Hydrogen Sulfide Regulates Inward-Rectifying K^+ Channels in Conjunction with Stomatal Closure[1][OPEN]

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Hydrogen sulfide (H_2S) is the third biological gasotransmitter, and in animals, it affects many physiological processes by modulating ion channels. H_2S has been reported to protect plants from oxidative stress in diverse physiological responses. H_2S closes stomata, but the underlying mechanism remains elusive. Here, we report the selective inactivation of current carried by inward-rectifying K^+ channels of tobacco (*Nicotiana tabacum*) guard cells and show its close parallel with stomatal closure evoked by submicromolar concentrations of H_2S. Experiments to scavenge H_2S suggested an effect that is separable from that of abscisic acid, which is associated with water stress. Thus, H_2S seems to define a unique and unresolved signaling pathway that selectively targets inward-rectifying K^+ channels.

Hydrogen sulfide (H_2S) is a small bioactive gas that has been known for centuries as an environmental pollutant (Reiffenstein et al., 1992). H_2S is soluble in both polar and, especially, nonpolar solvents (Wang, 2002), and has recently come to be recognized as the third member of a group of so-called biological gasotransmitters. Most importantly, H_2S shows both physical and functional similarities to the other gasotransmitters nitric oxide (NO) and carbon monoxide (Wang, 2002), and it has been shown to participate in diverse physiological processes in animals, including cardioprotection, neuromodulation, inflammation, apoptosis, and gastrointestinal functions among others (Kabil et al., 2014). Less is known about H_2S molecular targets and its modes of action. H_2S can directly modify specific targets through protein sulfhydration (the addition of an -SH group to thiol moiety of proteins; Mustafa et al., 2009) or reaction with metal centers (Li and Lancaster, 2013). It can also act indirectly, reacting with NO to form nitrosothiols (Whiteman et al., 2006; Li and Lancaster, 2013). Among its molecular targets, H_2S has been reported to regulate ATP-dependent K^+ channels (Yang et al., 2005), Ca^{2+}-activated K^+ channels, T- and L-type Ca^{2+} channels, and transient receptor potential channels (Tang et al., 2010; Peers et al., 2012), suggesting H_2S as a key regulator of membrane ion transport.

In plants, H_2S is produced enzymatically by the desulfhydration of L-Cys to form H_2S, pyruvate, and ammonia in a reaction catalyzed by the enzyme L-Cys desulfhydrase (Riemenschneider et al., 2005a, 2005b), DES1, that has been characterized in Arabidopsis (*Arabidopsis thaliana*; Alvarez et al., 2010). Alternatively, H_2S can be produced from D-Cys by D-Cys desulfhydrase (Riemenschneider et al., 2005a, 2005b) and in cyanide metabolism by β-cyano-Ala synthase (García et al., 2010). H_2S action was originally related to pathogenesis resistance (Bloem et al., 2004), but in the last decade it has been proven to have an active role in signaling, participating in key physiological processes, such as germination and root organogenesis (Zhang et al., 2008, 2009a), heat stress (Li et al., 2013a, 2013b), osmotic stress (Zhang et al., 2009b), and stomatal movement (García-Mata and Lamattina, 2010; Lisjak et al., 2010, 2011; Jin et al., 2013). Moreover, H_2S was reported to participate in the signaling of plant hormones, including abscisic acid (ABA; García-Mata and Lamattina, 2010; Lisjak et al., 2010; Jin et al., 2013; Scuffi et al., 2014), ethylene (Hou et al., 2013), and auxin (Zhang et al., 2009a).

ABA is an important player in plant physiology. Notably, upon water stress, ABA triggers a complex signaling network to restrict the loss of water through the transpiration stream, balancing these needs with those of CO_2 for carbon assimilation. In the guard cells that surround the stomatal pore, ABA induces an increase of cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), elevates cytosolic pH (pH_i), and activates the efflux of anions, mainly chloride, through S- and R-type anion channels. The increase in $[Ca^{2+}]_{cyt}$

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inactivates inward-rectifying K^+ channels (I_{KIN}); anion efflux depolarizes the plasma membrane, and together with the rise in pH_i, it activates K^+ efflux through outward-rectifying K^+ channels (I_{KOUT}; Blatt, 2000; Schroeder et al., 2001). These changes in ion flux, in turn, generate an osmotically driven reduction in turgor and volume and closure of the stomatal pore. All three gasotransmitters have been implicated in regulating the activity of guard cell ion channels, but direct evidence is available only for NO (Garcia-Mata et al., 2003; Sokolovski et al., 2005). Here, we have used two-electrode voltage clamp measurements to study the role of H_2S in the regulation of the guard cell K^+ channels of tobacco (*Nicotiana tabacum*). Our results show that H_2S selectively inactivates I_{KIN} and that this action parallels that of stomatal closure. These results confirm H_2S as a unique factor regulating guard cell ion transport and indicate that H_2S acts in a manner separable from that of ABA.

RESULTS AND DISCUSSION

To address whether H_2S-induced stomatal closure is mediated by changes in the activities of these channels, we recorded currents by two-electrode voltage clamp. I_{KIN} and I_{KOUT} currents were resolved on the basis of their voltage-dependent kinetics and challenging with H_2S donor GYY4137 (for *p*-methoxyphenyl(morpholino) phosphinodithioic acid). Follow impalement current-voltage recordings were carried out to confirm a stable baseline of channel activities for 5 to 10 min before the H_2S donor GYY4137 was added. We observed a complete response to the H_2S donor within 2 to 3 min of additions, indicating a halftime for response to the donor of less than 120 s. Most impalements could be held for 20 to 30 min only and therefore, did not allow us to assess current recovery after washout of the H_2S donor. Figure 1A shows current traces and the mean steady-state currents as a function of voltage (I-V curves) from guard cells before and after 5 min of exposure to 10 μM GYY4137. The I-V curves show the characteristic voltage dependence of both I_{KOUT} and I_{KIN} as previously described (Blatt, 1992; Gradmann et al., 1993; Garcia-Mata et al., 2003). In 10 mM KCl, voltages positive of -40 mV yielded I_{KOUT} that increased in amplitude with the voltage. Mean I_{KOUT} at +30 mV was +120 $\pm$ 28 and +91 $\pm$ 30 $\mu A\ cm^{-2}$ before and after exposure to GYY 4137, respectively, indicating a small but not very significant effect on the I_{KOUT}. Voltages negative of -100 mV were marked by a strong inward-directed current typical of I_{KIN}, and the current amplitude increased with negative-going voltages. We found that I_{KIN} at -220 mV was reduced by roughly 90% by H_2S donor treatments, yielding a mean I_{KIN} of $-21 \pm 8\ \mu A\ cm^{-2}$ compared with $-169 \pm 12\ \mu A\ cm^{-2}$ for the control. Exposure to the H_2S donor also affected the halftimes for I_{KIN} activation. Mean halftimes for I_{KIN} activation at -220 mV were 710 $\pm$ 70 ms in the control and 1,230 $\pm$ 230 ms after exposure to 10 μM GYY4137, indicating a significant change in gating of I_{KIN} (Fig. 1B).

Steady-state current through any ion channel depends on the ensemble conductance (*G*), which is the product of the number of functional channels at the plasma membrane (*N*), the single-channel conductance for a given ion species (γ_X), and the gating characteristics of the channel that describe the open probability of the channel (P_o). Plotting the conductance of I_{KIN} before and after exposure to H_2S as a function of voltage allowed a separation of the differences in the gating characteristics before and after H_2S donor treatments (Fig. 1C). The *G-V* curves were jointly fitted to a modified Boltzmann function (Eq. 1) to determine the maximum

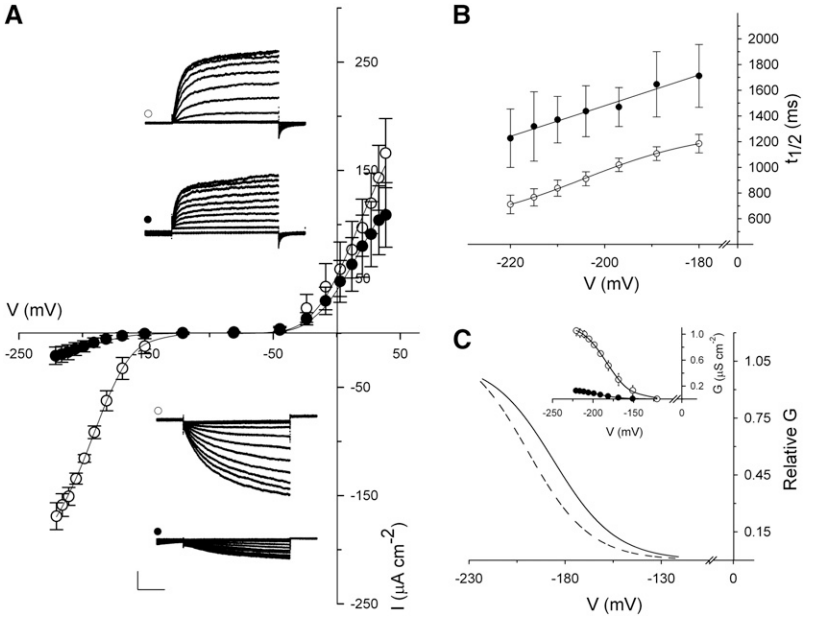

Figure 1. H_2S selectively affects I_{KIN}. A, Currents through I_{KOUT} and I_{KIN} above and below the voltage axis, respectively, recorded under voltage clamp from tobacco guard cells. Voltages were clamped from a holding voltage of -100 mV in 6-s steps between -220 and +120 mV and 4-s steps between -80 and +40 mV. Data are means $\pm$ SE of $n = 5$ guard cells bathed in 5 mM Ca^{2+}-MES buffer (pH 6.1) with 10 mM KCl before (white circles) and 5 min after (black circles) adding 10 μM GYY4137. Insets, Current traces for I_{KOUT} and I_{KIN} for control and H_2S treatment. Currents are cross referenced to the current-voltage curves by symbols. Bars = 2 s (horizontal); 50 $\mu A\ cm^{-2}$ (vertical). B, Mean activation halftimes as a function of voltage derived from current traces, including those in A, and cross referenced by symbols. C, Steady-state conductance as a function of voltage normalized to G_{max} in the control (black line) and with 10 μM GYY4137 (dashed line). Inset, Steady-state conductance as a function of voltage. Curves were jointly fitted to Boltzmann function (Eq. 1) with gating charge-δ held in common.

conductance (G_{max}) and the gating characteristics of I_{KIN} (Table I). For joint fittings, δ was held in common, and it yielded statistically and visually satisfactory fittings with a value of -1.66 ± 0.04. As expected from the I-V data, the H_2S donor suppressed the G_{max} significantly by up to 90% relative to the control. We cannot distinguish from these data whether this effect was mediated through a change in the number of channels available for activation (N) or the single-channel conductance (γ_K). Such detail would require single-channel analysis. However, we noted that the H_2S donor displaced $V_{1/2}$ by approximately −12 mV (Fig. 1C; Table I), indicating that the H_2S not only resulted in a decrease of the maximum conductance but also affected the voltage dependence for gating of I_{KIN}. An action on $V_{1/2}$ cannot be explained solely by an effect on N or γ_K. In short, H_2S selectively inactivated I_{KIN}.

The inactivation of the K^+ current is consistent with GYY4137 action in suppressing K^+ uptake and promoting stomatal closure, and it argues against earlier (and statistically undocumented) claims that H_2S donors promote stomatal opening (Lisjak et al., 2010, 2011). To assess the action of H_2S effect on stomatal movement, we measured apertures from stomata treated with different concentrations of the H_2S donor. Epidermal peels were placed in opening buffer under light of 150 μmol m^{-2} s^{-1} for 2 h to open the stomata before transfer to 5 mM Ca^{2+}-MES (pH 6.1) with 60 mM KCl supplemented with 0, 0.1, 1, or 10 μM GYY4137 for 90 min. Apertures were recorded immediately before and after H_2S treatments, and the data were normalized to the controls (Fig. 2). Exposure to the control buffer alone yielded stomatal apertures of 6.6 ± 1.8 μm; treatments with H_2S donor resulted in stomatal apertures ranging from 5.9 to 4.8 μm. Fitting the data to the hyperbolic decay function yielded an apparent K_i of 160 ± 40 nM for GYY4137. We carried out parallel measurements of I_{KIN} to determine its dose dependence after treating guard cells in buffer supplemented with 0, 0.1, 1, or 10 μM GYY4137. Figure 2 also shows the mean values for I_{KIN} again normalized to the control treatment. Increasing the concentration of H_2S donor, indeed, enhanced I_{KIN} inactivation. Fitting these data to hyperbolic decay function gave a K_i of 120 ± 70 nM for GYY4137, a value that did not significantly differ from that for stomatal closure compared with t test ($P = 0.735$). These results, thus, confirm the close kinetic relationship between I_{KIN} inactivation and stomatal closure in H_2S.

The similar effects of H_2S and ABA on I_{KIN} and stomatal aperture prompted us to explore the connection between H_2S and ABA signaling, which was suggested by García-Mata and Lamattina (2010). A similar set of protocols was used as above. Epidermal peels were pretreated for 2 h with opening buffer and light for 90 min before treatments in 5 mM Ca^{2+}-MES (pH 6.1) with 10 mM KCl with and without supplement of five distinct combinations of stomatal effectors: 10 μM GYY4137, 10 μM hypotaurine (HT), 10 μM GYY4137 + 10 μM HT, 20 μM ABA, and 20 μM ABA + 10 μM HT. HT interacts with free sulfide to form thiotaurine, effectively scavenging free H_2S in solution (Ortega et al., 2008). Figure 3A shows the percentage of stomatal closure induced by each treatment relative to the control. Measurements were carried out separately at the Consejo Nacional de Investigaciones Científicas y Técnicas and yielded starting apertures (4.9 ± 0.1 μm) that differed quantitatively from those recorded at the University of Glasgow. Qualitatively, however, the results were consistent between data sets. Exposure to 10 μM GYY4137 and 20 μM ABA resulted in 60% and 80% reductions in stomatal aperture, corresponding to apertures of 3.2 ± 0.1 and 2.2 ± 0.07 μm, respectively. Treatment with HT alone had no effect on stomatal aperture. When epidermal peels were treated with both 10 μM GYY4137 and HT, the effect of the H_2S donor was alleviated, yielding apertures of 4.6 ± 0.1 μm, similar to those of the control. Treatment of epidermal peels with 20 μM ABA + 10 μM HT partially suppressed the effect of ABA on aperture, resulting in a reduction to 70% pore width compared with control treatment.

Given the role of $[Ca^{2+}]_{cyt}$ in ABA signaling and control of I_{KIN} (Blatt, 2000; Garcia-Mata et al., 2003), we sought to test whether the H_2S-induced effect on I_{KIN} might be mediated by the Ca^{2+} intermediate. For this purpose, we loaded guard cells from the microelectrode with 50 mM EGTA, which chelates and buffers Ca^{2+} to suppress its elevation (Grabov and Blatt, 1998; Chen et al., 2010; Wang et al., 2012). After being impaled, guard cells were held for a period of 5 min to ensure loading with EGTA. Thereafter, the guard cells were either maintained in 5 mM Ca^{2+}-MES (pH 6.1) with 10 mM KCl or challenged with 10 μM GYY4137 for a period of 10 min. In the absence of H_2S donor, we observed no substantive effect on I_{KIN}. The mean amplitude at −200 mV was -217 ± 29 μA cm^{-2}. In the presence of H_2S donor, I_{KIN} was suppressed, yielding a mean current of -61 ± 11 μA cm^{-2} (Fig. 4A). EGTA did yield a small but not very significant recovery of I_{KIN} in the presence of the H_2S donor (Fig. 4B). These results indicate that H_2S acts in a manner that is largely independent of $[Ca^{2+}]_{cyt}$.

Table I. *Fitted gating characteristics for I_{KIN}*

Statistical differences after ANOVA as determined by Student Newman-Keuls test. *P* values are indicated for each parameter comparing control and H_2S treatments.

	$V_{1/2}$ ($P = 0.006$)	δ	G_{KIN} ($P < 0.001$)
	mV		*μs cm^{-2}*
Control	−183 ± 0.5	−1.67 ± 0.04	1.15 ± 0.01
+10 μM GYY4137	−195 ± 3		0.15 ± 0.01

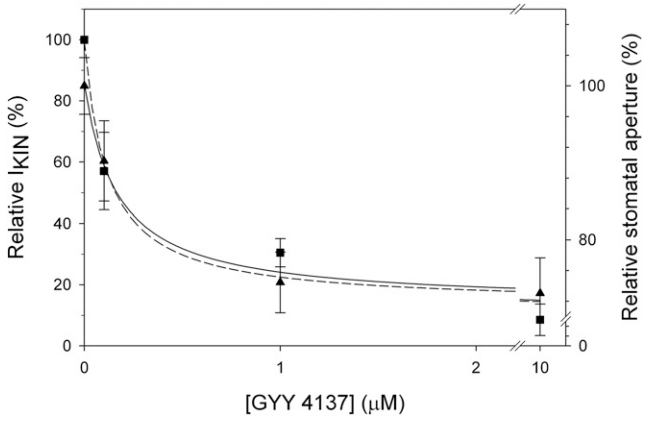

Figure 2. H_2S affects stomatal aperture and K^+ current in a dose-dependent manner with similar concentration dependencies. Mean stomatal apertures ± SE (n = 50) recorded from epidermal peels of tobacco (triangles) and mean I_{KIN} ± SE (n = 5) at −200 mV (squares) normalized to the controls without GYY 4137 treatment. A hyperbolic decay function was fitted to each set of data, yielding a K_i of 160 ± 40 nM GYY4137 for the aperture response (solid line) and 120 ± 70 nM GYY4137 for current inactivation (dashed line).

We also investigated the effect of the above compounds and their combinations on I_{KIN}, again following the same set of protocols. Figure 3B displays the mean percentage reduction of the I_{KIN} amplitude at −200 mV before and after the exposure to each of the treatments. H_2S resulted in almost complete loss of I_{KIN}, which is shown in Figure 1. ABA treatment reduced I_{KIN} by 62%, resulting in a mean current of -170 ± 39 μA cm^{-2} at −200 mV. Interestingly, exposure of guard cells to 10 μM HT yielded I_{KIN} of -292 ± 64 μA cm^{-2}, marginally greater in amplitude compared with -241 ± 40 μA cm^{-2} for the control, although this difference was not very significant. Suppression of the current by H_2S was blocked when guard cells were treated with the combination of H_2S donor and scavenger, resulting in I_{KIN} of similar amplitude as the control treatment. In contrast, the reduction of I_{KIN} evoked by ABA was not prevented by adding HT, which yielded a mean I_{KIN} of -174 ± 35 μA cm^{-2}. Altogether, these data indicate that H_2S acts in a manner paralleling that either of ABA or upstream of the hormone.

Stomatal movement is a highly coordinated process that is generally recognized to engage several signaling networks leading to the regulation of K^+ channels, anion channels, and H^+-ATPases at the plasma membrane as well as a complementary assembly of transporters at the tonoplast (Blatt, 2000). For ABA-evoked stomatal closure, this process includes inactivation of I_{KIN} through changes in $[Ca^{2+}]_{cyt}$ and activation of the I_{KOUT} mediated by a rise in pH_i (Blatt, 1990; Blatt and Armstrong, 1993; Garcia-Mata et al., 2003; Siegel et al., 2009). Our findings that H_2S differentially affects I_{KIN} and I_{KOUT} and that I_{KIN} inactivation is dose dependent with an apparent K_i in the low nanomolar range confirm a subcellular target for the action of this gasotransmitter. The timescale of the H_2S-triggered changes in channel gating is entirely in keeping with posttranslational regulation, which is in contrast with the slower effects of ABA that, over timescales of many minutes or hours, clearly rely on the transcription regulation and trafficking of the channel proteins (Pilot et al., 2003; Sutter et al., 2007; Eisenach, et al., 2012, 2014). These findings together with evidence that H_2S mediates stomatal closure and that its scavenging partially suppresses closure in ABA suggest a connection with the hormone, albeit a loose one. Notably, H_2S scavenging failed to reverse ABA-evoked inactivation of I_{KIN} (Fig. 3), and we found that

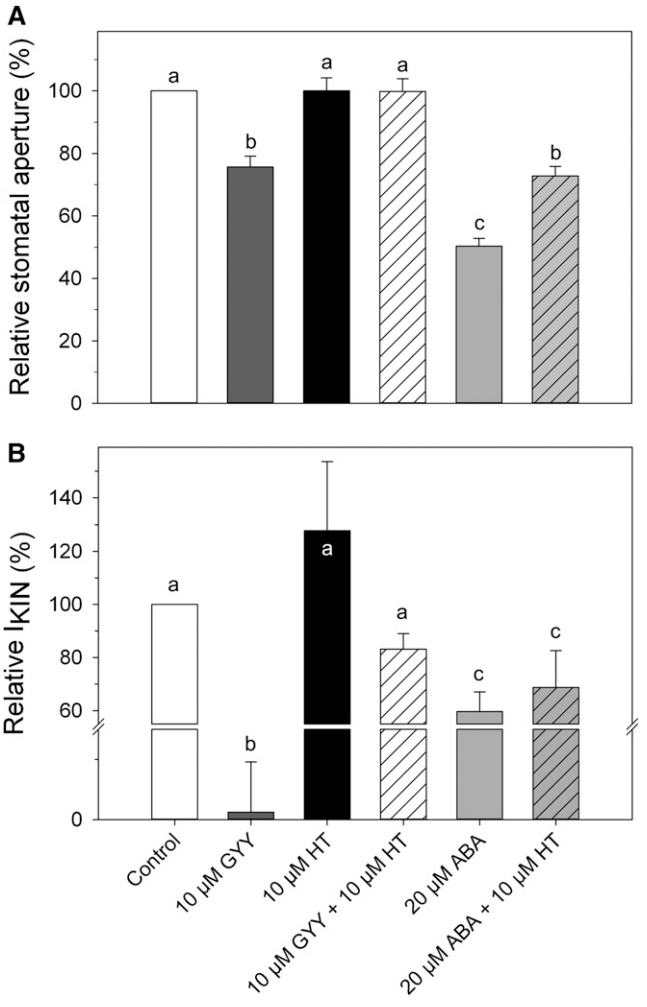

Figure 3. ABA and H_2S affect aperture and I_{KIN} in parallel. A, Mean stomatal apertures ± SE (n > 190 per treatment), including the control (100%), after treatments with 10 μM GYY4137 (GYY; dark-gray bar), 10 μM HT (black bar), 10 μM GYY4137 + 10 μM HT (white striped bar), 20 μM ABA (light-gray bar), or 20 μM ABA + 10 μM HT (light-gray striped bar). Letters indicates statistical differences by ANOVA ($P < 0.05$) as determined by Student Newman-Keuls test. B, Mean current ± SE (n = 5) for I_{KIN} recorded under voltage clamp as in Figure 1 and normalized to I_{KIN} at −200 mV in the control. Letters indicates statistical differences by ANOVA ($P < 0.05$) as determined by Student Newman-Keuls test.

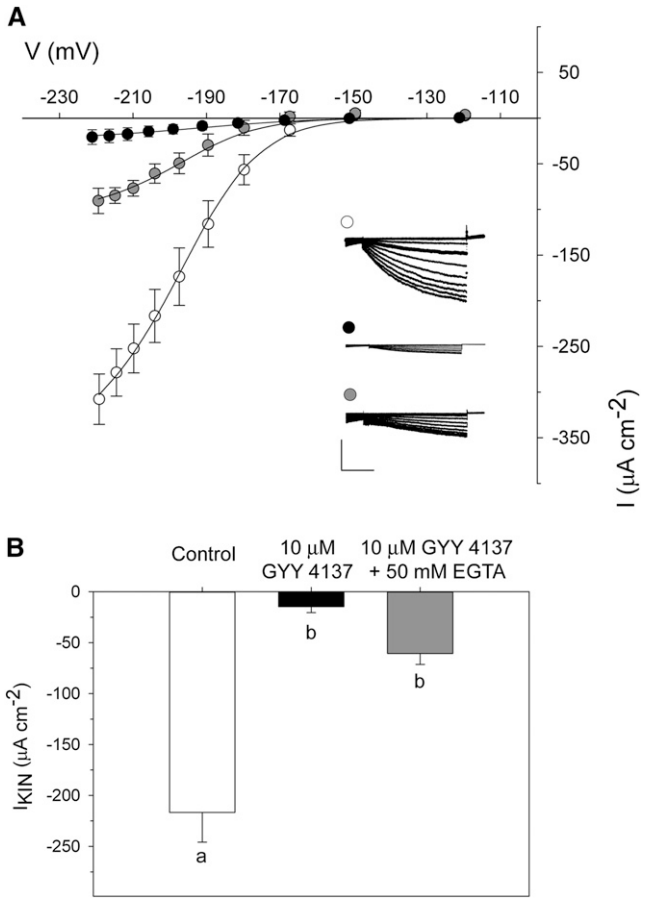

Figure 4. H_2S inactivates currents from I_{KIN} in a Ca^{2+}-independent manner. A, Current-voltage (I-V) curves for I_{KIN} recorded under voltage clamp as in Figure 1. Guard cells were bathed in 10 mM KCl (white circles) or 10 mM KCl supplemented with 10 μM GYY4137 (gray circles) and loaded from the microelectrode with 50 mM EGTA. The I-V curve for guard cells treated with only 10 μM GYY4137 (black circles) from Figure 1 is included for visual reference. Data are means ± SE of $n = 5$ guard cells for each data set. Curves were jointly fitted to Boltzmann function (lines), with $V_{1/2}$ and gating charge-δ held in common. Insets present current traces recorded under voltage clamp. Bars = 2 s (horizontal) and 200 μA cm^{-2} (vertical). B, Mean I_{KIN} at −205 mV from the current recordings for control (white bars) and 10 μM GYY4137 (black bars) treatments in the presence of EGTA and 10 μM GYY4137 without EGTA (gray bars). Lettering indicates statistical differences by ANOVA ($P < 0.001$) as determined by Student Newman-Keuls test.

stomatal closure was enhanced when treated with ABA and the H_2S scavenger compared with treatment with ABA alone. These observations are difficult to reconcile with a role for H_2S as an intermediate in ABA signaling per se and instead, suggest a partial overlap in signaling pathways.

This interpretation is in agreement with recent studies showing an ABA dependency of H_2S effect on stomatal movements (García-Mata and Lamattina, 2010; Scuffi et al., 2014). It also complements substantial evidence for a separate set of intermediates, especially ROS and NO, that trigger the elevation of $[Ca^{2+}]_{cyt}$ and are important for ABA-mediated stomatal closure (Pei et al., 2000; García-Mata and Lamattina, 2002, 2003), and it agrees with our finding that Ca^{2+} buffering did not substantially rescue I_{KIN}. Guard cells are thought to produce NO in response to ABA through the activity of nitrate reductases (Desikan et al., 2002), and NO action is also dependent on the secondary messengers cGMP and cADPR (Neill et al., 2002). Garcia-Mata et al. (2003) showed that NO promoted the inhibition of I_{KIN} and activated anion efflux through an enhanced sensitivity of internal Ca^{2+} release to Ca^{2+} influx across the plasma membrane. Notably, the effects of $[Ca^{2+}]_{cyt}$ on I_{KIN} gating and especially, $V_{1/2}$ are much more pronounced than observed with H_2S. Furthermore, NO is also able to modulate I_{KOUT} by direct nitrosylation of the channel or an associated regulatory protein (Sokolovski and Blatt, 2004), but we observed little evidence of an effect on I_{KOUT} current. Therefore, these observations implicate a parallel and as yet uncharacterized signaling pathway that acts on I_{KIN}, thereby overlapping with the well-known pathways leading from ABA to I_{KIN} and stomatal closure.

How might H_2S act to modulate I_{KIN}? Nitrosylation of Cys sulfhydryl groups on either the channel itself or on closely associated regulatory proteins has been suggested to mediate the NO-induced block of I_{KOUT} (Sokolovski and Blatt, 2004), and such modifications may be linked to ROS modification of residues within the voltage sensor domain (García et al., 2010). H_2S is also capable of covalently modifying protein targets, and the mechanism is equally relevant to I_{KIN} and the proteins that regulate these channels, including protein kinases and phosphatases (Thiel and Blatt, 1994; Li et al., 1998; Michard et al., 2005). In animals, for example, H_2S activates ATP-dependent K^+ channels through the sulfhydration of a Cys residue of the sulfonurea SUR protein (Babenko et al., 2000). Addition of the SUR inhibitor glibenclamide antagonized the H_2S response and prevented the hypotensive effect of H_2S (Zhao et al., 2001). In other cases, sulfhydration is suppressed by the reducing agent dithiothreitol and mutants defective in H_2S production (Mustafa et al., 2009). Of interest, glibenclamide has also been shown to abolish stomatal closure triggered by ABA and external Ca^{2+} through the inhibition of anion and I_{KOUT} (Leonhardt et al., 1999). More recent studies, however, have shown only partial suppression by glibenclamide of stomatal closure in ABA, whereas the response to H_2S was completely abolished (García-Mata and Lamattina, 2010). These findings suggest that ABC proteins, a major target of glibenclamide, may contribute to channel regulation in guard cells upon H_2S exposure. At present, however, there is not sufficient information from any system that would enable realistic predictions of the possible motifs for sulfhydration.

No doubt, future studies with transgenic Arabidopsis lines defective in H_2S, NO production, and ABA sensitivity should help clarify the role of H_2S in these processes (Hetherington and Woodward, 2003). What is clear, however, is that H_2S is active in selectively regulating I_{KIN} of guard cells over timescales consistent

with short-term posttranslational modification of specific target proteins. Furthermore, our evidence implicates H_2S in a signaling pathway that is separable from that of ABA, although both ABA and H_2S modulate stomatal behavior in parallel.

MATERIALS AND METHODS

Plant Material, Chemicals, and Stomatal Assays

Tobacco (*Nicotiana tabacum*) plants were grown in Levington F2+S compost under long-day conditions (16-h-light/8-h-dark cycle; temperature approximately 26°C and 22°C for day and night, respectively; relative humidity of 60% and 70% for day and night, respectively) under 100 μmol m^{-2} s^{-1} of light.

Epidermal peels were obtained from the abaxial side of tobacco leaves and placed in opening buffer comprised of 10 mM Na^+-MES (pH 6.1; 10 mM MES titrated to pH 6.1 with NaOH) with 60 mM KCl under light of 150 μmol m^{-2} s^{-1} for 2 h before treatment with the H_2S donor GYY4137 (Sigma) in the same buffer. Stomata were imaged before and after 90 min of H_2S treatment using an LD Achroplan 40× Objective and an Axio-Cam HRc Digital Camera (Zeiss). Apertures were tracked for individual stomata and quantified using IMAGEJ version 1.48 (image.nih.gov/ij/).

Guard Cell Electrophysiology

Currents were recorded under two-electrode voltage clamp using Henry's EP Software Suite (http://www.psrg.org.uk). Microelectrodes were constructed to give tip resistances of greater than 100 MΩ and filled with 200 mM K^+-acetate (pH 7.5) to minimize interference arising from anion leakage from the microelectrode (Blatt and Slayman, 1983; Blatt, 1987; Wang and Blatt, 2011). Electrolyte filling solutions were equilibrated against the resin-bound Ca^{2+} buffer BAPTA [for 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ca^{2+} sponge; Invitrogen] to prevent Ca^{2+} loading of the cytosol from the microelectrodes. K^+ channel currents were recorded from guard cells bathed under continuous superfusion with 5 mM Ca^{2+}-MES (pH 6.1; 5 mM MES titrated to pH 6.1 with $Ca(OH)_2$; [Ca^{2+}] = 1 mM) plus 10 mM KCl alone and supplemented with reagents as indicated. Recordings typically included a 2-s holding voltage at −100 mV and two to six steps to voltages between −220 and +40 mV. Surface areas of the impaled guard cells were calculated assuming a spheroid geometry (Blatt et al., 1987).

Current voltage analysis and fittings were carried out using Henry's EP Software Suite and SigmaPlot 11 (SPSS; Systat Software). Conductance-voltage curves were fitted by joint nonlinear least squares and the Marquardt-Levenberg algorithm using a modified Boltzmann function of the form

$$G = \frac{G_{max}}{1 + e^{\delta F(V_{1/2} - V)/RT}} \quad (1)$$

where G_{max} is the maximum conductance, V is the membrane voltage, $V_{1/2}$ is the voltage at which half-maximum activation of channels occurs, δ is the apparent gating charge, and F, R, and T have their usual meanings.

Statistical Analysis

Results are reported as means ± SE of n observations, with significance determined using Student's t test and ANOVA at $P < 0.05$.

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