ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 2, 2009

DOI: 10.1089/ars.2008.2132

Original Research Communication

A Source of Hydrogen Sulfide and a Mechanism of Its Release in the Brain

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Abstract

Hydrogen sulfide (H_2S) is recognized as a neuromodulator as well as neuroprotectant in the brain. H_2S can be produced from cysteine by enzymes such as cystathionine β-synthase. However, a mechanism for releasing H_2S under physiologic conditions has not been identified. Here we show that H_2S is released from bound sulfur, an intracellular store of sulfur, in neurons and astrocytes of mice and rats in the presence of physiologic concentrations of endogenous reducing substances glutathione and cysteine. The highest pH to release H_2S from another sulfur store, acid-labile sulfur, which is localized mainly in mitochondria, is 5.4. Because mitochondria are not in the acidic condition, acid-labile sulfur may not be a physiologic source of H_2S. Free H_2S is immediately absorbed and stored as bound sulfur. Our novel method, using silver particles to measure free H_2S, shows that free H_2S is maintained at a low level in basal conditions. Alkalinization of the cytoplasm is required for effective release of H_2S from bound sulfur, and this condition is achieved in astrocytes by the high concentrations of extracellular K^+ that are normally present when nearby neurons are excited. These data present a new perspective on the regulation of H_2S in the brain. *Antioxid. Redox Signal*. 11, 205–214.

Introduction

Hydrogen sulfide (H_2S), well-known toxic gas, acts as a modulator of synaptic activity in the brain and also as a smooth muscle relaxant (1, 14). H_2S can be produced from cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (10, 35, 36). CBS is expressed in the brain, where H_2S enhances the induction of hippocampal long-term potentiation (LTP) (1). H_2S also regulates the activities of serotonergic neurons, as well as the release of corticotropin-releasing hormone (7, 20), and relaxes smooth muscle (14, 46). Finally, H_2S protects neurons as well as cardiac muscle from oxidative stress (9, 18, 19, 32, 42) and regulates insulin secretion (3, 17, 45).

Despite the various effects of H_2S in many tissues, the major cellular sources of H_2S and the mechanism of its release are not well understood. At least two possibilities exist. One possibility is that H_2S is immediately released after its production by enzymes. Another possibility is that H_2S produced by enzymes is stored and is released in response to a physiologic signal. Two forms of sulfur stores in cells have been identified (24, 38). Acidic conditions release acid-labile sulfur, which is mainly from the iron-sulfur center of enzymes in mitochondria. Acid-labile sulfur in the brain of rats, humans, and bovines has been measured as brain sulfide (13, 29, 43). Another form of storage is called bound sulfur, which is localized to the cytoplasm and releases H_2S in reducing conditions (25). Because the activity of reducing substances is increased in alkaline conditions, H_2S can be released from bound sulfur when intracellular conditions become alkaline.

H_2S dissociates to H^+ and HS^- in solution. In physiologic saline at 37°C and pH 7.4, less than one fifth of H_2S exists as the undissociated form (H_2S), and the remaining four fifths exist as HS^- plus a trace of S^{2-} at equilibrium with H_2S (8, 44). Most of H_2S is dissolved as HS^- and S^{2-} in the alkaline conditions, whereas H_2S is evaporated in the acidic conditions. Because pK_1 is 6.76 at 37°C, ~85% of H_2S exists as H_2S gas, and the remaining 15%, as HS^- at pH 6.0. Although it has not been possible to determine which form of H_2S (H_2S, HS^-, or S^{2-}, the mix of free inorganic sulfides) is active, the term "hydrogen sulfide" has been used. The term "hydrogen sulfide for total free sulfides" is also used here.

Changes in intracellular pH have been widely studied both in neurons and in glia (6, 11, 23, 31). Increases in extracellular concentrations of potassium during neuronal excitation lead to the depolarization of astrocytes and the acti-

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vation of the electrogenic sodium bicarbonate (Na^+/HCO_3^-) cotransporter (23, 27). With the activation of the cotransporter, the intracellular pH of the astrocytes is increased.

The present study shows that both acid-labile and bound sulfur exist in the brain. Greater amounts of bound sulfur than of acid-labile sulfur are found, and free H_2S is maintained at a low level in basal conditions. We also show that H_2S is released from bound sulfur in homogenates of neurons and astrocytes in alkaline conditions. An alkaline shift in the cytoplasm of astrocytes is caused by high extracellular concentrations of K^+ released from neurons after excitation.

Materials and Methods

Measurement of the amounts of bound sulfur and acid-labile sulfur

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. All rats or mice used were killed by an overdose of diethyl-ether. For the measurement of acid-labile sulfur, rat tissues were homogenized with a 10 volumes (vol/vol) of 10 m*M* NaOH and centrifuged at 10,000 *g* for 10 min at 4°C. The pH of 300 μl of supernatant (brain, 8.0 mg protein/ml; liver, 20.7 mg protein/ml; heart, 10.4 mg protein /ml) was adjusted by 600 μl 20% phosphoric acid for pH 1.5; 1 *M* citrate buffer to 2.5, 3.2, and 6.0; by 1 *M* acetate buffer to 4.0, 4.7, and 5.4; or by 1 *M* Tris-acetate buffer to 7.4 in a 15-ml centrifuge tube (IWAKI, Tokyo) sealed with Parafilm M (American National CAL, Chicago, IL). After 30-min incubation at 37°C, 2 ml of gas was measured with gas chromatography (GC-2014; Shimadzu, Kyoto). Denaturants and detergents were added to 300 μl of brain supernatant (8.0 mg protein/ml) and incubated for 10 min at room temperature and for an additional 10 min at 37°C, and 2 ml of gas was analyzed for H_2S with gas chromatography.

For the measurement of bound sulfur, tissues of rats or mice were homogenized with a polytron homogenizer (PT 10-35; Kinematica, Lucerne, Switzerland) in ice-cold 1 *M* Tris-acetate (pH 7.4) buffer, and centrifuged at 10,000 *g* for 10 min at 4°C. Thirty-three microliters of 50 m*M* dithiothreitol (DTT) was added to 300 μl of supernatant (8.0 mg protein/ml) of homogenates in a 15-ml centrifuge tube. The tube was filled with N_2 gas and sealed with Parafilm M and incubated at 37°C for 15 min to 5 h. After incubation, citrate buffer (pH 6.0) was added to each tube and incubated at 37°C for 10 min. Two milliliters of gas was analyzed for H_2S with gas chromatography. Sodium sulfide (Na_2S) (Wako, Osaka) solution was used for calibration.

Measurement of free H_2S

A whole rat brain was homogenized with 10 volumes (vol/vol) of 0.5 *M* borate buffer (pH 9.0), and centrifuged at 10,000 *g* at 4°C for 10 min. Thirty milliliters of supernatant was mixed with 0.02 g of powdered silver (Ag) (Wako, Osaka). After 2 h of incubation at 37°C, the supernatant was removed, and the Ag was washed 3 times with 5 ml of 1% Triton X-100 and 5 times with 5 ml of water. The tube containing the Ag powder was filled with N_2 gas and sealed with Parafilm M. Five hundred μl of 0.5 *M* thiourea containing 0.01N H_2SO_4 was added to the Ag powder and vortexed for 1 min. Two milliliters of gas was analyzed for H_2S by gas chromatography.

Measurement of absorbed H_2S exogenously applied to the homogenates

One nanomol Na_2S was added to 300 μl of supernatants (8.0 mg protein/ml) obtained from tissue homogenates or fetal bovine serum (FBS), BSA, or lysozyme (Sigma-Aldrich, St. Louis, MO) in a 15-ml centrifuge tube. The tube was sealed and mixed for 10 sec, and then incubated at room temperature for 10 sec to 30 min. After incubation, 600 μl citrate buffer (pH 6.0) was added and incubated at 37°C for 10 min. Two milliliters of gas was analyzed for H_2S with gas chromatography.

Measurement of H_2S released from brain homogenates and cell lysates

A whole brain of adult C57BL/6n mice was homogenized with a 10 volumes of 100 m*M* Tris-HCl buffer (pH 8.4), 100 m*M* phosphate buffer (pH 7.9), or 20 m*M* phosphate buffer (pH 7.4). Primary cultures were washed 3 times and harvested (1.7 mg protein/ml) in 800 μl Tris-HCl (pH 8.4) or phosphate buffer (pH 7.9). The cells were sonicated with a SONIFIER (Branson Sonic Power, Danbury, MA). The rest of the procedure was the same, with the measurement of bound sulfur.

Cell culture

Cultures of astrocytes and neurons were prepared from embryonic day 17 rats or day 16 mice, as previously described (2, 21, 22, 39). In brief, cortices were stripped of meninges and treated with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.1% DNase I (Sigma-Aldrich) in L-15 medium (Invitrogen, Carlsbad, CA) at 37°C for 30 min. After adding fetal bovine serum, the suspension was passed through a 41-μm nylon mesh and plated at a density of 10^6 cells/dish on glass-bottomed 35-mm dishes (MatTek, Ashland, OR) coated with poly-D-lysine (Sigma-Aldrich). Astrocytes were maintained in minimum Eagle's medium (MEM) (Sigma-Aldrich) supplemented with 30 m*M* glucose, 2 m*M* glutamine, 1 m*M* sodium pyruvate (Sigma-Aldrich), 10% fetal calf serum, and 50 U/ml penicillin/streptomycin (Sigma-Aldrich) at 37°C with 10% CO_2. Neurons were maintained in the same medium for 3 days, and then exposed to 5 μ*M* cytosine arabinoside (AraC; Sigma-Aldrich) overnight. The medium was changed to Neurobasal (GIBCO, Grand Island, MI) supplemented with 2% B-27 supplement (GIBCO), 0.5 m*M* glutamine, and 50 U/ml penicillin/streptomycin. The medium was changed every 3–4 days. Cultures of neurons or astrocytes were used 12–15 days after the preparation. Approximately 95% of the cells were neurons or astrocytes in each culture, determined by cell morphology and immunolabeling as well as Western blot analysis with a neuronal marker MAP2 and a glial marker GFAP (20).

Subcellular fractionation

A whole rat brain was homogenized with a 10 volumes of 100 m*M* Tris buffer (pH 8.4), and centrifuged at 1,000 *g* for 5 min at 4°C. The pellets were recovered as a nuclear fraction. The supernatant was centrifuged at 17,000 *g* for 10 min at 4°C, and the pellets were recovered as mitochondrial fractions. The supernatant was then centrifuged at 100,000 *g* for 1 h at 4°C, and the precipitates were designated as a microsomal fraction, and the supernatant, as a cytoplasmic frac-

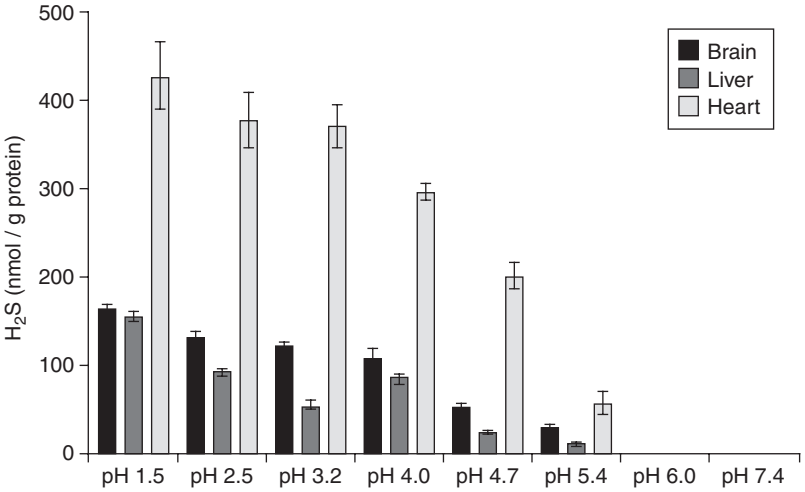

FIG. 1. The amounts of H_2S released from acid-labile sulfur in rat brain, liver and heart. The supernatants of brain (8.0 mg protein/ml), liver (20.7 mg protein/ml), and heart (10.4 mg protein/ml) homogenates were prepared, the pH was adjusted to the indicated values, and the homogenates incubated for 30 min at 37°C. The amounts of H_2S released at various pH values were measured with gas chromatography. All data are represented as the mean ± SEM of three experiments.

tion. Each fraction was resuspended with 10 m*M* Tris buffer (pH 8.4); whole brain, 2.9; nuclei, 6.9; mitochondria, 3.4; microsome, 3.2; and cytosol, 2.4 (in mg protein/ml).

Measurement of the intracellular pH

Carboxyseminaphthorhodofluor-1 (SNARF-1) was used to measure the intracellular pH (pH_i). Confluent mouse cultures incubated for 12–15 days were washed twice with Earle's balanced salt solution (EBS) consisting of 123 NaCl, 3 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 5 D-glucose, 26 $NaHCO_3$, and 1 NaH_2PO_4 (in m*M*). To load SNARF-1, cultures were incubated at 37°C for 30 min with 5% CO_2 in EBS containing 10 μM SNARF-1-acetoxymethyl ester (SNARF-1-AM) (Molecular Probes, Eugene, OR). The loading buffer was removed and replaced with fresh EBS. During the measurement of pH_i, EBS bubbled with 95% O_2/5% CO_2 was perfused at a rate of 1.25 ml/min at room temperature.

After a 10-min preincubation with 95% O_2/5% CO_2, intracellular SNARF-1 fluorescence was excited at 495 nm, and the emission detected at 580 and 635 nm for ratiometric analysis. The imaging was performed by using an upright microscope (DM LFS; Leica, Heidelberg, Germany) with a 40× water-immersion objective (0.5 NA; Leica) and CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). A sequence of images was acquired with 5-s intervals and 2×2 binning. A calibration curve was generated for each experiment by incubating cells with SNARF-1 with 10 μM nigericin in buffers of pH 5.6 and 9.0 [135 KCl, 15 NaCl, 1 CaC_2, 1 KH_2PO_4, 0.5 $MgSO_4$, and 10 HEPES (in m*M*)].

Statistics

The data were analyzed by using StatView software (Abacus Concepts, Berkeley, CA) for one-way ANOVA with *post hoc* testing by using the Fisher's PLSD multiple comparison test.

Results

Acid-labile sulfur in the brain

The release of H_2S from acid-labile sulfur has been measured in many tissues (13, 29, 41, 43), but the critical pH at which H_2S is released from acid-labile sulfur has not been determined. To address this problem, the amounts of H_2S released from homogenates of whole brain, liver, and heart were measured at various pH values with gas chromatography. Because the release of H_2S is maximal at 30 min after the exposure to acids, the amount of H_2S was measured at this time. The release of H_2S was maximal at pH 1.5, the lowest pH tested, and gradually decreased with higher pH up to 5.4 (Fig. 1). Although at pH 6.0, ~85% of the total inorganic sulfide in solution would be in the form of gaseous H_2S and thus free to equilibrate with the N_2 gas, no H_2S gas was detected with any of the three tissues tested. Thus, it appears that little or no H_2S is released at pH 6.0 or higher. Heart released more than twice as much H_2S as liver and brain at pH 5.4 and lower (37). Therefore, the critical pH to release H_2S from acid-labile sulfur is 5.4.

To examine the lability of sulfur to substances other than acids, the effect of several detergents and a protein denaturant was tested. The 4 *M* guanidine HCl and 2% SDS adjusted pH 6.0 released 109% and 75% of the tissue H_2S relative to the amount liberated by 1N HCl (100%) (Fig. 2A). Sodium deoxycholate, CHAPS, and CTAB also released H_2S, but their effects were much weaker than that of SDS. No detectable H_2S was released by Triton X100 and Tween 80.

To examine whether H_2S released by SDS was produced from acid-labile sulfur, H_2S released by SDS from cell lysates pretreated with HCl was compared with those without HCl pretreatment. HCl was added to the supernatant of brain homogenates, and all released H_2S was removed. The addition of SDS to the resultant cell lysates released only 6% of H_2S released by SDS without HCl pretreatment (Fig. 2B). This result suggests that H_2S released by SDS may originate from acid-labile sulfur. To explore this possibility further, the converse experiment was done, in which brain homogenates were first treated with SDS followed by HCl. The lysates pretreated with SDS released only 17% of the H_2S released by HCl from supernatant without SDS pretreatment (Fig. 2C). These observations show that H_2S released by SDS is from acid-labile sulfur. Similar observations were obtained with guanidine HCl (Fig. 2D and E).

Sources of H_2S released by reducing agents are distinct from those released by acid

DTT has been used to release H_2S from bound sulfur (24). To determine the amounts of bound sulfur in brain, liver, and heart, time courses of H_2S released in the homogenates

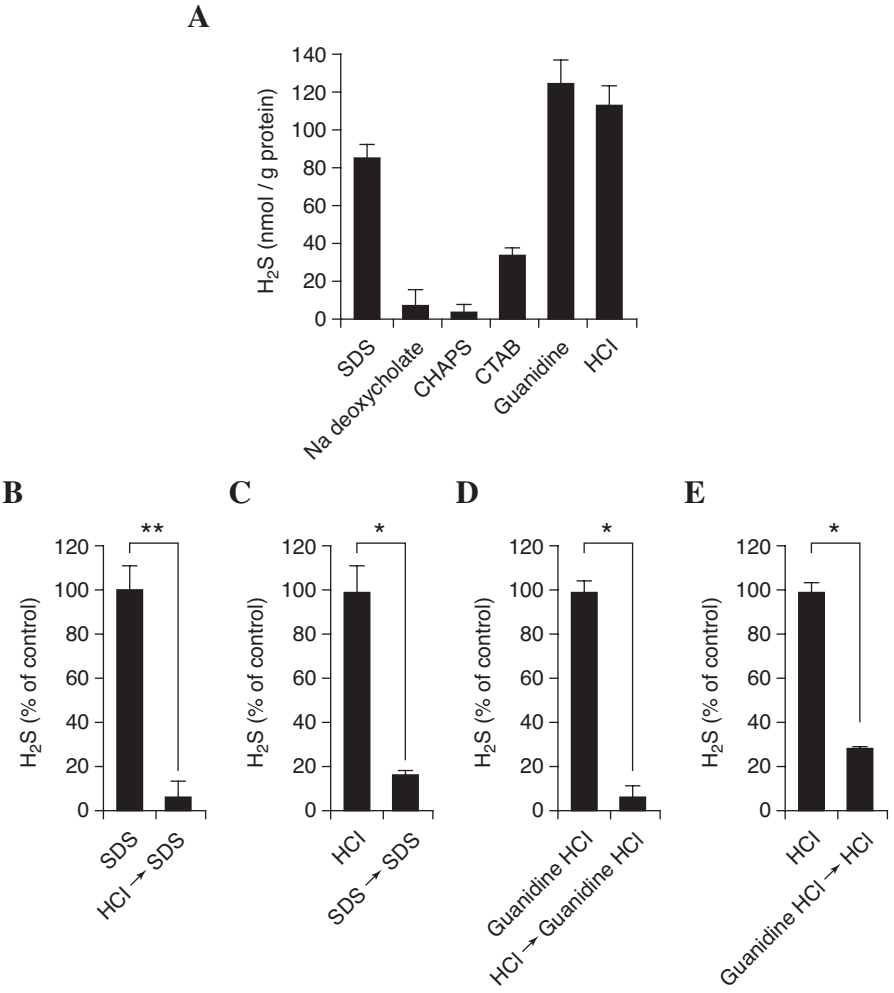

FIG. 2. H_2S released by detergents and a protein denaturant. (**A**) H_2S released by detergents and guanidine. The detergents and guanidine were added to the supernatants of rat brain homogenates (8.0 mg protein/ml) with 1 *M* citrate buffer (pH 6.0) to make the final concentrations of 4 *M* guanidine HCl, 2% Triton-X 100, 2% Tween 80, 2% SDS, 2% sodium deoxycholate, 2% CHAPS, or 0.6% CTAB. The resultant supernatants were incubated for 10 min at 37°C, and the amounts of H_2S released measured. (**B**, **C**) H_2S released by SDS from brain homogenates pretreated with HCl (**B**) or *vice versa* (**C**). Rat brain homogenates were incubated in the presence of 1N HCl (**B**) or 2% SDS (**C**) for 30 min (**B**) or 5 min (**C**) at 37°C, and H_2S was removed. The resultant reaction mixture was incubated in the presence of 2% SDS (**B**) or 1N HCl (**C**) for 5 min at room temperature, and released H_2S was determined. The amount of H_2S released by SDS alone (**B**) or HCl alone (**C**) is shown as 100%. $^{*}p < 0.0001$ and $^{**}p < 0.001$ by ANOVA. (**D** and **E**) H_2S released by guanidine HCl from brain homogenates pretreated with HCl (**D**) or *vice versa* (**E**). Rat brain homogenates were incubated in the presence of 1N HCl (**D**) or 4 *M* guanidine HCl (**E**) for 30 min (**D**) or 1 h (**E**) at 37°C, and H_2S was removed. The resultant mixture was incubated in the presence of 4 *M* guanidine HCl (**D**) or 1N HCl (**E**) for 1 h at room temperature, and released H_2S determined. The amount of H_2S released by guanidine HCl alone (**D**) or HCl alone (**E**) is shown as 100%. $^{*}p < 0.0001$ by ANOVA. All data are represented as the mean ± SEM of three experiments.

in the presence of DTT were examined. The release of H_2S from liver homogenates reached a peak 1 h after the application of DTT and reached a plateau for ~1 h, and then abruptly decreased (Fig. 3A). The decrease of H_2S may be caused for the following reason. The absorption of H_2S by the homogenates may overcome the release of H_2S by DTT (see Fig. 4A). In contrast, the H_2S release from brain homogenates steadily increased for at least 5 h after the application of DTT. Heart homogenates released H_2S for only the initial 2 h at a low level.

To examine whether H_2S released by acids and reducing agents originated from the same source of sulfur, H_2S released by DTT from brain homogenates from which H_2S had been released by HCl pretreatment was compared with DTT alone, and *vice versa*. When HCl was added to brain homogenates, and all free H_2S removed, the addition of DTT to the resultant supernatant released almost the same amount of H_2S as without HCl pretreatment (Fig. 3B). This observation indicates that H_2S released by DTT and by acids has different sources of sulfur. Conversely, DTT released almost the same amount of H_2S after pretreatment with HCl (Fig. 3C). These observations confirm that the pool of bound sulfur is distinct from acid-labile sulfur. Although H_2S release by acids is increased and reaches a plateau after 30-min exposure to acids, H_2S release by DTT lasts longer and is a greater amount in the alkaline condition (Fig. 3A). H_2S released by HCl alone was 161 ± 5 nmol/g protein ($n = 3$), whereas that by DTT alone was 1,481 ± 174 nmol/g protein ($n = 5$ at pH 7.4) at 3 h.

Free H_2S in the brain

Although the amount of acid-labile sulfur in the brain has been determined (13, 29, 43), the levels of free H_2S have not been measured. The methylene blue method, which is widely used to measure H_2S, is not appropriate for brain homogenates, because the method uses acidic conditions, which release H_2S from acid-labile sulfur in homogenates. We therefore developed a method of using small particles of silver to absorb H_2S as Ag_2S from brain homogenates. With this method, proteins adhered to the silver particles were removed with 1% Triton X100, and thiourea and H_2SO_4 were applied only to protein-free silver particles to release H_2S from silver sulfide produced on the surface of the particles. This new method excluded the possibility of measuring acid-labile sulfur. With this method, no H_2S was detected. Be-

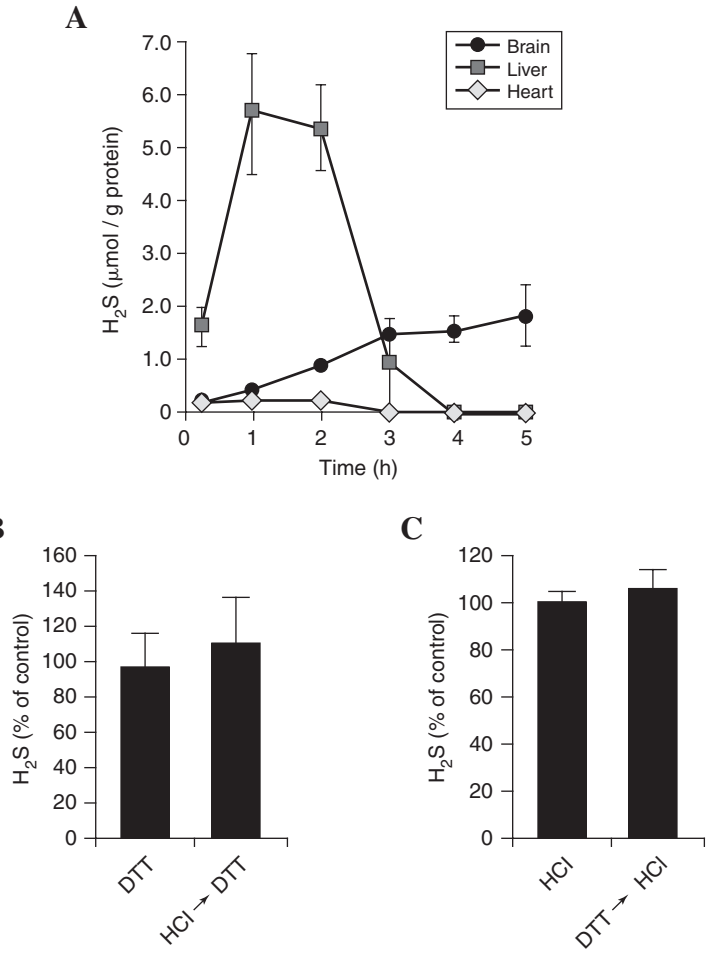

FIG. 3. Bound sulfur is distinct from acid-labile sulfur. (**A**) The time course of H_2S release from bound sulfur in rat brain, liver, and heart. Homogenates were incubated in the presence of 5 m*M* DTT for the hours indicated, and released H_2S determined. (**B, C**) H_2S released by DTT from rat brain homogenates (8.0 mg protein/ml) pretreated with HCl (**B**) or *vice versa* (**C**). Rat brain homogenates were incubated in the presence of 1N HCl (**B**) or 5 m*M* DTT (**C**) for 10 min (**B**) or 3 h (**C**) at 37°C, and H_2S was removed. The resultant reaction mixture in the presence of 5 m*M* DTT (**B**) The 1N HCl (**C**) was incubated for 10 min at 37°C, and released H_2S determined. The amount of H_2S released by DTT alone (**B**) or HCl alone (**C**) is shown as 100%. All data are represented as the mean ± SEM of three experiments.

cause the detectable level is 25 nmol H_2S in an assay tube, which corresponds to ~9.2 μM H_2S in the brain, free H_2S in the brain, if any, is <9.2 μM. The level of H_2S is maintained low in the basal condition.

Exogenously applied H_2S is absorbed as bound sulfur

Although some enzymes can produce H_2S, free H_2S is under detectable levels in the assay used in the present study. It is possible that enzymatically produced H_2S is immediately absorbed and stored as bound sulfur or acid-labile sulfur. To examine this possibility, an Na_2S solution was mixed and absorbed in homogenates of brain, liver, and heart. The H_2S that was not absorbed in homogenates remained as free H_2S, which was measured with gas chromatography. The residual free H_2S was not detected in liver homogenates, indicating that the liver homogenates immediately absorbed H_2S (Fig. 4A). The heart homogenates also promptly absorbed H_2S, and all H_2S was absorbed in 10 min. In contrast, absorption of H_2S by brain homogenates was much slower than those of liver and heart (Fig. 4A). These observations suggest that newly synthesized free H_2S may also remain in the brain. It is, therefore, possible that newly synthesized H_2S functions as a free entity for a longer time in the brain than heart and liver.

To compare the ability of tissue homogenates to absorb H_2S with other proteins, H_2S absorption by BSA, lysozyme, and fetal bovine serum was also measured. No absorption of H_2S by lysozyme or BSA was noted. Fetal bovine serum slowly absorbed H_2S, but much more slowly than brain homogenates (Fig. 4A).

To examine which pool of sulfur absorbed the exogenous H_2S and to show that the H_2S was not simply oxidized, the amounts of H_2S recovered from brain homogenates exposed to exogenously applied H_2S were measured after acid or DTT treatment. DTT released 45% more H_2S from preabsorbed homogenates than from homogenates without preabsorption (Fig. 4B). In contrast, TCA released H_2S from endogenous acid-labile sulfur but it did not release preabsorbed H_2S, suggesting that H_2S is not absorbed into an acid-labile sulfur pool in brain homogenates (Fig. 4C). To confirm this observation, the effects of SDS and guanidine HCl, which release H_2S from acid-labile sulfur, were also examined. Neither SDS nor guanidine HCl released preabsorbed H_2S (Fig. 4D and E). These observations indicate that exogenously applied H_2S is absorbed as bound sulfur but not as acid-labile sulfur in the brain.

Bound sulfur in the brain

Because free H_2S is immediately absorbed by brain homogenates and stored as bound sulfur (Fig. 4), H_2S produced by enzymes may also be stored as bound sulfur. It is possible that H_2S is released from bound sulfur in the presence of reducing molecules when intracellular pH is shifted to more-alkaline conditions, which lead to a more reduced state

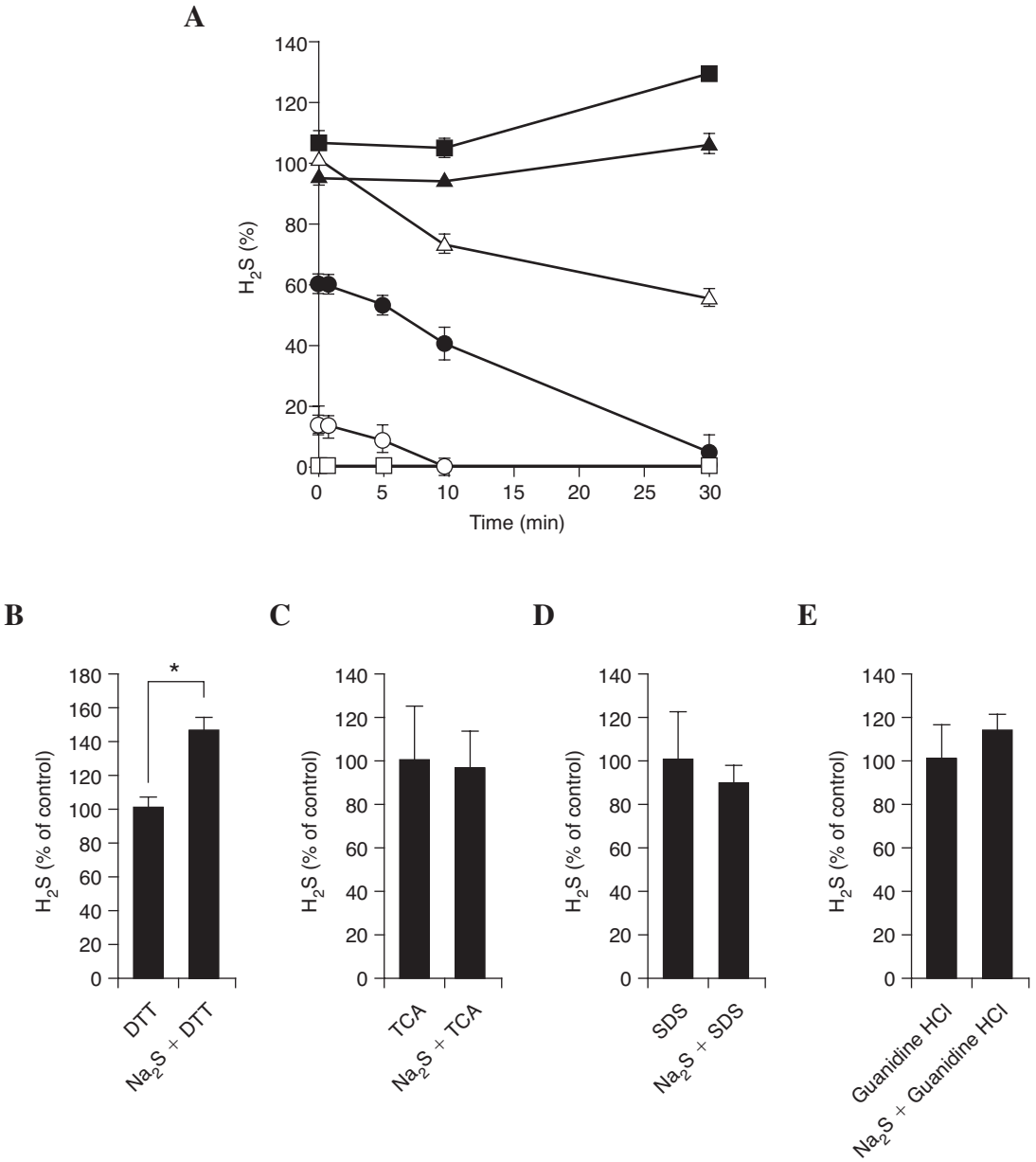

FIG. 4. Exogenously applied H_2S is absorbed in rat tissue homogenates as bound sulfur. (**A**) The time-course of H_2S absorption by tissue homogenates, serum, and proteins. Homogenates (8.0 mg protein/ml) of brain, liver or heart or solutions (8.0 mg protein/ml) of BSA, lysozyme, or fetal bovine serum were incubated in the presence of 3.33 μM Na_2S for the times indicated. The remaining H_2S, which was not absorbed, was released from the aqueous phase by converting most of the dissolved HS^- (the dominant form at pH 7.4) into H_2S with citrate buffer (pH 6.0) and measured. ●, brain; □, liver; ○, heart; ▲, BSA; ■, lysozyme; △, fetal bovine serum. (**B**) Absorbed exogenous H_2S was released by DTT. Brain homogenates were incubated in the presence of 3.33 μM Na_2S for 30 min. Bound H_2S was released by 50 mM DTT and measured. H_2S released from the supernatants of brain homogenates without exogenously applied Na_2S was shown as "DTT" (100%). $^*p < 0.008$ by ANOVA. (**C–E**) Absorbed exogenous H_2S was not released by TCA, SDS, or guanidine HCl. Brain homogenates were incubated in the presence of 3.33 μM Na_2S for 30 min, and the amounts of H_2S released by 100% TCA (**C**), 2% SDS (**D**), or 4 M guanidine HCl (**E**) were measured. H_2S released from the supernatants of brain homogenates without exogenously applied Na_2S are shown as TCA, SDS, and guanidine HCl, respectively. All data are represented as the mean ± SEM of three experiments.

(15, 30). To identify these conditions, H_2S released from brain homogenates was measured in the presence of physiologic concentrations of the major endogenous reducing substances, glutathione and cysteine. Because the reducing activity of thiols is greater in alkaline conditions than at a neutral pH (15, 30), the release of H_2S was examined in alkaline conditions that have been documented in cells. H_2S was released in the presence of 10 mM glutathione and 100 μM cysteine at pH 8.4, and to a greater extent in the presence of 250 μM cysteine (Fig. 5A). The localization of the bound sulfur was also examined; most of the H_2S was released by GSH from the cytoplasm, and less from microsomes (Fig. 5B).

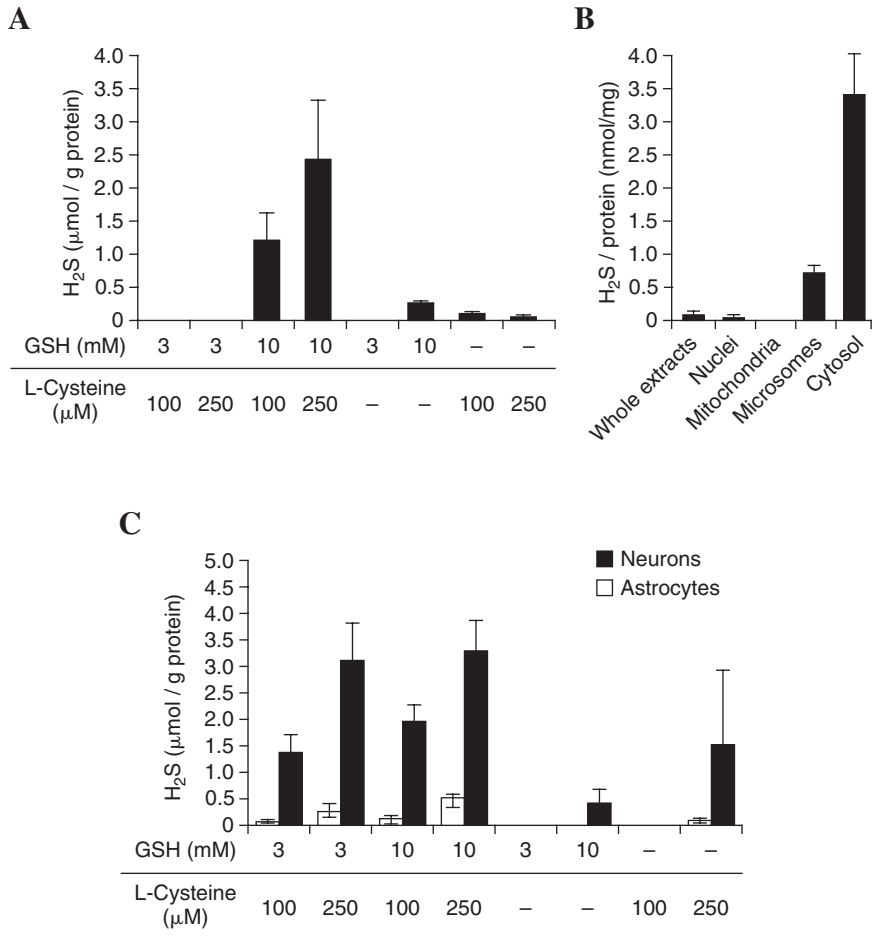

FIG. 5. H_2S released from bound sulfur in the brain. (**A**) H_2S released from mouse brain homogenates in the presence of glutathione and cysteine. The indicated concentrations of glutathione and cysteine were added to mouse brain homogenates adjusted to pH 8.4. The homogenates (1.7 mg protein/ml) were incubated at 37°C for 4 h, and amounts of H_2S released were measured. (**B**) Intracellular localization of bound sulfur. Adult rat brains were homogenized and fractionated. Three hundred microliters of each fraction was incubated in the presence of 10 m*M* GSH at 37°C for 4 h, and H_2S was measured. Protein concentrations of each fraction were as follow. A whole brain, 2.9; nuclei, 6.9; mitochondria, 3.4; microsome, 3.2; cytosol, 2.4 (in mg protein/ml). (**C**) H_2S released from primary cultures of mouse neurons and astrocytes in the presence of glutathione and cysteine. Indicated concentrations of glutathione and cysteine were added to the lysates of mouse primary cultures of neurons and astrocytes adjusted to pH 8.4. The lysates of neurons (0.6 mg protein/ml) or astrocytes (0.5 mg protein/ml) were incubated at 37°C for 4 h, and the amounts of H_2S released was measured. All data are represented as the mean ± SEM of three experiments.

To examine the cellular origin of the bound sulfur, H_2S release from primary cultures of neurons and astrocytes was investigated. Although both neurons and astrocytes released H_2S in the presence of 3 m*M* glutathione and 100 μM cysteine, neurons released >10 times as much H_2S than did astrocytes (Fig. 5C). H_2S released from a tube containing 10 m*M* glutathione and 250 μM cysteine in the presence of lysates of neurons, lysates of astrocytes, and in the absence of lysates were 0.610 ± 0.178, 0.177 ± 0.019, and 0.093 ± 0.034 nmol H_2S/tube ($n = 3$), respectively. Primary cultures of neurons and astrocytes require lower concentrations of glutathione and cysteine to release H_2S than do brain homogenates (Fig. 5A and C), perhaps because some substances that absorb H_2S in the brain homogenates that are not found in cultures of neurons and astrocytes. These observations show that brain cells can release H_2S in the presence of physiologic concentrations of glutathione and cysteine in an alkaline condition with pH 8.4, and that neurons contain more bound sulfur than do astrocytes.

K^+ induced alkalinization in astrocytes

Because physiologic concentrations of glutathione and cysteine are sufficient to induce H_2S release when cells are under the alkaline condition at pH 8.4, we explored the physiologic stimuli that may shift the intracellular pH to alkaline. Neurotransmitters (glutamate, glycine, noradrenaline, adrenaline, GABA, dopamine, serotonin, substance P, neurotensin, angiotensin), growth factors (BDNF, EGF, and TGF-α), amino acids (arginine, lysine, aspartate, histidine, glutamine, cystine), and NH_4Cl, dexamethasone, dibutyryl cAMP, dibutyryl cGMP, sodium nitroprusside, phenylephrine, arachidonic acid, decosahexanoic acid, melatonin, IBMX, linoleic acid, forskolin, GM-CSF, mannitol, phorbol 12-myristate 13-acetate, carbachol, propionic acid, platelet-activating factor, and KCl were examined by using primary cultures of mouse neurons and astrocytes. Only KCl was effective to alkalinize the intracellular pH.

Ten millimolar exogenous K^+, which can be obtained when neurons are repetitively excited (34), induced reversible alkalinization in astrocytes (Fig. 6) (5). One hundred fifteen of 185 cells responded to 10 m*M* K^+, and the average response was ÄpH 0.56 ± 0.03 (Fig. 6A). The average changes of the most-responsive 20 cells was ÄpH 1.27 ± 0.10 (Fig. 6B). We could not, however, detect the small amounts of H_2S released into the superfusing solution from cultures of astrocytes, because only small populations of cells reached pH 8.4. The development of a more-sensitive method to measure H_2S in the superfusing solution is required. Ten millimolar K^+ did not induce alkalinization in neurons, nor did any of the other conditions tested.

Discussion

The present study shows that the two forms of sulfur storage in the brain are defined as acid-labile sulfur and bound sulfur. Free H_2S in the brain is below detectable levels (9.2 μM) in basal conditions. Lysates of neurons and astrocytes

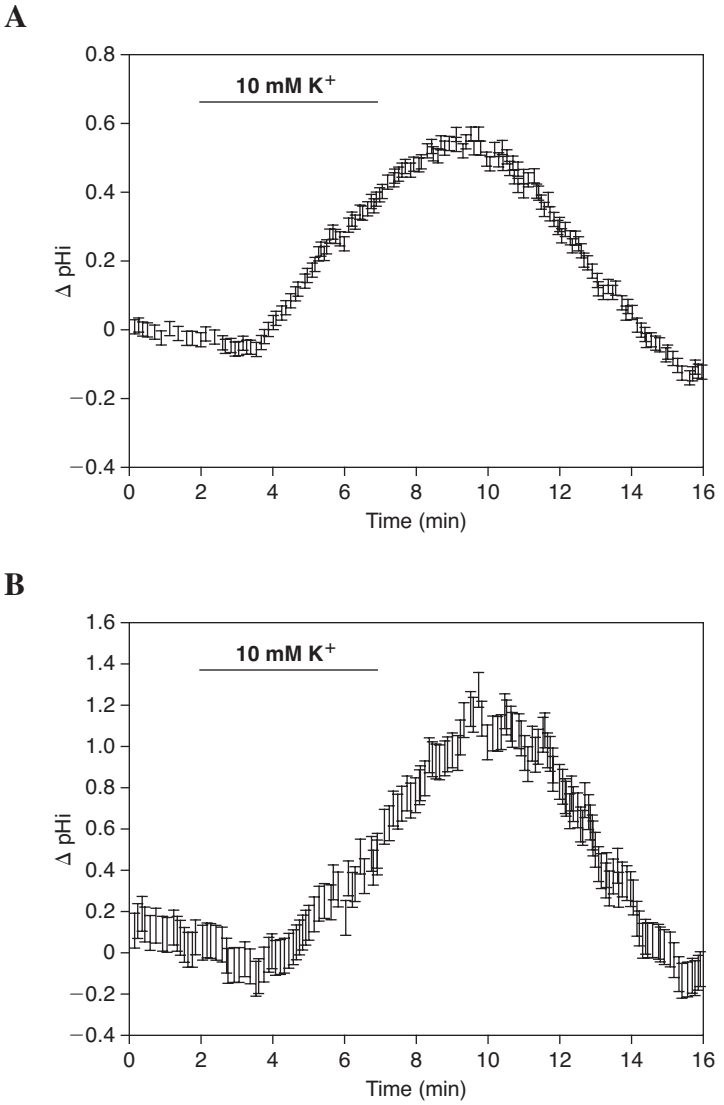

FIG. 6. K^+-induced intracellular alkalinization in mouse astrocytes. (**A**) Intracellular alkalinization induced by a high concentration of K^+ in astrocytes. Primary cultures of astrocytes prepared from embryonic day 16 mouse brain were stained with 10 μM SNARF-1 at 37°C for 30 min. Cultures were superfused with EBS solution bubbled with 95% O_2/5% CO_2 at 1.25 ml/min. 115 of 185 cells responded to 10 mM K^+, but 70 cells did not show any pH change. The average responses of 115 cells ± SEM are shown. (**B**) Intracellular alkalinization induced by a high concentration of K^+ in the most-responsive 20 astrocytes. The average responses of 20 cells ± SEM are shown.

release H_2S from bound sulfur in the presence of physiologic concentrations of glutathione and cysteine in alkaline conditions. In addition, high extracellular concentrations of potassium shift the intracellular pH of astrocytes to alkaline that is sufficient to release H_2S. These observations identify a mechanism for the release of H_2S from bound sulfur in astrocytes that surround excitable neurons.

We previously demonstrated several physiologic roles for H_2S in the nervous system (1, 19, 21). Endogenous levels of sulfide have been measured in the brains of rats, bovines, and humans (13, 29, 43), but high concentrations of hydrochloric acid or trichloroacetic acid were used to release H_2S in these studies. Therefore, the amounts measured were not of free H_2S, but of H_2S released from acid-labile sulfur. The present results show that the critical pH to release H_2S from acid-labile sulfur is 5.4 (Fig. 1). Acid-labile sulfur, which mostly consists of iron–sulfur complex of enzymes involved in oxidative phosphorylation, is localized mainly to mitochondria (25, 26). Because mitochondria usually do not become acidic, it may be difficult for H_2S to be released from acid-labile sulfur under physiologic conditions.

Exogenously applied free H_2S is readily absorbed and stored as bound sulfur (Fig. 4A). Although NaHS applied to culture medium was readily evaporated and only one third of the applied NaHS remained in 15 min (12, 18), a single application of NaHS protected neurons from oxidative stress (18), whereas multiple applications caused the toxic effect (12). It is probably because even though the concentrations of NaHS in the medium are decreased, the multiple applications cause the accumulation of bound sulfur to reach toxic levels.

In reducing conditions, H_2S is released from endogenously produced bound sulfur as well as exogenously applied H_2S absorbed and stored as bound sulfur (Figs. 3 and 4). H_2S is absorbed in brain homogenates more slowly than in liver and heart homogenates, and the release is also slower from brain homogenates than from those of liver and heart. Therefore, once H_2S is released from bound sulfur or from H_2S-producing enzymes, free H_2S may remain longer in the brain than in the liver and heart.

Although bound sulfur is known to release H_2S in the presence of DTT (25, 28), the release of H_2S in physiologic conditions has not been investigated. Bound sulfur is localized mainly to the cytoplasm in the brain (Fig. 5B). Our results show that H_2S is released from bound sulfur in the presence of the physiologic concentrations of glutathione and cysteine in alkaline conditions (Fig. 5A). Homogenates of

neurons release more H_2S than astrocytes, suggesting that neurons have a greater capacity than astrocytes to store H_2S as bound sulfur (Fig. 5C). Because cysteine is a substrate for CBS to produce H_2S, it is possible that some of H_2S released may be generated by CBS. However, this possibility is excluded because a large amount of H_2S was released in the presence of GSH only (Fig. 5B).

The reducing activity of glutathione and cysteine is greater in alkaline conditions (15, 30). Therefore, it is necessary to shift the intracellular pH to alkaline to release H_2S from bound sulfur in the presence of these endogenous reducing substances. When neurons are excited, sodium ions enter and potassium ions exit from cells, resulting in high potassium concentrations in the extracellular environment and the depolarization of surrounding astrocytes (34). To recover from the depolarized state to the quiescent condition, Na^+/HCO_3^- cotransporter is activated in astrocytes (23). Entrance of 1 Na^+ and 2 or 3 HCO_3^- is electrogenic, and HCO_3^- causes the alkalinization of the cell (5). In primary cultures, ~60% of the astrocytes responded to high concentrations of K^+, causing intracellular alkalinization, whereas the remaining astrocytes were quiescent (Fig. 6A). Ten percent of the astrocytes vigorously responded and shifted their intracellular 1.27 pH units (Fig. 6B). Because the basal intracellular pH is 7.0 ~ 7.2 and because pH 8.4 is required to release H_2S (Fig. 5), it appears that 10% of astrocytes can release H_2S. However, in the present study, the experiments were performed in the presence of only glutathione and cysteine as reducing substances. Because other endogenous reducing substances are found in the intracellular environment, the reducing activity in the cells may be greater than observed in the present study. The astrocytes used in the present study were homogeneous in phenotype, type I (39), but only 60% of the astrocytes responded to high concentrations of K^+, suggesting that the astrocytes were heterogeneous in function. Similarly, the same preparation of astrocytes had only a subset of cells that responded to NaHS (21, 39). Although the luminal side of mitochondria is in alkaline conditions, bound sulfur is not localized to mitochondria (Fig. 5B). Therefore, the contribution of mitochondria to release H_2S from bound sulfur may be little.

In conclusion, two forms of the sulfur pool inside cells release H_2S, acid-labile sulfur, and bound sulfur. Although free H_2S is under a detectable level in the brain, H_2S is released from bound sulfur in the presence of physiologic concentrations of glutathione and cysteine in slightly alkaline conditions. Extracellular potassium, at concentrations that are attained after neuronal excitation, causes the intracellular pH of astrocytes to increase to a level that supports the release of H_2S. The free exogenous H_2S is then able to play a physiologic role in neurotransmission and cell survival, as we and others previously demonstrated.

Acknowledgment

I thank Dr. Schubert for the critical reading of the manuscript. This work was supported by a grant from the National Institute of Neuroscience to H. K.

Abbreviations

Ag, silver; ANOVA, analysis of variance; Ag_2S, silver sulfide; AraC, cytosine arabinoside; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CBS, cystathionine β-synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CSE, cystathionine γ-lyase; CTAB, cetyltrimethylammonium bromide; DTT, dithiothreitol; EBS, Earle's balanced salt solution; EGF, epidermal growth factor; GABA, γ-aminobutyric acid; GM-CSF, granulocyte–macrophage colony-stimulating factor; GSH, glutathione; H_2S, hydrogen sulfide; H_2SO_4, sulfuric acid; IBMX, isobutylmethylxanthine; KCl, potassium chloride; LTP, long-term potentiation; NaHS, sodium hydrosulfide; Na_2S, sodium sulfide; NH_4Cl, ammonium chloride; NO, nitric oxide; SNARF-1, carboxyseminaphthorhodofluor-1; SNARF-1-AM, carboxyseminaphthorhodofluor-1-acetoxymethyl ester; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; TGF-α, transforming growth factor α; pH_i, intracellular pH.

Disclosure Statement

No competing financial interests exists.

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Date of first submission to ARS Central, May 19, 2008; date of final revised submission, August 25, 2008; date of acceptance, August 27, 2008.

This article has been cited by:

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