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Endogenous and exogenous hydrogen sulfide facilitates T-type calcium channel currents in $Ca_v3.2$-expressing HEK293 cells

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ABSTRACT

Hydrogen sulfide (H_2S), a gasotransmitter, is formed from L-cysteine by multiple enzymes including cystathionine-γ-lyase (CSE). We have shown that an H_2S donor, NaHS, causes hyperalgesia in rodents, an effect inhibited by knockdown of $Ca_v3.2$ T-type Ca^{2+} channels (T-channels), and that NaHS facilitates T-channel-dependent currents (T-currents) in NG108-15 cells that naturally express $Ca_v3.2$. In the present study, we asked if endogenous and exogenous H_2S participates in regulation of the channel functions in $Ca_v3.2$-transfected HEK293 ($Ca_v3.2$-HEK293) cells. DL-Propargylglycine (PPG), a CSE inhibitor, significantly decreased T-currents in $Ca_v3.2$-HEK293 cells, but not in NG108-15 cells. NaHS at 1.5 mM did not affect T-currents in $Ca_v3.2$-HEK293 cells, but enhanced T-currents in NG108-15 cells. In the presence of PPG, NaHS at 1.5 mM, but not 0.1–0.3 mM, increased T-currents in $Ca_v3.2$-HEK293 cells. Similarly, Na_2S, another H_2S donor, at 0.1–0.3 mM significantly increased T-currents in the presence, but not absence, of PPG in $Ca_v3.2$-HEK293 cells. Expression of CSE was detected at protein and mRNA levels in HEK293 cells. Intraplantar administration of Na_2S, like NaHS, caused mechanical hyperalgesia, an effect blocked by NNC 55-0396, a T-channel inhibitor. The *in vivo* potency of Na_2S was higher than NaHS. These results suggest that the function of $Ca_v3.2$ T-channels is tonically enhanced by endogenous H_2S synthesized by CSE in $Ca_v3.2$-HEK293 cells, and that exogenous H_2S is capable of enhancing $Ca_v3.2$ function when endogenous H_2S production by CSE is inhibited. In addition, Na_2S is considered a more potent H_2S donor than NaHS *in vitro* as well as *in vivo*.

1. Introduction

Hydrogen sulfide (H_2S), a gasotransmitter, is formed from L-cysteine by distinct enzymes including cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) and cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase [1]. Accumulating evidence has shown critical roles of H_2S in health and disease. H_2S appears to play dual roles in many tissues/organs including neuronal systems, being anti-/pro-inflammatory and cytoprotective/cytotoxic [2,3]. H_2S is now considered to play a pro-nociceptive role in somatic pain signaling and also in processing of visceral pain including colonic, pancreatic and bladder pain [2,4–9], although there are a few conflicting reports showing antinociceptive effects of H_2S donors in colorectal distension models [10]. Endogenous H_2S produced by CSE and/or CBS participates in pathophysiology of inflammatory and neuropathic pain [4,7,8,11–14]. H_2S targets a variety of molecules including distinct ion channels [2]. Among them, both $Ca_v3.2$ T-type Ca^{2+} channels (T-channels) and transient receptor potential ankyrin-1 (TRPA1) channels are considered to mediate H_2S-induced somatic and/or visceral pain signaling [5,8,9,15,16]. Our *in vivo* studies have shown that NaHS-induced somatic and visceral pain/hyperalgesia is reduced by gene silencing of $Ca_v3.2$ T-channels or TRPA1 channels [5,8,15,16]. We have also demonstrated that NaHS, an H_2S donor, facilitates T-channel-dependent Ba^{2+} currents (T-currents) in NG108-15 cells that naturally express $Ca_v3.2$ [4,17] and in isolated mouse dorsal root

Abbreviations: H_2S, hydrogen sulfide; CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; T-channel, T-type Ca^{2+} channel; TRPA1, transient receptor potential ankyrin-1; T-current, T-channel-dependent current; PPG, DL-propargylglycine; $Ca_v3.2$-HEK293, $Ca_v3.2$-transfected HEK293 cells.

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ganglion neurons that abundantly express $Ca_v3.2$ [6]. However, the effects of H_2S on T-currents have yet to be confirmed in $Ca_v3.2$-transfected cells, while NaHS-induced cytosolic Ca^{2+} mobilization has been demonstrated in TRPA1-transfected cells. In the present study, we thus determined if NaHS and Na_2S, H_2S donors, and/or DL-propargylglycine, an inhibitor of CSE, alter T-type Ca^{2+} currents in $Ca_v3.2$-transfected HEK293 cells. Here we provide, for the first time to our knowledge, direct evidence that exogenous H_2S and endogenous H_2S formed by CSE act to promote ion channel functions of $Ca_v3.2$ expressed in HEK293 cells.

2. Materials and methods

2.1. Chemicals

DL-Propargylglycine (PPG), Na_2S and NNC 55-0396 were purchased from Sigma–Aldrich (St. Louis, MO, USA), and NaHS was from Kishida Chemical (Osaka, Japan). All chemicals were dissolved in distilled water for *in vitro* experiments or in saline for *in vivo* experiments.

2.2. Cell culture and creation of HEK293 cells that stably express GFP-human $Ca_v3.2$

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chem., Osaka, Japan) supplemented with 10% fetal calf serum (FCS) (Thermo Electron, Melbourne, Australia), 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco, Carlsbad, CA, USA). NG108-15 cells (mouse neuroblastoma × rat glioma hybrid cells) were cultured in high glucose-containing DMEM (Wako Pure Chem.) supplemented with 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS.

$Ca_v3.2$ cDNA originally cloned from pregnant human uterus [18] was subcloned into pCruz GFP vector (Santa Cruz Biotech., Santa Cruz, CA, USA) and then transfected into HEK293 cells using the FuGENE6 Transfection Reagent (Roche Diagnostics K.K., Tokyo, Japan). To select a clone expressing GFP-$Ca_v3.2$ at a high level, fluorescence of GFP was observed with a fluorescent microscope, and T-channel currents (T-currents) in each clone were measured by a whole-cell patch clamp technique, as described below. The GFP-$Ca_v3.2$-transfected HEK293 cells ($Ca_v3.2$-HEK293) were maintained in the above-mentioned medium containing G418 (Sigma-Aldrich) at 250 μg/ml for keeping selection pressure.

2.3. Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings in $Ca_v3.2$-HEK293 and NG108-15 cells were performed as described previously [4]. Cells (1×10^4 cells) were seeded in plastic dishes (35 mm in diameter) and cultured for a day in each cell culture medium containing 1% FCS. The composition of the extracellular solution for patch-clamp experiments was (in mM): 2 CsCl, 160 tetraethylammonium (TEA)-Cl, and 10 HEPES, adjusted to pH 7.4, for measurement of Ca^{2+} currents in $Ca_v3.2$-HEK293 cells, or 97 *N*-methyl-D-glucamine (NMDG), 10 $BaCl_2$, 10 HEPES, 40 TEA-Cl and 5.6 glucose, adjusted to pH 7.4, for measurement of Ba^{2+} currents in NG108-15 cells. The composition of the intracellular solution was (mM): 110 CsCl, 10 EGTA, 10 HEPES, 3 Mg-ATP, and 0.6 Na-GTP, adjusted to pH 7.2, for $Ca_v3.2$-HEK293 cells, or 150 CsCl, 4 $MgCl_2$, 5 EGTA and 10 HEPES, adjusted to pH 7.2, for NG108-15 cells. The resistance of patch electrodes ranged from 3 to 5 MΩ. Series-resistance was compensated by 80%, and current recordings were low-pass filtered (<5 kHz). In $Ca_v3.2$-HEK293 and control HEK293 cells, the cell membrane voltage was held at −90 mV, and whole-cell Ca^{2+} currents were elicited by step pulses from −120 to 40 mV with increments of 5 mV. T-channel-dependent currents (T-currents) were elicited by a test pulse at −30 and −20 mV from the holding potential at −90 and −80 mV in $Ca_v3.2$-HEK293 and NG108-15 cells, respectively. T-currents were measured as the difference between currents at a peak and detected 150 ms after the beginning of the test pulse. Data were acquired and digitalized through Digidata (1440A, Axon Instrument, Foster City, CA, USA) and analyzed by a personal computer using pClamp10.2 software (Axon Instruments). After recording the control currents, PPG, a CSE inhibitor, or vehicle at 0.95 or 5 mM was added, and currents were recorded again 10 min later. Thereafter, in the presence of PPG, H_2S donors, NaHS at 0.1-1.5 mM or Na_2S at 0.03-0.3 mM, were added, and currents were recorded 2 and 5 min later.

2.4. Reverse-transcribed-polymerase chain reaction (RT-PCR)

$Ca_v3.2$-HEK293 cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA extracted from the cell lysate was reverse-transcribed and then amplified by PCR using RNA LA PCR kit (AMV) ver. 1.1 (Takara Bio, Otsu, Japan). The specific primers are as follows: human CSE (product size, 157 bp), 5′-CAC TGT CCA CCA CGT TCA AG-3′ (forward), 5′-GTG GCT GCT AAA CCT GAA GC-3′ (reverse); human GAPDH (product size, 226 bp) 5′-GAA GGT GAA GGT CGG AGT C-3′ (forward), 5′-GAA GAT GGT GAT GGG ATT TC-3′ (reverse). The PCR reactions for CSE and GAPDH were allowed to proceed for 35 and 25 cycles, respectively (94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s). The PCR products were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining. LNCaP prostate cancer cells were used as a positive control for CSE.

2.5. Western blotting

$Ca_v3.2$-HEK293 cells and the control HEK293 cells were collected with lysis buffer containing 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris–HCl and 10% glycerol (pH 6.8). The protein samples were separated by electrophoresis on a 12.5% SDS–polyacrylamide gel (Wako Pure Chem.) and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA). The primary antibodies used were: rabbit anti-CSE polyclonal antibody (Sigma–Genosys/Sigma–Aldrich) against a peptide corresponding to the amino acid sequence, (C)80GGTNRYFRR89 V, in rat CSE [19]) and anti-GAPDH antibody (Santa Cruz Biotechnol.). Immunolabelled proteins (CSE, 44 kDa; GAPDH, 37 kDa) were visualized by Chemi-Lumi One Super (Nakarai Tesque, Kyoto, Japan).

2.6. Evaluation of effects of H_2S donors on mechanical nociceptive threshold in mice

Male ddY mice weighing 18–25 g were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). The animals were housed in a temperature-controlled room under a 12-h day/night cycle at about 24 °C and had free access to food and water. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University, and in accordance with EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/enviroment/chemicals/lab_animals/legislation_eu.htm). Mice were placed on a raised wire mesh floor, covered with a clear plastic box (23 × 16 × 12 cm), and acclimated to the experimental environment. The mid-plantar surface of the right hindpaw was stimulated with von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6 and 1 g), and 50% paw withdrawal threshold was determined according to the up-down method [20].

2.7. Statistics

Data are represented as means ± S.E.M. Statistical significance for parametric data was evaluated by ANOVA followed by Tukey's test for multiple comparisons or by Student's *t*-test for two-group comparisons. For non-parametric analysis, the Kruskal–Wallis *H*-test followed by a least significant difference (LSD)-type test was employed for multiple comparisons. Significance was set at a level of $P < 0.05$.

3. Results

3.1. Effect of PPG, a CSE inhibitor, on T-currents in $Ca_v3.2$-HEK293 cells

Stable expression of GFP-$Ca_v3.2$ T-channels in HEK293 cells was confirmed by detection of GFP fluorescence and Ca^{2+} currents (Fig. 1A and B). The peak T-current was observed in response to test pulses around −30 mV from the holding potential at −90 mV in $Ca_v3.2$-HEK293 cells (Fig. 1B). The T-currents were partially, but significantly suppressed by treatment with PPG, an inhibitor of CSE, at 0.95 or 5 mM for 10 min (Fig. 1C and D). Expression of CSE at protein and mRNA levels was confirmed in both control HEK293 cells and $Ca_v3.2$-HEK293 cells, as in LNCaP cells (Fig. 1E).

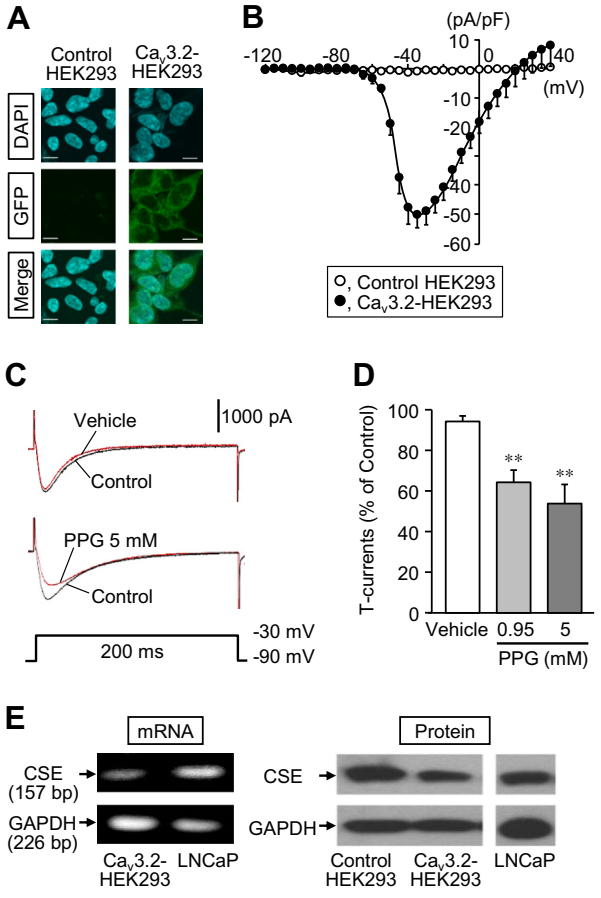

Fig. 1. Effects of PPG, a CSE inhibitor, on T-currents in GFP-$Ca_v3.2$-transfected HEK293 ($Ca_v3.2$-HEK293) cells. (A, B) Confirmation of expression of $Ca_v3.2$ in HEK293 cells. Fluorescence of GFP (green) (A) and inward currents (B) were detected in $Ca_v3.2$-HEK293 cells, but not in the control HEK293 cells. Nuclei were stained with DAPI (blue), and scale bars indicate 10 μm (A). (C, D) T-currents induced by a test pulse at −30 mV from a holding potential at −90 mV were measured before and 10 min after application of PPG. Data show mean ± S.E.M. for 7–8 (B) or 5–7 (D) different cells. $^{**}P < 0.01$ vs. vehicle. (E) Detection of CSE mRNA and protein in HEK293 cells. CSE protein was detected in both control and $Ca_v3.2$-HEK293 cells, as in LNCaP cells known to express CSE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Effect of H_2S donors on T-currents in $Ca_v3.2$-HEK293 cells in the absence or presence of PPG, a CSE inhibitor

In NG108-15 cells that naturally express $Ca_v3.2$ [17], PPG did not alter T-currents (Fig. 2A), being in contrast to its suppressive effect on T-currents in $Ca_v3.2$-HEK293 cells (see Fig. 1C and D). On the other hand, in the absence of PPG, stimulation with NaHS, an H_2S donor, at 1.5 mM for 2 min did not alter T-currents in $Ca_v3.2$-HEK293 cells (Fig. 2C), although it enhanced T-currents in NG108-15 cells (Fig. 2B), as reported previously [17]. Nonetheless, in the presence of PPG at 5 mM, NaHS at 1.5 mM, but not 0.1 or 0.3 mM, significantly enhanced the T-currents in $Ca_v3.2$-HEK293 cells (Fig. 2D). Similarly, another H_2S donor, Na_2S, even at 0.1 and 0.3 mM also significantly enhanced T-currents in the presence of PPG at 5 mM (Fig. 3C) and also at a lower concentration, 0.95 mM, in $Ca_v3.2$-HEK293 cells (Fig. 3B), while it had no effect on T-currents in the absence of PPG (Fig. 3A). Thus, the effective concentrations of Na_2S and NaHS were 0.1–0.3 mM and 1.5 mM or more, respectively, in $Ca_v3.2$-HEK293 cells, suggesting that Na_2S is more potent than NaHS in facilitating $Ca_v3.2$ functions.

3.3. Effect of intraplantar administration of Na_2S on the mechanical nociceptive threshold in mice

To confirm the difference in the potency of Na_2S and NaHS as the enhancer of $Ca_v3.2$ functions *in vivo*, we finally evaluated the pro-nociceptive activity of Na_2S in comparison with NaHS, known to exhibit T-channel-dependent mechanical allodynia/hyperalgesia [4,5,15]. Intraplantar (i.pl.) administration of Na_2S at 10–100 pmol/paw significantly decreased mechanical nociceptive threshold, as determined by the von Frey test, in mice (Fig. 3E), while the

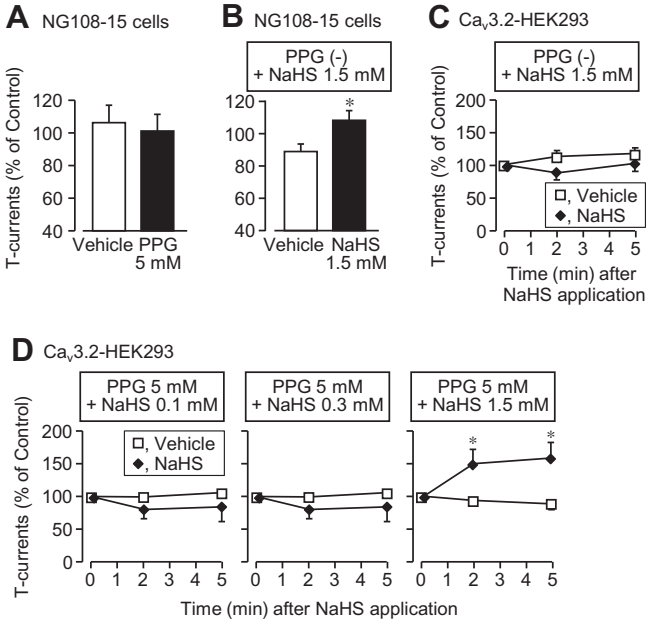

Fig. 2. Effects of NaHS, an H_2S donor, on T-currents in NG108-15 cells and $Ca_v3.2$-HEK293 cells in the presence or absence of PPG, a CSE inhibitor. (A, B) In NG108-15 cells, T-currents were determined before (control) and 10 min after addition of PPG (A), and before (control) and 2 min after application of NaHS in the absence of PPG. (C, D) In $Ca_v3.2$-HEK293 cells, T-currents were measured before (control) and 2 and 5 min after application of NaHS in the presence (D) or absence (C) of PPG at 5 mM. Data show mean ± S.E.M. for 12–18 (A), 6 (B) or 4–7 (C, D) different cells. $^{*}P < 0.05$ vs. vehicle.

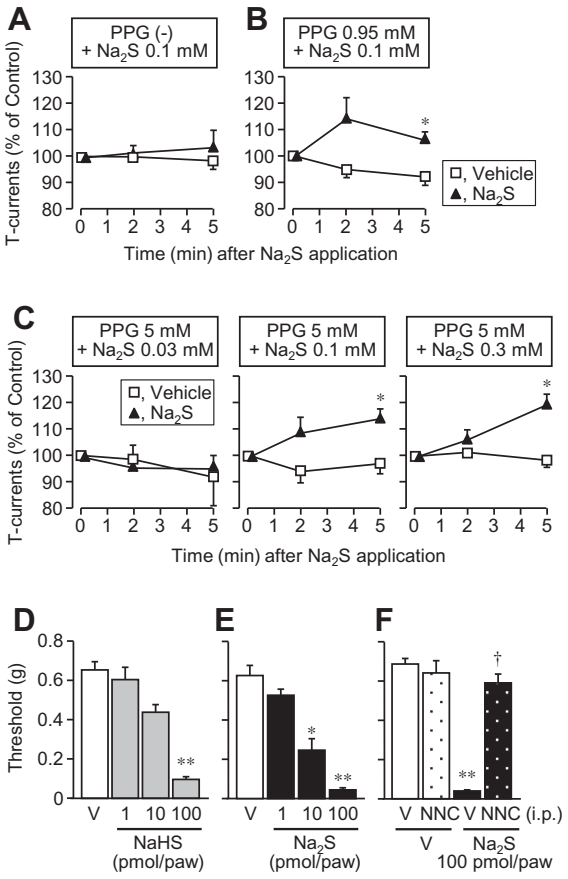

Fig. 3. Effects of Na_2S, another H_2S donor, on T-currents in the presence or absence of PPG, a CSE inhibitor, in $Ca_v3.2$-HEK293 cells, and on mechanical nociceptive threshold in mice as assessed by the von Frey test. (A–C) T-currents were measured before (control) and 2 and 5 min after application of Na_2S in the cells treated with PPG at 0.95 mM (B) and 5 mM (C) or with vehicle (A) for 10 min. Data show mean ± S.E.M. for 4 different cells. $^{*}P < 0.05$ vs. Vehicle. (D-F) The nociceptive threshold was evaluated 15–25 min after intraplantar (i.pl.) administration of NaHS (D) or Na_2S (E and F) in a volume of 10 μl. NNC 55-0396 (NNC), an inhibitor of T-channels, at 20 mg/kg or vehicle (V) was administered i.p. 30 min before i.pl. Na_2S (F). Data show mean ± S.E.M. for 4–5 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. V (D, E) or V + V (F); $^{\dagger}P < 0.05$ vs. V + Na_2S.

significant hyperalgesic effects of i.pl. NaHS were detected at 100, but not 10 pmol/paw (Fig. 3D). It was also confirmed that the hyperalgesic effect of Na_2S was completely blocked by pretreatment with NNC 55-0396, an inhibitor of T-channels, at 20 mg/kg (Fig. 3F).

4. Discussion

Our finding that the CSE inhibitor PPG suppressed T-currents, suggests that endogenous H_2S formed by CSE tonically enhances the function of $Ca_v3.2$ transfected into HEK293 cells that naturally express CSE. The results that H_2S donors, NaHS and Na_2S, enhanced T-currents in $Ca_v3.2$-HEK293 cells in the presence, but not absence, of PPG, indicate that endogenous H_2S is capable of promoting $Ca_v3.2$ function only in the absence of endogenous H_2S brought about by inhibition of CSE (Fig. 4). On the other hand, in NG108-15 cells, endogenous H_2S formed by CSE might not be enough to fully enhance $Ca_v3.2$ function, since the T-currents detected in NG108-15 cells were not affected by PPG, and enhanced by H_2S donors even in the absence of PPG.

Among three T-channel isoforms, $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$, only $Ca_v3.2$ is sensitized by H_2S and L-cysteine, and suppressed by Zn^{2+} at physiological concentrations and ascorbic acid (vitamin C) [21–23]. The functional regulation of $Ca_v3.2$ by all these chemicals is considered to involve their interaction with a histidine residue at position 191 (His^{191}) in the second extracellular loop of domain I of $Ca_v3.2$, which is not conserved in $Ca_v3.1$ or $Ca_v3.3$ [23,24]. $Ca_v3.2$ appears to be tonically exposed to inhibition by Zn^{2+} under physiological conditions. Therefore, Zn^{2+}-chelating agents and L-cysteine or H_2S known to interact with Zn^{2+} are capable of cancelling Zn^{2+} inhibition of $Ca_v3.2$, leading to facilitation of $Ca_v3.2$ function and nociceptor sensitization responsible for induction of hyperalgesia *in vivo* [5,22,24–26]. CSE, one of major enzymes involved in H_2S production, is expressed abundantly in the liver, kidney, uterus, brain, blood vessels as well as pancreatic islets [2]. The expression of CSE in HEK293 cells (see Fig. 1E) was not surprising, because HEK293 is a human embryonic kidney-derived cell line.

NaHS and Na_2S at 1.5 mM and 0.1–0.3 mM, respectively, enhanced T-currents in $Ca_v3.2$-HEK293 cells pretreated with PPG, indicating that Na_2S is almost ten-fold more potent than NaHS (see Figs. 2 and 3). In the present study, we also demonstrated that i.pl. administration of Na_2S produced T-channel-dependent hyperalgesia in mice (see Fig. 3E and F), as i.pl. NaHS did in the present study (see Fig. 3D) and the previous report [15]. Considering the effective doses of Na_2S and NaHS (10–100 mg/kg and 100 mg/kg, respectively), Na_2S appears to be more potent than NaHS in the *in vivo* nociception assay, but the potency difference *in vivo* is not as great as that *in vitro*. The difference of the relative potency of Na_2S and NaHS between the *in vitro* and *in vivo* experiments remains to be interpreted.

In addition to $Ca_v3.2$ T-channels, H_2S may target multiple ion channels including ATP-sensitive K^+ channels, Ca^{2+}-activated K^+ channels, cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, L-type voltage-dependent Ca^{2+} channels, TRPA1 channels, and so on [2]. Considering the present findings, impact of endogenous H_2S should be taken into consideration, when the effects of H_2S donors on target molecules including the above-mentioned ion channels are evaluated in HEK293 cells and in other cells that abundantly express H_2S-forming enzymes including CSE.

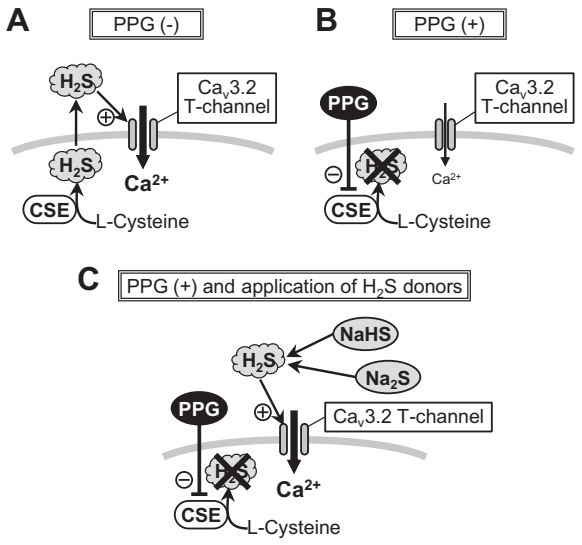

Fig. 4. A hypothetical scheme for sensitization of $Ca_v3.2$ T-channels by endogenous and exogenous H_2S in $Ca_v3.2$-HEK293 cells. CSE, cystathionine-γ-lyase; PPG, DL-propargylglycine. (A) In the absence of PPG, a CSE inhibitor, $Ca_v3.2$ is functionally upregulated by endogenous H_2S synthesized by CSE from L-cysteine. (B, C) In the presence of PPG, the function of $Ca_v3.2$ is downregulated because of decreased endogenous H_2S production (B), and exogenous H_2S derived from NaHS or Na_2S, H_2S donors, is capable of reversing the decreased $Ca_v3.2$ function (C).

In conclusion, the present study provides evidence that endogenous and exogenous H_2S facilitates T-channel function in $Ca_v3.2$-expressing HEK293 cells.

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