Hydrogen sulfide promotes calcium uptake in larval zebrafish

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Kwong RW, Perry SF. Hydrogen sulfide promotes calcium uptake in larval zebrafish. Am J Physiol Cell Physiol 309: C60–C69, 2015.—Hydrogen sulfide (H2S) is a signaling molecule that can modulate various physiological functions in vertebrates (17). Bio-synthesis of H2S is mediated primarily by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), which are expressed in various tissues in mammals, including the kidney, ileum, brain, and endothelium (6, 21). Although it has been reported that H2S concentration in the plasma or environment. calcium; ECaC; hydrogen sulfide; protein kinase A; zebrafish; ionocyte; NaR cell

HYDROGEN SULFIDE (H2S) is a signaling molecule that can modulate various physiological functions in vertebrates (17). Bio-synthesis of H2S is mediated primarily by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), which are expressed in various tissues in mammals, including the kidney, ileum, brain, and endothelium (6, 21). Although it has been reported that H2S concentration in the plasma or fish (26), critical measurements of intracellular H2S are lacking. Previous studies suggested that H2S is involved in cardiorespiratory control, oxygen sensing, and Na+ transport in mammals and fish (1, 13, 25, 27, 31, 33). In mammalian systems, it was also demonstrated that H2S exposure increased cytosolic Ca2+ levels (15, 22, 24). However, the mechanisms by which H2S modulates Ca2+ fluxes, and the physiological importance of H2S in regulating whole body Ca2+ content, remain unknown.

In typical freshwater ecosystems, the environmental Ca2+ levels range from 0.01 to 3 mM, yet plasma Ca2+ levels are remarkably constant, regardless of the ambient Ca2+ levels. To achieve Ca2+ homeostasis, freshwater fish modulate their Ca2+ transport functions in response to changing external Ca2+ levels (3, 23). In zebrafish, Ca2+ uptake is thought to be localized to a subset of ionocytes. The Ca2+-transporting ionocytes are Na+/K+-ATPase-rich cells (NaRcs), which express epithelial Ca2+ channels (ECaC) at the apical membrane, and plasma membrane Ca2+-ATPase (PMCA) and Na+/Ca2+ exchanger (NCX) at the basolateral membrane (7). It has been demonstrated that acclimation to low-Ca2+ water increases the capacity for Ca2+ uptake in zebrafish (14, 19, 29). Various mechanisms for chronic modulation of Ca2+ uptake have been identified, primarily involving regulated expression of ECaC (7).

In the present study, we examined the effects of exposure to H2S donors on whole body Ca2+ fluxes and evaluated the physiological role of endogenous H2S on Ca2+ balance in developing zebrafish. We demonstrated that H2S exposure increases Ca2+ influx via posttranslational activation of cAMP-PKA pathways and found that CBS isoform b (CBSb)-generated H2S promotes Ca2+ influx when fish are maintained in a low-Ca2+ environment.

MATERIALS AND METHODS

Zebrafish maintenance. Adult zebrafish (Danio rerio) were maintained in aerated, dechloraminated City of Ottawa tap water at 28°C (in mM; 0.25 [Ca2+], 0.78 [Na+], 0.02 [K+], 0.15 [Mg2+], where brackets denote concentration; pH 7.6). Fish were subjected to a constant 14:10-h light-dark photoperiod and fed daily until satiation with no. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL). Embryos were collected and reared in 50-ml petri dishes containing dechloraminated City of Ottawa tap water supplemented with 0.05% methylene blue. The petri dishes were kept in incubators at 28°C. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care and after the approval of the University of Ottawa Animal Care Committee (protocol BL-226).

Acclimation experiments. Control (normal) and low-Ca2+ water were prepared with double deionized water supplemented with CaSO4·2H2O, MgSO4·7H2O, NaCl, K2HPO4, and KH2PO4. The [Ca2+] of the normal and low-Ca2+ water were 250 and 25 μM, respectively; the levels were verified using flame emission spectrophotometry. All other ion concentrations were kept constant (in mM; 0.8 Na+, 0.16 Mg2+, and 0.3 K+). Fish were transferred to control or low-Ca2+ water at 1 day postfertilization (dpf) and were sampled for
subsequent experiments (detailed below) at 4 dpf, unless stated otherwise.

Measurement of Ca\textsuperscript{2+} influx. Influx of Ca\textsuperscript{2+} was measured using a radiotracer method, as described previously (14). In brief, fish were exposed to 0.2 \mu M \textsuperscript{45}Ca\textsuperscript{2+} (as CaCl\textsubscript{2}; Perkin Elmer) for 1 h. At the end of the flux period, fish were killed with an overdose (i.e., 4 g/l) of tricaine methanesulfonate (MS-222) and rinsed in isotope-free water; two fish were pooled as one sample (n = 1). Fish were digested with a tissue solubilizer (Solvable; Perkin Elmer) and later neutralized using glacial acetic acid. The radioactivity of the digest and the water samples was measured using a liquid scintillation counter (LS-6500; Beckman Coulter) following the addition of a scintillation cocktail (BioSafe-II; Research Products International). The Ca\textsuperscript{2+} influx (J\textsubscript{Ca}) was determined using the formula: J\textsubscript{Ca} = F/(SA × n × t), where F is the total radioactivity counted in the fish (counts/min), SA is the specific activity of the water (cpm/nmol), n is the number of fish, and t is the duration of the experiment in hours.

Treatment with H\textsubscript{2}S donors. To examine the acute effects of H\textsubscript{2}S exposure on Ca\textsuperscript{2+} influx, normal or low-Ca\textsuperscript{2+} water acclimated zebrafish were preexposed to the H\textsubscript{2}S donor sodium sulfide (Na\textsubscript{2}S\textsubscript{3-}) (1 or 10 \mu M) for 30 min. The chronic effects of H\textsubscript{2}S exposure on Ca\textsuperscript{2+} balance were examined using a stable and slow-releasing H\textsubscript{2}S donor GYY-4137 (Sigma-Aldrich) (16). Fish were exposed to 100 or 200 \mu M GYY-4137 starting at 1 dpf. Water was changed daily with the addition of GYY-4137. To examine whether exposure to H\textsubscript{2}S donors affect the Ca\textsuperscript{2+} uptake, influx of Ca\textsuperscript{2+} at 4 dpf (N = 6) was measured in normal Ca\textsuperscript{2+} water (250 \mu M). The concentrations of GYY-4137 used in the present study (100 and 200 \mu M) are estimated to yield stable H\textsubscript{2}S levels of ~5 and 10 \mu M, respectively (16).

Whole body Ca\textsuperscript{2+} content also was measured in fish exposed to GYY-4137. At 4 dpf, control or GYY4137-treated fish were killed with an overdose of MS-222 and then briefly rinsed in double-distilled water. Ten fish were pooled as one sample, and a total of six samples (N = 6) were analyzed in this experiment. The fish were digested with 5 N HNO\textsubscript{3} at 70°C for 24 h, and diluted appropriately with deionized water. The total [Ca\textsuperscript{2+}] was measured by flame emission spectrophotometry (Spectra AA 220FS; Varian) and verified using certified Ca\textsuperscript{2+} standards (Fisher Scientific).

Treatment with PKA and PKC inhibitors. The potential role of protein kinases in H\textsubscript{2}S-stimulated Ca\textsuperscript{2+} influx was evaluated using selective blockers for PKA and PKC. Fish at 4 dpf were exposed to 10 \mu M Na\textsubscript{2}S together with either 10 \mu M H-89 or bisindolylmaleimide I (BIS-1) (Cayman Chemical) for 30 min, and Ca\textsuperscript{2+} influx was measured as described above. H-89 and BIS-1 are reversible competitive inhibitors for PKA and PKC, respectively. Both H-89 and BIS-1 inhibit PKA or PKC activity by acting on the ATP binding sites on the kinase catalytic subunit. To test whether Ca\textsuperscript{2+} influx could also be directly modulated by cAMP, fish were exposed to a cAMP-elevating agent forskolin or 8-bromo-cyclic AMP (8-Br-cAMP) (both at 10 \mu M) for 30 min before Ca\textsuperscript{2+} uptake measurements. Coexposure to H-89 and forskolin also was performed to evaluate the effects of PKA inhibition on forskolin-stimulated Ca\textsuperscript{2+} influx. Additionally, influx of Ca\textsuperscript{2+} was examined following 30-min exposure to 100 \mu M isobutylmethylxanthine (IBMX). IBMX is a selective inhibitor of phosphodiesterase, which is an enzyme responsible for degrading cAMP. All influxes (N = 6) were performed in normal Ca\textsuperscript{2+} water (250 \mu M).

Pharmacological inhibition of H\textsubscript{2}S biosynthesis. To examine the involvement of endogenous H\textsubscript{2}S in basal Ca\textsuperscript{2+} influx, fish acclimated to normal (250 \mu M) or low-Ca\textsuperscript{2+} (25 \mu M) water were exposed to either 100 \mu M propargylglycine (PPG; an inhibitor of CSE) or 100 \mu M aminooxyacetic acid (AOA; an inhibitor of CBS) at 3 dpf. The water pH was adjusted to 7.6 with 0.1 N NaOH. Influx of Ca\textsuperscript{2+} was measured in the same exposure water at 4 dpf (N = 6).

Gene knockdown of H\textsubscript{2}S-biosynthesis enzymes. The involvement of endogenous H\textsubscript{2}S in regulating basal Ca\textsuperscript{2+} influx was examined further using a gene knockdown approach. Two H\textsubscript{2}S-biosynthesis enzymes, CSE and CBSb, were knocked down using antisense oligonucleotide morpholinos (GeneTools). The CSE morpholino (5'-GCC ACA AGA GTG AAC TGC TGT TCG T3') was designed to splice out exon 1 (57–219 bp of NC_007117.5), and the CBSb morpholino (5'-TTG TCC TGT GAG AAA ACG CAT T3') was designed to splice out exon 3 (385–491 bp of NM_001014345.2). The effectiveness of these morpholinos was confirmed previously (13, 33). The morpholinos were diluted in Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.1% SDS, 50 mM Tris-HCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] plus protease inhibitor cocktail (ThermoScientific). The extracted protein was loaded on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). After transfer, the membrane was blocked with 5% skimmed milk in Tris buffer plus 0.05% Tween 20 (TBST) for 2 h at room temperature. The membrane was then washed with TBST (three times and 5 min each; 3 × 5 min), the membrane was probed with 1:5,000 goat anti-rabbit antibodies (Invitrogen) for 2 h at room temperature. The membrane was then washed (5 × 5 min), and the bands were detected using enhanced chemiluminescence (SuperSignal West Femto chemiluminescent substrate; Pierce) with a ChemiDoc system (Bio-Rad). Subsequently, the membrane was reprobed with β-actin antibodies (1:4,000; Sigma) after being stripped with a reblot solution (ThermoScientific).

PCR analysis. Methods for RNA extraction, cDNA synthesis, and PCR analysis were described previously (14). In brief, total RNA from different tissues of adult zebrafish, or from larval zebrafish following experimental treatments (described below), were extracted using RNeasy kit (Qiagen). After treatment with DNase I (Biolabs), cDNA was synthesized with 1 \mu g of RNA using RevertAid H-minus reverse transcriptase (Thermo) and random hexamer primers. To evaluate the relative abundance of cse and cbsb in different tissues of adult zebrafish, 150 ng of cDNA template were used and amplified for 30 or 40 cycles. Potential changes in mRNA levels of caco, NCX isoform 1b (ncx1b), and PMCA isoform 2 (pmca2) following GYY4137 exposure or CBSb knockdown also were examined using real-time PCR (N = 6). Both ncx1b and pmca2 are known to be expressed in caco-positive cells (18). Real-time PCR analysis was performed using a Bio-Rad CFX96 qPCR system, as described previously (14); 18S RNA was used as an internal control. Primer pairs used in the present study are provided in Table 1.

Immunohistochemistry and confocal imaging. Fish were fixed overnight in a 4% paraformaldehyde solution in PBS at 4°C. After fixation, the fish were rinsed briefly with PBS containing 0.1% Tween (PBST) and then dehydrated using methanol. Following rehybridization with PBST, the fish were subjected to antigen retrieval, as described previously (8). Fish were then blocked with 3% BSA in PBS plus 0.8% Triton X for 2 h and then incubated with CBSb and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (labels NaRCs; Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) antibodies overnight at 4°C. Subsequently, fish were rinsed with PBST and then incubated with secondary antibodies (Alexa-488 conjugated anti-rabbit IgG and Alexa-596 conjugated anti-mouse IgG; Invitrogen) at 1:500 dilution in PBST for 2 h at room temperature. To label H\textsuperscript{-}ATPase-rich cells (HRCs), a
vital dye concanavalin A (ConA; Invitrogen) was used, as described previously (13). The images were acquired using a confocal laser scanning microscopy (AIR+; Nikon Instruments).

Statistical analysis. All statistical analyses were performed using SigmaPlot (version 11.2; Systat Software). Data were analyzed using either Student’s t-test, or one-way ANOVA followed by a post hoc Holm-Sidak test. Data are reported as means ± SE, and P ≤ 0.05 was taken as the level of significance.

RESULTS

**H₂S stimulates Ca²⁺ influx in a low-Ca²⁺ environment.** The effects of Na₂S on Ca²⁺ influx were examined in fish acclimated to either normal (250 μM) or low-Ca²⁺ (25 μM) water. Exposure to Na₂S at concentrations of 1 or 10 μM had no effect on Ca²⁺ influx in fish acclimated to normal Ca²⁺ water (Fig. 1A). In contrast, Na₂S exposure significantly increased Ca²⁺ influx in fish acclimated to low-Ca²⁺ water (Fig. 1B). A fivefold increase in mRNA levels of ecc4 was observed after acclimation to low-Ca²⁺ water (data not shown). Similarly, the density of NaRCs increased significantly in fish acclimated to low-Ca²⁺ water (19.8 ± 0.9 cells/100 μm²) compared with that in normal Ca²⁺ water (9.4 ± 0.8 cells/100 μm²).

**H₂S stimulates Ca²⁺ influx through its interaction with PKA.** Exposure to 10 μM Na₂S significantly increased Ca²⁺ influx at 4 dpf, a response that was abolished in the presence of the PKA inhibitor H-89 (Fig. 2A). In contrast, treatment of fish with a PKC inhibitor BIS-1 did not reduce the Na₂S-stimulated Ca²⁺ influx (Fig. 2B). Similarly, exposure to the cAMP-elevating agent forskolin significantly increased Ca²⁺ influx, which was reduced substantially by H-89 (Fig. 3A). Fish treated with forskolin exhibited a significant increase in cAMP levels by ~40-fold compared with the controls (forskolin treatment: 917.4 ± 114.2 pmol/ml, control: 24.2 ± 2.1 pmol/ml, N = 5; Y. Kumai and M. Tresguerres, personal communications). Influx of Ca²⁺ also was increased during exposure to 8-Br-cAMP (Fig. 3B) and the phosphodiesterase inhibitor IBMX (Fig. 3C). However, exposure to Na₂S did not further increase the 8-Br-cAMP stimulated Ca²⁺ influx (data not shown).

**Chronic exposure to H₂S donor GYY4137 increases Ca²⁺ influx and whole body Ca²⁺ levels.** The chronic effects of H₂S exposure on Ca²⁺ influx were evaluated using a stable H₂S donor GYY4137. Fish exposed to 200 μM GYY4137 exhibited a significant increase in Ca²⁺ influx at 4 dpf (Fig. 4A). An increase in whole body Ca²⁺ levels also was observed in fish exposed to 200 μM GYY4137 (Fig. 4B). Results from real-time PCR experiments demonstrated that exposure to 200 μM GYY4137 did not affect mRNA expression of ecc4, ncx1b, or pmca2 (Fig. 4C).

**Tissue-specific distribution of cse and cbsb in adults and their expression levels in larvae acclimated to low-Ca²⁺ water.** Figure 5A shows representative images of cse and cbsb mRNA expression in different tissues of adult zebrafish using RT-PCR. Tissues particularly abundant in cse mRNA were kidney, muscle, and intestine. For cbsb, mRNA appeared to be expressed primarily in the kidney and muscle with lower levels in the brain, gill, and heart. Acclimation to low-Ca²⁺ water did not affect mRNA expression of cse in larval zebrafish at 4 dpf (Fig. 5B). In contrast, mRNA expression of cbsb was significantly increased in fish acclimated to low-Ca²⁺ water (Fig. 5C).

**Table 1. Primers used in the present study**

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<th>Gene</th>
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cse, Cystathionine γ-lyase; cbsb, cystathionine β-synthase isofrom b; ecca, epithelial Ca²⁺ channels; pmca2, plasma membrane Ca²⁺-ATPase isofrom 2; ncx1b, Na⁺/Ca²⁺ exchanger isofrom b; FWD, forward; REV, reverse.

Fig. 1. Acute exposure to sodium sulfide (Na₂S) increases Ca²⁺ influx in fish acclimated to low-Ca²⁺ water (C) compared with normal-Ca²⁺ water (A) or low-
Ca²⁺ water (B). A, Control; B, Low-Ca²⁺; C, Na₂S. Values are means ± SE, n = 6. *ab Different letters represent a statistical difference from each other (one-way ANOVA, followed by a Holm-Sidak post hoc test; P < 0.05).
The H2S-biosynthesis enzyme CBSb is expressed in ionocytes. Whole-mount immunohistochemistry and confocal microscopy were performed to examine the localization of CBSb in larval zebrafish at 4 dpf (Fig. 6). Immunostaining of HRCs (purple; Fig. 6A) and NaRCs (red; Fig. 6B) was performed using a vital dye concanavalin A and Na+/K+-ATPase antibody, respectively. Immunostaining of CBSb (green; Fig. 6C) was observed in the skin covering the yolk sac. Both HRCs and NaRCs were found to express CBSb (Fig. 6D). Figure 6, E–H, are higher magnification images showing CBSb in HRCs and NaRCs.

Pharmacological inhibition of CBS activity reduces Ca2+ influx in a low-Ca2+ environment. The physiological importance of endogenous H2S in regulating Ca2+ influx was evaluated by pharmacological inhibition of H2S-biosynthesis enzymes. Treatment of fish with either PPG (an inhibitor of CSE) or AOA (an inhibitor of CBS) did not affect Ca2+ influx in fish acclimated to normal Ca2+ water (Fig. 7A). Exposure to AOA, but not PPG, significantly reduced Ca2+ influx in fish acclimated to low-Ca2+ water (Fig. 7B).

Knockdown of CBSb reduces Ca2+ influx in a low-Ca2+ environment. The potential role of CSE and CBSb in regulating Ca2+ influx was further evaluated using a morpholino knock-down.
down approach. Knockdown of CSE or CBSb did not affect Ca\(^{2+}\) influx in fish acclimated to normal Ca\(^{2+}\) water (Fig. 8A). A significant reduction in Ca\(^{2+}\) influx was observed in CBSb-deficient fish following acclimation to low-Ca\(^{2+}\) water (Fig. 8B). Results from real-time PCR suggested that mRNA expression of ecac, ncx1b, and pmca2 was not altered in 4 dpf fish experiencing CBSb knockdown (Fig. 8C).

As reported previously [Kumai et al. (13); Porteus et al. (33)], the CBSb morpholino was originally designed to splice out exon 3, but it appeared to reduce total CBSb mRNA levels, likely by premature mRNA degradation. In the present study, we further confirmed the effectiveness of the CBSb knockdown by Western blot using a specific antibody against the zebrafish CBSb. Western blot analysis demonstrated that the CBSb antibody detected a band at \(\sim 50\) kDa, and preabsorption of the CBSb antibody with corresponding immunizing peptide eliminated the immunoreactivity (Fig. 8D). Notably, the size of...
the CBSb protein appeared to be smaller than its predicted size (65 kDa). This smaller size possibly reflected posttranslational cleavage of CBS as reported in mammalian systems (9). Consistent with previous observations, the reduced cbsb mRNA expression by morpholino knockdown [Kumai et al. (13); Porteus et al. (33)] also resulted in a reduction in its protein expression (Fig. 8E). Quantitative analysis suggested that knockdown of CBSb led to 85% reduction in the protein expression levels of CBSb (Fig. 8F).

DISCUSSION

Overview. The gasotransmitter H2S is known to regulate the central nervous system, cardiorespiratory function, and epithelial Na+ transport in vertebrates (1, 13, 17, 25, 40). Using developing zebrafish as a model system, we demonstrated a novel stimulatory role for H2S in promoting Ca2+ influx, which appeared to involve rapid activation of the cAMP-PKA pathways. Production of endogenous H2S via the H2S-biosynthesis enzyme CBSb was found to be critically involved in increasing Ca2+ influx, particularly in fish acclimated to a low-Ca2+ environment.

The effects of exposure to H2S donors on Ca2+ fluxes and the role of cAMP/PKA pathways. In zebrafish, it is proposed that transepithelial Ca2+ uptake occurs predominantly through a subset of NaRCs, which express ECaC at the apical membrane, and NCX and PMCA at the basolateral membrane (7). It is also largely assumed that absorption of Ca2+ via ECaC is the rate-limiting step (32). Here we demonstrated that exposure to H2S donors Na2S or GYY4137 increased Ca2+ influx in larval zebrafish at 4 dpf. Additionally, whole body Ca2+ levels were increased following chronic exposure to GYY4137. Interestingly, the H2S-stimulated Ca2+ influx was observed only in fish that were acclimated to low-Ca2+ water. Several previous studies have demonstrated that acclimation to low-Ca2+ water increases the levels of ecac mRNA expression and numbers of ecac-expressing NaRCs in larval zebrafish (14, 19, 29). Thus it
seems possible that the capacity for H2S to stimulate Ca2+ uptake is higher when the preexisting levels of ECaC expression and/or the numbers of ECaC-expressing cells are high. In isolated rat colonic epithelial cells, H2S exposure was found to promote Ca2+ extrusion via NCX (34). Similarly, exposure to GYY4137 increased the expression and activity of NCX in a human HeLa cell line (22). In the present study, we observed that mRNA expression of ecac, pmca2, and ncc1b was unaffected following chronic exposure to GYY4137. Considering that Ca2+ influx also was elevated after acute exposure (30 min) to H2S donors, it seems likely that H2S increased the affinity for Ca2+ uptake in larval zebrafish through rapid activation of the preexisting Ca2+ channels, likely via post-translational modification (discussed below).

To examine the potential for cross talk between H2S and nitric oxide (NO) on modulating Ca2+ influx (41), Ca2+ influx was also evaluated following exposure to NO donors (S-nitroso-N-acetylpenicillamine) or knockdown of neuronal NO synthase. S-nitroso-N-acetylpenicillamine exposure did not affect Ca2+ influx in larval zebrafish. Similarly, influx of Ca2+ remained unchanged in fish experiencing neuronal NO synthase knockdown (K. McGregor, R. W. M. Kwong, and S. F. Perry, personal observations). These findings suggested that NO has little role in stimulating Ca2+ influx in larval zebrafish.

ECaC is known to contain several putative PKA and PKC phosphorylation sites, and in mammalian systems acute activation of these sites was shown to increase the activity of ECaC (2, 4). It has also been suggested that activation of the cAMP-dependent PKA signaling pathways promotes phosphorylation at the COOH-terminus of ECaC, thereby increasing its conductance to Ca2+ (4, 39). To examine whether stimulation of Ca2+ influx by H2S was dependent on PKA and/or PKC activity, Ca2+ influx was evaluated during exposure to Na2S with selective PKA or PKC inhibitors. The results demonstrated that treatment of fish with the PKA inhibitor H-89 prevented the effects of H2S on increasing Ca2+ influx. In contrast, exposure of the PKC inhibitor BIS-1 did not affect H2S-stimulated Ca2+ influx. These findings suggest that the stimulatory effects of H2S on Ca2+ influx were probably associated with activation of PKA. Because the activity of PKA is highly dependent on the cellular levels of cAMP, we also determined whether Ca2+ influx could be stimulated by the cAMP-elevating agent forskolin, 8-Br-cAMP or IBMX (i.e., a specific inhibitor of cAMP-degrading enzyme phosphodiesterase). The results suggested that exposure to these cAMP-elevating agents significantly increased Ca2+ influx in larval zebrafish. Interestingly, we also observed that the effects of forskolin on stimulating Ca2+ influx was substantially lower when the fish were acclimated to normal Ca2+ water; exposure to forskolin resulted in a 1.25-fold increase in Ca2+ influx in fish acclimated to normal Ca2+ water (data not shown), while it caused a 2-fold increase in Ca2+ uptake in fish acclimated to low-Ca2+ water. This finding reinforces our contention that the prevailing level of ECaC expression is probably a limiting factor in cAMP-stimulated Ca2+ influx. Taken together, the findings of the present study demonstrated that activation of the cAMP/PKA signaling pathways, either by H2S donors or by cAMP-elevating agents, acutely stimulates Ca2+ uptake in larval zebrafish.

H2S is known to have diverse physiological effects on various ion channels/transporters. For examples, H2S inhibits voltage-dependent Ca2+ channels in cardiomyocytes (36), whereas it activates transient receptor potential vanilloid channels (e.g., TRPV1) in urinary tract (35) and airway smooth muscle (38). Our laboratory previously demonstrated that exposure to H2S donors reduced Na+ influx in larval zebrafish (13). Similarly, morpholino knockdown of H2S-biosynthesis enzymes CSE or CBSb increased Na+ influx, suggesting that endogenously produced H2S inhibits Na+ uptake (13). H2S was found to reduce Na+ uptake via its interaction with HRCs, but not Na+/H+ cotransporter-expressing cells, which are two major types of ionocytes responsible for Na+ uptake in zebrafish (13). Interestingly, treatment with forskolin was shown to increase Na+ influx via both HRCs and Na+/H+ cotransporter-expressing cells, indicating that Na+ uptake by these ionocytes is stimulated by activation of the cAMP/PKA signaling cascade (12). The physiological significance of H2S reducing Na+ influx in HRCs, while simultaneously promoting Ca2+ influx in NaRCs, remains unclear. However, these ob-
The physiological involvement of H₂S-biosynthesis enzymes in the regulation of Ca²⁺ uptake. In mammals, the two major H₂S-biosynthesis enzymes, CSE and CBS, are expressed in a tissue-specific manner, with CBS being expressed primarily in the brain and CSE in peripheral tissues (30). In rainbow trout (Oncorhyncus mykiss), cse and cbs mRNA are expressed in various tissues, including gill, liver, brain, heart, and skeletal muscle (28). Here we observed that, in adult zebrafish, cse mRNA was expressed abundantly in the intestine, kidney, and muscle. cbsb mRNA appeared to be expressed primarily in the kidney and muscle, with lower levels in the brain, gill, and heart. In larval zebrafish, CSE is expressed in both NaRCs and HRCs on the skin covering the yolk sac (13). In the present study, we also observed that CBSb was expressed in NaRCs and HRCs on the skin of the yolk sac, suggesting that these ionocytes have the capacity to produce H₂S via either CSE (13) or CBSb (this study).

To further examine the physiological role of endogenous H₂S, the activity/expression of CSE or CBSb was suppressed using pharmacological or gene knockdown approaches. We observed that treatment of fish with the CSE inhibitor PPG or morpholino knockdown of CSE did not affect Ca²⁺ influx. In contrast, treatment with the CBS inhibitor AOA or morpholino knockdown of CBSb significantly reduced Ca²⁺ influx. Notably, the reduction of Ca²⁺ influx by AOA exposure or by CBSb knockdown only was observed in fish that were acclimated to low-Ca²⁺ water. These findings suggest that H₂S...
produced by CBSB is important in promoting Ca\textsuperscript{2+} influx in a low-Ca\textsuperscript{2+} environment. The precise mechanism(s) by which endogenous H\textsubscript{2}S promotes Ca\textsuperscript{2+} influx is not clear. Because the mRNA levels of eaac, nxc\textsubscript{1b}, and pmca\textsubscript{2} remained unchanged in fish experiencing CBSB knockdown, it suggests that H\textsubscript{2}S stimulated Ca\textsuperscript{2+} influx through posttranscriptional regulation, presumably involving PKA signaling. Nevertheless, mRNA expression of cbsb was substantially increased in fish acclimated to low-Ca\textsuperscript{2+} water. The elevated cbsb expression may contribute to an increased H\textsubscript{2}S synthesis, which may promote ECA\textsubscript{C} phosphorylation to increase Ca\textsuperscript{2+} conductance via cAMP-dependent PKA signaling pathways (4, 39).

**Concluding remarks.** In summary, the present study demonstrated that exposure to H\textsubscript{2}S donors increased Ca\textsuperscript{2+} influx and whole body Ca\textsuperscript{2+} content. H\textsubscript{2}S stimulated Ca\textsuperscript{2+} influx by activating the cAMP-PKA pathways, which likely modified the conductance of ECA\textsubscript{C} to Ca\textsuperscript{2+}. Furthermore, we showed that H\textsubscript{2}S generated by CBSB is important in promoting Ca\textsuperscript{2+} influx, particularly under a low-Ca\textsuperscript{2+} environment. Interestingly, a recent study showed that H\textsubscript{2}S exposure reduced Na\textsuperscript{+} influx in larval zebrafish (13), suggesting that H\textsubscript{2}S may have divergent physiological functions in regulating ion movements in fish. Further investigation is required to fully elucidate the cell- and tissue-specific regulation of ion transport by H\textsubscript{2}S. The precise molecular targets for endogenous H\textsubscript{2}S in modulating Na\textsuperscript{+} and Ca\textsuperscript{2+} uptake have yet to be examined. Future experiments should also examine the effects of H\textsubscript{2}S and cAMP on intracellular Ca\textsuperscript{2+} levels in iocytes. Although the biochemistry and physiology of H\textsubscript{2}S remain poorly understood, there is a growing interest in using H\textsubscript{2}S inhibitors or donors as potential therapeutic options for various diseases (37). The present study suggests that the zebrafish could be a useful model to examine the actions and physiological consequences of H\textsubscript{2}S in vivo.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: R. W. K. and S. F. P. conceived and designed the research; R. W. K. performed the experiments; R. W. K. and S. F. P. analyzed the data; R. W. K. and S. F. P. interpreted the results of the experiments; R. W. K. prepared the figures; R. W. K. and S. F. P. drafted the manuscript; R. W. K. and S. F. P. edited and revised the manuscript; R. W. K. and S. F. P. approved the final version of the manuscript.

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