle cells (Jaggar & Nelson, 2000). Conceivably, effects of H_2S on Ca^{2+} waves may be concentration dependent, with higher non-physiological H_2S concentrations, which can induce an alkaline shift in pH, stimulating RyR channels sufficiently to increase Ca^{2+} wave frequency (Heppner *et al.* 2002).

In pressurized piglet cerebral arterioles, iberiotoxin or ryanodine alone did not alter arterial diameter. This finding is consistent with previous measurements performed *in vitro* and *in vivo* indicating that baseline Ca^{2+} spark and K_{Ca} channel activity are too low in piglet cerebral arteriole smooth muscle cells to oppose pressure-induced vasoconstriction (Jaggar *et al.* 2002; Ahmed *et al.* 2004; Kanu & Leffler, 2007). We show that H_2S produces hyperpolarization and vasodilatation

in pressurized arterioles. Iberiotoxin partially reversed H_2S-induced hyperpolarization and iberiotoxin and ryanodine both attenuated H_2S-induced vasodilatation. These data indicate that H_2S stimulates Ca^{2+} sparks that activate K_{Ca} channels, leading to hyperpolarization and vasodilatation. We have previously demonstrated that H_2S-induced vasodilatation in pressurized piglet cerebral arterioles is also partially inhibited by glibenclamide, a K_{ATP} channel-specific inhibitor (Liang *et al.* 2011). Therefore, our data indicate that H_2S dilates by activating both K_{Ca} and K_{ATP} channels in smooth muscle cells. Our previous *in vivo* study showed that glibenclamide can completely block H_2S (10 μM)-induced piglet pial arteriole dilatation in cranial window experiments (Leffler *et al.* 2011). The greater effect of glibenclamide *in vivo* could be explained by different experiment conditions and potential impact of other cells. The cranial windows are surgically implanted and closed. Under cranial window, cerebral arterioles are in contact with meninges, neurons and particularly astrocytes. Topically applied H_2S may affect not only pial arterioles but also astrocytes or neurons. Those cells also could affect pial arterial dilatation. Oxygen partial pressure is ~40 mmHg in cranial window experiments, but ~150 mmHg in the pressurized arterial experiments. Finally, the pressurized arterioles in the present study had mean diameters of ~150 μm, while the arterioles measured *in vivo* were much smaller at ~50 μm. Any or all of these differences may allow detection of a role for K_{Ca} channels in dilatation to H_2S that was not detected in the previous study *in vivo*. Collectively, these data indicate that H_2S can dilate piglet cerebral arterioles via the activation of both K_{Ca} and K_{ATP} channels, the former mediated through Ca^{2+} spark activation. Recent evidence indicates that K_{ATP} channels are the primary vasodilatory target of H_2S in non-pressurized mouse and rat aorta and mesenteric arteries (Cheng *et al.* 2004; Mustafa *et al.* 2011). Therefore, vascular origin, size, age and species may underlie different vasodilatory mechanisms for H_2S and specific ion channels targeted.

In summary, we demonstrate that H_2S elevates $[Ca^{2+}]_{SR}$, which stimulates Ca^{2+} sparks, leading to an increase in transient K_{Ca} current frequency in cerebral arteriole smooth muscle cells. The H_2S-induced elevation in K_{Ca} channel activity produces membrane hyperpolarization that reduces global $[Ca^{2+}]_i$, leading to vasodilatation.

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Author contributions

G.H.L. performed and analysed experiments, contributed to study design and wrote the manuscript; Q.X. performed experiments; J.H.J. and C.W.L. designed the study and wrote the manuscript. All authors approved the final version.

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