Choline Release and Inhibition of Phosphatidylcholine Synthesis Precede Excitotoxic Neuronal Death but Not Neurotoxicity Induced by Serum Deprivation*

Received for publication, December 30, 1999, and in revised form, March 10, 2000
Published, JBC Papers in Press, March 28, 2000, DOI 10.1074/jbc.M910468199

Teresa Gasull‡, Nuria DeGregorio-Rocosolano§, Agustin Zapata, and Ramon Trullas¶

From the Neurobiology Unit, Institut d’Investigacions Biomèdiques de Barcelona, Consejo Superior de Investigaciones Científicas, Institut d’Investigacions Biomèdiques August Pi i Sunyer, Rosselló 161, 08036 Barcelona, Spain

* This work was supported by Direcció General de Ensenància Superior e Investigacions Científicas Grant SAF98-0063, Plan Nacional I+D, Ministerio de Educación y Cultura of Spain (to R. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Programa de Reincorporación de la Secretaría de Universidades e Investigación de Spain.

§ Holder of a Formació de Personal Investigació fellowship from the Subdirecció General de Formació y Promoció del Conocimiento, Ministerio de Educación y Cultura of Spain.

¶ To whom correspondence should be addressed. Tel.: 3493-3638303; Fax: 3493-3638324; E-mail: rtonbi@ibib.csic.es.

The abbreviations used are: GLU, L-glutamate; NMDA, N-methyl-D-aspartate; PC, phosphatidylcholine; SM, sphingomyelin; Cho, choline; PI, phosphatidylinositol; MLH, modified Locke Hepes buffer; SD, serum deprivation; PLD, phospholipase D; PtdBut, phosphatidylbutanol; HCh-3, hemicholinium 3; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; PLA₂, phospholipase A₂; CDP-choline, choline 5′-diphosphate-choline.

N-methyl-D-aspartate (NMDA) receptor overactivation has been proposed to induce excitotoxic neuronal death by enhancing membrane phospholipid degradation. In previous studies, we have shown that NMDA releases choline and reduces membrane phosphatidylcholine in vivo. We now observed that glutamate and NMDA induce choline release in primary neuronal cortical cell cultures. This effect is Ca²⁺-dependent and is blocked by MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate). In cortical neurons, the NMDA receptor-mediated choline release precedes excitotoxic cell death but not neuronal death induced by either osmotic lysis or serum deprivation. Glutamate, at concentrations that release arachidonic acid, does not release choline in cerebellar granule cells, unless these cells are rendered susceptible to excitotoxic death by energy deprivation. The NMDA-evoked release of choline is not mediated by phospholipases A₂ or C. Moreover, NMDA does not activate phospholipase D in cortical cells. However, NMDA inhibits incorporation of [methyl-³H]choline into both membrane phosphatidylcholine and sphingomyelin. These results show that the increase in extracellular choline induced by NMDA receptor activation is directly related with excitotoxic cell death and indicate that choline release is an early event of the excitotoxic process produced by inhibition of phosphatidylcholine synthesis and not by activation of membrane phospholipid degradation.

1-L-Glutamate (GLU) receptor overactivation induces pathological membrane permeability changes that result in excitotoxic neuronal cell death (1, 2). Excitotoxicity has been postulated to underlie the neuronal death observed in brain ischemia, traumatic brain injury and in some chronic neurodegenerative disorders, including Alzheimer’s and Huntington’s diseases (3–5). It has been established that Ca²⁺ influx through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors is an initial event that plays a central role in glutamate-evoked neuronal excitotoxicity. However, the immediate Ca²⁺-dependent pathogenic targets that trigger NMDA receptor-mediated neurotoxicity are not fully understood.

Excitotoxic neuronal death induced by glutamate has been hypothesized to occur via both necrosis and apoptosis, depending on the intensity and duration of glutamate exposure (6–8). Apoptosis is considered to be an active process of cell destruction characterized by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing followed by phagocytosis. In contrast, necrotic cell death is considered to be a passive process of cell death produced by cell swelling, injury to cytoplasmic organelles, and membrane lysis. Nonetheless, in neurons, apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths (8, 9). Mature cortical or granule cell cultures exposed to mild excitotoxic insults undergo a cell death that is mediated by post-translational activation of caspases and exhibits apoptotic morphology but does not require new RNA and protein synthesis (7, 10). As the exposure time or concentration of NMDA receptor agonist increases, excitotoxic cell death changes its shape from apoptotic to necrotic. However, despite the fact that apoptosis and necrosis may share some biochemical mechanisms, the major distinction between them is that in necrosis there is cytoplasmic membrane breakdown and release of cell contents that causes an inflammatory reaction in vivo. We hypothesized that the cytoplasmic membrane breakdown associated with necrosis, instead of being a passive process of cell destruction, is produced by alteration of biochemical processes involved in membrane building.

Choline-containing phospholipids such as phosphatidylcholine (PC) and sphingomyelin (SM) are major structural components of cell membranes. It has been postulated that the cytoplasmic membrane damage associated with excitotoxic neuronal cell death is caused by increased hydrolysis of membrane phospholipids by various phospholipases (11). Previous results obtained in our laboratory using in vivo microdialysis demonstrated that NMDA receptor activation increases extracellular choline (Cho) levels (12). The NMDA-evoked increase in extracellular Cho concentration was associated with a reduction of membrane phosphatidylcholine and with delayed excitotoxicity in prefrontal cortex. In the present experiments, we investigated the mechanisms involved in the increase of extracellular Cho induced by NMDA. The objectives were to...
determine whether choline release is directly related with excitotoxic cell death and to assess whether NMDA receptor-mediated choline release results from membrane phospholipid degradation. We now report that the increase in extracellular Cho observed after NMDA receptor activation precedes necrotic but not apoptotic cell death. Moreover, the increase in extracellular Cho induced by NMDA receptor activation is produced by inhibition of phosphatidylcholine synthesis and not by an increase in phosphatidylcholine hydrolysis.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of brain cortical and cerebellar granule neurons were performed essentially as described previously (13). Cortical and cerebellar granule cells were obtained from Harlan Sprague-Dawley E18 rat fetuses and 7-postnatal day rat pups, respectively. Frontal-lateral cortical lobes or cerebellum were dissected, and cells were chemically dissociated in the presence of trypsin and DNase I and plated in poly-L-lysine-coated (10 μg/ml) wells. Cells were seeded in 24-well plates at a density of 8 × 10^5 cells/cm² in 750 μl of 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. In experiments measuring phospholipase D activity, cells were plated in six-well plates at the same density. For cortical cell cultures, medium was supplemented with t-glucose to reach a final concentration of 25 mM. Cytosine β-D-arabinofuranoside (10 μM) was added to cerebellar and cortical cells 24 and 72 h after seeding, respectively. Cultures were incubated at 37 °C in a 5% CO₂, 95% air atmosphere. Medium remained unchanged until experiments were performed (8–10 days in vitro). For experiments using Hoechst 33342 or propidium iodide (PI) staining, cells were incubated at 37°C but allowed sufficient production of phosphatidylbutanol (PtdBut).

Test conditions were performed 1 h after incubation, medium was removed, cells were washed twice with 1-ml aliquots of Locke Hepes buffer (154 mM NaCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM KCl, 5.6 mM t-glucose in 5 mM Hepes buffer, pH 7.4). In treated with serum deprivation (SD), medium was replaced by fresh medium without fetal bovine serum. In experiments examining the effects of phospholipase inhibitors, these compounds were added 20 min before treatment with NMDA. Cho levels were measured in the incubation media at the indicated times after each treatment. Neuronal cell death was determined with PI staining at the indicated times after treatment. In experiments in cortical cell cultures, medium was removed, cells were washed twice, and incubations with different treatments were performed. Both control and energy-compromised cerebellar granule cultures were exposed to either GLU or vehicle for 30 min in 300 μM MLH without glucose renders cerebellar cell death was calculated as follows: % cell death = 100 × (X – F₀₋₁)/ (Fₘₐₓ – F₀₋₁), where X is fluorescence at any given time. Cells were kept in the incubator between measurements. In some experiments, the percentage of dead cells was measured counting the number of PI-stained cells and dividing by the total number of cells using simultaneous fluorescence and phase contrast observation in an epifluorescence microscope. In these experiments, cells were incubated with 10 μM PI for 30 min and fixed in 3.7% paraformaldehyde for 20 min at room temperature before the addition of a final glycerol protective layer.

Determination of Cho—Extracellular Cho levels were measured in the incubation medium using a high-performance liquid chromatograph coupled to an enzyme reactor and an electrochemical detector (BAS, West Lafayette, IN). Cho was separated in a BAS/Sepektik microbore column with a mobile phase consisting of 50 mM NaH₂PO₄, 0.005% Kathon, and 0.5 mM EDTA, pH 8.5 (adjusted with NaOH), at a flow rate of 140 μl/min. Cho was enzymatically converted to hydrogen peroxide by the choline oxidase enzyme reaction, and the resulting hydrogen peroxide was detected on a platinum electrode at +500 mV (versus an Ag/AgCl reference electrode, BAS-LC4B). Results were expressed in pmol/mg of protein. Protein content was measured in each well using the BCA protein assay reagent (Pierce).

Measurement of Cells with Apoptotic Morphology—Nuclear condensation or pyknosis, a hallmark of cells undergoing apoptotic cell death, was examined in permeabilized cells with Hoechst 33342 staining. Following treatments, cells were washed twice with phosphate-buffered saline and fixed for 5 min in ice-cold methanol. Once fixed, cells were incubated with Hoechst 33342 (0.1 μg/ml) for 15 min at room temperature. Cells were then washed three times with phosphate-buffered saline, and a final Mowiol protective layer was added. Cells with pyknotic and total cell nuclei were counted (3 fields per well) by direct observation using an epifluorescence microscope.

Assay of Phospholipase D Activity—Phospholipase D (PLD) (EC 3.1.4.4) activity was assayed in the presence of alcohol using the transphosphatidylation reaction, which is catalyzed specifically by PLD (16). PLD normally hydrolyses phosphatidylcholine producing free choline and phosphatidic acid. In the presence of a primary alcohol, the enzyme catalyzes a transphosphatidylation reaction that transfers the phosphatidyl moiety to the alcohol, forming a phosphatidylalcohol (17). The concentrations of ethanol generally used to determine PLD activity (18) have been reported to inhibit NMDA receptor activation (19). In fact, ethanol and butanol induced a concentration-dependent inhibition of NMDA-evoked Cho release (results not shown). Thus, to measure PLD activity after NMDA receptor activation, we used butanol at a concentration (0.15%) that did not inhibit NMDA-evoked Cho release but allowed sufficient production of phosphatidylbutanol (PtdBut). Cells were prelabelled for 4 h in 1.5 ml of MLH with [32P]Plasphate (5 μCi/well). Ten minutes earlier, cells were incubated with 1 h with 1.5 ml of MLH containing 0.15% butanol and vehicle, 10 μM ionomycin, or 100 μM NMDA. Incubation buffer was removed and cells were sonicated in ice-cold 100 μM NMDA. Lipid extraction was performed by adding chloroform and H₂O (final proportion of methanol/chlorofol/H₂O, 2:2:1 (v/v/v)) and separating phases by centrifugation (10 min at 400 g). The lower organic phase was removed, dried under vacuum and dissolved in 10 μl of chlorofol/methanol (1:1 (v/v)). Lipid extraction was performed by adding chloroform and H₂O (final proportion of methanol/chlorofol/H₂O, 2:2:1 (v/v/v)) and separating phases by centrifugation (10 min at 400 g). The lipids present in the lower organic phase were dried under vacuum centrifugation and dissolved in 10 μl of chlorofol/methanol (4:1 (v/v)). Lipids were separated on silica TLC plates (HPTLC 60, 10 × 20 cm, Merck) using ethyl acetate/2,2,4-trimethylpentane/acetonic acid (75:25:2, v/v/v) as mobile phase. Phospholipid spots were identified by exposure to iodine and co-migration with standards. Radioactivity co-migrating with PtdBut and radioactivity present in the total lipid fraction was determined.

Determination of PC and SM Synthesis—To determine the effect of NMDA on de novo synthesis of PC and SM, we measured incorporation of [methyl-3H]Cho into membrane PC and SM. As a positive control, we used hemicholinium 3 (Hch-3) a choline kinase inhibitor. Cells were incubated for 1 h in MLH with [methyl-3H]Choline chloride (0.5 μCi/ well) in the presence of vehicle, 1 mM HCh-3, or 100 μM NMDA. Immediately after incubation, medium was removed, ice-cold HCl:methanol (1:10 (v/v)) was added and the methanol cell suspension was sonicated. Lipid extraction was performed by adding chloroform and H₂O (final proportion of methanol/chlorofol/H₂O, 2:2:1 (v/v/v)) and separating phases by centrifugation (10 min at 400 g). The lipids present in the lower organic phase were dried under vacuum centrifugation and dissolved in 10 μl of chlorofol/methanol (4:1 (v/v)). Lipids were separated on silica TLC plates (HPTLC 60, 10 × 20 cm, Merck) using chloroform/methanol/acetic acid/water (75:45:3:1 (v/v/v/v)) mobile phase. PtdSer and PtdChol were used as markers. PC and SM were separated by co-migration with standards. The spots identified as PC and SM were scraped, and the radioactivity was measured. Results are expressed as dpm of [3H]Cho incorporated into PC and SM.

Time Course Analysis of Incorporation of [methyl-3H]Cho into Anabolenes of the CDP-Choline Pathway—Cortical cells were treated with vehicle or NMDA (100 μM) and 0.5 μM of [methyl-3H]Choline chlo-
FIG. 1. NMDA and GLU induce a concentration and Ca$^{2+}$-dependent increase in extracellular Cho levels in primary neuronal cortical cell cultures that is blocked by (+) MK-801. A, cortical cell cultures were incubated in 300 μL of MLH containing varying concentrations of NMDA (■) or GLU (□) for 1 h. Incubation buffer was removed after 1 h of treatment to determine extracellular Cho levels. Basal extracellular Cho levels were 210 ± 52 pmol/mg protein. B, cortical cell cultures were incubated in 300 μL of MLH containing NMDA (100 μM) or GLU (200 μM) either in the presence of vehicle or (+) MK-801 (10 μM), or in the absence of extracellular Ca$^{2+}$. Data are mean ± S.E. of at least three experiments, with triplicate samples in each, *, significantly different from control (p < 0.05).

FIG. 2. Choline release precedes cell death induced by NMDA. Cell death (A) and extracellular Cho concentration ([choleine]ext) (B) were measured in cortical cell cultures at different times during continuous incubation with 300 μL of MLH without serum (•) or 300 μL of MLH without serum plus 100 μM NMDA (■). Cell death was measured by propidium iodide fluorescence and expressed as a percentage of maximum cell death, obtained with digitonin as described under “Experimental Procedures.” Data are mean ± S.E. of at least three experiments with triplicate samples. *, significantly different from control serum-deprived cells at the corresponding time; #, significantly different from preceding value in the same group (p < 0.05).

### RESULTS

**NMDA Induces a Concentration and Ca$^{2+}$-dependent Increase in Extracellular Cho in Cortical Cells That Is Blocked by MK-801**—Incubation of cortical cell cultures with different concentrations of Glutamate or NMDA for 1 h induced a concentration-dependent release of Cho (Fig. 1A) (E$_{max}$ = 277 ± 14% and 336 ± 19% and EC$_{50}$ with 95% confidence intervals = 7 (2–22) μM and 17 (7–39) μM for glutamate and NMDA, respectively). NMDA (100 μM) and GLU (200 μM) increased extracellular Cho levels to 277 ± 25% (n = 9) and 264 ± 40% (n = 5) of control values, respectively (Fig. 1B). The effect of both GLU and NMDA on extracellular Cho was completely blocked by MK-801 (10 μM) (Fig. 1B). Removal of extracellular Ca$^{2+}$ inhibited the increase in extracellular Cho evoked by NMDA, indicating that this effect is dependent on extracellular Ca$^{2+}$ entry (Fig. 1B).

The NMDA-evoked Increase in Extracellular Cho Precedes Excitotoxic Cell Death but Not Cell Death Induced by Trophic Factor Withdrawal in Cortical Cells—Previous studies in cortical cell cultures have shown that NMDA induces excitotoxic cell death that is predominantly necrotic when the culture medium contains physiological ion concentrations (20). To determine whether the increase in extracellular Cho induced by NMDA is produced before or after cell death, we performed time course studies. First, the effects of NMDA on excitotoxic neuronal death and extracellular Cho levels were investigated in serum-free Locke Hepes buffer (Figs. 1 and 2), because both serum and basal Eagle’s medium contain Cho. In addition, exposure of cortical cell cultures to fresh serum-containing medium triggers excitotoxicity (21). Fig. 2A shows the time course of neuronal cell death induced by NMDA in serum-deprived cortical cell cultures. A significant cell death was observed in serum-deprived controls (38 ± 1%, after 24 h of serum deprivation). Simultaneous treatment with SD and with 100 μM NMDA for 24 h produced 57 ± 1% cell death, an effect...
that is significantly different from the observed in cultures treated with SD alone. Thus, exposure of cortical cells to 100 μM NMDA for 24 h produced a cell death of approximately 19%. Whereas both SD and NMDA were neurotoxic, the cell death induced by SD was significant after the first half-hour of treatment compared with controls not deprived of serum. In contrast, cell death induced by NMDA was not significant until 6 h of continuous treatment, compared with controls deprived of serum for 6 h (Fig. 2A). Exposure of cortical cell cultures to 100 μM NMDA induced a marked increase in extracellular concentration of Cho. This effect was observed 30 min after addition of NMDA and reached a maximum after 4 h of continuous treatment with NMDA. 30 min, 1 h, 2 h, and 4 h after addition of NMDA, extracellular Cho was increased approximately 2-, 6-, 12-, and 4-fold, respectively (Fig. 2B). Exposure of control cells to SD alone did not modify extracellular Cho levels during the first 4 h; however, a small but significant increase in extracellular Cho levels was observed between 4 and 24 h of treatment (Fig. 2B). The NMDA-evoked increase in extracellular Cho was observed well before cytoplasmic membrane breakdown associated with necrotic cell death. Excitotoxicity induced by NMDA was not significant until after 6 h of continuous treatment (Fig. 2A). However, the increase in extracellular Cho occurred within the initial 4 h after the addition of NMDA (Fig. 2B). In contrast, trophic factor withdrawal produced a significant cell death but did not modify extracellular Cho levels. Cell death induced by SD was significant after 30 min of treatment (Fig. 2A), but no significant increase was observed in extracellular Cho until 24 h of SD (Fig. 2D).

To assess whether the NMDA-evoked Cho release depends on the presence of extracellular Cho and to determine whether it is produced only before excitotoxic cell death, we investigated the effect of NMDA (100 μM) on extracellular Cho levels in cortical cell cultures incubated with serum-containing conditioned medium. In these experiments, NMDA was added directly to conditioned medium that contains Cho and serum. The results obtained confirmed that the increase in extracellular Cho levels precedes excitotoxic cell death but not cell death induced by growth factor withdrawal (Figs. 3). No significant cell death was observed after 1 h of continuous treatment with either NMDA or SD (Fig. 3A). However, a pronounced cell death was observed 24 h after continuous exposure to both treatments (43 ± 3% and 56 ± 4% for SD and NMDA, respectively) (Fig. 3A). Moreover, exposure of cortical cells for 1 or 24 h to 100 μM NMDA in conditioned medium produced an increase in extracellular Cho of 79 ± 8 pmol/well over control levels at 1 and 24 h, respectively (Fig. 3B). In contrast, SD did not significantly modify extracellular Cho content after either 1 or 24 h of treatment (Fig. 3B).

To gain further insight on the type of cell death that is preceded by Cho release, we measured the number of condensed nuclei (pyknosis), an index of apoptosis. SD for 12 h produced a significantly higher pyknosis than NMDA. Cortical cells were continuously exposed to NMDA (100 μM) (filled bar) or SD (gray bar) for 12 h. Control cells (open bar) remained undisturbed in their own conditioned medium. NMDA (100 μM) was added directly to conditioned medium. In cells treated with serum deprivation, medium was replaced by fresh medium without fetal bovine serum. A, percentage of pyknotic nuclei after 12 h of treatment. Results are mean ± S.E. of three experiments. *, significantly higher (p < 0.05 versus control); #, significantly higher (p < 0.05 versus NMDA-treated cells). B–D, microphotographs showing control cells (B), cells treated with 100 μM NMDA for 12 h (C), and cells treated with SD for 12 h (D). Pyknotic nuclei are marked by arrows.
Cells were preincubated for 40 min with MLH with or without D-glucose and subsequently exposed to either 20 μM GLU (filled bars) or vehicle (open bars) for 30 min. After GLU treatment, cells were washed twice, conditioned medium was added, and cells were returned to the incubator. A, cell death was measured by PI fluorescence 24 h after GLU exposure and expressed as a percentage of maximum cell death, obtained with digitonin. B, Cho levels were measured in the incubation buffer at the end of the 30-min period of control and glutamate treatments. Results are mean ± S.E. of at least three experiments. *, p < 0.05 versus control.

**Fig. 5.** Cho release is associated with excitotoxic cell death. The effect of GLU on cell death (A) and choline release (B) was investigated in control and energy-compromised cerebellar granule cells. Cells were preincubated for 40 min with MLH with or without 5 mM glucose and subsequently exposed to either 20 μM GLU (filled bars) or vehicle (open bars) for 30 min. After GLU treatment, cells were washed twice, conditioned medium was added, and cells were returned to the incubator. A, cell death was measured by PI fluorescence 24 h after GLU exposure and expressed as a percentage of maximum cell death, obtained with digitonin. B, Cho levels were measured in the incubation buffer at the end of the 30-min period of control and glutamate treatments. Results are mean ± S.E. of at least three experiments. *, p < 0.05 versus control.

**Fig. 6.** NMDA-induced increase in extracellular Cho is not mediated by activation of phospholipases A₂, C, and D. A, effect of PLA₂ inhibitors 4-bromophenacyl bromide (4-BPB) and aristolochic acid (ARIST) and the phospholipase C inhibitor D-609 on Cho release induced by NMDA in cortical cells. Experiments were performed in 300 μM of MLH. Cells were preincubated with 4-bromophenacyl bromide (10 μM) or aristolochic acid (100 μM) for 20 min before the addition of NMDA (100 μM). Inset, effect of D-609, a phospholipase C inhibitor (42), on Cho release induced by NMDA. Experiments were performed in Krebs-Ringer’s bicarbonate solution (1.4 mM KCl, 120 mM NaCl, 1.3 mM KH₂PO₄, 20 mM NaHCO₃, 1.2 mM CaCl₂, 10 mM d-glucose) with 5 mM phosphate, pH 7.4, because Hepes buffer renders D-609 toxic. Cortical cells were preincubated with D-609 (100 μM) for 20 min before exposure to NMDA (100 μM). Choline levels were measured in the incubation medium after 1 h of continuous exposure to NMDA. B, cortical cells were incubated with [³²P]orthophosphate (5 μCi/well) in 1.5 ml of MLH in six-well plates for 4 h. Residual radioactivity was washed, and cells were subsequently incubated in 1.5 ml of MLH with vehicle, NMDA (100 μM), or ionomycin (10 μM) for 1 h in the presence of 0.15% butanol. Immediately after treatment, lipids were extracted and separated by TLC. Results are expressed as mean ± S.E. dpm ([³²P]PtdBut fraction/ dpm incorporated in the total lipid fraction) × 100 of three experiments. *, p < 0.05 versus control.

**Fig. 6.** NMDA-induced increase in extracellular Cho is not mediated by activation of phospholipases A₂, C, and D. A, effect of PLA₂ inhibitors 4-bromophenacyl bromide (4-BPB) and aristolochic acid (ARIST) and the phospholipase C inhibitor D-609 on Cho release induced by NMDA in cortical cells. Experiments were performed in 300 μM of MLH. Cells were preincubated with 4-bromophenacyl bromide (10 μM) or aristolochic acid (100 μM) for 20 min before the addition of NMDA (100 μM). Inset, effect of D-609, a phospholipase C inhibitor (42), on Cho release induced by NMDA. Experiments were performed in Krebs-Ringer’s bicarbonate solution (1.4 mM KCl, 120 mM NaCl, 1.3 mM KH₂PO₄, 20 mM NaHCO₃, 1.2 mM CaCl₂, 10 mM d-glucose) with 5 mM phosphate, pH 7.4, because Hepes buffer renders D-609 toxic. Cortical cells were preincubated with D-609 (100 μM) for 20 min before exposure to NMDA (100 μM). Choline levels were measured in the incubation medium after 1 h of continuous exposure to NMDA. B, cortical cells were incubated with [³²P]orthophosphate (5 μCi/well) in 1.5 ml of MLH in six-well plates for 4 h. Residual radioactivity was washed, and cells were subsequently incubated in 1.5 ml of MLH with vehicle, NMDA (100 μM), or ionomycin (10 μM) for 1 h in the presence of 0.15% butanol. Immediately after treatment, lipids were extracted and separated by TLC. Results are expressed as mean ± S.E. dpm ([³²P]PtdBut fraction/ dpm incorporated in the total lipid fraction) × 100 of three experiments. *, p < 0.05 versus control.
The present work demonstrates that NMDA receptor-mediated release of Cho precedes excitotoxic cell death but not cell death induced by SD. We found that, in cortical cell cultures, both NMDA and GLU induce a concentration-dependent increase in extracellular Cho (Fig. 1A). This effect is dependent on the presence of extracellular Ca\(^{2+}\) and it is blocked by (+) MK-801 (10 \(\mu M\)), a selective antagonist of the NMDA subtype of glutamate receptors (24), indicating that Ca\(^{2+}\) entry through NMDA receptors is necessary for the excitatory amino acid induced increase of extracellular Cho (Fig. 1B).

We observed that the increase in extracellular Cho evoked by NMDA is previous to excitotoxic cytoplasmic membrane damage and cell death (Figs. 2 and 3). Exposure of cortical cells to NMDA in Locke Hepes buffer produces a significant increase in extracellular Cho 30 min after addition of NMDA, whereas no significant cell death was observed until 6 h of continuous treatment with NMDA (Fig. 2). This difference in time course between NMDA-evoked Cho release and cell death indicates that Cho release is an early event in the process of excitotoxic cell death.

To determine whether the absence of Cho and serum in the incubation medium has an influence on the effect of NMDA on Cho release and cell death, we performed experiments in which NMDA was directly added to the medium in which cells had grown (conditioned medium) that contains serum and Cho. Thus, in these experiments, the effect of NMDA could be isolated from that of SD. As in experiments performed in Locke Hepes buffer, we found that addition of 100 \(\mu M\) NMDA to conditioned medium increased extracellular Cho (79 ± 12 pmol/well over control values) but did not produce cell death during the first 1 h of exposure (Fig. 3). Continuous exposure of cortical cells to NMDA for 24 h increased extracellular Cho levels further and induced a cell death of 56 ± 4% (Fig. 3). These results confirm those obtained in experiments in which treatment with NMDA was performed simultaneously with SD and provide further evidence showing that NMDA receptor-mediated Cho release precedes excitotoxic cell death.

Subjecting cortical cells to SD caused an amount of cell death that was equivalent to the observed after treatment with GLU or NMDA (Fig. 3A). However, extracellular Cho did not increase during SD (Figs. 2B and 3B). Under our experimental conditions, cell death induced by NMDA was mostly necrotic (Fig. 4). This is in agreement with recent evidence showing that in cortical cells, even delayed excitotoxic cell death, previously considered apoptotic, is in fact necrotic (20). In contrast, cell death induced by SD was mostly apoptotic (Fig. 4), in accordance with previous findings in cortical cell cultures showing that cell death induced by SD is dependent on protein synthesis and fulfills morphological criteria for apoptosis (25). The difference between SD and NMDA in releasing Cho before cell death indicates that Cho release discriminates between necrotic cell death induced by excitotoxicity and apoptotic cell death induced by SD.

To determine whether NMDA receptor-mediated Cho release is directly related with excitotoxicity or is just an epiphenomenon, we investigated the effects of NMDA and GLU in cerebellar granule cell cultures. Cerebellar granule cells are resistant to excitotoxic cell death (3). However, energy deprivation makes cerebellar granule cells susceptible to excitotoxicity by GLU (14). To test the hypothesis that there is a direct link between Cho release and excitotoxicity, we investigated whether the neurotoxic effect of GLU in energy-compromised cerebellar granule cells was preceded by an increase in extracellular Cho. Exposure of cerebellar granule cells to NMDA (500 \(\mu M\)) for 1 h (results not shown) or GLU (20 \(\mu M\)) for 30 min, in the presence of a physiological concentration of glucose, did not produce significant cell death 24 h after treatment (Fig. 5A). Under these conditions, neither NMDA (results not shown) nor GLU released Cho (Fig. 5B), but both NMDA and GLU released arachidonic acid (13). In contrast, exposure of energy-compromised cerebellar granule cells to GLU for 30 min increased extracellular Cho levels by 56% during GLU exposure and induced 61 ± 3% cell death 24 h after treatment with GLU (Fig. 5). These results indicate that glutamate receptor
Excitotoxicity and Choline Release

activation is followed by Cho release only if the activation leads to neurotoxicity. Based on these findings, we can conclude that NMDA receptor-mediated Cho release is directly related with excitotoxic cell death.

These results in cerebellar granule cells are in contrast with those observed in cortical cells, in which NMDA receptor activation increases extracellular Cho and produces subsequent cell death even in the presence of physiological concentrations of glucose. The mechanism responsible for the high sensitivity of cortical cells to release Cho after NMDA receptor activation may account for the greater susceptibility of these cells to excitotoxicity compared with cerebellar granule cells. The difference between cortical and cerebellar granule cells to release Cho after NMDA receptor activation is consistent with our previous results in vivo showing that NMDA increases dialysate Cho levels in prefrontal cortex but not in cerebellum (12).

The extracellular Cho measured after exposure of cells to NMDA in a Locke Hepes buffer that does not contain Cho indicates that this Cho must proceed from the cell. In brain, Cho is stored almost completely in a metabolized form (26). Intracellular free Cho concentration is kept low because once Cho is inside the cell, it is rapidly phosphorylated by choline kinase (26, 27). In fact, in our experimental conditions, osmotic lysis of cortical cells did not produce a significant increase in free Cho (75 and 78 pmol/well Cho in control and lysed cells, respectively). Thus, we investigated whether NMDA-evoked Cho release is produced by breakdown of Cho containing compounds. The main compounds containing Cho in neurons are acetylcholine, in cholinergic cells, and the phospholipids PC and SM (28, 29). Neither cortical nor cerebellar granule cell cultures showed detectable levels of extracellular acetylcholine even in the presence of high concentrations of the acetylcholinesterase inhibitor neostigmine (not shown), indicating that acetylcholine is not the source of Cho released by NMDA. Our previous studies showed that sustained activation of NMDA receptor in vivo is associated with a significant decrease of membrane PC (12). Thus, we investigated whether the phospholipases $\text{PLA}_2$, PC-phospholipase C and PLD that are involved in PC breakdown are also implicated in the effect of NMDA on Cho release. NMDA receptor activation has been reported to increase phospholipase C activity (34–36).

The $\text{PLA}_2$ inhibitors 4-bromophenacyl bromide and aristolochic acid did not inhibit NMDA-evoked Cho release (Fig. 6A). This observation, combined with the fact that cerebellar granule cells exposed to GLU, in the presence of glucose, release arachidonic acid (13) but not Cho (Fig. 5B), strongly indicates that $\text{PLA}_2$ is not involved in the release of Cho induced by NMDA. Likewise, the phosphatidylethanolamine-specific phospholipase C inhibitor D-609 (37) did not inhibit the increase in extracellular Cho induced by NMDA (Fig. 6A, inset). Finally, we investigated the possibility that Cho released by NMDA could be produced by activation of PLD. There is evidence indicating that activation of PLD by glutamate is exclusively mediated through metabotropic receptors (18, 38, 39). Accordingly, we found that NMDA does not activate PLD in neuronal cortical cell cultures (Fig. 6B). However, the concentrations of alcohol generally used to determine PLD activity by the transphosphatidylation reaction (18) inhibit NMDA receptor activation (19). It could therefore be possible that the concentration of primary alcohol used in the enzymatic assay might block the effect of NMDA on PLD activity. This possibility was ruled out in our experiments because we used a concentration of alcohol in the transphosphatidylation reaction that does not significantly inhibit NMDA-evoked Cho release. Based on these results, we conclude that the NMDA receptor-mediated increase in extracellular Cho is not produced by activation of membrane phospholipid degradation.

Because the increase in extracellular Cho is not produced by NMDA receptor-induced phospholipid catabolism, inhibition of phospholipid synthesis by NMDA emerged as an alternative mechanism to explain the NMDA-evoked Cho release. A decrease in PC synthesis with no significant alteration in PC breakdown would lead to a decrease in membrane PC content and also a subsequent increase in extracellular Cho, because if conversion of phosphocholine to PC is inhibited, Cho leaks outside the cell after rapid dephosphorylation. Indeed, the finding that NMDA causes a marked inhibition of PC synthesis (Fig. 7) provides a strong support for this interpretation. Furthermore, consistent with this interpretation is the fact that inhibitors of phospholipid hydrolysis potentiate the effect of NMDA on Cho release (Fig. 6A). Inhibitors of phospholipid catabolism diminish the cellular supply of diacylglycerol, an essential and limiting substrate in the last step in PC biosynthesis, inhibiting, in turn, the biosynthesis of PC. Thus, the...
potentiation of NMDA-evoked Cho release by inhibitors of phospholipid hydrolysis can be attributed to an enhanced inhibition of PC synthesis produced by these compounds. Moreover, recent studies in our laboratory have shown that NMDA does not inhibit Cho uptake (40). Therefore, the NMDA-evoked inhibition of PC synthesis must be produced by an action on one or several of the enzymes of the Kennedy cycle (41).

Analysis of incorporation of [methyl-3H]Cho into the intermediates of the CDP-choline pathway in the presence and absence of NMDA indicated that the reduction of radiolabeled mediators of the CDP-choline pathway in the presence and/or several of the enzymes of the Kennedy cycle (41). Inhibition of PC synthesis must be produced by an action on one of these compounds. Moreover, we propose that NMDA receptor-mediated potentiation of NMDA-evoked Cho release by inhibitors of phosphatidylcholine breakdown. These findings provide evidence for a new mechanism of excitotoxicity that may discriminate between necrotic and apoptotic neuronal cell death. Moreover, we propose that NMDA receptor-mediated inhibition of phosphatidylcholine synthesis is a key early event in the excitotoxic cascade that leads to necrotic cell death.

REFERENCES

1. Olney, J. W., Ho, O. L., and Rhee, V. (1971) Exp. Brain Res. 14, 61–76
2. Rothman, S. M., and Olney, J. W. (1995) Trends. Neurosci. 18, 57–58
3. Meldrum, B., and Garthwaite, J. (1990) Trends Pharmacol. Sci. 11, 579–387
4. Coyle, J. T., and Puttfarcken, P. (1993) Science 262, 689–695
5. Lipton, S. A., and Rosenberg, P. A. (1994) N. Engl. J. Med. 330, 613–622
6. Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., and Nicotera, P. (1995) Neuron 15, 961–973
7. Benfoco, E., Krain, D., Ankarcrona, M., Nicotera, P., and Lipton, S. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7162–7166
8. Cheung, N. S., Pascoe, C. J., Giardina, S. F., John, C. A., and Beart, P. M. (1998) Neuropharmacology 37, 1419–1429
9. Leist, M., and Nicotera, P. (1997) Biochem. Biophys. Res. Commun. 236, 1–9
10. Du, Y., Bales, K. R., Dedel, R. C., Hamilton-Byrd, E., Horn, J. W., Czilli, D. L., Simmons, L. K., Ni, B., and Paul, S. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11657–11662
11. Farooqui, A. A., Yang, H. C., Rosenberger, T. A., and Horrocks, L. A. (1997) J. Neurochem. 69, 889–901
12. Zapata, A., Capdevila, J. L., and Trullas, R. (1998) J. Neurosci. 18, 3597–3605
13. Liu, S., and Fain, J. N. (1992) J. Biol. Chem. 267, 3679–3685
14. Lysko, P. G., Cox, J. A., Vigano, A., and Henneberry, R. C. (1989) Brain Res. 499, 258–266
15. Rudolph, J. G., Lemasters, J. J., and Crews, F. T. (1997) Neurosci. Lett. 221, 149–152
16. Llafi, S., and Fain, J. N. (1992) Mol. Pharmacol. 42, 55–60
17. Wada, R. M. (1967) Biochem. J. 105, 206–210
18. Boss, V., and Conn, P. J. (1994) J. Neurochem. 62, 2340–2343
19. Roche, K. M., Wong, G., and Skolnick, P. (1993) Alcohol Clin. Exp. Res. 17, 54–60
20. Wang, B. J., Koh, J. Y., Demarco, A. J., Ying, H. S., Jaquin, M., and Choi, D. W. (1997) Neuroscience 77, 393–401
21. Yan, G. M., Ni, B. H., Wellen, M., Wood, K. A., and Paul, S. M. (1994) Science 266, 43–51
22. Ko, K. M., Wang, G., and Skolnick, P. (1983) Brain Res. 603, 207–214
23. Fosslom, L., Basile, A. S., and Skolnick, P. (1985) Mol. Pharmacol. 48, 981–987
24. Wong, E. H., Kemp, J. A., Priestley, T., Knight, A., Woodruff, G., and Iversen, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7104–7108
25. Koh, J. Y., Wang, B. J., Lohn, D., and Choi, D. W. (1995) Science 268, 573–575
26. Klein, J., Kippen, A., and Loffelholz, K. (1990) J. Neurochem. 55, 1231–1236
27. Millington, W. R., and Wurtman, R. J. (1982) J. Neurochem. 28, 1748–1752
28. Blusztajn, J. K., and Wurtman, R. J. (1983) J. Neurochem. 41, 418–426
29. Zeisel, S. H., and Blusztajn, J. K. (1984) Annu. Rev. Nutr. 14, 269–296
30. Dumuis, A., Sebbon, M., Haynes, L., Pin, J. P., and Bockeart, J. (1988) Nature 336, 68–70
31. Sanfeliz, J., Hunt, A., and Patel, A. J. (1990) Brain Res. 526, 241–248
32. Lazarewicz, J. W., Wroblewski, J. T., Palmer, M. E., and Costa, E. (1988) Neuropharmacology 27, 765–770
33. Lazarewicz, J. W., Wroblewski, J. T., and Costa, E. (1990) J. Neurochem. 53, 1875–1881
34. Smith, S. S., and Li, J. (1993) Mol. Pharmacol. 43, 1–5
35. Shimohama, S., Akaike, A., Tamura, Y., Matsushima, H., Kume, T., Fujimoto, S., Takenawa, T., and Kimura, J. (1995) J. Neurosci. Res. 41, 418–426
36. Hokin, L. E., Dixon, J. F., and Los, G. V. (1996) Adv. Enzyme Regul. 36, 229–244
37. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) Cell 71, 765–776
38. Hoffer, T., Cappel, E., Klein, J., and Loffelholz, K. (1993) J. Neurochem. 61, 1569–1572
39. Boss, V., Nutt, K. M., and Conn, P. J. (1994) Mol. Pharmacol. 45, 1177–1182
40. Zapata, A., Capdevila, J. L., and Trullas, R. (2000) Synapse 35, 272–280
41. Vance, D. E. (1991) in Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. E., and Vance, J., eds) pp. 205–240, Elsevier Science Publishers, Amsterdam
42. Li, Y., Maher, P., and Schubert, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7748–7753
Choline Release and Inhibition of Phosphatidylcholine Synthesis Precede Excitotoxic Neuronal Death but Not Neurotoxicity Induced by Serum Deprivation
Teresa Gasull, Nuria DeGregorio-Rocasolano, Agustin Zapata and Ramon Trullas

J. Biol. Chem. 2000, 275:18350-18357. doi: 10.1074/jbc.M910468199 originally published online March 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M910468199

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 14 of which can be accessed free at http://www.jbc.org/content/275/24/18350.full.html#ref-list-1