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Original Research Communication

Differentiated Astrocytes Acquire Sensitivity to Hydrogen Sulfide That Is Diminished by the Transformation into Reactive Astrocytes

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ABSTRACT

Hydrogen sulfide (H_2S) enhances the induction of hippocampal long-term potentiation (LTP) and induces calcium waves in astrocytes. Based on these observations, H_2S has been proposed to be a synaptic modulator in the brain. Here we show that differentiated astrocytes acquire sensitivity to H_2S that is diminished by their transformation into reactive astrocytes. Although sodium hydrosulfide hydrate (NaHS), a donor of H_2S, did not increase the intracellular concentration of Ca^{2+} in progenitors, exposure of progenitors to leukemia inhibitory factor (LIF), which induces differentiation into glial fibrillary acidic protein (GFAP)-positive astrocytes, greatly increased the sensitivity to NaHS. In contrast, epidermal growth factor (EGF), transforming growth factor-α (TGF-α), dibutyryl cyclic AMP (db cAMP). and interleukin-1β (IL-1β) induced the conversion to reactive astrocytes with diminished sensitivity to NaHS. This suppressive effect of EGF on the sensitivity to NaHS was inhibited by cycloheximide, indicating that *de novo* protein synthesis was required for the suppression of H_2S sensitivity. *Antioxid. Redox Signal.* 8, 257–269.

INTRODUCTION

It has been demonstrated that hydrogen sulfide (H_2S) is a synaptic modulator in the brain (4, 25). H_2S can be produced by cystathionine β-synthase (CBS) (11). H_2S enhances the induction of hippocampal long-term potentiation (LTP), a synaptic model of learning and memory, by potentiating the responses of neurons to *N*-methyl-D-aspartate (1). We recently demonstrated that H_2S increases intracellular concentrations of Ca^{2+} in astrocytes, a subtype of glia, and that this response propagates to the neighboring astrocytes (39). Astrocytes, which express functional channels and receptors for many neurotransmitters, surround the synapse and are key modulators of neuronal activity. Astrocytes and neurons may communicate with each other in both directions (13, 41). A synapse that is regulated by both neurons and surrounding glia has been called a tripartite synapse (3), and H_2S may be involved in this type of synaptic regulation (39).

Astrocytes, which are generated from multipotent neural stem cells (31), are able to proliferate, and gliogenesis continues in the developing brain (16). The fate decision between neurogenesis and gliogenesis is regulated by various mediators, such as growth factors and cytokines. Leukemia inhibitory factor (LIF) causes both multipotent neural stem cells and progenitors of astrocytes to differentiate into glial fibrillary acidic protein (GFAP)-positive astrocytes in culture (56). Embryonic brains of mice deficient in LIF receptors contain few GFAP-positive astrocytes (26, 54), and mice deficient in LIF have a significant decrease in hippocampal astrocytes (9), suggesting that LIF may also induce differentiation into astrocytes *in vivo*.

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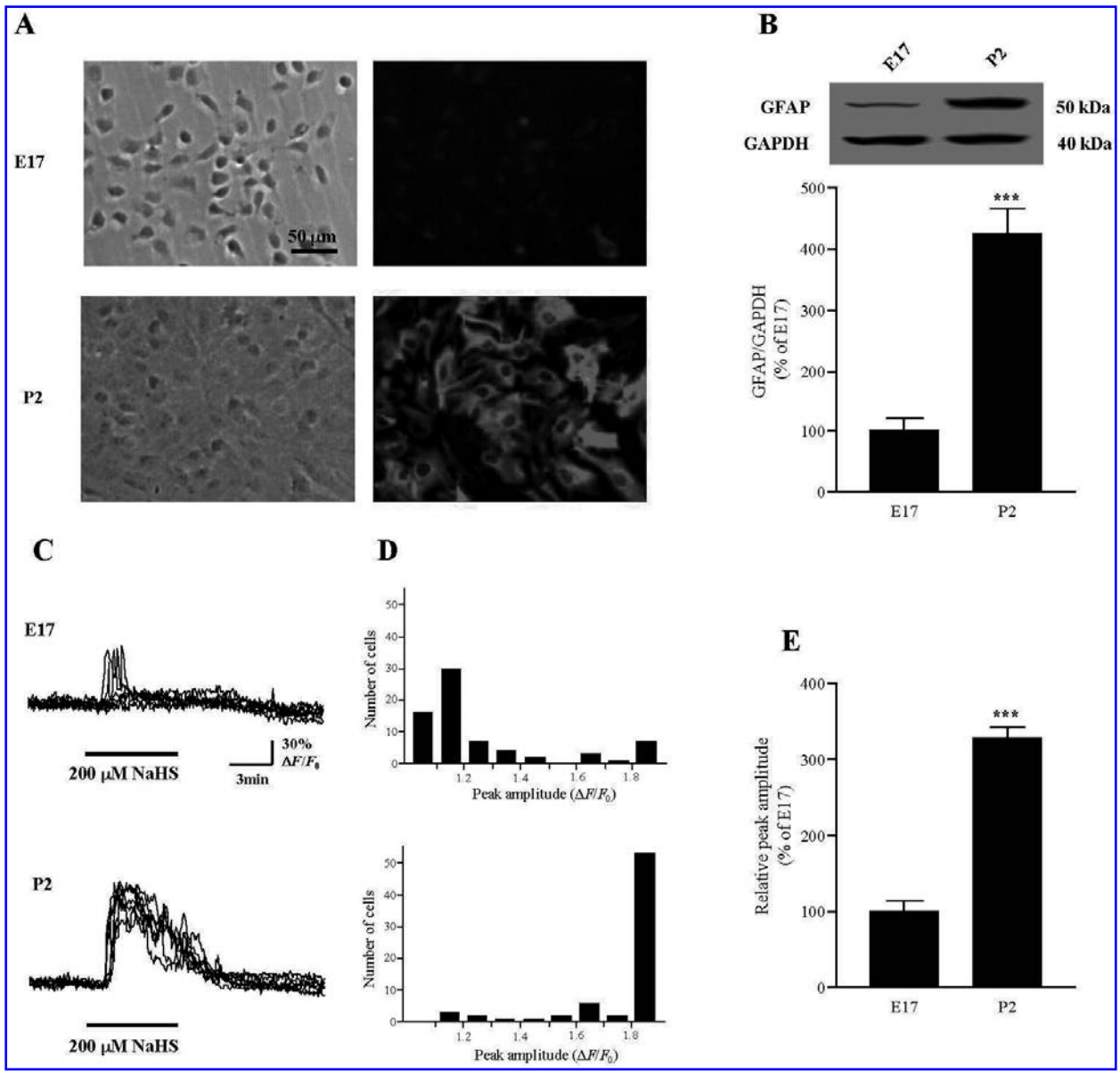


FIG. 1. Differentiated astrocytes acquire sensitivity to H_2S. (**A**) Morphology and immunoreactivity for GFAP of 6-day-old cells prepared from E17 and P2 rats. The left panels show phase-contrast micrographs. The right panels show the same fields stained with an antibody against GFAP. Note that cells prepared from E17 rats were round and were slightly stained for GFAP, whereas most cells from P2 were flat and GFAP positive. (**B**) Western blot analysis of GFAP in 6-day-old cells prepared from E17 and P2 rats. Western blot analysis of GFAP and GAPDH (*top*) and the levels of GFAP normalized to GAPDH were expressed as a percentage of the level at E17 taken as 100% (*bottom*). GFAP normalized to GAPDH is shown as the value in cells from E17 rats taken as 100%. Data are presented as the mean ± SEM (***$p < 0.001$ by Student's *t* test). (**C**) Calcium imaging of responses to H_2S of cells prepared from E17 and P2 rats. Representative responses of seven individual 6-day-old cells to 200 μ*M* NaHS were superimposed. Cells obtained from E17 rats responded slightly to NaHS, whereas most cells from P2 rats responded robustly to NaHS. (**D**) Distribution of cells with different peak amplitudes of responses to H_2S. Seventy individual cells were sorted according to their peak amplitudes. Most cells prepared at E17 had a peak amplitude ($\Delta F/F_0$) of ≤1.2, whereas 76% of cells from P2 showed a peak amplitude >1.8. (**E**) The relative peak amplitudes of responses of cells prepared from E17 and P2 rats. Data are presented as the mean ± SEM of 70 cells from the same cultures shown in **D**. ***$p < 0.001$ by Student's *t* test.

In the adult brain, proliferation-competent astrocytes can participate in wound healing as glial scar formation or reactive gliosis in response to insults, including infarct lesions and ischemic injury (20, 28, 43). Astrocytes in the glial scar undergo a profound phenotypic change, and the resultant reactive cells are central regulators of the inflammatory response in the central nervous system (CNS) (10). Reactive astrocytes are characterized by increased process extension and the upregulated expression of GFAP (47, 51). Transforming growth factor-α (TGF-α) is a protein of the epidermal growth

factor (EGF) family and shares the same receptor with EGF (29). The observation that the synthesis of TGF-α and EGF receptor is increased in reactive astrocytes (23) in conjunction with the finding with TGF-α–inducible transgenic mice in which reactive astrocytes are increased by the induction of TGF-α, suggests that enhanced TGF-α synthesis is sufficient to induce reactive astrocytes (49). Increases in intracellular cAMP also induce reactive astrocytes and upregulate the level of GFAP (15, 19). Finally, an inflammatory mediator, interleukin-1β (IL-1β), which is produced by microglia and contributes to scar formation, is critical for the induction of reactive astrocytes (18, 30).

We previously showed that the sensitivity of astrocytes to H_2S changes as a function of time after the preparation of cultures (39). How the sensitivity of astrocytes to H_2S is regulated is, however, not well understood. The present study focused on how the differentiation of astrocytes alters their sensitivity to H_2S. LIF enhances the differentiation of progenitors into GFAP-positive astrocytes and increases the sensitivity to NaHS. In contrast, TGF-α, EGF, dibutyryl cyclic AMP (db cAMP), and IL-1β, which upregulate the expression of GFAP and induce reactive astrocytes, all decrease the sensitivity to H_2S. Therefore, the sensitivity of astrocytes to H_2S may depend on their state of differentiation. The sensitivity of differentiated astrocytes is lost on their transformation into reactive astrocytes.

MATERIALS AND METHODS

Chemicals

Sodium hydrosulfide hydrate (NaHS), EGF, TGF-α, db cAMP, L-glutamic acid, adenosine 5′-triphosphate (ATP), and mouse anti-GFAP monoclonal antibody (clone GA-5, Cy3 conjugated) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.); LIF, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody from Chemicon (Temecula, CA, U.S.A.); IL-1β from Wako (Osaka, Japan); cycloheximide (CHX) from Nacalai Tesque (Kyoto, Japan); and peroxidase-conjugated anti-mouse IgG antibody from Jackson ImmunoResearch (West Grove, PA, U.S.A.).

Cell cultures

Primary cultures were prepared from Sprague–Dawley rats at embryonic day 17 (E17) or postnatal day 2 (P2). Cultures prepared from P2 rats were used only for the experiments of which the data are shown in Fig. 1. The cerebral cortex was excised, and the meninges were dissected in L15 medium (Invitrogen, Carlsbad, CA, U.S.A.). The tissue was chopped and digested with 0.25% trypsin (Sigma-Aldrich) and 0.1% DNase I (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free PBS for 15 min at 37°C. After mechanical dissociation, cells were plated at a density of 1,600 cells/mm^2 onto poly-D-lysine–coated 35-mm dishes (BD Biosciences, San Jose, CA, U.S.A.). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS; Sigma-Aldrich), 5% horse serum (HS; Invitrogen), and penicillin/streptomycin (P/S; 50 U/ml; Sigma-Aldrich) at 37°C in 10% CO_2. For the induction of differentiation by LIF, the medium was changed to DMEM supplemented with 0.5% FBS, 0.5% HS, and P/S containing 40 ng/ml LIF, and 3-day-old cells were incubated for 7 days. For the induction of reactive astrocytes, the medium was changed to DMEM supplemented with 5% FBS, 5% HS, and P/S containing EGF (40 ng/ml), TGF-α (40 ng/ml), db cAMP (1 m*M*), and IL-1β (20 ng/ml), and 24-day-old cells were incubated for 1 to 3 days. In some cases, CHX (1 μ*M*) was added to 24-day-old astrocytes in the presence or absence of EGF (40 ng/ml), and cells were incubated for 2 days in DMEM supplemented with 5% FBS, 5% HS, and P/S.

Calcium imaging

Cells were washed once with basal salt solution (BSS) consisting of 130 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 5.5 m*M* glucose, and 10 m*M* Hepes-NaOH (pH 7.3), and loaded with 1 μ*M* Calcium Green-1/acetoxymethyl ester (Molecular Probes, Eugene, OR) in BSS for 40 min at 37°C. After washing, cells were incubated at room temperature (20 to 24°C) for 20 min in BSS, and then perfused at a rate of 2 ml/min at room temperature. Calcium imaging was performed by using an upright microscope (DM LFS; Leica, Heidelberg, Germany) with a ×20 water– immersion objective (0.5 NA; Leica) and CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). Fluorescent images with 147-ms exposure were acquired by using ARGUS HiSCA (Hamamatsu Photonics). A sequence of images was acquired at 4-s intervals and 4 × 4 binning. Changes in calcium concentrations were monitored as changes in the fluorescence intensity (F) relative to the control images (F_0) that were acquired before stimulation. NaHS was used as a source of H_2S. NaHS dissociates to Na^+ and HS^- in solution, and then HS^- associates with H^+ and produces H_2S. It does not matter whether the H_2S solution is prepared by bubbling H_2S gas or by dissolving NaHS. In physiologic saline, approximately one third of the H_2S exists as the undissociated form (H_2S), and the remaining two thirds exists as HS^- at equilibrium with H_2S (50). The use of NaHS enables us to define the concentrations of H_2S in solution more accurately and reproducibly than by bubbling H_2S gas. The influence of less than 1 m*M* sodium ion on the experiments is negligible, because BSS contains 130 m*M* sodium ion. NaHS at concentrations used in the present study does not change the pH of BSS. For these reasons, NaHS has been widely used for studies of H_2S (6, 27, 55). The solutions containing NaHS were prepared immediately before use from a 2 *M* NaHS water solution, which was stocked in 1.5-ml Eppendorf tube filled with N_2 gas and stored at −80°C.

Immunocytochemistry

After washing with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 (Wako) for 5 min. Cells were incubated with Cy3-conjugated anti-GFAP monoclonal antibody (diluted 1:2,000) in PBS containing 2% bovine serum albumin (BSA) for 1 h in the dark. After washing with PBS, air-dried cells were covered with a glass coverslip and

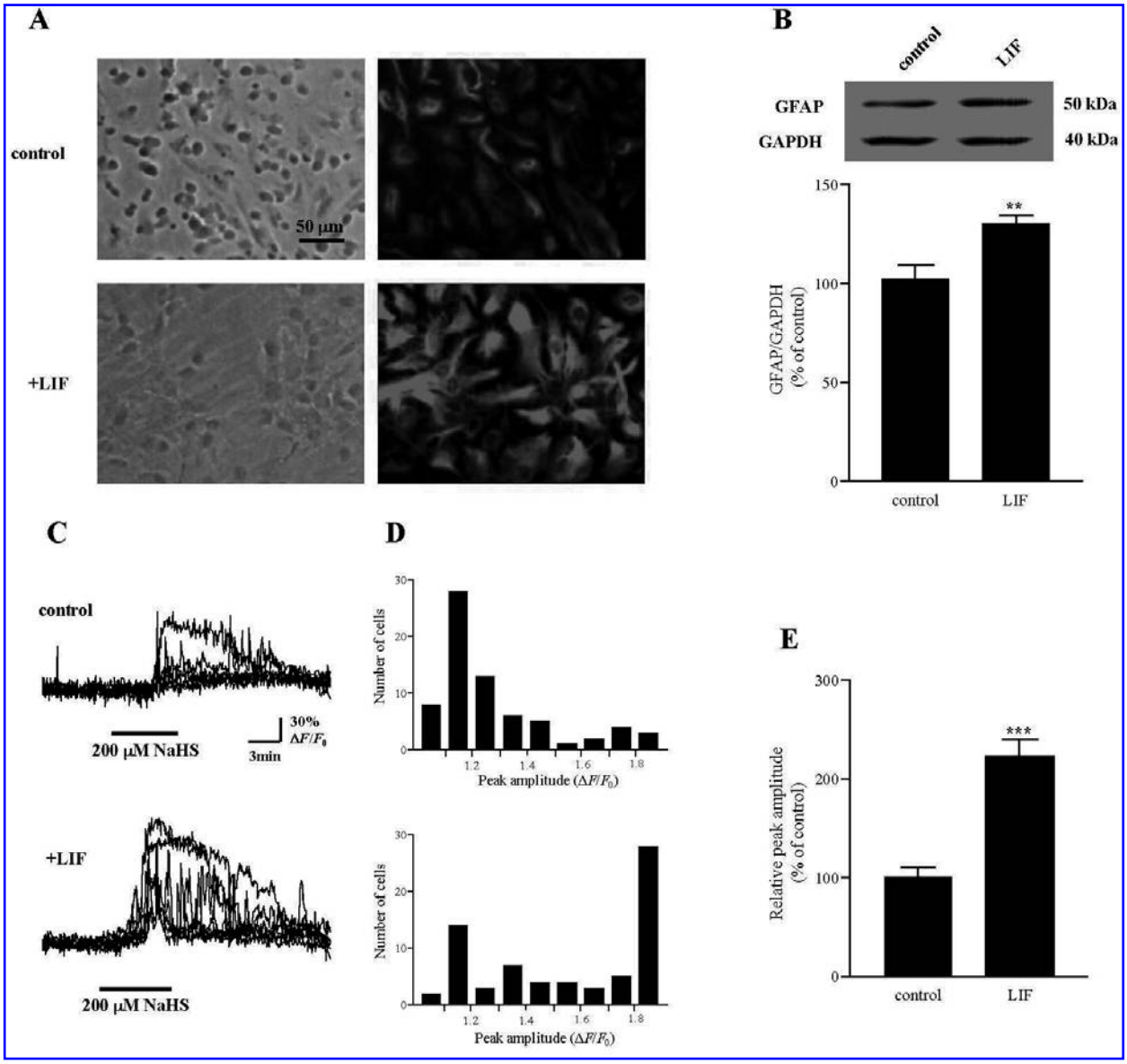


FIG. 2. LIF induces the differentiation of astrocytes that are sensitive to H_2S. (**A**) Morphology and immunoreactivity of GFAP in LIF-treated cells. The left panels show phase-contrast fields. The right panels show the same fields with fluorescent anti-GFAP. Note the greater numbers of GFAP-positive cells in LIF-treated cultures than in control cultures. (**B**) Western blot analysis of GFAP in LIF-treated cells. Western blot analysis of GFAP and GAPDH (*top*) and the levels of GFAP normalized to GAPDH were expressed as a percentage of the control (*bottom*). Data are presented as the mean ± SEM. $^{**}p < 0.01$ by Student's *t* test. (**C**) Calcium imaging of responses to H_2S of astrocytes treated with LIF. Representative responses of seven individual 10-day-old cells prepared from E17 rats to 200 μ*M* NaHS were superimposed. (**D**) Distribution of cells with different peak amplitudes of responses to H_2S. Seventy individual cells were sorted according to their peak amplitudes. (**E**) The relative peak amplitudes of Ca^{2+} responses of cells exposed to LIF. The relative peak amplitudes of responses of LIF-treated cells to the peak amplitude of control cells taken as 100% are shown. Data are presented as the mean ± SEM of 70 cells by using the same series of cells as shown in **D**. $^{***}p < 0.001$ by Student's *t* test.

aqueous mounting medium PERMAFLUOR (Beckman Coulter, Fullerton, CA). Stained samples were observed and photographed with a Olympus IX70 (Olympus, Tokyo, Japan) equipped with a CCD camera (Olympus). All experiments were performed at room temperature.

Western blot analysis

After washing with ice-cold PBS, cells were scraped with a sample buffer containing 125 m*M* Tris HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 4% glycerol, 10% β-mer-

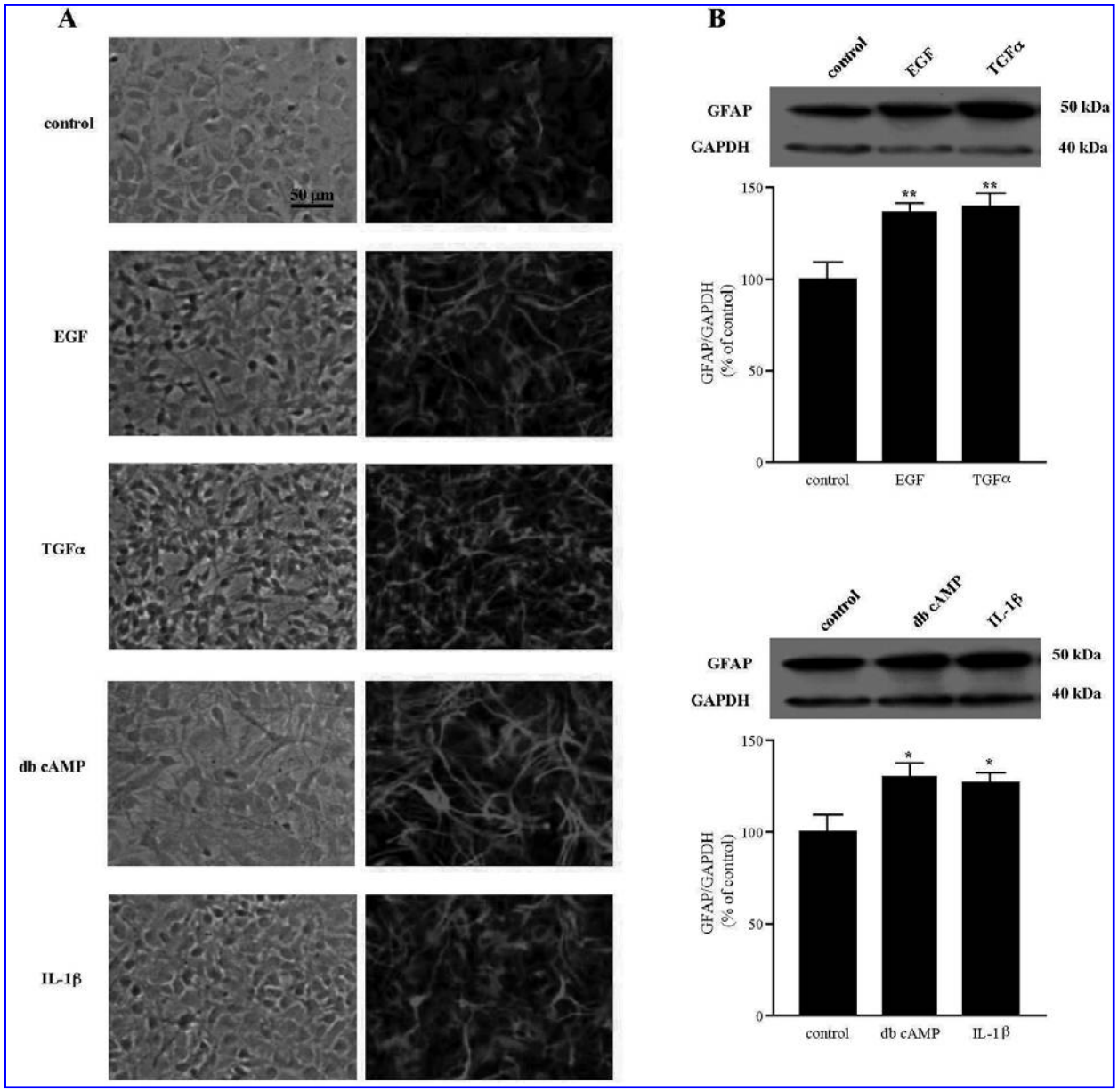


FIG. 3. Induction of reactive astrocytes by EGF, TGF-α, db cAMP, and IL-1β. (**A**) Morphology and immunoreactivity for GFAP of 26-day-old astrocytes prepared from E17 rats treated with EGF, TGF-α, db cAMP, or IL-1β for 2 days. Note that the astrocytes exposed to these factors exhibited a reactive phenotype characterized by elongated processes. (**B**) Western blot analysis of the expression of GFAP. Western blot analysis of GFAP and GAPDH (*top*) and the amounts of GFAP normalized to GAPDH were expressed as a percentage of the value of the control (*bottom*). Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ vs. control by Bonferroni's multiple comparison test after one-way ANOVA.

captoethanol, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and bromophenol blue. Cell lysates were sonicated and boiled for 5 min. Ten micrograms of protein per lane was applied and electrophoresed in a 12.5% SDS-polyacrylamide gel. The resultant proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking with Tween-containing Tris-buffered saline (TTBS; 10 m*M* Tris HCl, 150 m*M* NaCl, 0.05% Tween-20, pH 7.4) containing 5% nonfat skim milk overnight at 4°C, blotted membranes were incubated with a mouse anti-GFAP monoclonal antibody (diluted 1:2,000) and a mouse anti-GAPDH monoclonal antibody (diluted 1:600) in the blocking buffer for 2 h at room temperature. Membranes were then incubated with a peroxidase-conjugated anti-mouse secondary antibody (diluted 1:10,000), and immunoreactive bands were detected by using Immobilon Western (Millipore). The intensity of the bands was assessed by using a Fuji Las-1000 luminescent image analyzer (Fujifilm, Tokyo, Japan) with Image Gauge software (Fujifilm).

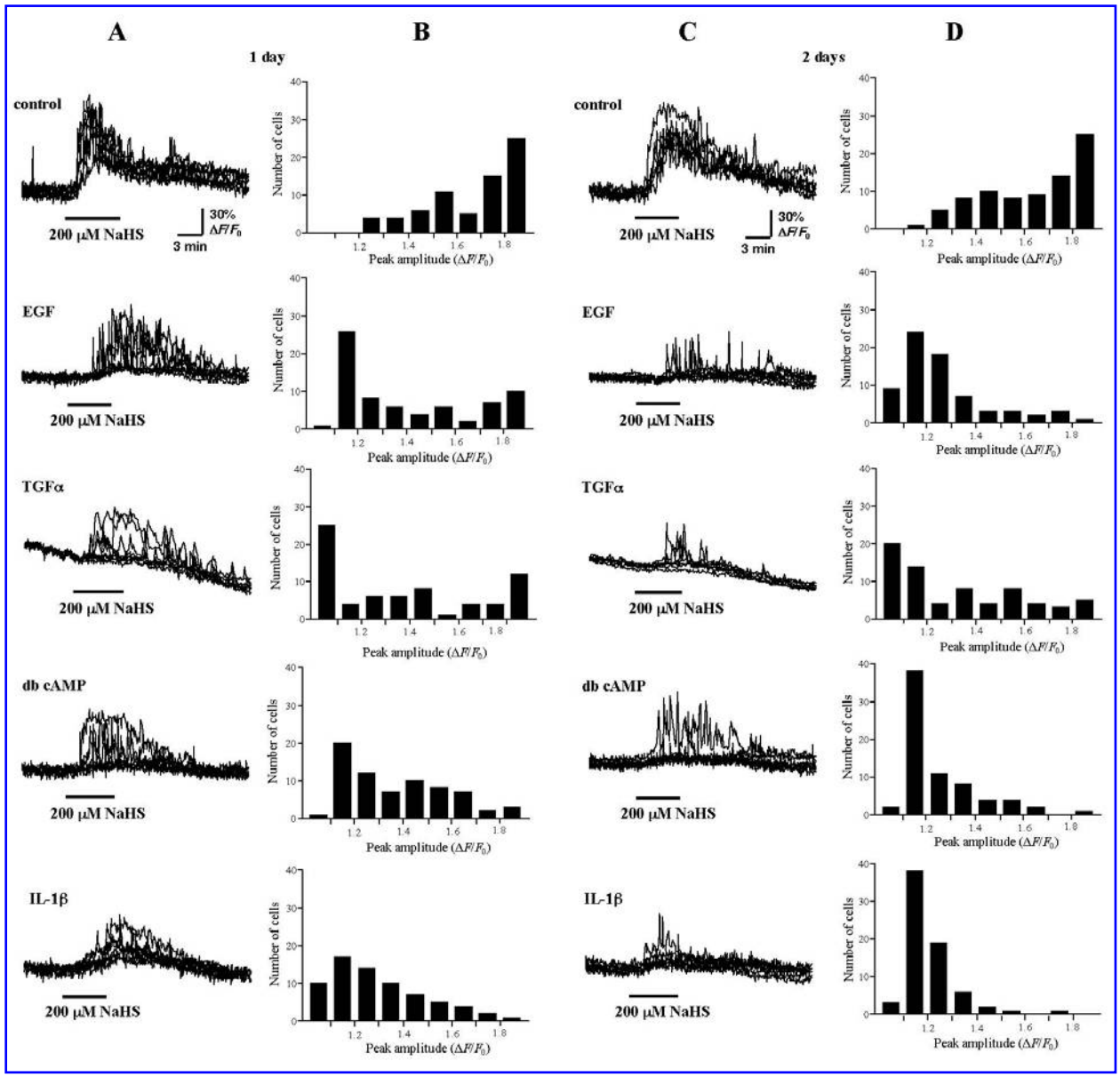


FIG. 4. The sensitivity of astrocytes to H_2S is reduced by EGF, TGF-α, db cAMP, and IL-1β. (**A, C, E**) Calcium imaging of responses to H_2S of 24-day-old astrocytes prepared from E17 rats treated with EGF, TGF-α, db cAMP, or IL-1β for 1 day (**A**), 2 days (**C**), and 3 days (**E**). Representative responses of seven individual cells to 200 μ*M* NaHS were superimposed. (**B, D, F**) Distribution of cells with the different peak amplitudes of responses to H_2S. Seventy individual cells were sorted according to their peak amplitudes. (**G**) The peak amplitudes of responses of cells exposed to EGF, TGF-α, db cAMP, or IL-1β. Data are presented as the mean ± SEM of 70 cells that are the same as shown in **B**, **D**, and **F**. ***$p < 0.001$ vs. control by Bonferroni's multiple comparison test after one-way ANOVA.

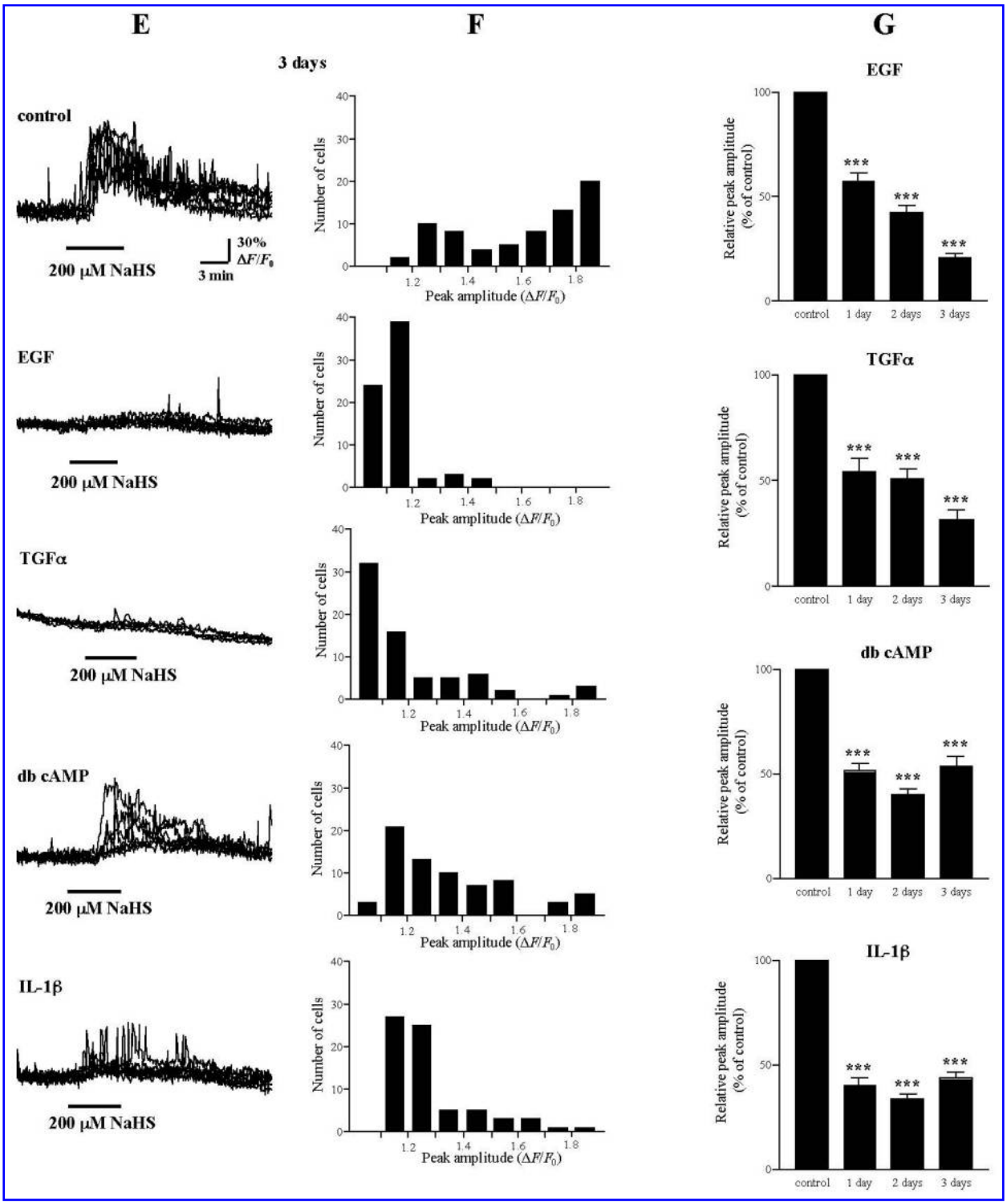
Statistics

All statistical analyses were performed by using Graphpad Prism software (San Diego, CA). Differences between two groups were analyzed with Student's *t* test. Differences among more than three groups were analyzed with one-way analysis of variance (ANOVA) with the *post hoc* Bonferroni test.

RESULTS

Differentiated astrocytes respond to H_2S

H_2S increases intracellular Ca^{2+} and induces Ca^{2+} waves in primary cultures of astrocytes. Responses to H_2S were rarely observed in the initial 6 days after starting the hippocampal cell cultures, but the cells responded to H_2S after 10 days of



FIGS. 4E–G.

culture (39). GFAP, which is a marker for astrocytes (7), is not detectable until E17, and GFAP-positive astrocytes are dominant only after 10 days of culture (2, 53). Taking these observations into account, it is possible that the sensitivity to H_2S depends on the differentiation of progenitors into astrocytes. To examine this possibility, the levels of GFAP and sensitivity to 200 μ*M* NaHS were compared between 6-day-old cultures prepared from E17 rats and those from P2 rats. Immunohistochemistry showed that cells obtained at E17 were round and slightly GFAP positive, whereas the majority

of cells at P2 were flat, non–process-bearing, and GFAP positive (Fig. 1A). Western blot analysis showed that the level of GFAP in cells at P2 was approximately 4 times greater than that at E17 (Fig. 1B). Most cells prepared at E17 responded only weakly to NaHS, having peak amplitude ($\Delta F/F_0$) between 1.1 and 1.2 (Fig. 1C and D). In contrast, cells prepared from P2 responded well to NaHS, with the majority of cells having peak amplitude >1.8 (Fig. 1C and D). Overall, cells prepared from P2 rats show approximately 3 times greater sensitivity to NaHS than did cells prepared from E17 embryos (Fig. 1E). Because cells that were obtained by trypsinizing the well-responding confluent cells and incubated for only 1 h responded well to NaHS, the possibility that confluent cells acquire sensitivity to H_2S was excluded (data not shown). These observations suggest that the sensitivity to H_2S may be acquired on the differentiation or maturation of precursor cells into astrocytes.

LIF induces the differentiation of precursor into astrocytes and increases their sensitivity to H_2S

Further to examine whether the sensitivity to H_2S depends on the differentiation into astrocytes, the responses of LIF-treated cells to H_2S were tested. Three days after the preparation from E17 cerebral cortex, 40 ng/ml LIF was applied to the cells, which were cultured for an additional 7 days. Cells treated with LIF were flat and GFAP positive, whereas cells cultured in the absence of LIF were round with little expression of GFAP (Fig. 2A). Western blot analysis showed that the level of GFAP was approximately 30% greater in LIF-treated cells than in those cultured in the absence of LIF (Fig. 2B). Forty percent of cells treated with LIF ($n = 70$) strongly responded to 200 μ*M* NaHS, having a peak amplitude ($\Delta F/F_0$) greater than 1.8, whereas 46% of cells cultured in the absence of LIF only weakly responded to NaHS, with a peak amplitude ($\Delta F/F_0$) of 1.2 (Fig. 2C and D). Cells treated with LIF are approximately twice as sensitive to NaHS as are control cells (Fig. 2E). These observations confirm that the sensitivity to H_2S depends on the differentiation of precursor cells into astrocytes.

The sensitivity to H_2S is diminished in reactive astrocytes

Because the calcium oscillation in astrocytes is modified when cells are transformed into reactive astrocytes (37, 38), it is possible that the sensitivity to H_2S may be changed in reactive astrocytes. Because EGF, TGF-α, db cAMP, and IL-1β are known to induce reactive astrocytes (15, 18, 23), the sensitivity of reactive astrocytes to H_2S was examined after exposing 24-day-old astrocyte cultures to these substances. When these astrocytes were exposed to EGF, TGF-α, db cAMP, or IL-1β for 2 days, 26-day-old astrocytes extended long and thin processes, showing the characteristics of reactive astrocytes, and these substances upregulated the levels of GFAP (Fig. 3A and B). Twenty-six-day-old astrocytes in the absence of EGF, TGF-α, db cAMP, or IL-1β were flat and polygonal, forming a monolayer (Fig. 3A).

One day after the application of EGF or TGF-α, the distribution of the peak amplitude of the responses to 200 μ*M* NaHS was shifted to lower amplitudes, and the effect of both growth factors lasted during at least 3 days of exposure (Fig. 4). The suppressive effects of EGF and TGF-α on the responses to NaHS were increased during up to 3 days of exposure, whereas those of db cAMP and IL-1β increased for only 2 days and then were slightly attenuated (Fig. 4G). These observations suggest that the sensitivity to H_2S is diminished when astrocytes are transformed into reactive astrocytes.

Because exposure to cAMP transiently increases the basal intracellular Ca^{2+} levels (5), it is possible that NaHS is not able to increase the intracellular Ca^{2+} levels greater than the elevated basal levels. To examine this possibility, responses to glutamate and ATP after 2-day exposure to EGF, TGF-α, db cAMP, or IL-1β was tested. Responses to NaHS were greatly suppressed by exposure to EGF, TGF-α, db cAMP, or IL-1β (Fig. 5B), whereas responses to glutamate and ATP were rather increased (Fig. 5C and D). These results exclude the possibility that suppression of responses to NaHS is due to the elevated basal Ca^{2+} levels by pretreatment with growth factors and cytokines.

Because EGF activates tyrosine kinase and phosphorylates tyrosine during a short period (34), it is possible that the suppressive effect of EGF on the sensitivity of astrocytes to NaHS is an acute effect of EGF rather than a longer-term effect. This possibility was examined by applying EGF to astrocytes 10 min before the application of NaHS. The acute application of EGF did not suppress the sensitivity of astrocytes to NaHS (Fig. 6), nor did EGF alone induce any calcium responses of astrocytes.

De novo protein synthesis is required for the suppressive effect of EGF on sensitivity to H_2S

Because the suppressive effect of EGF on the sensitivity of astrocytes to NaHS was caused by their transformation into reactive astrocytes (Fig. 4), it was possible that *de novo* protein synthesis was required for the effect of EGF. To test this possibility, the effect of EGF was examined in the presence of an inhibitor of protein synthesis, cycloheximide. The cotreatment with cycloheximide plus EGF for 2 days suppressed the transformation into reactive astrocytes but did not significantly change the sensitivity to NaHS (Fig. 7). The decreases of peak amplitude induced by EGF were completely abrogated in the presence of cycloheximide (Fig. 7B–D). Although cycloheximide alone slightly reduced the peak amplitude of the responses to 200 μ*M* NaHS, the morphologic changes of astrocytes were not induced by cycloheximide (Fig. 7A). These observations suggest that *de novo* protein synthesis is required for the suppression of the sensitivity to NaHS induced by EGF and support the argument that EGF is acting as a trophic factor for glial activation.

DISCUSSION

The present study showed that H_2S induces an increase in the intracellular concentration of Ca^{2+} in differentiated astrocytes and that this response to H_2S is diminished in reactive astrocytes. The sensitivity of astrocytes to H_2S may therefore be regulated by glial differentiation and depend on the reactive state of the astrocytes.

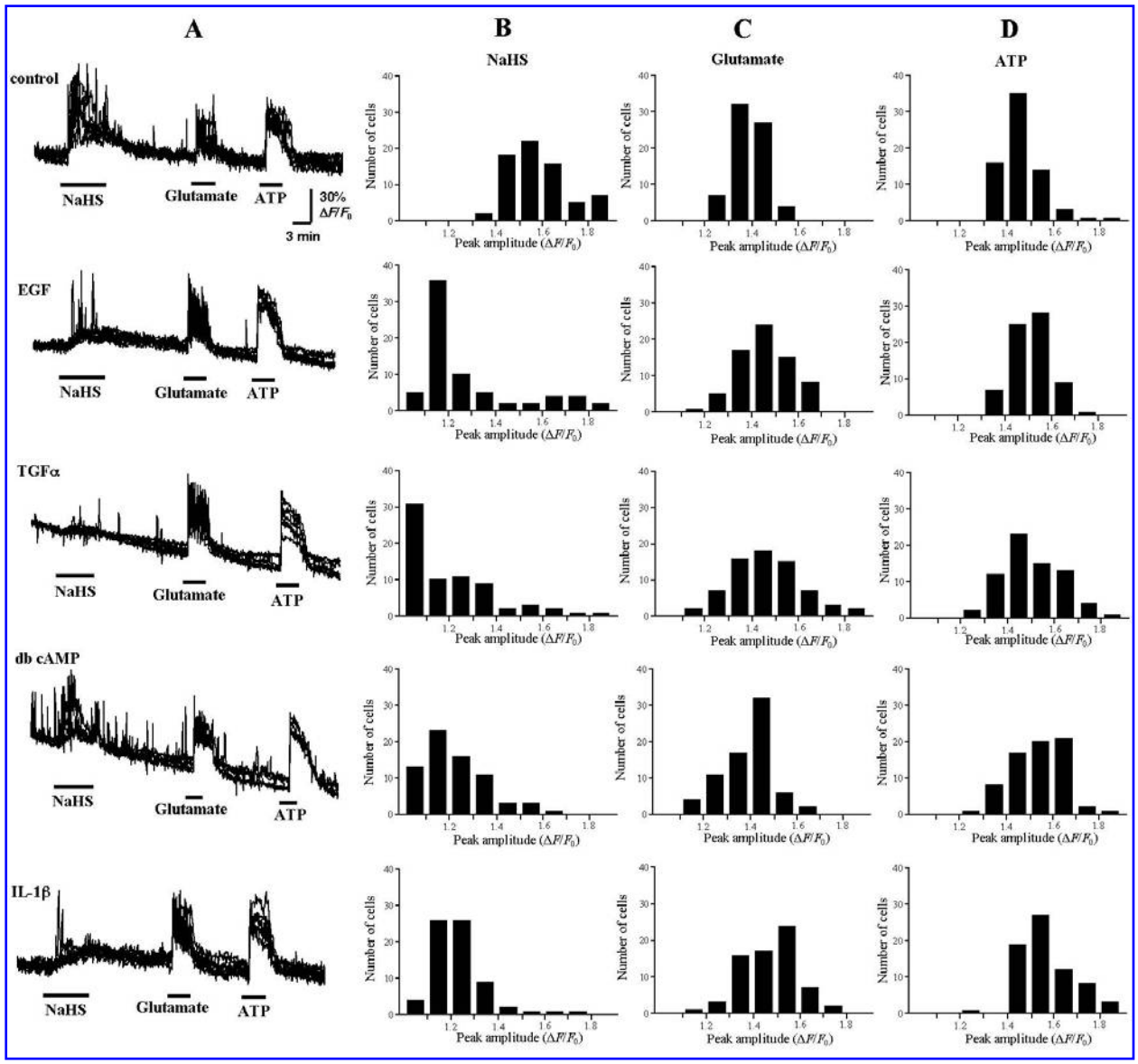


FIG. 5. Comparison of responses to H_2S with those to glutamate and ATP of astrocytes pretreated with EGF, TGF-α, db cAMP, or IL-1β. (**A**) Calcium imaging of responses to NaHS, glutamate, and ATP of 26-day-old astrocytes prepared at E17 rats pretreated with 40 ng/ml EGF, 40 ng/ml TGF-α, 1 m*M* db cAMP, or 20 ng/ml IL-1β. Representative responses to 200 μ*M* NaHS, 100 μ*M* glutamate, and 50 μ*M* ATP of seven individual cells were superimposed. (**B–D**) Distribution of cells with the different peak amplitudes of responses to NaHS (**B**), glutamate (**C**), and ATP (**D**). Seventy individual cells were sorted according to their peak amplitudes. Note that responses to glutamate and ATP were rather augmented by 2-day exposure to EGF, TGF-α, db cAMP, or IL-1β.

H_2S does not increase intracellular Ca^{2+} in primary cultures of neurons (39) or microglia (data not shown), indicating that the response to H_2S is specific for astrocytes. Glia and neurons are generated from common precursor cells in the developing brain. Progenitor cells for neurons, astrocytes, and oligodendrocytes exist in the rat cerebral cortex at embryonic days 15 to 16 (33). GFAP-positive cells in 6-day-old cultures prepared from newborn rats respond to NaHS, whereas cells in 6-day-old cultures prepared at E17 express little GFAP and only weakly respond to NaHS (Fig. 1). Our previous finding that the changes in the sensitivity of cells to H_2S depend on the number of days in culture may reflect the acquisition of the sensitivity by differentiation into astrocytes.

LIF induces differentiation of GFAP-negative progenitors into GFAP-positive astrocytes (56), which acquire sensitivity to H_2S (Fig. 2). This observation confirms that only differentiated astrocytes respond to H_2S. LIF stimulates the differentiation of both astrocyte progenitors (40) and oligodendrocyte-type 2 astrocyte (O-2A) progenitors (24) into type-1 and type-2 astrocytes, respectively. Our primary cultures of astro-

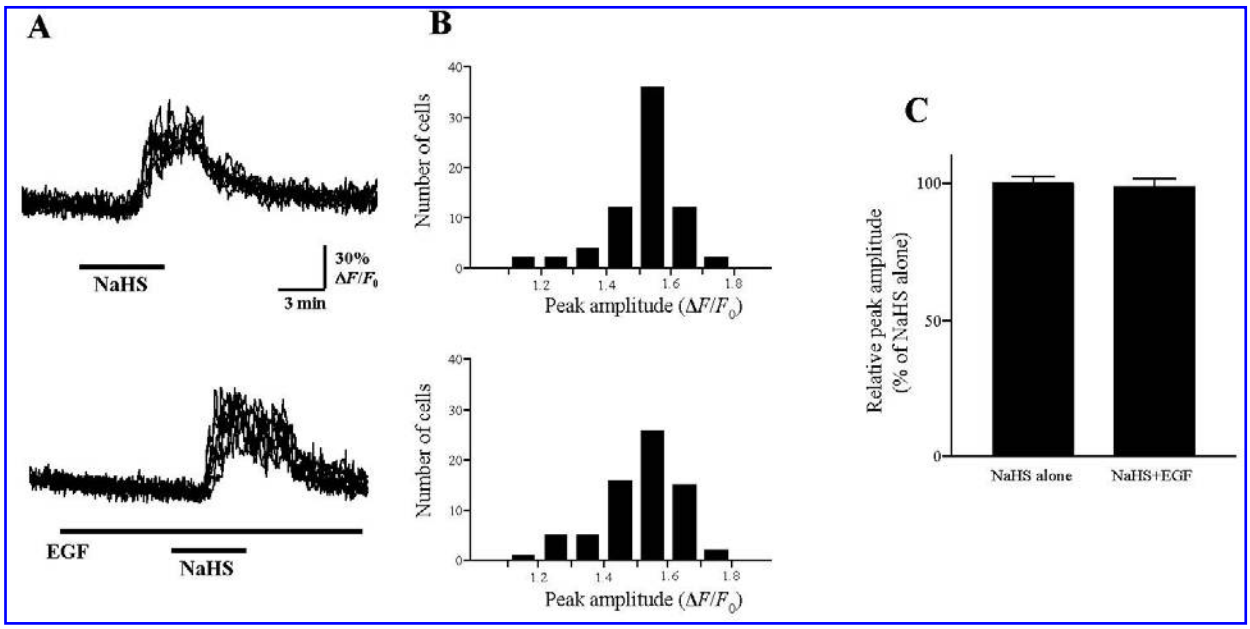


FIG. 6. EGF has no effect on acute responses to H_2S. (**A**) Calcium imaging of responses to H_2S in the presence of EGF. Representative responses of seven individual cells were superimposed. The 33-day-old astrocytes obtained from E17 rats were preincubated with 40 ng/ml EGF for 10 min, and 200 μ*M* NaHS was applied. (**B**) Distribution of cells with their different peak amplitudes of responses to H_2S. Seventy individual cells were sorted according to their peak amplitudes. Note that the distribution of the peak amplitudes of responses to NaHS alone was similar to that of the responses to NaHS in the presence of EGF. (**C**) The peak amplitude of Ca^{2+} responses of cells treated with EGF and NaHS. Data are presented as the mean ± SEM of 70 cells by using the same series of cells as shown in **B**, and significance was assessed by using Student's *t* test.

cytes, which respond to H_2S, show the morphologic characteristics of type-1 astrocytes (*i.e.*, they were flat and non–process-bearing) (Fig. 2).

Astrocytes transform into reactive astrocytes in response to brain injuries, including trauma, neurodegenerative diseases, and viral infections (43). The levels of many cytokines and growth factors are changed after brain injury. EGF is secreted by microglia (8, 42), and TGF-α is synthesized by injured neurons (32). The expression of EGF receptors is upregulated in reactive astrocytes after brain injury (23), and IL-1β is produced by reactive astrocytes and microglia (17, 30). In culture models of reactive astrocytes, EGF, TGF-α, db cAMP, and IL-1β induce reactive astrocytes (15, 18, 23). The present study shows that the treatment of astrocytes with EGF, TGF-α, db cAMP, or IL-1β for 1–3 days induces reactive astrocytes with diminished sensitivity to NaHS. Our previous study showed that H_2S may be involved in the signal transmission between neurons and astrocytes by increasing intracellular concentrations of Ca^{2+} and inducing calcium waves in astrocytes (39). It is possible that astrocytes may exchange signals with neurons under normal conditions but that they stop responding to H_2S when they are transformed into reactive astrocytes after brain injury.

EGF immediately induces the release of Ca^{2+} from intracellular Ca^{2+} stores by activating phospholipase C, which produces inositol 1,4,5-trisphosphate and increases the influx of extracellular Ca^{2+} through voltage-independent Ca^{2+}-specific channels (21, 36, 45, 48). In addition to the short-term effect of EGF alone, EGF evokes Ca^{2+} responses only after bradykinin stimulation in Swiss 3T3 mouse fibroblasts (44). EGF also enhances the increase in intracellular Ca^{2+} by ATP in cerebellar astrocytes (14). The present results, however, show that EGF did not rapidly suppress the sensitivity of astrocytes to NaHS (Fig. 6), indicating that suppression of the responses of astrocytes to NaHS does not represent a short-term effect of EGF, but is rather a long-term change that requires *de novo* protein synthesis (Fig. 7). Considering our previous finding that H_2S increases the influx of Ca^{2+} and causes the release of Ca^{2+} from intracellular stores (39), it is possible that EGF may change the expression of molecules that are involved in the regulation of Ca^{2+} channels and their signal transduction. Other examples in which long-lasting exposure to EGF is required to see a biologic effect include reducing the increase in intracellular Ca^{2+} caused by 30–50 m*M* KCl in GH_3 rat pituitary cells and by hydrogen peroxide in corneal endothelial cells (22, 35).

Characteristics of reactive astrocytes are the process-bearing stellate morphology as well as the elevation of GFAP levels (47, 51). Stellation and the elevation of GFAP levels, however, separately occur. Cultures of astrocytes prepared from GFAP-deficient mice are still capable of exhibiting stellate morphology, indicating that GFAP is not essential for stellation (46). The stellation is caused by exposure to db cAMP for a few hours, and the rapid morphologic changes are accompanied by redistribution but not by quantitative changes of cytoskeletal proteins, including GFAP (52). *De novo* synthesis of GFAP starts about 24 h after the treatment with db cAMP (12). Suppression of responses to NaHS by db cAMP is apparent after 1-day exposure when *de novo* protein synthesis occurs and the levels of GFAP are significantly increased (Fig. 4).

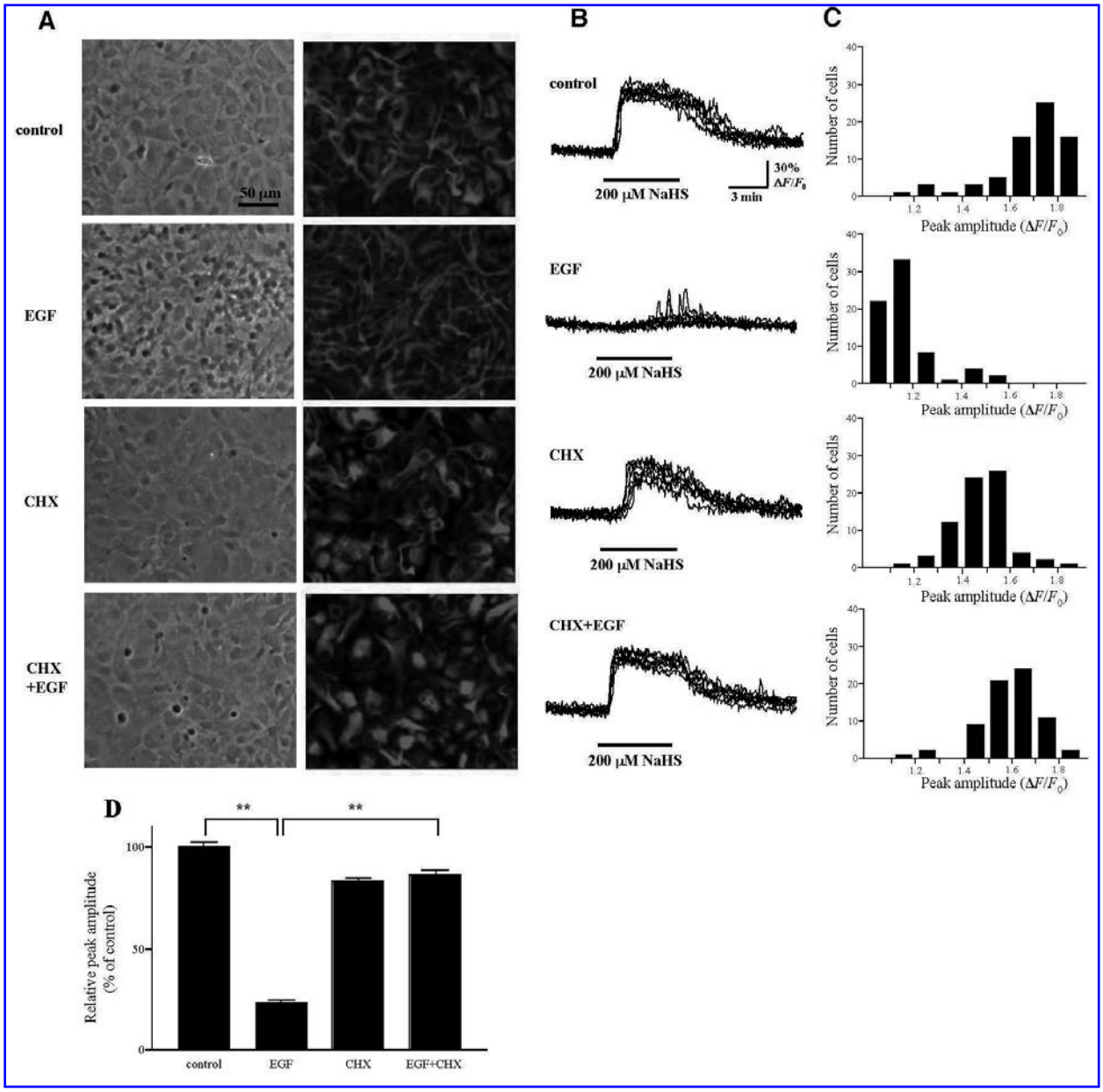


FIG. 7. ***De novo*** **protein synthesis is required for suppressive effect of EGF on the sensitivity to H_2S.** (**A**) Morphology and immunoreactivity of GFAP in 26-day-old astrocytes treated with EGF and cycloheximide (CHX) for 2 days. The left panels show phase contrast. The right panels show fluorescent fields of the same cells stained with an antibody against GFAP. Note that the morphologic changes of astrocytes were induced only by EGF alone but not by CHX in the presence or absence of EGF. (**B**) Calcium imaging of the responses to H_2S of cells treated with EGF and CHX. Representative responses of seven individual 26-day-old cells prepared from E17 rats to 200 μ*M* NaHS were superimposed. (**C**) Distribution of the peak amplitudes of responses to H_2S. Seventy individual cells were sorted according to their peak amplitudes. (**D**) The peak amplitudes of the Ca^{2+} responses of cells exposed to EGF and CHX. Data are presented as the mean ± SEM of 70 cells by using the same series of cells as shown in **C**. $^{**}p < 0.01$ vs. EGF by Bonferroni's multiple comparison test after one-way ANOVA.

In conclusion, astrocytes acquire sensitivity to H_2S by differentiation, and their transformation into reactive astrocytes in response to growth factors and cytokines diminishes their sensitivity to H_2S.

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ABBREVIATIONS

ANOVA, analysis of variance; BSA, bovine serum albumin; BSS, basal salt solution; CBS, cystathionine β-synthase; CHX, cycloheximide; CNS, central nervous system; db cAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; E17, embryonic day 17; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HS, horse serum; H_2S, hydrogen sulfide; IL-1β, interleukin-1β; LIF, leukemia inhibitory factor; LTP, long-term potentiation; NaHS, sodium hydrosulfide hydrate; O-2A, oligodendrocyte-type 2 astrocyte; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; P2, postnatal day 2; SDS, sodium dodecylsulfate; TGF-α, transforming growth factor-α; TTBS, Tween-containing Tris-buffered-saline.

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