Primary cultured rat cerebellar granule neurons underwent apoptosis when switched from medium containing 25 mM K⁺ to one containing 5 mM K⁺. N-methyl-D-aspartate (NMDA) protected granule neurons from apoptosis in medium containing 5 mM K⁺. Inhibition of apoptosis by NMDA was blocked by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY294002, but it was unaffected by the mitogen-activated protein kinase kinase inhibitor PD 98059. The antiapoptotic action of NMDA was associated with an increase in the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), an increase in the binding of the regulatory subunit of PI 3-kinase to IRS-1, and a stimulation of PI 3-kinase activity. In the absence of extracellular Ca²⁺, NMDA was unable to prevent apoptosis or to phosphorylate IRS-1 and activate PI 3-kinase. Significant inhibition of NMDA-mediated neuronal survival by ethanol (10–15%) was observed at 1 mM, and inhibition was half-maximal at 45–50 mM. Inhibition of neuronal survival by ethanol corresponded with a marked reduction in the capacity of NMDA to increase the concentration of intracellular Ca²⁺, phosphorylate IRS-1, and activate PI 3-kinase. These data demonstrate that the neurotrophic action of NMDA and its inhibition by ethanol are mediated by alterations in the activity of a PI 3-kinase-dependent antiapoptotic signaling pathway.

Glutamate functions as the major excitatory neurotransmitter in the mammalian central nervous system by activating ionotropic and metabotropic glutamate receptors (1, 2). The NMDA receptor is an important subtype of ionotropic glutamate receptors that functions as a ligand-gated ion channel and initiates cation influx upon activation by glutamate or NMDA (3, 4). Activated NMDA receptors are about 10 times more permeable to Ca²⁺ than to Na⁺ and contain regulatory sites for Mg²⁺, Zn²⁺, glycine, polyamines, and phenacyclidine (3, 4). An increase in [Ca²⁺]ᵢ, is central to many of the properties of NMDA receptor activation in the central nervous system, including its role in development, neuroplasticity, and neurotoxicity (4, 5).

NMDA receptors can produce neurotrophic and/or neurotoxic effects in brain, depending on the stage of development and the degree of receptor activation. During restricted developmental periods, NMDA receptor activation has been shown to be important for maintaining neuronal survival, synaptogenesis, synaptic plasticity, learning, and memory (5, 6). However, excessive NMDA receptor activation can result in neuronal damage and cell death (7). Recent studies have shown that glutamate-induced cell death in cerebellar granule neurons is composed of sequential necrosis and apoptosis (8). After exposure to glutamate, a subpopulation of granule neurons undergoes rapid necrotic cell killing that is associated with a loss of mitochondrial function. Neurons surviving the early necrotic phase recover their mitochondrial potential and energy levels and sustain delayed apoptotic cell death. Glutamate-induced apoptosis has been shown to involve a Ca²⁺-dependent activation of p38 mitogen-activated protein kinase in cerebellar granule neurons (9).

In contrast with the excitotoxic effects of high concentrations of NMDA, low doses of NMDA enhance the survival of cerebellar granule neurons (10, 11). NMDA promotes the survival of cerebellar granule neurons cultured in the presence of physiological [K⁺] (5 mM) by inhibiting apoptosis (12). The trophic effects of NMDA in cultured cerebellar granule neurons have been proposed to mimic the ability of glutamatergic innervation in vivo to prevent elimination of postmigratory granule neurons after the formation of synaptic contacts (12). The mechanism underlying the antiapoptotic action of NMDA in cerebellar granule neurons is unknown.

The sedative properties of ethanol have led to investigation of its effect on NMDA receptor function. Acute ethanol exposure causes decreases in NMDA-activated ion currents in hippocampal neurons (13), NMDA-induced [Ca²⁺]ᵢ responses, and cGMP production in cerebellar granule neurons (14), and NMDA-evoked neurotransmitter release in rat brain slices (15). Prenatal ethanol exposure decreases NMDA-sensitive [³H]glutamate receptor binding (16) and functional responses to NMDA and metabotropic glutamate receptors (17, 18) in rat hippocampus. In cerebellar granule neurons, both the neurotrophic (19, 20) and excitotoxic (19) actions of NMDA are inhibited by ethanol. Further studies have indicated that NMDA receptor activation can protect against ethanol-induced cell death in newly established cultures of cerebellar granule neurons (21).

We have recently demonstrated that ethanol can also induce apoptosis by inhibiting IGF-1 signaling in cerebellar granule neurons (22).
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neurons (22). Ethanol blocks the antiapoptotic action of IGF-I by inhibiting its ability to promote IRS-1 phosphorylation and stimulate PI 3-kinase. Inhibition of IGF-I signaling by ethanol occurs downstream of the ligand binding site (22, 23) and is mediated by blocking IGF-I receptor autophosphorylation (23). Ethanol also inhibits IGF-I-induced cell proliferation in Balb/c 3T3 cells (23) and IGF-I and IGF-2-mediated cell growth in C6 rat glioblastoma cells (24). Inhibition of cell growth by ethanol in C6 glioblastoma cells is accompanied by a block of IRS-1 phosphorylation and PI-3 kinase activation (24).

In the present study, we have examined the mechanism by which NMDA promotes neuronal survival in cerebellar granule neurons and the involvement of these processes in the neurotoxic action of ethanol. Our data show that the ability of NMDA to inhibit apoptosis in cerebellar granule neurons is associated with a Ca2+-dependent increase in the tyrosine phosphorylation of IRS-1, the binding of PI 3-kinase to IRS-1, and the activation of PI 3-kinase. Ethanol inhibits NMDA-induced [Ca2+]i increases and blocks the capacity of NMDA to protect cerebellar granule neurons from apoptotic cell death. Inhibition of NMDA-mediated neuronal survival by ethanol corresponds with a marked reduction in the phosphorylation of IRS-1, the binding of PI 3-kinase to IRS-1, and the activation of PI 3-kinase. These data demonstrate that NMDA prevents apoptosis through activation of PI 3-kinase and that ethanol induces cell death in cerebellar granule neurons by inhibiting NMDA-induced activation of a PI 3-kinase-dependent anti-apoptotic signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture media and solutions were purchased from Life Technologies, Inc. DNase, trypsin inhibitor, cytochrome c, poly-L-lysine (molecular mass, >300 kDa), trypsin blue, cycloheximide, MTT tetrazolium salt, phenol, chloroform, isooamyl alcohol, isopropanol, molybdenum blue spray reagent, phosphatidylinositol, and phosphatidylinositol 4-phosphate were all from Sigma. NMDA, PD 98059 was from Alexis (San Diego, CA). PD 98059 was from Alexis (San Diego, CA). Anti-phosphotyrosine antibody (5 μg/ml) was stained with 0.65% molybdenum oxide in 4.2M sulfuric acid and applied to nitrocellulose membranes. Secondary antibody horseradish peroxidase-conjugated goat anti-rabbit antibody, nitrocellulose membranes, and the enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantitated with a Bio-Rad Imaging Densitometer.

**Culture of Granule Neurons**—Rat cerebellar granule neurons were prepared, essentially as described by Doble et al. (10). The cerebella were obtained from Sprague-Dawley rat pups (postnatal day 7–8) and cross-chopped (400 μm thick) with a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, United Kingdom); they were then trypsinized with 0.025% trypsin/EDTA (including 0.01% DNase I) for 15 min at 37 °C. Tryptsin digestion was stopped by addition of trypsin inhibitor (0.05%). After trituration, cerebellar granule neurons were plated at a density of 1 × 106 cells/cm2 on 25-mm circular glass coverslips or in plastic multiwells and flasks coated with poly-L-lysine (10 μg/ml) in BME containing 10% fetal bovine serum, 25 mM KCl, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After 18–24 h, 10 μg cytochrome c was added to the neuronal cultures to inhibit replication of nonneuronal cells. Cerebellar granule neurons were maintained in culture and used at 6–7 days in vitro.

**Neuronal Survival**—Cerebellar granule neurons were washed twice with serum-free BME containing 25 mM KCl (high K+ [HK]) or 5 mM KCl (low K+ [LK]). Cells were then treated at excitatory concentrations and incubated for a further 24 h. Ethanol-treated cultures were covered with Parafilm to keep the ethanol concentration constant without interfering with the permeability to gases. After 24 h, the cells were rinsed once with PBS (100 mM phosphate, 0.9% NaCl, pH 7.4) and incubated with Hoechst dye 33342 (10 μg/ml) for 10 min or with 0.4% trypan blue for 5 min at room temperature (24°C). Cell morphology and trypan blue staining were assessed by phase contrast microscopy, and nuclear DNA staining was examined by digital fluorescence imaging microscopy (Olympus). Measurements were obtained from three randomly chosen fields on three different cell preparations.

**Cell viability was also assessed with the MTT tetrazolium salt assay, essentially as described by Mantyh et al. (25). Briefly, MTT tetrazolium salt (0.6 mg/ml) was added to neurons grown in 24-well plates and incubated for 2–3 h at 37 °C. The reaction media were then gently aspirated, and isopropanol containing 0.1 N HCl was added to solubilize the blue formazan product. Formazan-isopropanol mixtures were then transferred to 96-well plates and quantified using a Multiskan plate reader at 570 nm (Elbio, Oy, Helsinki, Finland).**

DNA Analysis—Genomic DNA was isolated from neurons grown in T25 or T75 flasks. Cells were rinsed with BME, scraped, collected in Tris-buffered saline (100 mM Tris, 0.9% NaCl, pH 7.5), and lysed with buffer containing 50 mM Tris, 20 mM EDTA, 100 mM NaCl, 1% SDS, 100 μg/ml proteinase K, and 20 μg/ml DNase-free RNase for 3 h at 50 °C. The reaction mixtures were extracted with 1 volume of Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8.1)-saturated phenol-chloroform-isooamyl alcohol (25:24:1), and the aqueous phases were mixed with 1/10 volume of 3 M sodium acetate (pH 5.2) and equal volumes of ice-cold isopropanol for 24 h at −20 °C. DNA was then pelleted, air-dried, and dissolved in Tris-EDTA buffer. Total DNA was resolved on a 1.2% agarose gel, stained with 1 μg/ml ethidium bromide, and photographed under UV light.

**Immunoprecipitation and Western Blotting**—Cells were lysed in buffer containing 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 0.1 mg/ml aprotinin (pH 7.5). Equal amounts of protein (500 μg) from each sample were immunoprecipitated with the addition of a rabbit anti-rat IRS-1 antibody (5 μg) and protein A-agarose. Immunocomplexes were dissolved with Laemmli buffer (26), separated on a 10% reducing SDS-PAGE gel, and electrophoerized onto nitrocellulose membranes. The membranes were probed with anti-phosphotyrosine antibody (PY20) (1:1000) and rabbit anti-rat PI 3-kinase (1:1000) antibody, followed by a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:5000). The membranes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) and quantitated with a Bio-Rad Imaging Densitometer.

**PI 3-Kinase Assay**—PI 3-kinase activity was measured, essentially as described previously (27, 28), with minor modifications. Briefly, IRS-1 immunoprecipitates were washed twice with lysis buffer and resuspended in Tris-buffered saline (100 mM Tris, 0.9% NaCl, pH 7.5) containing 1 mM EDTA. The kinase assay was run in a reaction mixtures containing 100 mM MnCl2, phosphatidylinositol (2 μg/ml), and (γ-32P)ATP (30 μCi) for 10 min at 24 °C. The reaction was stopped by addition of 8 N HCl and chloroform:methanol (1:1). The mixtures were centrifuged, and the lower organic phase was applied to a silica gel thin layer chromatography plate. The plate was developed in a chloroform:methanol:water:ammonium hydroxide mixture (60: 47: 3:1, v/v). The spots were visualized with an X-ray film and autoradiographed. Phosphatidylinositol 4-phosphate was stained with 0.65% molybdenum oxide in 4.2 μl sulfuric acid and used to determine the comigration of phosphatidylinositol 3-phosphate.

**Intracellular Calcium Measurements**—Neurons were grown on glass coverslips in the presence of HK medium and used for [Ca2+]i measurements at 6–7 days in vitro. Measurements of [Ca2+]i were obtained from cells loaded with Fura2 by incubation with Fura2/AM (2.5 μM in 0.025% plunic acid) for 15 min at 37 °C in HEPES buffer (121 mM NaCl, 5 mM NaHCO3, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, and 2% bovine serum albumin, pH 7.4). After loading, cells were washed once with HEPES buffer containing 2% bovine serum albumin, and then twice with HEPES buffer containing 0.25% bovine serum albumin. Fluorescence images were obtained, essentially as described previously (29). Coverslips with attached Fura2-loaded granule neurons were transferred to a chamber with 1 ml of HEPES buffer and mounted on the stage of an inverted Olympus epifluorescence microscope. The stage, oil immersion objective, and chamber were thermostatically regulated at 37 °C. A cooled charge-coupled device camera (Photometrics Ltd.) was used as the imaging device. For [Ca2+]i measurements, fluorescence images were obtained from the entire field of view at the emission wavelength of 460–600 nm. [Ca2+]i was calculated from the fluorescence measurements using the ratio method, as described previously (29).

**Statistical Analysis**—The data represent the mean ± S.E. from three different neuronal culture preparations. Statistical significance was determined by paired Student’s t test.
RESULTS

NMDA Inhibits Apoptosis in Cerebellar Granule Neurons—Cerebellar granule neurons were cultured for 7 days in medium containing 10% fetal calf serum and 25 mM KCl (HK). Neurons were then switched to serum-free medium containing either HK, 5 mM KCl (LK), or LK+NMDA (100 μM). Cell survival was assessed at 24 h by counting apoptotic and nonapoptotic neurons after staining nuclear chromatin with the fluorescent dye Hoechst dye staining. *, statistically significant difference from LK cultures alone (p < 0.05), calculated by paired Student’s t test. The results represent the mean ± S.E. of data from 75–90 cells.

Ethanol Inhibits the Antiapoptotic Action of NMDA—Ethanol inhibited the antiapoptotic action of NMDA in cerebellar granule neurons maintained in HK (Figs. 1 and 2). We have shown previously that ethanol does not inhibit the neurotrophic action of elevated K+ or potentiate cell death produced by LK (22). The dose-dependent effects of ethanol and NMDA on neuronal survival were investigated further using the MTT assay. Fig. 3 shows that the EC50 for NMDA-mediated neuroprotection was 5.3 μM, with maximal protection obtained with 100 μM NMDA. Ethanol produced near complete inhibition of neuronal survival at all concentrations of NMDA (Fig. 3). At high concentrations of NMDA (>200 μM), the ability to promote neuronal survival was decreased. This is consistent with previous findings in which excessive NMDA receptor stimulation has been shown to be toxic to cerebellar granule neurons (8, 9).

Fig. 4 shows the concentration dependence of ethanol-in-duced inhibition of NMDA-mediated neuronal survival. Significant inhibition by ethanol (10–15%) was observed at 1 mM, and complete inhibition was obtained at 200 mM. The IC50 for ethanol-induced inhibition was 45–50 mM. A similar dose dependence for ethanol-induced inhibition of NMDA-mediated neuronal survival has been shown in cerebellar granule neurons that have been kept in LK medium for 4 days in vitro (20). Combined Action of NMDA and IGF-I on Neuronal Survival—We have recently shown that IGF-I-mediated cell survival in cerebellar granule neurons is half-maximal at 2 ng/ml and that ethanol potently inhibits the ability of IGF-I to prevent apoptosis in cerebellar granule neurons (22). In view of the similar nature of ethanol interaction with the neurotrophic action of NMDA, we examined whether there is cross-talk between NMDA and IGF-I signaling pathways. Fig. 5 compares the ability of submaximal concentrations of NMDA (1 and 5 μM) and IGF-I (1 and 2 ng/ml) to inhibit apoptosis in cerebellar granule neurons. Addition of 1 μM NMDA or 1 ng/ml IGF-I sustained neuronal survival in 30–32% of cells, and 5 μM NMDA or 2 ng/ml IGF-I protected 44–50% of cerebellar gran-
Previous studies have shown that Ca$^{2+}$ entry plays an important role in the neurotrophic and neurotoxic actions of NMDA in cerebellar granule neurons (30, 31). Fig. 6 shows that NMDA increases [Ca$^{2+}$]$_i$ by 80–85%. NMDA-induced [Ca$^{2+}$]$_i$ increases were also completely inhibited by addition of EGTA (data not shown). To assess the contribution of Ca$^{2+}$ entry to the neurotrophic action of NMDA, the ability of NMDA to inhibit apoptosis was examined in the absence of extracellular Ca$^{2+}$. Removal of extracellular Ca$^{2+}$ with EGTA blocked the capacity of NMDA to prevent apoptosis in LK medium (31). Ethanol substantially inhibited Ca$^{2+}$ entry to the neurotrophic action of NMDA, the ability of NMDA to inhibit apoptosis was examined in the absence of extracellular Ca$^{2+}$. Removal of extracellular Ca$^{2+}$ with EGTA blocked the capacity of NMDA to prevent apoptosis in LK medium (31). Ethanol substantially inhibited Ca$^{2+}$ entry to the neurotrophic action of NMDA. These data demonstrate that the ant apoptotic action of NMDA depends on the stimulation of Ca$^{2+}$ entry and that inhibition of NMDA-mediated neuronal survival by ethanol is associated with a block of NMDA-induced [Ca$^{2+}$]$_i$ increases.

The downstream signaling pathways responsible for the Ca$^{2+}$-dependent neurotrophic action of NMDA were examined by investigating the effect of selective protein kinase inhibitors. Addition of the PI 3-kinase inhibitor LY294002 (30 μM), inhibited the ability of NMDA to prevent DNA fragmentation (Fig. 2) and promote neuronal survival (Fig. 7) in LK medium. In contrast, the mitogen-activated protein kinase kinase inhibitor PD98059 (50 μM) had no effect on the antiapoptotic action of NMDA (Figs. 2 and 7). These data are consistent with recent studies of the signaling pathways involved in the antiapoptotic action of IGF-I in cerebellar granule neurons (22, 31, 49) and suggest that NMDA-induced neuronal survival requires activation of PI 3-kinase but does not involve mitogen-activated protein kinase kinase activation.

**NMDA Phosphorylates IRS-1 and Activates PI 3-Kinase**—IGF-I inhibits apoptosis in cerebellar granule neurons by activating PI 3-kinase through an IRS-1-linked signaling pathway (22, 31, 34, 49). To determine whether IRS-1 is involved in the activation of PI 3-kinase by NMDA, cerebellar granule neurons in LK medium were stimulated with NMDA (100 μM) for 10 min, and then proteins were immunoprecipitated from cell lysates with an antibody to IRS-1 and separated by SDS-PAGE. The proteins were then probed with antibodies to phosphotyrosine and PI 3-kinase. NMDA increased the phosphorylation of IRS-1 (Fig. 8A) and the binding of PI 3-kinase to IRS-1 (Fig. 8B) by about 4-fold and 3-fold, respectively. The phosphorylation of IRS-1 and the binding of PI 3-kinase to IRS-1 corresponded with a 4-fold increase in NMDA-stimulated PI 3-kinase activity (Fig. 9).

Both ethanol (100 mM) and removal of extracellular Ca$^{2+}$ with EGTA inhibited the ability of NMDA to phosphorylate IRS-1, stimulated the binding of PI 3-kinase to IRS-1, and activated PI 3-kinase (Figs. 8 and 9). Incubation of cerebellar granule neurons in HK also stimulated the phosphorylation of IRS-1 and increased the activity of PI 3-kinase (data not shown).

**DISCUSSION**

In the present study, we have shown that NMDA inhibits LK-induced apoptosis in cerebellar granule neurons through the activation of PI 3-kinase. Activation of PI 3-kinase by NMDA depends on the stimulation of Ca$^{2+}$ entry and is associated with an increase in the tyrosine phosphorylation of IRS-1 and the binding of PI 3-kinase to IRS-1. Ethanol significantly inhibits the ability of NMDA to increase [Ca$^{2+}$], and blocks its capacity to protect cerebellar granule neurons from apoptotic cell death. Inhibition of NMDA-mediated neuronal survival by ethanol also correlates with a marked reduction in NMDA-stimulated IRS-1 phosphorylation and NMDA-induced PI 3-kinase activity. These findings demonstrate that the antiapoptotic action of NMDA (and its inhibition by ethanol) involves cross-talk with the IGF-I signaling pathway.

Ca$^{2+}$ entry has been shown to play an important role in the neurotrophic action of NMDA in cerebellar granule neurons (11). Inhibition of NMDA-mediated Ca$^{2+}$ entry has also been proposed to contribute to ethanol neurotoxicity because ethanol inhibits the trophic effect of NMDA and NMDA-induced [Ca$^{2+}$]$_i$ increases with similar potency (20). However, little is known about the downstream signaling pathways responsible for the Ca$^{2+}$-dependent neurotrophic action of NMDA and their potential to serve as targets for the neurotoxic action of ethanol. Some data have suggested that promotion of neuronal survival by NMDA and HK in cerebellar granule neurons involves activation of Ca$^{2+}$/calmodulin-dependent protein kinase (32) or release of a parathyroid hormone-related protein (33). Expression of parathyroid hormone/parathyroid hormone-related protein receptor mRNA is also up-regulated in an activity-dependent manner by NMDA and HK (33). There have also been inconsistent findings pertaining to the role of PI 3-kinase in HK-mediated neuronal survival in cerebellar granule neurons, with one study indicating a requirement for PI 3-kinase activation (31), whereas another showed that the neurotrophic action of HK does not involve activation of PI 3-kinase (34).

Our data show that the ability of NMDA-stimulated Ca$^{2+}$ entry to inhibit apoptosis depends on the activation of PI 3-kinase and is associated with the phosphorylation of IRS-1. We have recently shown that ethanol does not inhibit HK-induced apoptosis.
neuronal survival in cerebellar granule neurons, under conditions where it blocks the antiapoptotic action of IGF-I by inhibiting its ability to promote IRS-1 phosphorylation and stimulate PI 3-kinase (22). Previous studies have established that inhibition of IGF-I signaling by ethanol occurs distal from the ligand binding interaction (22, 23) and is mediated by blocking IGF-I receptor autophosphorylation (23). This indicates that ethanol can induce apoptosis by inhibiting individual components of distinct signaling pathways and blocking their capacity to activate PI 3-kinase.

IRS-1 is one of the major substrates phosphorylated in response to stimulation by insulin or IGF-I, and it functions

FIG. 5. Interaction between neurotrophic action of NMDA and IGF-I. Cerebellar granule neurons were rinsed twice with BME and incubated for 24 h in media containing 25 mM K\(^+\) (HK), 5 mM K\(^+\) (LK), or 5 mM K\(^+\) in the presence of the indicated concentrations of NMDA and IGF-I. Cell survival was determined by the MTT assay. The results represent the mean ± S.E. of data from three separate incubations.

FIG. 6. Effect of ethanol on NMDA-induced [Ca\(^{2+}\)]\(_i\) increases. Cerebellar granule neurons were grown on glass coverslips in HK medium and used for [Ca\(^{2+}\)]\(_i\) measurements at 6–7 days in vitro. After pretreatment in the absence or presence of ethanol (100 mM) for 5 min or 24 h, Fura2-loaded cerebellar granule neurons were exposed to NMDA (50 μM) for 15 min. The [Ca\(^{2+}\)]\(_i\), increase produced by NMDA (50 μM) was examined in the presence of Mg\(^2+\)-free HEPES buffer containing 10 μM t-serine. NMDA-induced [Ca\(^{2+}\)]\(_i\), increases in ethanol-treated cells were examined in the continued presence of ethanol. The results are expressed as the [Ca\(^{2+}\)]\(_i\), increase above basal levels (40–50 nM) and represent the mean ± S.E. of data from 28–30 cells.

FIG. 7. Signaling pathways involved in NMDA-mediated neurotrophism. Cerebellar granule neurons were rinsed twice with BME, incubated for 24 h in media containing 5 mM K\(^+\) (HK), 5 mM K\(^+\) (LK), 5 mM K\(^+\) and 100 μM NMDA (LK+NMDA), 5 mM K\(^+\) and 100 μM NMDA + 2 μM EGTA (LK+NMDA+EGTA), 5 mM K\(^+\) and 100 μM NMDA + 2 μM LY294002 (LK+NMDA+LY), or 5 mM K\(^+\) and 100 μM NMDA + 50 μM PD98059 (LK+NMDA+PD). Cell survival was determined by the MTT assay. The results represent the mean ± S.E. of data from three separate incubations.
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FIG. 9. NMDA stimulates PI 3-kinase activity. Cerebellar granule neurons were rinsed twice in BME and incubated for 2 h in medium containing 5 mM K+ and then incubated with no additions (LK), 100 μM NMDA (NMDA), 100 μM NMDA + 100 mM ethanol (NMDA+EtOH), or 100 μM NMDA + 2 mM EGTA (NMDA+EGTA) for 10 min. The cells were lysed, immunoprecipitated with an antibody to IRS-1, and assayed for PI 3-kinase activity using phosphatidylinositol as a substrate. Phosphatidylinositol 3-phosphate was separated using thin layer chromatography and quantified using a densitometer. Results are representative of two or three independent experiments.

targets of ethanol action. Thus, Fyn kinase binds and phosphorylates IRS-1 (47) and regulates behavioral sensitivity to ethanol and the ability of ethanol to interfere with NMDA receptor function (48). Fyn-deficient mice have been shown to be more sensitive to the hypnotic effect of ethanol and do not exhibit enhanced tyrosine phosphorylation of the NMDA receptor after ethanol treatment (48). In addition, acute tolerance to ethanol inhibition of NMDA-mediated excitatory postsynaptic potentials is not observed in hippocampal slices from mice lacking Fyn kinase.

NMDA-induced neuronal survival was inhibited by LY294002 but unaffected by PD98509. This is consistent with previous studies of the neurotrophic action of IGF-I in cerebellar granule neurons (22, 31, 34) and indicates that the antiapoptotic response of NMDA requires activation of PI 3-kinase and is independent of mitogen-activated protein kinase kinase. The signaling pathways through which PI 3-kinase activation promotes neuronal survival have recently started to be elucidated. For example, studies have shown that the serine-threonine protein kinase Akt (protein kinase B) is an important mediator of PI 3-kinase-dependent neuronal survival (49). The lipid products of PI 3-kinase bind to Akt and promote membrane translocation (50), thereby enabling its phosphorylation and activation by a recently identified family of Akt kinases (51, 52). Activated forms of Akt have been found to phosphorylate BAD (53, 54), which leads to its association with 14-3-3 proteins and prevents BAD from binding to and inhibiting the survival proteins Bcl-XL and Bcl-2 (55).

The ability of prenatal ethanol exposure to induce brain damage that can result in severe mental retardation linked to fetal alcohol syndrome, or associated attention disorders and hyperactivity, has been well documented (56, 57). The cerebellar region of the developing central nervous system is particularly sensitive to ethanol-induced cell injury, with decreases in cerebellar size, the number of granule and Purkinje neurons, and delayed neuronal maturation and synapse formation, all consequences of ethanol exposure (58, 59). However, the mechanisms underlying the teratogenic effects of ethanol in brain have remained ill defined. Most in vitro studies of the effects of ethanol on neuronal survival in cerebellum have assessed cell death by using markers of plasma membrane integrity (19, 60, 61). Although these probes provide a measure of cell viability, they do not distinguish between apoptotic and necrotic forms of cell death or define any of the events mediating these two processes. Moreover, there have been contradictory reports concerning the effects of ethanol on neuronal survival, with some indicating that ethanol has a direct neurotoxic action (60) and others showing that ethanol acts by inhibiting neurotrophic support (19, 20, 22). Here, we show that ethanol can induce apoptotic cell death in cerebellar granule neurons by inhibiting the ability of NMDA to activate a PI 3-kinase-dependent neuronal survival pathway.

In conclusion, the data presented here demonstrate that the ability of NMDA to inhibit apoptosis in cerebellar granule neurons is mediated by a Ca2+-dependent activation of PI 3-kinase. The activation of PI 3-kinase by NMDA is associated with an increase in both the phosphorylation of IRS-1 and the binding of PI 3-kinase to IRS-1. Ethanol blocks the antiapoptotic action of NMDA and inhibits NMDA-mediated [Ca2+]i increases, IRS-1 phosphorylation, and PI 3-kinase activation. These findings describe a new role for PI 3-kinase and IRS-1 in NMDA signaling and reveal an additional mechanism whereby ethanol induces apoptosis in cerebellar granule neurons by inhibiting NMDA-induced PI 3-kinase activation.
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