Hydrogen sulfide inhibits Kir2 and Kir3 channels by decreasing sensitivity to the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2)

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Inwardly rectifying potassium (Kir) channels establish and regulate the resting membrane potential of excitable cells in the heart, brain, and other peripheral tissues. Phosphatidylinositol 4,5-bisphosphate (PIP2) is a key direct activator of ion channels, including Kir channels. The gasotransmitter carbon monoxide has been shown to regulate Kir channel activity by altering channel–PIP2 interactions. Here, we tested in two cellular models the effects and mechanism of action of another gasotransmitter, hydrogen sulfide (H2S), thought to play a key role in cellular responses under ischemic conditions. Direct administration of sodium hydrogen sulfide as an exogenous H2S source and expression of cystathionine γ-lyase, a key enzyme that produces endogenous H2S in specific brain tissues, resulted in comparable current inhibition of several Kir2 and Kir3 channels. This effect resulted from changes in channel-gating kinetics rather than in conductance or cell-surface localization. The extent of H2S regulation depended on the strength of the channel–PIP2 interactions. H2S regulation was attenuated when channel–PIP2 interactions were strengthened and was increased when channel–PIP2 interactions were weakened by depleting PIP2 levels. These H2S effects required specific cytoplasmic cysteine residues in Kir3.2 channels. Mutation of these residues abolished H2S inhibition, and reintroduction of specific cysteine residues back into the background of the cytoplasmic cysteine-lacking mutant rescued H2S inhibition. Molecular dynamics simulation experiments provided mechanistic insights into how potential sulfhydration of specific cysteine residues could lead to changes in channel–PIP2 interactions and channel gating.

The gaseous mediator, hydrogen sulfide (H2S), is increasingly garnering the reputation of a major physiological messenger molecule with robust effects in ischemia-related insults to the heart, brain, and other peripheral tissues (1). H2S is generated from L-cysteine by three distinct enzymes: cystathionine β-synthase, cystathionine γ-lyase (CSE), and 3-mercaptopropionate sulfurtransferase/cysteine aminotransferase (2), and signals via sulfhydration (also known as persulfidation), a post-translational modification of reactive cysteine residues analogous to N-nitrosylation by nitric oxide (3). In these studies, sulfhydration was detected by techniques such as a Biotin-switch assay (4), a Tag-switch assay (5), and mass spectrometry (6). In experiments in vivo and in vitro, the endogenous production of H2S was modulated by several chemicals and/or H2S was applied exogenously to tissue/cells, using sulfide salts, mainly sodium hydrosulfide (NaHS). Collectively, H2S has been shown to play a role in diverse physiological processes, such as cellular necrosis, apoptosis, oxidative stress, or inflammation (7). One theme emerging from these studies is that H2S increases the excitability of neuronal compartments (8–12), whereas it depresses excitability of cardiac tissues (13, 14), correlating to neurotoxic (12, 15) and cardioprotective (16–20) effects, respectively. Therefore, exactly how H2S is affecting differentially the ion channels expressed in cells that rapidly control membrane excitability has become a central question. In the cardiovascular system for example, H2S stimulates the activity of the ATP-sensitive Kir6 (KATP) channel through the direct sulfhydration of an N-terminal cysteine (Cys-43) residue, hyperpolarizing and dilating blood vessels (13).

The KATP channel belongs to the Kir family of K+ channels that establish and regulate the resting membrane potential of cardiomyocytes, smooth muscle cells, pancreatic cells, neurons, and various other excitable cells (21). A wealth of electro-

2 The abbreviations used are: CSE, cystathionine γ-lyase; NaHS, sodium hydrosulfide; Kir, inwardly rectifying potassium channel; KATP, ATP-sensitive K+ channels; PIP2, phosphatidylinositol 4,5-bisphosphate; Wort, wortmannin; PIPS5, PIPS-kinase; PAG, propargylyglycine, MD, molecular dynamics; TEVC, two-electrode voltage clamp; ANOVA, analysis of variance; MSBT, 2-(methylsulfonyl)benzo[1]thiazole; pS, picosiemen; pF, picofarad; PDB, Protein Data Bank; Ci-VSP, voltage-activated PIP5 phosphatase; diC8, diocatanyol; aa, amino acid; I/R, ischemia and reperfusion; P(4,5)P2, phosphatidylinositol 4,5-bisphosphate; TIRF, total internal reflection fluorescence; CFP, cyan fluorescent protein; eGFP, enhanced GFP.
Physiological, structural, and biochemical studies have revealed that a biological phospholipid, phosphatidylinositol 4,5-bis-phosphate (PiP2), interacts with Kir channels as a key direct activator (22). The cysteine residue (Cys-43) implicated in the effect of H2S on vascular KATP happens to be conserved throughout the Kir subfamily and is positioned next to a positively-charged residue critical for coordinating PiP2 binding in Kir3.2 channels. Therefore, sulfhydration of this nearby cysteine may be comparable with other post-translational modifications, such as phosphorylation of specific channel residues that directly or allosterically alter channel interactions with PiP2 (23). The effect of H2S on phosphoinositide control of channel activity is thus a critical molecular theme to be clarified. More specifically, we explored the putative effect of H2S on cytoplasmic cysteine residues of Kir channels (both conserved and distinct).

Here, we demonstrate that the effect of H2S on Kir channels (Kir2 and Kir3), heterologously expressed in Xenopus laevis oocytes or CHO-K1 cells, is inhibitory, as opposed to being stimulatory on KATP channels. We used two approaches to study the effects of H2S on Kir channels by exogenous application of NaHS to Kir2 and Kir3 channels or by co-expressing CSE, the principal enzyme producing H2S in peripheral tissues. We report that channel inhibition does not involve changes in cell-surface localization or single-channel conductance.

Instead, open-time kinetics decrease while closed-time kinetics increase. We show that H2S-mediated inhibition of Kir channels is dependent on the strength of channel–PiP2 interactions by employing the voltage-clamp technique on whole oocytes as well as patch-clamp in excised membrane patches from oocytes. Using site-directed mutagenesis, we determined which cysteine residues are involved in the inhibitory effect of H2S on this channel. Finally, using molecular dynamics simulation experiments, we provide mechanistic insight as to how potential sulfhydration of these specific cysteine residues could lead to changes in channel–PiP2 interactions and channel gating.

Results
NaHS inhibits Kir3.2 channel activity in Xenopus oocytes

In contrast to previous studies on stimulation of vascular KATP (Kir6.1) channels (13), NaHS (2 mM) treatment inhibited Kir3.2* channel activity (* indicates the E152D mutant that increases channel activity) (24). NaHS administration to Kir3.2* channels expressed in Xenopus oocytes at increasing time intervals demonstrated a time-dependent inhibition of Kir3.2* currents, reaching a maximum (54.44 ± 19.20% inhibition) at 100 min from −11.68 ± 2.80 to −6.07 ± 1.40 μA (p < 0.01) at a test potential of −80 mV (Fig. 1, A and C). Further
treatment up to 400 min did not elicit additional inhibitory effects (Fig. 1C).

Therefore, the effect of NaHS was observed and compared when oocyte groups were treated for 100 min in successive experiments. To explore whether the NaHS effect was direct on the Kir3.2 protein, we attempted unsuccessfully to employ the Tag-switch assay (for description see under “Experimental procedures”). To test for possible changes in cell-surface localization induced by NaHS, an HA-tagged Kir3.2 was used in a chemiluminescence assay adopted to provide high sensitivity at the single oocyte level (see “Experimental procedures”) (25, 26).

NaHS did not alter significantly the cell-surface localization compared with untreated controls (Fig. 1D).

**NaHS inhibits Kir3.2 channel activity in CHO-K1 cells by altering the channel-gating kinetics**

Although NaHS inhibition of Kir3.2* channels in *Xenopus* oocytes took over 100 min, the same effect was observed in mammalian cells within 300 s (Fig. 2, A–C). Thus, whole-cell patch-clamp recording of Kir3.2* channels expressed in CHO-K1 cells showed that a 300-s treatment with 100 μM NaHS decreased currents by ~40%, from ~52 ± 11 to ~31 ±
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8.5 pA/pF \((n = 8, p < 0.01)\) at a test potential of \(-120\) mV. NaHS also inhibited currents mediated by wildtype Kir3.2 channels by \(\sim 60\%\) from \(-11 \pm 6\) to \(-4.5 \pm 3\) pA/pF \((n = 10, p < 0.01; \text{Fig. 2B})\). Consistent with the notion that \(\text{H}_2\text{S}\) is a cell-permeable signaling molecule, treatment with NaHS inhibited Kir3.2* channel currents in a time-dependent manner, with the maximal effect observed at \(\sim 300\) s (Fig. 2C). The inhibition remained stable, even when NaHS was washed out by the control bath solution. Next, we sought to determine whether \(\text{H}_2\text{S}\) decreased Kir3.2 currents by reducing the number of channels at the cell surface, the conductance of each channel, or the probability that a single channel would open. To assess the effect of NaHS on the number of Kir3.2 channels at the cell membrane, we expressed CFP-tagged Kir3.2 in CHO-K1 cells and monitored cyan fluorescence by total internal reflection fluorescence (TIRF) microscopy. Although the number of CFP pixels decreased by \(\sim 15\%\) during a 600-s treatment with 100 \(\mu\text{M}\) NaHS, the change could be attributed to photobleaching of the fluorophore, as it also occurred in cells that were only exposed to control solutions (Fig. 2D). Next, the conductance and open probability of Kir3.2* channels were assessed in CHO-K1 cell-attached patches. A 300-s treatment with 100 \(\mu\text{M}\) NaHS did not change the conductance of single channels \((\sim 30\) pS) studied at an effective test potential of \(-120\) mV (Fig. 2E and F, and Table 1). However, treatment with NaHS did decrease the open probability of Kir3.2* channels by \(\sim 40\%\) from 0.101 to 0.062 (Table 1). Studying the time that single channels dwell in the open or closed state showed that the decrease in open probability resulted from a diminution in the time that Kir3.2* channels spent open and an increase in the duration between channel openings (Fig. 2G and Table 1).

### Table 1

| Single channel parameters from cell-attached recordings of Kir3.2* expressed in CHO-K1 cells | Control | 100 \(\mu\text{M}\) NaHS |
|----------------------------------------|--------|----------------------|
| \(n\) | 88,440 | 86,625 |
| Amplitude, pA | 1.20 \pm 0.26 | 1.18 \pm 0.25 |
| Half-width, ms | 0.44 \pm 0.29 | 0.27 \pm 0.17* |
| Inter-event interval, ms | 3.52 \pm 0.65 | 8.18 \pm 0.5** |
| \(\mu\) | 0.101 | 0.062 |
| \(\gamma, \text{pS}\) | 30 | 29.5 |

#### Inhibition of other Kir3 and Kir2 channels but activation of cardiac Kir6 channels by NaHS

Similar to hydrogen sulfide-mediated inhibition of Kir3.2* channels, NaHS treatment diminished currents from oocytes expressing cardiac Kir3.4 channels, both homomeric Kir3.4* \((^{*} \text{denotes S143T, a mutant shown to boost Kir3.4 activity})\) (27), heteromeric Kir3.4/Kir3.1 channels (3A), and Kir2.3 channels, another Kir subfamily member (Fig. 3B). Interestingly, Kir2.1 activity was unaffected by NaHS treatment (Fig. 3B). Conversely, NaHS treatment augmented the activity of the cardiac Kir6.2 channel, consistent with previous experiments on the vascular Kir6.1 channel, when it was either co-expressed with SUR2A subunits or expressed alone as the recombinant Kir6.2Δ36 channel (Fig. 3C) in *Xenopus* oocytes. Kir6.2Δ36 refers to a C-terminal truncation mutant of Kir6.2 rendering these channels active as homomers (28) by removing a 3-aa motif (25) that serves as an endoplasmic reticulum retention signal.

#### NaHS inhibition of Kir3.2* channels is dependent on channel–PIP2 interactions

A common feature of Kir channels is that they all require the membrane phospholipid PIP2 to maintain their activity (29). The strength of Kir–PIP2 interactions determines the sensitivity of Kir channels to several regulatory factors such as pH, protein kinase C, and G-proteins (30, 31). Kir2.1 channels, with the highest apparent affinity to PIP2 among the channels tested, were unaffected by NaHS treatment, suggesting that the extent of the NaHS effect may depend on the strength of channel–PIP2 interactions. To test this hypothesis, we first altered cytoplasmic PIP2 levels and monitored NaHS-mediated inhibition of Kir3.2* channels. First, NaHS-mediated inhibition of Kir3.2* channels diminished when the intracellular pool of PIP2 was increased through the co-expression of PIP5K, a kinase that phosphorylates phosphatidylinositol 4-phosphate, into PI(4,5)P2 (Fig. 4A). Conversely, depleting intracellular levels of PIP2 through co-administration of wortmannin (Wort), a known blocker of PI4Ks at micromolar concentrations, permitted greater inhibition of Kir3.2* currents by NaHS (Fig. 4A). Furthermore, the NaHS-mediated inhibition was enhanced by Ci-VSP (voltage-activated PIP5 phosphatase)-dependent inhibition of Kir3.2* channels (Fig. 4B), another approach used to deplete the intracellular pool of PIP2. Consistent with these results, bolstering Kir3.2*–PIP2 interactions by co-expressing the G-protein subunit, G\(\beta\gamma\), also attenuated NaHS inhibition of the Kir3.2* current (Fig. 4C). Next, we tested what would happen to the extent of NaHS-mediated inhibition when Kir3.2 channels themselves were altered intrinsically using known point mutations that increase channel–PIP2 interactions. For example, the E152D point mutation in Kir3.2* was shown previously to strengthen channel–PIP2 interactions (30), and thus it was not surprising to see that NaHS had a greater inhibitory effect on wildtype Kir3.2 homomorphic channels with a weaker apparent PIP2 affinity than Kir3.2* channels (Fig. 4D), results consistent with those obtained in the CHO-K1 cells (see Fig. 2B). Moreover, the inhibitory effect of NaHS was virtually abolished when the point mutation (I1234L) in the background of Kir3.2* channels further strengthened the apparent channel–PIP2 affinity (Fig. 4D).

#### NaHS inhibits Kir2.3 in macropatch recordings at micromolar concentrations

NaHS incubation of whole oocytes expressing Kir channels led to significant inhibition of various Kir2.x and Kir3.x channel activities, although it stimulated Kir6.2 activity. We proceeded to apply NaHS to the cytoplasmic side of excised inside-out patches expressing Kir channels to examine directly \(\text{H}_2\text{S}\) effects, isolating the channel and other membrane-delimited elements from the rest of the cellular cytoplasm. We used Kir2.3, whose relative phosphoinositide-interaction profile has been characterized as moderate in previous experiments, thus yielding reasonable currents in excised patches. Kir2.3 was inhibited by NaHS in TEVC recordings (Fig. 3B). First, we tested the effect of applying NaHS at the micromolar range of
concentrations and chose to use a single (sub-maximal) concentration of soluble PIP2, diC8 (25 μM), to compare the relative NaHS effects. Not surprisingly, Kir2.3 channel activity was inhibited (Fig. 5, A and B) by NaHS in a dose-dependent manner (IC50 82.95 μM), and channel activity was maximally inhibited by 64%. Interestingly, NaHS also elicited dose-dependent inhibition of Kir2.3 when the channels were maximally activated with long chain PIP2 (Fig. 5C), but it showed a lesser extent of inhibition (37% maximal inhibition) than when Kir2.3 channels were activated by 25 μM diC8 PIP2 (see Fig. 5, A and B), consistent with the decrease of NaHS effects when channel–PIP2 interactions are strengthened, as seen in the whole oocyte experiments (Fig. 4, A, C, and D). Conversely, we performed dose-response measurements of PIP2 before and after inhibition by NaHS (100 μM, close to IC50 in Fig. 5, A and B) to examine how NaHS treatment might affect channel–PIP2 interactions. We determined that NaHS treatment to the patch (Fig. 5, D and E) right-shifted the PIP2 dose-response curve, decreasing the apparent affinity of PIP2 demonstrated by the increase of EC90 from 6.20 to 9.60 μM. Interestingly, direct NaHS treatment dramatically reduced the efficacy of PIP2, indicated by an ~29% reduction in maximal activity following NaHS treatment.

**Figure 3.** H2S effects on Kir channel activity. A, H2S inhibits cardiac Kir3.4 (S143T) homomeric channel and Kir3.4/Kir3.1 heteromeric channel activity. Treatment with NaHS led to ~42% inhibition of Kir3.4* channels (0.58 ± 0.20, n = 13) and ~67% inhibition of Kir3.4/Kir3.1 channels (0.33 ± 0.14, n = 10). B, treatment with NaHS significantly inhibited Kir2.3 channels (0.64 ± 0.27, n = 17) but did not significantly alter Kir2.1 channel activity (1.01 ± 0.24, n = 13). C, currents from both groups of oocytes expressing Kir6.2/SUR2A or Kir6.2Δ36, in which the 36 C-terminal amino acids were removed from Kir6.2 to permit cell-surface expression in the absence of sulfonylurea receptor (SUR2A) subunits, increased ~6-fold (normalized current values of 6.39 ± 1.82, n = 12, and 6.48 ± 3.96, n = 9, respectively) upon incubation with 2 mM NaHS, consistent with hyperpolarizing findings in Mustafa et al. (13), in which Kir6.1 was expressed in HEK293 cells. Unpaired Student’s t test was used to assess the significant differences between NaHS-untreated (control) and NaHS-treated groups. * indicates comparison to the untreated group. Single, double, and triple symbols indicate p < 0.05, p < 0.01, and p < 0.001, respectively. N.S., non-significant.

**NaHS inhibition of Kir3.2* channels is dependent on specific cytoplasmic cysteine residues**

H2S is a gasotransmitter that has been shown to signal via sulphydrylation, a post-translational modification of reactive cysteine residues and conversion of the Cys–SH group to an –SSH group. It was previously shown that NaHS is able to sulfhydrate a specific cysteine residue, of vascular KATP channels (Kir6.1), leading to eventual hyperpolarization and vasodilation of superior mesenteric arteries, and its substitution with serine (C43S) abolished the NaHS effects (13). This specific cysteine residue is conserved throughout Kir subfamily members and is positioned adjacent to a positively charged cytoplasmic residue shown in the Kir3.2 crystal structure to interact electrostatically with one of the negatively charged phosphate groups of PIP2, critical for channel gating (32). In the cytoplasmic region of the Kir3.2 channel, there are three putative cysteine residues Cys-190, Cys-221, and Cys-321 that may be sulfhydrated in addition to Cys-65, the conserved cysteine residue corresponding to Cys-43 in Kir6.1 channels (Fig. 6A). In addition to the four cytoplasmic cysteine residues, Kir3.2 channels also have two extracellular cysteine residues, Cys-134 and Cys-166, located in the extracellular loop region and involved in intersubunit disulfide...
A functional Kir3.2* “Cys-less” mutant lacking all four cytoplasmic cysteine residues characterized in previous experiments (33) was refractory to NaHS inhibition (0% inhibition) (Fig. 6B). The NaHS effect (~37% inhibition) was partially restored by reintroducing individual cysteines to the Kir3.2* Cys-less mutants at positions 65 (~18% inhibition) or 321 (~25% inhibition) (Fig. 6B). The NaHS effect on Kir3.2* was fully restored through reintroduction of both specific cysteines, 65 and 321 (Fig. 6B). The raw currents (non-normalized) for this experiment are shown in a supplemental figure (Fig. S1).

CSE co-expression mimics exogenous NaHS effects on Kir3.2* channels

CSE is an enzyme central to the production of H2S in cardiac tissue and specific regions of the brain (hippocampus and striatal neurons) in physiological and pathophysiological conditions (2, 3), and therefore it is most likely to be co-expressed in neurons with Kir3.2 channels and Kir2.x channels (as well as Kir3.4 channels in cardiac cells). Among the three enzymes that produce H2S, CSE seems to be the most inducible and is mod-
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regulated in diverse conditions such as inflammation mediated by tumor necrosis factor and lipopolysaccharides, metabolites such as glucocorticoids and glucose, as well as dietary restriction and endoplasmic reticulum stress thereby making it an attractive therapeutic target (2, 3). Co-expression of CSE with Kir3.2* in Xenopus oocytes recapitulated the inhibition of Kir3.2* produced by exogenously applied H₂S (NaHS), in which increasing the cRNA amount co-injected (ranging from 0.5 to 8.0 ng) resulted in a dose-dependent inhibition of Kir3.2* currents (Fig. 8A). Acute treatment with 5 mM propargylglycine (PAG) for 100 min, an inhibitor of CSE activity, reversed CSE inhibition of Kir3.2*, whereas it did not affect significantly Kir3.2* channels expressed in the absence of CSE (Fig. 8B). The Cys-less Kir3.2* channel, refractory to exogenous NaHS treatment, was also unaffected by CSE co-expression (Fig. 8C).

Molecular dynamics experiments show sulfhydration of specific cysteine residues alters channel gating and interactions with PIP₂

To gain a better mechanistic understanding about how sulfhydration of a specific cysteine residue (the functional effects demonstrated experimentally in Fig. 6B) may affect channel–PIP₂ interactions as well as channel gating, and because we could not biochemically demonstrate using the Tag-switch assay direct sulfhydration upon NaHS exposure, we turned to molecular dynamics (MD) simulations with Kir3.2 co-crystallized with PIP₂ (PDB code 3SYQ) to model the sulfhydration-induced Kir channel inhibition (32, 34). More specifically, we induced sulfhydration of three cysteine residues, Cys-65, Cys-321 (positive effects in Fig. 6B), or Cys-221 (no effect in Fig. 6B) and performed a 25-ns MD simulation run to observe whether dynamic changes occurred to (a) the G-loop gate, integral for Kir3.2 channel gating, and (b) the channel–PIP₂ interactions. First, we surveyed the minimal distances between one of the two phosphates on the PIP₂ inositol ring and the lysine residue, Lys-64, previously characterized as critical for Kir3.2-PIP₂ channel interactions (32), and we observed an increase in the distances when Cys-65 (red) or Cys-321 (gold) residues were sulfhydrated but not when Cys-221 (green) was sulfhydrated (Fig. 9A). Snapshots were taken at the times during the simulations when the distance between Lys-64 and PIP₂ was the largest. Dashed lines in the model snapshots taken from the molecular dynamics experiment detail how the Lys-64 residue moved away from the phosphate on the PIP₂ headgroup when Cys-65 or Cys-321 was sulfhydrated (Fig. 9B). Consistent with these

Figure 5. Hydrogen sulfide inhibition of Kir2.3 involves reductions in potency and efficacy of PIP₂ activation and requires micromolar concentrations of NaHS in macropatches. A, representative concentration-response experiment of NaHS in the presence of 25 μM diC₈ PIP₂. B, H₂S inhibits Kir2.3 channel activity in macropatches activated submaximally by soluble PIP₂ (25 μM diC₈). Direct administration of varying concentrations of NaHS to the cytoplasmic surface of inside-out patches expressing Kir2.3 channels leads to concentration-dependent inhibition of Kir2.3 channel activity (IC₅₀ = 82.55 μM and A₁ value = 0.36) when they are pulsed (activated) by 25 μM soluble dioctanoyl PIP₂ (diC₈), C, H₂S also inhibits Kir2.3 channel activity in macropatches maximally activated by LC-PIP₂ (full-length PIP₂), but to a lesser extent than 25 μM diC₈ pulses (Fig. 3A). Direct administration of varying NaHS concentrations to the cytoplasmic surface of inside-out patches expressing Kir2.3 channels led to concentration-dependent inhibition of Kir2.3 channel activity (IC₅₀ = 73.21 μM and A₁ value = 0.71) when they were activated by full-length PIP₂. D, representative experiment (summarized in E) of Kir2.3 expressing inside-out patches subject to various diC₈ PIP₂ concentrations (0, 10, 25, 50, 100, 200, and 400 μM) before and after exposure to NaHS (100 μM) for 200 s. E, summary data of three experiments such as that shown in D showing that H₂S (100 μM NaHS) reduces the efficacy (increased EC₅₀ values from 6.20 μM to 9.60 μM) and potency (A₂ value decreases from 1.04 to 0.74) of Kir2.3 channel activation by soluble PIP₂ (diC₈).
observations, scoring the average channel–PIP2 channel interactions (the limit was specified as being within 0.7 nm) revealed that sulfhydration of Cys-65 or Cys-321 (and not Cys-221) residues decreased interactions between the PIP2 and the channel (Table 2). We then sought to determine whether sulfhydration affects channel gating, and we monitored the minimal distance of the G-loop gate between the α carbons of Thr-317 on opposite Kir3.2 subunits. We observed a substantial decrease when the Cys-65 or Cys-321 (but not the Cys-221) residue was sulfhydrated (Fig. 9C). The change in G-loop gate conformation is highlighted by model snapshots (Fig. 9D) of the Thr-317 α carbon moving from no sulfhydration (gray) to Cys-65 (red), Cys-221 (green), or Cys-321 (yellow). These snapshots reflect the same time points during the simulations that gave the largest distances between Lys-64 and PIP2 (as was shown in Fig. 9A). In stark contrast, we observed an increase in channel–PIP2 interactions when a homology model of Kir6.2, based on the Kir3.2 structure (PDB code 3SYQ), was subjected to sulfhydration at Cys-42 (homologous to Cys-65 in Kir3.2) (Fig. 10 and Table 2). The sulfhydration of the Cys-42 also led to increases in distance between the analogous α carbons (Thr-294) in an MD simulation, suggesting that the G-loop gate opened (Fig. 10A), highlighted by the shift of Thr-294 α carbons (red) away from the non-sulfhydrated Kir6.2 (gray) (Fig. 10B).

Discussion

The “dual-functional effect” exerted by hydrogen sulfide is exemplified by neuronal versus cardiovascular cells that are deprived of oxygen. H2S mediates ischemic damage pursuant to stroke in neuronal tissue (15), whereas it serves as a powerful cardioprotective agent upon comparable conditions. One categorical correlation that emerged in the studies highlighting this “dual-physiological effect” by the gaseous mediator is that while H2S depolarizes cerebral components (8–12), it hyperpolarizes cardiomyocytes (13, 14). In other words, H2S increases the excitability of cerebral tissue, worsening seizure-like symptoms and rendering neurons more susceptible to ischemic insult (12, 15). In contrast, H2S depresses the excitability of the heart, in which H2S is cardioprotective in ischemia and reperfusion (I/R) injury models when administered either through a pre-I/R (16, 18, 19) or post-I/R (20) treatment regime. The link between the effect of H2S on tissue excitability and the clinical phenotype is especially evident when the blocking of a single ion channel, KATP, by glibenclamide abrogated the cardioprotective effects H2S had on the I/R injury models (19, 20). Thus, subsequent efforts have been made to identify key ion channels that contribute to the depolarizing effect of H2S on neuronal cells. To date, the identity of H2S-sensitive ion channels in specific neuronal tissues has remained elusive (8–12). Relying on ion channel
blockers with limited specificity has made it difficult to reliably correlate the effect of H\textsubscript{2}S on observed depolarizations in neurons to specific ion channels.

Here, we show for the first time that NaHS inhibits Kir2.3 and Kir3.3 channels expressed heterologously in Xenopus oocytes and CHO-K1 cells (Figs. 1 and 2), although it activates the closely related cardiac and pancreatic subunit of the K\textsubscript{ATP} channel subtype, Kir6.2 (confirming previous experiments to show the effect H\textsubscript{2}S has on other Kir6.x subtypes) (Fig. 3). The significant inhibition of Kir2.x and Kir3.x channels is surprising given their homology to Kir6.x and is consistent with the depolarizations observed in NaHS-treated native cerebral components. As with other known modulators of Kir channel activity, we find that the effect of H\textsubscript{2}S on Kir channels proceeds by altering the strength of channel–PIP\textsubscript{2} interactions. Bolstering channel–PIP\textsubscript{2} interactions (Fig. 4, A, C and D) reduces the effect of H\textsubscript{2}S on Kir channels, whereas Kir channels became more susceptible to H\textsubscript{2}S when channel–PIP\textsubscript{2} interactions were weakened (Fig. 4, A and B). These results are all consistent with our excised macropatch findings showing that exposure to NaHS decreases PIP\textsubscript{2} potency and efficacy in activating Kir channels. We also show here that the inhibitory effect of H\textsubscript{2}S on the Kir3.2\textsuperscript{+} channel is mediated by two of four cytoplasmic cysteine residues, Cys-65 and Cys-321 (but not Cys-190 and Cys-221) in each of four channel subunits that form tetrameric Kir3.2 channels. The substitution of all four cytoplasmic cysteines abolished the inhibitory effect of H\textsubscript{2}S (Fig. 6B and Fig. S1), without altering the strength of channel–PIP\textsubscript{2} interactions (Fig. 7). The reintroduction of cysteines into positions 65 or 321 in the background of the Kir3.2\textsuperscript{+} Cys-less mutant partially restored H\textsubscript{2}S-mediated inhibition, and reintroduction of both cysteines fully restored the extent of H\textsubscript{2}S-mediated inhibition (Fig. 6B and Fig. S1). Our computational MD simulation experiments that modeled the structural changes upon sulfhydration of the Kir3.2 channel provided predictions that are in agreement with the experimental data. Direct sulfhydration of specific cysteines, Cys-65 and Cys-321 (and not Cys-190), led to increased distances between the Lys-64 residue and PIP\textsubscript{2} phosphates (Fig. 9A.

**Figure 7. Strength of channel–PIP\textsubscript{2} interactions is similar between Kir3.2\textsuperscript{+} and Kir3.2\textsuperscript{+} (Cys-less) channels.**

A, voltage protocols (in mV) used for inhibition (top) and recovery (bottom) of Kir3.2\textsuperscript{+} currents by activation and deactivation of co-expressed Ci-VSP, respectively. B, Kir3.2\textsuperscript{+} and Kir3.2\textsuperscript{+} (Cys-less) displayed faster current inhibition compared with Kir3.2\textsuperscript{+}(I234L). C, values for inhibition for Kir3.2\textsuperscript{+} of 2.56 ± 0.147 s and Kir3.2\textsuperscript{+}(Cys-less) of 2.78 ± 0.09 s were not significantly different. However, both r values were significantly less than that of Kir3.2\textsuperscript{+}(I234L) of 10.1 ± 1.76 s. One-way repeated-measures ANOVA and Holm-Sidak post-test (p < 0.05) were used to assess for statistical significance; ***, p < 0.001 compared with control. D, Kir3.2\textsuperscript{+} and Kir3.2\textsuperscript{+} (Cys-less) showed slower recovery time than Kir3.2\textsuperscript{+}(I234L) at −80 mV. N.S., non-significant.
and Table 2) as well as changes in the G-loop gate favoring the closed conformation of the channel (Fig. 9, C and D) providing mechanistic insight into the inhibitory effect of H$_2$S.

Previous experiments have applied micromolar (5–200 μM) concentrations of NaHS, within the physiological range of reported hydrogen sulfide in mammals (15), and our experiments in the mammalian cell line CHO-K1 (Fig. 2) were consistent with effects seen with micromolar concentrations. Yet, in our two-electrode voltage-clamp (whole-cell) studies in Xenopus oocytes, 10-fold higher concentrations of NaHS (2,000 μM) were needed to elicit a significant response. We attribute this need for higher concentrations and extended treatment (for 100 min) to the expansive size of the Xenopus oocyte, as well as the stockpiling of β-globin in these cells that could serve as a major buffer for H$_2$S (35). It is noteworthy that this stringent NaHS administration led to channel-specific inhibitory effects, as H$_2$S was unable to affect Kir2.1 channel activity as well as the Kir3.2* (I234L) mutant, two channels displaying higher apparent affinities to PIP$_2$, whereas it activated (rather than inhibited) Kir6.2 channels. Still, to ensure we are not observing toxic effects of NaHS on Kir channels, we demonstrated that the inhibitory effect of NaHS on Kir3.2* channels could be mimicked by facilitating endogenous production of hydrogen sulfide through the co-expression in Xenopus oocytes of CSE, a key producer of endogenous H$_2$S in native cardiovascular and specific neuronal tissues. Perhaps the most convincing evidence that the whole-cell oocyte studies did not report an artifact was the requirement of micromolar range of NaHS concentrations when applied directly to the cytoplasmic side of Xenopus patches expressing Kir 2.3 channels (i.e. in inside-out macropatches, separating the membrane-delimited elements from the rest of the cellular cytoplasm). As expected, Kir2.3 channel activity was greatly inhibited (Fig. 5, A and B) by much lower concentrations of NaHS in a dose-dependent manner ($IC_{50}$ 82.95 μM), and channel activity was maximally inhibited by ~64%, comparable with the inhibition measured in whole oocytes incubated with high levels of NaHS (Fig. 3B). The oocyte inside-out macropatch results are also consistent with the CHO-K1 cell data in terms of the micromolar NaHS effective concentrations but are also consistent with other aspects of the results obtained in the two-electrode voltage-clamp experiments. Interestingly, maximally activating Kir3.2* channels in excised membrane macropatches with long chain PIP$_2$ (2 μM) decreased NaHS-mediated inhibition of the current (~37% maximal inhibition) compared to when Kir2.3 channels are activated by a submaximal concentration of water-soluble short-chain PIP$_2$ (25 μM diC8 PIP$_2$, Fig. 5, A and B). This finding is consistent with the diminished NaHS inhibition observed when channel–PIP$_2$ interactions were strengthened in the whole-oocyte experiments (Fig. 4). Also, PIP$_2$ efficacy and potency were weakened by NaHS treatment (Fig. 5E), as indicated by the ~29% reduction in maximal channel activity and a rightward shift of the diC8 PIP$_2$ dose-response curve, respectively, following NaHS treatment. All these electrophysiologi-
cal findings are consistent with the MD computational simulation results, in which conformational changes are induced by sulfhydration of cysteine residues, leading to weakened channel–PIP2 interactions and the closing of the G-loop gate (Fig. 9). Interestingly, our computational predictions are also in agreement with the activation of Kir6.2 by H2S (Fig. 3C), showing that sulfhydration of Cys-42 (works in a way opposite to modification of cysteines in the Kir3.2 model) in a homology model of Kir6.2 (based on the Kir3.2 structure) leads to strengthening of channel–PIP2 interactions and opening of the G-loop gate (Fig. 10).

We demonstrate the effect of H2S on Kir2.x and Kir3.x is inhibitory, in the opposite direction of the previously characterized Kir6.x channel activation despite their homology. The inhibition of Kir2.x and Kir3.x channels by H2S could be a critical factor in the NaHS-mediated depolarization of neuronal cells. The activation of Kir channels (Kir6.) is expected to limit the excitability of cardiomyocytes or colonic smooth muscle cells (36). Although our findings provide a potential explanation for H2S-mediated depolarization of neuronal cells, they do not rule out a role for effects of H2S on other ion channels or membrane receptors. Future studies evaluating the collective effect of H2S on specific native brain tissues will need to at least
address the differential expression of Kir channels (37). One explanation as to why H2S raises the excitability of specific tissues may be because its depolarizing influence via inhibition of Kir2.x and Kir3.x channels is simply greater than its opposing influence on Kir6.x channels (whereas the hyperpolarizing influence of H2S dominates in specific tissue cell types, like native cardiomyocytes and smooth muscle cells).

Here, we show that the inhibitory effect of H2S is PIP2-dependent. Thus, increasing the strength of channel–PIP2 interactions limits H2S-mediated inhibition of Kir2.x and Kir3.x channels while weakening channel–PIP2 interactions augments H2S inhibition. Our work identifies new physiological targets of H2S and offers mechanistic insights into the inhibition of Kir channels by H2S.

Experimental procedures

Materials

diC8 forms of PI(4,5)P2 were purchased from Echelon (Salt Lake, UT). They were dissolved in water to make 6.0 mM stock solutions, which were divided into aliquots and stored at −80 °C. Further dilutions were made in bath solutions on the day of the experiment. LC arachidonyl-stearyl PI(4,5)P2 was purchased from Avanti (Alabaster, AL). Aqueous stock and working solutions were prepared and sonicated as described (38, 39). NaHS was purchased from Strem (Newburyport, MA) and dissolved in water to make 100 mM stock solutions, which were divided into aliquots and stored at −20 °C. Further dilutions were made in incubation solution on the day of the experiment. DL-PAG was purchased from Sigma, was stored at 100 mM stocks, and was used at 5 mM working concentrations. Wortmannin was purchased from Tocris (Bristol UK) and dissolved in DMSO from Sigma to a stock concentration of 20 mM, stored at −80 °C, and was diluted to a 5 μM working concentration in experiments.

Molecular biology

A Cys-less mutant (C65V, C190S, C221S, and C321V) of Kir3.2C (33) was provided by the Slesinger Laboratory (Icahn School of Medicine at Mount Sinai, New York). Because Kir3.2C (425 aa) is a longer splice variant with an additional 11 aa at the C terminus, we generated a Cys-less Kir3.2 (414 aa) isoform by PCR. The Kir3.2(E152D) (referred to as Kir3.2*) mutation that boosts homomeric currents (Yi et al. (24)) was introduced, and the resulting Kir3.2* (Cys-less) channel was subcloned in the oocyte and mammalian expression vector pXoom, so that it may be compared with the Kir3.2* subtype. Desired point and double point mutations were introduced by the commercial QuikChange (Agilent Technologies) method. All mutations were confirmed by DNA sequencing (Genewiz). CSE constructs in pcDNA3 were cut with KpnI and EcoRI and subcloned into the pGEMHE vector.

X. laevis oocyte expression

All pGEMHE constructs were linearized using the Nhel restriction enzyme, whereas pXoom constructs were linearized using the Xhol restriction enzyme and in vitro–transcribed using the mMessage mMachine® kit (Ambion) kit. Complementary RNA (cRNA) concentrations were quantified by optical density. Xenopus oocytes were surgically extracted, dissociated, and defolliculated by collagenase treatment and microinjected with 50 nl of a water solution containing the desired cRNAs. The use of X. laevis frogs for this study was approved by the IACUC at Virginia Commonwealth University. All constructs used in this study were injected to achieve between 5 and 20 ng per oocyte, depending on the channel and co-injection of CSE, Ci-VSP, or PIP5K cRNA. Oocytes were incubated for 2–4 days at 18 °C.

Kir3.2 surface localization assay in Xenopus oocytes

Oocytes expressing Kir3.2, in which the HA epitope was inserted in the extracellular loop between Ile-126 and Glu-127 (26), were incubated for 100 min in the presence and absence of 2 mM NaHS, in dishes sealed with Parafilm. At the end of this incubation, both groups of oocytes were transferred to OR2(+) medium (5 mM KCl, 5 mM NaOH, 1 mM MgCl2, 1.8 mM CaCl2, 82.5 mM NaCl, 5 mM HEPES, adjusted to pH 7.4, 200 mosM)
containing 1% bovine serum albumin (BSA) and 0.5 μg/ml rat anti-HA high affinity antibody 3F10 (Roche Applied Science, catalog no. 11 867 423 001) and incubated for 2 h at room temperature under gentle shaking. Oocytes were then extensively washed (OR2+/BSA solution), incubated with an HRP-coupled goat anti-rat secondary antibody (KPL, catalog no. 474-1612, 2 μg/ml in OR2+/BSA) for 1 h at room temperature, washed as above, and transferred to OR2(+) without BSA. Individual oocytes were placed in 96-well plates (one oocyte/well) and incubated at room temperature for 2 min with 120 μl of SuperSignal Elisa Pico chemiluminescent substrate (Thermo Fisher Scientific, catalog no. 37070). Chemiluminescence intensity for each oocyte was quantified in a POLARStar OMEGA plate reader (BMG*LABTECH) and expressed as a percentage of the mean chemiluminescence intensity obtained for the non-NaHS-exposed oocytes. Not injected oocytes, subjected to the same treatment as above, served as a negative control. Surface labeling for un-injected oocytes was less than 0.5%.

**Electrophysiology**

Whole-oocyte currents were measured by conventional TEVC with a GeneClamp 500 amplifier (Axon Instruments) or TEC-03X (NPI) amplifiers. Agarose cushion microelectrodes were filled with 1.5% (w/v) agarose in 3 M KCl and were used with resistances between 0.1 and 1.0 mehm. Oocytes were held at 0 mV, and currents were assessed by 800-ms ramps from −80 to +80 mV. Currents at −80 mV were recorded. Barium-sensitive basal currents were defined as the difference between the steady-state currents while perfusing a high-potassium (HK) solution or a barium solution. The HK solution contained the following: 96 mM KCl, 1 mM NaCl, 1 mM MgCl₂, 5 mM KOH/HEPES, pH 7.4. The barium solution consisted of HK + 3 mM BaCl₂. Five to 20 oocytes from the same batch were recorded for each group, and the experiments were repeated in at least two batches. A specialized voltage protocol was designed to activate Ci-VSP submaximally when they were co-injected in several experiments, whereby Ci-VSP was activated at +10 mV, and current levels were monitored by brief pulses to −80 mV. Recovery was analyzed by holding at −80 mV following a 1-min +80-mV depolarization protocol that fully inhibited channel current. Data acquisition and analysis were carried out using pClamp9 (Molecular Devices) and OriginPro (Microcal) software.

**NaHS treatment in TEVC experiments**

Channels were expressed in X. laevis oocytes. After ~72 h of expression, the Vehicle group of injected oocytes was transferred to a Petri dish containing 2 mM NaCl and recorded subsequently added to HK solution, whereas the +NaHS group was transferred to a Petri dish containing 2 mM NaHS added to HK, sealed with Parafilm, and left to incubate for 100 min prior to recording. Barium-sensitive basal currents from both groups, 1) Vehicle and 2) +NaHS in the presence of HK solution, were assessed at −80 mV using two-electrode voltage clamp. Barium-sensitive currents were normalized to average basal current of vehicle. All experiments were tested in at least two batches.

**Wortmannin treatment and recording in TEVC experiments**

Channels were expressed in X. laevis oocytes. After ~72 h of expression, the Vehicle group of injected oocytes was transferred to a Petri dish containing 2 ml of HK and 5 μl of DMSO. The +Wort group of injected oocytes was transferred to a Petri dish containing 2 ml of HK and 5 μl of 10 mM solution of Wort (in DMSO). Oocytes (5–10) from Vehicle and +Wort oocytes were then assayed as described above under "Electrophysiology" (TEVC), 120 min following incubation. Oocytes from +Wort dish were then transferred to solution containing same solution as the Vehicle group ( +5 μl of DMSO in HK).

Subsequently, NaHS was added to both groups for 100 min at 2 mM working concentration (as described) and assayed through TEVC recordings.

**Inside-out macropatch**

The vitelline membrane was manually peeled using forceps to gain access to the oocyte plasma membrane. Pipettes were pulled using a Flaming-Brown micropipette puller and then fire-polished to give a final resistance of 0.8 to 1.2 megohms. Currents were acquired at 10 kHz and filtered at 2 kHz using pClamp software and the Axopatch 200A amplifier (Axon Instruments). Inward currents were monitored using a ramp protocol from −100 to +100 mV, with the holding potential at 0 mV. Following giga-seal formation, the pipette was pulled away from the membrane to achieve an inside-out patch and perfused directly using a multibarrel gravity-driven perfusion apparatus. Pipette solution contained the following (in mM): 96 KCl, 10 HEPES, 1.8 CaCl₂, 1 MgCl₂, 1 NaCl, pH 7.4. Bath solution contained the following (in mM): 76 KCl, 20 NaCl, 5 EGTA, 10 HEPES, pH 7.4.

**NaHS treatment in macropatch experiments**

**NaHS dose-response curve (diC8 PIP₂)—**Once the inside-out patch (technique described above) expressing Kir2.3 channels was isolated and once the channel activity ran down, the patch was subjected to 25 μM diC8 PIP₂ until the current reached a steady state, after which the patch was subjected to NaHS treatment in pipette solution for 200 s and reactivated by diC8 PIP₂. Subsequently, the patch was subjected to various concentrations of NaHS for a 200-s duration, and currents produced by subsequent 25 μM diC8 PIP₂ pulses were recorded and compared.

**NaHS dose-response curve (LC-PIP₂)—**We used the same technique as we did to generate the NaHS dose-response curve pulsed by 25 μM diC8 PIP₂, but now we applied ~10 μM solubilized long-chain PIP₂ until currents reached steady state and then we subjected them to varying concentrations of NaHS following 200-s intervals.

**diC8 PIP₂ dose-response curve—**Inside-out patches expressing Kir2.3 channels were allowed to run down and were pulsed by varying diC8 PIP₂ concentrations, and then the same patch was exposed to 100 μM NaHS for 200 s and pulsed by the same range of diC8 PIP₂ concentrations.

**Culture of CHO-K1 cells**

CHO-K1 cells (RRID: CVCL_0214) were purchased from ATCC, identity-authenticated by cytochrome oxidase one
analysis, demonstrated to be mycoplasma-free by Hoechst DNA stain and agar by culture, and maintained in F12K medium supplemented with 10% FBS (ATCC). Plasmids were transfected into cells with Lipofectamine 2000 according to the manufacturer’s instructions (Thermo Fisher Scientific). CHO cells for patch-clamp studies were co-transfected with eGFP for identification and studied 24–36 h post-transfection at room temperature.

**Patch-clamp recording**

Whole-cell currents were recorded with an Axopatch 200B amplifier and pCLAMP software (Molecular Devices, Sunnyvale, CA) at filter and sampling rates of 5 and 25 kHz, respectively. CHO-K1 cells were bathed in a solution containing (in mM): 130 NaCl, 4 KCl, 1.2 MgCl₂, 2 CaCl₂, and 10 HEPES; pH was adjusted to 7.4 with NaOH. Recording electrodes were fabricated from borosilicate glass (Warner, Hamden, CT) and fire-polished to have resistances of 3–4 megohms when filled with a solution containing (in mM): 130 KCl, 4 KCl, 5 EGTA, 5 K₂ATP, and 10 HEPES; pH was adjusted to 7.4 with KOH. The whole-cell capacitance of CHO cells was 7–12 pF; series resistance was typically <5 megohms, and the voltage-error of <3 mV was not adjusted. Cells for study were selected based on eGFP fluorescence. Currents were evoked from CHO cells with 500-ms test pulses from −120 to −20 mV from a holding potential of −80 mV, with 10-mV increments. To assess the time dependence of NaHS block, currents were assessed by 500-ms test pulses to −120 mV, from a holding potential of −80 mV every 10 s.

Single Kir3.2⁺ channels were studied in cell-attached patches from cells perfused with the bath solution described above. Recording electrodes were fabricated to a resistance of 8–9 megohms and were also filled with bath solution. Channels were studied at +40 mV, to give an effective membrane potential of −120 mV and a driving force of −40 mV. All the experiments were performed at room temperature, and all patch-clamp data were analyzed in pClamp (Molecular Devices) and OriginLab software (Northampton, MA).

**TIRF microscopy**

Single protein particle complexes at the surface of live CHO cells were identified and studied by TIRF microscopy as described previously (30). Briefly, the critical angle for TIRF was 120 mV, from a holding potential of −120 mV and a driving force of −40 mV. All the experiments were performed at room temperature. TIRF microscopy data were analyzed in pClamp (Molecular Devices, Sunnyvale, CA) at filter and sampling rates of 5 and 25 kHz, respectively. CHO-K1 cells were bathed in a solution containing (in mM): 130 NaCl, 4 KCl, 1.2 MgCl₂, 2 CaCl₂, and 10 HEPES; pH was adjusted to 7.4 with NaOH. Recording electrodes were fabricated from borosilicate glass (Warner, Hamden, CT) and fire-polished to have resistances of 3–4 megohms when filled with a solution containing (in mM): 130 KCl, 4 KCl, 5 EGTA, 5 K₂ATP, and 10 HEPES; pH was adjusted to 7.4 with KOH. The whole-cell capacitance of CHO cells was 7–12 pF; series resistance was typically <5 megohms, and the voltage-error of <3 mV was not adjusted. Cells for study were selected based on eGFP fluorescence. Currents were evoked from CHO cells with 500-ms test pulses from −120 to −20 mV from a holding potential of −80 mV, with 10-mV increments. To assess the time dependence of NaHS block, currents were assessed by 500-ms test pulses to −120 mV, from a holding potential of −80 mV every 10 s.

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**Preparation of MSBT**

Periodic acid (1.3 g, 5.80 mmol) was dissolved in CH₃CN (15 ml) by vigorous stirring at room temperature for 1 h. Then CrO₃ (27.5 mg, 0.27 mmol, 10 mol %) was added to the solution. The mixture was stirred at room temperature for 5 min to give a clear orange solution. The H₅IO₆/CrO₃ solution was then added dropwise over a period of 45 min to a solution of 2-(methylthio)benzo[π]thiazole (0.5 g, 2.76 mmol) in ethyl acetate (30 ml) at 0 °C. After the addition was completed, the reaction mixture was stirred at room temperature for 12 h. The reaction was quenched by addition of saturated Na₂SO₃ solution (2 ml) and filtered, and the solids were washed with ethyl acetate (60 ml). The filtrate was washed, respectively, with saturated aqueous Na₂SO₃ solution and brine and then dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure. The crude reaction mixture was purified by flash chromatography (hexane/ethyl acetate) to obtain MSBT (0.470 g, 82%) as a white solid.

**Hydrogen sulfide regulation of Kir channels**

Presence of sulphhydration was attempted with a recently designed modified Tag-switch assay (5). First, Kir3.2⁺ was expressed and purified from Pichia pastoris as described previously (30). We also purchased from Sigma recombinant glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) purified from Escherichia coli. Then, ~0.6 μg/μl of each of the two proteins was exposed to 1 mM NaHS for 16 min at room temperature to trigger sulphhydration of cysteines. Next, 10 mM methylsulfonyl benzothiazole (MSBT-A) exposure for 15 min at room temperature was used to form R-S-BT and R-S-S-BT adducts at the R-SH and R-S-SH moieties of the cysteines, respectively. Excess MSBT-A was removed by Micro Bio-Spin P-6 gel columns. A combination nucleophile and reporter molecule, CN-biotin, was employed at 2 mM for 1 h at room temperature in a reaction that is highly preferential to R-S-S-BT over all other moieties, resulting in a biotin tag detectable on sulphhydrated cysteines only. Excess CN-biotin was removed by Micro Bio-Spin P-6 gel columns. We aimed to detect biotinylated purified proteins by Western blotting using anti-biotin antibodies. Unfortunately, regardless of H₂S treatment, both polyclonal (Abcam catalog no. ab53496) and monoclonal (Sigma catalog no. B7653) anti-biotin antibodies detected GAPDH (Sigma catalog no. SRE0024), a well-known H₂S target that was used as a positive control. We next turned to BSA (Sigma catalog no. A7030) that has also been used as another positive control for H₂S treatment. BSA could not be detected by the monoclonal anti-biotin antibody (even though it was detected by the polyclonal antibody), thus showing specificity for biotin over untreated BSA. Unfortunately, H₂S treatment of BSA also failed to yield detection of sulphhydrated BSA by the specific monoclonal anti-biotin antibody. Purified Kir3.2 was exposed to 1 mM NaHS, but sulphhydration of Kir3.2 anticipating a band corresponding to ~47 kDa could not be detected with either anti-biotin antibody. From these negative experiments, we concluded that the Tag-switch assay in our hands lacked the needed sensitivity to detect sulphhydration of protein targets.
Hydrogen sulfide regulation of Kir channels

(s, 3H); MS-ESI (m/z): 214 [M + H]+. Preparation of 2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d][1,2,4]triazin-4-yl)pentanamido)ethyl 2-cyanoacetate (CN-biotin). This compound was synthesized by following the procedure reported in the literature (5), and the spectral data were matched with the reported compound.

Molecular dynamics experiments

The Kir3.2* control channel or the sulfhydrated Kir3.2* (added –SH to Cys-65, Cys-221-negative control, or Cys-321) channel with four PIP2 molecules were subjected to MD simulations, in which GROMACS version 4.5 was used to conduct the simulation, applying the GROMOS96 53a6 force field. The topology files and charges for the PIP2 atoms were calculated using the PRODRG web server, as described in previous work. Meanwhile, the topology file of the sulfhydrated Cys residue was generated based on existing force field parameters. The channel–PIP2 structures were immersed in an explicit POPC bilayer using the VMD membrane package and solvated with SPC water molecules with 150 mM KCl. To mimic the activated state, we applied a constant depolarizing electric field of −0.128 V·nm⁻¹. Energy minimization was performed, followed by a 300-pS position-restrained (1000 kJ/mol·nm⁴) MD run. Subsequently, the four systems were subjected to a 25-ns MD simulation. For analysis, the SIMULAID program was used to analyze/cluster structures and to calculate interaction networks, including hydrogen bonds, salt bridges, and hydrophobic contacts.

Statistical analysis

Data are reported as mean ± S.D.; n denotes the number of oocytes. Unpaired (two-sample) t tests were performed in most statistical analyses of TEVC experiments, and normalized currents from the oocyte group exposed to NaHS were compared with that of the vehicle (untreated) group with an overall significance level of p < 0.05 using OriginPro 2016 (Microcal). Currents were normalized to the average current of vehicle (untreated) group. To determine whether there are significant changes upon exposing oocyte groups to NaHS at increasing time periods, as well as increasing RNA injection amounts of CSE, one-way repeated-measures ANOVA were performed, and pairwise comparisons were made by the Holm-Sidak method with an overall significance level of p < 0.05 in Origin-Pro 2016 (Microcal). Inhibition (±S.D.) of currents by NaHS in inside-out macropatches was normalized to current elicited by PIP2 (prior to NaHS treatment), and non-linear fitting of current inhibition was done in OriginPro 2016 using the Growth/Sigmoidal category, DoseRep function, and Levenberg Marquardt iteration function to the following Equation 1,

\[ y = A_1 + \frac{A_2 - A_1}{1 + 10^{\log_{10}(s - x)}} \]  

(Eq. 1)

where y is the normalized current; x is the concentration of NaHS or dc8 applied to the patch; A1 is minimum value; A2 is the maximum value; and p is the parameter for the variable hill slope.

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