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NMDA Receptor Modulation by a Conditioned Medium Derived from Rat Cerebellar Granule Cells

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Abstract

Our previous studies have shown that the response to the excitotoxic action of glutamate by cultured cerebellar granule cells depends upon the cell density or the volume of medium in which they have been grown: the higher the cell density or the lower the volume, the higher the response to glutamate. We have hypothesized that this variable response is due to the formation in culture of a glutamate-sensitizing activity GSA more abundantly in conditioned medium derived from high-density or low-volume cultures than that present in low-density or high volume cultures and capable of restoring sensitivity in previously resistant granule cells. In order to elucidate the mechanism of action of glutamate-sensitizing activity, we measured the extent and function of NMDA receptors in low- and high-volume cultures and assessed the effect of glutamate-sensitizing activity on the same receptors. We found that under high-volume conditions the extent of MK-801 binding, the amount of NMDA receptor type 1, the currents evoked in whole cells after an NMDA pulse and the response of cultured cells to this ligand were markedly reduced compared with low-volume cultures. Addition of glutamate-sensitizing activity to high-volume cultures increased their glutamate sensitivity, the NMDA-evoked currents, the extent of MK-801 binding and the amount of NMDA receptor type 1 protein present. The corresponding mRNA transcripts, on the contrary, were unchanged in high-volume, low-volume and high-volume GSA-treated cultures.

Introduction

A wide variety of *in vitro* and *in vivo* experiments have unequivocally shown that the excitatory amino acid glutamate and the its complex receptor system play a crucial role both in neuronal functions, such as learning and memory processes, and in pathological events occurring after oxygen or glucose deprivation (Choi, 1988, 1990; Garthwaithe 1989; Garthwaithe *et al.*, 1992; Coyle and Puttfarchen, 1993). Neurotransmitter receptors, including those belonging to the glutamate family, are subject to a complex system of modulation(s) exerted at the transcriptional (Pratt *et al.*, 1993; Bessho, *et al.*, 1994; Resink *et al.*, 1995a), translational (Leahy *et al.* 1994; Resink *et al.*, 1995b) and post-translational (Marcaida *et al.*, 1995) levels. Each of these modulations may be operative during development or in the adult and is generally exerted by some ligands (hormones, growth factors, neuropeptides etc.), by an agonist co-released with the neurotransmitter or by the neurotransmitter itself (for review see Hollmann and Heinemann, 1994).

We have recently reported a peculiar type of modulation of the glutamate response (Ciotti *et al.*, 1996). It has been found that the *in vitro* response of rat cerebellar granule cells to the cytotoxic action of glutamate is not an invariant property of these neurons but changes markedly according to the density of plating or, if this parameter is kept constant, is dependent upon the volume of culture medium in which the neurons are grown. Thus, a 3- to 4-fold decrease in cell density or an increase in the volume of the culture medium of an identical 3- to 4-fold factor results in a reduced response to the otherwise lethal action of glutamate by 40–50%. We found that such a variable response is due to the production and release by cultured cells of a substance operationally defined as glutamate-sensitizing activity (GSA). Adding a conditioned medium, obtained from low-volume or high-density cultures of cerebellar granule cells, to sister cultures previously grown in a 4-fold higher volume, increases their sensitivity to glutamate by 30–50% or accelerates the response of granule cells by several days (Ciotti *et al.*, 1996).

Production of GSA is directly proportional to the mass of cultured cells and its activity is equally strictly dependent upon its concentration: if one keeps the cell mass constant, the lower the volume of medium the higher the sensitivity of the neurons to glutamate toxicity. We have also demonstrated that the action of GSA is specific, since GABAergic neurons present in the same cultures do not respond to its sensitizing action, and that it is inhibited by actinomycin D, suggesting that the effect of GSA requires transcription. Finally, it has been reported that GSA is not attributable to factors (acidic fibroblast growth factor, α-tumour necrosis factor, Tri-Iodo-L-thyronine) or substances (NMDA, glycine) previously reported to

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exert some sort of trophic or sensitizing activity. While these studies were in progress it was reported that the GABA receptor subunit of cerebellar granule cells is also under the control of some environmental cues, among which cell density of plating plays an important role (Beheringer *et al.*, 1996).

In order to elucidate the mechanism of action of GSA, we undertook a study to assess the level of action of this substance, and tried to correlate these molecular studies with a parallel analysis of the electrophysiological properties of glutamate receptors. As a first approach we focused our attention on the complex molecular system of transduction of glutamate message formed by NMDA receptors. The results obtained demonstrate that the cell mass/volume dependency of the response to glutamate by NMDA receptors is detectable at the levels of cell toxicity, of MK-801 binding to NMDA subunit, of the corresponding protein product, and of the electrophysiological properties of NMDA channel evaluated on the whole neurons. On the contrary, the mRNA coding for the NMDA receptor type 1 (NMDAR1) subunit is unchanged. Most of these diverse effects are also partially inducible by the addition of conditioned medium containing GSA.

A preliminary account of this research has been communicated (Zona *et al.*, 1995b)

Materials and methods

Cerebellar granule cell cultures and preparation of conditioned medium for GSA evaluation

Cultures enriched in granule neurons were obtained from dissociated cerebella of 8-day-old Wistar rats according to the procedure described by Levi *et al.* (1984). Cells were plated in Eagle's medium (BME, Gibco) supplemented with 10% fetal calf serum (Gibco), 25 mM KCl, 2 mM glutamine (Gibco) and 100 μg/ml gentamicin on dishes (Nunc) coated with poly-L-lysine, of various diameters (12, 35, 90 mm) according to the experimental test. In all instances the cell density was 1.0×10^6 cells per ml of BME for the low-volume condition and 1.0×10^6 per 4.0 ml of BME for the high-volume condition. Conditioned medium was prepared by growing 15×10^6 cells in 15 ml medium. After 8 days conditioned medium was removed and kept at –20°C until use.

Toxicity and neuron death

After 8 days in culture under the high- or low-volume condition, cells were washed once in Mg^{2+}-free Locke solution (154 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7,4) and exposed at room temperature to 0.1 mM glutamate or 0.5 mM NMDA, in Mg2+-free Locke solution for 30 min. After incubation with the specific agonist, cells were washed twice with standard Locke solution, and cells were returned to the incubator in the presence of the incubation medium in which cells had been grown. Twenty-four hours later, viable cells were assessed by counting the number of intact nuclei as described by Soto and Sonnencheim (1985), modified for counting cerebellar granule cells with the procedure of Volontè *et al.* (1994).

Whole-cell recording

Membrane currents were recorded from the cell soma of granules that had been cultured for 8–10 days. The whole-cell configuration of the patch-clamp method was used (Hamill *et al.*, 1981). Recordings were performed with pipettes pulled from capillary tubes with a Narishige micropipette puller. The pipettes were then fire-polished and filled with electrolytes. The electrodes had a resistance of 4–5 MΩ.

Patch-clamp electrodes were connected to a List EPC-7, List Electronic, Darmstadt, Germany, patch-clamp amplifier, which was controlled by a computer stimulation and data acquisition system (PDP-11/23-based Indec). The membrane currents were recorded, filtered, digitized at 10 kHz, and stored on a hard disk. All experiments were performed at room temperature. In order to compare the results obtained from cells cultured in the high-volume condition, the low-volume condition and the high-volume condition plus conditioned medium, we recorded granules that had similar diameter (8–10 mm) and after the same number of days *in vitro* (DIV; 8–10 days). Cell capacitance was measured for each cell from the amplifier and from transient currents produced by 10 mV hyperpolarizing voltage steps. The values of the peak current were normalized to cell capacitance and only responsive cells were included in the mean. Compensation of series resistance (50–70%) was routinely used. Different solutions were applied by gravity using small tubes (diameter <1 mm), which were placed at a distance of <0.5 cm from the patched cell. The response to a given drug started 300–500 ms after the opening of the tap that controlled drug perfusion. During the experiment, cells were continuously superfused with a control solution. Experiments were carried out in a bath medium that contained 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM glucose and 10 mM HEPES (pH 7.3 with HCl). The whole-cell recording pipette used for recording the membrane currents contained 130 mM CsCl, 20 mM tetraethylammonium chloride, 5 mM EGTA, 10 mM glucose, 10 mM HEPES, 1 mM $MgCl_2$, 0.24 mM $CaCl_2$ and 2 mM ATP (pH 7.3 with CsOH). The NMDA (0.5 mM) was dissolved in the bath medium, which was Mg^{2+}-free and contained 3 μM of glycine.

Binding of [^{3}H]MK-801 to cerebellar granule cells

[^{3}H]MK-801 binding to NMDA receptors was performed according to Damschroder-Williams *et al.* (1995) with some modifications.

Cerebellar granule cells were prepared and cultured in 24-well plates as previously described (Ciotti *et al.*, 1996). Binding was assayed at 4 DIV (2 days after addition of the conditioned medium) or at 8 DIV, 2 days after addition of the conditioned medium. The plates were rinsed twice with PBS and 0.5 ml of incubation mixture [containing PBS buffer (pH 7.4), 30 mM glycine and 10 nM [^{3}H]MK-801 (20.3 mCi/mmol; NEN Dupont)] was added to each well. Non-specific binding was determined in the presence of 10 mM unlabelled MK-801. The assay was performed at 37°C for 15 min. A preliminary series of investigations performed under three conditions (4°C, 120 min incubation; 25°C 120 min incubation and 37°C, 15 min incubation) indicated that the last of these three conditions was the most suitable for the binding assay. Binding was terminated by washing the wells three times with 1 ml ice-cold PBS. Finally, 0.5 ml of 1.0 N NaOH was added to each well to solubilize the cells. Aliquots of this suspension were used for determination of the radioactivity and the amount of protein. Specific binding was calculated as total binding minus non-specific binding. The protein concentration was measured according to Lowry *et al.* (1951).

Membrane preparation

Cultured granule cells (15×10^6 in 15 ml for the low-volume condition and in 45 ml for the high-volume condition) were rinsed with cold PBS and scraped from the culture dish in an ice-cold solution of 250 mM sucrose and 1 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 20 mg/ml aprotinin and 2.5 mg/ml pepstatin, and transferred to a glass homogenizer on ice. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fraction was removed and centrifuged at 50 000 *g* for 90 min to obtain the crude

membrane fraction. The membranes were dissolved in Laemmli's sample buffer for subsequent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Equal amounts of proteins (40, 70 and 100 Mg/lane were separated on 6.8% polyacrylamide gels. Electrophoresis was performed essentially according to Laemmli (1970). After SDS–PAGE, proteins were electroblotted onto nitrocellulose transfer membrane (Hybond-C, Amersham).

Western blot analysis

Following transfer, the nitrocellulose sheets were immersed in a solution of 5% (wt/vol) non-fat dry milk in PBS containing 0.02% Tween (blocking solution) for 2 h at room temperature. The sheets were then incubated at 4°C overnight with a polyclonal antiserum against the NMDAR1 receptor (Chemicon International Inc, Temicula, CA, USA). For detection of glutamate receptor 2/3 and of 6/7 subunits, rabbit affinity-purified antisera from UBI Upstate Biotechnology Inc., Lake Placid, USA, were used. After washing twice for 10 min with PBS containing 0.02% Tween, the membrane filters were incubated for 1 h at room temperature with horseradish peroxidase-conjugated protein A diluted 1:10 000 in PBS, 0.02% Tween. The bound conjugated protein A was visualized by the luminescence method (ECL, Amersham) according to the manufacturer's instructions. Quantification of protein bands was performed by densitometric analysis of the films. Values reported are means ± SEM and were analysed for statistical significance using a paired difference *t*-test. The significance of the influence of growth conditions was evaluated ($P < 0.05$)

Reverse transcriptase and PCR

Oligo dT-selected RNA (0.1 mg), extracted from 15×10^6 cells using a commercially available kit (Quick Prep-Micro mRNA purification kit, Pharmacia Uppsala, Sweden), was incubated with Molony murine leukaemia virus reverse transcriptase (200 U; Promega Madison, WI) in 20 ml of first strand buffer, 10 mM dithiothreitol, 0.5 mM of each deoxynucleoside triphosphate (dNTP) and 100 pmol of random examer for 90 min at 37°C. Reverse transcription products (cDNA) were used immediately, or stored at –20°C. The polymerase chain reaction (PCR) was carried out in a reaction volume of 100 ml containing 10 ml of 10× PCR buffer (Promega), 0.2 mM dNTP (Pharmacia), 25 pmol of each primer, 2.5 mCi [α-^{32}P]dCTP and 2 U of *Taq* DNA polymerase (Promega). PCR cycles were usually performed at 94°C for 60 s, 55°C for 60 s, 72°C for 90 s for NMDAR1, β-actin and GAPDH primers. PCR co-amplification of β-actin and of GAPDH mRNAs was used as a measure of RNA input and as a control for variations in the reverse transcription reaction and PCR conditions among different experiments. Increasing amplification rounds of PCR were performed to determine the range of cycle numbers at which the reactions were in the linear phase of amplification. Subsaturating cycle numbers were chosen for each primer pair and used to compensate for the different quantity of transcripts. The PCR products were electophoretically separated on 1.8% agarose gel, which was then dried and exposed to Kodak X-OMAT film overnight.

All primers were designed from published sequences. They were: NMDAR1: sense 5′-ATCTGGCCAGGAGGAGAG-3′ and antisense 5′-TTCATGGTCCGCGCCAGC-3′, amplifying sequences corresponding to nucleotides 1137–1411 of rat the NMDAR1 gene (Moriyoshi *et al.*, 1991); β-actin: sense primer, 5′-TGCTCGACAACGGCTCCGGCATGT-3′ and 5′-CCAGCCAGGTCCAGACGCAGGAT-3′ antisense primer (Salvatore *et al.*, 1995); GAPDH sense primer, 5′-CCATGGAGAAGGCTGGGGCC-3′ and antisense primer 5′-CAAAGTTGTAATGGATGACC-3′ position 351–546 of the mouse GAPDH gene (Sabath *et al.* 1990). The specificity of the NMDAR1 subunit, β-actin and GAPDH genes was confirmed by sequence analysis of the PCR products (data not shown). Quantitative analysis was performed using PhosphorImager 400A (Molecular Dynamics Inc. Sunnyvale, CA, USA) with Image Quant version 3.2 software.

Results

Reduction of glutamate and NMDA toxicity under high-volume conditions

Previous studies have demonstrated that the onset of sensitivity to glutamate of cerebellar granule cells depends upon the plating density or the medium volume in which the cells are grown (Ciotti *et al.*, 1996). In cerebellar granule cells glutamate toxicity is almost completely prevented (Atlante *et al.*, 1996) by MK-801, a specific antagonist of the NMDA type of glutamate receptor, indicating that these receptors play a crucial role in the toxicity exerted by this excitatory neurotransmitter. We therefore focused our studies on NMDA receptors and compared their properties in neurons grown in the high-volume condition, in which granule cells exhibit a much higher resistance to glutamate toxicity, with neurons grown in a low-volume condition (generally adopted in most laboratories experimenting on granule cells), in which 80–90% of neurons die after a pulse of 0.1–1.0 mM glutamate. Cells were exposed to 0.1 mM L-glutamate or 0.5 mM NMDA for 30 min and their viability was determined 24 h after exposure as described in Materials and methods. Figure 1 shows the results of these experiments. As can been seen, granule cells grown in the high-volume condition exhibited resistance both to glutamate and to NMDA which was much higher than that shown by sister

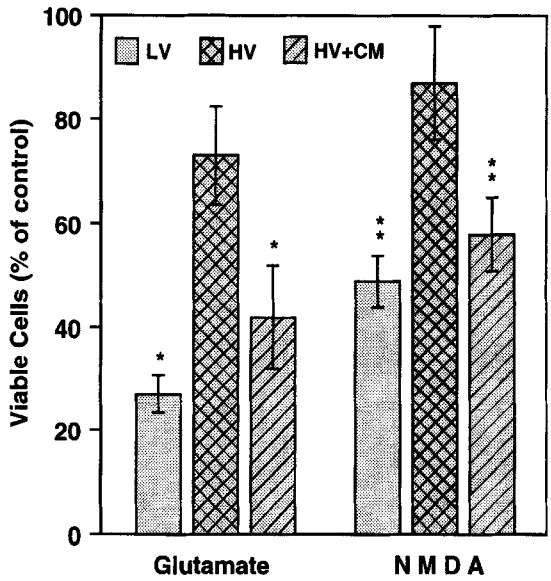


FIG. 1. Viability of cerebellar granule cells cultured in the high-volume condition (HV), the low-volume condition (LV) and the high-volume condition + conditioned medium after excitotoxic treatment with 100 μM glutamate or 500 μM NMDA. Viable cells were calculated as the ratio (%) between intact nuclei counted after the glutamate or NMDA pulse and those counted in a sister control culture. Data are the average (±SEM) of six experiments performed on six cell preparations each performed in duplicate. Statistically significant differences were calculated by one-way analysis of variance. Student's *t*-test indicated that the estimate for each culture condition in the two treated groups was significantly different from the corresponding high-volume condition. $*P = 0.000$ $**P = 0.005$.

cultures grown in low-volume conditions. In the high-volume condition the number of viable neurons after glutamate exposure was 73.0 ± 9.5 versus 27.0 ± 3.6 in low-volume conditions, and was 87.0 ± 11.0 versus 49.0 ± 5.0 after NMDA incubation under the same culture conditions. Moreover, as can be seen in Figure 1, addition of a conditioned medium obtained from low-volume cultures to sister cultures previously grown for 6 days in the high-volume condition induced a response to glutamate or to NMDA similar (although more variable in extent) to that detected in low-volume cultures. These findings, as thoroughly discussed in a previous report, demonstrate that the response to glutamate is not an invariant property of granule cells but that it varies *in vitro* and is attributable to the production and release of a hitherto unknown substance operationally defined as GSA.

Whole-cell NMDA current in low-volume, high-volume and high-volume plus conditioned medium

In order to complement and extend the neurotoxicity assays, the current in response to NMDA application was examined in cerebellar granule cells by whole-cell patch-clamp recordings employing established procedures (Hamill *et al.*, 1981).

Figure 2 shows currents recorded in whole-cell configuration of the patch-clamp technique evoked by 500 μM of NMDA in the presence of glycine (3 mM) in cerebellar granule cells grown in low-volume (Fig. 2A), high-volume (Fig. 2B) and conditioned medium (high-volume + conditioned medium, Fig. 2C). The concentration of 500 μM of NMDA was found to be saturating in all three types of cells and was therefore adopted for all measurements reported in this paper. When the three different types of cerebellar neurons were voltage-clamped at –60 mV, bath application of NMDA for 2–10 s induced inward currents in almost all tested neurons. The responses from the different cell types were blocked to a similar extent both by the NMDA antagonist D-2-phosphonopentanoic acid and at the negative potentials by Mg^{2+}, which blocks the NMDA-channel (data not shown). Current given by NMDA was detected in 16 of 16 cells cultured in the low-volume condition, in 16 of 18 cells cultured in the high-volume condition and in 19 of 21 cells cultured in conditioned medium. The NMDA-induced current, normalized to cell capacitance, in cells cultured in the high-volume condition (4.0 ± 2.2 Pico Ampere×Pico $Farad^{-1}$) was typically smaller than that induced in the low-volume condition (12.5 ± 3.8 $pAxpF^{-1}$), which is consistent with a reduction in the number and/or in the responsiveness of NMDA receptors in cells grown in the high-volume condition. Moreover, when we assessed the effect of conditioned medium we found that it was able to partially restore the NMDA-evoked current in cerebellar granule cells, previously grown for 6 days in the high-volume condition (7.0 ± 3.2 $pAxpF^{-1}$) .

Effect of growth conditions on binding of [^{3}H]MK-801

The reduced responsiveness induced by NMDA that was observed in granule cells grown in the high-volume condition might have been due to lowered expression of NMDA receptor. In order to investigate this possibility, the binding of [^{3}H]MK-801 to membranes of granule cells cultured in the low-volume or high-volume condition or in the high-volume condition with conditioned medium was measured. Two incubation times were adopted: addition of the conditioned medium to high-volume cultures at 2 DIV and measurement at 4 DIV, and addition of the conditioned medium at 6 DIV and measurement at 8 DIV. As shown in Figure 3A the binding of [^{3}H]MK-801 to membrane from low-volume cultures was 61% higher than in high-volume cultures. In the presence of conditioned medium [^{3}H]MK-801 binding increased by 143% over that in high-volume cultures, indicating increments even higher than those detectable under the low-volume condition. If analogous binding experiments were performed at 8 DIV (Fig. 3B) binding of MK-801 was 71% higher in low-volume than in the high-volume cultures. The finding that total binding was slightly lower

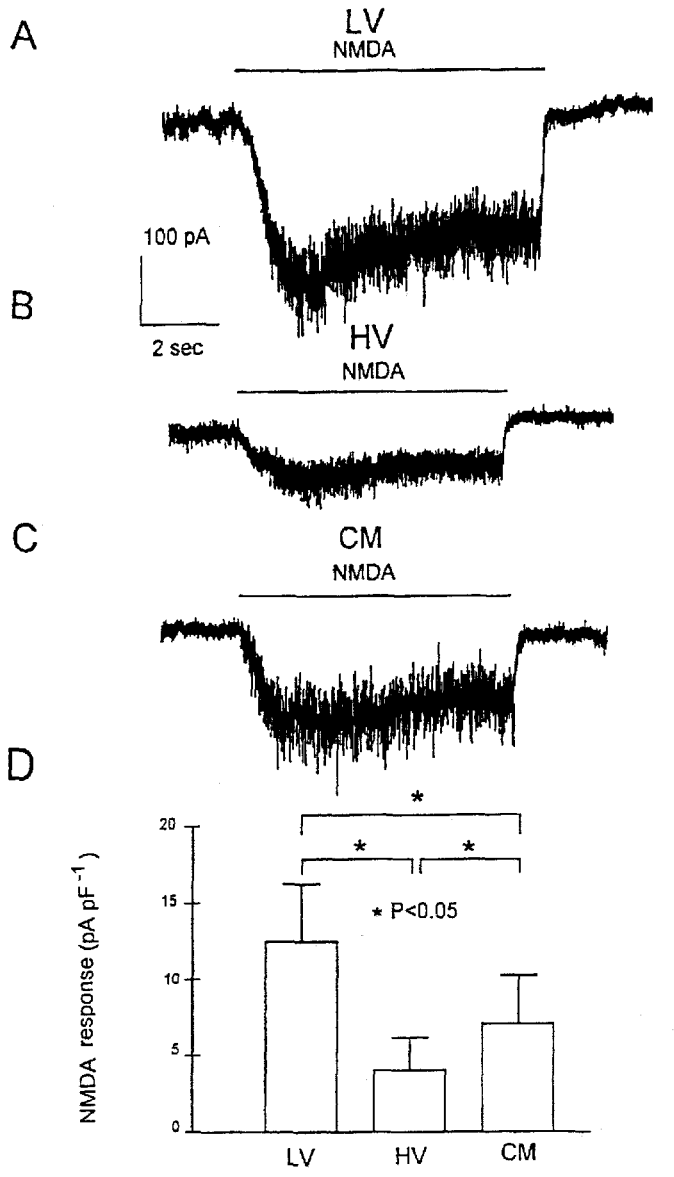
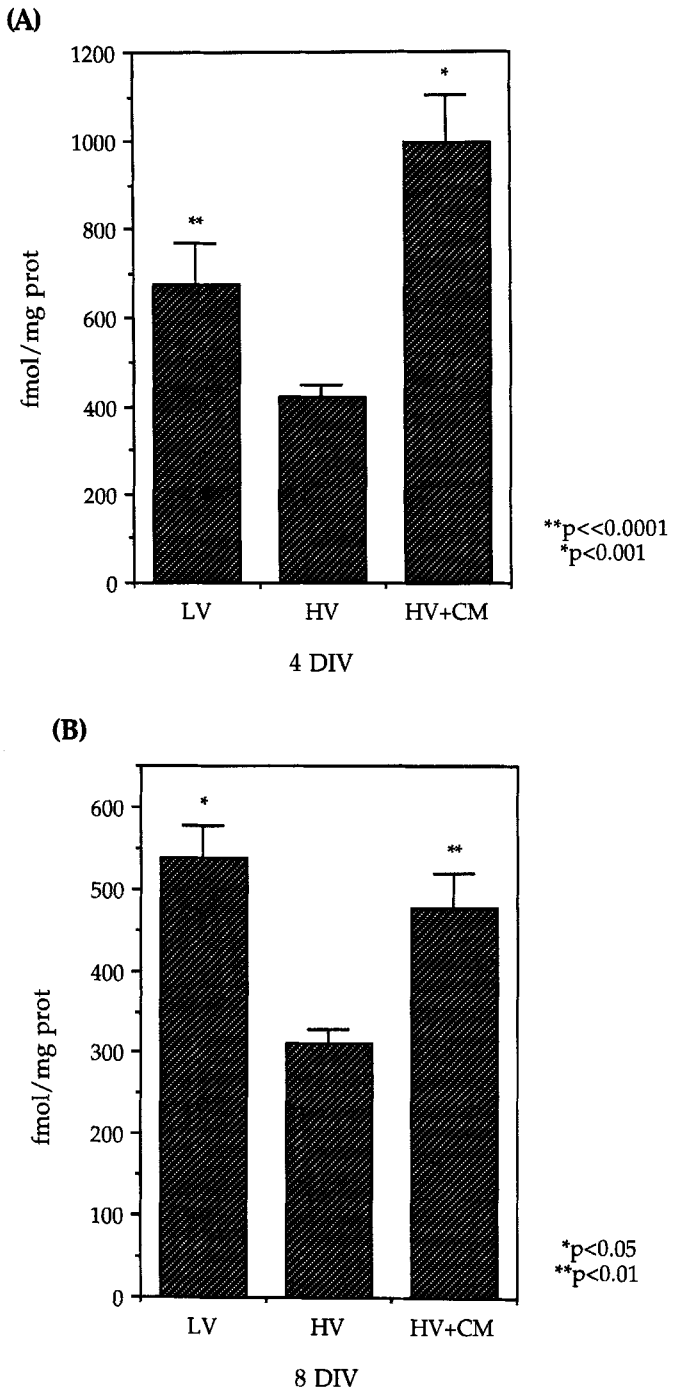


FIG. 2. Representative whole-cell response to NMDA (500 μM) recorded at –60 mV in the presence of glycine (3 μM) from cells cultured in the low-volume condition (LV; A), the high-volume condition (HV; B) and the high-volume condition + conditioned medium (CM; C). Bar indicates duration of NMDA application. (D) Histogram of data on peak current amplitude normalized to cell capacitance from the three cell types. The response of cells cultured in the low-volume condition was significantly greater than that of cells cultured in the high-volume condition ($P < 0.05$, analysis of variance), and the response of cells cultured with conditioned medium was significantly greater than that of cells cultured in the high-volume condition without conditioned medium ($P < 0.05$), although the current did not attain the level reached in the low-volume condition. P values were calculated by analysis of variance followed by a Student–Newman–Keuls test. The data were derived from five cell preparations.

at 8 DIV than at 4 DIV while glutamate and NMDA sensitivities were higher at this time could be due to the fact that the expression of NMDAR1, which is responsible for MK-801 binding, is not fully consistent with the functional expression of the whole subunits responsible for mediating the toxic action of NMDA and glutamate. Alternatively, the availability of the site responsible for binding was lower at 8 DIV, although the actual content in the membrane was identical or event higher at this time of development in culture.

As can be seen in Figure 3, addition of conditioned medium caused an increase of 52% over the high-volume conditions. The effect of conditioned medium was therefore much higher when it was added at 2 DIV than when it was added at 6 DIV. The possible reasons for this difference will be discussed below and it was probably the consequence of several causes.



Effects of growth conditions on NMDA receptor protein and mRNA levels

The decreased response to NMDA and the lowered binding of [^{3}H]MK-801 to the NMDA binding site detected in cells grown in the high-volume condition could have been due to a different conformational state, to some post-translational modification or to a decreased number of binding sites. To test the last possibility, polyclonal antibodies raised against C-terminus of the obligatory NMDAR1 subunit protein of NMDAR1 were used. The antibodies stained a single protein band of 115 kDa. (Fig. 4) The quantitative evaluation of western blots, performed at three concentrations, showed that the relative amount of the NMDAR1 subunit was reduced in membrane preparations from cells grown in high-volume culture by 44% compared with cells grown in low-volume culture (55.0 ± 4.6 versus 100, $P < 0.05$). Figure 4 also shows that the conditioned medium had a partial but statistically significant influence on the amount of NMDAR1 of high-volume cultures since it increased the amount of protein detectable under the high-volume conditions alone by 40%.

In order to assess whether the differential expression of glutamate receptor was also exerted on other types of glutamate receptors, we measured the levels of AMPA(α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)/kainate subunits detected with two polyclonal antibodies cross-reacting with glutamate receptors 2/3 and 6/7 under the same experimental conditions. As can be seen in Figure 5, no changes in the protein levels of these subunits were detected either in low-volume or high-volume cultures. Thus, since no difference was found under these two opposing conditions, tests on the possible action of conditioned medium were not undertaken.

In order to better understand the mechanism underlying the effect of growth condition, we determined the level of mRNA coding for the NMDAR1 receptor subunit by semiquantitative PCR analysis. The level of expression of NMDAR1 was measured as the ratio between the amplification product of this mRNA and that of the β-actin or GAPDH mRNA products amplified in the same reaction in samples derived from low- and high-volume cultures. As can be seen in Figure 6, no considerable differences under the two culture conditions were observed as far as this mRNA was concerned, suggesting that the reduced level of

FIG. 3. Effect of growth conditions on the binding of [^{3}H]MK-801 in primary cultures of cerebellar granule cells. The binding assay was performed at 4 DIV after 2 days of incubation with the conditioned medium (A) or at 8 DIV after 2 days of incubation with the conditioned medium (B), as described in Materials and methods. Specific binding was calculated after subtraction of the non-specific binding measured in the presence of a 1000-fold excess of the unlabelled ligand. Triplicate tests were run for each binding test for total binding and duplicate tests were run for non-specific binding. At 4 DIV binding differed significantly (A) between the high- and low-volume conditions ($P < 0.0001$) and between the high-volume condition and the high-volume condition + conditioned medium ($P < 0.001$). Binding of [^{3}H]MK-801 to membranes from cerebellar granule cells at 8 DIV (B) grown in the high-volume conditions was significantly different ($P \leq 0.05$) from that for cells grown in the low-volume condition and the high-volume condition + conditioned medium ($P < 0.01$).Values reported represent means and SEM of 26 experiments (B) and of seven experiments (A), each performed with a different cell preparation.

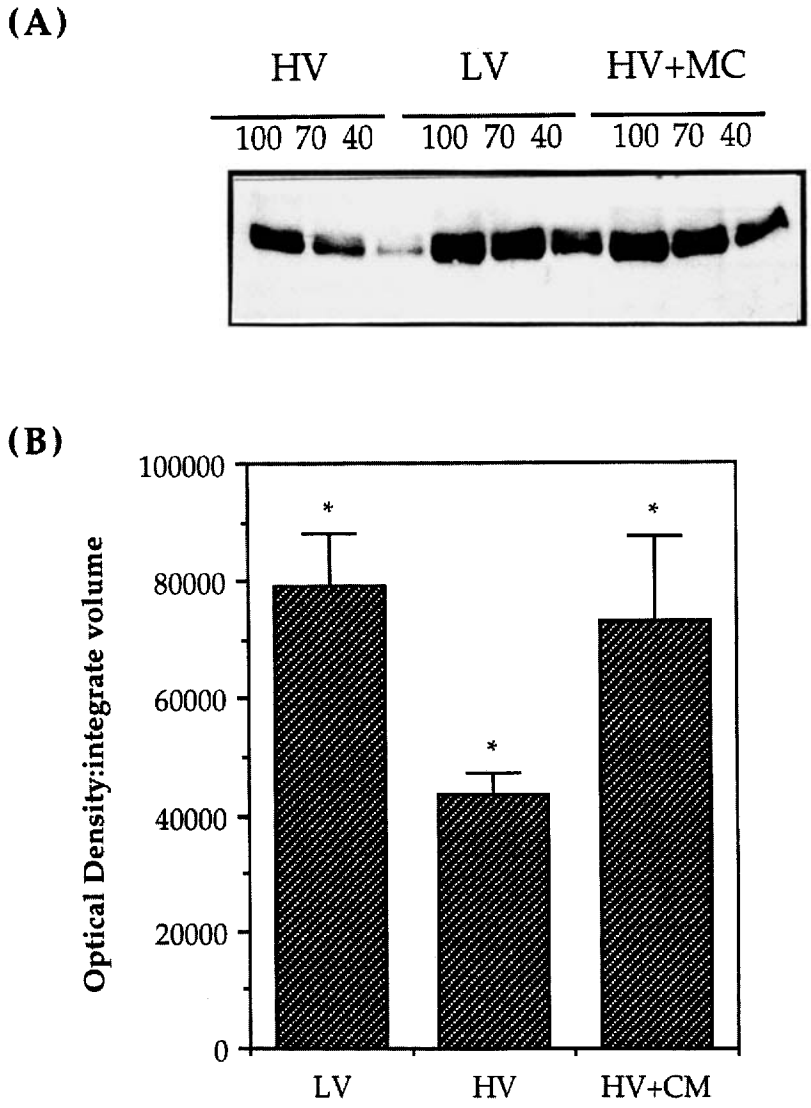


FIG. 4. (A) Western blotting analysis of NMDAR1 receptors subunit in cerebellar granule cells. Cerebellar granule cells were grown under the high- or low-volume condition for 6 DIV, when conditioned medium was added to some of the high-volume cultures. Cells were further incubated for 2 DIV and subsequently harvested for western blotting analysis. NMDAR1 were determined using a quantitative immunoblotting assay as described in Materials and methods. The figure shows a representative autoradiogram from an immunoblot in which membranes (40, 70 and 100 mg/lane) from cells grown in the low-volume condition, the high-volume condition or the high-volume condition + conditioned medium were loaded. (B) Estimates calculated from data in A are expressed as integrated volumes of optical densities, and represent the average of 13 experiments each run in duplicate (four experiments) or in triplicate (nine experiments) on different cell preparations, as shown in this figure. Low-volume versus high-volume condition, $P < 0.01$; high-volume condition + conditioned medium versus high-volume condition, $P < 0.05$ (see Materials and methods for details of statistical analysis).

NMDAR1 revealed by western blotting presumably does not involve a decrease in transcriptional rate of the mRNA coding for this subunit.

Discussion

Modulation of functional expression of NMDA receptors in low- and high-volume conditions

The data presented clearly show that culturing cerebellar granule cells under low or high-volume conditions modulates their response to glutamate and to NMDA. Such modulation can be the consequence of an altered concentration of NMDA receptors on the surface of cerebellar granule cells or of a different function of an equal number of these receptors. In order to clarify this problem we undertook a series of experiments centred on different types of measurements. The results obtained demonstrate that the volume-dependent modulation of NMDA receptors is observed at the level of the neurotoxicity assay, of NMDA-elicited currents, of the binding of a specific NMDA antagonist such as MK 801, and at the level of protein content. On the contrary, no substantial difference is observed at the level of the mRNA coding for one of the subunits constituting NMDA receptors (NMDAR1). These findings indicate that the differential sensitivity to glutamate and to NMDA of high- and low-volume cultures is most probably due to differential expression of NMDAR1. A discrepancy between NMDAR1 mRNA and protein level similar to that observed in this study has been described in other conditions involving the exposure of granule cells to NMDA (Resink *et al.*, 1995a, b). It appears unlikely that such a discrepancy is due to a general decrease

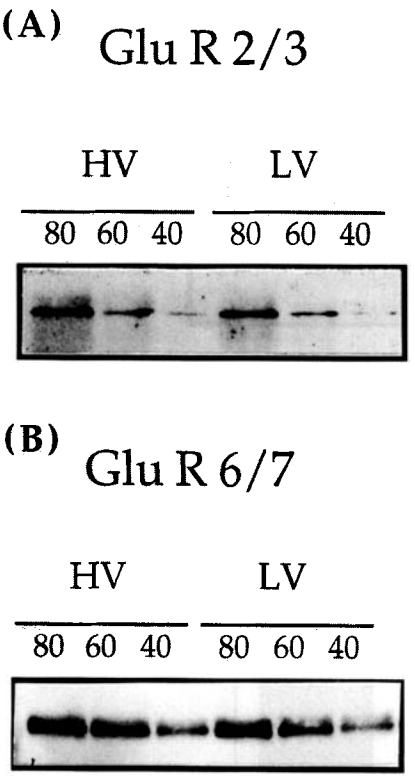


FIG. 5. Western blotting analysis of glutamate receptor 2/3 (GluR2/3) (A) and GluR6/7 (B) under low-volume (LV) and high-volume (HV) conditions. The assay was performed on the same membrane preparations as those used for the experiments reported in Figure 4, with three protein quantities (80, 60, 40 μg). Notice that the intensity of the bands is identical under low- and high-volume conditions. This conclusion was supported by optical density analysis. The autoradiogram is representative of six experiments performed on different cell preparations.

in the rate of protein synthesis in cells grown in the high-volume condition, as indicated by the lack of effect of culture condition on the subunit content of AMPA/kainate receptors.

Since previous studies (Ciotti *et al.*, 1996) have indicated that actinomicin D largely prevents the glutamate-sensitizing activity of GSA, we must conclude that the action of this transcriptional inhibitor is not exerted at the level of mRNA coding for NMDAR1 but could involve some splice variants of NMDAR1, some subunit of NMDAR2 or some other step(s) of GSA action.

In conclusion, our data suggest that the regulation of the NMDAR1 subunit under high- and low-volume culture conditions involves mRNA translation and possibly post-translational modifications. The diverse kinds of transcriptional, post-transcriptional and transductional modulation of NMDAR1 receptors, as well as of other types of glutamate receptors, has considerably broadened the types of modulation(s) that could contribute to differential glutamate sensitivity

The NMDA receptor channel is a hetero-oligomer structure composed of a NMDAR1 subunit and one or more NMDAR2 subunits (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992; Kutsuwada *et al.*, 1992). Although the NMDAR1 subunit is common to all NMDA receptors, the NMDAR2 subunit exhibits a variable distribution, which is believed to be the basis of the functional diversity of NMDA receptors in the brain (Monyer *et al.*, 1992). The results obtained in this study demonstrate that the NMDAR1 subunit is differentially expressed under low- and high-volume culture conditions, but we cannot exclude the possibility that differences in NMDA receptor composition could involve changes in NMDAR2 subunit type and possible post-translational modifications. It is noteworthy in this connection that protein glycosylation is important in neocortical neurons for the functional expression of voltage-activated channels during early stages of cell growth in culture (Zona *et al.*, 1990).

The different amplitude of the current evoked by NMDA in the low- and high-volume conditions could be due either to different conductance of a similar number of channels or to different numbers of channels, each having similar conductance. The results obtained with the western blot experiments reported in Figure 4 and the results on MK-801 binding shown in Figure 3 favour this latter hypothesis, although other possibilities cannot be ruled out. Thus, previous studies (Marcaida *et al.*, 1995) have reported that the extent of MK-801 binding can be markedly influenced by post-translational modifications of an equal number of NMDA receptors; moreover, the extent of MK-801 binding is a measure of the amount of NMDAR1 subunits exposed on the external surface of the membrane and not, as the western or northern blot experiments suggest, of the total cellular or protein content mRNA of this subunit. Our data, therefore, could indicate that the amount facing the outside environment and available for MK-801 binding is markedly different. Alternatively, they could indicate that the currents and the MK-801 binding are different due to post-translational modifications of an equal number of NMDAR1 subunits conferring different permeability and different binding capacity. Future studies should make it possible to discriminate among these possibilities.

Effect of conditioned medium containing GSA on NMDA receptors

The data on NMDA currents (Fig. 2) clearly indicate that under the high-volume condition currents are 70% reduced with respect to current elicited in cells grown in low-volume and that GSA present in conditioned medium of low-volume cultures is capable of restoring significantly, but not totally, the NMDA currents detectable under the low-volume condition. An analogous situation occurs when we assess the action of GSA in conferring sensitivity to glutamate or to NMDA on previously resistant cultures grown under high-volume conditions (Fig. 1). Moreover, the binding experiments performed with the specific antagonist MK-801 demonstrate that the differential response of high-volume cultures to GSA is more pronounced when the conditioned medium is added at 2 rather than 6 DIV. This finding suggests that the action(s) of GSA mainly, if not exclusively, consists of accelerating the acquisition of a glutamate-sensitive phenotype. According to this view, addition of a conditioned medium containing GSA at 2 DIV results in accelerated maturation with exposure of glutamate receptors and acquisition of a glutamate-sensitive phenotype. Such acceleration is more pronounced at 2 DIV since, at this time, cells are still in the linear phase of glutamate receptor exposure. When, on the contrary, GSA is added at 6 DIV, cerebellar granule cells have already partially acquired this phenotype—also due to the contribution of GSA released by the growing neurons—and the action of externally added GSA is much less pronounced.

The lesser action of externally added GSA on NMDA currents and on NMDAR1 content detected in the western blot experiments compared with that revealed in the low-volume cultures was probably due to experimental conditions. Incubation of externally added GSA lasts, for experimental reasons, for 2 DIV rather than for the whole period of incubation under the low-volume condition, which is 8 DIV. Moreover, the necessary manipulations for the conservation of the conditioned medium after its collection may lead to partial loss of its activity. Finally, studies now in progress demonstrate that the

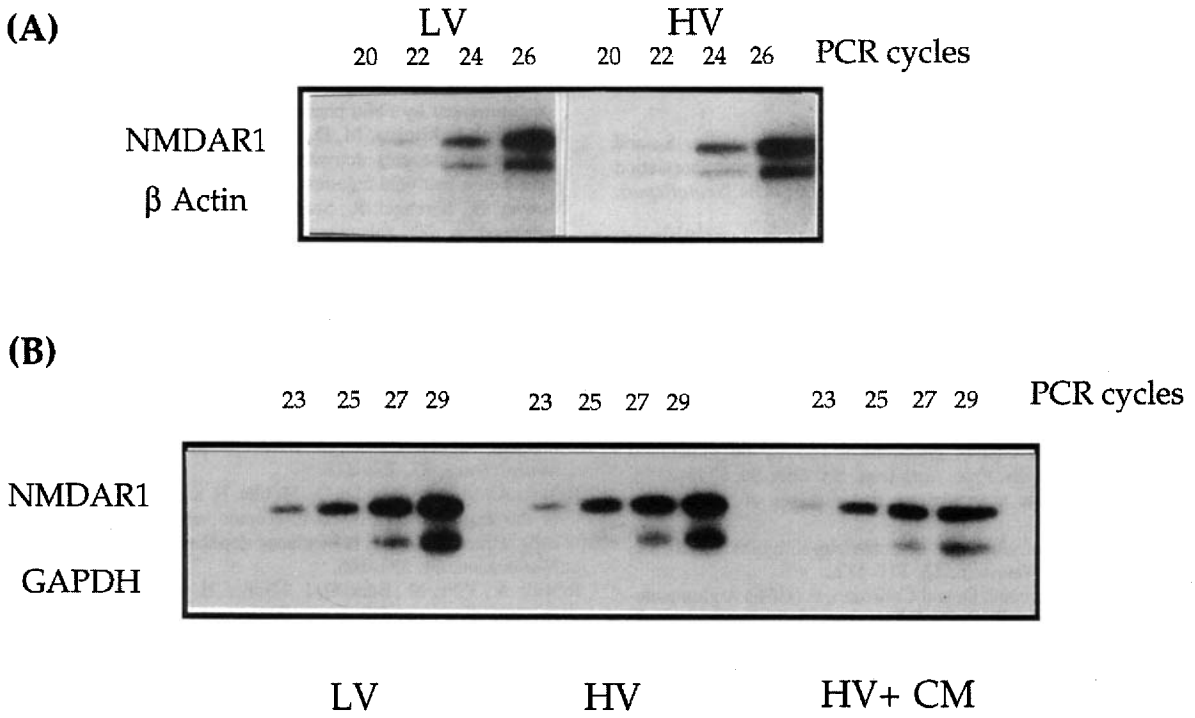


FIG. 6 Autoradiogram showing the effect of growth condition on NMDAR1 mRNA. RNA isolated from cerebellar granule cells grown in the low-volume condition (LV), the high-volume condition (HV) and the high-volume condition + conditioned medium (CM) was analysed for the presence of the NMDAR1 subunit mRNA by a semiquantitative reverse-transcription-coupled polymerase chain reaction. The expression level of NMDAR1 mRNA was measured as the ratio between the amplification product of this mRNA and the β-actin mRNA (A) or the GAPDH enzyme (B) product amplified in the same reaction. This experiment was performed three times for β-actin and twice for GAPDH on different cell preparations.

conditioned medium also acts on kainate receptors and voltage dependent sodium channels (Zona *et al.*, manuscript in preparation), suggesting that its sensitizing action is actually the resultant of several distinct effects exerted at the level of not only NMDA receptor but also of other membrane or submembranous structures involved in the cytotoxic response. Thus, measures of a single component of the NMDA receptor, such as NMDAR1, do not embrace the whole effect exerted by GSA on the response to NMDA toxicity or cell currents elicited by this ligand. It is of particular interest in this connection that the functional expression of NMDA receptors in granule cells has been found to parallel the appearance of voltage-operated sodium channels under two experimental paradigms: induction of glutamate sensitivity in previously resistant granule cells (Calissano *et al.*, 1993; Zona *et al.*, 1993); and in the present set of experiments under low- and high-volume conditions (Zona *et al.*, in preparation). This finding is in agreement with the notion that the synchronous expression of these two sets of channels—ligand- and voltage-operated—is instrumental in the proper functioning of NMDA channels, since their full opening and function is strictly dependent upon the depolarization of the membrane in which they operate. The finding that these two types of channels are co-expressed suggests in turn that GSA message is intracellularly addressed to up-regulate the expression of NMDA receptors and of voltage-operated Na^+ channels, or that the effect of the conditioned medium is due to more than one single substance acting on different signal transduction pathways.

Production of GSA

Insulin-like growth factor-1 (IGF-1) has been previously reported to confer glutamate sensitivity on previously resistant cerebellar granule cells (Calissano *et al.* 1993). When the presence of a GSA in the conditioned medium had been detected, it was initially hypothesized that it could have been due to *in vitro* production by cultured cerebellar granule cells of IGF-1. Subsequent studies, however, have demonstrated that such sensitizing activity is not directly due to IGF-1 but rather to the production and release in culture medium of GSA due to the presence of a trophic factor such as IGF-1. In other words, this somatomedin would exert a pleiotrophic action on these neurons allowing the production of GSA (Ciotti *et al.*, 1996). The greater the amount of cerebellar neurons that survive due to such pleiotrophic action of IGF-1, the larger the amount of GSA produced and released in culture. Since IGF-1 has been shown to have the most effective trophic action on cerebellar granule cells among the several growth factors tested (Calissano *et al.* 1993; Ciotti *et al.*, 1996), its presence would allow the greatest production of GSA.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
DIV	days *in vitro*
GSA	glutamate-sensitizing activity

NMDAR1 NMDA receptor type 1
PCR polymerase chain reaction

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