Journal of Cellular Biochemistry 72:135–144 (1999)

Expression of N-Methyl-D-Aspartate Receptors Using Vaccinia Virus Causes Excitotoxic Death in Human Kidney Cells

Mónica García-Gallo, M. Margarita Behrens, Jaime Renart,* and Margarita Díaz-Guerra

Instituto de Investigaciones Biomédicas ''Alberto Sols'' CSIC-UAM, 28029-Madrid, Spain

Abstract N-Methyl-D-Aspartate (NMDA) receptors containing NR1 and NR2A subunits have been expressed with high efficiency in Human Embryonic Kidney 293 cells with the aid of a recombinant vaccinia virus. This expression system produced functional receptors that sustained calcium influxes dependent on receptor agonists and inhibited by receptor antagonists. Immunocytochemistry of the recombinant receptors demonstrated that they were properly arranged in membrane structures. The entrance of calcium through the recombinant receptors induced delayed toxicity, demonstrated by approximately a three-fold increase in the number of dead cells obtained 12 h after the antagonist 2-amino-phosphopentanoic acid (DL-AP5) was removed from the culture. This result correlated with more than 88% inhibition in the expression of a reporter gene 24 h after antagonist removal. Calcium toxicity was completely abolished by specific antagonists of the NMDA receptor. Treatment of cell extracts with N-glycosydase showed that both receptor subunits were N-glycosylated. Tunicamycin prevented calcium toxicity; gel electrophoresis studies showed that this protection was likely due to degradation of the NR1 subunit. J. Cell. Biochem. 72:135–144, 1999.

Key words: Recombinant NMDA receptors; vaccinia virus; calcium toxicity; N-glycosylation; tunicamycin

Excitotoxicity is thought to be the main mechanism of brain injury during ischemia and acute brain trauma, and it has also been related to neurodegenerative disorders like Alzheimer's and Huntington's diseases as well as AIDS-associated dementia [reviewed by Lipton 1993]. It is well established that this phenomenon is due to glutamate receptor overstimulation by its natural agonists, producing a massive entry of Ca^{2+} in neurons [Choi 1987, 1988, 1995; Michaels and Rothman, 1990; Tyaminski et al., 1993]. The main candidate for this calcium influx is the NMDA receptor, that exhibits a great permeability to this ion [Mori and Mishina 1995]. However, it is now clear that α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)/kainate receptors may also play a direct role in excitotoxicity, not only by a rapid membrane depolarization that eliminates Mg^{2+} inhibition of the NMDA receptor, but also due to high Ca^{2+} permeability of AMPA/kainate receptors that lack the GluR2 subunit [Brerson et al., 1994; Turetsky et al., 1994]. The net accumulation of intracellular Ca^{2+} is a critical determinant of neuronal injury [Lu et al., 1996], although it is presently unknown how this ion mediates the changes in cellular function that ultimately cause cell death.

In order to understand the mechanisms underlying excitotoxicity, it would be very interesting to know whether this phenotype is exclusively due to the presence of glutamate receptors in neurons or whether there are also other neuronal specific steps in the death pathway. It has been shown that heterologous expression of NMDA receptor subunits can lead to cell death [Anegawa et al., 1995; Cik et al., 1993]. Al-

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; DL-AP5, 2-amino-phosphonopentanoic acid; DMEM, Dulbecco's modified Eagle medium; h.p.i., hours post infection; HEK, human embryonic kidney; NMDA, N-Methyl-D-Aspartic acid; PBS, phosphate buffered saline; VV, vaccinia virus.

Contract grant sponsor: Comisión Interministerial de Ciencia y Tecnología; Contract grant number: PB93–0143; Contract grant sponsor: Comunidad de Madrid, Spain; Contract grant number: AE00287/95.

M. Margarita Behrens' present address is Department of Neurology & Center for the Study of the Nervous System Injury, Washington University School of Medicine, St. Louis, MO 63110.

*Correspondence to: Jaime Renart, Instituto de Investigaciones Biomédicas, Arturo Duperier, 4, 28029-Madrid, Spain. E-mail: jrenart@iib.uam.es

Received 24 November 1997; Accepted 27 July 1998

though pharmacological and biophysical results obtained with recombinant receptors must be interpreted with caution [Sucher et al., 1996], the heterologous systems might be very useful to identify the players involved in excitotoxicity that are not neuronal specific.

In this investigation, we have used a powerful expression system, based on vaccinia virus (VV), to produce recombinant NMDA receptors in non neuronal cells. Vaccinia is a poxvirus widely studied [Moss, 1996] that has a number of useful characteristics as an expression vector, among others the capability of nearly 100% infection of targeted cells. VV permits highly efficient expression of recombinant proteins which are correctly localized and modified in cells of various species, including mammals, birds, and amphibians [reviewed in Smith, 1993]. Recent reports have shown that VV can be used to direct the expression of heterologous proteins in neurons of the CNS of frogs, both in vivo and in vitro [Wu et al., 1995], or in anatomically selected populations of neurons in rat hippocampal slices, while maintaining intact synaptic circuitry and function [Pettit et al., 1995]. Inhibition of host nucleic acids and protein synthesis is produced early in infection and, therefore, expression corresponds to viral or viral-driven genes. In this work, Human Embryonic Kidney (HEK293) cells were first infected with a recombinant VV that expresses the bacteriophage T7 RNA polymerase gene [Fuerst et al., 1987] and then transfected with plasmids carrying rat NR1 and NR2A cDNAs under the control of the T7 promoter; as VV replication is cytoplasmic, high levels of expression of heterologous proteins can be obtained by transient transfection without the need of recombinant viruses. We present here our results on the expression of NMDA receptors using this technology and the characterization and properties of this new excitotoxicity system.

MATERIALS AND METHODS

Chemicals

NMDA receptor antagonists 2-amino-phosphonopentanoic acid (DL-AP5) and MK-801 were from Tocris-Cookson (Bristol, UK); NMDA and tunicamycin were from Sigma (St. Louis, MO). N-glycosydase F was from Boehringer-Mannheim Biochemicals (Mannheim, Germany). $^{45}CaCl_2$ (36 mCi/mg) was obtained from DuPont-NEN (Boston, MA). All other products were of the highest quality available.

Cell Culture

HEK293 cells (ATCC CRL 1573) were grown in DMEM medium plus 10% fetal bovine serum, 40 μg/ml gentamicin, and 2 mM glutamine. African green monkey kidney cells (BSC40) were grown as before but using newborn calf serum. Cells were grown at 37°C in 5% CO_2. When indicated NMDA (1 mM), DL-AP5 (2 mM), or MK-801 (10 μM) were added to the culture. Tunicamycin (2 μg/ml) was added to the transfection mix and kept for the duration of the experiment. VV was always manipulated in the P2 facility of the Instituto de Investigaciones Biomédicas.

Plasmids and Viruses

Plasmids coding for NR1 [Moriyoshi et al., 1991] and NR2A [Ishii et al., 1993] subunits of the rat NMDA receptor were kindly supplied by Dr. S. Nakanishi. This NR1 subunit is the NR1–1a splice variant in the nomenclature of Hollmann et al. [1993]. Both plasmids contain the coding sequence in the pBS plasmid (Stratagene, La Jolla, CA), under the control of the T7 promoter. pcDLUC was provided by Dr. M. Esteban and contains the luciferase gene under the control of T7 and CMV promoters in the pcDNAI/Neo plasmid (Invitrogen, La Jolla, CA). The recombinant virus vTF7–3 [Fuerst et al., 1987] expressing the bacteriophage T7 RNA polymerase gene under the control of the p7.5 VV promoter was provided by Dr. J. Ortín. This virus was grown and titrated in BSC-40 cells according to standard procedures [Earl et al., 1991].

Expression of the NMDA Receptor

For expression experiments, HEK293 cells (8×10^5, in 12-well plates) were infected with vTF7–3 virus at a multiplicity of four plaque forming units per cell for 1 h at 37°C. After adsorption, virus inoculum was removed, cells were washed once with DMEM with no serum and transfected using Transfectace liposomes [Rose et al., 1991] with a 1:1 relation of dimethyldioctadecyl ammonium bromide and dioleyl L-α-phosphatidyl ethanolamine [J. Ortín, personal communication]. Transfection of NR1 and NR2A genes was performed with 2 μg each

plasmid and 0.2 μg of the luciferase reporter gene. Control transfections were done with 4 μg of the NR1 plasmid together with the reporter gene as before. Lipids and DNA were incubated for 15 min in DMEM without serum and then added to the infected cells for 5–6 h. Then, DMEM with 10% serum and 2 mM DL-AP5 was added and cells were incubated for the indicated times. Cells were scraped off the plate with the medium, centrifuged 1 min at 13,000 rpm, and the cell pellet lysed with 100 μl of lysis buffer (25 mM glycylglycine, pH 7.8; 15 mM $MgSO_4$; 4 mM EGTA; 1 mM DTT; 1% Triton X100). Extracts were again centrifuged for 5 min before assaying luciferase activity according to Braiser et al. [1989]. The same extracts were used for immunoblot analysis.

Calcium Influx Through the NMDA Receptor

Cells infected/transfected as before were washed three times with HCSS medium (120 mM NaCl; 5.4 mM KCl; 0.8 mM $MgCl_2$; 1.8 mM $CaCl_2$; 15 mM Glucose; 20 mM Hepes; 10 mM NaOH; 0.002% Phenol Red) containing 10 mM $CaCl_2$ and 10 μM glycine and then incubated for 30 min at room temperature in 250 μl of the same medium plus 1.25 μM $^{45}CaCl_2$ (final concentration in the assay 5 μCi/ml [Lu et al., 1996]). Agonists and antagonists were also added as stated. Cells were then washed four times with HCSS/calcium/glycine medium as before. Cells were lysed with 0.4 ml of 2% SDS for 20 min at room temperature with occasional swirling, and counted.

Assessment of Cell Death

Transfections were carried out as described. After 24 h of infection, cells were washed twice with DMEM without serum, and then the same media was added but containing agonists and antagonists as stated. Incubation proceeded for an additional 12 or 24 h. Luciferase activity was assayed in cell extracts as before, and cell death was estimated as the decrease of luciferase activity compared to controls [Memon et al., 1995]. Cell viability was measured by propidium iodide staining by addition of 5 μg/ml of this DNA intercalator to the growth media just before visualizing cells in an inverted fluorescence microscope, counting total and stained cells. Mean values ± standard deviations of the percentage of dead cells were obtained from four fields corresponding to two independent experiments.

Immunocytochemistry

Cells were grown in coverslips and treated as described [Ehlers et al., 1995]. Briefly, cells were washed with phosphate buffered saline (PBS) and fixed for 15 min at room temperature with 4% formaldehyde in PBS plus 4% sucrose. The coverslips were either washed with PBS and processed immediately, or stored at 4°C in PBS plus 0.05% sodium azide. Cells were permeabilized 5 min at room temperature with 0.25% Triton X100 in PBS and then blocked for 1 h at 37°C in PBS plus 1% gelatin. The same conditions were used for incubation with mouse anti-NR1 (Pharmigen, San Diego, CA; at 1:100), rabbit anti-NR2A/B (Chemicon, Temecula, CA, at 1:50) or both antibodies. Rabbit anti-NR2A/B antibodies were pre-adsorbed for 30 min at 4°C to a dry acetone precipitate obtained from HEK293 cells infected/transfected with the NR1 gene as before [Harlow and Lane, 1988]. Cover slips were washed four times (10 min each) with PBS, and incubated as before with 1:100 dilution of goat anti-rabbit (rhodamine labeled, Jackson Laboratories, Bar Harbor, ME) or goat anti-mouse (BODIPY-labeled, Molecular Probes, Eugene, OR) antibodies. After four additional washes with PBS, coverslips were mounted with Mowioll. Cells were photographed using a Zeiss Axiophot fluorescence microscope.

Other Methods

N-glycosidase F treatment was done in extracts from cells infected/transfected for 48 h in the presence of DL-AP5. Thirty μg of protein in 24 μl lysis buffer were adjusted to 50 mM sodium phosphate, pH 7.2; 20 mM EDTA; 0.1% 2-mercaptoethanol; 0.4% SDS (final volume of 30 μl), and incubated with 20 units/ml of enzyme for 14 h at 37°C, according to Cik et al. [1993].

Polyacrylamide gel electrophoresis and immunodetection of proteins transferred to Immobilon-P membranes (Millipore, Bedford, MA) were performed by standard procedures using the antibodies mentioned above or a rabbit polyclonal anti-vaccinia virus serum [Demkowicz et al., 1992] kindly supplied by Dr. M. Esteban. This serum recognizes both early and late viral proteins. Immunocomplexes were detected with the ECL kit from Amersham (Little Chalfont, Bucks., UK), according to the manufacturers instructions. Protein determination was done using the BCA reagent from Pierce (Rockford, IL).

Aminoacids in the culture media were determined by HPLC after filtration through Ultrafree-MC filters (Millipore) according to Hernanz and Polanco [1991].

RESULTS

Expression of Luciferase in the Presence of NMDA Receptor Subunits

A simple assay to examine the effect of transiently expressed genes on cell death has been recently developed, based on cotransfection of reporter genes [Memon et al., 1995]. In order to verify the suitability of this method to study excitotoxicity, we first characterized the pattern of expression of a luciferase reporter gene cotransfected with plasmids coding the NMDA receptor subunits NR1 and NR2A, using conditions where excitotoxicity is inhibited. In order to maximize coexpression of luciferase and the receptor subunits in the same cells, cultures of HEK293 infected with high multiplicity of the recombinant vTF7–3 virus were cotransfected with both receptor subunits cDNAs and limited amounts of the reporter gene.

Infection/transfection proceeded in the presence of the NMDA receptor antagonist DL-AP5. Under these conditions there was a nearly linear increase in accumulation of luciferase up to 24 h after infection: 25.6 ± 4.1, 58.2 ± 1.3, and 92.1 ± 0.9 relative luciferase units (RLUs) per cell at 5, 10, and 24 h, respectively. Levels of luciferase activity remained stable for the next 24 h with a value of 93.5 ± 0.8 RLUs per cell at 48 h after infection. Similar results were also found when transfecting only NR1 with the reporter gene (data not shown). In these conditions, changes in luciferase activity measured 48 h after infection/ transfection could be considered a good indicator of the amount of viable cells (see below).

Expression and Localization of NMDA Receptor Subunits

To study the kinetics of expression of the two receptor subunits, extracts obtained in the above experiment were subjected to immunodetection analysis. Anti-NR1 antibody detected a prominent band of 116 kDa both in cells transfected with NR1 (Fig. 1, lanes 1–4, upper panel) or NR1 plus NR2A (Fig. 1, lanes 5–8, upper panel). The smear observed for NR1 is probably due to protein glycosylation (see below), since nonspecific proteolysis was ruled out by the fact that

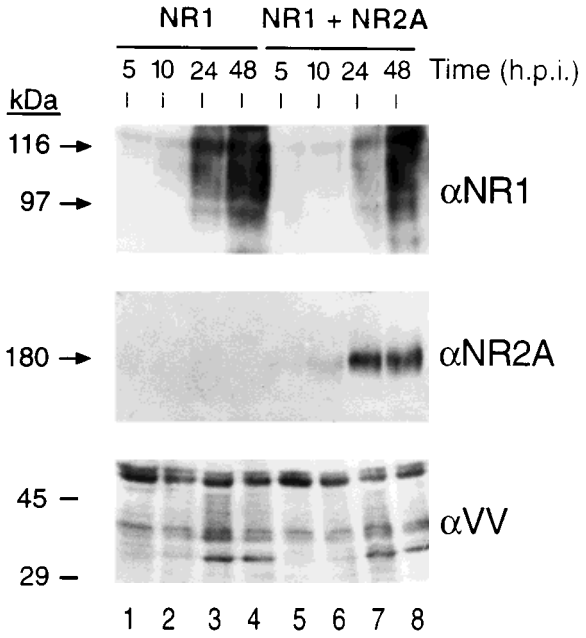

Fig. 1. Time-course of expression of NMDA receptor subunits. Cells were infected/transfected with NR1 and NR2A genes, or NR1 alone, together with pcDLUC in the presence of 2 mM DL-AP5 as described (see Materials and Methods). Cell extracts (30 μg) were fractionated in 6% SDS-polyacrylamide gels, transferred to PVDF membranes, and immunoblotted as described using monoclonal antibodies specific for the NR1 subunit (upper panel), rabbit polyclonal antiserum against NR2A (middle panel), or a rabbit polyclonal anti-VV serum (lower panel). Results presented are representative of several independent experiments. These samples were also used to measure luciferase activity as mentioned in the text.

NR2A or VV early and late proteins showed no degradation in the same samples (Fig. 1, middle and lower panels). The anti-NR2A antibody detected a broad band of 180 kDa only in cells transfected with the NR2A gene (Fig. 1, lanes 5–8, middle panel). The time-course of receptor subunits and luciferase accumulation was similar up to 24 h after infection, reflecting that all three genes depend on an early/late VV promoter. Both subunits were detectable five hours after infection/transfection; however, between 24 and 48 h, the kinetics of accumulation presented some differences. NR1 had a significant increase, whereas NR2A levels raised moderately in this period, more in accordance with the luciferase activity mentioned earlier (Fig. 1, lanes 7 and 8, upper and middle panels).

Immunocytochemistry of cells doubly transfected with NR1 and NR2A genes as before is shown in Figure 2. In permeabilized cells, intense fluorescence corresponding to NR1 (Fig. 2A,C) or NR2A subunit (Fig. 2B,D) was ob-

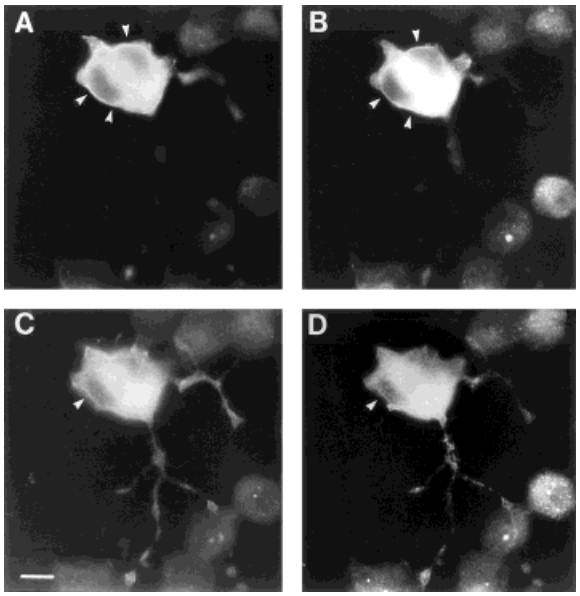

Fig. 2. Immunocytochemistry of cells expressing NMDA receptor subunits. Cells grown in coverslips were infected/transfected with NR1 and NR2A genes. Infections proceeded for 24 h in the presence of 2 mM DL-AP5. Cells fixed and permeabilized as described were incubated with mouse anti-NR1 (**A,C**) or rabbit anti-NR2A/B (**B,D**). Samples were visualized with a fluorescence microscope using a 100× oil immersion objective, and upper (A and B) or lower (C and D) focusing planes were photographed. Arrowheads indicate regions where colocalization of both subunits is more evident. Scale bar is 10 μm.

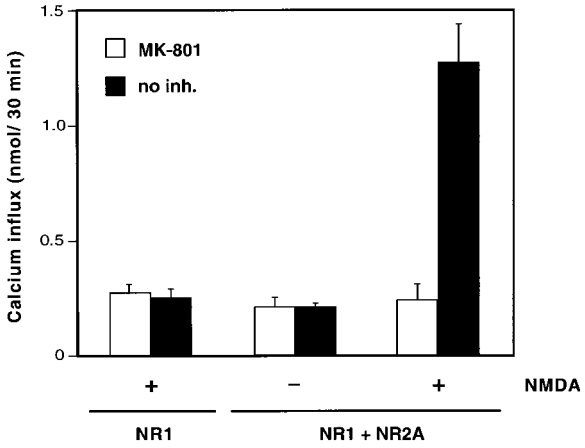

Fig. 3. Calcium influx through the NMDA receptor. Infected cells were transfected with NR1 alone or NR1 plus NR2A as before. After 24 h of infection in the presence of 2 mM DL-AP5, the antagonist was removed, and influx of calcium was measured as indicated in Methods during 30 min in the presence or absence of 1 mM NMDA or the antagonist MK-801 (100 μM). Mean values and standard deviations corresponding to three independent experiments are given.

served in approximately 60% of the total cell population. Although some cells could be detected that produce only one receptor subunit, approximately 40% of them synthesized the two subunits that colocalized. Some protein was detected in the cytoplasm, but the pattern of staining was compatible with a localization mainly in the plasma membrane, as shown by taking photographs at different focusing planes (Fig. 2A,C for NR1 and 2B,D for NR2A).

Functionality of Recombinant NMDA Receptors

The recombinant proteins expressed by the VV system produced NMDA receptors that were functional as shown by Ca^{2+} influx experiments (Fig. 3). Expression of the NR1 subunit alone did not produce any $^{45}Ca^{2+}$ accumulation. On the contrary, coexpression of both NR1 and NR2A subunits produced receptors that allowed calcium entry into the cells in a NMDA-dependent manner, demonstrated by a six-fold increase in the amount of $^{45}Ca^{2+}$ accumulated in the presence of the agonist. Influx of $^{45}Ca^{2+}$ was blocked by the use of MK-801, a noncompetitive and irreversible antagonist with affinity for the open channel.

Induction of Cell Death by Treatment With Receptor Agonists

The experiments above showed that luciferase activity could be used to estimate cell death dependent on calcium influx. To this aim, cells were infected/transfected as before and incubated for 24 h in the presence of DL-AP5. At this time, when the steady state of luciferase was reached, cells were treated with NMDA for the next 24 h in the absence or presence of receptor antagonists, and luciferase activity measured. Figure 4 shows that reporter gene expression was reduced more that 88% in cells producing both subunits compared to controls that expressed NR1 alone. This reduction was abolished by the presence of DL-AP5 or MK-801 in the media.

As these results were independent of the presence of NMDA (Fig. 4), we measured the concentration of glutamate in the culture medium from cells infected/transfected for 48 h. The concentration of L-glutamate in the media was found to be 120 μM, larger than the EC_{50} for the NR1/NR2A heteromeric recombinant receptor [Kutsuwada et al., 1992], and enough to induce cell death in cultured neurons [Choi, 1987]. No differences in L-glutamate concentration was observed between media collected from

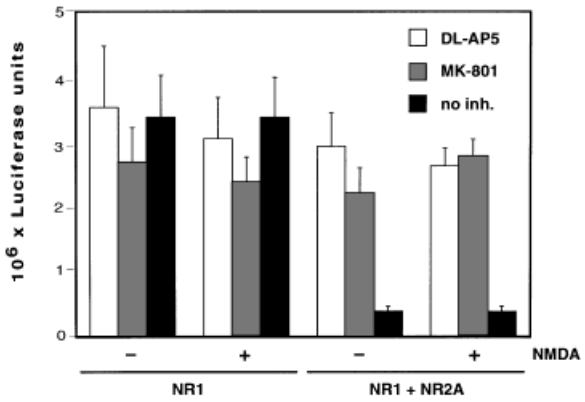

Fig. 4. Estimation of cell death by decreased luciferase activity. Cells were infected/transfected as before for 24 h in the presence of 2 mM DL-AP5. After removing the antagonist, infection proceeded for another 24 h in the presence or absence of 1 mM NMDA, with or without the NMDA receptor antagonists DL-AP5 (2 mM) or MK-801 (10 μM). Luciferase activity was measured in cell extracts and mean values of luciferase units obtained per well with standard deviations corresponding to three independent experiments are given.

cells expressing only NR1 or NR1 and NR2A and, in this case, the results were independent of the presence of antagonists (L-glutamate concentration in the medium prior to the experiment was 3.5 μM).

In order to demonstrate that the decrease in accumulation of luciferase was due to reduced viability of cells expressing the NMDA receptor subunits, we measured the percentage of dead cells by staining with propidium iodide. Cells infected/transfected as before were treated with NMDA for 12 h in the presence or absence of DL-AP5. In cells expressing the NR1 subunit alone, the percentages of dead cells were, respectively, 9.3 ± 1.6 and 13.6 ± 0.8 in the absence or presence of the receptor antagonist. These values were due to VV cytophatic effects since control cells infected but not transfected presented comparable percentages of death (9.9 ± 1.8 and 10.5 ± 0.5, respectively). Although cells expressing NR1 and NR2A subunits showed similar values in the presence of DL-AP5 (12.4 ± 0.2), they presented a percentage of 35.8 ± 1.0 dead cells when the receptor antagonist was omitted. This three-fold increase in the number of dead cells confirms the results obtained with the reporter gene expression and demonstrate delayed cytotoxicity mediated by NMDA receptors in our expression system.

The reduction in reporter gene expression also correlated with a decrease in the accumulation of receptor subunits, as explained below.

Requirement of N-Glycosylation for NMDA Receptor Functionality

As mentioned earlier, NR1 immunoblots persistently showed a smear between 116 kDa and 97 kDa which was not due to nonspecific proteolysis. Likewise, NR2A was also detected as a thick band. This is a common behavior of glycoproteins, and indeed, NR1 subunit has ten possible N-glycosylation sites [Moriyoshi et al., 1991], whereas NR2A subunit has six putative sites [Ishii et al., 1993].

To analyze the glycosylation status of the receptor subunits in our expression system, extracts from cells infected/transfected for 48 h in the continuous presence of DL-AP5 were treated with N-glycosidase F and subjected to immunodetection (Fig. 5A). The size of the proteins observed after treatment with the glycosidase (Fig. 5A, lanes 2 and 4) was reduced compared to the untreated samples (Fig. 5A, lanes 1 and 3), and coincident with the expected molecular weight of the recombinant polypeptides (97,000 and 165,000, respectively for NR1 and NR2A genes).

After showing that both receptor subunits were N-glycosylated, we were interested in studying the effect of glycosylation inhibition on NMDA receptor synthesis and function. Cells expressing the receptor subunits were treated with tunicamycin, an inhibitor of the GlcNAc-1-P transferase and analyzed by immunoblotting (Fig. 5B). In the presence of tunicamycin, the NR1 peptide was undetectable, both in single or double transfections, independently of the addition of DL-AP5 (Fig. 5B, lanes 3, 4, 7, and 8, upper panel). In the case of the NR2A subunit, a sharper band of lower molecular weight than that of the corresponding protein in non-treated cells was observed (Fig. 5B, lanes 7 and 8, lower panel). The size of the NR2A subunit (165 kDa) obtained in cells treated with tunicamycin was similar to that observed after N-glycosidase treatment (Fig. 5A, lanes 4 and 5, lower panel). This figure also shows the reduction in the levels of subunits due to cell death. In the absence of tunicamycin, whereas in NR1 transfected cells levels of this protein were independent of the presence of DL-AP5 (Fig. 5B, lanes 1 and 2, upper panel), in doubly transfected cells, both NR1 and NR2A protein levels were diminished if DL-AP5 was omitted (lanes 5 and 6, upper and lower panel, respectively).

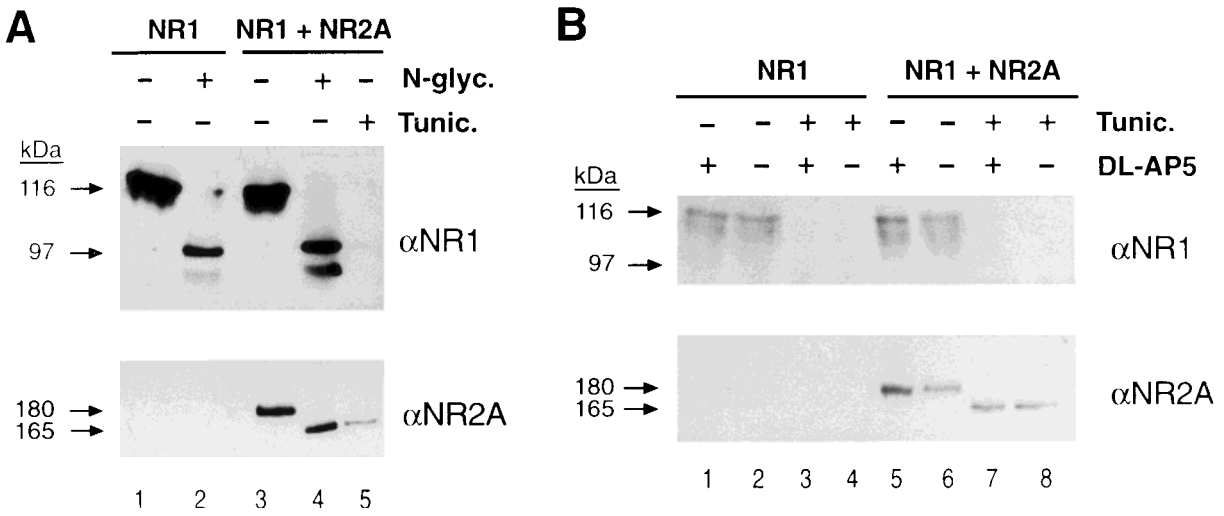

Fig. 5. Analysis of N-glycosylation of NMDA receptor subunits. **A**: In vitro deglycosylation. Protein extracts (30 µg) prepared from cells infected/transfected for 48 h in the continuous presence of 2 mM DL-AP5 as before, were treated with N-glycosidase F (lanes 2 and 4) or left untreated (lanes 1 and 3). For comparison, the same amount of protein prepared from cells infected/transfected similarly but in the presence of 2 µg/ml of tunicamycin was analyzed in lane 5. **B**: Inhibition of in vivo glycosylation. Cells were infected/transfected as before for 24 h in the presence of 2 mM DL-AP5 and for additional 24 h with 1 mM NMDA in the presence or absence of DL-AP5 as above. When indicated, 2 µg/ml tunicamycin was added for the duration of the experiment. Cell extracts (30 µg) were fractionated and immunoblotted as before. Upper panels: Mouse anti-NR1 antibodies. Lower panels: Rabbit anti-NR2A/B antibodies. These results are representative of several independent experiments.

Inhibition of N-glycosylation has a dramatic effect on NMDA receptor function. In cells expressing the receptor subunits there was no cell death in the presence of tunicamycin, whereas more than 94% of the cells died in the absence of the antibiotic (Fig. 6). There was a significant effect of tunicamycin in luciferase activity (compare values obtained in cells transfected with both subunits in the presence DL-AP5, with or without tunicamycin) that is probably due to partial inhibition of protein synthesis described to occur in cells treated with this antibiotic [Elbein, 1981].

Considering the above results, we conclude that glycosylation is essential for the expression of functional NMDA receptors, and that degradation of nonglycosylated NR1 subunit is probably the mechanism accounting for the decrease in cell death observed in our expression system in the presence of tunicamycin.

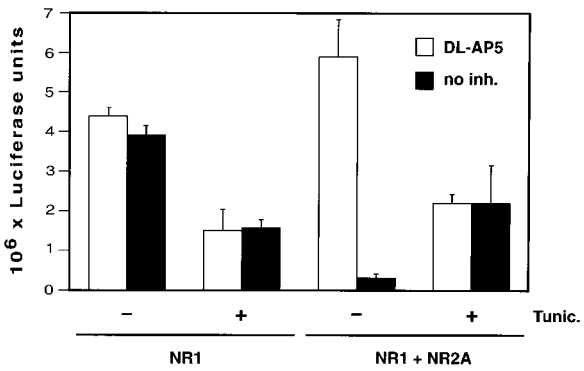

Fig. 6. Effect of inhibition of receptor N-glycosylation in cell viability. Assay of luciferase activity was performed in samples prepared as in Figure 5B. Mean values with standard deviations corresponding to three independent experiments are given.

DISCUSSION

The results presented here show that the VV expression system, based on the synthesis of the T7 RNA polymerase in mammalian cells, efficiently promotes the expression of NMDA receptor subunits NR1 and NR2A. Two different types of experiments demonstrate that these subunits form functional receptors: first, they allow calcium influxes that are dependent on agonists (NMDA) and are inhibited by specific antagonists (MK-801). Second, when challenged with agonists, cell death is induced. This death is dependent on functional NMDA receptors, as transfection of only the NR1 subunit does not induce death, and antagonists specific for the NMDA receptor (DL-AP5 and MK-801) arrest cell death.

High concentrations of DL-AP5 are used in this system (2 mM) to completely protect cells from toxic calcium influx. This value represents

approximately 20-fold excess over the IC_{50} of DL-AP5 in our experimental conditions (using the Cheng-Prussoff equation with EC_{50} of 1.7 μM for glutamate [Kutsuwada et al., 1992], a K_i for DL-AP5 of 1.93 μM [Benveniste and Mayer, 1991] and 120 μM as the concentration of glutamate). Other systems have also been reported in which similar high concentrations of DL-AP5 were necessary to completely protect cells from toxic calcium influx [Anegawa et al., 1995; Choi, 1988; Freese et al., 1990]. The concentration of glutamate in the media of infected cells appears to be high enough to induce cell death in the absence of NMDA. Other authors have observed similar glutamate levels in media from noninfected growing HEK293 cells [Anegawa et al., 1995], ruling out that these levels were due to this expression system. A different situation is found in the experiment where calcium influxes were measured (Fig. 3), since in this case incubation was performed for only 30 min in HCSS, a medium without glutamine or glutamate, we find a strict dependence on NMDA addition.

N-glycosylation sites in the NMDA receptor subunits have been deduced from their primary sequence. Extensive studies on the glycosylation of the glutamate receptor GluR1 have been made by Hollmann et al. [1994] to demonstrate that, in this protein, all six putative N-glycosylation sites are actually glycosylated. In *Xenopus laevis* oocytes, tunicamycin does not prevent the synthesis of functional GluR1 although maximal currents are usually decreased by 40–50% [Hollmann et al., 1994]. However, there are also some other reports on the importance of N-glycosylation for the functionality of NMDA receptors. Kawamoto et al. [1995] have reported that nonglycosylated forms of the mouse NR1 subunit have lost the capacity of binding antagonists to the glycine site. Another group [Chazot et al., 1995] has shown that tunicamycin treatment of cells expressing recombinant receptors protects them from excitotoxic cell death in a dose-dependent manner: 0.1 μg/ml of tunicamycin reduced mortality to approximately 50% of values obtained for untreated cells, while 0.5 μg/ml reduced mortality to 13%. Using the lower concentration of antibiotic, Chazot and coworkers did not find any change in the number of binding sites for MK-801/mg of protein, but a decrease in affinity, while still being able to detect some glycosylation in the NR1 protein by immunoblotting. However, they did not further characterized binding of MK-801 at higher concentrations of the drug, were glycosylation of NR1 is absent. In a different context, data recently published point to the great importance of the protein primary structure in the binding of agonists and antagonists to the NMDA receptors. Site-directed mutagenesis has identified binding of glycine and glutamate to homologous regions in, respectively, the NR1 [Hirai et al., 1996] and NR2B subunits [Laube et al., 1997], consisting of pockets formed by the interaction between the N-terminal extracellular domain and the loop separating transmembrane domains M3 and M4 of each protein. Interestingly, these regions in the different subunits of the glutamate receptor present significant sequence similarity with a family of bacterial amino acid-binding proteins, suggesting that there is a phylogenetically conserved amino acid binding fold that created the binding pockets of diverse types of glutamate receptors [Laube et al., 1997]. Results obtained in our expression system suggest that the protection from cell death elicited by 2 μg/ml tunicamycin is due to the absence of the NR1 subunit, most likely as a consequence of degradation of this protein. It has to be kept in mind that tunicamycin is a powerful inducer of the Endoplasmic Reticulum (ER) stress response [Wang et al., 1996] that can inhibit progression through the ER and promote degradation of some proteins while sparing others.

The method we have used to study cell death deserves some consideration. We have observed that luciferase activity remains constant from 24 to 48 h after infection with the recombinant VV expressing T7 polymerase. This is likely due to continuous synthesis and degradation of the enzyme, as it has been shown that luciferase has a half-life of only 3 h in mammalian cells [Thompson et al., 1991]. This relatively short half-life makes luciferase a good probe for cell integrity or death, as any small alteration in the equilibrium between synthesis and degradation would be readily detectable. Direct measurement of cell death by propidium iodide staining confirms the adequacy of this method.

The results presented here were obtained with a heterogeneous population of cells expressing or not the NMDA receptor subunits after transient transfection. The fact that only 40% of the cells synthesize both subunits reveal the power of the expression system derived from VV. However, more efficient expression

could be still obtained by using recombinant VV coding the NMDA receptor subunits. Several reports have already shown the use of these recombinants viruses to test proteins relevant to neuronal functionality, like the Kv1.3 K^+ channel [Spencer et al., 1997] and calcium-calmodulin-dependent protein kinase II [Pettit et al., 1994] among others. More important, recombinant vaccinia viruses have been shown to be good candidates for expression of heterologous proteins in the nervous system while maintaining intact synaptic function [Pettit et al., 1995; Wu et al., 1995].

The high efficiency of the VV system for expressing recombinant NMDA receptors makes it a very useful alternative for biochemical and electrophysiological studies.

ACKNOWLEDGMENTS

We thank Dr. S. Nakanishi for the gift of the receptor subunit plasmids, Dr. M. Esteban for plasmid pcDLUC and serum against vaccinia virus, Dr. A. Hernanz (Hospital La Paz, Madrid) for performing the amino acid HPLC analysis and Carmen Moratilla for technical help. We are also grateful to Dr. Juan Lerma (Instituto Cajal, CSIC) and Dr. Jesús Avila (Centro de Biología Molecular "Severo Ochoa," CSIC-UAM) for helpful discussions and critical review of the manuscript.

REFERENCES

Anegawa NJ, Lynch DR, Verdoorn TA, Pritchett DB. 1995. Transfection of N-methyl-D-aspartate receptors in a non-neuronal cell line leads to cell death. J Neurochem 64: 2004–2012.

Benveniste M, Mayer ML. 1991. Structure-activity analysis of binding kinetics for NMDA receptor competitive antagonists: The influence of conformational restriction. Br J Pharmacol 104:207–221.

Braiser AR, Tate JE, Habener JF. 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques 7:1116–1122.

Brerson JR, Manzolillo PA, Miller RJ. 1994. Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured Purkinje cells. J Neurosci 14:187–197.

Chazot PL, Cik M, Stephenson FA. 1995. An investigation into the role of N-glycosylation in the functional expression of a recombinant heteromeric NMDA receptor. Mol Membr Biol 12:331–337.

Choi DW. 1987. Ionic dependence of glutamate neurotoxicity. J Neurosci 7:369–379.

Choi DW. 1988. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623–634.

Choi DW. 1995. Calcium: still center-stage in hypoxic-ischemic neuronal death. Trends Neurosci 18:58–60.

Cik M, Chazot PL, Stephenson FA. 1993. Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: a biochemical characterization. Biochem J 296:877–883.

Demkowicz WE, Maa JS, Esteban M. 1992. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. J Virol 66:386–398.

Earl PL, Cooper N, Moss B. 1991. Preparations of cell cultures and vaccinia virus stocks. In: Ausubel FM Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, editors. Current protocols in molecular biology. New York: Green Publishing Associates and Wiley Interscience, pp 16.16.1–16.16.7.

Ehlers MD, Tingley WG, Huganir RL. 1995. Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. Science 269:1734–1737.

Elbein AD. 1981. The tunicamycins—useful tools for studies on glycoproteins. Trends Biochem Sci 6:219–221.

Freese A, DiFligia M, Koroshetz WJ, Beal MF, Martin JB. 1990. Characterization and mechanism of glutamate neurotoxicity in primary striatal cultures. Brain Res 521:254–264.

Fuerst TR, Earl PL, Moss B. 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol 7:2538–2544.

Harlow E, Lane D. 1988. Antibodies. A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Hernanz A, Polanco I. 1991. Plasma precursor amino acids of central nervous system monoamines in children with coeliac disease. Gut 32:1478–1481.

Hirai H, Kirsch J, Laube B, Betz H, Kushe J. 1996. The glycine binding site of the N-methyl-D-aspartate receptor subunit NR1: Identification of novel determinants of coagonist potentiation in the extracellular M3-M4 loop region. Proc Natl Acad Sci USA 93:6031–6036.

Hollmann M, Boulter J, Maron C, Beasley L, Sullivan L, Pecht G, Heinemann S. 1993. Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. Neuron 10:943–954.

Hollmann N, Maron C, Heinemann S. 1994. N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. Neuron 13:1331–1343.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, Nakanishi S. 1993. Molecular characterization of the family on the N-Methyl-D-Aspartate receptor subunits. J Biol Chem 268:2836–2843.

Kawamoto S, Uchino S, Hattori S, Hamajima K, Mishina M, Nakajima-Iijima S, Okuda K. 1995. Expression and characterization of the zeta 1 subunit of the N-methyl-D-aspartate (NMDA) receptor channel in a baculovirus system. Brain Res Mol Brain Res 30:137–148.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, Mishina M. 1992. Molecular diversity of the NMDA receptor channel. Nature 358:36–41.

Laube B, Hirai H, Sturgess M, Betz H, Kuhse J. 1997. Molecular determinants of agonist discrimination by NMDA receptor subunits: Analysis of the glutamate binding site on the NR2B subunit. Neuron 18:493–503.

Lipton SA. 1993. Prospects for clinically tolerated NMDA antagonists: open-channel blockers and alternative redox states of nitric oxide. Trends Neurosci 16:527–532.

Lu YM, Yin HZ, Chiang J, Weiss JH. 1996. Ca^{2+}-permeable AMPA/kainate and NMDA channels: High rate of Ca^{2+} influx underlies potent induction of injury. J Neurosci 16:5457–5465.

Memon SA, Petrak D, Moreno MB, Zacharchuk CM. 1995. A simple assay for examining the effect of transiently expressed genes on programmed cell death. J Immunol Methods 180:15–24.

Michaels RL, Rothman SM. 1990. Glutamate neurotoxicity in vitro, antagonist pharmacology and intracellular calcium concentrations. J Neurosci 10:283–292.

Mori H, Mishina M. 1995. Review: Neurotransmitter receptors VIII: Structure and function of the NMDA receptor channel. Neuropharmacol 34:1219–1237.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno M, Nakanishi S. 1991. Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31–37.

Moss B. 1996. Poxviridae: The viruses and their replication. In: Fields BN Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B, Straus SE, editors. Fundamental virology, third edition. Philadelphia, PA: Lippincott-Raven Publishers, pp 1163–1197.

Pettit DL, Perlman S, Malinow R. 1994. Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. Science 266:1881–1885.

Pettit DL, Koothan T, Liao D, Malinow R. 1995. Vaccinia virus transfection of hippocampal slice neurons. Neuron 14:685–688.

Rose JK, Buonocore L, Whitt MA. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques 10:520–525.

Smith GL. 1993. Expression of genes by vaccinia virus vectors. In: Davidson AJ, Elliot RM, editors. Molecular virology: A practical approach. Oxford: IRL Press, pp 257–283.

Spencer RH, Sokolov Y, Li H, Takenaka B, Milici AJ, Aiyar J, Nguyen A, Park H, Jap BK, Hall JE, Gutman GA, Chandy KG. 1997. Purification, visualization, and biophysical characterization of Kv1.3 tetramers. J Biol Chem 272:2389–2395.

Sucher NJ, Awobuluyi M, Choi Y-B, Lipton SA. 1996. NMDA receptors: From genes to channels. Trends Pharmacol Sci 17:348–355.

Thompson JF, Hayes LS, Lloyd DB. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 103:171–177.

Turetsky DM, Canzoniero LMT, Sensi SL, Weiss JH, Goldberg MP, Choi DW. 1994. Cortical neurons exhibiting kainate-activated Co^{2+} uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. Neurobiol Dis 1:101–110.

Tyaminski M, Wallace MC, Spiegelman I, Uno M, Carlen PL, Tator CH, Charlton MP. 1993. Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. Neuron 11:221–235.

Wang X-Z, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi L-J, Boorstein R, Kreibich G, Hendershot LM, Ron D. 1996. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol 16:4273–4280.

Wu G-Y, Zou D-J, Koothan T, Cline HT. 1995. Infection of frog neurons with vaccinia virus permits in vivo expression of foreign proteins. Neuron 14:681–684.