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A Systemic Administration of NMDA Induces Immediate Early Gene *pip92* in the Hippocampus

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Abstract: In the mammalian CNS, aspartate and glutamate are major excitatory amino acids, and their receptors are believed to mediate a wide range of physiological and pathological processes, including neurotransmission, plasticity, excitotoxicity, and various forms of neurodegeneration. The immediate early gene *pip92* has been identified in serum-stimulated BALB/c 3T3 fibroblasts, activated T lymphocytes treated with cycloheximide, and fibroblast growth factor-stimulated hippocampal cells during neuronal differentiation. In this study we have demonstrated that *pip92* is expressed in the mouse brain after a single intraperitoneal injection of NMDA. The distribution of *pip92* mRNA levels in the NMDA-treated mouse brain was investigated using in situ RT-PCR. The region-specific activation of *pip92* in the CNS was observed 3 h after NMDA injection, and high levels of *pip92* mRNA were detected in the hippocampal dentate gyrus and piriform cortex regions. In addition, the activation of *pip92* by NMDA was mediated by activation of mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK) and p38 kinase, but not extracellular signal-regulated kinase (ERK) in the mouse hippocampus and immortalized rat hippocampal progenitor cells. This study suggests that *pip92* is likely to play an important role in neuronal cell death induced by excitotoxic NMDA injury in the CNS. **Key Words:** NMDA—*pip92*–Excitotoxic injury—c-Jun N-terminal kinase—p38—Hippocampus.
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In many cell types, extracellular stimuli such as serum, growth factors, phorbol esters, neurotransmitters, cytokines, Ca^{2+}, UV light, and cellular redox agents regulate critical cellular events such as growth, differentiation, and apoptosis through activation of protein kinase cascades. In the brain, excitatory neurotransmission elevates the calcium concentration in neuronal cells and activates the transcription of immediate early genes (Ghosh and Greenberg, 1995). Glutamate and aspartate, the major excitatory neurotransmitters in the brain, become toxic to the CNS via excess activation of ionotropic receptors sensitive to NMDA, kainate, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Choi et al., 1987). Cell death induced by glutamate is believed to be involved in neuronal loss associated with both acute, e.g., stroke, and chronic, e.g., Alzheimer's disease, neurodegenerative insults (Rothman, 1984; Choi and Rothman, 1990).

The mitogen-activated protein kinase (MAPK) cascade is considered to be a major signaling pathway that links signals from the cell surface to the nuclear events (Hill and Treisman, 1995). This signaling pathway involves transient formation of Ras-GTP, activation of Raf kinases at the membrane, and sequential activation of MAPK kinase (MEK) and MAPK/extracellular signal-regulated kinase (ERK) (Marshall, 1994). Activated ERKs translocate to the nucleus, phosphorylate, and thus activate transcription factors such as cyclic AMP response element binding protein and Elk1, resulting in the induction of new gene transcripts. In addition, other

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Abbreviations used: ACPT-II, (1*R*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-trichlorocarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ERK, extracellular signal-regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein; MEK, mitogen-activated protein kinase kinase; MEKK, mitogen-activated protein kinase kinase kinase; M-MLV, Moloney murine leukemia virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBQX, 6-nitro-7-sulfamoylbenzo-[*f*]quinoxaline-2,3-dione; SEK1, stress-activated protein/extracellular signal-regulated kinase kinase 1; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor.

members of the MAPK superfamily—c-Jun N-terminal kinase (JNK) and p38—are activated by inflammatory cytokines and cellular stresses such as UV and high osmolarity (Kyriakis et al., 1994; Matsuda et al., 1995).

The immediate early gene *pip92* (also known as *chx1* or *ETR101*) is rapidly and transiently induced by serum, platelet-derived growth factor, nerve growth factor, fibroblast growth factor, and 12-*O*-tetradecanoylphorbol 13-acetate and is functionally implicated in the neuronal differentiation process (Charles et al., 1990; Chung et al., 1998). *pip92* encodes a short-lived, proline-rich protein with no significant sequence similarity to any known protein. However, little is known about the function of its encoded protein. The serum response element (SRE) is the major regulatory element located in the *pip92* promoter, which is recognized by serum response factor (SRF) and ternary complex factor (TCF), a family of Ets-domain transcription factors (Latinkic and Lau, 1994). Elk-1 is a member of the TCF family, which is phosphorylated by ERK and therefore activated to form a ternary complex with SRF and SRE (Hill and Treisman, 1995; Treisman, 1995). Electrophoretic mobility shift analysis has demonstrated that Elk-1 binds to Ets sites in the *pip92* promoter (Latinkic and Lau, 1994). JNK and p38, as well as ERK, could also phosphorylate Elk-1 (Gille et al., 1995; Whitmarsh et al., 1995, 1997; Janknecht and Hunter, 1997). Although there have been several reports about situations in which JNK and/or p38 activation occurs without influencing cell death (Liu et al., 1996; Lenczowski et al., 1997), high levels of JNK and p38 activities have been correlated with the induction of apoptosis in many instances (Raitano et al., 1995; Xia et al., 1995; Chen et al., 1996*a*,*b*; Verheij et al., 1996; Xu et al., 1996; Zanke et al., 1996; Goillot et al., 1997; Rodrigues et al., 1997; Yang et al., 1997).

Based on these findings, it is possible that JNK and p38 are involved in *pip92* induction during the cell death process. However, little is known about its role and the induction mechanism of *pip92* in neuronal cell death induced by cytotoxic stress or neurodegenerative insults. To investigate the role of *pip92* in the cellular response to extracellular cytotoxic stimuli, we have tested the effect of a systemic administration of NMDA on *pip92* expression in mouse brain and analyzed the possible signaling pathways involved. Here we report for the first time that *pip92* mRNA levels are increased in a region-specific manner in mouse CNS after a single intraperitoneal NMDA injection. Our results indicate that the activation of p38 and JNK, but not ERK, is involved in the induction of *pip92* during NMDA-mediated neuronal cell death, suggesting that different MAPK pathways are involved in expression of the immediate early gene *pip92* induced by growth factors and excitotoxic stimuli.

EXPERIMENTAL PROCEDURES

Materials

Fetal bovine serum, Dulbecco's modified Eagle's medium, and geneticin were purchased from Life Technologies (Grand Island, NY, U.S.A.). PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA, U.S.A.). (+)-MK-801 maleate, 6-nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3-dione (NBQX), and (1*R*,3*R*,4*S*)-1-aminocyclopentane-1,3,4-trichlorocarboxylic acid (ACPT-II) were purchased from Tocris (Bristol, U.K.). NMDA and all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Dominant-negative MEKK (MEK kinase) and SEK1 (stress-activated protein/ERK kinase 1) mutant, *p*ΔMEKK(3′)KR-5′EE-CMV, pSEK1(AL)/EE-CMV, and pEE-CMV DNAs were kindly provided by D. Templeton. Plasmid expressing glutathione *S*-transferase (GST)–MAPK-activated protein (MAPKAP) kinase-2 was obtained from C. K. Huang. Plasmid expressing GST–c-Jun was provided by P. Angel.

Specimen preparation

Adult ICR mice (weighing 30–35 g) were injected intraperitoneally with 70 mg/kg NMDA prepared in phosphate-buffered saline (pH 7.4) and killed 3 h after treatments as described elsewhere (Shin et al., 1998). As a control, an equal volume of vehicle was injected into the mice. The mice were perfused with phosphate-buffered saline through the heart. Then the whole brain was removed to prepare for the molecular biological and histological investigations. The protocols for animal experimentation were approved by the Committee on Animal Care and Experimentation at Kosin University College of Medicine. The general molecular biological methods, such as nucleic acid extraction, RT-PCR, and cloning, were performed as described elsewhere (Ausubel et al., 1995).

Oligonucleotide primers

Based on the sequences of mouse *pip92* cDNA (Charles et al., 1990) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (Sabath et al., 1990), two sets of oligonucleotide primers (5′-CTACTCTCGTCGAACAAGCATGGAAG-3′, 5′-GCCACCAGAGCTCTCAGAAGGCCACC-3′ for *pip92*; 5′-GCCACCCAGAAGACTGTGGATGGC-3′, 5′-CATGTAGGCCATGAGGTCCACCAC-3′ for G3DPH) were synthesized (Bioneer, Korea).

Cell culture and transfection

Rat neuronal hippocampal progenitor H19-7 cells were generated by transduction with the retroviral vectors containing temperature-sensitive SV40 large T antigen that is functionally active at 33°C and inactive at 39°C (Eves et al., 1992). The H19-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained at 33°C under 200 μg/ml G418 selection. Transient transfections were performed using LipofectAMINE reagents (GibcoBRL) according to the manufacturer's protocol. Plasmid pCMV-GAL, which contains the *Escherichia coli* β-galactosidase gene driven by the cytomegalovirus promoter, was used as an internal control to determine transfection efficiency. When specified, cells were pretreated with 50 μ*M* noncompetitive NMDA antagonist [(+)-MK-801], 30 μ*M* selective AMPA/kainate antagonist (NBQX), 100 μ*M* competitive antagonist of the metabotropic glutamate receptor (ACPT-II), 50 μ*M* synthetic p38 kinase inhibitor (SB203580), or 30 μ*M* MEK inhibitor (PD98059) 30 min before NMDA stimulation.

RNA preparations and northern blot analysis

Total cellular RNAs from H19-7 cells were isolated by the single-step extraction procedure using guanidinium isothiocyanate as described elsewhere (Chomczynski and Sacchi, 1987).

Northern blot analysis to measure *pip92* mRNA levels was done as described previously (Chung et al., 1998).

Assessment of cell survival by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) extraction assay

Cell survival was quantified by a tetrazolium salt-extraction method as described elsewhere (Hansen et al., 1989). To each well containing 250 μl of medium in a 24-well plate, 62.5 μl of the 5 mg/ml stock solution of MTT was added. After 2 h of incubation at 37°C, 250 μl of extraction buffer containing 20% sodium dodecyl sulfate and 50% *N,N*-dimethyl formamide (pH 7.4) was added. After an overnight incubation at 37°C, the optical density at 570 nm was measured using a Spectra MAX 340 ELISA Reader (Molecular Devices, Sunnyvale, CA, U.S.A.), using the extraction buffer as the blank.

In vitro RT-PCR

Total RNA was prepared from the brain using RNAzol B solution (BIOTEX). The RT conditions were as follows: 4 μg of total RNA, 4 μl of Moloney murine leukemia virus (M-MLV) 5× RT buffer, 4 μl of 10 m*M* deoxynucleotide triphosphates, 100 pmol of oligo$(dT)_{16}$, 20 units of RNasin, 100 units of M-MLV reverse transcriptase, and RNase-free water to a total volume of 20 μl. The RT mixture was incubated for 1 h at 42°C. The PCR conditions were as follows: 1 μl of RT products, 3 μl of *Taq* 10× buffer, 1 μl of 10 m*M* deoxynucleotide triphosphates, 10 pmol of sense and antisense primer, 1 unit of *Taq* polymerase, and deionized water to a total volume of 30 μl. PCR amplification (30 cycles) was carried out at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s.

In situ RT-PCR

Mouse brains were cryosectioned 10 μm thick and mounted on 3-aminopropyltriethoxysilane-coated slides. Slides were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline, and dehydrated in serial concentrations of ethanol. Genomic DNA was removed using RNase-free DNase (8 U/100 μl). In situ RT mix contained 5 μl of M-MLV 5× RT buffer, 5 μl of 10 m*M* deoxynucleotide triphosphates, 100 pmol of oligo$(dT)_{16}$, 20 units of RNasin, 100 units of M-MLV reverse transcriptase, and RNase-free water to a total volume of 25 μl. The section was washed with distilled water and dehydrated with 100% ethanol. The slide seal for in situ PCR (TaKaRa, Japan) was located around the tissue section on the slide and covered with PCR mixture. In situ PCR mix contained 2.5 μl of *Taq* 10× buffer, 2.5 μl of 10× PCR dig-labeling mix (Boehringer Mannheim), 25 pmol of sense and antisense primers, 2.5 units of *Taq* polymerase, and deionized water to a total volume of 25 μl. Cycling conditions were the same as in vitro PCR conditions. The in situ PCR amplification was carried out in an OmniGene thermocycler (Hybaid, U.K.), and the products were visualized using the digoxigenin detection kit (Boehringer Mannheim). Slides were analyzed using an optical microscope. Positive-staining cells were purple, whereas negative cells usually appeared colorless or slightly brown.

Protein kinase assay for ERK, JNK, and p38

Dissected tissues were homogenized using buffer A containing 20 m*M* Tris (pH 7.9), 137 m*M* NaCl, 5 m*M* Na_2EDTA, 10% glycerol, 1.0% Triton X-100, 0.2 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 20 μ*M* leupeptin, 1 m*M* Na_3VO_4, 1 m*M* EGTA, 10 m*M* NaF, 1 m*M* tetrasodium pyrophosphate, and 1 m*M* β-glycerophosphate. Confluent cells were harvested and lysed using the same buffer. Cell lysates were subjected to centrifugation at 12,000 *g* for 10 min at 4°C. Soluble fraction was incubated for 1 h at 4°C with antibodies against JNK1, ERK2, or p38. Immunocomplex kinase assays were performed as described previously (Chung et al., 1999). The phosphorylated substrates were visualized by autoradiography. Myelin basic protein (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), GST–c-Jun (kindly provided by Dr. P. Angel), and GST–MAPKAP kinase-2 (kindly provided by Dr. C. K. Huang) were used as the substrate for ERK, JNK, and p38 kinase, respectively. Bacterial GST–fusion proteins were prepared with the GST purification module (Pharmacia) according to the manufacturer's protocol.

Western blot analysis with anti-ERK, JNK, or p38 antibody

Western blot analysis was performed by using anti-ERK (Promega, Madison, WI, U.S.A.), anti-JNK (Promega), or anti-p38 antibody (Santa Cruz), as described previously (Chung et al., 1999). The MAPK bands were visualized by enhanced chemiluminescence (Amersham, U.K.).

RESULTS

Single intraperitoneal injection of NMDA induced an immediate early gene, *pip92,* in mouse brain

In rats, systemic administration of NMDA was commonly used to induce epileptic seizures and excitotoxic CNS injury (Feigenbaum et al., 1989; Nitsch and Frotscher, 1992; Metsis et al., 1993; Bing et al., 1997). In this study we used a single intraperitoneal injection of convulsive-dosage NMDA to induce excitotoxic injury in the mouse brain. We have observed that injection of the same dosage of NMDA induced severe neuronal cell death in the entire hippocampal region of the CNS (data not shown). In addition, apoptosis was observed up to 40 days after NMDA injection, and the expression of a few genes, for example, the immediate early gene c-*fos* and c-*jun* and the apoptosis-related gene *Fas/Fas* ligand (*Fas*L), was observed in the hippocampal area (Shin et al., 1998). To determine the NMDA-induced expression of *pip92* transcripts in the CNS, total RNAs from the whole brain were prepared at the indicated time after NMDA injection, and the occurrence of those transcripts

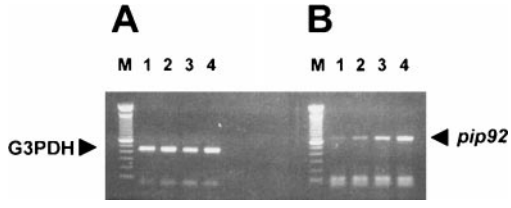

FIG. 1. Induction of mouse brain *pip92* mRNA after treatment with NMDA. After a single intraperitoneal NMDA injection (70 mg/kg), mice were killed at the indicated time, and the total brain RNAs were extracted. RT-PCR was performed using specific primer sets of mouse G3PDH as a control for equal RNA loading (**A**) and *pip92* cDNA (**B**). Lanes 1, 2, 3, and 4 represent control, 1 h, 2 h, and 3 h after NMDA treatment, respectively. Lane M represents the 100-bp DNA ladder size marker (Gibco/BRL). These results are representative of two independent experiments.

was measured by RT-PCR. As shown in Fig. 1, a single systemic administration of 70 mg/kg NMDA induced *pip92* gene transcripts in the CNS, and their levels increased in a time-dependent manner for up to 3 h after addition of NMDA.

Region-specific *pip92* induction by NMDA in mouse CNS

The distribution of *pip92* mRNA levels in the NMDA-treated mouse brain was investigated using in situ RT-PCR. The region-specific activation of *pip92* in the CNS is shown at 3 h after NMDA injection in Fig. 2. Densely labeled *pip92* messages in the hippocampal dentate gyrus and moderate signals in the piriform cortex were detected in the NMDA-treated mice (Fig. 2B). Microscopy with increasing magnification revealed discrete cellular levels of *pip92* labeling in each area. However, the corresponding brain section of a control mouse did not show any significant in situ *pip92* signal (Fig. 2A).

NMDA induced activation of stress-activated MAPKs, such as JNK and p38, but not ERK in mouse hippocampus

To clarify the downstream signaling cascades for the induction of *pip92* in the mouse hippocampus in response to NMDA, we isolated hippocampal tissue from the brain after a single intraperitoneal injection of 70 mg/kg NMDA. Using whole-cell lysates from the hippocampus, we measured the activation of endogenous ERK, JNK, and p38 by NMDA. As shown in Fig. 3, both JNK and p38 activities increased from 30 min to 2 h post-NMDA stimulation. However, we did not detect any significant levels of ERK activation by NMDA. As a control for protein loading, we measured the amount of nonactivated MAPKs by western blot analysis. In all samples, ERK, JNK, and p38 kinase were present at the same levels, respectively (data not shown).

JNK- and/or p38-dependent *pip92* induction by NMDA in conditionally immortalized rat hippocampal H19-7 cells

To confirm the selective activation of p38 and JNK during the excitotoxic NMDA injury, we tested the effect of blocking MAPK signaling pathways on NMDA-induced cell death and *pip92* induction in neuronal H19-7 cells. The H19-7 cells are derived from embryonic day 17 rat hippocampal cells that have been conditionally immortalized with temperature-sensitive SV40 large T antigen (Eves et al., 1992). The chemical inhibitors PD98059 and SB203580, which can selectively inhibit the activation of MEK and p38 kinase, respectively, and

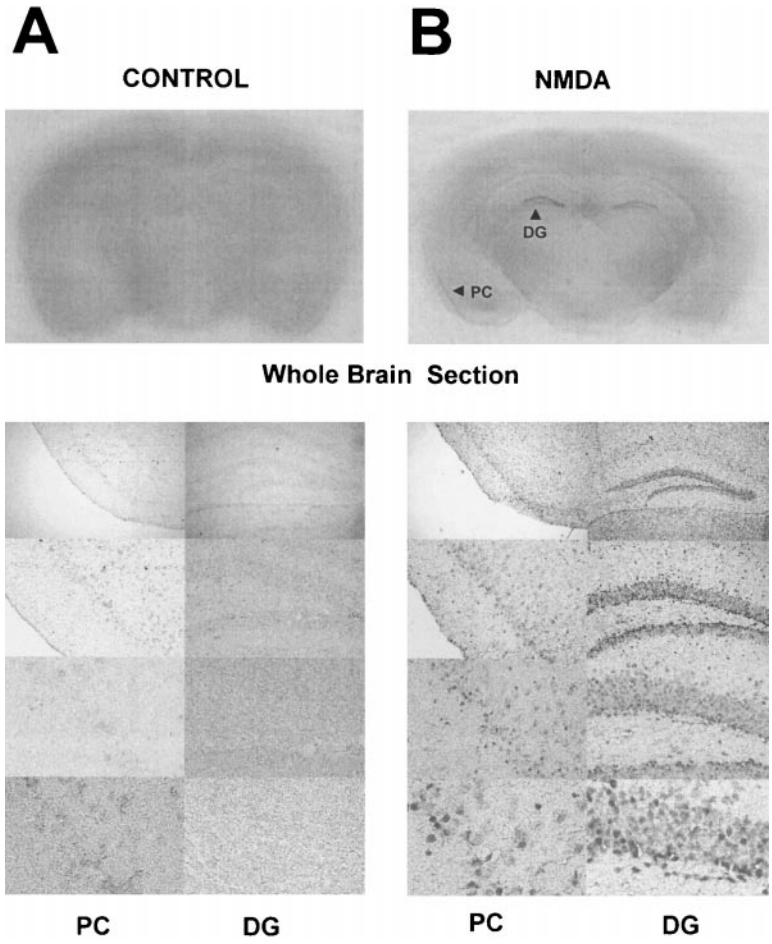

FIG. 2. Localization of *pip92* mRNA in mouse brain after a single intraperitoneal injection of NMDA. At 3 h after a single intraperitoneal injection of (**B**) NMDA (70 mg/kg) or (**A**) vehicle, mice were killed, and brain sections were prepared and followed by immunodetection of dig-dUTP-incorporated and amplified *pip92* cDNA. **Top left panel:** Gross structure of brain section in a vehicle-injected or vehicle-treated control mouse after 3 h (A). **Top right panel:** Gross structure at 3 h after NMDA treatment (B). **Bottom left** and **right panels:** Micrographs are arranged with increasing order of magnification of enlarged structures of the piriform cortex region (PC; left) and hippocampal dentate gyrus region (DG; right) of (A) vehicle-treated control or (B) NMDA-treated mice, respectively. These results are representative of two independent experiments.

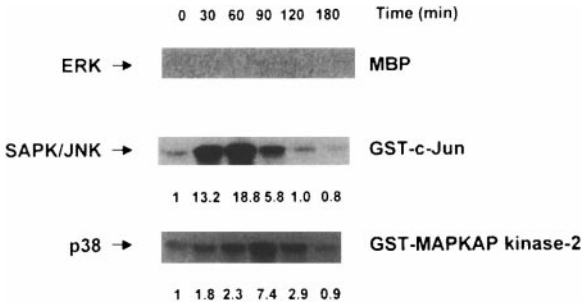

FIG. 3. Effect of a single intraperitoneal injection of NMDA on activation of MAPKs in mouse hippocampus. After a single intraperitoneal NMDA injection (70 mg/kg), mice were killed at the indicated time, and their hippocampal tissues were dissected. Total cell lysates were prepared, and the protein kinase activities of ERK, stress-activated protein kinase (SAPK)/JNK, and p38 kinase were measured by immunoprecipitation using anti-ERK, anti-JNK1, and anti-p38 antibody followed by an in vitro solid-phase kinase assay. Myelin basic protein (MBP), GST–c-Jun, and GST–MAPKAP kinase-2 were used as ERK, JNK, and p38 substrates, respectively. The numbers at the bottom of the SAPK/JNK and p38 panels indicate quantitated values of fold induction measured using a densitometer. These results are representative of two independent experiments.

kinase-inactive dominant-negative mutant cDNAs in the JNK signaling cascades, such as SEK1 and MEKK, were used. We have examined the effect of NMDA on the viability of H19-7 cells. We observed that treatment of the H19-7 cells with 100 μM NMDA for 12 h induced ~75% cell death, as measured by the MTT extraction assay (Table 1). In accordance with the pattern of hippocampal tissue isolated after a systemic NMDA administration, the same pattern of selective activation of JNK and p38, but not ERK, was observed by 100 μM NMDA treatments in the H19-7 cells (data not shown). When 50 μM SB203580 (a p38 kinase inhibitor) was added to the cells, we observed a complete inhibition of endogenous p38 activities induced by NMDA (data not shown). As well, addition of 30 μM MEK inhibitor, PD98059, to the H19-7 cells was shown to block the basic fibroblast growth factor-induced activation of ERK completely, resulting from the inhibition of MEK activity (Kuo et al., 1997).

Pretreatment of H19-7 cells with 50 μM SB203580 resulted in a significant protection from NMDA-induced cell death (Table 1). However, when 30 μM PD98059 was added with NMDA, we did not find any significant inhibition of cell death, compared with that induced by NMDA alone. Addition of the same concentration of MEK and p38 kinase inhibitor alone to the cells did not produce a significant cell death. When we transfected the H19-7 cells with kinase-inactive dominant-negative mutant SEK1 and MEKK, there was a significant decrease of cell death, compared with the cells transfected with the parental control vector alone (pEE-CMV). These data implied that NMDA-mediated neuronal cell death is mediated at least in part by the activation of JNK and p38 kinase, but not ERK. Consistent with the inhibition pattern of NMDA-induced cell death by blocking JNK and p38 kinase activities, we found that NMDA-induced *pip92* expression in H19-7 cells is also regulated by JNK and p38 kinase. Northern blot analysis showed that *pip92* mRNA levels are induced highly by 100 μM NMDA (Fig. 4). Induction of *pip92* mRNAs by NMDA was decreased by pretreatment with SB203580 but not PD98059. In a similar way, transient transfection of kinase-inactive SEK1 and MEKK cDNAs into the H19-7 cells also produced a significant inhibition of *pip92* ex-

TABLE 1. *Effect of inhibition of the MAPKs ERK, JNK, and p38 on NMDA-induced neuronal cell death in immortalized hippocampal H19-7 cells*

Treatment	Cell survival (%)
No treatment	100
NMDA	24.4 ± 3.6
SB203580	91.2 ± 5.7
PD98059	93.2 ± 7.1
NMDA + SB203580	58.7 ± 8.6
NMDA + PD98059	26.6 ± 5.2
NMDA + pCMV	27.8 ± 3.9
NMDA + mut. MEKK	47.5 ± 4.1
NMDA + mut. SEK1	53.4 ± 6.8

After addition of 50 μM p38 kinase inhibitor (SB203580) or 30 μM MEK inhibitor (PD98059) to the H19-7 cells for 30 min, 100 μM NMDA was added and incubated for 12 h. To test the effect of kinase-inactive SEK1 and MEKK mutants on NMDA-induced cell death, 5 μg of pEE-CMV (pCMV), pΔMEKK(3′)KR-5′EE-CMV (mut. MEKK), or pSEK1(AL)/EE-CMV (mut. SEK1) DNAs was transiently transfected into the cells for 24 h using LipofectAMINE, followed by addition of 100 μM NMDA for 12 h. Cell viability was measured by MTT extraction. Results are mean ± range of data from three independent experiments done in triplicate.

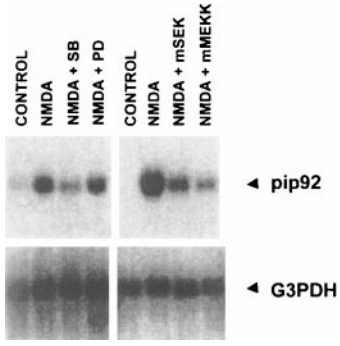

FIG. 4. Effect of the inhibition of the MAPKs, ERK, JNK, and p38 on NMDA-induced *pip92* expression in rat embryonic hippocampal neuronal H19-7 cells. After pretreatment of H19-7 cells with 50 μM p38 kinase inhibitor [SB203580 (NMDA + SB)] or 30 μM MEK inhibitor [PD98059 (NMDA + PD)] for 30 min as indicated, 100 μM NMDA was added and incubated for 3 h. To test the effect of mutant SEK1 (NMDA + mSEK) and MEKK (NMDA + mMEKK) on NMDA-induced *pip92* expression, 5 μg of the respective mutant DNAs was transfected into the cells, followed by addition of 100 μM NMDA for 3 h. Northern blot analysis was performed to measure *pip92* mRNA levels. As a control for equal RNA loading, total RNAs were hybridized to ^{32}P-labeled G3PDH cDNA probe. These results are representative of three independent experiments.

pression induced by NMDA, suggesting that p38 and JNK, but not ERK, are involved in the induction of *pip92* during NMDA-mediated neuronal cell death.

NMDA exerts its cytotoxic action through an ionotropic NMDA receptor subtype in hippocampal neuronal H19-7 cells

To ensure which subtype of glutamate receptors is activated during the induction of immediate early gene *pip92* by NMDA in H19-7 cells, we tested the effect of various selective antagonists of glutamate receptor subtypes on NMDA-induced cell death. Various NMDA receptor antagonists, such as the noncompetitive NMDA antagonist (+)-MK-801, the selective AMPA/kainate antagonist NBQX, and the competitive antagonist of the metabotropic glutamate receptor ACPT-II, were used to examine by which receptor subtype the cell death-promoting signals of NMDA are transmitted in the H19-7 cells. When the NMDA-selective MK-801 was pretreated for 30 min, the cell death induced by NMDA was greatly reduced, whereas the AMPA/kainate and the metabotropic glutamate antagonists did not show any significant inhibition of NMDA-induced cytotoxicity (Fig. 5). These data suggest that NMDA exerts its cytotoxic effect via the activation of ionotropic glutamate receptors during the excitotoxic cell death process in neuronal H19-7 cells.

DISCUSSION

Glutamate receptors have been divided into two major subtypes: ionotropic and metabotropic glutamate receptors. The ionotropic receptors are further categorized into NMDA, AMPA, and kainate receptors based on their agonist names (Watkins et al., 1990). The AMPA/kainate receptors activate ligand-gated cation channels that are permeable to Na^+ and K^+, whereas NMDA receptors are ~10 times more permeable to Ca^{2+} than to Na^+. In many cases, glutamate neurotoxicity, especially later phases of neuronal degeneration, can be attributed to excessive stimulation of the NMDA subtype of glutamate receptors (Choi et al., 1988; Ankarcrona et al., 1995). Pretreatment with the NMDA receptor antagonist 7-aminophosphonoheptanoic acid reduced acute ischemic hippocampal damage in vivo (Simon et al., 1984). Unilateral injections of NMDA (25 nmol/0.5 μl) into posterior striatum of 7-day-old rats produced consistent and reproducible lesions characterized by confluent necrosis, gross tissue deformation, and reactive gliosis predominantly affecting the corpus striatum, dorsal hippocampus, and overlying neocortex in the lesioned hemisphere (McDonald et al., 1990). This pattern of damage corresponds to NMDA receptor distributions within the immature brain.

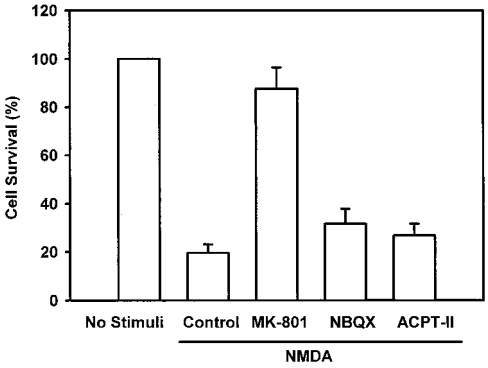

FIG. 5. Effect of various glutamate receptor subtype antagonists on NMDA-induced neuronal cell death in rat hippocampal H19-7 cells. After H19-7 cells were pretreated with (+)-MK-801 (50 μM), NBQX (30 μM), or ACPT-II (100 μM) for 30 min, 100 μM NMDA was added and incubated for 12 h. Cell viability was measured by MTT extraction. Results are mean ± range (bars) of data from three independent experiments.

In many studies where the effect of NMDA on the release of neurotransmitters was examined in vivo, the substance was applied to a local region through a probe. This might reflect the fact that it is difficult for NMDA to penetrate into the CNS because of the blood–brain barrier in adult animals. However, several studies have indicated that neonatal rodents are particularly sensitive to systemic administration of excitatory amino acids. Systemic administration of monosodium glutamate to neonatal mice led to acute neuronal necrosis in the developing brain (Olney, 1969). Schoepp et al. (1990) reported that NMDA administered systemically to immature rats caused motor convulsions that were diminished by pretreatment with DL-2-amino-5-phosphonovaleric acid or (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid, a competitive antagonist of NMDA receptors. A significant and pronounced increase of the extracellular taurine level in adult rat hippocampal CA1 and striatum was observed after an intraperitoneal injection of 10 mg/kg NMDA, whereas a similar effect of NMDA was also noted when 300 μM NMDA was applied locally to both regions via probes (Shibanoki et al., 1993). Systemic applications of kainate and NMDA are known to induce seizure behavior in rats within 90 min, followed by severe neuronal cell death in hilar and pyramidal neurons of the hippocampus within 3 days (Dragunow and Preston, 1995). A single systemic application of a convulsive dose of NMDA to mice also results in both short- and long-term excitotoxic effects on the CNS (Dragunow and Preston, 1995). These findings suggest that NMDA administered systemically acts directly on the specific receptors located in the brain after penetration through the blood–brain barrier, even if such penetration is strongly restricted by the blood–brain barrier system. Brace et al. (1997) have shown that both NMDA and kainate not only damage neurons and myelin but also compromise the integrity of the blood–brain barrier.

The immediate early gene *pip92* (also known as *chx1* or *ETR101*) was independently cloned from serum-stimulated BALB/c 3T3 fibroblasts (Charles et al., 1990) and activated T lymphocytes treated with cycloheximide (Coleclough et al., 1990). Human *pip92* cDNA was cloned from the myeloid leukemia cell line HL-60 (Shimizu et al., 1991). *pip92* is rapidly and transiently

induced by stimulation with serum growth factors and the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate in fibroblasts and by treatment with nerve growth factor or membrane depolarization in PC12 cells (Charles et al., 1990). The *pip92* promoter has been cloned, and its transcription is induced by serum in mouse 3T3 fibroblasts via the SRE.

The SRE, studied most extensively in the c-*fos* promoter, consists of a CArG box that binds to the SRF. When SRF is bound to the c-*fos* SRE, it recruits a TCF to an upstream Ets-like binding site (Hill and Treisman, 1995; Treisman, 1995). In the *pip92* promoter, the SRE consists of at least one Ets protein binding site and a CArG site. Gel shift analysis demonstrated that the Ets sites in the *pip92* promoter bind to Elk-1 on phosphorylation of two sites, Ser^{383} and Ser^{389}, by ERKs (Latinkic and Lau, 1994; Chung et al., 1998). It was shown that, unlike the c-*fos* SRE, the *pip92* SRE can interact independently with Elk-1 without prior formation of the SRF–DNA binary complex (Latinkic and Lau, 1994; Latinkic et al., 1996). Recently it has been shown that Elk-1 is phosphorylated and therefore activated by JNK and p38, as well as ERK (Gille et al., 1995; Whitmarsh et al., 1995, 1997). However, it is unclear whether Elk-1 is involved in *pip92* induction by NMDA. Recently we have determined that *pip92* is rapidly and transiently expressed by the translational inhibitor anisomycin in NIH 3T3 fibroblast cells and that its expression is mediated by the JNK- and p38-dependent activation of Elk-1 (K. C. Chung et al., manuscript in preparation). In addition to Elk-1, the other family member of the TCF, Sap-1a, has been reported to be phosphorylated by p38 and involved in c-*fos* expression through the SRE in human embryonic kidney 293 cells (Janknecht et al., 1993). It is also possible that Sap-1 and/or other unidentified members of TCF regulate *pip92* expression by NMDA via its SRE. Answers to these questions will enhance our understanding of the mechanism of how mitogenic signals and excitotoxic insults elicit the nuclear response.

pip92 encodes a short-lived, proline-rich protein with no significant sequence similarity with any known protein. However, to date, little is known about the function of its encoded protein. Recently, it has been shown that *pip92* is selectively induced by differentiating factors in rat hippocampal neuronal cells, suggesting that it is a key component in neuronal differentiation (Chung et al., 1998). Those studies also reveal the presence of at least two pathways, one MAPK-dependent and the other MAPK-independent, for the induction of *pip92* in response to basic fibroblast growth factor or to activated Raf-1 in neuronal H19-7 cells. In addition to differentiation, the present study demonstrates that *pip92* is likely to play an important role during the NMDA-induced excitotoxic injury process. The region-specific induction of *pip92* in the hippocampus is mediated by activation of the MAPKs JNK and p38, but not ERK, suggesting that different MAPK pathways are involved in *pip92* expression induced by growth factors and excitotoxic stimuli. p38 and JNK are known to be involved in cell death induced by nerve growth factor deprivation in PC12 cells, by ceramide in U937 and BAE cells, and by anti-IgM antibody in human B lymphocytes (Xia et al., 1995; Graves et al., 1996; Verheij et al., 1996). In cultured chick fetal forebrain neurons, p38 activity was down-regulated by insulin, which can help these cells survive (Heidenreich and Kummer, 1996). Recently, activation of p38 kinase has been reported in glutamate-induced apoptosis in rat cerebellar granule cells, suggesting an essential role of p38 kinase in mediating the death-promoting activity of glutamate (Kawasaki et al., 1997). In cultured cortical neurons, ERK activity was enhanced by stimulation of metabotropic and kainate types of glutamate receptors but not by stimulation of the NMDA receptor (Fiore et al., 1993). Consistent with this finding, by using three different glutamate receptor subtype antagonists we have determined that the cytotoxic effect of 100 μM NMDA is mediated by the activation of ionotropic NMDA receptor in the H19-7 cells.

Many actions of NMDA are coupled to the influx of extracellular Ca^{2+} mediated directly or indirectly by its receptor present in neurons. Transient changes in intracellular Ca^{2+} levels are known to trigger several cellular responses, including the change of gene expression. We have observed that treatment of H19-7 cells with thapsigargin, which causes Ca^{2+} to leak out of internal stores and elevates the intracellular Ca^{2+} levels (K. C. Chung et al., manuscript in preparation), increases the transcription of *pip92* in neuronal H19-7 cells.

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