Hydrogen Sulfide Is Produced in Response to Neuronal Excitation

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Although hydrogen sulfide (H_2S) is generally thought of in terms of a poisonous gas, it is endogenously produced in the brain. Physiological concentrations of H_2S selectively enhance NMDA receptor-mediated responses and alter the induction of hippocampal long-term potentiation (LTP). Here we use cystathionine β-synthase (CBS) knock-out mice to clearly show that CBS produces endogenous H_2S in the brain and that H_2S production is greatly enhanced by the excitatory neurotransmitter L-glutamate, as well as by electrical stimulation. This increased CBS activity is regulated by a pathway involving Ca^{2+}/calmodulin. In addition, LTP is altered in CBS knock-out mice. These observations suggest that H_2S is produced by CBS in response to neuronal excitation and that it may regulate some aspects of synaptic activity.

Key words: hydrogen sulfide; neuromodulator; calcium ion; calmodulin; neuronal excitation; glutamate; LTP

Hydrogen sulfide (H_2S) is a well known toxic gas, and most studies about H_2S have been devoted to its toxic effects (Reiffenstein et al., 1992). However, relatively high endogenous levels of H_2S have been measured in the brains of rats, humans, and bovine (Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990), suggesting that H_2S may have a physiological function. Endogenous H_2S can be formed from L-cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) (Stipanuk and Beck 1982; Griffith, 198[illegible]; Swaroop et al., 1992). CBS is expressed in the brain, and the CBS inhibitors hydroxylamine and amino-oxyacetate suppress the production of H_2S, whereas the CBS activator S-adenosyl-L-methionine (SAM) enhances H_2S production. Physiological concentrations of H_2S specifically potentiate the activity of NMDA receptor and alter the induction of long-term potentiation (LTP) in the hippocampus (Abe and Kimura, 1996). cAMP-mediated pathways may be involved in the modulation of NMDA receptor by H_2S (Kimura, 2000). H_2S can also regulate the release of corticotropin-releasing hormone from the hypothalamus (Russo et al., 2000). Based on these observations, it has been proposed that CBS can produce H_2S in the brain and that H_2S may function as a neuromodulator (Abe and Kimura, 1996).

Two other gases, nitric oxide (NO) and carbon monoxide (CO), are endogenously produced by enzymes localized in the brain (Maines, 1988; Palmer et al., 1988; Verma et al., 1993). Both NO and CO have been proposed as retrograde messengers in hippocampal LTP, a synaptic model of learning and memory (O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992; Bliss and Collingridge, 1993; Stevens and Wang, 1993; Zhuo et al., 1993). The activities of NO synthase are regulated by Ca^{2+}/calmodulin, and NO is released when NMDA receptors are activated by L-glutamate (Garthwaite et al., 1988; Bredt and Snyder, 1990). The regulation of CO production by neuronal excitation is not understood (Baranano et al., 2001).

CBS knock-out mice have been established (Watanabe et al., 1995). Animals homozygous for the CBS mutant gene are born at the expected frequency from matings of heterozygotes, but a majority of them die within 5 weeks after birth. They have less body weight than the wild-type mice, but the weight and morphology of the brain is normal (Watanabe et al., 1995; our unpublished observation). We used the CBS knock-out mice to show that CBS produces the endogenous H_2S in the brain. We also found a novel regulation for H_2S production by Ca^{2+}/calmodulin and determined the 19 amino acid calmodulin binding domain in CBS. In addition, it is shown that L-glutamate, as well as electrical stimulation, enhances the production of H_2S from brain slices and that LTP is altered in CBS knock-out mice. These observations suggest that endogenous H_2S is produced when CBS is activated by the Ca^{2+} influx, which occurs with neuronal excitation, and that H_2S may function as a neuromodulator or transmitter (Baranano et al., 2001).

MATERIALS AND METHODS

Determination of genotype by PCR. CBS knock-out heterozygous mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Exons 3 and 4 were deleted and exchanged with the neomycin-resistant gene in knock-out mice (Watanabe et al., 1995). Genomic DNA was isolated from mouse tails and amplified by PCR with three primers: 5′-CGG ATG ACC TGC ATT CAT CT-3′; 5′-GAA GTG GAG CTA TCA GAG CA-3′; and 5′-GAG GTC GAC GGT ATC GAT A-3′.

Purification of CBS from brain homogenates. For the measurement of H_2S production, CBS was purified by calmodulin Sepharose 4B from brain homogenates. Brain homogenates in 3 vol of Tris-buffered saline (TBS) [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2 mM PMSF, 1 mM EGTA, and protease inhibitor cocktail (Roche, Basel, Switzerland)] were centrifuged at 10,000 × *g* for 60 min at 4°C, and the supernatant was recovered. Immediately before applying to calmodulin Sepharose column, $CaCl_2$ was added to the final concentration of 4 mM. After washing with five bed volumes of 1 mM $CaCl_2$ containing TBS, CBS was eluted with 2 mM EGTA containing TBS. The resultant eluent was dialyzed with TBS. The protein concentrations of the eluent were estimated by Protein Assay (Bio-Rad, Hercules, CA).

Brain cell suspensions. Brain cell suspensions were prepared from the brain of 8-d-old mice by a modified method described by Garthwaite et al. (1988). The fresh tissues were minced and digested with 5 ml of 0.25 mg/ml trypsin in Ca^{2+}/Mg^{2+}-free basic salt solution [10 mM HEPES,

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pH7.2, 150 mM NaCl, 5 mM KCl, 11 mM glucose, and 0.75% BSA (type III; Sigma, St. Louis, MO)] at 37°C for 30 min. The reaction was terminated by adding 5 ml of Ca^{2+}/Mg^{2+}-free basic salt solution containing 40 μg/ml deoxyribonuclease I and 0.25 mg/ml soy bean trypsin inhibitor (Sigma). After washing, cells were resuspended at the density of 10^5 cells/ml. After 1 hr of preincubation at 37°C in air, 100 μl of cell suspensions were transferred to a 1.5 ml microtube, and agonists or antagonists were applied. Stimulation was terminated by adding 2 μl of 10 M NaOH.

Measurement of H_2S. The amounts of endogenous H_2S in the brain and H_2S produced by cell suspensions were measured by a gas chromatograph (Hoshika and Iida, 1977) (GC-14B; Shimazu, Kyoto, Japan). Briefly, 100 μl of homogenates consisting of 1 vol of brain and 3 vol of 10 mM NaOH in a 1.5 ml microtube were filled with N_2 gas and sealed with parafilm (American National Can, Chicago, IL). H_2S gas was released by adding 100 μl of 100% trichloro acetic acid to the tube with a 1 ml syringe and then incubated at 37°C for 30 min. Three hundred microliters of gas were removed from the reaction tube and applied to a gas chromatograph.

H_2S produced by purified CBS was measured as follows: 100 μl of 50 mM Tris, pH 8.6, 2 mM pyridoxal 5′-phosphate, and 1 mM L-cysteine, with 4.6 μg of total protein of purified CBS was incubated at 37°C for 30 min. Concentrations of Ca^{2+} in the reaction mixture were determined by an ion meter (F-23; Horiba, Kyoto, Japan). The procedures to measure the amounts of H_2S released were the same as above. The quantitation of H_2S was done using NaHS as a standard.

Measurement of free L-cysteine in the brain. The amounts of L-cysteine were measured by using a reverse-phase HPLC with fluorescence detection (Waters 2690 separation module and 474 scanning fluorescence detector; Waters, Milford, MA). The brain extracts were boiled and extracted with phenol–chloroform and then centrifuged at 15,000 × *g* for 10 min. The supernatant was labeled by AccQ-Tag system (Waters) and applied to the HPLC. Quantitation was done with an external standard of L-cysteine.

The electrical stimulation of brain slices and the induction of LTP. For the study of H_2S production induced by electrical stimulation, slices of cerebral cortices (300 μm) were prepared from the 4-week-old mice and maintained in a chamber at 30°C in artificial CSF (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose (bubbled with 95% O_2–5% CO_2). A bipolar stimulating electrode was placed at the white matter, and four 100 Hz pulses (100 μsec duration) at 200 msec intervals and 3 V of stimulus intensity were applied for 30 sec or 1 min. After stimulation, each slice was transferred into a 1.5 ml microtube, and the amount of H_2S was measured.

For LTP experiments, hippocampal slices (400 μm) were prepared from the 12- to 16-d-old CBS knock-out mice and the wild-type littermates and maintained in a chamber at 30°C in ACSF that contained 10 μM bicuculline to suppress inhibitory synaptic responses. A bipolar stimulating electrode was placed in the stratum radiatum in the CA1/CA2 border region, and the evoked EPSP was extracellularly recorded from the stratum radiatum in the CA1 region with a glass capillary microelectrode (1–5 MΩ) filled with 0.5 M NaCl. A single test stimulation (100 μsec) was applied at intervals of 10 sec. The initial EPSP slopes of 0.15–0.20 mV/msec were used. To induce LTP, five sets with 10 sec intervals of theta-burst stimulation (10 bursts of four pulses at 100 Hz with 200 msec interburst intervals) were applied. Changes in field potential were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA).

RESULTS

CBS produces the endogenous brain H_2S

Relatively high endogenous levels of brain H_2S have been measured in rats, humans, and bovine (Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990), and H_2S production from L-cysteine in brain homogenates is suppressed by CBS-specific inhibitors, such as hydroxylamine and aminooxyacetate, and is increased by the CBS activator SAM (Abe and Kimura, 1996). Based on these observations, we proposed that CBS is an enzyme that produces endogenous H_2S in the brain. A critical experiment to support the hypothesis is to measure the endogenous levels of H_2S in the brains of CBS knock-out mice. Although the homozygotes of CBS knock-out mice show growth retardation

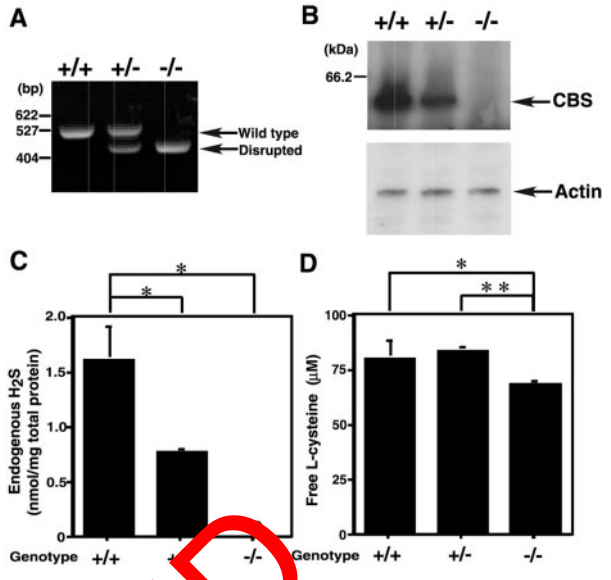

Figure 1. Brains of CBS knock-out mice do not contain endogenous H_2S. *A*, Determination of the genotype of CBS knock-out mice by PCR. PCR amplification with three primers gave fragments of 500 bp for the wild type and 320 bp for the disrupted mutant. *B*, Determination of CBS levels in the brain by Western blot analysis. Protein (15 μg) obtained from the brain homogenates of homozygous (−/−) and heterozygous (+/−) CBS knock-out and the wild-type (+/+) mice were analyzed by Western blotting with antibodies against CBS and actin. *C*, Determination of endogenous H_2S levels in the brain. Endogenous H_2S levels in the brains of homozygous (−/−) and heterozygous (+/−) CBS knock-out mice and the wild type (+/+) were determined by gas chromatography. *D*, Endogenous L-cysteine levels in the brain. Endogenous L-cysteine levels in the brains used for *C* were determined by HPLC. Data in *C* and *D* represent the mean ± SEM of five experiments for the heterozygous mice and the wild type and three experiments for the homozygous CBS knock-out mice. $^*p < 0.05$; $^{**}p < 0.001$; Student's *t* test.

(Watanabe et al., 1995), the morphology and weights of their brains are normal (data not shown). The genotypes of CBS knock-out mice were determined by PCR and confirmed by Western blot analysis (Fig. 1*A*,*B*). Because CBS knock-out mice have a high incidence of death during the third and fourth postnatal weeks (Watanabe et al., 1995), 2-week-old mice were used to measure endogenous brain H_2S. H_2S in the brains of the homozygous CBS knock-out mice was under detectable levels (Fig. 1*C*). The H_2S level of the heterozygous mice (0.76 ± 0.04 nmol/mg protein; $n = 5$) was less than one-half of the wild-type mice (1.60 ± 0.32 nmol/mg protein; $n = 5$; $p < 0.05$ by the Student's *t* test). Because the above data could be attributable to differences in substrate concentration, the amounts of L-cysteine in the brains of CBS knock-out mice were measured and compared with those of the wild type. Although the levels of L-cysteine in homozygous (68.0 ± 2.4 μM; $n = 5$) CBS knock-out mice are less than those of the wild-type (79.8 ± 8.8 μM; $n = 5$; $p < 0.05$ by the Student's *t* test) and heterozygous (83.1 ± 2.8 μM; $n = 5$; $p < 0.001$) mice, the lack of brain H_2S in CBS knock-out mice cannot be attributable to the slightly lower level of L-cysteine (Fig. 1*D*). These observations clearly show that CBS produces the endogenous H_2S in the brain.

Regulation of CBS activity by Ca^{2+}/calmodulin

CBS is dependent on pyridoxal 5′-phosphate and heme, and its activity is enhanced by SAM (Finkelstein et al., 1975; Kery et al., 1994). No other regulators for this enzyme have been found. CBS

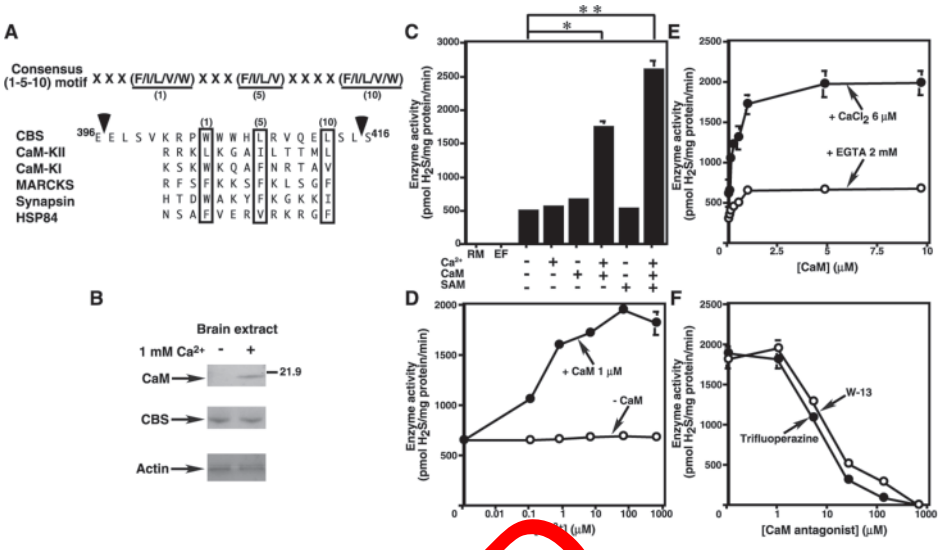

Figure 2. H_2S production by CBS is regulated by Ca^{2+}/calmodulin. *A*, A comparison of a consensus sequence of calmodulin binding domain of CBS with other calmodulin binding proteins. *CaM-KII*, Calmodulin-dependent kinase II; *CaM-KI*, calmodulin-dependent kinase I; *MARCKS*, myristoylated alanine-rich protein kinase C substrate; *HSP84*, heat shock protein 84 kDa. *B*, Immunoprecipitation assay for calmodulin binding to CBS. Ten milligrams of brain homogenate were immunoprecipitated with an antibody against CBS and analyzed by Western blotting with antibodies against CBS and calmodulin. Actin in the lysate served as a control. *C*, H_2S production from purified CBS is regulated by Ca^{2+} and calmodulin. Protein (4.6 μg) of CBS purified by calmodulin Sepharose 4B column was incubated with 1 mM cysteine and 2 mM pyridoxal 5′-phosphate in the presence or absence of 6 μM Ca^{2+} and/or 1 μM calmodulin, and H_2S production was measured. *RM*, Reaction mixture alone; *EF*, enzyme fraction alone. $^*p < 0.01$; $^{**}p < 0.001$; Student's *t* test. *D*, *E*, Ca^{2+} (*D*) or calmodulin (*E*) concentration-dependent H_2S production from purified CBS. Protein (4.6 μg) of CBS purified by calmodulin Sepharose 4B was incubated with 1 mM cysteine and 2 mM pyridoxal 5′-phosphate in the presence or absence of 1 μM calmodulin (*D*) or 6 μM Ca^{2+} (*E*), and the production of H_2S with different concentrations of Ca^{2+} (*D*) or calmodulin (*E*) was measured. *F*, Inhibition of H_2S production by calmodulin inhibitors. Purified CBS (4.6 μg) was incubated with 1 mM cysteine and 2 mM pyridoxal 5′-phosphate in the presence of 1 μM calmodulin and 6 μM Ca^{2+}, and the effects of trifluoroperazine (●) or W-13 (○) on H_2S production were examined. All data from *C–F* represent the mean ± SEM of five experiments.

contains a consensus sequence conserved in calmodulin binding proteins (Rhoads and Friedberg, 1997) (Fig. 2*A*). Therefore, the potential interaction between CBS and calmodulin was examined by immunoprecipitation assays with brain extracts. Because calmodulin binding is Ca^{2+} dependent, brain extracts were immunoprecipitated with an antibody against CBS in the presence or absence of 1 mM Ca^{2+}. Calmodulin coimmunoprecipitated with CBS in the presence of 1 mM Ca^{2+} but not in the absence of Ca^{2+} (Fig. 2*B*). CBS was not coimmunoprecipitated with an unrelated antibody against focal adhesion kinase in the same experiment (data not shown). This observation shows that CBS interacts with calmodulin in the presence of Ca^{2+}.

Because CBS interacts with calmodulin, H_2S production by CBS could be regulated by Ca^{2+}/calmodulin. To examine this possibility, H_2S production by CBS purified from brain homogenates by calmodulin Sepharose 4B affinity chromatography was investigated. CBS was purified 45-fold as determined by the ratio of activity to protein relative to the crude brain homogenates. H_2S was then measured in the presence of 1 mM L-cysteine and 2 mM pyridoxal 5′-phosphate, plus or minus 0.6 μM Ca^{2+} or 1 μM calmodulin. In the presence of Ca^{2+} and calmodulin, CBS produced H_2S at a rate 3.5 times greater than those without Ca^{2+} and calmodulin (Fig. 2*C*) ($p < 0.01$ by the Student's *t* test). In the presence of SAM, the enhanced CBS activity by Ca^{2+} and calmodulin was potentiated (Fig. 2*C*) ($p < 0.001$). Calmodulin or SAM alone very weakly activated the production of H_2S, but Ca^{2+} alone did not have any effect on CBS activity. These observations show that CBS is regulated by Ca^{2+}/calmodulin.

The minimal Ca^{2+} concentration required for the maximal activation of CBS was determined with purified CBS in the presence of 1 μM calmodulin. Ca^{2+} potentiates H_2S production in the presence of calmodulin, with an ED_{50} value of 290 nM, whereas H_2S production remained at the basal level in the absence of calmodulin (Fig. 2*D*). The dose–response curve of calmodulin required for CBS activity was also determined in the presence of 6 μM Ca^{2+}. Calmodulin potentiates H_2S production with the ED_{50} of 140 nM (Fig. 2*E*). To confirm that H_2S production by CBS requires Ca^{2+}/calmodulin, the effect of calmodulin inhibitors on H_2S production from purified CBS was examined. A potent calmodulin inhibitor, trifluoroperazine, suppressed H_2S production, with IC_{50} value of 8 μM (Fig. 2*F*). A specific calmodulin inhibitor, W-13, suppressed H_2S production, with an IC_{50} value of 51 μM (Fig. 2*F*). Those observations confirm that H_2S production by CBS is regulated by Ca^{2+}/calmodulin.

Calmodulin binding domain in CBS

The above observations show that CBS produces endogenous H_2S in the brain and that CBS is regulated by Ca^{2+}/calmodulin. To determine the Ca^{2+}/calmodulin regulatory domain within CBS, deletion mutants of CBS was prepared by transfecting COS-7 cells with expression plasmids containing mutant CBS cDNAs. The mutant (1–415), which has the C-terminal 141 amino acids of the wild-type CBS deleted (Kery et al., 1998), contains a consensus sequence for calmodulin binding (Fig. 2*A*), but the mutant (1–396), lacking the C-terminal 160 amino acids, is deficient in the consensus sequence. These mutants produced by COS-7 cells are shown in Figure 3*A*.

To determine whether or not the 19 amino acid sequence that contains the calmodulin binding consensus sequence interacts with calmodulin, an immunoprecipitation experiment was performed with lysates of COS-7 cells containing the wild-type CBS, mutant (1–415) or mutant (1–396). Lysates of COS-7 cells were immunoprecipitated with the antibody against CBS, and Western blot analysis was performed with the antibody against calmodulin. Both the wild type and mutant (1–415) coimmunoprecipitated with calmodulin, but the mutant (1–396) did not (Fig. 3*B*). These data show that the 19 amino acid sequence of CBS is required for the interaction with calmodulin.

Because CBS interacts with calmodulin at a 19 amino acid consensus sequence, it was asked whether this consensus sequence is critical for the regulation of H_2S production. Lysates of COS-7 cells containing the wild-type or CBS mutants were incubated with 1 mM L-cysteine and 2 mM pyridoxal 5′-phosphate in the presence or absence of 0.6 μM Ca^{2+}/1 μM calmodulin, and

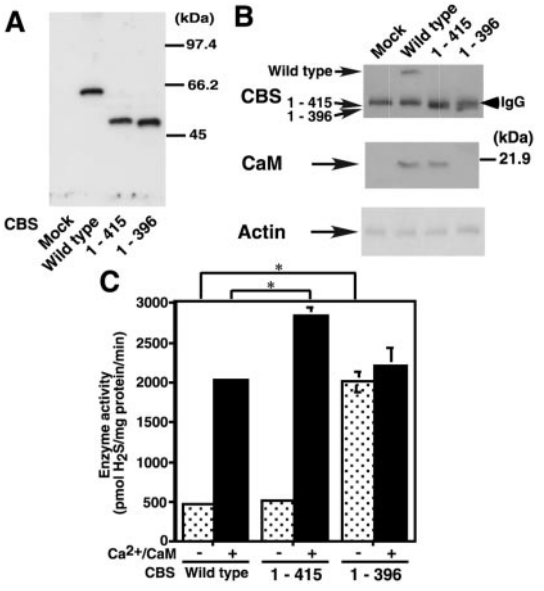

Figure 3. Determination of the calmodulin binding domain of CBS. *A*, Western blot analysis of deletion mutants of CBS. COS-7 cells were transiently transfected with expression plasmids containing the wild-type CBS (amino acid 1–556), mutant (*1–415*), and mutant (*1–396*). The cell lysates were analyzed by Western blotting with the antibody against CBS. *B*, Immunoprecipitation assay for calmodulin (*CaM*) binding to CBS mutants. Lysates (1.5 mg) described in *A* were immunoprecipitated with an antibody against CBS and analyzed by Western blotting with antibodies against CBS and calmodulin. Actin in the lysates served as control. Note that CBS mutants (*1–415*) and (*1–396*) appeared with a strong band of IgG heavy chain. *C*, Ca^{2+}/calmodulin-dependent production of H_2S from the wild-type and mutant CBS. Protein (1.5 μg) obtained from each cell lysate described in *A* was incubated with 1 mM L-cysteine, 2 mM pyridoxal 5′-phosphate in the presence or absence of 6 μM Ca^{2+} 1 μM calmodulin, and the production of H_2S was measured. The data represent the mean ± SEM of five experiments. $^*p < 0.01$; Student's *t* test.

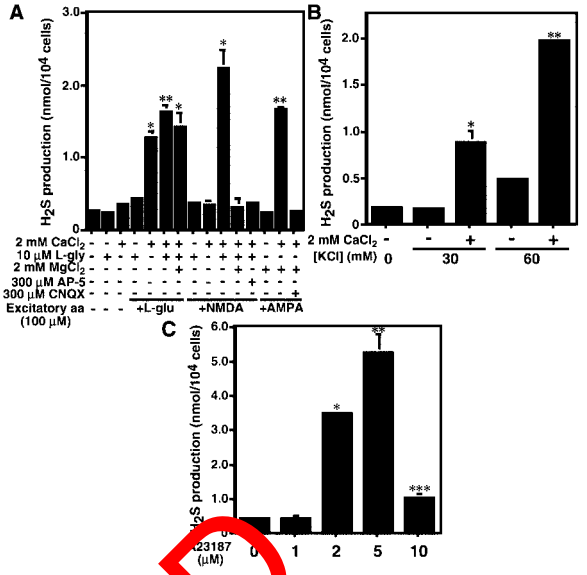

Figure 4. H_2S production is enhanced by L-glutamate, depolarization, and Ca^{2+} ionophore. *A*, L-Glutamate and its analogs enhance the production of H_2S. Cells at 1×10^4 in 100 μl of cell suspensions obtained from mouse cerebral cortices were incubated with glutamate analogs and their inhibitors at 37°C for 5 min, and the production of H_2S was measured by gas chromatography. $^*p < 0.01$; $^{**}p < 0.001$; Student's *t* test. *B*, Membrane depolarization enhances the production of H_2S. K^+ at 30 or 60 mM was applied to brain cell suspensions in the presence or absence of 2 mM Ca^{2+} at 37°C for 5 min, and the production of H_2S was measured. $^*p < 0.05$; $^{**}p < 0.001$; Student's *t* test. *C*, H_2S production in brain cell suspensions induced by Ca^{2+} influx. H_2S produced in brain cell suspensions by the application of A23187 was measured. $^*p < 0.001$; $^{**}p < 0.01$; $^{***}p < 0.05$; Student's *t* test. All data in *A–C* represent the mean ± SEM of five experiments.

H_2S production was determined. The mutant (1–396), which is deficient in 19 amino acid consensus sequence, produced H_2S at almost the same rate as the wild-type enzyme even in the absence of Ca^{2+}/calmodulin (Fig. 3*C*, $p < 0.01$ by the Student's *t* test). The wild type and mutant (1–415) produced only a basal rate of H_2S in the absence of Ca^{2+}/calmodulin. These observations suggest that the 19 amino acid sequence suppresses the CBS activity in the absence of Ca^{2+}/calmodulin. Once calmodulin binds to the sequence, CBS is released from the suppressed state to become active. A similar model has been proposed for the regulation of CBS activity by SAM (Shan et al., 2001).

H_2S production is enhanced by L-glutamate, Ca^{2+} ionophore, and electrical stimulation

Because H_2S production by CBS is regulated by Ca^{2+}/calmodulin, H_2S production may be controlled by neuronal activity. To examine this possibility, we prepared brain cell suspensions (Garthwaite et al., 1988) and measured H_2S production induced by the application of L-glutamate and its analogues. The production of H_2S was greatly enhanced by stimulation with L-glutamate ($p < 0.001$ by the Student's *t* test), NMDA ($p < 0.01$), or AMPA ($p < 0.001$) in the presence of 2 mM Ca^{2+} (Fig. 4*A*). The Ca^{2+}-dependent activation by NMDA requires L-glycine but is suppressed in the presence of Mg^{2+}. The effect of NMDA was inhibited by an NMDA-specific inhibitor, AP-5, and that of AMPA was inhibited by an AMPA-specific inhibitor, CNQX (Fig. 4*A*). These observations indicate that H_2S is produced when Ca^{2+} enters into the cells by the activation of at least two classes of ionotropic glutamate receptors.

When the neuronal membrane is depolarized, voltage-activated Ca^{2+} channels are opened and Ca^{2+} enters into the cells (Llinas, 1988). Because high concentrations of K^+ depolarize the membrane, the effect of high concentrations of K^+ on the H_2S production was examined using brain cell suspensions. In the presence of 2 mM Ca^{2+}, 30 ($p < 0.05$ by the Student's *t* test) and 60 ($p < 0.001$) mM K^+ greatly enhanced H_2S production (Fig. 4*B*). To confirm that H_2S production from brain cell suspensions was induced by Ca^{2+} influx, the effect of the Ca^{2+} ionophore A23187 on H_2S production was examined. Concentrations up to 5 μM A23187 dose-dependently potentiate H_2S production ($p < 0.01$ by the Student's *t* test), whereas 10 μM A23187 showed weaker potentiation (Fig. 4*C*) ($p < 0.05$). These observations suggest that H_2S production is induced by Ca^{2+} entry after the depolarization of the membrane.

Based on the above observations, it was asked whether H_2S is produced from slices of cerebral cortices by L-glutamate and electrical stimulation. L-Glutamate at 100 μM enhances H_2S production three times above the basal level (Fig. 5*A*) ($p < 0.05$ by the Student's *t* test). Electrical stimulation for 0.5 and 1 min causes H_2S production at approximately twice (1.62 ± 0.40 nmol/mg protein; $n = 3$; $p < 0.05$) and three (2.18 ± 0.50 nmol/mg protein; $n = 3$; $p < 0.05$) times the basal level (0.76 ± 0.04 nmol/mg protein; $n = 3$), respectively, whereas longer stimulation for 2 min did not effectively increase H_2S production ($p < 0.01$) (Fig. 5*B*). These observations show that H_2S is produced when neurons in slices are excited by electrical stimulation.

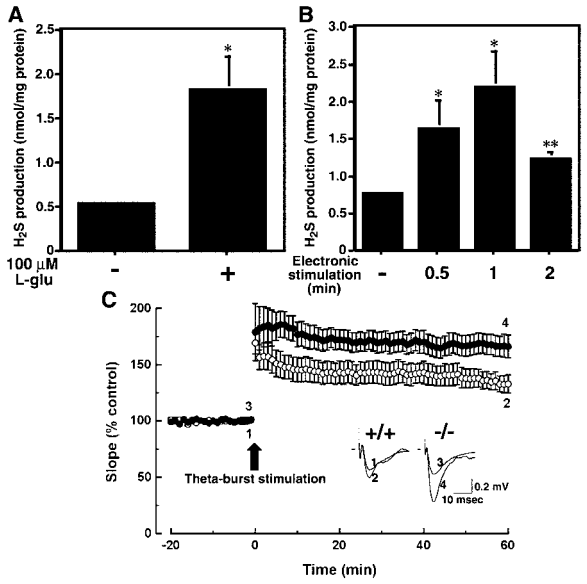

Figure 5. The production of H_2S and the induction of LTP in brain slices. *A*, *B*, The production of H_2S induced by L-glutamate and electrical stimulation. H_2S produced in the slices of cerebral cortices by the application of 100 μM L-glutamate (*A*) and by electrical stimulation for 0, 0.5, 1, and 2 min (*B*) was measured. Data are represented as the mean ± SEM of three experiments. $^*p < 0.05$; $^{**}p < 0.01$; Student's *t* test. *C*, LTP is altered in the absence of H_2S. Five sets of theta-burst stimulation (10 trains of 4 pulses of 100 μsec each at 200 msec intervals) applied at 10 sec intervals to hippocampal slices of CBS knock-out mice (●) and the wild-type mice (○). The field EPSP slopes were expressed as the percentage of baseline values before stimulation. Representative records at the times denoted by the *numbers* are shown as *insets*. The mean field EPSP slope (166.1 ± 10.1%; $n = 9$) 60 min after stimulation in the slices of CBS knock-out mice is significantly different ($p < 0.037$; Student's *t* test) from those in the wild-type mice (132.1 ± 9.3%; $n = 6$).

LTP is altered in CBS knock-out mice

Because exogenously applied H_2S modifies the induction of LTP in hippocampal slices (Abe and Kimura, 1996), we examined whether or not LTP is altered in CBS knock-out mice. Theta-burst stimulation was applied to induce LTP (Larrent et al., 1998), and changes in the slopes of EPSPs were measured. After stimulation, the augmented field EPSP slope in CBS knock-out mice gradually decreased and reached a plateau of 166.1 ± 10.1% ($n = 9$) of that before stimulation (Fig. 5*C*). In contrast, in slices of wild-type mice, the field EPSP slope reached a plateau of 132.1 ± 9.3% ($n = 6$) of that before stimulation (Fig. 5*C*). The statistical difference between EPSP slopes at 60 min after stimulation in CBS knock-out mice and the wild-type mice is significant ($p < 0.037$ by the Student's *t* test). These observations show that LTP is altered in the absence of H_2S and suggest the involvement of H_2S in synaptic activity.

DISCUSSION

The enzymatic activity of CBS has two metabolic outcomes (Mudd et al., 1989; Warenycia et al., 1989). Most studies have been devoted to a pathway in which CBS catalyzes the reaction with substrate homocysteine to produce cystathionine (Mudd et al., 1989). In another pathway, CBS catalyzes the reaction with L-cysteine as a substrate to produce H_2S and pyruvate (Stipanuk and Beck, 1982). The latter reaction had not been studied in the nervous system until we proposed that CBS can produce endogenous H_2S in the brain (Abe and Kimura, 1996). Because the activities of CBS in both metabolic pathways are regulated by SAM (Finkelstein et al., 1975; Abe and Kimura, 1996), a model for CBS regulation has been proposed in which the C-terminal domain of CBS bends to and covers its own catalytic domain, suppressing enzymatic CBS activity. Once SAM binds to the regulatory domain of CBS, a conformational change occurs that frees the catalytic domain, and CBS becomes active (Shan et al., 2001). Our present observations suggest that a similar mechanism may also function in the regulation of CBS by Ca^{2+}/calmodulin. In the absence of Ca^{2+}/calmodulin, the C-terminal domain may cover the catalytic domain, and CBS activity remains at a basal level. When Ca^{2+}/calmodulin binds to the 19 amino acid sequence, the catalytic domain is exposed by opening of the C-terminal domain, and CBS becomes active. This model is supported by our observation that the CBS mutant (1–396), which is deficient in the 19 amino acid Ca^{2+}/calmodulin binding sequence, is constantly active, even in the absence of Ca^{2+}/calmodulin (Fig. 3*C*).

Physiological basal concentrations of H_2S applied exogenously with a weak tetanic stimulation, which by itself does not induce LTP, facilitate the induction of LTP (Abe and Kimura, 1996). The present study shows that LTP is augmented in CBS knock-out mice (Fig. 5*C*). Physiological basal concentrations of H_2S enhance the NMDA receptor-mediated responses, whereas higher concentrations of H_2S specifically suppress EPSPs (Abe and Kimura, 1996). When H_2S is applied by superfusion, NMDA receptors on the postsynaptic membrane may be activated before the suppression of EPSPs, resulting in the facilitated induction of LTP. In contrast, electrical stimulation might produce H_2S at nerve endings, which could suppress EPSPs before diffusing across the synaptic cleft to activate postsynaptic NMDA receptor. Because there is no endogenous H_2S in CBS knock-out mice, LTP must be augmented.

Although H_2S is a toxic gas, most toxicology work has been done with whole animals, and less is known about its direct effect on cells (Reiffenstein et al., 1992). Because the neuronal excitation is local and lasts only for a short time, the increase in the concentrations of H_2S might not be toxic. For example, higher concentrations of H_2S than the basal level suppress EPSPs, but this suppression is reversible (Abe and Kimura, 1996). In addition, H_2S in the brain is tightly regulated to maintain endogenous concentrations at less than the toxic levels. For example, concentrations >10 μM A23187 and the electrical stimulation longer than 2 min did not efficiently enhance H_2S production (Figs. 4*C*, 5*B*).

In conclusion, endogenous H_2S in the brain is produced by CBS, and the production of H_2S by CBS is regulated by Ca^{2+}/calmodulin. The production of H_2S is greatly enhanced by the activation of glutamate receptors, as well as by electrical stimulation, and the loss of H_2S alters LTP. These observations suggest that H_2S may regulate some aspects of synaptic activity.

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