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Molecular Mechanism for H_2S-Induced Activation of K_{ATP} Channels

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Abstract

Hydrogen sulfide (H_2S) is an endogenous opener of K_{ATP} channels in many different types of cells. However, the molecular mechanism for an interaction between H_2S and K_{ATP} channel proteins remains unclear. The whole-cell patch-clamp technique and mutagenesis approach were used to examine the effects of H_2S on different K_{ATP} channel subunits, rvKir6.1 and rvSUR1, heterologously expressed in HEK-293 cells. H_2S stimulated coexpressed rvKir6.1/rvSUR1 K_{ATP} channels, but had no effect on K_{ATP} currents generated by rvKir6.1 expression alone. Intracellularly applied sulfhydryl alkylating agent (*N*-ethylmaleimide, NEM), oxidizing agent (chloramine T, CLT), and a disulfide bond–oxidizing enzyme (protein disulfide isomerase) did not alter H_2S effects on this recombinant channels. CLT, but not NEM, inhibited basal rvKir6.1/rvSUR1 currents, and both abolished the stimulatory effects of H_2S on K_{ATP} currents, when applied extracellularly. After selective cysteine residues (C6S and C26S but not C1051S and C1057S) in the extracellular loop of rvSUR1 subunits were point-mutated, H_2S lost its stimulatory effects on rvKir6.1/rvSUR1 currents. Our results demonstrate that H_2S interacts with Cys6 and Cys26 residues of the extracellular N terminal of rvSUR1 subunit of K_{ATP} channel complex. Direct chemical modification of rvSUR1 subunit protein constitutes a molecular mechanism for the activation of K_{ATP} channels by H_2S. *Antioxid. Redox Signal.* 12, 1167–1178.

Introduction

Hydrogen sulfide (H_2S) is generated endogenously in mammalian cells from L-cysteine by pyridoxal-5′-phosphate–dependent enzymes, including cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (36–38). Pronounced physiologic effects of H_2S have been reported, including modulating neuronal activity (11), protecting the heart from ischemic damage (18), regulating cellular apoptosis and proliferation (43, 45, 46), inducing vasorelaxation and reducing blood pressure (44, 50), and altering insulin secretion (47). Among the most acknowledged molecular mechanisms for the cellular effects of H_2S is the activation of ATP-sensitive K^+ (K_{ATP}) channels. After our first evidence that H_2S stimulated K_{ATP} channels in vascular smooth muscle cells (SMCs) (50), many subsequent studies confirmed the interaction of H_2S and glibenclamide-sensitive K_{ATP} channels in vascular SMCs (9, 32), cardiac myocytes (18), pancreatic β cells (47), and the gastrointestinal tract (10). Our previous single-channel studies on cell-free membrane patches also showed that the activation of K_{ATP} channels by H_2S was not mediated by cytosolic second messengers (32, 47). However, the molecular mechanisms underlying a direct H_2S action on K_{ATP} channel protein complex are unclear.

Direct chemical interaction of gasotransmitters, such as nitric oxide (NO) and carbon monoxide (CO), with proteins or ion channels has been reported (41). NO covalently modifies free cysteine residues in proteins through *S*-nitrosylation (29). *S*-nitrosylation of Ca^{2+}-activated K^+ (K_{Ca}) channel proteins (β subunit) by NO directly changes the functional activity of these channels (7, 41). Chemical modification of histidine residues of K_{Ca} channel proteins (α subunit) by CO through the formation of hydrogen bond, a process of carboxylation, has been indicated (39, 41). H_2S also may induce chemical modification of selective proteins to alter their functions.

K_{ATP} channels play important roles in the regulation of membrane excitability by coupling cellular metabolic activity. These channels are composed of the pore-forming Kir6.1 or Kir6.2 subunit and regulatory sulfonylurea receptors (SUR1, SUR2A, and SUR2B) (15, 16, 42). The present study aimed at investigating the interaction of H_2S with cloned rat vascular K_{ATP} channel subunits (rvKir6.1/rvSUR1), and

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underlying molecular mechanisms. To accomplish this goal, we used the patch-clamp technique to characterize the effects of H_2S on heterologously expressed K_{ATP} channels, rvKir6.1 alone or together with rvSUR1. To test the hypothesis that H_2S may interact with cysteine residues of K_{ATP} channel subunits, we examined the effects of cysteine-specific oxidizing and reducing chemicals on the effect of H_2S on K_{ATP} channels. A mutagenesis approach that replaces cysteine residues with structurally similar serine residues also was adopted. Our study identified the subunit of K_{ATP} channel complex that is reactive to H_2S, and pinpointed the topologic location of H_2S-sensitive cysteine residues in extracellular loop of the K_{ATP} channel subunit.

Materials and Methods

Recording of K_{ATP} currents

The whole-cell configuration of the patch-clamp technique was used, as described previously (32, 50). In brief, a 35-mm Petri dish with attached cells was mounted on the stage of an inverted phase-contrast microscope (Olympus IX70, Olympus, Tokyo, Japan). A home-made superfusion chamber was inserted into the Petri dish. The cells inside the superfusion chamber were superfused continually at a flow rate of 3–5 ml/min. The time required for a complete solution change from the onset of a drug application was estimated at 15–25 s, as described (32). Pipettes were pulled from soft microhematocrit capillary tubes (Fisher, Nepean, Ontario, Canada) with tip resistance of 2–4 MΩ when filled with pipette solution. Currents were recorded with an Axopatch 200-B amplifier (Molecular Devices, Sunnyvale, CA), controlled by a Digidata 1200 interface and a pCLAMP software (Version 6.02, Molecular Devices). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter. At the beginning of each experiment, the junction potential was electronically adjusted to zero. Test pulses were made stepwise with 10-mV increments from −150 to +50 mV. The holding potential was set at −30 mV. Current–voltage curves were constructed by using the sustained current amplitude at the end of 600-ms test pulses, unless otherwise specified. The pipette solution contained (m*M*): 140 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 2 EGTA, 10 HEPES, and 0.5 Na_2-ATP (pH adjusted to 7.3 with KOH). The bath solution contained (m*M*): 135 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 10 glucose (pH adjusted to 7.3 with NaOH), unless otherwise specified. To amplify the K_{ATP} inward current, some experiments used a special protocol with membrane potential held at −60 mV and the $[K^+]_o$ of the bath solution being changed from 5.4 to 140 m*M* with equimolar NaCl removed from the bath solution. All experiments were conducted at room temperature (20–22°C).

Site-directed mutagenesis of rvSUR1 subunit

The transcripts of Kir6.1 and SUR1 from rat mesenteric artery smooth muscles were previously identified in our laboratory by using primer pairs designed based on the known specific cDNA sequences. Because these K_{ATP}-subunit cDNAs were derived from **r**at **v**ascular tissues, they were named rvKir6.1 (1,311 bp) and rvSUR1 (4,780 bp). The nucleotide sequences of these clones were deposited in GenBank as *AB043637* (rvKir6.1) and *AB052294* (rvSUR1) (8). In this study, rvKir6.1 and rvSUR1 cDNAs were used in the gene transfection. *In vitro* site-directed mutagenesis of selected cysteine residues of the wild-type rvSUR1 gene (GenBank *AB052294*) was performed by using a QuikChange site-directed mutagenesis kit (Stratagene).

Each single-site mutant replaces one of the four extracellular cysteine residues of rvSUR1 subunit with a serine residue (C6S, C26S, C1051S, and C1075S) (Fig. 1). For example, C6S stands for the replacement of the cysteine residue, the sixth amino acid from the N-terminus of the rvSUR1 protein, with a serine. To produce rvSUR1-C6S mutant, a forward oligonucleotide primer of 5′-CTT TGG CCT TCT CCG GCA CCG AGA AC-3′ and a reverse primer of 5′-GTT CTC GGT GCC GGA GAA GGC CAA AG-3′ were designed. For rvSUR1-C26S mutagenesis, the forward primer is 5′-CTC AAC AAC GGC TCC TTC GTG GAC GCG-3′, and the reverse primer, 5′-CGC GTC CAC GAA GGA GCC GTT GTT GAG-3′. As for the production of the rvSUR1-C1051S mutant, the forward primer is 5′-CGC CAG GAA CTC CTC CTC CCT CAG CCA GGA AT-3′, and the reverse primer, 5′-ATT CCT GGC TGA GGG AGG AGT TCC TGG CG-3′. The rvSUR1-C1057S mutant was induced with a forward primer of 5′-CTC AGC CAG GAA TCT GCC CTG GAC CAA TC-3′ and a reverse primer of 5′-GAT TGG TCC AGG GCA GAT TCC TGG CTG AG-3′.

A double-site mutated mutant (*i.e.*, rvSUR1-C6S-C1057S) was introduced on the top of the rvSUR1-C6S mutant with the use of the previously mentioned mutagenic primer pair for the rvSUR1-C1057S mutagenesis. The rvSUR1-C6S mutant

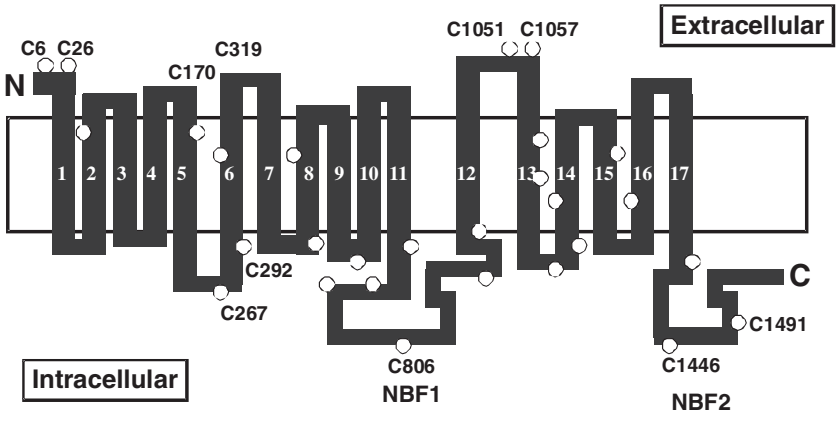

FIG. 1. Topologic distribution of the cysteine residues of the rvSUR1 subunit. In total, 28 cysteine residues exist in the SUR1 subunit; among them, only four are located on the extracellular loop of SUR1. Cys6 and Cys26 are located near the N-terminus; whereas Cys1051 and Cys1057 are located between transmembrane helices 12 and 13 in transmembrane domain 3. Possible intramolecular disulfide bridges can be formed between these cysteine residues.

was confirmed with relevant enzymatic analysis and dideoxynucleotide DNA sequencing.

Mutagenesis and amplification reactions were performed with a PCR Mastercycler (Eppendorf, Mississauga, Canada) in a 50-μl reaction volume, containing 50 ng of the circular plasmid DNA containing pcDNA3/rvSUR1, 1.5 m*M* $MgCl_2$, 125 ng of each primer, 1 ml of 10 m*M* mixed dNTPs, 1x reaction buffer and 1 μl of *pfu Turbo* DNA polymerase (2.5 U/μl). Incorporation and extension of the mutagenic primers were done with the following cycling program: 26 cycles of 30-s denaturation at 95°C, 1 min of annealing at 55°C, and 20 min. of extension at 68°C. The nonmutated parental DNA template was digested with *Dpn*I restriction endonuclease for 1 h at 37°C, and 5 μl of the PCR mix was used to transform with DH5α-competent bacteria by heat shock at 37°C before the bacteria culture and plasmid purification process. The identity of the mutated cDNAs (contained in the pcDNA3 vector) was confirmed by both enzymatic analysis and dideoxynucleotide DNA sequencing. The absence of additional mutations in these cDNAs and in the parental cDNAs also was verified. The mutagenesis process was controlled with a pWhitescript control plasmid, and its specific mutagenesis control primers provided with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Heterologous expression of wild-type or mutated rvKir6.1 and rvSUR1

HEK-293 cells (CRL 1573, Batch 203970 from American Type Culture Collection, Rockville, MD) were cultured in 60-mm Petri dishes at 37°C in a humidified incubator with 95% air and 5% CO_2 in MEM culture medium containing L-glutamine and supplemented with 10% fetal bovine serum and penicillin/streptomycin. The culture medium was changed every 3 days. HEK-293 cells used in the present study were passages 33 to 37. The cultured cells were trypsinized and plated into a new dish with the cell density of 8×10^4/60-mm dish before gene transfection.

The wild-type rvKir6.1, inserted into a mammalian expression pHA3/neo vector (a gift from Dr. Anderson from University of Saskatchewan) (19), was transfected into HEK-293 cells by using a FuGENE 6 transfection reagent (Roche), as described previously, with modifications (8). In brief, 2 μg cDNA and 6 μl reagent were mixed in 500 μl of FBS-free MEM. After incubation for 30 min at room temperature (20 to 22°C), the mixture was added to HEK-293 cells in 3.5 ml FBS-free MEM (cell density, 8×10^4/60-mm dish). The medium was changed to FBS-containing MEM 6 h after the transfection. Geneticin (G418) selection was performed at the concentration of 0.5 mg/ml. Mock transfection (vector-only transfection) also was performed. Nontransfected HEK-293 cells were included as negative control for the antibiotic selection. After 2 weeks of the antibiotic selective culturing, surviving gene-transfected cell colonies were individually picked into a 24-well culture plate for proliferation. When they grew into >90% confluence in 10-cm culture dishes, the cells were harvested for confirmation of heterologous gene expression. The wild-type rvSUR1 or mutated rvSUR1 cDNAs were inserted into a pcDNA3/Zeocin vector (Invitrogen). Coexpression of rvKir6.1 with these rvSUR1 subunits was performed by using dual selections with 0.5 mg/ml geneticin and 0.15 mg/ml Zeocin. In short, HEK-293 cells were stably transfected with pcDNA3.1/Hygro-rvKir6.1 plasmid by using hygromycin (50 μg/ml) as the selective antibiotic. Hygromycin-resistant colonies were isolated and then transfected with pcDNA3.1/Zeo-rvSUR1 plasmid by using Zeocin (50 μg/ml) as the selective reagent. The hygromycin- and Zeocin-resistant cell colonies were cloned, and the coexpression of rvKir6.1 and rvSUR1 subunits was confirmed with both Western blotting and the patch-clamp techniques, as we described previously (8).

Chemicals and data analysis

The H_2S solution was prepared daily by directly bubbling the saline with pure H_2S gas (Praxair, Mississauga, Ontario, Canada) for 30–40 min to make the saturated H_2S solution (0.09 *M* at 30°C) (50). Glibenclamide, diazoxide, *N*-ethylmaleimide, chloramine T, protein disulfide isomerase, and other chemicals were purchased from Sigma Chemical Co. Data were expressed as mean ± SEM and analyzed by using a paired-sample *t* test or one-way ANOVA. Differences were considered statistically significant when $p < 0.05$.

Results

Characterization of heterologously expressed rvKir6.1/rvSUR1 K_{ATP} channels

A low concentration of ATP in the pipette (0.5 m*M*) stimulated heterologously coexpressed rvKir6.1/rvSUR1 K_{ATP} currents with the inward currents increased from -304.2 ± 48.8 to -577.1 ± 66.6 pA at -150 mV, and the outward currents increased from 267.8 ± 39.3 to 528.1 ± 102.8 pA at +60 mV ($n = 12$; $p < 0.05$). Elevation of the pipette solution ATP concentration to 3 and 5 m*M* inhibited the K_{ATP} current ($n = 6$; $p < 0.05$) (Fig. 2A and B). Inclusion of 1 m*M* GDP in the pipette also increased K_{ATP} currents significantly (Fig. 2C). Thus, in all of the subsequent studies, 1 m*M* GDP and 0.5 m*M* ATP were always included in the pipette solution.

K_{ATP} channel opener diazoxide at 100 μM stimulated rvKir6.1/rvSUR1 K_{ATP} currents, which were inhibited by glibenclamide (Fig. 3A and B). Both the diazoxide and glibenclamide effects on K_{ATP} channels were reversed on washout. Changing the $[K^+]$ concentration of the bath solution from 5.4 to 140 m*M* increased the inward K_{ATP} currents (at -60 mV) from -0.2 ± 0.2 to -11.9 ± 1.8 pA/pF ($n = 7$; $p < 0.05$). Subsequently applied glibenclamide (5 μM) inhibited the currents to -7.3 ± 1.3 pA/pF ($n = 7$; $p < 0.05$) (Fig. 3C and D). The inhibition of the K_{ATP} currents by glibenclamide was reversed after washout (Fig. 3D). These results demonstrated the successful heterologous expression of K_{ATP} channels with classical pharmacologic sensitivities to ATP/GDP, glibenclamide, and diazoxide.

Differential effects of H_2S on rvKir6.1 current and rvKir6.1/rvSUR1 currents

H_2S had no effect on K_{ATP} currents generated by heterologously expressed rvKir6.1 alone (Fig. 4A and B). Conversely, H_2S significantly increased K_{ATP} currents generated by coexpression of rvKir6.1 and rvSUR1 ($n = 6$; $p < 0.05$) (Fig. 4C and D). It appears that H_2S targets the sulfonylurea subunit of the K_{ATP} channel complex. With symmetric $[K^+]$ across the cell membrane, rvKir6.1/rvSUR1 K_{ATP} currents were also increased by H_2S (100 μM) and reversibly inhibited by glibenclamide (Fig. 4E and F).

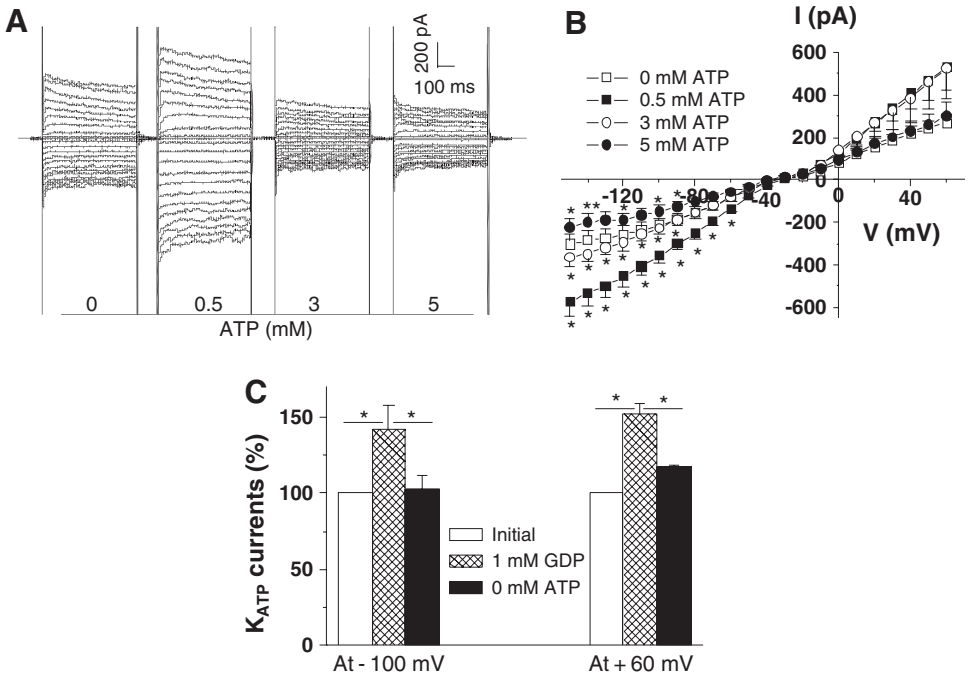

FIG. 2. Sensitivity of the rvKir6.1/rvSUR1-encoded K_{ATP} channels in HEK-293 cells to ATP and GDP. All K_{ATP} currents were recorded with 600-ms depolarizing pulses (0.1 Hz) from −150 to +50 mV, with a holding potential of −30 mV. (**A**) Representative K_{ATP} current traces with different ATP concentrations in the pipette. (**B**) Summarized current–voltage (I-V) relations of K_{ATP} channels with different ATP concentrations in the pipette solution ($n = 6$–13 for each group; $^*p < 0.05$ *vs.* other groups). (**C**) Comparison of the changes of inward (–100 mV) and outward (+60 mV) K_{ATP} currents in cells dialyzed with 1 m*M* GDP ($n = 9$) and 0 m*M* ATP ($n = 13$) ($^*p < 0.05$ *vs.* other groups).

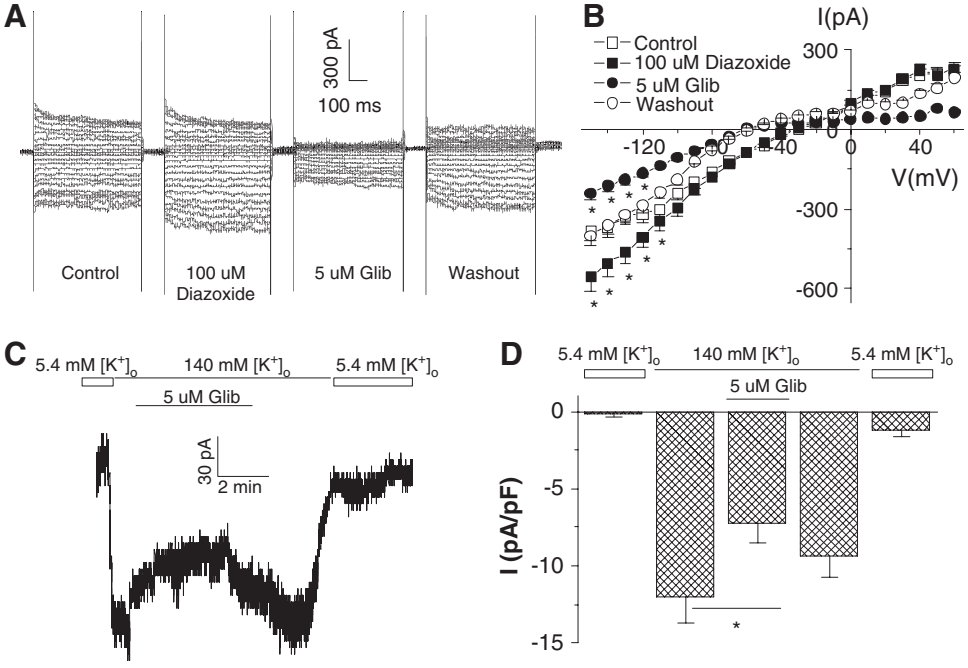

FIG. 3. Pharmacologic properties of rvKir6.1/rvSUR1-encoded K_{ATP} channels in HEK-293 cells. (**A**) Representative K_{ATP} current traces during 600-ms depolarizing pulses (0.1 Hz) from −150 to +50 mV, with a holding potential of −30 mV. (**B**) Summarized current–voltage (I-V) relations of K_{ATP} channels in the absence and then the presence of 100 μM diazoxide and 5 μM glibenclamide (Glib), and after washout of chemicals. (**C**) K_{ATP} currents recorded at a stable membrane potential of −60 mV with different extracellular K^+ concentrations. (**D**) Summary of the effects of glibenclamide (Glib) on K_{ATP} channels under the same recording conditions as in C ($n = 7$; $^*p < 0.05$).

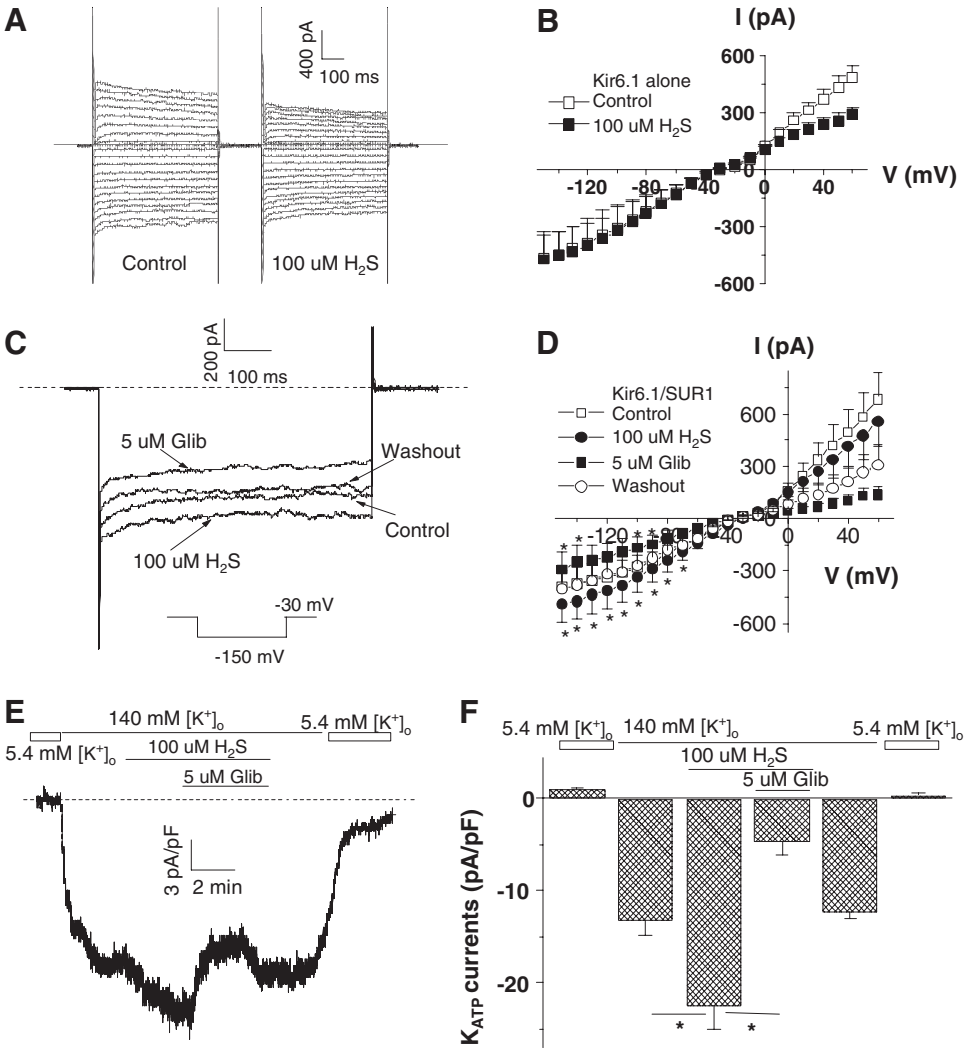

FIG. 4. Effects of H_2S on heterologously expressed K_{ATP} channels in HEK-293 cells. (**A**) Representative Kir6.1-encoded K_{ATP} current traces during 600-ms depolarizing pulses (0.1 Hz) from −150 to +50 mV, with a holding potential of −30 mV. (**B**) Summarized current–voltage (I-V) relations of Kir6.1-encoded K_{ATP} channels in the absence and then the presence of 100 μM H_2S ($n = 7$). (**C**) Representative rvKir6.1/rvSUR1-encoded K_{ATP} current traces under the same recording condition as in **A**. (**D**) Summarized I-V relations of rvKir6.1/rvSUR1-encoded K_{ATP} channels in the absence ($n = 6$) and then in the presence of 100 μM H_2S ($n = 6$) and 5 μM Glib ($n = 6$), and after washout ($n = 4$) (*$p < 0.05$). In (**E, F**), the rvKir6.1/rvSUR1-encoded K_{ATP} current was recorded at a stable membrane potential of −60 mV with different extracellular K^+ concentrations. Representative K_{ATP} current is shown in **E,** and the summary, in **F**. ($n = 10$; *$p < 0.05$).

Interaction of H_2S and sulfhydryl groups of K_{ATP} channels

To determine the topologic location of the H_2S target(s) on K_{ATP} channels, chemical modification of K_{ATP} channels was conducted.

Effect of sulfhydryl alkylating agent. NEM, a membrane-impermeable cysteine-specific alkylating agent, induces the cross-linking of protein thiol groups. Regardless of intra- or extracellular application, NEM did not change the heterologously expressed rvKir6.1 current in HEK-293 cells (data not shown). Extracellularly applied NEM also did not significantly alter basal currents of the coexpressed rvKir6.1/rvSUR1 channels (from -18.8 ± 5.0 to -22.4 ± 5.1 pA/pF at −60 mV; $n = 5$). However, the presence of NEM in the bath solution completely abolished the effect of H_2S on the rvKir6.1/rvSUR1 current (Fig. 5A and B). H_2S still increased the rvKir6.1/rvSUR1 K_{ATP} currents from -9.9 ± 0.8 to -22.3 ± 1.5 pA/pF ($n = 5$; $p < 0.05$) when NEM was included in the pipette solution (Fig. 5C through E).

Effect of sulfhydryl oxidizing agent. CLT is a potent oxidant of –SH groups and methionine (24). When applied extracellularly in the bath, CLT (1 mM) significantly inhibited the rvKir6.1/rvSUR1 K_{ATP} currents from -18.6 ± 3.0 to -10.1 ± 1.9 pA/pF ($n = 5$; $p < 0.05$). The presence of CLT in the bath solution also completely blocked the effect of H_2S on the K_{ATP} currents (Fig. 6A and B). In contrast, when CLT was included in the pipette, the stimulatory effect of H_2S on the

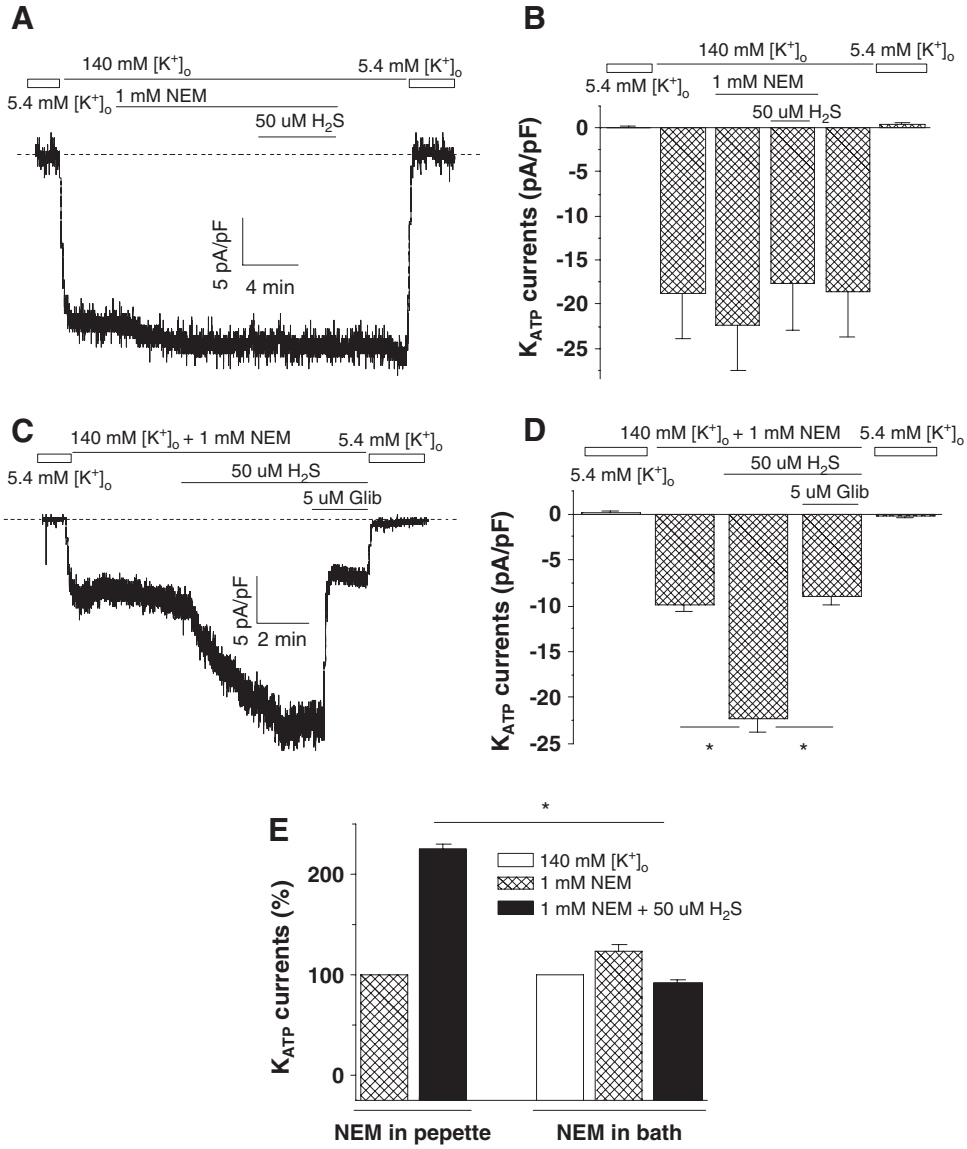

FIG. 5. Effects of *N*-ethylmaleimide and H_2S on rvKir6.1/rvSUR1-encoded K_{ATP} channels in HEK-293 cells. All recordings were conducted at −60 mV with different extracellular K^+ concentrations. (**A**) Representative original K_{ATP} current recording with bath application of NEM at 1 m*M*. (**B**) Summary of the effects of extracellular NEM from four cells. (**C**) Representative original K_{ATP} current with intracellular NEM (1 m*M*). (**D**) Summary of the effects of intracellular NEM from five cells (*$p < 0.05$). (**E**) Comparison of the alterations of H_2S-stimulated K_{ATP} channels between the effects of intracellular NEM ($n = 5$) and extracellular NEM ($n = 4$) (*$p < 0.05$).

K_{ATP} current was not altered (Fig. 6C and D). These results indicate that H_2S may bind to extracellular –SH groups or the methionine residue of the rvSUR1 subunit. The applied high concentration of CLT at millimolar range is prone to oxidizing methionine. Unlike NEM, extracellularly applied CLT inhibited the K_{ATP} currents by 45% (Fig. 6E), indicating the involvement of the methionine oxidation in addition to –SH groups.

Effect of disulfide bond–oxidizing enzyme. In cells dialyzed with disulfide bond–oxidizing enzyme, protein disulfide isomerase (PDI at 1 μM), the stimulatory effect of H_2S and the inhibitory effect of CLT on K_{ATP} currents were not altered ($n = 5$; $p < 0.05$) (Fig. 7A and B). No difference was noted in the H_2S effects on the K_{ATP} currents with and without PDI application ($n = 5$) (Fig. 7C).

H_2S effects on mutated rvKir6.1/rvSUR1 channels

After rvKir6.1 was coexpressed with one of rvSUR1 mutants (C6S, C26S, C1051S, C1057S), large inward K_{ATP} currents were still generated in HEK-293 cells. H_2S at 100 μM significantly increased the K_{ATP} currents of the rvKir6.1/rvSUR1-C1057S channels (Fig. 8A) or rvKir6.1/rvSUR1-C1051S channels (Fig. 8B). In contrast, the stimulatory effect of H_2S on K_{ATP} channels was completely lost with the rvKir6.1/rvSUR1-C26S channel (Fig. 8C) or with the rvKir6.1/rvSUR1-C6S channel (Fig. 8D). These results indicate that

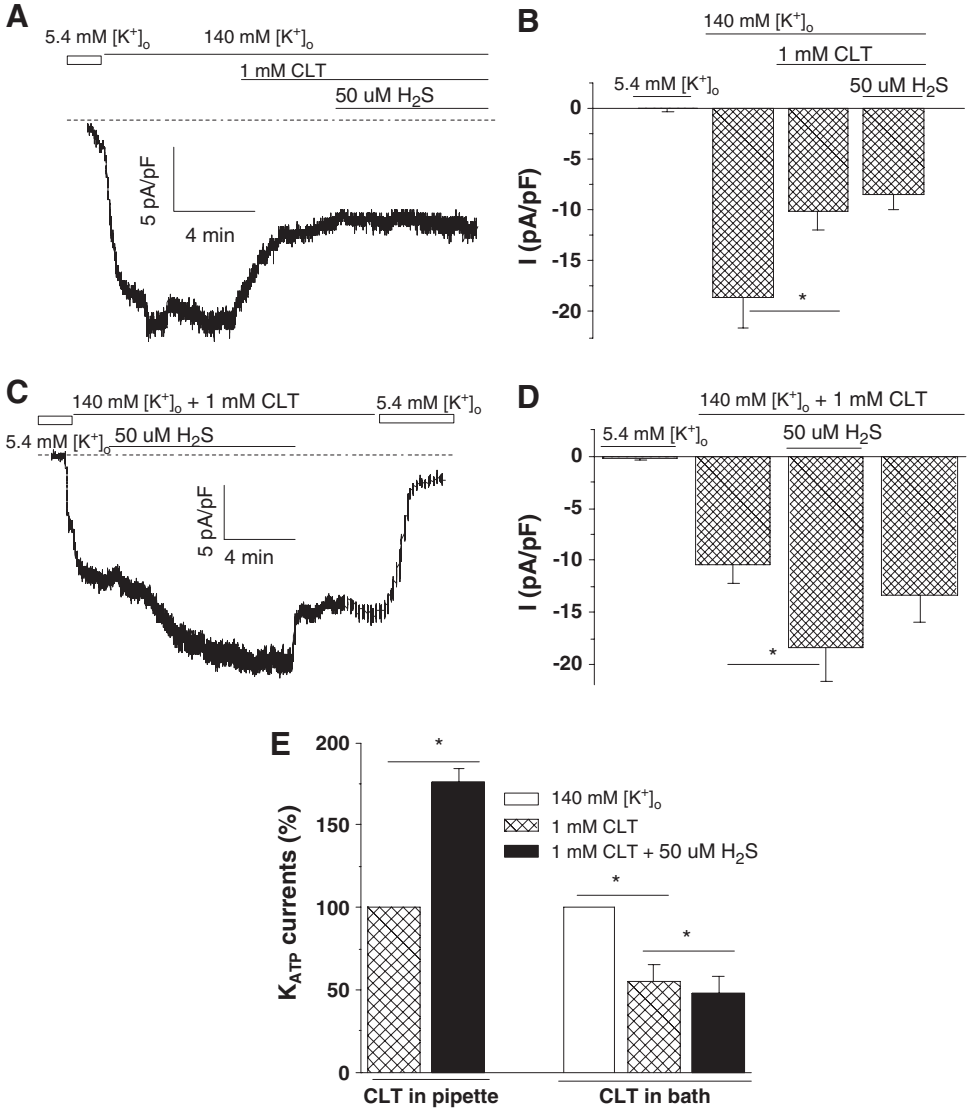

FIG. 6. Effects of chloramine-T (CLT) and H_2S on rvKir6.1/rvSUR1-encoded K_{ATP} channels in HEK-293 cells. All recordings were conducted at −60 mV with different extracellular K^+ concentrations. (**A**) Representative original K_{ATP}-current recording with extracellular CLT at 1 m*M*. (**B**) Summary of the effects of extracellular CLT from five cells ($^*p < 0.05$). (**C**) Representative original K_{ATP}-current recording with intracellular CLT at 1 m*M*. (**D**) Summary of the effects of intracellular CLT from five cells ($^*p < 0.05$). (**E**) Comparison of the alterations of H_2S-stimulated K_{ATP} channels between the effects of intracellular CLT ($n = 5$) and extracellular CLT ($n = 5$) ($^*p < 0.05$).

Cys6 and Cys26, but not Cys1051 and Cys1057, are necessary for H_2S-induced increase in K_{ATP} currents. In double-mutated rvKir6.1/rvSUR1 (C6S and C1057S) channels, H_2S (even at 200 μM) did not stimulate K_{ATP} currents ($n = 7$) (Fig. 9A and B). Furthermore, this mutated channel lost the sensitivity to glibenclamide ($n = 5$) and protopine (Fig. 9C and D), which are two specific agents to modulate selectively the rvSUR1 subunits of the functional rvKir6.1/rvSUR1 channel complex (17).

Discussion

Whereas the stimulation of K_{ATP} channels by H_2S has been reported, our present study is the first to provide a molecular mechanism underlying this effect of H_2S. H_2S interacts with the SUR subunit of K_{ATP} channel complex to cause the channel to open. The first line of evidence for this conclusion is that H_2S stimulated the activity of coexpressed rvKir6.1/rvSUR1 channel complex, but not rvKir6.1 alone–encoded channel. SUR is a regulatory subunit of K_{ATP} channel complex, endowing the channel with a sensitivity to high-affinity sulfonylurea inhibition (2, 3) and activation by MgADP and K^+ channel openers (KCOs) (25, 35) and zinc (4, 27), respectively. We previously reported that protopine selectively inhibited the heterologously expressed K_{ATP} channel complex by targeting the SUR1 subunit, rather than the Kir6.1 subunit (17). Thus, H_2S may regulate different cellular functions by targeting the SUR1 subunit of K_{ATP} channels in the concerned cell types.

Cysteine residues, especially those located extracellularly, are important for the functional integrity of the involved proteins by modulating protein configuration and activity by

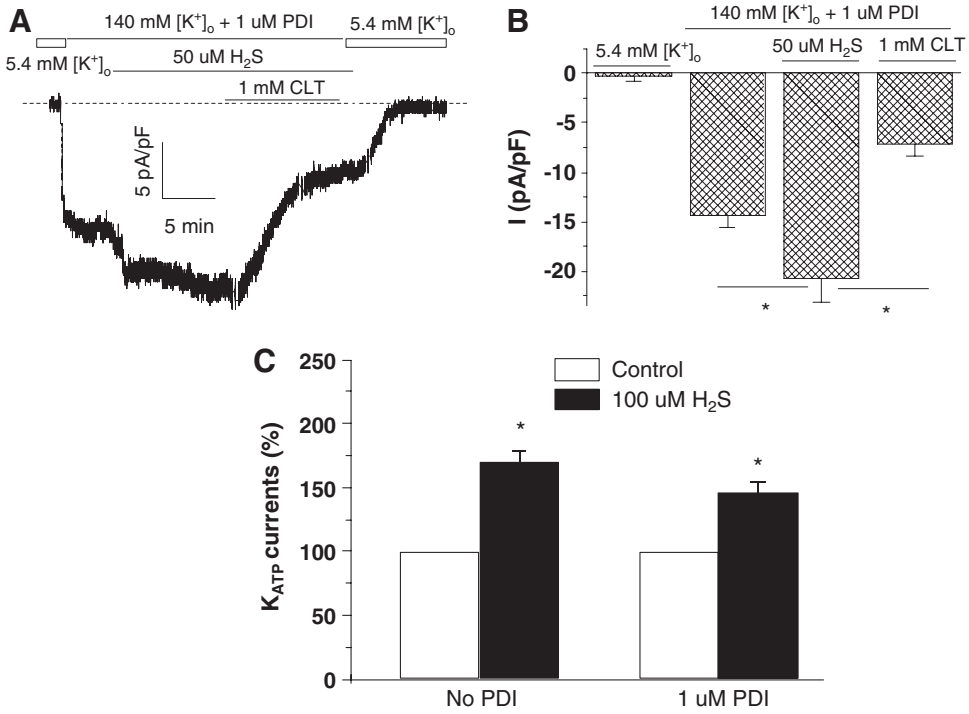

FIG. 7. Effects of protein disulfide isomerase (PDI) and H_2S on rvKir6.1/rvSUR1-encoded K_{ATP} channels in HEK-293 cells. All recordings were conducted at −60 mV with different extracellular K^+ concentrations. (**A**) Representative original K_{ATP} current recording with intracellular PDI at 1 m*M*. (**B**) Summary of the effects of intracellular PDI from five cells ($^*p < 0.05$). (**C**) Comparison of the alterations of H_2S-stimulated K_{ATP} channels in the absence and presence of intracellular PDI ($n = 5 \sim 10$; $^*p < 0.05$).

reacting on their free –SH groups (5, 6). These reactions include a thiolation/dethiolation (the formation of an adduct of HS^- with free –SH groups or the breakdown of disulfide bonds), S-nitrosylation (29), and chelation of transition metals (Fenton-type reaction and Haber-Weiss reaction) (13, 49). The initial clues for the notion that H_2S may interact with cysteine residues of the rvSUR1 subunit were derived from our chemical modification studies, which applied different chemical agents to opposite topologic sides of the cell membrane. These chemicals include NEM, CLT, and PDI.

The current density of the rvKir6.1/rvSUR1 K_{ATP} channels in cells dialyzed with NEM (-9.9 ± 0.8 pA/pF; $n = 5$) (Fig. 5D) is much lower than that in the presence of extracellular NEM (-22.4 ± 5.1 pA/pF; $n = 5$) (Fig. 5B) under the same recording conditions. This indicated that internally applied NEM inhibited rvKir6.1/rvSUR1 K_{ATP} channel activity. It was reported that intracellularly applied NEM inhibited whole-cell K_{ATP} currents in CRI-G1 insulin-secreting cells (21) and suppressed single K_{ATP} channel activity in inside-out patches of cardiomyocytes (14) and skeletal muscle (40). These results provide evidence for the presence of essential sulfhydryl residues, associated with the normal functioning of the K_{ATP} channel, which are situated at the internal surface of the membrane, possibly near the channel pore. Conversely, the observation that bath-applied NEM did not increase basal K_{ATP} currents may be explained by the lack of free sulfhydryl groups on extracellular loop of K_{ATP} channels. In the presence of NEM in the bath solution, the H_2S-induced increase in the rvKir6.1/rvSUR1 current was abolished (Fig. 5B). This can be explained by the notion that H_2S may break extracellular disulfide bond of rvSUR1, which exposed free sulfhydryl groups for NEM alkylation and then inhibited the open channels, leading to the abolishment of H_2S effects.

CLT can oxidize the thioether group of methionine residues of protein to methionine sulfoxide at high concentration ($\sim$100–300 μM), and oxidize sulfhydryl groups of cysteine residues to cystine at a low concentration ($<$100 μM) (33). In our study, basal rvKir6.1/rvSUR1 currents were similarly reduced by CLT applied intracellularly or extracellularly. A similar observation was reported before (40). Interestingly, extracellularly, but not intracellularly, applied CLT abolished the stimulatory effects of H_2S on K_{ATP} currents, indicating that cysteine residues on the extracellular loop of rvSUR1 were the potential targets of H_2S, although methionine residues also may be affected.

PDI is an enzyme in the endoplasmic reticulum, facilitating the folding and oxidation of proteins. PDI is reported to catalyze the breakage and reformation of disulfide bridges (34). Intracellularly applied PDI did not alter basal K_{ATP} currents and did not prevent the stimulatory effect of H_2S and the inhibitory effects of CLT on K_{ATP} currents (Fig. 7A and B). These data indicate that sulfhydryl groups, located on the intracellular loops of the SUR subunit, may not be the target of H_2S.

With indications from the effects of the aforementioned chemical agents, we focused our investigation on the interaction of H_2S with extracellular cysteine residues of rvSUR1 subunits of coexpressed K_{ATP} channels. Subsequently, a gene-mutation approach was adopted. In selectively mutated rvKir6.1/rvSUR1-C6S and rvKir6.1/rvSUR1-C26S, the stimulatory effect of H_2S on K_{ATP} currents was eliminated. Dou-

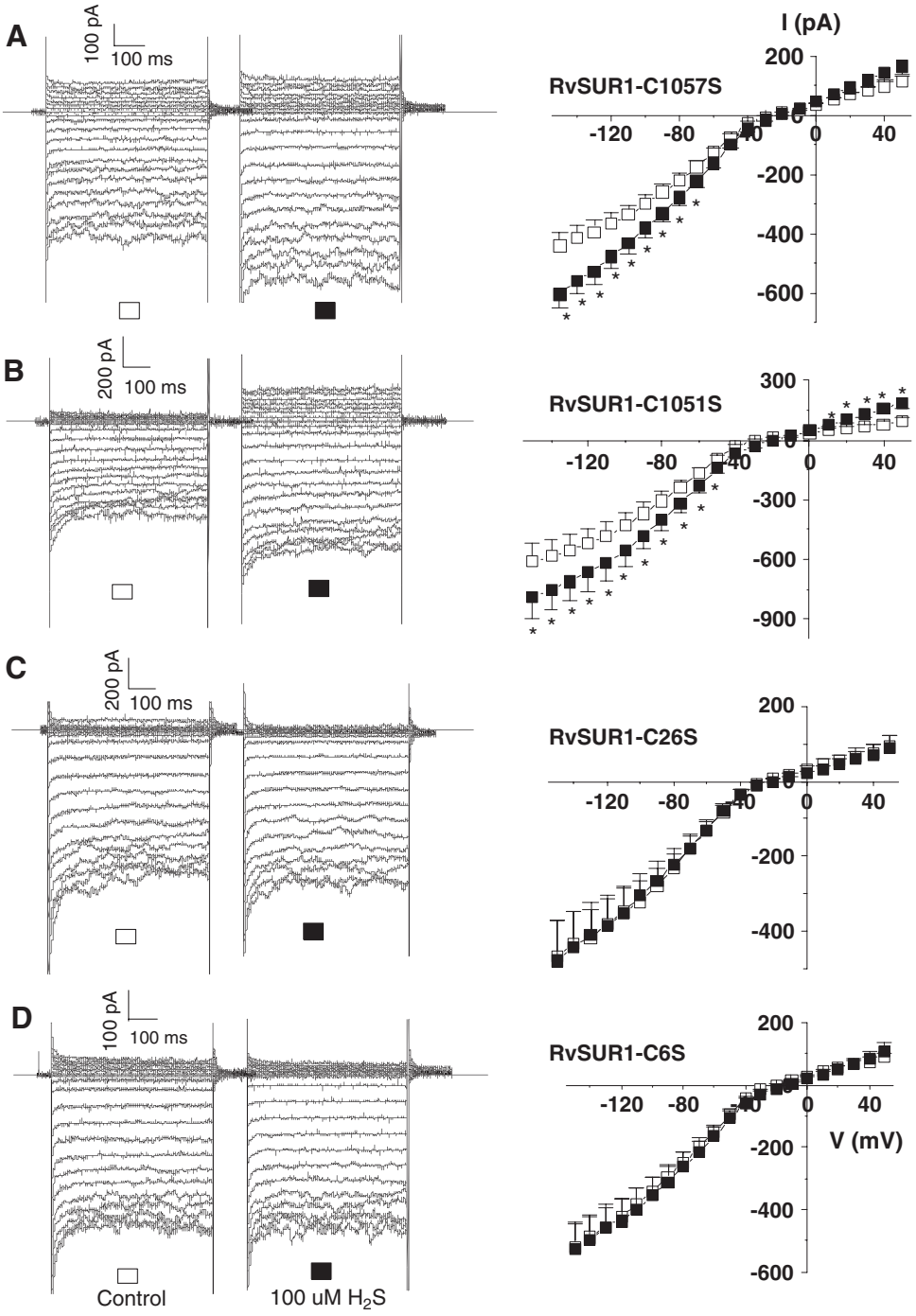

FIG. 8. Effects of H_2S on K_{ATP} channels in HEK-293 cells encoded by rvKir6.1 and the mutated rvSUR1 gene. All K_{ATP} currents were recorded with 600-ms depolarizing pulses (0.1 Hz) from −150 to +50 mV with a holding potential of −30 mV. (**A**) Representative K_{ATP}-current traces in the *left* and the summarized current–voltage (I-V) relation in the *right* panel, with the mutated rvSUR1-C1057S gene ($n = 12$; $^*p < 0.05$). (**B**) Representative K_{ATP}-current traces in the *left* and the summarized I-V relation in the *right* panel with the mutated rvSUR1-C1051S gene ($n = 12$; $^*p < 0.05$). (**C**) Representative K_{ATP}-current traces in the *left* and the summarized I-V relation in the *right* panel, with the mutated rvSUR1-C26S gene ($n = 11$). (**D**) Representative K_{ATP}-current traces in the *left* and the summarized I-V relation in the *right* panel, with the mutated rvSUR1-C6S gene ($n = 6$). The *open squares* (□) and *solid squares* (■) stand for the control and H_2S treatment at 100 μM, respectively, in all illustrations.

ble-mutation of rvSUR1-C6S-C1057S also abolished the stimulatory effect of H_2S. To this end, we demonstrated that two cysteine residues of the rvSUR1 subunit, Cys6 and Cys26, located at the extracellular loop of the N-terminus, are the target sites of H_2S action.

The functional correlation of the rvKir6.1/ rvSUR1 K_{ATP} channel

K_{ATP} channels are composed of a pore-forming inwardly rectifying K^+ channel (Kir6.x) tetramer and a regulatory

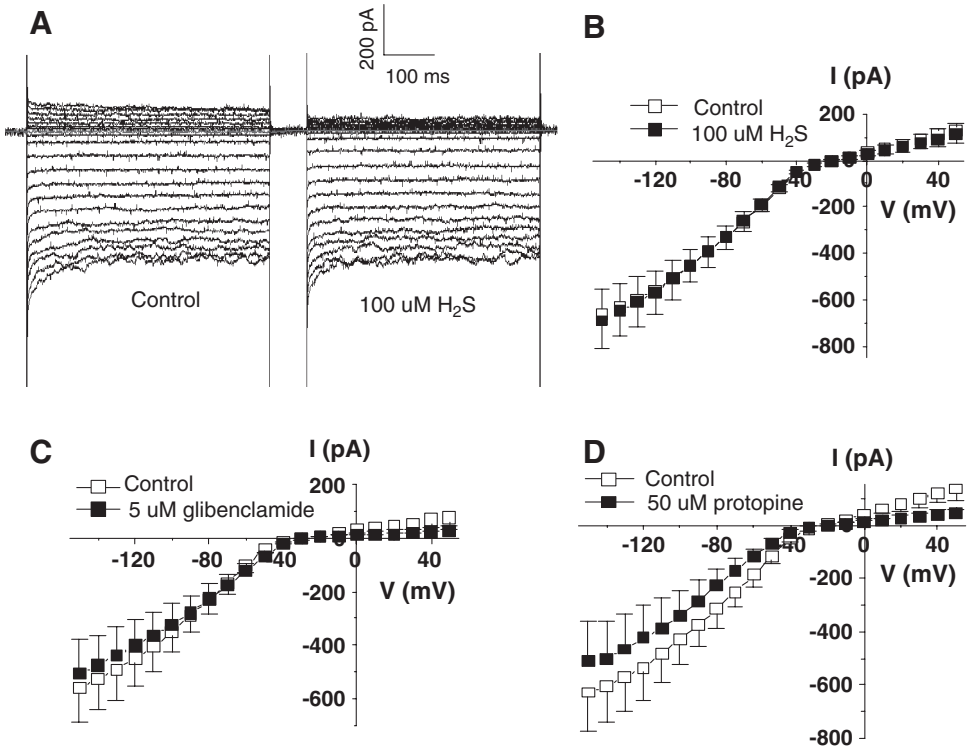

FIG. 9. Effects of H_2S on K_{ATP} channels in HEK-293 cells encoded by rvKir6.1 and the double-mutated rvSUR1 gene (C1057S + C26S). All K_{ATP} currents were recorded with 600-ms depolarizing pulses (0.1 Hz) from −150 to +50 mV, with a holding potential of −30 mV. (**A**) Representative original K_{ATP}-current recordings in the absence and presence of 100 μM H_2S. (**B**) Summarized I-V relations of rvKir6.1/rvSUR1-C1057S-C26S–encoded K_{ATP} channels in the absence and presence of 100 μM H_2S ($n = 6$). (**C**) Summarized I-V relations of rvKir6.1/rvSUR1-C1057S-C26S–encoded K_{ATP} channels in the absence and presence of glibenclamide ($n = 5$). (**D**) Summarized I-V relations of rvKir6.1/rvSUR1-C1057S-C26S–encoded K_{ATP} channels in the absence and presence of protopine ($n = 5$).

sulfonylurea receptor (SURs) tetramer. The latter confers sulfonylurea and nucleotide (ATP and ADP) sensitivity (15, 30). Different combinations of Kir6.x and SURs yield the tissue-specific K_{ATP} channel subtypes with different electrophysiologic and pharmacologic features (16, 42). Thus, Kir6.2/SUR1 constitutes K_{ATP} channels in pancreatic β cells and in brain tissues, including cholinergic basal forebrain neurons (1, 22). Kir6.2/SUR2A forms K_{ATP} channels in cardiac and skeletal muscles. Kir6.2/SUR2B is the K_{ATP} isoform in nonvascular SMCs and other types of neurons, whereas Kir6.1/SUR2B is the isoform of K_{ATP} channels in vascular SMCs (12).

Kir6.1/SUR1 constitutes functional K_{ATP} channels in frog retinal glial cells (28), dentate gyrus granule cells (26, 28), and some glucose-receptive neurons within the rat ventromedial hypothalamus (20). Based on the pharmacologic sensitivity to diazoxide, P-1075, glibenclamide, 5-HD, and HMR-1098, Kir6.1/SUR1 is believed to be the molecular makeup of mitochondrial K_{ATP} channels in cardiac myocytes and other cell types (23, 48). This notion is further endorsed by the identification of both Kir6.1 and SUR1 proteins in mitochondria of P_{12} cells (31).

The SUR1 subunit plays important roles in the nervous system for the regulation of neuronal excitability and also in pancreatic β cells for insulin secretion. Our present study focused on the effect of H_2S on the coexpressed rvKir6.1/rvSUR1 channels for the following reasons:

1. The expression of Kir6.1 and SUR1 genes in rat mesenteric artery smooth muscle was confirmed in our previous study (8). Use of these cloned K_{ATP} channel subunits will better correlate our previous observations that H_2S stimulates native K_{ATP} channels in vascular smooth muscle cells (32, 50).
2. Kir6.1 has a ubiquitous tissue expression and confers the relative ATP insensitivity, which is one of the fingerprints of K_{ATP} channels in glial cells, vascular smooth muscle cells, and mitochondria.
3. Because Kir6.1/SUR1s are putative protein complexes for K_{ATP} channels in glial cells and in mitochondria, the exploration of electrophysiologic and pharmacologic characteristics of rvKir6.1/rvSUR1 channels will help us to understand the effect of H_2S on glial as well as mitochondrial functions.

A proposed model of K_{ATP} channel modulation by H_2S

Under resting conditions, a dynamic balance between free –SH groups and disulfide bonds formation is reached, which sustains basal K_{ATP} channel activity. Sulfhydryl-modifying agents, such as NEM and CLT, bind to external cysteine residues so that the binding site for H_2S is occupied and the effect of H_2S on K_{ATP} channel activity is abolished. NEM itself has no effects on basal K_{ATP} currents, which indicates the lack of free –SH groups of the SUR subunit on the external side of the

cell membrane. When H_2S is applied, the disulfide bonds of the SUR subunit are broken, and free –SH groups are exposed to offer binding sites for NEM. That may be the reason that H_2S stimulatory effects can be suppressed by NEM. Unlike NEM, CLT can bind to both free –SH groups and nonfree –SH groups. That may be the reason that CLT inhibits basal K_{ATP} currents. In a similar way to NEM, CLT abolished H_2S stimulation on K_{ATP} currents. The deletion of extracellular Cys6 or Cys26 of SUR1 subunits causes the loss of channel sensitivity to H_2S. It appears that H_2S either binds to cysteine residues of the extracellular loop of the SUR1 subunit of K_{ATP} channels at the location of Cys6 and Cys26 or breaks the disulfide bond involving Cys6 and Cys26 and then changes the K_{ATP} channel complex configuration, leading to the opening of the pore-forming Kir6.1 subunit and increased K_{ATP} currents. The discovery of H_2S targeting on the SUR subunit may help us to design specific agents to modulate the function of K_{ATP} channels selectively and to facilitate the understanding of the general mechanisms governing the molecular interaction of H_2S with other proteins.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

CBS = cystathionine β-synthase
CLT = chloramine T
CSE = cystathionine γ-lyase
Cys = cysteine
Diaz = diazoxide
Gli = glibenclamide
H_2S = hydrogen sulfide
K_{ATP} channels = ATP-sensitive K^+ channels
NEM = *N*-ethylmaleimide
PDI = protein disulfide isomerase
–SH group = sulfhydryl group

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