Amino Acids (2002) 23: 19–25
DOI 10.1007/s00726-001-0104-8

Amino
Acids
Printed in Austria

Inhibition of phosphatidylcholine synthesis is associated with excitotoxic cell death in cerebellar granule cell cultures

T. Gasull, N. DeGregorio-Rocasolano, M. Enguita, J. M. Hurtan, and **R. Trullas**

Neurobiology Unit, Institut d'Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas (IDIBAPS), Barcelona, Spain

Received June 29, 2001
Accepted August 6, 2001
Published online June 3, 2002

Summary. Glucose deprivation (GD) enhances the sensitivity of cerebellar granule cells to die by excitotoxicity. Neither 70 min of GD, a treatment that depletes cell energy resources, nor exposure to 20 μM glutamate (GLU) for 30 min, induce significant cell death in cultures of cerebellar granule cells. However, the combined treatment with GLU and GD induces choline (Cho) release before excitotoxic cell death. We investigated whether the neurotoxic effect of this treatment is related with inhibition of phosphatidylcholine (PC) synthesis. We found that exposure to GLU for 30 min, to GD for 70 min, and to the combination of both, inhibited PC synthesis at the end of treament by 71%, 92% and 91%, respectively. The inhibition of PC synthesis was accompanied by a decrease in the incorporation of [^{3}H]Cho into phosphocholine and by an increase of the intracellular content of free [^{3}H]Cho, indicating that these treatments inhibit the synthesis of PC by inhibiting choline kinase activity. However, only the combined treatment with GLU and GD induced a prolonged inhibition of PC synthesis that extented after the end of treatment. These results show that excitotoxic death is associated with sustained inhibition of PC synthesis and suggest that this effect of the combined treatment with GLU and GD on PC synthesis is produced by an action on an enzymatic step downstream of choline kinase activity.

Keywords: Excitotoxicity – Neuronal death – Phosphatidylcholine – Glutamate

Introduction

We have previously reported that overactivation of N-methyl-D-aspartate (NMDA) receptors releases choline (Cho) and inhibits the incorporation of [^{3}H]Cho into phosphatidylcholine (PC) in cortical cell cultures (Gasull et al., 2000, 2001). The release of Cho by NMDA begins immediately after exposure to neurotoxic concentrations of this drug and is directly related with excitotoxic necrotic, but not apoptotic, cell death (Gasull et al., 2000). The effect of NMDA receptor overactivation on Cho release is not exclusive of cortical neurons. Thus, exposure to neurotoxic concentrations of glutamate (GLU) also induces Cho release in energy deprived cerebellar granule cells (Gasull et al., 2000). In these cells, excitotoxicity by GLU is completely blocked by antagonists of the NMDA subtype of GLU receptors (Novelli et al., 1988; Schramm et al., 1990). All these previous results indicate that NMDA receptor overactivation induces Cho release by inhibiting PC synthesis. However, the mechanism by which NMDA inhibits PC synthesis is still unknown. Moreover, a cause-effect relationship between inhibition of PC synthesis by NMDA and necrotic cell death remains to be stablished.

The major route of PC synthesis in mammalian non-hepatic cells is the CDP-choline pathway, also named Kennedy pathway (Kennedy, 1989; Vance, 1991). This pathway involves three enzymatic steps: phosphorylation of Cho to phosphocholine, conversion of phosphocholine to CDP-choline and transfer of the phosphocholine moiety of CDP-choline to diacylglycerol (DAG). These three steps are catalysed by the sequential action of choline kinase (CK), CTP : phosphocholine cytidylyltransferase (CT) and CDP-choline, 1,2-diacylglycerol cholinephosphotransferase (CPT). The steps catalyzed by CK and CTP require the energy donors ATP and CTP, respectively.

Granule cells are resistant to excitotoxic cell death (Meldrum and Garthwaite, 1990). However, energy deprivation renders these cells susceptible to excitotoxicity by GLU (Novelli et al., 1988). We previously reported that neither exposure to 20 μM GLU for

30 min nor 70 min of GD, a treatment that depletes cell energy resources, induces significant Cho release or cell death in cerebellar granule cells. Only the combined treatment with GLU and GD induces both Cho release and cell death in these cells (Gasull et al., 2000). In the present study we investigated whether the neurotoxic effect of this combined treatment is related with inhibition of PC synthesis. To do so, we measured the effect of GD, exposure to GLU alone and the combined treatment with GLU and GD on the incorporation of [^{3}H]Cho into PC and its anabolites in cerebellar granule cells.

Material and methods

Cell culture

Primary cultures of cerebellar granule neurons were prepared essentially as described previously (Viu et al., 1998). Cerebellum was dissected from 7-day postnatal Harlan Sprague-Dawley rat pups and cells were chemically dissociated in the presence of trypsin and DNase I. Cells were seeded in poly-L-lysine-coated (100 μg/ml) 24 well plates at a density of 6×10^5 cells/cm^2. Cells were plated and grown in basal Eagle´s medium supplemented with 0.1 mg/ml gentamicin, 2 mM L-glutamine, 25 mM KCl and 10% heat-inactivated fetal bovine serum. Cultures were incubated at 37°C in a 5% CO_2, 95% air atmosphere. Cytosine β-D-arabinofuranoside (10 μM) was added 24 h after seeding and medium remained unchanged until experiments were performed (8 days in vitro).

Materials

Glutamic acid monosodium salt, propidium iodide, digitonin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, phosphatidylcholine, choline, cytidine 5′-diphosphocholine sodium salt, trypsin, DNase I, poly-L-Lysine, cytosine-β-arabinofuranoside and L-glutamine were from Sigma (Madrid, Spain). Basal Eagle´s medium, gentamicin and fetal bovine serum were from Life Technologies, Inc (Barcelona, Spain). TLC silica gel 60 plates 20 × 20 cm and HPTLC silica gel-60 plates 10 × 20 cm, with concentrating zone were from Merck (Darmstadt, Germany). [methyl-^{3}H]choline chloride was from NEN (Boston, USA).

Treatments

Cells were preincubated for 40 min in 300 μl of modified Locke-Hepes buffer without Mg^{2+} (MLH) (154 mM NaCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM KCl, in 5 mM Hepes buffer, pH 7.4) with or without 5.6 mM D-glucose. Control and energy-compromised cells were then exposed to either 20 μM GLU or vehicle for 30 min. To determine PC synthesis during exposure to the treatments, [^{3}H]Cho (1 μCi/well) was added simultaneously with GLU and cells were harvested at the end of treatment. After treatment, cells were washed twice with 1 ml aliquots of Locke-Hepes buffer (LH) (154 mM NaCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM KCl, 5.6 mM D-glucose, 1.2 mM Mg^{2+} in 5 mM Hepes buffer, pH 7.4), conditioned media was added and cells were returned to the incubator. Neuronal death was measured 30 min, 1 h or 24 h after the end of glutamate exposure. To determine PC synthesis after the end of treatments, [^{3}H]Cho (3 μ Ci/well) was added 30 min after the addition of conditioned medium.

Cell viability studies

Cell death was assessed using Propidium Iodide (PI), a compound that is excluded by the plasma membrane of viable cells. PI fluorescence was measured in 24 well plates using a CytoFluor 2350 scanner (Millipore, Barcelona, Spain) with 530 nm (25 nm band pass) excitation and 645 nm (40 band pass) emission filters. The percentage of non-viable cells was measured using a modification of the method described by Rudolph et al. (1997). In brief, a background fluorescence reading F(−1) was obtained immediately after addition of PI (30 μM) to the medium of undisturbed cells. Conditioned medium containing PI was removed and saved before the addition of treatments. After the end of treatments, cells were incubated in its own conditioned medium and fluorescence readings were obtained at different times. At the end of the experiment, cells were permeabilized by incubating with 375 μM digitonin for 10 min at 37°C to obtain the maximum fluorescence corresponding to 100% of cell death (f(max)). Percentage cell death was expressed as $\%F(max) = 100 * (X - F(-1))/(F(max) - F(-1))$, where X is fluorescence at any given time. Cells were kept in the incubator between measurements.

MTT assay

Mitochondrial enzymes have the capacity to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into the insoluble product formazan. Therefore, MTT assay provides an indication of mitochondrial metabolic function. MTT assay was performed at different times during and after GD following the procedure previously described by Atabay et al. (1996). In brief, cells were incubated for 30 min at 37°C with 1 ml of LH containing 0.4 μM MTT. The buffer was then removed and 300 μl of dimethyl sulphoxide were added to extract the insoluble blue formazan complex. Optical density of samples was measured at 490 and 630 nm and MTT production was calculated as the difference between the optical density obtained at 490 nm and that obtained at 630 nm. Values are expressed as percent of control cells.

Extraction of lipids and water-soluble metabolites

After treatment, the incubation buffer was removed, methanol was added and the methanolic cell suspension sonicated. Lipid extraction was performed by adding chloroform, incubating 15 min at room temperature and adding H_2O (final proportion of methanol/chlorofom/H_2O, 6 : 6 : 5, v/v/v). To separate the aqueous and the lipidic phases tubes were shaken vigorously and centrifuged (5 min at 2,000 ×g). The upper aqueous phases containing radioactive soluble metabolites and the lower organic phases containing radioactive lipids were transferred to new tubes. The lipids present in the organic phase and the compounds present in the upper aquous phase were dried under vacuum centrifugation.

Analysis of PC by thin layer chromatography (TLC)

Lipids were dissolved in 10 μl of chloroform/methanol (4 : 1, v/v) and spotted onto silica gel plates. [^{3}H]Cho PC was separated in silica gel plates (HPTLC silica gel-60 plates 10 × 20 cm, with concentrating zone, Merck) using the solvent chloroform/methanol/acetic acid/water (75 : 45 : 3 : 1, v/v/v/v). The spots corresponding to PC were detected by exposure to iodine and identified by co-migration with standards. The spots identified were scraped and the radioactivity was measured.

Analysis of water soluble metabolites of the Kennedy pathway by TLC

Water soluble metabolites of the Kennedy pathway were dissolved in 10 μl of ethanol/H_2O (1 : 1, v/v), spotted onto silica gel plates (TLC silica gel-60 plates, 20 × 20 cm, Merck) and separated using the solvent methanol/0.9%NaCl/ammonia (50 : 50 : 5, v/v/v). In this chromatographyc system [^{3}H]Cho, [^{3}H]phosphocoline and [^{3}H]CDP-choline migrate with Rf values of 0.07, 0.33 and 0.55, respectively. The areas corresponding to [^{3}H]Cho, [^{3}H]phosphocoline and [^{3}H]CDP-choline in the silica gel plate were scrapped and the radioactivity was measured.

Data analysis

Results are expressed as mean ± S.E. Statistical significance of differences was examined using independent *t* tests.

Results

GLU induces cell death in cerebellar granule cells exposed to GD

Exposure of cerebellar granule cells to 20 μM GLU for 30 min, GD for 70 min, or the combined treatment with GLU and GD did not induce significant cell death one hour after the end of treatment (Fig. 1A). Likewise, treatment with 20 μM GLU for 30 min or GD for 70 min did not induce significant cell death 24 h after treatment when compared with control cells. In contrast, the combined treatment with GLU and GD killed 60% of the cells 24 h after treatment (Fig. 1B).

Exposure to GLU, GD or the combination of both treatments inhibits the incorporation of [^{3}H]Cho into PC when measured at the end of treatment

Previous results have shown that only the combined treatment with GLU and GD induce Cho release (Gasull et al., 2000). To determine whether the Cho release and the cell death evoked by the combined treatment with GLU and GD are related with inhibition of PC synthesis, we measured the incorporation of [^{3}H]Cho into PC in granular cell cultures treated with 20 μM GLU for 30 min, GD for 70 min or a combination of both treatments. We found that exposure to 20 μM GLU alone for 30 min, GD for 70 min, and the combination of both treatments inhibited PC synthesis when measured at the end of treatment by 71%, 92% and 91%, respectively. The inhibition of PC synthesis by glutamate was more pronounced in the absence of glucose (Fig. 2).

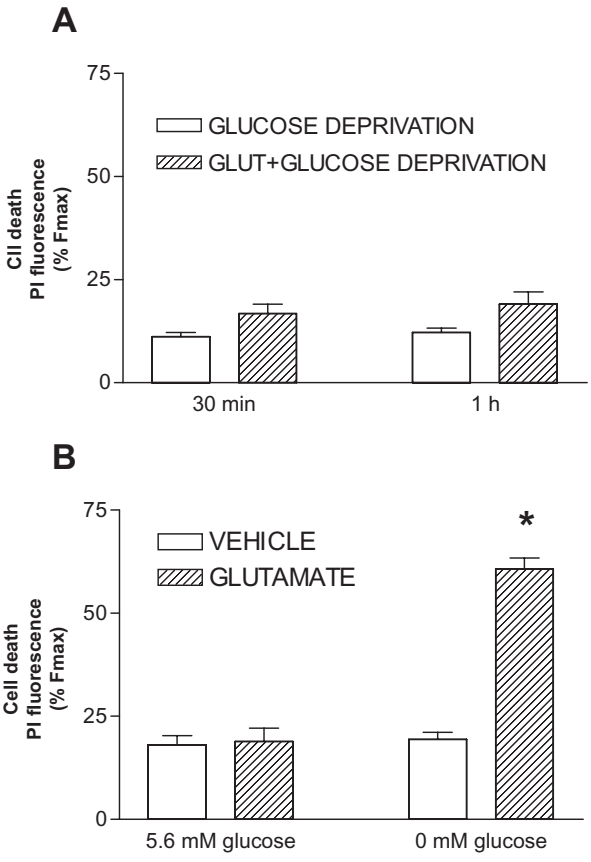

Fig. 1. Effect of GLU, GD or the combination of both treatments on cell death in cerebellar granule cells. Cerebellar granule cells were preincubated for 40 min in 300 μl of MLH containing 0 or 5.6 mM D-glucose. Cells were then incubated for 30 min in the presence of either 20 μM GLU or vehicle. After treatment, conditioned medium was added and cell death was measured 30 min and 1 h after the end of treatments (**A**) or 24 h after the end of treatments (**B**). Data are mean ± SEM of three experiments. * Significantly different from control cells ($p < 0.05$)

Exposure to GLU, GD or the combination of both treatments inhibits the incorporation of [^{3}H]Cho into phosphocholine and CDP-choline when measured at the end of treatment

To investigate which reaction of the Kennedy pathway is inhibited by treatment with GLU, GD or the combination of both treatments, we measured the incorporation of [^{3}H]Cho to phosphocholine and CDP-choline immediately after the end of treatment. We found that exposure to 20 μM GLU for 30 min, GD for 70 min or 20 μM GLU + GD decreased the incorporation of [^{3}H]Cho into phosphocholine by 77%, 93% and 96%, respectively, and the incorporation of [^{3}H]Cho into CDP-choline by 76%, 82% and 80%, respectively (Fig. 3B and 3C). In addition, exposure to 20 μM

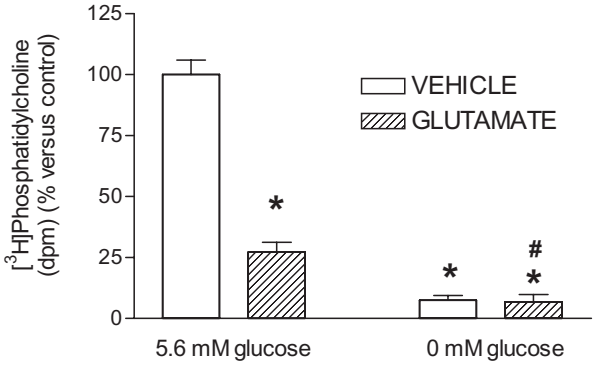

Fig. 2. Exposure to GLU, GD or the combination of both treatments inhibits the incorporation of [^{3}H]Cho into PC when measured at the end of treament. Cerebellar granule cells were preincubated for 40 min in 300 μl of MLH with 0 or 5.6 mM D-glucose and subsequently exposed for 30 min to either 20 μM GLU or vehicle in the presence of [^{3}H]Cho (1 μ Ci/well). At the end of treatment, cells were obtained and processed to obtain the lipidic fraction. PC was separated from the other lipids by TLC and the radioactivity incorporated into PC was determined. Data are mean ± SEM of three experiments. * Significantly different from control cells ($p < 0.05$); # Significantly different from cells exposed to glutamate in the presence of 5.6 mM glucose ($p < 0.05$)

GLU alone or GD alone increased threefold the intracellular content of free [^{3}H]Cho whereas exposure to 20 μM GLU + GD increased seven times the intracellular content of free [^{3}H]Cho (Fig. 3A).

GD decreases mitochondrial activity. This effect is reversed after the addition of conditioned medium

Mitochondrial enzymes transform MTT into the insoluble product formazan and, thus, MTT levels provide an indication of mitochondrial metabolic function. In addition, a decrease in MTT metabolism correlates well with the decrease in energy level observed after treatment with glutamate (Ankarcrona et al., 1995). We found that 70 min of GD decreased mitochondrial activity in cerebellar granule cells as measured by a 67% decrease in the ability of these cells to metabolise MTT to formazan. However, 15 min after the addition of glucose the ability of cerebellar granule cells to metabolise MTT to formazan is decreased only by 25% (Fig. 4). Therefore, mitochondrial activity recovers soon after the addition of conditioned medium containing glucose.

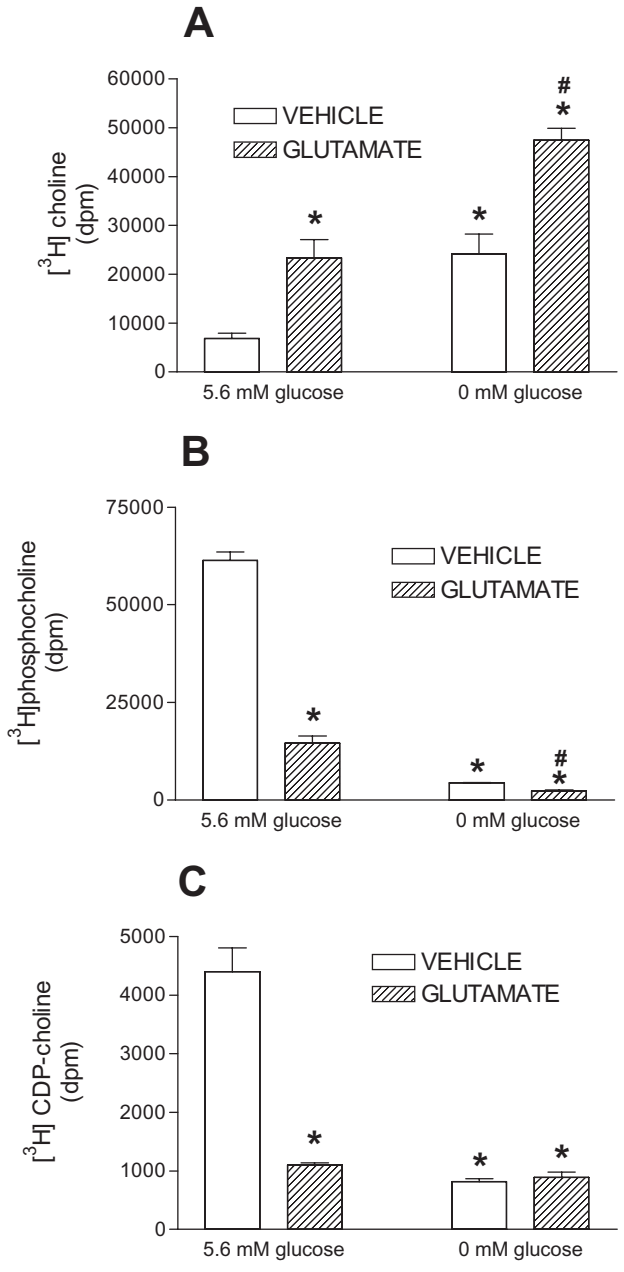

Fig. 3. Exposure to GLU, GD or the combination of both treatments inhibits the incorporation of [^{3}H]Cho into phosphocholine and CDP-choline when measured at the end of treament. Cerebellar granule cells were treated as described in Fig. 2. At the end of treatment, cells were harvested and processed to obtain the water soluble fraction. Cho, phosphocholine and CDP-choline were separated by TLC and the radioactivity incorporated into these compounds was determined. Data are mean ± SEM of three experiments. * Significantly different from control cells ($p < 0.05$); # Significantly different from cells exposed to glutamate in the presence of 5.6 mM glucose ($p < 0.05$)

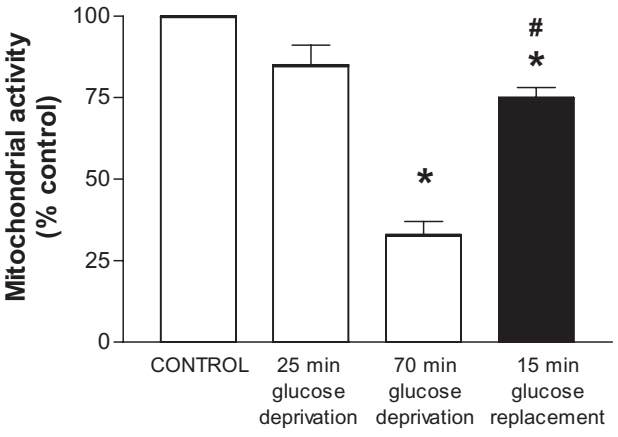

Fig. 4. Effect of GD on mitochondrial activity. Cerebellar granule cells were left undisturbed in conditioned medium (*CONTROL*), incubated in MLH (modified Locke-Hepes Buffer without glucose) for 25 min or 70 min (Glucose Deprivation), or incubated for 15 min after replacement of conditioned medium to cells treated with 70 min glucose deprivation (Glucose replacement). Mitochondrial activity was assessed at the end of treatments using the MTT assay. Data are mean ± SEM of three experiments. * Significantly different from control cells ($p < 0.05$); # Significantly different from 70 min incubation with MLH ($p < 0.05$)

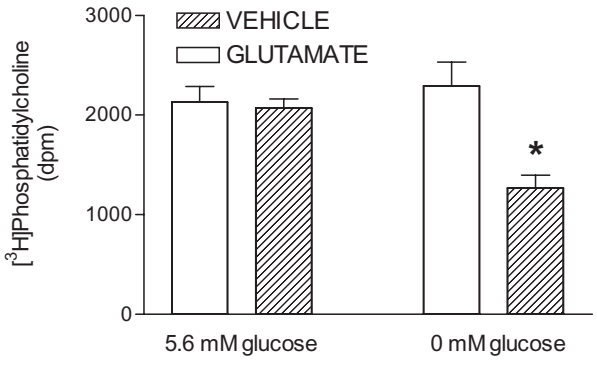

Fig. 5. Only the combined treatment with GLU and GD induces inhibition of the incorporation of [^{3}H]Cho into PC that extends after the end of treatment. Cerebellar granule cells were preincubated for 40 min in 300 μl of MLH containing 0 or 5.6 mM D-glucose. Cells were then incubated for 30 min in the presence of either 20 μM GLU or vehicle. After treatment, conditioned medium was added, cells were incubated for 30 min, [^{3}H]Cho (3 μCi/well) was added, and cells were further incubated for 30 min. Cells were then obtained and processed to obtain the lipidic fraction. PC was separated from the other lipids by TLC and the radioactivity incorporated into PC was determined. Data are mean ± SEM of three experiments. * Significantly different from control cells ($p < 0.05$)

Only the combined treatment with GLU + GD induces inhibition of PC synthesis that lasts at least 30 min after the end of treatment

The previous results indicate that inhibition of PC synthesis at the choline kinase step during the period of treatment is not sufficient to induce excitotoxic cell death in cerebellar granule cells. To determine whether a more lasting inhibition of PC synthesis might be related with excitotoxic cell death, we started to measure the incorporation of [^{3}H]Cho into PC 30 min after the end of treatments, when cells had been incubated for 30 min in the presence of conditioned medium. We found that exposure to either 20 μM GLU or GD did not inhibit the incorporation of [^{3}H]Cho into PC. In contrast, exposure to 20 μM GLU + GD decreased by 45% the incorporation of [^{3}H]Cho into PC (Fig. 5).

Discussion

We have proposed that NMDA-receptor mediated inhibition of PC synthesis is a key early event in the excitotoxic cascade that leads to necrotic cell death in cortical cell cultures (Zapata et al., 1998; Gasull et al., 2000). To determine whether this also happens in cells that are resistant to die by excitotoxicity we investigated the relationship between inhibition of PC synthesis and necrotic cell death in cerebellar granule neuronal cultures. In these cells, exposure to either 20 μM GLU for 30 min, GD for 70 min, or the combination of both treatments inhibited PC synthesis by 71%, 92% and 91%, respectively, when measured at the end of treatment (Fig. 2). However, only the combined treatment with GLU and GD induced significant Cho release (Gasull et al., 2000), and cell death (Fig. 1B). These results indicate that a transient inhibition of PC synthesis evoked by either GD or GLU is not sufficient to induce choline release or cell death.

To investigate which enzymatic step of the CDP-choline pathway is inhibited during the treatment with GLU, GD or the combination of both, we measured the incorporation of [^{3}H]Cho into phosphocholine and CDP-choline at the end of treatments. Our results suggest that the inhibition of PC synthesis during the exposure of cerebellar granule cells to either 20 μM GLU for 30 min, GD for 70 min or the combination of both treatments is produced by inhibition of CK. Thus, all these treatments decreased the incorporation of [^{3}H]Cho into phosphocholine and CDP-choline and increased the intracellular content of free [^{3}H]Cho (Fig. 3). In normal conditions, intracellular levels of free Cho are low because Cho inside the cell is rapidly

phosphorilated by CK (Klein et al., 1990; Millington and Wurtman, 1982). In addition, it is well known that accumulation of substrates and decrease of the levels of the product of a given enzyme usually indicates inhibition of its enzymatic activity. Since all treatments investigated decreased the levels of [^{3}H]phosphocholine, the product synthesized by the enzyme CK, and increased the levels of intracellular [^{3}H]Cho, a substrate of CK, these results indicate that all these treatments inhibit the activity of CK. We also found that all treatments investigated decreased the levels of [^{3}H]CDP-choline, the product synthesized by CT (Fig. 3C). However, this effect is accompanied with a decrease of the levels of the CT substrate [^{3}H]phosphocholine (Fig. 3B), indicating that inhibition of the incorporation of [^{3}H]Cho into CDP-choline is produced by a decreased availability of the substrate [^{3}H]phosphocholine. This observation provides further evidence to indicate that all treatments investigated inhibit the enzymatic step catalyzed by CK, at least during the time cells are exposed to the treatments.

CK requires ATP to synthesize phosphocholine. Thus, it is most likely that the inhibition of CK activity and, in turn, the inhibition of PC synthesis in cells exposed to GD alone or GLU alone is due to energy depletion. Thus, soon after the addition of conditioned medium, cells recover ATP levels, CK activity and the normal synthesis of PC. Indeed, we found that 70 min of GD decreased by 58% the mitochondrial activity in cerebellar granule cells. Mitochondrial activity increased soon after the addition of the conditioned medium containing glucose (Fig. 4). This result is in line with a previous report demonstrating that GD induces energy depletion (Novelli et al., 1988). In addition, it has been reported that exposure to GLU alone induces energy depletion in cerebellar granule cell cultures (Ankarcrona et al., 1995; Marcaida et al., 1997).

In our experimental conditions we measured inhibition of PC synthesis at the end of treatments. However, when assessing cell death in cerebellar granule cells, conditioned medium was added at the end of treatment and cells were incubated in this conditioned medium, containing glucose, for 24 h before assessing cell death. Therefore, we also investigated the effect of GLU + GD on PC synthesis and CK activity following the end of treatment and before cell death. We measured the incorporation of [^{3}H]Cho into PC 1 h after the end of treatments. PC synthesis remained inhibited only after the combined treatment with GLU + GD (Fig. 5). This treatment induces cell death in cerebellar granule cells 24 h after exposure. We found that exposure to 20 μM GLU + GD did not induce significant cell death when measured 30 and 60 min after the addition of conditioned medium (Fig. 1A). This observation is different from the reported using fluorescein diacetate staining to asses toxicity (Lysko et al., 1989); however this discrepancy might be explained by slightly different sensitivities of our culture systems to glutamate, particularly if we consider that 20 μM GLU is the minimum concentration able to produce complete toxicity (Lysko et al., 1989; Boje et al., 1993).

In summary, exposure of cerebellar granule cells to 20 μM GLU alone for 30 min or GD for 70 min induce a reversible inhibition of PC synthesis by an action on CK activity. None of these treatments induced cell death. However, the combined treatment with GLU and GD induces a more prolonged inhibition of PC synthesis that is associated with cell death. These results suggest that this effect of the combined treatment with GLU and GD on PC synthesis is produced by an action on an enzymatic step downstream of choline kinase activity.

Acknowledgments

This work was supported by a grant from CICYT, BFI2001-1035, Plan Nacional I + D, Ministerio de Ciencia y Tecnología (MCyT) of Spain to R. Trullas. N. DeGregorio-Rocasolano has a F.P.I. fellowship from "Subdirección General de Formación y Promoción del Conocimiento", MCyT of Spain. Marta Enguita has F.P.I. fellowship from IDIBAPS, Generalitat de Catalunya.

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Authors' address: Dr Ramon Trullas, Neurobiology Unit, IIBB/CSIC, IDIBAPS, Rosselló 161, E-08036 Barcelona, Spain, Fax: (3493) 3638303, E-mail: rtonbi@iibb.csic.es