Actions of a hydrogen sulfide donor (NaHS) on transient sodium, persistent sodium, and voltage-gated calcium currents in neurons of the subfornical organ

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Kuksis M, Ferguson AV. Actions of a hydrogen sulfide donor (NaHS) on transient sodium, persistent sodium, and voltage-gated calcium currents in neurons of the subfornical organ. J Neurophysiol 114: 1641–1651, 2015. First published July 15, 2015; doi:10.1152/jn.00252.2015.—Hydrogen sulfide (H$_2$S) is an endogenously found gasotransmitter that has been implicated in a variety of beneficial physiological functions. This study was performed to investigate the cellular mechanisms underlying actions of H$_2$S previously observed in subfornical organ (SFO), where H$_2$S acts to regulate blood pressure through a depolarization of the membrane and an overall increase in the excitability of SFO neurons. We used whole cell patch-clamp electrophysiology in the voltage-clamp configuration to analyze the effect of 1 mM NaHS, an H$_2$S donor, on voltage-gated potassium, sodium, and calcium currents. We observed no effect of NaHS on potassium currents; however, both voltage-gated sodium currents (persistent and transient) and the N-type calcium current had a depolarized activation curve and an enhanced peak-induced current in response to a series of voltage-step and ramp protocols run in the control and NaHS conditions. These effects were not responsible for the previously observed depolarization of the membrane potential, as depolarizing effects of H$_2$S were still observed following block of these conductances with tetrodotoxin (5 μM) and α-conotoxin-GVIA (100 nM). Our studies are the first to investigate the effect of H$_2$S on a variety of voltage-gated conductances in a single brain area, and although they do not explain mechanisms underlying the depolarizing actions of H$_2$S on SFO neurons, they provide evidence of potential mechanisms through which this gasotransmitter influences the excitability of neurons in this important brain area as a consequence of the modulation of multiple ion channels.

Calcium channel; sodium channel; hydrogen sulfide; subfornical organ; voltage clamp

HYDROGEN SULFIDE (H$_2$S), a well-known toxic gas and recently characterized gasotransmitter, has been found to have a role in neuronal and cellular excitability throughout the body. Since its first documentation as a beneficial neurological mediator in the hippocampus (Abe and Kimura 1996), H$_2$S has now been implicated in many physiological pathways in the central nervous system and periphery. Endogenously, H$_2$S is produced through one of three different pathways. In the brain, cystathionine β-synthase (CBS) is highly expressed and produces H$_2$S from cysteine (Abe and Kimura 1996), while in the periphery, cystathionine γ-lyase (CSE) (Hosoki et al. 1997; Kaneko et al. 2006; Mani et al. 2014; Patel et al. 2009), or the 3-mecapto-pyruvate sulfur transferase (3MST)-cysteine aminotransferase (CAT) pathway, is more common (Kuo et al. 1983). These pathways contribute to endogenous concentrations of H$_2$S, which have been measured to be anywhere between 10 nM and 160 μM, representing both the total tissue levels (nM range) and the concentrations found in local active pools of H$_2$S (μM range) (Furne et al. 2008).

Within this range of physiological concentrations, H$_2$S has been found to have a wide range of effects in the body (Koenitzer et al. 2007). In the nervous system H$_2$S acts to have both neuroprotective (Hu et al. 2009; Kimura 2010b; Kimura et al. 2006, 2010; Kimura and Kimura 2004) and, of particular relevance to this study, neuromodulatory effects. H$_2$S functions to manipulate the activity of ion channels to influence membrane potential, action potential characteristics, and overall neuronal excitability. Initially, H$_2$S was shown to have effects on the NMDA receptor (Abe and Kimura 1996). Since then, H$_2$S has been implicated in the regulation of many ion channels such as ATP-sensitive potassium (K$_{ATP}$) channels (Peers et al. 2012; Zhao et al. 2001), Ca$^{2+}$-activated K$^+$ channels (BKCa) (Sidikova et al. 2010; Telezhkin 2010), as well as the delayed rectifier K$^+$ current ($I_{K}$) (Feng et al. 2013). Furthermore, T-type and L-type Ca$^{2+}$ channels have also been shown to be acted upon by H$_2$S (Garcia-Bereguiain et al. 2008; Kawabata et al. 2007; Messinger et al. 2009), as well as one type of sodium channel (Na$_{a,1.5}$) (Streve et al. 2011). Finally, there is growing evidence to suggest that transient potential receptor vanilloid 1 (TRPV1) channels (Trevisani et al. 2005) and chloride channels (Kimura et al. 2006) may also be modulated by this signaling molecule.

Previous work in our laboratory has identified a role of H$_2$S in the subfornical organ (SFO), functioning to regulate blood pressure by influencing the excitability of the neurons (Kuksis et al. 2014). The SFO is a circumventricular organ (CVO) vitally important to the sensing of circulating signals regulating homeostatic state. CVOs are uniquely positioned outside the blood brain barrier and are able to sense signals that other brain areas cannot. This information is integrated in areas such as the SFO and relayed to the relevant autonomic control centers. The SFO functions in pathways that control cardiovascular function, and because H$_2$S is a contributor to central cardiovascular regulation (Gan et al. 2012; Kuksis et al. 2014), we looked to investigate if the SFO was a site at which H$_2$S acted to regulate cardiovascular function. We observed a significant effect on blood pressure upon microinjection of H$_2$S into SFO, presumably brought about by the observed change in neuronal excitability (membrane depolarization, action potential potentiation). However, the ionic mechanism of the function of H$_2$S...
was left undiscovered and will be the focus of the present study.

As mentioned above, in addition to the work of our own laboratory, there have been many other well-documented physiological effects of H$_2$S and the process of attempting to elucidate the mechanisms underlying these effects has begun. While previous studies have certainly uncovered the enormous range of ion channels that H$_2$S acts on, importantly, much of this work has been conducted using certain cell lines or by isolating one conductance using specific blockers. By only evaluating one conductance at a time, the physiological relevance of the findings may be compromised. As such, we have conducted a study on a population of dissociated SFO neurons in which all of the voltage-gated ion channels are intact. We have individually evaluated the effect of H$_2$S on five voltage-gated conductances: the transient and persistent sodium conductances, the N-type calcium conductance, and transient and delayed rectifier potassium conductances. This study has uncovered potential mechanisms of action of H$_2$S in the SFO but will also argue the notion that H$_2$S has multiple effects on the same cell and that attributing a physiological function to one specific aspect of the signaling mechanism of hydrogen sulfide may be misleading.

METHODS

Ethics Statement

Male Sprague-Dawley rats (Charles River, Quebec, Canada) were used in all experiments. All animal procedures were approved by the Queen’s University Animal Care Committee: Protocol 100821 entitled Central Nervous System Pathways Integrating Cardiovascular and Metabolic Function.

SFO Neuron Preparation

SFO neurons were dissociated as previously described in the literature (Ferguson et al. 1997; Kuksis et al. 2014). Rats weighing 125–150 g were decapitated, and their brains were placed in an ice-cold, oxygenated artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 2.5 KCl, 1.24 KH$_2$PO$_4$, 2.27 CaCl$_2$, 1.3 MgSO$_4$, 20 NaHCO$_3$, and 10 glucose. Under a dissecting microscope, the SFO was microdissected away from the rest of the brain and placed in a drop of Hibernate media (Brain Bits, Springfield, IL). This process was repeated twice more to retrieve two more SFOs, and all three SFOs were then placed into 5 ml of Hibernate media containing 10 mg papain (Worthington Biochemical, Lakewood, NJ). To allow for the structural components of the SFO to be broken up, they were then incubated for 30 min at 31°C. They were then rinsed twice with Hibernate media supplemented with B27 (Invitrogen) and then triturated three times to fully dissociate the neurons. The solution was then spun at 200 g for 8 min in a centrifuge. After the supernatant was carefully removed, the cells were resuspended in Neurobasal A media (Invitrogen) supplemented with B27, 100 μM penicillin-streptomycin (Invitrogen), and 0.4 mM L-glutamine (Invitrogen). Aliquots were then made of ~90-μl drops of the solution on 35-mm plastic bottom dishes (MatTek, Ashland, MA). These plates were incubated at 37°C in 5% CO$_2$ for 2.5–3 h to allow for the newly dissociated neurons to sink down and adhere to the bottom of the plate. Afterwards, each dish was filled with ~2 ml of Neurobasal A (containing B27). The cells were then returned to the incubator. Dissociated neurons were maintained in culture for at least 24 h before being used, and recordings always took place within 4 days of the dissociation process.

Electrophysiology

Whole cell voltage-clamp and current-clamp recordings were obtained from dissociated SFO neurons, the majority of which did not have any processes as a result of the dissociation process. A Multi-clamp 700B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) was used to collect the data and filter it at 2 kHz. The recording stimulation and parameters were controlled and the data were collected using Signal (Version 6) and Spike2 (Version 7.05b) (Cambridge Electronics Design, Cambridge, UK). A Cambridge Electronics Design Micro 1401 interface was used to digitize the data at 5 kHz. A Flaming Brown micropipette (P47; Sutter Instrument, Novato, CA) was used to make electrodes from borosilicate glass (World Precision Instruments, Sarasota, FL), which had a resistance of 3–6 MΩ. Recording solutions varied as to their make-up; however, they were always adjusted to a pH of 7.2 using NaOH for the aCSF and CsOH or KOH for the internal. During the recordings, series resistance was monitored and never changed by >25% throughout the recording. Different voltage-gated currents were isolated using variable recording solutions and/or voltage protocols.

SFO neurons were perfused with aCSF at 37°C at an average rate of 1.5 ml/min. Perfusion setup utilized a gravity perfusion system and a vacuum pump. With the use of an MP-225 micromanipulator (Sutter Instrument), the recording electrode was positioned in the bath and slowly lowered to touch the cell membrane of the targeted neuron. Negative pressure was then applied to allow the formation of a GΩ seal, and then whole cell access was gained by applying a consistent pulse of negative pressure.

Measurement of the delayed rectifier potassium currents ($I_{K}$). To measure the voltage-gated delayed rectifier K$^+$ current, external and internal recording solutions that blocked voltage-gated Na$^+$ conductances were used. The external solution contained the following (in mM): 140 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 5 glucose, 5 mannitol, and 0.0005 tetrodotoxin. The internal solution contained the following (in mM): 125 potassium gluconate, 2 MgCl$_2$, 5.5 EGTA, 10 KCl, 0.1 CaCl$_2$, 10 HEPES, and 2 Na$_3$ATP. These solutions resulted in a junction potential of +15.9 mV, which was corrected for in all data analysis. We did not block voltage-gated Ca$^{2+}$ currents, as the broad-spectrum calcium channel blocker Cd$^{2+}$ reacts with H$_2$S and precipitates out of solution. We did, however, compare the voltage-gated potassium currents measured in control aCSF to those in Ca$^{2+}$-free aCSF in the same cells ($n = 6$) and observed currents similar in magnitude, shape, and activation, indicating that there was little contribution of a calcium-dependent component to the overall voltage-activated K$^+$ current (sustained current, control 901.4 ± 162.2 pA vs. Ca$^{2+}$-free 962.7 ± 220.6 pA, paired t-test, $P > 0.5$). Furthermore, the magnitude of the voltage-gated Ca$^{2+}$ current (≤0.1 nA, at +20-mV voltage step) is negligible compared with the size of the voltage-gated potassium currents (>1.25 nA, at +20-mV voltage step) and could therefore be ignored in the measurement of peak $I_K$.

We then utilized a voltage-step protocol, where we held the neuron at −75 mV, stepped to −40 mV, and then induced 250-ms voltage steps from −80 to +20 mV in 10-mV increments. This protocol worked to inactivate the transient potassium currents ($I_{K}$) before the test voltage steps, ensuring that the only current active was the $I_K$. This was confirmed by running this protocol and then bath applying TEA and observing the blockage of all induced current. The outward sustained current in the final 50 ms of the step was measured and used as a measurement of the $I_K$. Currents were normalized and plotted on an I-V curve.

Measurement of the transient potassium currents ($I_{K}$). To measure the voltage-gated transient K$^+$ current, the same external and internal recording solutions mentioned above were used. We utilized the same voltage-step protocol as mentioned above and then used another protocol where we held the neuron at −75 mV and then induced 250-ms voltage steps from −80 to +20 mV in 10-mV increments. The currents in the first protocol (where only $I_K$ is active) were then

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subtracted from the currents induced by the second protocol (where both \(I_a\) and \(I_K\) are active), which resulted in a current which was entirely the \(I_Na\) current. The peak current was measured, normalized, and plotted on an \(I-V\) curve.

**Measurement of the transient sodium current (\(I_{NaT}\)).** To measure the transient sodium current (\(I_{NaT}\), external and internal recording solutions that blocked voltage-gated \(K^+\) conductances were used. We did not block voltage-gated \(Ca^{2+}\) currents, as the broad-spectrum calcium channel blocker \(Cd^{2+}\) reacts with \(H_2S\) and precipitates out of solution. Furthermore, the magnitude of the voltage-gated \(Ca^{2+}\) current (<0.075 nA at \(-30\) mV voltage step) could be considered negligible compared with the size of the \(I_{NaT}\) (>1.0 nA at the \(-30\) mV) and also had a shape distinct from that of the \(I_{NaT}\). We therefore were able to ignore the \(Ca^{2+}\) current in the measurement of peak \(I_{NaT}\).

The external solution contained the following (in mM): 25 NaCl, 130 tetraethylammonium chloride, 1 MgCl2, 1 CaCl2, 1 CsCl, 10 HEPES, and 5 glucose. The internal solution contained the following (in mM): 125 CsMeSO4, 2 MgCl2, 5.5 EGTA, 10 CsCl, 0.1 CaCl2, 10 HEPES, and 2 Na2ATP. The reduced extracellular Na+ concentration ([Na+]o) (compared with normal conditions) was used to reduce the magnitude of \(I_{NaT}\), allowing the activation profile to gradually increase and take multiple steps to reach 100% conductance. We therefore did not compensate for series resistance, as an activation curve could already be accurately measured, and control vs. \(H_2S\) comparisons were made within the same neurons. The currents were leak subtracted using the specified protocol in Signal (Version 6). These solutions resulted in a junction potential of \(+18.1\) mV, which was corrected for in all data analysis. We then utilized a voltage-step protocol, where we held the neuron at \(-75\) mV and then induced 250-ms voltage steps from \(-80\) to \(-20\) mV in 10-mV increments. Peak inward currents were measured, and normalized conductance values were calculated using the following formula:

\[
G_{Na} = \frac{I_{Na}}{V_m - E_{Na}}
\]

where \(E_{Na}\) is the calculated reversal potential for \(I_{Na}\) (+67.5 mV). Conductance (\(G\)) values were plotted against test potential and fit with a Boltzmann function. The \(V_{50}\) and slope values of the Boltzmann functions in the control and NaHS conditions were compared with a paired \(t\)-test.

We also evaluated the inactivation profile of the transient sodium conductance in the same neurons. We ran an inactivation step protocol by holding the neuron at \(-75\) mV, inducing 100-ms voltage steps from \(-110\) to \(-20\) mV in 10-mV increments and then finally stepping the voltage to the test potential of \(-10\) mV for 500 ms where the measurement was taken. Normalized conductance values were again calculated using the equation stated above.

Finally, to evaluate the potential effect of \(H_2S\)-induced changes in sodium current activation and magnitudes on our previously observed NaHS-induced changes in excitability, we sought to evaluate any potential effect of bath application of NaHS (1 mM) on spontaneous action potential threshold in SFO neurons under the current-clamp configuration. Neurons were perfused with aCSF that contained the following (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 5 mannitol, and 5 glucose. The internal pipette solution contained the following (in mM): 125 potassium gluconate, 2 MgCl2·6H2O, 5.5 EGTA, 10 KCl, 2 NaATP, 10 HEPES, and 0.1 CaCl2. Action potential threshold was defined as the point at which the slope of the action potential reached exceeded 1 and was compared by averaging a group of spontaneous action potentials in control and NaHS conditions and comparing the mean threshold value with a paired \(t\)-test.

**Measurement of the persistent sodium current (\(I_{NaP}\)).** To measure the persistent sodium current (\(I_{NaP}\), the same external and internal recording solutions were used as described above. We used a fast voltage ramp from \(-100\) to \(+40\) mV at a rate of 140 mV/s and confirmed that the current was tetrodotoxin (TTX) sensitive and \(\omega\)-conotoxin-GVIA (\(\omega\)-CTX) insensitive (current was completely abolished by application of 5 \(\mu\)M TTX; mean values were compared at the peak voltage in control 0.6 ± 0.4 nA to the same voltage in TTX 0.06 ± 0.1 nA; \(n = 3\)). The conductance values (calculated with the above equation) were plotted against test potential and fit with a Boltzmann function. The \(V_{50}\) and slope values of the Boltzmann functions in the control and NaHS conditions were compared with a paired \(t\)-test.

**Measurement of voltage-gated calcium current.** To measure the voltage-gated calcium current, external and internal recording solutions that blocked voltage-gated \(Na^+\) and \(K^+\) conductances were used. The external solution contained the following (in mM): 120 NaCl, 10 BaCl2, 10 tetraethylammonium chloride, 1 4-aminopyridine, 1 MgCl2, 10 HEPES, 5 glucose, and 0.0005 TTX. The internal solution contained the following (in mM): 125 CsMeSO4, 2 MgCl2, 5.5 EGTA, 10 CsCl, 0.1 CaCl2, 10 HEPES, and 2 Na2ATP. These solutions resulted in a junction potential of \(+11.5\) mV, which was corrected for in all data analysis. Barium was used as a charge carrier to avoid the possibility of current rundown due to calcium-induced inhibition of the calcium channels. Accordingly, we observed no rundown of currents during the length of the recording. We utilized a voltage-step protocol, where we held the neuron at \(-75\) mV and then induced 250-ms voltage steps from \(-80\) to \(+20\) mV in 10-mV increments. Peak inward currents were measured, plotted against test potential, and fit with a Boltzmann function. To avoid the complication of changes in driving force associated with a typical voltage-step protocol, we then measured the tail current by measuring the peak current induced when stepping from each test pulse back to the holding potential (\(-75\) mV). This current was then plotted against the preceding voltage step (where it was stepped from) and fit with a Boltzmann function. The \(V_{50}\) and slope values of the Boltzmann functions in the control and NaHS conditions were compared with a paired \(t\)-test.

**Measurement of \(H_2S\)-induced depolarization magnitude in the presence of \(N\)-type calcium channel blockers or sodium channel blockers.** To evaluate the potential contribution of \(H_2S\)-induced changes in current magnitudes on the previously observed depolarization of the membrane potential, we bath applied NaHS (1 mM) to dissociated SFO neurons in the presence of the \(N\)-type calcium channel blocker \(\omega\)-CTX or the broad-spectrum sodium channel blocker TTX. Dissociated neurons were recorded in current-clamp using aCSF containing the following (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 5 mannitol, and 5 glucose, and an internal solution containing the following (in mM): 125 potassium gluconate, 2 MgCl2·6H2O, 5.5 EGTA, 10 KCl, 2 NaATP, 10 HEPES, and 0.1 CaCl2. The neurons were then perfused with NaHS (1 mM) for 50 s. The magnitude of the depolarization was observed, and the neuron was allowed to recover. The neurons were then perfused with aCSF containing either \(\omega\)-CTX (100 mM) for >10 min to ensure complete block of the \(N\)-type calcium channel (the main calcium current in SFO) or with TTX (5 \(\mu\)M) for >5 min to ensure complete blockage of the sodium channels. The blockage of the sodium channel was then confirmed through a voltage-clamp protocol, which showed the complete elimination of the induced sodium current. The neurons were then put back into current clamp and perfused with NaHS (1 mM), and the magnitude of the depolarization was measured. The magnitude of the depolarization in the control phase was compared with that in the presence of the blocker with a paired \(t\)-test.

**\(H_2S\) Administration**

This study has utilized NaHS as a donor of \(H_2S\). Our reported concentration of \(H_2S\) is 1 mM; however, estimates of the amount that
actually exists as free H$_2$S in solution in a closed system varies between 6 (personal communication, J. Wallace) and 18.5% of the original NaHS concentration (Dombkowski et al. 2004). Our studies utilized an open system to deliver NaHS to the cells, in which there would be an even greater rate of dissipation. We would therefore expect an even lower percentage than these estimates (made for a closed system). We always prepared our NaHS immediately before use, but 50% is still lost every 5 min that NaHS is exposed to open air (DeLecon et al. 2012). As such, we estimate that the actual concentrations of H$_2$S that we used during the course of this study were closer to 30–100 µM (or 3–10% of NaHS concentration). Because of this uncertainty, we have reported the donor concentrations of NaHS that we have used.

Following this same logic, the endogenous concentrations of H$_2$S have been suggested to lie anywhere between 10 nM and 160 µM in the body (Furne et al. 2008; Kimura 2010a; Kimura et al. 2010; Li et al. 2011), which would mirror 150-nM to 1-mM concentrations of NaHS (assuming 10% values). In accordance with such calculations, previous studies investigating H$_2$S effects on ion channels using specific cell expression systems have shown effects of H$_2$S on ion channel activity beginning at 1 mM NaHS (Li et al. 2010; Sekiguchi et al. 2014; Streng et al. 2008). As such, we believe that the concentrations of H$_2$S used in these studies likely fall within high physiological and pharmacological concentration ranges.

Although H$_2$S has been suggested to influence the pH of a solution, when we dissolved NaHS (1 mM) in solution, the change in pH was negligible (<0.1 pH units). These observed changes agree with previously observed values (Dombkowski et al. 2004).

Chemicals and Peptides

Salts used in preparation of the aCSF and the internal solution, and NaHS were obtained from Sigma-Aldrich (Oakville, ON, Canada). TTX and w-CTX were obtained from Alomone Labs (Jerusalem, Israel).

RESULTS

The Voltage-Gated K$^+$ Conductances $I_A$ and $I_K$ Are Not Influenced by NaHS

Voltage-gated potassium conductances are well known modulators of neuronal excitability and have previously been implicated as a site of action by which H$_2$S functions to alter neuronal excitability (Feng et al. 2013). We first investigated two specific voltage-gated potassium conductances, the delayed rectifier potassium conductance ($I_K$) and the transient (A-type) potassium conductance ($I_A$). We ran voltage-step protocols designed to activate the $I_A$ and $I_K$ currents in SFO neurons. These protocols were run in the presence of TTX (5 µM) to ensure there was no voltage-gated sodium current being conducted. As described in the methods, we used the sustained current over the final 50 ms of the step as a measurement of the $I_K$ and a subtraction protocol to isolate the $I_A$. Since $I_K$ does not reach a peak in the range of the voltage protocol we used, we were not able to normalize the curve and fit it with a Boltzmann function. Instead, we compared the current amplitudes at each step and found no significant difference in the control compared with the NaHS conditions. Interestingly, we observed no effect on the activation curve of $I_K$ (Fig. 1A), nor did we observe a change in the magnitude of the peak current ($P > 0.05$). Next, we compared the activation curve and magnitude of peak current of the $I_A$ current. We did this by fitting each cell with a Boltzmann function and then averaging the $V_{50}$ and slope values. We compared these values with a paired $t$-test, and observed no shift in the activation curve (control: $V_{50}$: $-15.1 \pm 2.5$ mV, slope: $14.7 \pm 1.5$; NaHS: $V_{50}$: $-14.8 \pm 2.4$ mV, slope: $13.7 \pm 0.7$; $V_{50}$: $P > 0.05$, slope: $P > 0.05$). Furthermore, we observed no change in peak current magnitude ($P > 0.05$) in control conditions compared with NaHS ($n = 8$). These data are illustrated in

![Image](http://jn.physiology.org/lookup/doi/10.1152/jn.00252.2015)
Fig. 1C and summarized in Fig. 1D. It is possible that there is a Ca\(^{2+}\)-sensitive component of these K\(^{+}\) currents, as well. Ono et al. (2005) have suggested that there may be a Ca\(^{2+}\)-dependent component of the I\(_A\) but did not report any effects on the I\(_K\). The currents measured in those studies appear similar in shape and magnitude and have similar activation curves to those recorded in the present study, and we did not observe any significant effect of NaHS on these currents.

The Transient Na\(^{+}\) Current (I\(_{NaT}\)) Is Influenced by NaHS

Next, we looked to investigate another important modulator of neuronal excitability, the transient sodium current (I\(_{NaT}\)). We used whole cell patch-clamp recordings in the voltage-clamp configuration to evaluate the effect of NaHS on the I\(_{NaT}\). We utilized a voltage-clamp protocol (see METHODS), bath applied NaHS, and observed a rightward shift in the activation curve in seven of seven neurons tested (as illustrated in Fig. 2A and summarized in Fig. 2B). We fit each cell with a Boltzmann function, averaged the V\(_{50}\) and slope values, and compared the control and NaHS conditions with a paired t-test. We found the V\(_{50}\) and slope shifted from \(-46.8 \pm 3.5\) and \(3.1 \pm 0.4\) mV, respectively, in the control phase to \(-38.8 \pm 3.1\) and \(3.1 \pm 0.3\) mV, respectively in the NaHS phase (V\(_{50}\): \(*P < 0.001;\) slope: \(P > 0.05\)). These data are summarized in Fig. 2D. We then used a voltage-step protocol to evaluate the inactivation curve of the transient sodium current in the same neurons.

\(\text{Fig. 2. NaHS shifts the activation curve and the inactivation curve and increases the peak conductance of transient sodium current (I}_{NaT}\). A: raw traces of current induced at 5 different voltage steps in control (black), NaHS (red), and recovery (blue) conditions. Note the shift in activation in the NaHS state. B: mean activation curves (n = 7) of normalized conductance (G) plotted against test potential for control (black) and NaHS (red) conditions. Data are fitted with a Boltzmann function that can be seen to be shifted in the NaHS state (V\(_{50}\) = \(-40.7 \pm 0.7\) slope = \(5.2 \pm 0.7\)) compared with the control state (V\(_{50}\) = \(-47.7 \pm 0.7\) slope = \(5.1 \pm 0.6\)), (V\(_{50}\): \(*P < 0.001;\) slope: \(P > 0.05\)). C: mean inactivation curves (n = 7) of normalized conductance plotted against test potential for control (black) and NaHS (red) conditions. Data are fitted with a Boltzmann function which can be seen to be shifted in the NaHS state (V\(_{50}\) = \(-56.9 \pm 0.4\) slope = \(5.3 \pm 0.4\)) compared with the control state (V\(_{50}\) = \(-63.8 \pm 0.4\) slope = \(5.6 \pm 0.3\); V\(_{50}\): \(*P < 0.001;\) slope: \(P > 0.05\)). D: bar graph illustrating the mean V\(_{50}\) and slope (k) for both the activation and inactivation curves, the average peak conductance in the control (black) and NaHS (red) states for all responding neurons (n = 7; \(*P < 0.05\)), and the average action potential threshold (n = 6). Error bars indicate SE.\)
In seven of seven neurons, we found the $V_{50}$ and slope shifted on average from $-68.7 \pm 2.9$ to $6.6 \pm 0.5$ mV, respectively, in the control phase to $-60.0 \pm 2.7$ and $5.8 \pm 0.3$ mV, respectively, under NaHS conditions ($V_{50}$: *$P < 0.01$; slope: $P > 0.05$). The mean inactivation curves of these neurons were $-9.8 \pm 2.6$ nS in the control state and $-12.0 \pm 2.6$ nS in the NaHS state ($n = 6$; *$P < 0.05$; Fig. 2D).

We had previously observed changes in overall excitability of SFO neurons in response to bath application of NaHS (1 mM), and we sought to further investigate this notion by evaluating the threshold of spontaneous action potentials in control and NaHS (1 mM) conditions. Recording in the current-clamp configuration, we found that the action potential threshold was depolarized from $-43.8 \pm 1.3$ mV in the control condition to $-36.6 \pm 3.0$ mV under NaHS conditions ($n = 6$; *$P < 0.05$; Fig. 2D). These findings agree with our previously observed increases in excitability, as well as the presently reported effects of NaHS on the activation curve of the transient sodium current.

**The Persistent Na$^+$ Current ($I_{NaP}$) Is Influenced by NaHS**

Similarly, the $I_{NaP}$ has been suggested to regulate the electrical activity of SFO neurons (9). As such, we investigated the effect of NaHS on this conductance. We activated $I_{NaP}$ specifically by using a fast voltage ramp (140 mV/s) in with TEA present in the aCSF. This protocol activated an inward current near $-70$ mV, which peaked near $-40$ mV and was identified as the $I_{NaP}$. We ran the protocol in control conditions and in the presence of H$_2$S and observed a rightward shift in the activation curve of the current in nine of nine neurons tested, as illustrated in Fig. 3Ai. Each point was then averaged across all neurons, and each neuron was fit with a Boltzmann function. The average Boltzmann curves ($V_{50}$; control: $-64.1 \pm 0.4$ mV, NaHS: $-54.0 \pm 0.4$ mV, slope: $P < 0.01$; NaHS: $5.1 \pm 0.3$ mV, slope: $P < 0.05$) with 95% confidence intervals are shown in Fig. 3Aii. A conductance was then calculated for each current value and then normalized. The $V_{50}$ and slope values of each function were compared with a paired $t$-test, and the data are summarized in Fig. 3Aiii. These values are summarized in Fig. 3C. Similarly, there was also an increase in the peak conductance elicited by the voltage ramp in eight of nine neurons tested. The average conductance of these neurons in control conditions was $0.09 \pm 0.06$ nS compared with $0.13 \pm 0.04$ nS in the NaHS condition ($*P < 0.05$; $n = 8$; Fig. 3C).

**NaHS Shifts the Activation Curve of the Voltage-Gated Calcium Current**

To measure the voltage-gated calcium current in SFO neurons, we utilized a series of voltage steps in aCSF containing TEA, 4-AP, and TTX, resulting in the measurement of isolated voltage-activated calcium currents. We ran the step protocol in the control aCSF conditions, and then again in the presence of NaHS, and found that 9 of 10 neurons tested showed a shift in the activation curve to the right (as illustrated in Fig. 4Ai and summarized in Fig. 4Aii). To avoid changes in driving force complicating the calculation of the activation curve, we then measured the tail current at each step. Because the measurements of the tail current were taken for each step at the same potential (the holding potential), the driving force for each step would be constant, as illustrated in Fig. 4Bi. We plotted the measurement of the tail current against the magnitude of the

![Image](http://jn.physiology.org/doi/10.1152/jn.00252.2015)
NaHS EFFECTS ON VOLTAGE-GATED ION CHANNELS IN SFO NEURONS

Fig. 4. NaHS enhances the N-type calcium current and shifts the activation curve. Ai: raw traces of current induced at 3 different voltage steps in control (black), NaHS (red), and recovery (black) conditions. Notice the shift in activation as well as enhancement of peak current in the NaHS state. Aii: average normalized current values for all responding neurons (n = 9) plotted against test potential. Aiii: bar graph illustrating the mean V_{50}, slope, and peak step-induced current in NaHS (red) and control (black) conditions (VC: *P < 0.05; slope: P > 0.05; peak I: *P < 0.05). Error bars indicate SE.

preceding voltage step and fit the data with a Boltzmann curve for each neuron, and compared the mean V_{50} and slope values with a paired t-test. The V_{50} and slope values shifted from −14.0 ± 0.9 and 8.1 ± 0.8 mV, respectively, in the control condition to −8.4 ± 1.2 mV and 8.5 ± 1.0, respectively, in the NaHS condition (n = 9; V_{50}: *P < 0.05; slope: P > 0.05; illustrated in Fig. 4Bii, and summarized in Fig. 4Aiii). Furthermore, the peak current increased in 7 of 10 neurons tested. The average peak current in these neurons increased from −0.248 ± 0.069 nA in the control condition to −0.330 ± 0.082 nA in the NaHS condition (n = 7; *P < 0.05; Fig. 4Aiii).

NaHS-Induced Changes in Na\(^+\) and Ca\(^{2+}\) Currents Are Not Responsible for NaHS-Induced Depolarization of the Membrane Potential

Finally, we investigated whether the observed effects of NaHS on the magnitude and activation of the transient and persistent sodium currents and the N-type calcium current were responsible for the NaHS-induced depolarization that we observed in our previous studies. We bath applied NaHS (1 mM) in control conditions, and then again in the same neuron in the presence of the specific blocker (either 100 nM ω-CTX or 5 μM TTX) and compared the magnitude of the depolarizations. When the N-type calcium channel (responsible for the majority of the voltage-gated calcium current) was blocked, the average magnitude of depolarization was 6.4% larger than in the control phase (n = 4; P > 0.05). When sodium channels were blocked, the average magnitude of depolarization was 16% smaller than in the control phase (n = 4; P > 0.05). Neither comparison reached significance and these data are summarized in Fig. 5.

DISCUSSION

This study has characterized the actions of NaHS, a fast H\(_2\)S donor, on specific ion channels in SFO neurons. It is the first to investigate the effect of H\(_2\)S on a range of voltage-gated ion channels in the same neuron and to make comparisons between effects on these different currents. Our voltage-clamp analyses have identified intriguing common effects on the activation profiles of H\(_2\)S in both voltage-gated sodium and calcium channels, which would be expected to have significant impact on the excitability of SFO neurons.
Our previous work with H$_2$S in the SFO demonstrated not only physiological actions of H$_2$S in SFO increasing blood pressure but also actions on single SFO neurons to depolarize membrane potential and increase the excitability of these neurons. Increases in excitability are often caused by modulation of voltage-gated ion channels, and work done in trigeminal ganglion neurons found that H$_2$S increased excitability of the neurons by inhibiting the $I_K$ conductance (Feng et al. 2013). As such, we first investigated two voltage-gated potassium conductances, the $I_K$ and the $I_A$. Interestingly, we observed no effect on the activation profile or the magnitude of current elicited by a series of voltage steps in control conditions compared with NaHS conditions. This was surprising, considering that H$_2$S has been shown to influence potassium conductances in other brain areas, but our data show that this is clearly not the case in the SFO. These effects in other brain areas were

![Graph showing NaHS-induced depolarization and blockage of Na$^+$ or Ca$^{2+}$ currents.](http://jn.physiology.org/)

Fig. 5. NaHS-induced depolarization of the membrane is not attenuated by blockage of Na$^+$ or Ca$^{2+}$ currents. A: current-clamp trace of a single neuron in control conditions (left) and in the presence of $\omega$-conotoxin-GV1A ($\omega$-CTX; 100 nM; dark grey bar; right). Red bars indicate application of NaHS (1 mM). Note the similar magnitude of depolarization in both cases. B: current-clamp trace of a single neuron in control conditions (left) and in the presence of tetrodotoxin (TTX; 5 μM; light grey bar; right). Red bars indicate application of NaHS (1 mM). Note the similar magnitude of depolarization in both cases. C: bar graph illustrating the percent of the control depolarization when NaHS was applied in the presence of either $\omega$-CTX or TTX. The depolarization was not statistically different from the control in the presence of either blocking agent ($\omega$-CTX: $P > 0.05$; TTX: $P > 0.05$).
often seen as effects on a Ca$^{2+}$-dependent K$^+$ conductance. We did, however, investigate if this type of current may have been present in SFO neurons by comparing the K$^+$ currents in Ca$^{2+}$-free aCSF to those in our normal aCSF. We found that the currents were similar in size, shape, and activation profile ($n = 6$, data not shown) in both solutions. In other neurons that have a large Ca$^{2+}$-dependent potassium component, the outward current can be seen to continue to increase throughout the voltage step and has not reached its peak at 500 ms (Galvan and Sedlmeir 1984; Li and Ferguson 1996), whereas the potassium currents observed in our recordings reached their peak in <100 ms. We therefore concluded that there was no major contribution of Ca$^{2+}$-dependent K$^+$ channels to the voltage activated K$^+$ current, to which we saw no effect. As a result, we sought to investigate the effects of H$_2$S on other voltage-gated ion channels that could potentially be responsible for the observed increases in excitability.

We next explored the effects of H$_2$S on the properties of the voltage-gated sodium channel by isolating the transient and persistent sodium currents using specific voltage-step or ramp protocols in solutions designed to isolate the currents. We found that the activation curve of both transient and persistent sodium currents was shifted to the right as a result of H$_2$S application by ~7.0 and 10.7 mV, respectively. These effects were uniform (100% of neurons responded) and reversible. Furthermore, both currents appeared to be enhanced by the application of H$_2$S. The transient sodium current plays a key role in action potential initiation. Paradoxically, a shift in the activation curve of this current to a more depolarized range should function to make the cell less excitable, while increasing the amount of current passed through the membrane would make the cell more excitable. Our previous work found that H$_2$S caused a substantial depolarization of the membrane, as well as overall increased excitability of the membrane. The depolarization of the membrane was so rapid and substantial (10–15 mV) that it would on occasion cause sodium channels to become inactivated, thereby decreasing the excitability of the membrane and thus the ability of the neuron to fire action potentials. The data we present here may provide an explanation for this paradox. By shifting the threshold for action potential generation (i.e., the activation curve) closer to the new depolarized membrane potential, it would maintain the ability of the neuron to fire action potentials. Furthermore, shifting the inactivation curve to the right would raise the potential at which sodium channels become inactive and allow for more channels to be available to open at the action potential threshold. We have also reported that the amount of current passed upon opening of the sodium channels increased as a result of H$_2$S. Under these conditions, opening of the channels would result in a greater upstroke of the action potential.

The persistent sodium current, on the other hand, makes up ~2% of the total sodium current but still significantly contributes to the modulation of membrane excitability (Stafstrom et al. 2007). Our recording solutions contained reduced sodium concentrations, so the persistent sodium current measured was actually substantially smaller than what could be expected in vivo. This current is thought to pass through the same channels as I$_{NaP}$ but has a different form due to a different gating mechanism (the incomplete inactivation of the sodium channel). Specifically, I$_{NaP}$ has been shown to amplify subthreshold oscillation, modulate synaptic potentials (i.e., neurotransmitter release), and facilitate repetitive firing (French et al. 1990; Stafstrom et al. 1984; Yu et al. 2005). The role of I$_{NaP}$ in regulating neuronal excitability is clear, and such a profound effect on the activation of this conductance by H$_2$S could have important physiological effects. Similar to the potential effects of a depolarized activation of I$_{NaP}$, the depolarized activation of I$_{NaT}$ could lead to lowered membrane excitability. I$_{NaP}$ activation only being available at a more depolarized potential would reduce subthreshold oscillations, would reduce the facilitating effect of I$_{NaP}$ on synaptic potentiation, and would also reduce the incidence of repetitive firing in the neuron. As was the case with the I$_{NaT}$, however, it is possible that the depolarized membrane is related to the shift in activation of I$_{NaP}$ and makes the shift less meaningful. Again, the peak I$_{NaP}$ passing through the channels is enhanced and could increase the incidence of subthreshold oscillations, potentiate synaptic transmission and increase repetitive firing in the neurons.

It is perhaps not surprising that both sodium currents were influenced by H$_2$S, as it is believed that both currents are passed through the same channel. These sodium channels are composed of α- and β-subunits, where each α-subunit is composed of four homologous domains with six transmembrane segments (Catterall et al. 2011). The positively charged S4 segment in each domain acts as the voltage sensor for the channel (Catterall et al. 2011) and is the segment that we might speculate is being affected by H$_2$S. Both currents are affected, which may suggest that the actions are not occurring at the inactivation gate (the site which facilitates I$_{NaP}$). However, it is possible that the enhancement of the current may be caused by an effect at the pore. H$_2$S is a reducing agent, and it is possible that H$_2$S may act to reduce key residues in these subunits, leading to a conformational change of the ion channel. It may also be possible that changes in the kinetics of the channel may be responsible for the increase in magnitude of the observed current.

We next evaluated effects of H$_2$S on calcium currents in SFO neurons by using solutions designed to specifically isolate this conductance and performing voltage-step protocols to activate this current. The calcium current elicited was confirmed to be of the N-type, as it was almost completely blocked after application of the specific N-type calcium channel blocker ω-CTX. Similar to the sodium channel, the activation curve of the N-type calcium channel was shown to shift to the right. We were able to avoid one of the challenges of measuring a calcium activation curve, the changing driving force. In our solutions, the observed reversal potential for calcium was significantly less positive, +30 mV, than it has been theorized to be in vivo, and as such, our voltage protocol resulted in diminishing current after the ~5-mV step (or +5 mV in the presence of H$_2$S) due to the diminished driving force. We used a previously employed technique of measuring the tail current, which results from repolarizing the membrane potential to the holding potential after completion of the step. This technique ensures that there is a constant driving force for all measurements of current. From this, we were able to plot an accurate activation curve, which was shown to shift to the right. Furthermore, the magnitude of this current was also enhanced by application of H$_2$S.

Our use of ω-CTX confirmed that the majority of the calcium current we were recording was indeed N-type current; however, our experimental protocols did not account for other
types of calcium current present in the SFO. A previous study from our laboratory found that 22% of calcium current was of the L type, while another 10% was of the R type (Washburn and Ferguson 2001). It is certainly possible that H2S may have effects on these types of conductances in addition to its effects on the N-type calcium current, and further study should be devoted to identifying any potential effects pertaining to these conductances. Our study, however, focused on the N-type calcium current, which does make up the majority of the calcium current found in SFO neurons.

The best established physiological function of calcium channels is the regulation of synaptic potentiation and neurotransmitter release through Ca2+ influx mediation (Catterall 2001). Although this study was performed in dissociated neurons where there were no synaptic connections, it can be theorized that N-type Ca2+ channels expressed on the axon terminals of SFO neurons may be influenced by H2S in a similar manner. The depolarization of the activation curve would cause the presynaptic neuron to become less excitable, due to the depolarized membrane potential required for activation of the current. Once that channel does become activated, however, there is a larger influx of current through the channel, and therefore greater neurotransmitter release into the synapse at once, and subsequently an increased likelihood of postsynaptic initiation of an action potential. This suggests that H2S may alter N-type Ca2+ channel properties to act as a “low-pass” filter for synaptic transmission. Secondly, N-type calcium currents have also been implicated in the regulation of tonic firing in neuronal cell bodies (Kasten et al. 2007; Wong et al. 2011). Enhancement of the current by H2S may therefore act to increase the excitability of neurons by increasing the likelihood of tonic firing. Similarly, although the depolarized activation curve may make it more difficult for these channels to open, during an action potential, the membrane potential is dramatically depolarized, and the comparatively minor shift in activation may actually be less functionally relevant compared with the increase in current magnitude seen as a result of H2S administration.

Calcium channels are thought to be made up of as many as five subunits (Hanlon and Wallace 2002). It has been suggested that the α-subunits, specifically the S4 (voltage sensing) and the S2 and S3, of sodium channels have conserved charge residues compared with subunits in the calcium channel (Tanabe et al. 1987). This could be a possible explanation for how calcium channels have a similar shift in the activation curve as do sodium channels. It is possible that H2S acts at a residue that is conserved in both types of channels and elicits similar effects. H2S also acts to enhance the peak current passing through both types of channels. We speculate that this may be a result of H2S actions on a subunit, which comprises of the pore of the sodium and calcium channel.

Taken together, it can be seen that H2S has a range of effects on a variety of ion channels. We observed profound effects on voltage-gated sodium and calcium channels but interestingly no effect on potassium channels. Our previous work found that a significant depolarization of the membrane occurs when neurons are subjected to H2S depolarizations that were still observed in our current studies following pharmacological block of each of these conductances. We were, however, able to uncover a potential mechanism for one manner by which H2S may act to influence SFO neuron excitability. Our observed effects on the action potential threshold certainly support the notion that NaHS influences the excitability of these neurons. Furthermore, these effects are consistent with our observed effects on the transient sodium conductance. It is not surprising that we observed a shift in action potential threshold after observing a shift in the activation curve of the transient sodium conductance, as this is likely the main current that contributes to the action potential threshold. Further work will need to be done to investigate if our observed effects of NaHS on the persistent sodium current and calcium current may play a further role in altering the excitability of the neurons. While we were unable to uncover the mechanism for the previously observed depolarization, this perhaps underscores that while H2S is certainly a powerful modulator of neuronal function, attempting to attribute its effects on either excitability or membrane potential to actions on a single conductance may be at best a little naive. Our observed changes in membrane potential were accompanied by effects on two types of voltage-gated channels passing three different currents. However, the currents are not reliant upon one another and for all intents and purposes could be considered three separate ways by which H2S acts to influence neuronal excitability.

In conclusion, our experiments have identified potential mechanisms through which H2S modulates the excitability of SFO neurons through actions on persistent sodium, transient sodium, and N-type calcium currents. To uncover the mechanism of the depolarization observed in our previous work, future studies will be required to identify other, non-voltage-gated conductances that are affected by H2S. Previous work has found that H2S does in fact affect TRPV1 channels, chloride channels, and KATP channels, and it is possible that these may be responsible for the previously observed depolarization. Furthermore, our experiments did not block all calcium conductances present in SFO, and it is possible that some of the more minor calcium currents (L-type, R-type) could contribute to the observed depolarization. Thorough investigation of all conductances in SFO will be required to get a complete picture of the mechanisms through which H2S modulates both the excitability and membrane potential of SFO neurons.

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