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Original Research Communication

Hydrogen Sulfide Raises Cytosolic Calcium in Neurons Through Activation of L-Type Ca^{2+} Channels

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ABSTRACT

Hydrogen sulfide (H_2S) concentration can be maintained in cell cultures within the range reported for rat brain by repetitive pulses of sodium hydrogen sulfide. Less than 2 h exposure to H_2S concentrations within 50 and 120 μM (*i.e.*, within the upper segment of the reported physiological range of H_2S in rat brain), produces a large shift of the intracellular calcium homeostasis in cerebellar granule neurons (CGN) in culture, leading to a large and sustained increase of cytosolic calcium concentration. Only 1 h exposure to H_2S concentrations within 100 and 300 μM raises intracellular calcium to the neurotoxic range, with nearly 50% cell death after 2 h. L-type Ca^{2+} channels antagonists nimodipine and nifedipine block both the H_2S-induced rise of cytosolic calcium and cell death. The *N*-methyl-D-aspartate receptor antagonists (+)-MK-801 and DL-2-amino-5-phosphonovaleric acid afforded a nearly complete protection against H_2S-induced CGN death and largely attenuated the rise of cytosolic calcium. Thus, H_2S-induced rise of cytosolic calcium eventually reaches the neurotoxic cytosolic calcium range, leading to glutamate-induced excitotoxic CGN death. The authors conclude that H_2S is a major modulator of calcium homeostasis in neurons as it induces activation of Ca^{2+} entry through L-type Ca^{2+} channels, and thereby of neuronal activity. *Antioxid. Redox Signal.* 10, 31–41.

INTRODUCTION

THE HIGH ENDOGENOUS LEVELS of hydrogen sulfide (H_2S), 10–160 μM, measured in human, rat, and bovine brains, have led to the suggestion that H_2S can function as an endogenous neuromodulator (1, 5, 14, 17). It has been reported that physiological concentrations of H_2S enhance NMDA receptor-mediated responses and the induction of hippocampal long-term synaptic potentiation (1, 16). In addition, it has been recently shown that H_2S is a mediator of cerebral ischemic damage (28), and is involved in the development of recurrent febrile seizures (20). These effects of H_2S are in contrast with its potential to protect neurons from oxidative stress (18, 19), and to act as an inhibitor of peroxynitrite-mediated processes (36). As shown by Kimura *et al.* (19), in addition to increasing glutathione levels, neuroprotection by H_2S also implies K_{ATP} and Cl^- channel activation. Although occupational exposure to H_2S (up to 10 ppm) is neither a reproductive toxicant nor a behavioral developmental neurotoxicant in rats (12), exposures to higher H_2S concentrations have been shown to leave persistent neurological sequelae in humans (33) and demyelination of nerve fibers (34). However, the molecular mechanisms underlying neuronal degeneration remain unclear. Therefore, the possibility that the neuromodulator range is relatively close to the neurotoxic range of H_2S concentrations deserve to be experimentally assessed. In single H_2S pulse experiments done to cells in culture dishes, hydrogen sulfide is expected to decrease rapidly by diffusion to the surrounding atmosphere. Therefore, an approach based in the application of repetitive hydrogen sulfide pulses rather than a single pulse approach is needed to simulate the effects of chronic exposure of cells to hydrogen sulfide.

Cytosolic calcium plays a major role in synaptic activity and overall neuronal excitability, such that neuronal survival or

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death is strongly dependent on the maintenance of cytosolic calcium homeostasis within a narrow concentration window in neurons (13). Production of H_2S in brain is mainly supported by cystathionine β-synthase (3), highly expressed in hippocampus and cerebellum (1), which catalyzes the condensation of L-cysteine and homocysteine to produce cystathionine and H_2S (8). In spite of the increasing acceptance of hydrogen sulfide as a neuromodulator and of the reported alterations of cytosolic calcium homeostasis in astrocytes and microglial cells (22, 26), there is no report on the putative alterations of cytosolic calcium homeostasis in neurons. We suspected that this could be due to the fact that H_2S rapidly decays in the culture media after the application of a single pulse to a standard cell culture dish (half-time <10 min).

Recently, it has been noticed that the treatment of astrocytes with a single bolus of physiological concentrations of H_2S increases intracellular Ca^{2+} concentrations, and induced Ca^{2+} waves that propagated to neighbor astrocytes through a direct interaction with Ca^{2+} channels (26). However, these authors suggested further studies in order to identify the specific type of Ca^{2+} channel targeted by H_2S. As L-type voltage-operated Ca^{2+}-channels display a very high sensitivity to oxidative stress in cerebellar granule neurons (15), these neurons are a good model system to test the hypothesis that L-type Ca^{2+} channels can be a highly sensitive target for H_2S modulation. Mature CGN can be maintained without significant loss of cell viability for >15 days in culture in 25 m*M* K^+ (24, 25), and express several types of voltage-operated Ca^{2+} channels, although several studies have shown that in mature CGN grown in 25 m*M* KCl medium L-type Ca^{2+} channels are mainly responsible for the sustained rise in $[Ca^{2+}]_i$ which promotes neuronal survival (13, 23, 32).

This paper is focused on the alteration of cytosolic calcium homeostasis in CGN by H_2S concentrations maintained close to the range reported for rat brain with repetitive NaSH pulses, and the results pointed out that exposure of cerebellar granule neurons in culture to these concentrations of H_2S for 1–2 h produced a large rise of cytosolic calcium, which is fully prevented by the L-type calcium channels blockers nimodipine and nifedipine, thus revealing a high sensitivity of the activity of these calcium channels to H_2S.

MATERIALS AND METHODS

Preparation of rat cerebellar granule neurons

Cultures of cerebellar granule neurons (CGN) were obtained from dissociated cerebella of 7-day-old Wistar rats, as described previously (15, 24, 25, 31). Cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 25 m*M* KCl on 35-mm dishes (Corning, NY) coated with poly-D-lysine, at a density of 2.5×10^6 cells/dish. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cytosine arabinofuranoside (10 μM) was added to fresh culture medium 48 h after plating to prevent replication of non-neuronal cells. Seven days after plating, the culture medium was replaced with the following serum-free DMEM:F12 medium (1:1) supplemented with 30 m*M* glucose, 5 μg/mL insulin, 0.1 mg/mL apo-transferrin, 20 n*M* progesterone, 50 U/mL penicillin, 50 μg/mL streptomycin, 0.1 mg/mL pyruvate, 2 m*M* L-glutamine, and containing 25 m*M* KCl. All experiments were performed using CGN at 8 days *in vitro*.

Estimation of viable cells was obtained by measuring the amount of colored formazan by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells, and was also assessed by trypan blue exclusion as described previously (15, 24, 25, 31). At least 500 neurons per plate were counted for the estimations done with the trypan blue exclusion method.

Protein concentration was determined by the method of Bradford (7), using the Bio-Rad (Hercules, CA) protein assay reagent and bovine serum albumin as standard.

Chemicals

APV, DMEM, DMEM:F12, EGTA, ethydium bromide, fetal bovine serum, L-glutamate dehydrogenase ($NADP^+$) from *Proteus* sp., apo-transferrin, HEPES, insulin, progesterone, penicillin, streptomycin, pyruvate, glutamine, cytosine arabinofuranoside, poli-D-lysine, MTT, ionomycin, NADH, $NADP^+$, nimodipine, nifedipine, NaSH, TES, Tris, and trypan blue were obtained from Sigma (St. Louis, MO). (+)-MK-801 was obtained from RBI (Natick, MA). Glutamate-pyruvate transaminase, oligo dT_{15}, and dithiothreitol were supplied by Roche Diagnostics GmBH (Mannheim, Germany). TriZol was supplied by Invitrogen (Paisley, UK). M-MLV reverse transcriptase has been purchased from Ambion (Austin, TX). Ribonuclease inhibitor was supplied by Fermentas International, Inc. (Ontario, Canada). Fura-2 acetoxymethyl ester, Pluronic-F127 and Br-A23187 were obtained from Molecular Probes (Eugene, OR). The primers for cystathionine β-synthase and cystathionine γ-lyase were produced and supplied by MWG-Biotech AG (Ebersberg, Germany). All other chemicals used were analytical grade reagents from Merck (Darmstadt, Germany) or Sigma.

Expression of the mRNAs of cystathionine β-synthase and cystathionine γ-lyase

The expression of the mRNAs of these enzymes was monitored by reverse transcription-PCR (RT-PCR). Total RNA was extracted from CGN in culture using TriZol reagent (Invitrogen), following the protocol indicated by the manufacturer. cDNA synthesis was performed using 1 μg of total RNA with M-MLV reverse transcriptase (Ambion) and oligo dT_{15} as primer. Briefly, 10 μL containing the RNA and oligo dT_{15} are heated 10 min at 65°C and then cooled at 4°C during 5 min. After mixing with 10 μL of a solution containing the retrotranscriptase (50 U), 2.5 m*M* desoxyribonucleotides (dNTPs), 20 m*M* dithiothreitol, and 10 U of ribonuclease inhibitor (Fermentas), the sample is subjected to the following cycle: 10 min at 25°C, 60 min at 42°C, and 15 min at 70°C.

The following primers were used for the PCR reaction of cystathionine β-synthase (Gen Bank NM012522): FW 5′-CACCTGACCGACACACTGGGC-3′; RV 5′-CTCACGGGCTGCCAGGAAGTTTAG-3′. Temperature and annealing time were 64°C and 10 s, respectively, and a total number of 30 cycles were run. The PCR product had a size of 171 bp, as expected with the sequence published in Gen Bank.

The following primers were used for the PCR reaction of cystathionine γ-lyase (Gen Bank AB052882): FW 5′-TCCGGATGGAGAAACACTTC-3′; RV5′-CTGCCTTTAAAGCTTGACC-3′. Temperature and annealing time were 57°C and 10 s, respectively, and a total number of 35 cycles were run. The PCR product had a size of 400 bp, as expected with the sequence published in Gen Bank.

PCR products were analyzed in 3% agarose gels pre-stained with ethydium bromide (0.2 μg/mL), and bands were viewed under ultra-violet excitation using a BioRad transilluminator (Fig. 1A).

Measurement of H_2S concentration in the culture medium

H_2S was measured using the colorimetric method described in Ref. 10. Briefly, 0.05 mL of culture medium is diluted to a total volume of 0.6 mL of 50 m*M* TES [2-{(2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino} ethanesulfonic acid] premixed with 0.1 mL of 1% zinc acetate, and to this mixture are sequentially added 0.1 mL of *N*,*N*-dimethyl-p-phenylenediamine (20 m*M* in 7.2 *M* HCl) and 0.08 mL $FeCl_3$ (30 m*M* in 1.2 *M* HCl), vortexed, and incubated for 20 min at room temperature. Thereafter, 0.2 mL of 10% trichloroacetic acid is added, vortexed, and incubated during 5 min at room temperature. The supernatant after 5 min centrifugation at 10,000 *g* in an Eppendorf microcentrifuge was used for absorbance readings at 670 nm.

The calibration of the absorbance at 670 nm versus H_2S concentration (Fig. 1B) was done by mixing aliquots of a freshly prepared 0.2 *M* NaSH solution with 0.6 mL of 50 m*M* TES premixed with 0.1 mL zinc acetate to minimize H_2S diffusion. Thereafter, the protocol was followed as indicated above.

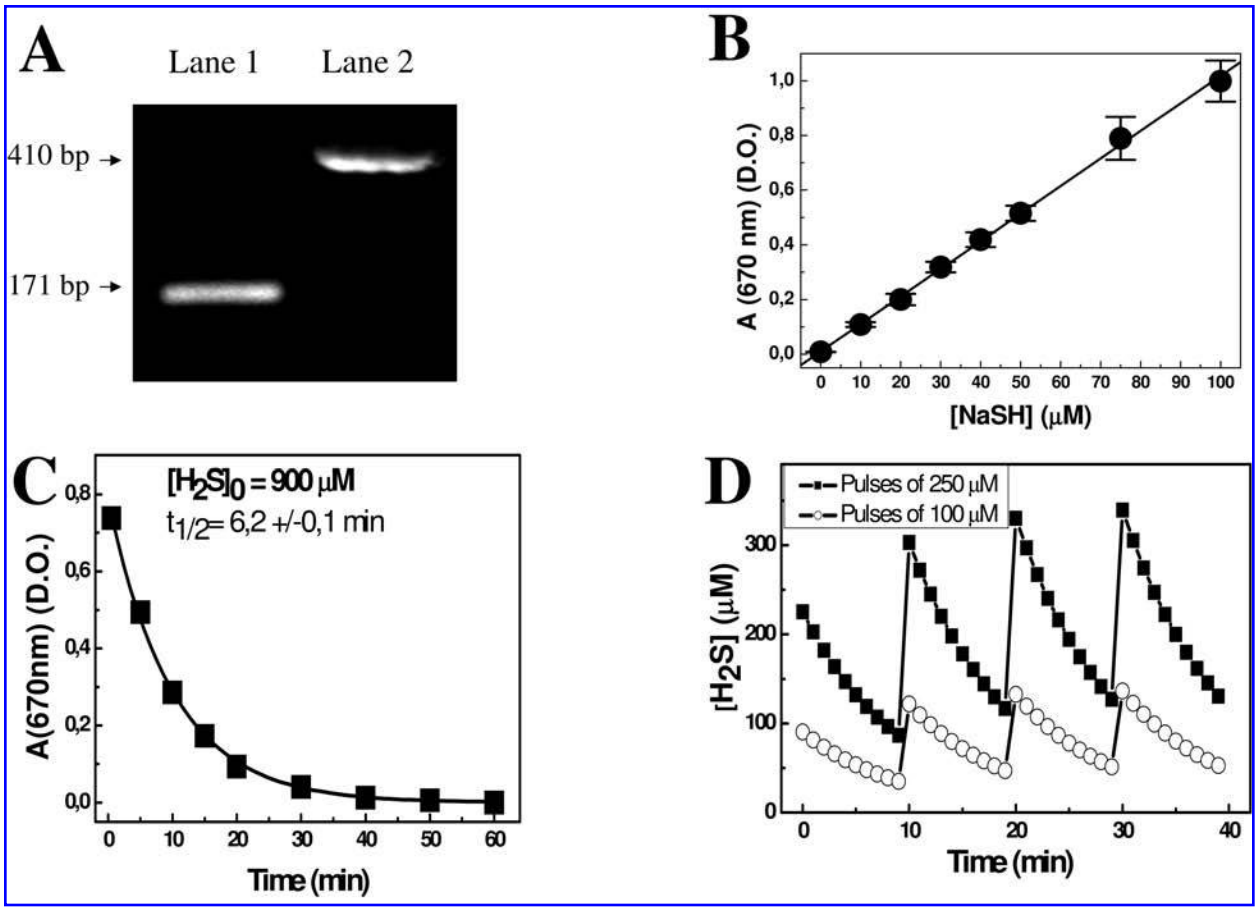

FIG. 1. (A) RT-PCR shows the expression of the mRNAs of cystathionine-β-lyase (lane 1) and cystathionine-γ-lyase (lane 2) in CGN. See Materials and Methods for details. The molecular size of the mRNAs encoding for these two enzymes are given on the side of the agarose gel pre-stained with ethydium bromide. (**B**) Calibration of the absorbance at 670 nm versus H_2S concentration (added to the solution in the form of NaSH). The line is the best linear least-squares fit of the data ($R = 0.999$; $p < 0.001$) to the following equation: $A_{670\,nm} = 0.01 + 0.01 \cdot [NaSH]$ (*μM*). (**C**) Kinetics of H_2S decay from the culture medium in 35-mm dishes. The kinetics of H_2S decay was monitored as follows: at time zero 0.9 m*M* NaSH was added to 2 mL of the culture medium at 37°C, and aliquots were pooled at the times indicated in the abscissae. The results shown (*solid squares*) are the average of a triplicate experiment, and the standard deviation is approximately the size of the data-points. The *solid line* is the best nonlinear least squares fit of the data to a single exponential decay, which yielded a half-time of 6.2 ± 0.1 min. (**D**) Control of H_2S concentration in the extracellular medium by serial repetitive NaSH pulses. Time dependence of the H_2S concentration in the culture medium at 37°C after serial repetitive pulses of 100 *μM* (*open circles*) and 250 *μM* NaSH (*solid squares*). NaSH pulses were applied every 10 min.

Measurement of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured basically as indicated in previous works (15, 31). Briefly, CGN were loaded with Fura-2 by incubation in DMEM-F12 for 45–60 min with 5 μM Fura-2-acetoxymethyl ester (Fura-2 AM) and 0.025% Pluronic-F127 at 37°C. Afterwards, CGN were washed twice with Locke's buffer (4 m*M* $NaHCO_3$, 10 m*M* HEPES, 5 m*M* glucose, 2.3 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 134 m*M* NaCl/25 m*M* KCl, pH 7.4 at 37°C) and the culture dish was placed in a thermostatic controlled plate (Warner Instrument Co., Hamden, CT) of a Nikon Diaphot 300 (Tokyo, Japan) inverted microscope, equipped with an epifluorescence attachment and excitation filter wheel. To measure the intracellular calcium concentration, fluorescence ratio images were obtained with excitation filters of 340 and 380 nm and a dichroic mirror DM510 and absorption filter (emission side) of 520 nm. Digital images were taken with a Hamamatsu Hisca CCD camera (Hamamatsu City, Japan) and Lamdba 10–2 filter wheel controller, and subsequently analyzed with Argus/Hisca software. $[Ca^{2+}]_i$ was calculated as indicated in (15, 31), with the equation:

$$[Ca^{2+}]_i = K_d \cdot \{(R\text{-}R_{min})/(R_{max} - R)\} \cdot \beta \qquad [1]$$

where R is the measured fluorescence ratio (340/380), and R_{max} and R_{min} are the ratio values (340/380) for Ca^{2+}-bound and Ca^{2+}-free Fura-2 in loaded CGN. R_{max} and R_{min} were experimentally determined from steady-state fluorescence ratio (340/380) measurements after sequential addition to the culture medium of Fura-2-loaded CGN of (1) BrA23187 (5 μg/mL) or ionomycin (45 μg/mL), and (2) 10 mM EGTA, respectively. The average values obtained for R_{max} and R_{min} were 3.8 ± 0.4 and 0.20 ± 0.02 ($n > 500$ cells), respectively, and the obtained average value for the ratio of fluorescence values for Ca^{2+}-free/Ca^{2+}-bound indicator at 380 nm (β) obtained with our instrument setup was 1.85 ± 0.15. A value of 224 n*M* has been used for K_d, the dissociation constant of the complex fura-2:Ca^{2+} (35).

Measurements of L-glutamate released to the extracellular medium

The concentration of L-glutamate in Locke's buffer, the extracellular medium used for the treatment of CGN with repetitive NaSH pulses, was determined using L-glutamate dehydrogenase ($NADP^+$), as in Ref. 27. Briefly, 1.8 mL of extracellular medium was carefully withdrawn from the 35 mm diameter culture dish to minimize cell resuspension during experimental handlings, centrifuged at 500 *g* for 5 min to remove the cells that could have been detached during the treatments, and placed in a fluorimeter cuvette thermostated at 30°C. Thereafter, the extracellular medium was brought to pH 8.3 with 50 m*M* Tris, and supplemented with 2 m*M* $NADP^+$ and 50 U of glutamate dehydrogenase. Glutamate released by CGN was calculated from the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) due to NADPH produced by the oxidative deamination of released L-glutamate by glutamate dehydrogenase. The H_2S pulses used in this study did not interfere with L-glutamate detection using this enzymatic assay, since up to 500 μM H_2S did not increase the fluorescence of Locke's buffer supplemented with $NADP^+$, therefore excluding reduction of $NADP^+$ by H_2S, nor significantly altered (*i.e.*, <10% difference) the increase of fluorescence after the application of several 2.5 nmoles pulses of L-glutamate. To further assess the good performance of the enzyme assay in the measurements of L-glutamate released by CGN, a standard of exogenous L-glutamate (2.5 or 5 nmoles) was added at the end of each experiment as a routine control. The glutamate concentration in the medium was calculated by interpolation into the linear plot of fluorescence intensity versus glutamate concentration in Locke's buffer, done up to 25 μM with the appropriate additions of L-glutamate from a standard solution freshly prepared by weighing.

Population analysis

For population analysis, at least four different preparations of CGN and at least three plates of each preparation were used. The number of neurons counted for each experimental condition was ≥150, and the fluorescence ratio (340/380) values were always taken from the soma of Fura-2-loaded CGN. Three-dimensional plots were built up with Origin™ software (Northampton, MA). Statistical analysis was done using the analysis of the variance (ANOVA) on the Instat software program (Graph-PAD Software, San Diego, CA). Data shown are mean ± standard deviation (s.d.) or standard error (s.e.). Significant difference was accepted for $p < 0.05$.

RESULTS

The application of repetitive NaSH pulses allows the exposure of neurons in culture to pathophysiological H_2S concentrations for different time periods

A single pulse application of NaSH to the 35-mm diameter plates used for neuronal culture rapidly generates nearly the same concentration of H_2S, which then decays as this gas diffuses out from the culture medium (Fig. 1C). As expected for a simple diffusion process, the decay of H_2S can be fit to a single exponential decay, showing a half-time of 6.2 ± 0.1 min. Therefore, the application of only one pulse of NaSH to cell culture plates leads to a very short exposure of cells to H_2S, which cannot be claimed to simulate the chronic exposure of neurons to H_2S within the brain. However, it is possible to maintain the H_2S concentration in the cell culture medium within the pathophysiological range reported for brain through the application of repetitive NaSH pulses, as shown in Fig. 1D. As also noticed by Kimura *et al.* (17), there is not a significant change on pH, at most 0.1 pH units after six 250 μM NaSH pulses. The results shown in Fig. 1D demonstrate that application of 100 μM NaSH pulses every 10 min maintains the H_2S concentration within 50 and 120 μM [*i.e.*, within the upper concentration segment of the physiological H_2S range reported for rat brain (1, 5, 14, 17)], and that application of 250 μM NaSH pulses every 10 min maintains the H_2S concentration within the range 200–300 μM, reported for rat blood plasma (20).

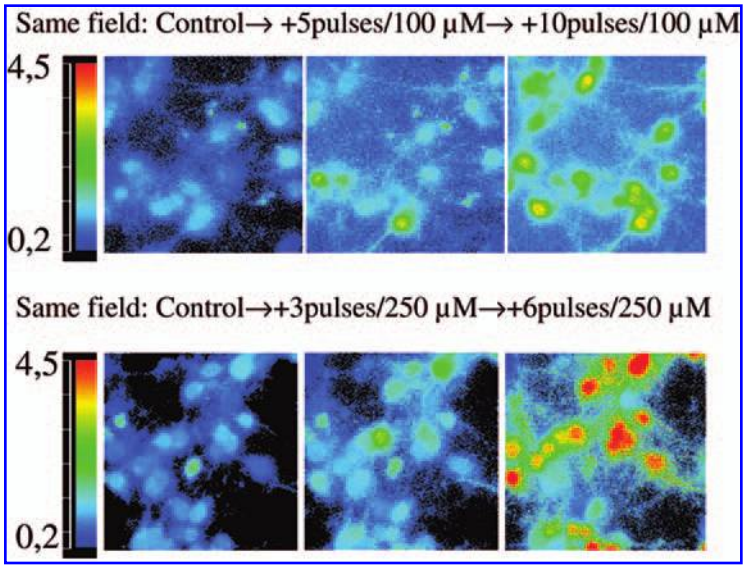

FIG. 2. Repetitive NaSH pulses promoted a sustained increase of the cytosolic calcium concentration in CGN in culture. *Top panels*. Pseudo-color representative fluorescence ratio (340/380) images of Fura-2-loaded CGN in Locke's K25 buffer of control CGN (*left image*), of CGN treated with five 100 μM NaSH pulses (*center*), and of CGN treated with ten 100 μM NaSH pulses (*right image*). *Bottom panels.* Pseudo-color representative fluorescence ratio (340/380) images of Fura-2-loaded CGN in Locke's K25 of control CGN (*left image*), of CGN treated with three 250 μM NaSH pulses (*center*), and of CGN treated with six 250 μM NaSH pulses (*right image*). In all cases, NaSH pulses were applied every 10 min and images were acquired 5 min after the application of the last pulse. CGN loading with Fura-2-AM and fluorescence ratio (340/380) images acquisition was performed as indicated in Materials and Methods.

The period of time and the H_2S concentration range can be fixed at will by simply changing the number and size of the pulses of NaSH.

Treatment of CGN with repetitive pulses of NaSH produced a sustained rise of the cytosolic calcium concentration

It is to be noted that in spite of the ability of CGN to express the mRNA encoding for cystathionine β-synthase and cystathionine γ-lyase (Fig. 1A), the enzymes that produce H_2S, the level of H_2S in the cell culture medium in standard dishes of 35 mm diameter was always lower than 10 μM, measured with cell lysates following the protocol indicated in Ref. 1 (*data not shown*). Obviously, this is due to its rapid diffusion to the surrounding atmosphere and as a consequence it must be supplemented to the neurons in culture.

Figure 2 illustrates that only 5–10 pulses of 100 μM NaSH or only 3–6 pulses of 250 μM NaSH applied every 10 min are needed to produce a sustained rise of cytosolic calcium in CGN

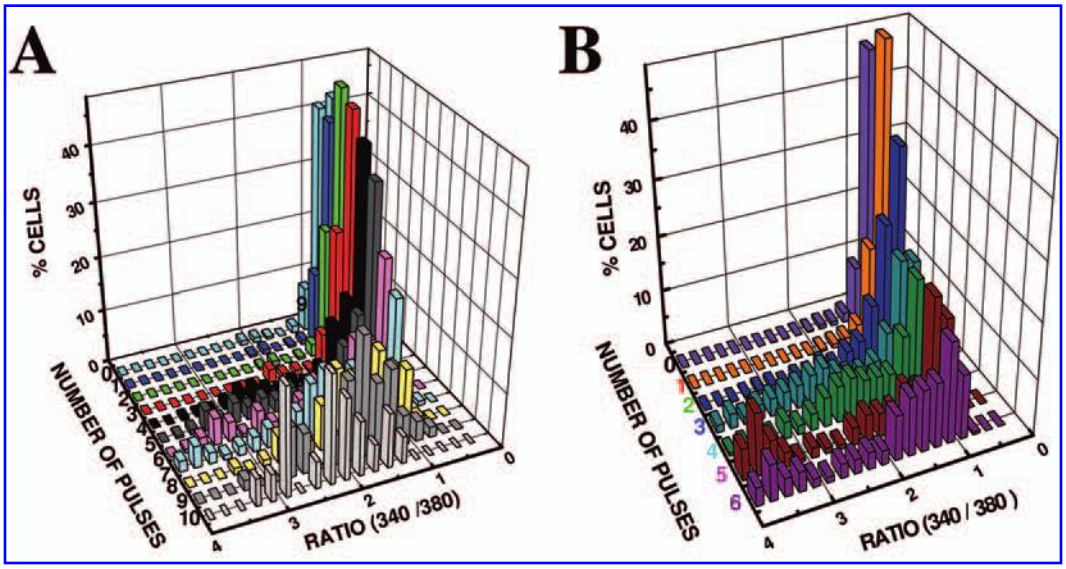

FIG. 3. Effect of the number of NaSH pulses upon the distribution of the population of CGN between different fluorescence ratio (340/380) intervals. (**A**) Three-dimensional plot of the results obtained for repetitive 100 μM NaSH pulses applied every 10 min. The histograms are elaborated with $n > 150$ neurons for each number of pulses, and the results obtained with three different CGN preparations were accumulated. (**B**) Three-dimensional plot of the results obtained for repetitive 250 μM NaSH pulses applied every 10 min. The histograms are elaborated with $n > 1000$ neurons for each number of pulses, and the results obtained with ten different CGN preparations were accumulated. The number of neurons analyzed in this latter case is much higher than those analyzed for (**A**), because repetitive pulses of 250 μM NaSH were used for most of the rest of the experiments done in this study. In all the cases, the images were acquired 5 min after the application of the NaSH pulse.

TABLE 1. POPULATION ANALYSIS AND MEANS OF THE FLUORESCENCE RATIO (340/380) VALUES OF FURA-2-LOADED CGN AFTER TREATMENT WITH REPETITIVE NaSH PULSES*

	Subpopulation A		*Subpopulation B*	
	(%)	*Mean ± s.d.***	*(%)*	*Mean ± s.d.***
Repetitive 100 μM NaSH pulses				
Control	100	0.83 ± 0.2	—	—
6 pulses	28 ± 2	0.97 ± 0.07	72 ± 2	1.54 ± 0.45
10 pulses	—	—	100	2.3 ± 0.4
Repetitive 250 μM NaSH pulses				
Control	100	0.84 ± 0.2	—	—
3 pulses	50 ± 5	1.02 ± 0.17	50 ± 5	2.4 ± 0.6
6 pulses	60 ± 5	1.22 ± 0.25	40 ± 5	3.6 ± 0.6

*Calculated from the results shown in Fig. 3.
**s.d., standard deviation.

in culture. The rise can be seen in neuronal soma and also within the dendrites in CGN treated with repetitive 100 μM NaSH pulses (Fig. 2). All these results were confirmed with duplicate plates of four different CGN preparations. Thus, exposure of CGN for <2 h to concentrations of H_2S within the reported physiological range produced a sustained shift of calcium homeostasis towards higher cytosolic calcium concentrations. It is well known that CGN in culture tend to spontaneous clus-

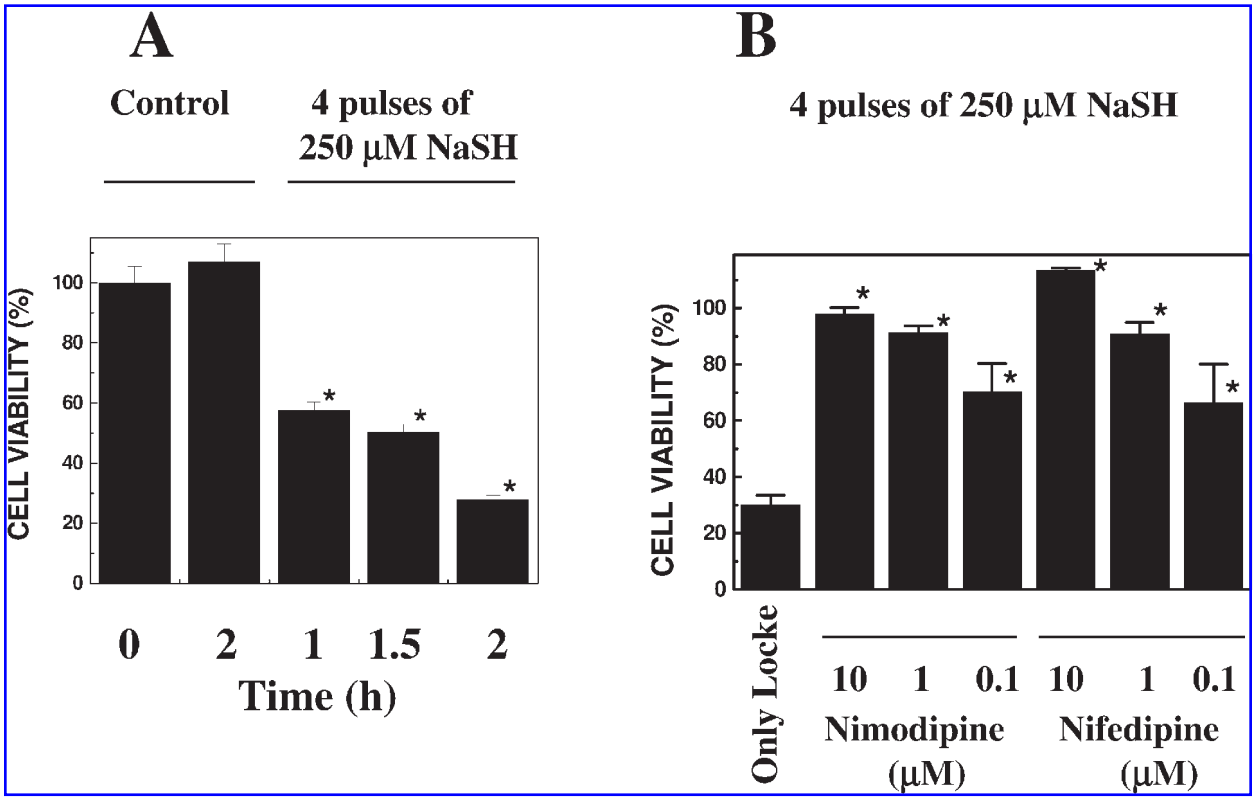

FIG. 4. Repetitive pulses of 250 μM NaSH induced CGN death and L-type calcium channel blockers nimodipine and nifedipine protected against NaSH-induced CGN death. (**A**) Cell viability was determined with the MTT assay, as indicated in Materials and Methods, after four pulses of 250 μM NaSH applied with 10 min intervals between pulses and at the times indicated in the Figure after the first 250 μM NaSH pulse. An asterisk (*) over the bar means statistically significant difference with respect to control CGN treated with water pulses instead of NaSH pulses ($p < 0.05$). (**B**) CGN in the absence and in the presence of the indicated concentrations of nimodipine and nifedipine were treated with four pulses of 250 μM NaSH applied with 10 min intervals between pulses. Cell viability was determined with the MTT assay 2 h after the first 250 μM NaSH pulse. The results shown in (**A**) and (**B**) are the means (±s.e.) of experiments done by triplicate with three different CGN preparations ($n = 9$). An asterisk (*) over the bar means statistically significant difference with respect to CGN treated with four pulses of 250 μM NaSH in the absence of L-type calcium channel blockers ($p < 0.05$).

ters in neuronal granules, yielding a heterogeneous population of neurons in terms of the number and type of neuronal–neuronal contacts. As we noticed some heterogeneity in the quantitative rise of cytosolic calcium induced by NaSH pulses (Fig. 2), the statistical relevance of the sustained rise of cytosolic calcium induced by H_2S was assessed by the population analysis displayed in Fig. 3. In addition, the histograms shown in this figure revealed the smooth and progressive shift to higher cytosolic calcium (higher ratio values) caused by increasing the number of 100 μM NaSH pulses, and the appearance of a subpopulation of neurons with saturated Fura-2 ratio values, *i.e.* ratio values >3.2 or $[Ca^{2+}]_i > 1$ μM, when CGN were treated with 250 μM NaSH pulses. For the sake of clarity, only the results of the statistical analysis of the ratio values of Fura-2-loaded CGN after treated with a selected number of pulses of 100 and 250 μM NaSH are listed in Table 1. These results indicated that the $[Ca^{2+}]_i$ rises from 100 ± 17 nM (ratio value = 0.9 ± 0.1) to 580 ± 150 nM (ratio value = 2.3 ± 0.4) when CGN are exposed to H_2S concentrations within the reported physiological range for nearly 2 h. In addition, these data also show that the $[Ca^{2+}]_i$ of nearly half-the-cell population (40 ± 5%) is ≥1 μM when CGN are treated with 250 μM NaSH pulses for ~1 h, suggesting that it may have reached the neurotoxic range of cytosolic calcium.

H_2S-induced $[Ca^{2+}]_i$ deregulation produces CGN death that is prevented by L-type calcium channels blockers nimodipine and nifedipine

Cell viability was measured to assess that $[Ca^{2+}]_i$ reached the neurotoxic range after treatment of CGN with repetitive 250 μM NaSH pulses. Cell viability was determined using the MTT

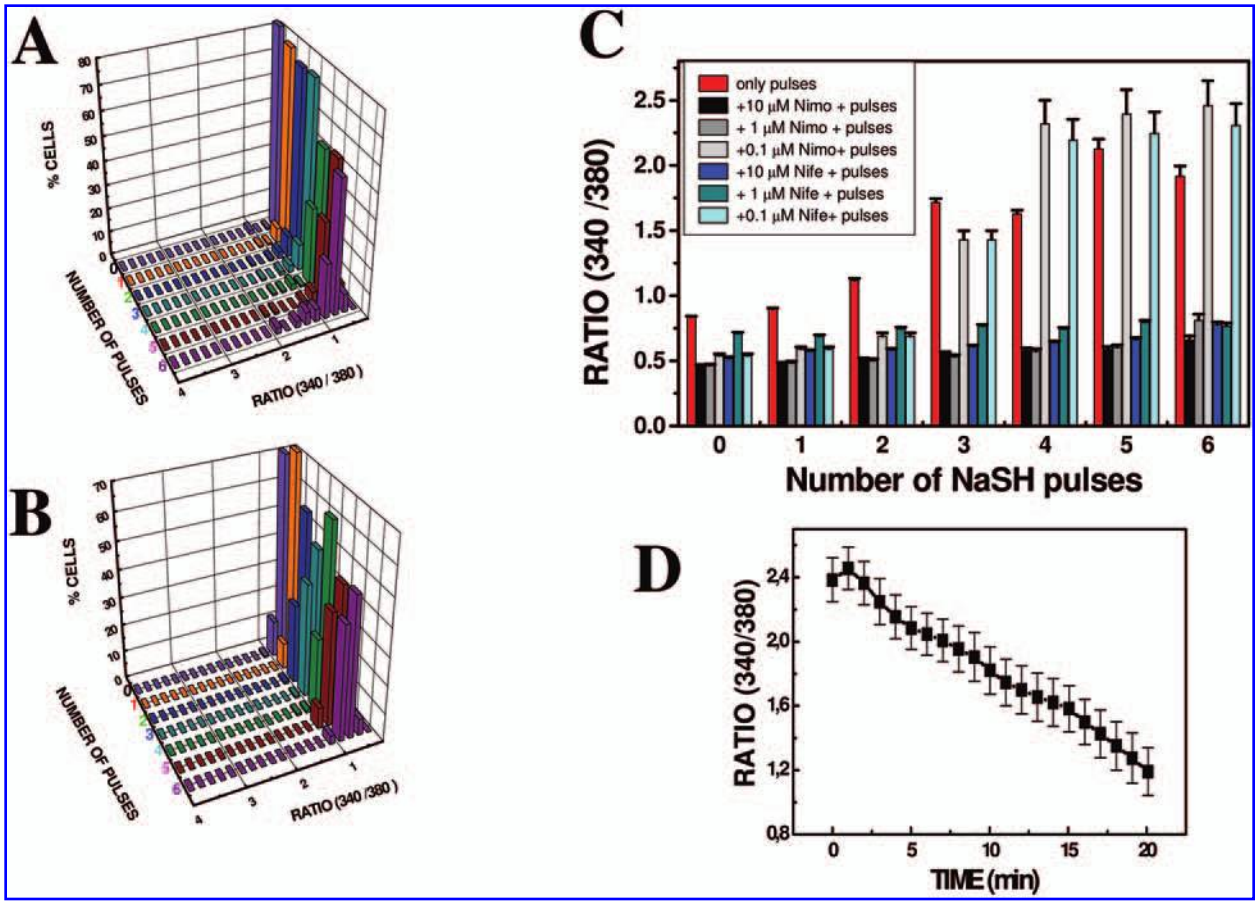

FIG. 5. L-type calcium channel blockers nimodipine and nifedipine protected against the sustained increase of the cytosolic calcium concentration in CGN in culture induced by repetitive NaSH pulses. Three-dimensional plot of the results obtained for repetitive 250 μM NaSH pulses applied every 10 min to CGN in the presence of 1 μM nimodipine (**A**) or 1 μM nifedipine (**B**). The histograms were elaborated with $n > 150$ neurons for each number of pulses, and the results obtained with three different CGN preparations were accumulated. (**C**) Fluorescence ratio (340/380) values (mean ± s.e., $n > 150$ neurons) of CGN treated with the repetitive number of 250 μM NaSH pulses indicated in the abscissae in the absence and presence of 0.1, 1.0, and 10 μM nimodipine or nifedipine. *Inset*: Color-coded bars for the different conditions, nimodipine and nifedipine have been abbreviated as Nimo and Nife, respectively. (**D**) Fura-2-loaded CGN showing a sustained rise of cytosolic calcium after treatment with three pulses of 250 μM NaSH, applied with 10 min intervals between pulses, still retain their ability to decrease the fluorescence ratio (340/380) values down to the ratio values measured for untreated CGN after addition of 10 μM nifedipine or nimodipine. The data obtained for nifedipine and nimodipine overlap within the experimental errors. Measured fluorescence ratio (340/380) values (*solid squares*, mean ± s.e. of triplicate experiments, $n > 100$ neuronal somas) are plotted versus the time after addition of nifedipine or nimodipine.

assay as indicated in Materials and Methods, and the results confirmed that treatment of CGN with repetitive 250 μM NaSH pulses produced a large loss of cell viability at a time as short as 1 h after the application of the first NaSH pulse (Fig. 4A). To further validate this conclusion, cell death was also assessed using the trypan blue exclusion method which yielded the same results and release of lactate dehydrogenase to the extracellular medium (*data not shown*). Thus, treatment of CGN with repetitive 250 μM NaSH pulses produced rapid CGN death.

Previous publications have shown that in CGN the Ca^{2+} entry through L-type calcium channels plays a major role in the fine tuning of cytosolic calcium homeostasis in the neuronal soma (13, 15, 23, 32). As shown in Fig. 4B, the well known L-type calcium channels blockers nimodipine and nifedipine efficiently protected against H_2S-induced CGN death, in both cases with an $IC_{50} < 1$ μM. In fact, micromolar concentrations of both nimodipine and nifedipine afforded nearly complete protection ($\geq$90%).

Therefore, the possibility that H_2S-induced rise of $[Ca^{2+}]_i$ in CGN is caused by an stimulation of Ca^{2+} entry through the L-type calcium channels deserved to be studied. To this end, Fura-2-loaded CGN were treated with repetitive 250 μM NaSH pulses in the presence of 1 μM nimodipine or nifedipine and the results obtained are shown in Figs. 5A and B. The comparison of these results with those shown in panel B of Fig. 3 demonstrates that both nimodipine and nifedipine protect CGN against $[Ca^{2+}]_i$ deregulation by repetitive 250 μM NaSH pulses. To further reinforce this important experimental observation, these experiments were done in the presence of three different concentrations of either nimodipine and nifedipine, 0.1, 1.0, and 10 μM, and the average fluorescence ratio 340/380 has been plotted versus the number of 250 μM NaSH pulses applied to the neurons (Fig. 5C). Also, the presence of 1 μM nimodipine or nifedipine fully prevents the rise of $[Ca^{2+}]_i$ upon repetitive application of 100 μM NaSH pulses, up to 12 pulses were tested *(data not shown)*. These results pointout that concentrations as low as 0.1 μM of both nimodipine and nifedipine afford partial protection against $[Ca^{2+}]_i$ deregulation by repetitive 250 μM NaSH pulses. Moreover, the application of nimodipine within 5 min after the third pulse of 250 μM NaSH allows Fura-2-loaded CGN to recover to the fluorescence ratio values measured before H_2S treatment (Fig. 5D). Full reset of the fluorescence ratio (340/380) to the ratio value measured before the treatment of CGN with NaSH pulses (0.9 ± 0.1) was achieved within 20 min after the addition of nifedipine or nimodipine. As the neurons showing fluorescence ratio values as high as 2.4 have not lost Fura-2 loading, plasma membrane permeability breakdown has not yet taken place and the cells have not lost their ability to maintain $[Ca^{2+}]_i$ homeostasis. Consistent with this observation, application of nimodipine or nifedipine immediately after the third pulse of 250 μM NaSH blocks cell death, as the drop of cell viability after 2 h was at most 10%

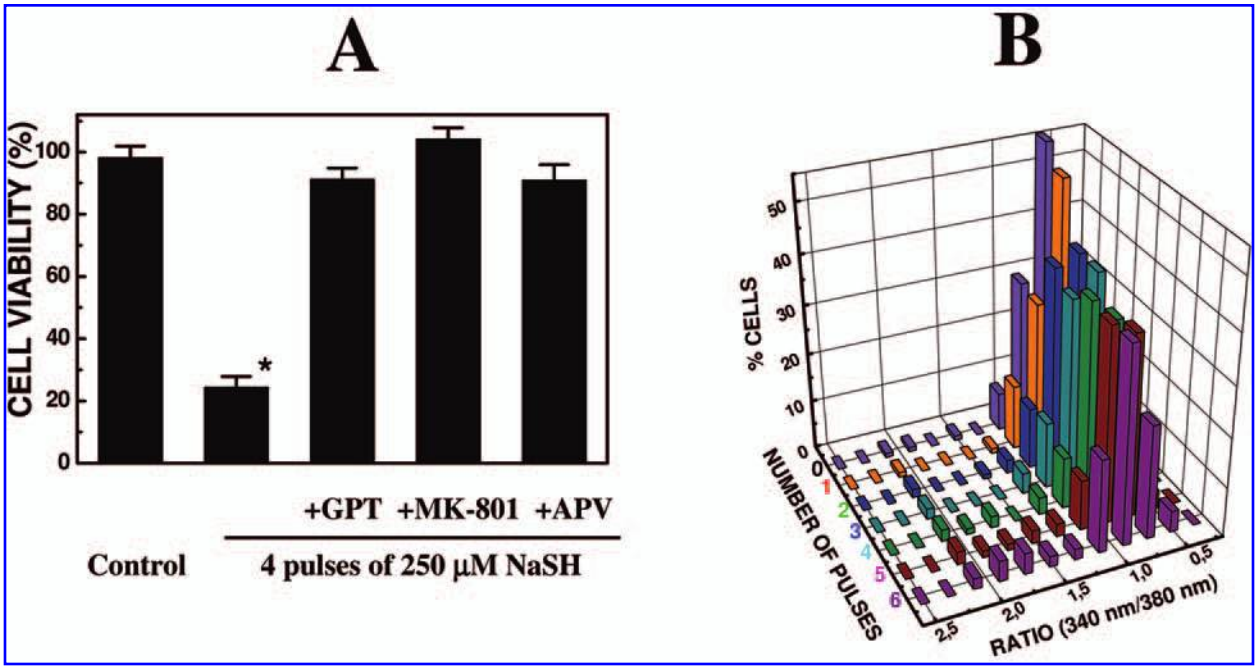

FIG. 6. Glutamate removal from the extracellular medium and NMDA receptor antagonists (+)-MK-801 and APV protected against CGN death induced by repetitive NaSH pulses. (**A**) CGN in the absence and in the presence of 50 U glutamate-pyruvate transaminase (GPT) plus 3 mM pyruvate or in the presence of 10 μM (+)-MK-801 or 50 μM APV were treated with four pulses of 250 μM NaSH applied with 10 min intervals between pulses. Cell viability was determined with the MTT assay 2 h after the first 250 μM NaSH pulse. The results shown are the means ($\pm$s.e.) of experiments done by triplicate with three different CGN preparations ($n = 9$). An asterisk (*) over the bar means statistically significant difference with respect to control ($p < 0.05$). (**B**) Three-dimensional plot of the results obtained for repetitive 250 μM NaSH pulses applied every 10 min to CGN in the presence of 10 μM (+)-MK-801. The histograms were elaborated with $n > 150$ neurons for each number of pulses, and the results obtained with three different CGN preparations were accumulated.

in triplicate measurements done with three different CGN preparations ($n = 9$). Therefore, H_2S rises intracellular calcium before the CGN are committed to death, and these results indicate that cell death was ensued by an event triggered by the rise of $[Ca^{2+}]_i$ mediated by L-type calcium channels. All together, these results strongly support that L-type calcium channels are a primary and highly sensitive molecular target for H_2S.

Rapid H_2S-induced CGN death is mediated by glutamate release

CGN are glutamatergic neurons and express NMDA receptor (4, 29), and a rise of $[Ca^{2+}]_i$ is well known to induce release of neurotransmitters by stimulating presynaptic vesicle fusion with the synaptic plasma membrane. As extracellular glutamate can further deregulate $[Ca^{2+}]_i$ homeostasis in CGN by acting through NMDA receptors, which leads to excitotoxic neuronal death (6, 9), the possible involvement of glutamate release in H_2S-induced CGN death deserved to be studied. Glutamate release to the extracellular medium has been measured, as indicated in Materials and Methods. Treatment of CGN with repetitive 250 μM H_2S pulses increased the concentration of glutamate in the extracellular medium up to 10–15 μM within 90 and 120 min from the first H_2S pulse, whereas the concentration of glutamate in the extracellular medium of control CGN (treated with water pulses) was 1.0 ± 0.2 μM after 120 min from the first water pulse (average results of nine measurements done with three different CGN preparations).

Figure 6A shows that H_2S-induced neuronal death can be completely blocked by supplementation of the culture medium with 50 U of glutamate-pyruvate transaminase (GPT) plus 3 mM pyruvate, a system that has been used to efficiently remove released glutamate below the neurotoxic level for CGN in culture (2). In addition, H_2S-induced neuronal death was more than 90% decreased by the NMDA receptor channel blocker (+)-MK-801 and by the L-glutamate competitive antagonist APV (Fig. 6A), at concentrations used to block NMDA receptor-mediated calcium entry in neurons, namely, 10 μM for (+)-MK-801 in CGN (30) and 50 μM for APV (37).

As shown in Fig. 6B, in the presence of 10 μM (+)-MK-801, the application of 250 μM NaSH pulses largely attenuated the rise of $[Ca^{2+}]_i$, from a mean ratio value of 0.84 (control) up to a mean ratio value of 1.25–1.30 (after six pulses), thus preventing $[Ca^{2+}]_i$ from reaching the neurotoxic calcium range.

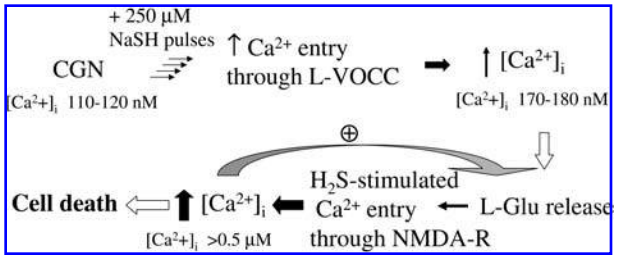

FIG. 7. Schematic outline of CGN death induced by the application of repetitive 250 μM NaSH pulses. L-VOCC, L-type voltage-operated calcium channels; L-Glu, L-glutamate; NMDA-R, NMDA receptor.

To ascertain that the L-glutamate concentrations released to the medium [*i.e.*, 10–15 μM (see above)] can account for the rise of the ratio (340/380) to values ≥ 3.0 in CGN treated with three to four repetitive 250 μM NaSH pulses, we took advantage of the fact that CGN treated with three to four repetitive 250 μM NaSH pulses in Locke's buffer with low potassium (5 mM KCl) produced only a minor increase of the ratio value, from 0.47 ± 0.12 to 0.56 ± 0.10, and no significant increase of extracellular L-glutamate. Upon application of 10 μM L-glutamate to CGN treated with three to four repetitive 250 μM NaSH pulses in Locke's buffer with low potassium (5 mM KCl) the ratio (340/380) of Fura-2-loaded CGN increased up to values >3.0 in <5 min, whereas the application of 100 μM L-glutamate was needed to produce a similar increase of the ratio (340/380) within 5 min in Fura-2-loaded CGN not treated with repetitive NaSH pulses. Thus, these results are in excellent agreement with the stimulation of the NMDA receptor-mediated responses by H_2S reported in Refs. 1 and 16.

DISCUSSION

It is shown in this work, for the first time to the best of our knowledge, that H_2S concentrations within the reported physiological range act as a modulator of neuronal cytosolic calcium homeostasis. Due to the large implications of sustained alterations of cytosolic calcium on neuronal excitability and synaptic activity, the sustained rise of cytosolic calcium induced by H_2S in CGN is expected to have a deep impact in the activity of the cerebellum, as CGN are the most abundant neurons within this area of the brain. Similar effects have been previously reported for glial cells (22, 26). The same authors concluded that treatment with a single pulse of physiological H_2S concentrations had no effect on the calcium homeostasis of rat hippocampal neurons in culture (26). The results reported in this paper also show that treatment with a single pulse of physiological H_2S concentrations had no effect on the calcium homeostasis of CGN in culture. Nevertheless, one single H_2S pulse generates only a brief exposure of neurons in culture, since the H_2S concentration decays with a half-time of 6.2 ± 0.1 min, whose duration is too short to reasonably assume that it can be used to simulate the exposure to H_2S within the brain. Therefore, we have developed an experimental protocol for generation of steady state H_2S concentrations within the 50–160 μM physiological range based on the application of repetitive NaSH pulses every 10 min. Less than 2 h exposure to physiological H_2S concentrations have been found to be enough to produce a large shift of the intracellular calcium homeostasis in CGN in culture, leading to an increase of cytosolic calcium concentration from 100 ± 15 to 580 ± 150 nM. Only 1 h exposure of CGN in culture to H_2S concentrations between 200 and 300 μM [*i.e.*, in the range reported for rat blood plasma (20)], produces a rise of $[Ca^{2+}]_i$ up to the neurotoxic calcium range and death of nearly 50% of the neurons after only 2 h. Thus, H_2S modulates the fine tuning between the major calcium transport systems involved in the control of calcium homeostasis in these neurons. As calcium entry through L-type calcium channels had been shown to be particularly relevant to raise the cytosolic calcium concentration in CGN in culture (13, 15, 23, 32), the pos-

sibility of their modulation by H_2S deserved to be considered at first.

The well-known L-type calcium channel blockers nimodipine and nifedipine protect against H_2S-induced alteration of cytosolic calcium homeostasis in CGN in culture. H_2S-induced rise of $[Ca^{2+}]_i$ in CGN is almost completely blocked by 1 μM nimodipine and nifedipine, and is partially attenuated by concentrations as low as 0.1 μM of both blockers. Moreover, both afford full protection against H_2S-induced CGN death, and when applied shortly after H_2S-induced rise of $[Ca^{2+}]_i$ up to 650 nM (fura-2 fluorescence ratio of 2.4), the loss of cell viability is minimum (<10%), and CGN are still able to recover to normal cytosolic calcium homeostasis within 15 min. Therefore, L-type calcium channels are a highly sensitive molecular target for H_2S, being more active in the presence of this gas and are responsible to trigger the sustained rise of $[Ca^{2+}]_i$ in CGN exposed to H_2S in culture. As the activation of L-type calcium channels develops slowly (*i.e.*, required an exposure of CGN to H_2S for 30–60 min to be clearly seen), the possibility that this could be a consequence of H_2S-induced imbalance of the activity of protein kinases and phosphatases in the neurons, as suggested earlier for NMDA receptor (16), or only of neuronal reactive oxygen species production should be explored in future studies, since the activity of L-type calcium channels is modulated by ROS (15) and by several protein kinases (11). Owing to the high relevance of the calcium entry through L-type calcium channels for the fine tuning of cytosolic calcium homeostasis in neurons, these results give a strong support to the concept of H_2S as a major modulator of the neuronal activity level, which has been suggested earlier by others (1, 5, 17).

The exposure to H_2S concentrations less than two-fold higher than the concentrations reported for the rat brain produced a rapid death of neurons (*e.g.*, nearly 50% neuronal death in <1 h exposure). Thus, the reported physiological range of H_2S in rat brain is very close to the neurotoxic range of this gas for CGN in culture (200–300 μM), which in turn is not significantly different from the H_2S concentration reported for rat blood plasma (20). Moreover, higher levels of H_2S in rat blood plasma, ≈370 μM, have been reported for a rat model of recurrent febrile seizures (20). The relevance of this fact for brain damage in cerebral trauma or neurodegeneration, though speculative at present, merits to be considered. In this regard, it is to be noted that our results show that H_2S-induced neuronal death is excitotoxic and is mediated by L-glutamate release to the extracellular medium and subsequent NMDA receptor activation, as it is prevented by rapid enzymatic removal of glutamate from the extracellular medium and also by the NMDA receptor antagonist APV and by the NMDA receptor channel blocker (+)-MK-801. It is to be noted that the extracellular concentrations of L-glutamate measured in this study after treatment of CGN with repetitive 250 μM NaSH pulses, namely 10–15 μM, have been shown earlier to be high enough to cause excitotoxic CGN death in high-K^+ culture medium [*i.e.*, KCl ≥20 mM (2)]. The large attenuation of the H_2S-induced rise of $[Ca^{2+}]_i$ when the application of repetitive NaSH pulses is done in the presence of (+)-MK-801 further supports this conclusion, as activation of NMDA receptors by L-glutamate leads to a large calcium influx into the neuronal cytosol. The increase of the ratio (340/380) up to 1.25–1.30 observed after the application of repetitive 250 μM NaSH pulses in the presence of (+)-MK-801 allows the calculation that activation of L-type Ca^{2+} channels by H_2S leads to a sustained rise of $[Ca^{2+}]_i$ up to 170–180 nM, which is sufficient to trigger L-glutamate release in CGN treated with repetitive 250 μM NaSH pulses. Our results also point out that the NMDA receptor response to L-glutamate is markedly enhanced in CGN treated with H_2S, as it has been previously shown in other neuronal cultures (1, 16). The scheme presented in Fig. 7 outlines the proposed mechanism for CGN death induced by the application of repetitive 250 μM NaSH pulses, highlighting that activation of calcium entry through NMDA receptors forms a positive feedback cycle leading to further increase cytosolic calcium until the neurotoxic range. During the last decade, disruption of the blood/brain barrier and excitotoxic neuronal death have been shown by many laboratories to be particularly relevant in neuronal damage associated with ischaemia/reperfusion episodes in the brain (21). On the other hand, a minimum threshold level of cytosolic calcium is needed for neuronal survival in culture (13), and for CGN in culture it has been shown that this threshold level can be attained just by raising the concentration of K^+ in the extracellular medium from 5 to 25 mM (13, 25). Moreover, the enhanced calcium entry through L-type calcium channels upon partial plasma membrane depolarization by 25 mM K^+ can account for CGN survival in culture and suppression of low K^+-induced apoptosis (13). Therefore, our results suggest that a minimum H_2S level in the brain may be needed to maintain the threshold level of cytosolic calcium concentrations needed for survival of, at least, some groups of neurons. On these grounds, it will be particularly relevant to study whether this low level of brain H_2S concentrations is attained in individuals with neurodegenerative diseases.

In summary, concentrations of H_2S within the reported physiological range have a strong stimulating effect on the calcium currents through L-type calcium channels of CGN in culture, and as a consequence H_2S is a major modulator of the cytosolic calcium homeostasis in these neurons. The sustained rise of cytosolic calcium induced by <1 h exposure of CGN to H_2S concentrations only 1.5 to 2-fold higher than those reported for normal rat brain promotes L-glutamate-induced excitotoxic neuronal death through NMDA receptor stimulation. Because the molecular mechanisms involved in the neuromodulation and neurotoxicity of H_2S are also present in neurons from other relevant areas of the brain, it can be anticipated that future studies will show that H_2S has a widespread impact in many brain functions. Finally, the neurotoxic H_2S range for CGN in culture is very close to the range of concentrations reported for this gas in rat blood and not far from that reported for rat brain.

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ABBREVIATIONS

APV, DL-2-amino-5-phosphonovaleric acid; Br-A23187, 4-bromo-calcimycin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; CGN, cerebellar granule neurons; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylenediaminetetraacetic

acid; GPT, glutamate-pyruvate transaminase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, concentration needed to produce 50% of the maximal inhibitory effect; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; RT-PCR, reverse transcription-polymerase chain reaction; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino} ethanesulfonic acid; Tris, tris-(hydroxymethyl)aminomethane; U, amount of enzyme that releases 1 micromol of product per min.

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