Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: a biochemical characterization

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The *N*-methyl-D-aspartate R1 (NMDAR1) and NMDAR2A subunits were expressed transiently either alone or in combination in human embryonic kidney (HEK) 293 cells. The biochemical and pharmacological properties of the cloned receptors were compared with those of adult rat brain NMDA receptors using both immunological methods with a newly developed anti-NMDAR2A-(1435–1445) antibody and [^{3}H]MK801 radioligand binding activity. Anti-NMDAR2A-(1435–1445) antibodies recognized specifically four immunoreactive species with M_rs of 180000, 122000, 97000 and 54000 in rat brain, but only a single band of M_r 180000 in HEK 293 cells singly transfected with plasmid pCISNMDAR2A. N-deglycosylation of HEK cell membranes yielded a 165000-M_r immunoreactive species, which is in agreement with the size predicted from the cDNA sequence for the mature NMDAR2A subunit. Co-expression of NMDAR1 and NMDAR2A subunits in HEK 293 cells resulted in cell death. Thus conditions were established for the optimum expression of heteromeric receptors in viable cells, including a requirement for DL-2-amino-5-phosphonopentanoic acid (AP5) in the culture medium post-transfection. Cells transfected with pCISNMDAR1 and pCISNMDAR2A combined yielded a 10-fold increase in the number of [^{3}H]MK801 binding sites compared with single subunit expression. MK801 had similar affinity for the expressed receptors as for those found in adult rat and mouse brain. These results demonstrate that the NMDAR1 and NMDAR2A receptor subunits co-assemble to form a heteromeric complex with properties similar to those of the native receptors of adult mammalian forebrain. Furthermore, the conditions reported for maximal transient expression provide a basis for further structure–activity studies.

INTRODUCTION

L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. It mediates its effects via interaction with the glutamate receptors, two main classes of which have been distinguished on the basis of their transduction mechanisms; namely the G-protein-coupled metabotropic and the fast-acting, ionotropic glutamate receptors (reviewed in Nakanishi, 1992). Within the ionotropic glutamate receptors, two pharmacological subclasses were defined: those activated by *N*-methyl-D-aspartate, i.e. the NMDA receptors, and those insensitive to NMDA, i.e the non-NMDA receptors. The NMDA receptors are of particular interest because they are subject to multiple allosteric regulation by a strychnine-insensitive glycine binding site, polyamines and Mg^{2+} and Zn^{2+} cations. Additionally, they have been implicated both in the induction of long-term potentiation, an activity that may underlie learning and memory, and in neuronal degeneration (reviewed in Madison et al., 1991).

Until recently, there was little information available regarding the molecular properties of vertebrate NMDA receptors. In 1991, Moriyoshi et al. cloned by functional expression a cDNA encoding the NMDA receptor subunit NMDAR1. Multiple alternative splice variants of this subunit, together with a second subunit type, NMDAR2 (alternative nomenclature NMDARε1), with isoforms 2A–2D, were later identified by cDNA homology hybridization (e.g. Kutsuwada et al., 1992; Monyer et al., 1992; Meguro et al., 1992; Ishii et al., 1993). Cloned NMDA receptors have been characterized electrophysiologically following the expression of different subunit combinations in *Xenopus* oocytes or in mammalian cells (e.g. Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Ishii et al., 1993). These studies, together with localization of the different mRNAs by *in situ* hybridization (e.g. Kutsuwada et al., 1992; Monyer et al., 1992), suggested that the native receptor is heteromeric and that it is probably composed of an NMDAR1 subunit in combination with either one of the NMDAR2 subunits in as yet unknown proportions.

In order to study the molecular properties of the NMDA receptors, it is important also to characterize both cloned and native receptors biochemically. The *Xenopus* oocyte system is not amenable for such studies, and therefore we have utilized expression in mammalian cells. Indeed, we have previously reported the expression and immunological characterization of NMDAR1 homo-oligomers, albeit expressed at low efficiency, in human embryonic (HEK) 293 cells (Chazot et al., 1992). In the present paper we now report the first biochemical characterization of NMDAR1/NMDAR2A heterometric receptors in HEK 293 cells and, importantly, also, describe optimum conditions for their expression with minimal cell death.

MATERIALS AND METHODS

Materials

[^{3}H](+)-5-Methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine (MK801; 28.8 Ci/mmol) was from Du Pont (U.K.) Ltd. (Stevenage, Herts., U.K.). The peptide NMDAR1-(929–938), amino acid sequence LQLCSRHRES, was from Multiple Peptide Systems (San Diego, CA, U.S.A.). The peptides NMDAR2A-(1435–1445) (amino acid sequence YKKMPSIESDV),

Abbreviations used: AP5, DL-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DKA, 5,7-dichlorokynurenic acid; HEK, human embryonic kidney; MAPS, multiple antigen peptide system; MK801 (+)-5-methyl-10,11-dihydrodibenzo[a,d]cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate.

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NMDAR2B-(1446–1456) and the multiple antigen peptides (MAPs) YKKMPSIESDVAA-MAP and MAP-YKKMPSIESDV were obtained from Peptide and Protein Research (University of Exeter, Devon, U.K.); biotinylated anti-rabbit immunoglobulin, streptavidin–peroxidase complex, rabbit immunoglobulin, horseradish peroxidase-linked whole antibody and the Enhanced Chemiluminescence (ECL) detection system were from Amersham International (Amersham, Bucks., U.K.). N-Glycosidase F was from Boehringer Mannheim (Lewes, E. Sussex, U.K.). MK801 maleate was from Research Biochemicals Inc. (Natick, MA, U.S.A.); DL-2-amino-5-phosphonopentanoic acid (AP5), 5,7-dichlorokynurenic acid (DKA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Neuramin (Bristol, U.K.). HEK 293 cells were a gift from Dr. T. G. Smart (School of Pharmacy, London, U.K.) and the pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, CA, U.S.A.). All other materials were from commercial sources.

Plasmid construction and cell transfection

The cDNA encoding the full-length rat NMDAR1 was excised from the pN60 plasmid (Moriyoshi et al., 1991) and cloned directionally into the *Sma*I/*Xba*I sites of the pCIS mammalian expression vector (Gorman et al., 1990) as previously described (Chazot et al., 1992). The cDNA encoding full-length mouse NMDARε1 (NMDAR2) was subcloned into the pCIS via a two-step procedure. A 4.8 kb *Bam*HI/*Eco*RI fragment of the cDNA encoding NMDAε1 was excised from pBKSAε1 (Meguro et al., 1992) and ligated into the *Bam*HI/*Eco*RI polycloning site of pBlueskript II KS+ to yield pBKSANDMDARε1-(138–1464). A 2.6 kb *Xba*I/*Hin*dIII fragment of the cDNA encoding NMDAε1 was excised from pBKSAε1 and cloned directionally into pCIS to yield pCISNMDAε1-(1–895). A 2.6 kb *Hin*dIII/*Hin*dIII fragment was excised from pBKSANMDARε1-(138–1464) and ligated in the *Hin*dIII site in the correct orientation of pCISNMDAε1-(1–895) to yield pCISNMDAε1, which now contained the cDNA encoding the full-length NMDAε1 subunit. Note that there is an alternative nomenclature for the NMDAε1 subunit, NMDAR2A (Monyer et al., 1992) and this will now be used throughout the rest of this paper.

Cell transfection was performed as previously described (Chazot et al., 1992). Briefly, HEK 293 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12). At 24 h prior to transfection, cells were plated to a density of 4×10^6 per flask. Cells were transfected using the calcium phosphate precipitation method and subsequently grown in glutamine-free DMEM/F12 in the presence or absence of NMDA receptor ligands (Gorman et al., 1990). Cells were harvested up to 62 h post-transfection and processed as described (Chazot et al., 1992).

Brain membrane preparation

Forebrains were dissected from adult rats (Wistar strain) and adult mice (Balb/c strain), snap-frozen in liquid nitrogen and stored at −80 °C until use. Membranes were prepared as described in Chazot et al. (1983).

Radioligand binding assays

[^{3}H]MK801 radioligand binding assays were performed using a polyethyleneimine filtration assay with 10 μM MK801 for the determination of non-specific binding (Chazot et al., 1992). Numbers of binding sites stated in the text and Figure legends are all $B_{max.}$ values. Where single point [^{3}H]MK801 radioligand concentrations were used, correction to $B_{max.}$ was made using the determined dissociation constants as in Table 3 or in Chazot et al. (1992).

Antibody production and characterization by immunoblotting

Anti-NMDAR1-(929–938) antibodies were generated and characterized as previously reported (Chazot et al., 1992). Polyclonal antibodies were raised in rabbits to the MAPS peptides, NMDAR2A-(1435–1445)-Ala-Ala-MAP and MAP-NMDAR2A-(1435–1445), i.e. peptides were linked to the branched lysine by both the N- and the C-terminus. Anti-peptide antibody production was monitored by an enzyme-linked immunoadsorbent assay with peptide NMDAR2A-(1435–1445) as the antigen (Stephenson and Duggan, 1991). Maximal antibody titres of 1:2500 were obtained, where the antibody titre is defined as the serum concentration that gives half the maximal absorbance at $\lambda = 492$ nm. Antibodies were affinity-purified on the MAP-YKKMPSIESDV peptide–CH Sepharose 4B affinity column with a yield of 100 μg of purified antibody/ml of immune serum (Duggan and Stephenson, 1990). The best results were obtained with the antigen NMDAR2A-(1435–1445)-AA-MAP, and this was thus used for all subsequent receptor characterizations.

Immunoblotting was carried out essentially as described by Duggan et al. (1991) using SDS/PAGE in 7% polyacrylamide mini-slab gels under reducing conditions and with a final antibody concentration of 10 μg of protein/ml. Additionally, in some immunoblots, anti-(rabbit Ig) horseradish peroxidase-linked whole antibody was used in preference to the streptavidin peroxidase/biotinylated antibody complex. The specificity of the immunoreaction was demonstrated by peptide blockade where purified antibodies were preincubated for 16 h at 4 °C before immunoblotting with 100 μg of NMDAR1-(929–938), NMDAR2A-(1435–1445) or NMDAR2B-(1446–1456)/ml in phosphate-buffered saline, or with phosphate-buffered saline alone.

N-deglycosylation of recombinant NMDAR2A

Homogenates of HEK 293 cells transfected with NMDAR2A alone (60 μg of protein) were incubated in the presence or absence of N-Glycanase (final concentration 20 units/ml) for 4 h at 37 °C in 50 μM sodium phosphate, pH 6.0, containing 0.4% (w/v) SDS, 20 mM EDTA and 0.1% (v/v) β-mercaptoethanol. The reaction was terminated by addition of SDS/PAGE sample buffer, and samples were analysed by immunoblotting as described above. Control samples were treated exactly as above, except that the enzyme was omitted.

Protein determination

Protein concentrations were determined using the method of Lowry et al. (1951) with BSA as the standard protein.

Cell death

Cell death was determined by the uptake of Trypan Blue. Briefly, cells were harvested 20 h post-transfection, and centrifuged for 10 min at 1500 *g* at 4 °C. The pellet was resuspended in Hanks' balanced salt solution (10 ml). The number of cells which accumulated Trypan Blue was determined as a percentage of the total cell number using a haemocytometer according to the manufacturer's recommendations (Sigma Chemical Co., Poole, Dorset, U.K.).

RESULTS

Anti-NMDAR2A antibodies and their use in the detection of the NMDAR2A subunit in transfected cells and in rat brain

In a previous study, we described the generation of an anti-NMDAR1 antibody using as immunogen, the synthetic peptide NMDAR1-(929–938) coupled to keyhole limpet haemocyanin as a carrier protein. This antibody was used to immunologically identify the NMDAR1 glutamate receptor subunit expressed in HEK 293 cells and in adult rat brain. Anti-NMDAR1-(929–938) antibodies recognized specifically a band of M_r 117000 in immunoblots from cells transfected with pCISNMDAR1 which was coincident with that from adult rat brain membranes. Using the higher resolution SDS/7%-PAGE, the 117000-M_r seen in rat brain membranes was resolved into two species which differed by M_r 5000 (Figure 1b, lane 8). Additionally, in the transfected cells, a 97000-M_r immunoreactive species was also identified which was non-N-glycosylated NMDAR1 subunit (Chazot et al., 1992). We now report the production of anti-NMDAR2A antibodies adopting the multiple antigen peptide system (MAPS) approach (Tam, 1992). This method negates the use of a carrier protein for the production of antibodies against small synthetic peptides. Multiple peptides are instead covalently attached to a non-immunogenic branched lysine backbone and then used directly as the antigen (Tam, 1992).

In immunoblots with adult rat forebrain membranes, affinity-purified anti-NMDAR2A-(1435–1445) antibodies recognized four major species with M_r values of 180000 ± 4000, 122000 ± 2000, 97000 ± 2500 and 54000 ± 2000 ($n = 5$) (Figure 1a, lane 1; Figure 1b, lane 4). These bands were all blocked by prior incubation of the antibody with either the corresponding MAPS or the free NMDAR2A-(1435–1445) peptide (Figure 1b, lane 6). With the HEK 293 cells transfected with pCISNMDAR2A alone, a single specific immunoreactive species of M_r 180000 ± 3000 ($n = 5$) was detected in immunoblots (Figure 1a, lanes 2 and 4; Figures 1b, lane 3). In control experiments using either rat liver homogenates or untransfected cells, no reactivity was detected in immunoblots (Figure 1b, lanes 1, 2). Following treatment with N-Glycanase of membranes prepared from HEK cells transfected with NMDAR2A, the M_r of the immunoreactive species was decreased to 165000 ± 3000, compared with control samples which retained the M_r 180000 immunoreactive species (Figure 1a, lanes 4 and 5). The size of the non-N-glycosylated subunit is consistent with that deduced from the NMDAR2A cDNA (Meguro et al., 1992; Monyer et al., 1992). N-deglycosylation of rat brain membranes followed by immunoblotting resulted in the loss of immunoreactive bands in both tests and control samples, despite the inclusion of protease inhibitors.

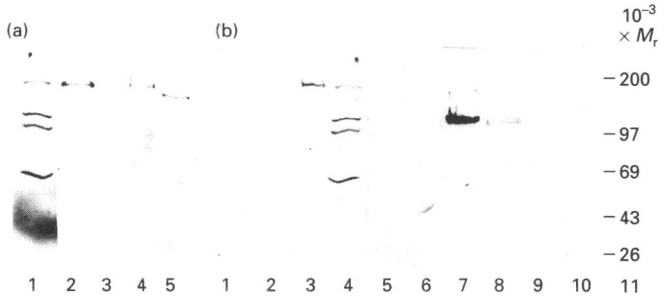

Figure 1 Immunoblots of rat brain membranes and HEK 293 cells transfected with pCISNMDAR1 and pCISNMDAR2A

Membranes were prepared from rat forebrain membranes and HEK 293 cells transfected with pCISNMDAR2A alone or with pCISNMDAR1 and pCISNMDAR2A in combination as described in the Materials and methods section. Immunoblotting was carried out by SDS/7%-PAGE under reducing conditions, transfer to nitrocellulose and using the ECL detection method. Affinity-purified antibodies were employed at final concentrations of 10 μg/ml anti-NMDAR1-(929–938) and 10 μg/ml anti-NMDAR2A-(1435–1445). (**a**) Blotting with anti-NMDAR2A-(1435–1445) antibodies only. Lanes: 1, rat forebrain membranes (35 μg of protein); 2, HEK 293 cells transfected with pCISNMDAR2A alone (60 μg of protein); 3, HEK 293 cells transfected with pCISNMDAR2A alone after preincubation of the antibody with NMDA-(1435–1445) (100 μg/ml) prior to immunoblotting (60 μg of protein); 4, HEK 293 cells transfected with pCISNMDAR2A alone and incubated for 4 h at 37 °C (60 μg of protein); 5, as for 4 with the inclusion of N-glycosidase F in the incubation medium. (**b**) Lanes 1–6 show blots with anti-NMDAR2A-(1435–1445) (10 μg/ml) and lanes 7–10 show blots with anti-NMDAR1-(929–938) (5 μg/ml). Lane 1, liver membranes (60 μg of protein); 2, non-transfected HEK 293 cells (60 μg of protein); 3, 5, 7 and 9, HEK 293 cells transfected with pCISNMDAR1 and pCISNMDAR2A; 4, 6, 8 and 10, rat forebrain membranes; 11, prestained protein standards. For lanes 3–10, 60 fmol (B_{max} value) of [^{3}H]MK801 binding series per gel lane were applied. For lanes 5, 6, 9 and 10, the antibody was preincubated with the appropriate peptide (100 μg/ml) prior to immunoblotting.

Importance of NMDA receptor blockade for successful expression of heteromeric NMDAR1/NMDAR2A receptors in mammalian cells

In initial studies it was observed that, after 16 h, HEK cells co-transfected with pCISNMDAR1 and pCISNMDAR2A and cultured in DMEM/F12 media became detached from the culture dishes and lost viability compared with HEK cells transfected with either of the constructs alone. No immunoreactive bands were found in immunoblots using membranes prepared from the co-transfected HEK cells, in contrast to single subunit expression where M_r 117000 (NMDAR1) and M_r 180000 (NMDAR2A) immunoreactive species were observed (Figure 2). Furthermore, [^{3}H]MK801 binding activity was not detected in the co-transfected cells. Previous experience in transfection studies has revealed that successful transfections using HEK 293 cells were obtained with DMEM/F12 cell culture medium (results not shown). This medium is glutamine-free but it does contain

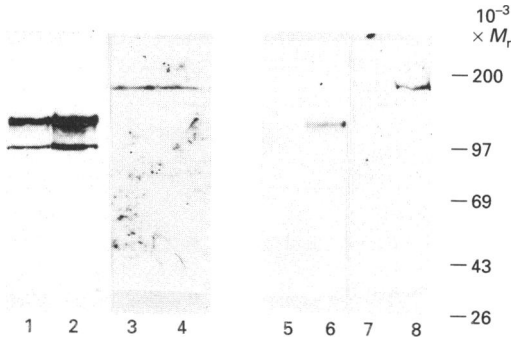

Figure 2 Demonstration by immunoblotting of the effect of AP5 in the culture medium following HEK 293 cell transfection with pCISNMDAR1 and pCISNMDAR2A

HEK 293 cells were transfected with either pCISNMDAR1 and pCISNMDAR2A alone or in combination, and subsequently grown in the presence or absence of AP5 (200 μM) for 48 h. Membranes were prepared from the transfected cells and immunoblotting carried out as described in the Materials and methods section. Lanes 1 and 2, cells transfected with pCISNMDAR1 alone and blotted with anti-NMDAR1-(929–938) antibodies; lanes 3 and 4, cells transfected with pCISNMDAR2A alone and blotted with anti-NMDAR2A-(1435–1445) antibodies; lanes 5–8, HEK 293 cells transfected with both plasmids and blotted with anti-NMDAR1-(929-938) antibodies (lanes 5 and 6) or with anti-NMDAR2A-(1435–1445) antibodies (lanes 7 and 8). Lanes 1, 3, 5 and 7, cells grown in the absence of AP5; lanes 2, 4, 6 and 8, cells grown in the presence of AP5. The positions of prestained protein standards are shown on the right.

sufficient concentrations of L-glutamate to open NMDA receptor channels with a predicted resultant influx of Ca^{2+} which may result in cell death. Thus, following co-transfection, HEK cells were grown in the presence of the NMDA receptor antagonist AP5 (200 μM); under these conditions the cells remained viable and both the NMDAR1 and NMDAR2A polypeptides were detected immunologically for up to 62 h. Similarly, [^{3}H]MK801 binding activity was detectable, with up to a 10-fold increase in binding with respect to HEK cells expressing the NMDAR1 subunit alone. Values ($\pm$S.D.; $n = 3$) obtained for fmol of [^{3}H]MK801 specific binding sites/mg of protein and corrected to $B_{max.}$ using K_D values as in Table 3 and Chazot et al. (1992) were: 90.4$\pm$20.1 (pCISNMDAR1 alone); 45.2$\pm$45.1 (pCISNMDAR1/pCISNMDAR2A in the absence of AP5); 1056.7$\pm$20.0 (pCISNMDAR1/pCISNMDAR2A in the presence of AP5). No specific [^{3}H]MK801 binding was detected in HEK cells expressing only the NMDAR2A polypeptide.

Prevention of cell death by pharmacological manipulation

Since the NMDA receptor is modulated allosterically by different classes of compounds, a series of quantitative experiments was carried out in which the effect in the culture media post-cotransfection of the presence of different pharmacological agents, all known to antagonize NMDA receptor activation, was investigated. Cell viability was monitored by Trypan Blue exclusion. The results are summarized in Table 1. Optimal results with respect to cell survival were obtained with a combination of AP5 and the antagonist of the strychnine-insensitive glycine site, DKA, where no significant difference between the percentage of viable cells in control and test samples was found. Mg^{2+} (10 mM) and DKA alone both partially protected against cell death. No effect was observed for the non-NMDA-receptor antagonist (CNQX).

The integrity of the NMDAR1 and NMDAR2A polypeptides and their assembly to form heteromeric receptors as determined by immunoblotting and [^{3}H]MK801 radioligand binding, was maintained under all of these protection conditions (results not shown). Indeed, with respect to these molecular properties, there was no difference observed between the presence of AP5 alone and that of AP5 plus DKA (results not shown). Routine transfection and culturing in the presence of 200 μM DKA is prohibitively expensive; therefore, since the molecular properties of the cloned receptor were unchanged under the two conditions, further optimization for the co-transfection studies was carried out in the presence of AP5 alone.

Table 1 The effect of NMDA receptor ligands on cell survival following co-transfection with pCISNMDAR1 and pCISNMDAR2A

HEK 293 cells were transfected by the calcium phosphate method with pCISNMDAR1 alone (10 μg), pCISNMDAR2A alone (10 μg) or co-transfected with both pCISNMDAR1 and pCISNMDAR2A (1:3 ratio with a total of 10 μg of DNA) and cultured for 20 h in the presence of different pharmacological agents. Cells were harvested, collected by centrifugation and the percentage cell viability determined by Trypan Blue exclusion as in the Materials and methods section. Values shown are the means $\pm$ S.D. for 10 fields of view for three independent cell counts from each transfection and for n (in parentheses) separate transfections. The efficiency of cell transfection was in the range 15–20% (Chazot et al., 1992). Results were analysed using a two-tailed Student's t-test with a criterion of significance of $P < 0.05$. In comparison to cells grown in the absence of AP5, the degree of significance for the presence of AP5 alone was $P < 0.0001$; DKA alone, $P < 0.0001$ and Mg^{2+} alone, $P < 0.0002$. In comparison to cells grown in the presence of AP5, the degree of significance for AP5 + DKA was $P < 0.02$.

Culture conditions	Cell mortality (%)
Untransfected cells	3.3$\pm$1.8 (2)
Cells transfected with pCISNMDAR1 alone	3.2$\pm$0.9 (3)
Cells transfected with pCISNMDAR2A alone	2.4$\pm$0.1 (2)
Co-transfected cells	21.7$\pm$3.0 (11)
Co-transfected cells + 2 × AP5 (200 μM each)	8.3$\pm$2.6 (5)
Co-transfected cells + 2 × DKA (200 μM each)	12.8$\pm$3.7 (4)
Co-transfected cells + 2 × Mg^{2+} (10 mM each)	13.6$\pm$3.7 (3)
Co-transfected cells + 2 × [AP5 (200 μM) + DKA (200 μM)]	2.8$\pm$1.6 (3)
Co-transfected cells + 2 × [AP5 (200 μM) + Mg^{2+} (10 mM)]	5.7$\pm$1.9 (4)
Co-transfected cells + 2 × [DKA (200 μM) + Mg^{2+} (10 mM)]	5.2$\pm$1.8 (3)
Co-transfected cells + 2 × CNQX (100 μM each)	21.8$\pm$4.6 (3)

Determination of the optimum conditions for the expression of NMDAR1/NMDAR2A heteromeric receptors in mammalian cells cultured post-transfection in the presence of AP5

Because of difficulties experienced with toxicity as described above, the experimental conditions following HEK 293 cell transfection were investigated further in order to maximize the transient expression of NMDA receptors. Firstly, a time course for the expression of the receptors post-transfection was carried out (Figure 3). The time-dependent appearance of immunoreactivity was identical for both single subunit and co-transfection experiments (Figures 3a–3c). Immunoblotting of cell homogenates showed that immunoreactivity was detected at 3 h post-transfection (note that for the exposure time shown in Figure 3 this is not seen), and reached significant levels at 16 h. Maximum immunoreactivities were obtained at 24 h and remained at approximately the same level for up to 62 h. It was noted also that overexpression of NMDAR1 in single transfection experiments, as defined by the presence of non-N-glycosylated polypeptide, occurred at 24 h. This was concomitant with the time at which cell division was attenuated (Figure 3d). Specific [^{3}H]MK801 binding activity to NMDAR1/NMDAR2A expressed receptors followed a similar time course with a peak of binding sites by 24 h which remained stable for up to 62 h (Figure 3e).

The pCISNMDAR1/pCISNMDAR2A ratio used for transfection was also investigated. Earlier studies had shown that 10 μg of total plasmid DNA was optimum for the expression of receptors (results not shown). Therefore in all experiments the ratio was varied such that the total plasmid DNA used for cell transfection corresponded to this value (10 μg); the time of cell harvesting was 48 h. Both [^{3}H]MK801 binding assays and immunoblots were performed on the resultant transfected cells. The results for [^{3}H]MK801 binding are shown in Table 2. The immunoblots showed a profile which correlated directly with the amount of DNA of each respective subunit for transfection (results not shown). Maximum expression was achieved with pCISNMDAR1/pCISNMDAR2A ratios of 1:3 and 1:5. It was noted that, under these conditions, no overexpression of the NMDAR1 subunit, as defined by the detection of the non N-glycosylated polypeptide in immunoblots, occurred (Figure 3c).

In summary, the optimum conditions in the presence of AP5 post-transfection which were adopted as standard for all sub-

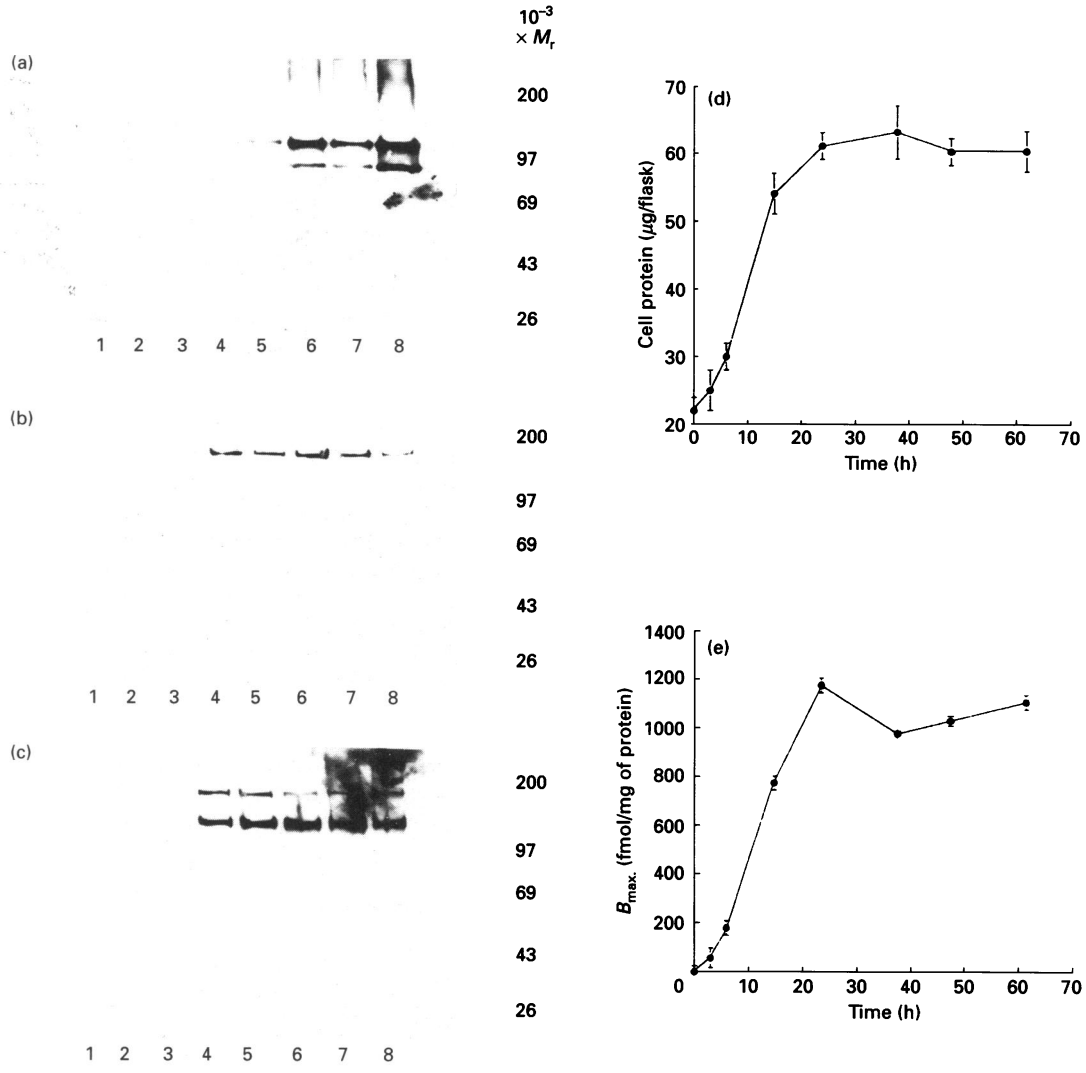

Figure 3 Time-dependence of the expression of NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells

HEK 293 cells were transfected with pCISNMDAR1 (**a**), pCISNMDAR2A (**b**) or both combined (**c**), cultured in the presence of AP5 and harvested at 0, 3, 6, 15, 24, 38, 48 and 62 h (lanes 1–8 respectively). At each time point, the cells were analysed by immunoblotting (**a**–**c**), for the concentration of total protein (**d**) and by [^{3}H]MK801 specific ligand binding activity (**e**), all as in the Materials and methods section. (**a**)–(**c**) are immunoblots with transfection conditions as detailed above; affinity-purified antibodies used were anti-NMDAR1-(929–938) (**a**), anti-NMDAR2A-(1435–1445) (**b**) and both antibodies (**c**). (**d**) shows the time-dependence for cell protein concentration and (**e**) shows the time-dependence for the expression of [^{3}H]MK801 radioligand binding activity, again with co-transfection conditions detailed above.

Table 2 Effect of varying the pCISNMDAR1/pCISNMDAR2A ratio used for co-transfection on the expression of heteromeric receptors in HEK 293 cells

HEK 293 cells were co-transfected with pCISNMDAR1 and pCISNMDAR2A in different ratios such that the total DNA used for transfection was always 10 μg. Cells were cultured post-transfection in the presence of AP5, harvested at 48 h and assayed for [^{3}H]MK801 specific ligand binding activity, all as described in the Materials and methods section. Values are the means ± S.D. for three separate experiments.

Sample	Increase in [^{3}H]MK801 binding activity (fold)
NMDAR1 alone	1
NMDAR1/NMDAR2A, 1:1	2.7 ± 1.8
NMDAR1/NMDAR2A, 1:2	5.0 ± 1.1
NMDAR1/NMDAR2A, 1:3	9.0 ± 0.9
NMDAR1/NMDAR2A, 1:5	8.2 ± 0.3

sequent experiments were a pCISNMDAR1/pCISNMDAR2A ratio of 1:3, the presence of 200 μM AP5 in the culture medium, and cell harvesting at 24 h post-transfection.

The radioligand binding properties of cloned NMDAR1/NMDAR2A hetero-oligomers: comparison with native receptors

[^{3}H]MK801 bound to NMDAR1/NMDAR2A heteromeric receptors at a single high-affinity site, with $K_D = 6.1 \pm 1.4$ nM and $B_{max.} = 1053 \pm 185$ fmol/mg of protein ($n = 10$). The affinity of this site is comparable with that determined for the NMDA receptor in adult rat and mouse forebrain and for the singly expressed NMDAR1 subunit (Table 3; Chazot et al., 1992). The number of binding sites present in the co-transfection experiments showed an approximate 10-fold increase with respect to the NMDAR1 expressed alone. It was noted, however, that when equal numbers of [^{3}H]MK801 binding sites for rat forebrain

Table 3 Comparison of the properties of [^{3}H]MK801 radioligand binding to natural and NMDAR1/NMDAR2A recombinant receptors

$B_{max.}$ values are the means ± S.D. for at least 10 determinations. Inhibitory constants are the means ± S.D. for three determinations. Binding results were analysed by using the Enzfitter program (Elsevier-Biosoft, Cambridge, U.K.).

Source	K_D (nM)	$B_{max.}$ (fmol/mg of protein)
Recombinant	6.1 ± 1.4	1053 ± 185
Mouse forebrain	5.4 ± 0.2	1809 ± 157
Rat forebrain	5.0 ± 0.8	1820 ± 192

membranes and co-transfected HEK 293 cells were applied per gel lane and immunoblotted with both specificity antibodies, the signals obtained were qualitatively always stronger for the recombinant system (Figure 1b, lanes 3 and 7 for cloned receptors and lanes 4 and 8 for rat brain membrane receptors). This would suggest that even under the established optimum conditions of expression herein described, there is still incomplete assembly of the NMDA receptor polypeptides.

DISCUSSION

Currently, detailed molecular properties of mutant and wild-type neurotransmitter receptors of the ligand-gated ion channel superfamily are obtained by the electrophysiological characterization of their properties following transient expression either in the *Xenopus* oocyte or in mammalian cells. Biochemical studies, however, because of the higher concentrations required, utilize mammalian cell expression only. In this paper we describe the establishment of optimum conditions for the transient expression of wild-type NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells, thus providing a methodological base for further biochemical analyses of the molecular properties of this important neurotransmitter receptor protein. Such an investigation was required because it was found that the transient expression of NMDAR1/NMDAR2A receptors under standard transfection conditions resulted in rapid cell death and the loss of receptors, as measured both by [^{3}H]MK801 radioligand binding activity and by immunoblotting with receptor-subtype-selective antibodies. The NMDA receptor channel is permeable to Ca^{2+}; NMDA-receptor-mediated Ca^{2+} influx in the central nervous system is neurotoxic (reviewed in Rothman and Olney, 1987). At resting membrane potentials of −80 mV, the NMDA receptor channel is subject to blockade by Mg^{2+}. Mg^{2+} ions are present at active concentrations (i.e. 0.7 mM) in the culture medium; however, the resting membrane potential of HEK 293 cells is in the range of −15 to −50 mV, potentials at which the voltage-dependent Mg^{2+} block is greatly reduced (Ascher and Nowak, 1987). Thus it was proposed that the mechanism of observed cell death in co-transfected cells compared with cells showing single subunit expression was due to high-level expression of functional receptors, activation by L-glutamate in the culture medium, sustained Ca^{2+} influx and resultant cell death. This was corroborated by the inclusion of the antagonist AP5 in the culture medium following co-transfection; this permitted cell survival, the biochemical characterization of the expressed receptor and a comparison with the properties of native receptors. Although not well documented, AP5 is also employed for expression of heteromeric NMDA receptors in *Xenopus* oocytes, where the electrophysiological properties of the heteromeric receptors have been characterized extensively. It was against this background that all subsequent experiments were carried out.

Initially, the anti-(NMDA receptor subunit) antibodies were characterized. Because of the predicted size of the NMDAR2A subunit from its cDNA sequence, the concentration of acrylamide used for SDS/PAGE was decreased to 7% from the 10% used for earlier studies (Chazot et al., 1992). Under these conditions, the 117000-M_r species recognized by anti-NMDAR1-(929–938) antibodies in adult rat forebrain membranes, but not in transfected cells, was resolved into two components (Figure 1). Several alternative splice variants of the NMDAR1 gene have been described, of which four share the same epitope and are predicted to have M_rs within this range (Sugihara et al., 1992). The observation of an additional immunoreactive species is consistent with detection of an NMDAR1 splice variant and not a proteolytic fragment, since it is not detected in the transfected cells. The anti-NMDAR2A antibody recognized a single band in cells transfected with the corresponding cDNA. The size of the N-deglycosylated recombinant subunit agreed with that predicted from the cDNA sequence (Moriyoshi et al., 1992; Monyer et al., 1992). Anti-NMDAR2A-(1435–1445) antibodies recognized four polypeptides in rat brain membranes. The amino acid sequence chosen for anti-peptide antibody production has 73% identity with the NMDAR2B subunit sequence, and this polypeptide is predicted to be the same size as NMDAR2A. Indeed, the immunoreactive signal in rat brain membranes was blocked by both peptides NMDAR2A-(1435–1445) and NMDAR2B-(1446–1456) (results not shown). Therefore, since the 180000-M_r band corresponds in size with that found for the recombinant protein, it was identified as the NMDAR2 subunit. Alternatively, it may comprise a mixture of both NMDAR2A and NMDAR2B subunits which are not resolved in this SDS/PAGE system. The assignment of this immunoreactive species agrees with two recent reports. Benke et al. (1993) showed that a 175000-M_r protein was specifically photoaffinity-labelled by the novel NMDA ligand ^{125}I-CGP 55802A, the sodium salt of (*E*)-2-amino-10-[(4-azido-2-hydroxy-3-iodobenzoyl)amino]-4-phosphonomethyldec-3-enoic acid. Tingley et al. (1993) reported that a 180000-M_r phosphorylated protein was co-immunoprecipitated with an anti-NMDAR1 antibody from primary cultures of rat cortical neurones. Both groups suggested that these may be the NMDAR2A and/or NMDAR2B polypeptides. The lower-M_r species that were immunoreactive with anti-NMDAR2A-(1435–1445) antibodies may be brain-specific proteins which share the same antigenic determinant as the NMDAR2 subunits. Alternatively, they may be splice forms of the NMDAR2A/2B genes that have not yet been discovered by cloning methodology.

The standard conditions recommended for mammalian cell transfection and transient expression of proteins (e.g. Sambrook et al., 1989) were not appropriate for NMDA heteromeric receptors because of toxicity problems (see the Results section). Therefore we established optimum conditions both for the expression of the respective polypeptides (determined immunologically) and for their co-assembly into functional receptors; the latter being assessed by [^{3}H]MK801 binding activity. That the subunits do co-assemble to form heteromeric receptors was confirmed by the 10-fold increase in [^{3}H]MK801 binding activity upon co-expression compared with single subunit expression. This is in agreement with both electrophysiological studies, where co-expression yielded enhanced L-glutamate-gated ion conductance (e.g. Kutsuwada et al., 1992; Monyer et al., 1992) and antibody studies for the non-NMDA receptors which demonstrated the existence of heteromeric receptors (Wenthold

et al., 1992). Low but significant levels of [^{3}H]MK801 binding were detected for single-subunit NMDAR1 expression but not NMDAR2A expression, suggesting that the MK801 binding site is localized to the NMDAR1 subunit. In support of this, Sonders et al. (1990) reported that an azido derivative of MK801 specifically photoaffinity-labelled a 120000-M_r polypeptide (NMDAR1) in rat brain membranes. In that study there was no evidence for incorporation of radioactivity into a higher-M_r species (180000), although radioactivity was observed at the gel origin which may have represented aggregated receptor (Sonders et al., 1990).

Interestingly, the pCISNMDAR1/pCISNMDAR2A ratio used for transfection influenced the efficiency of functional receptor assembly. This may either be a reflection of the relative efficiency of the respective constructs of different sizes (i.e. pCISNMDAR1/pCISNMDAR2A, 1:1.5) in entering the permeabilized cells, or it may relate to subunit ratios in the assembled heteromeric protein. With respect to the latter, this would simply mean with the information currently available that there were more NMDAR2A than NMDAR1 subunits present per oligomer.

The pharmacological specificity of the NMDAR1/NMDAR2A cloned receptor was similar to that found in native forebrain compared to cerebellar receptors (e.g. Ebert et al., 1991).

The finding that levels of receptor expression in HEK 293 cells that were similar to those found in native brain membranes leads to cell death means that this parameter can be used as an independent index of efficient receptor assembly. Notably, blockade of the strychnine-insensitive glycine-binding site alone permitted a degree of cell survival in the co-transfected cells. This is further evidence to support a role of glycine as a co-agonist at the NMDA receptor (Kleckner and Dingledine, 1988). A further interesting point is that NMDA receptors are known to undergo desensitization and down-regulation by L-glutamate (summarized in Ascher and Nowak, 1987). Neuronal nicotinic receptors also desensitize, but they are up-regulated in response to chronic exposure to the agonist, nicotine (Wonnacott, 1990). In the experiments described here, clearly the continued presence of L-glutamate in the culture medium leads to no such adaptive response in either direction, which suggests a requirement for other factors and regulation at a gene level which is not permissive in the HEK 293 cells.

In summary, we have described in this paper the identification of the NMDAR2A receptor polypeptide in native and recombinant systems using a newly developed antibody. Additionally, we have reported the optimization of conditions for the expression of heteromeric NMDA receptors in mammalian cells at levels which are amenable for biochemical analysis. The availability of this system will now permit more detailed investigations into the regulation and functional significance of NMDA receptor diversity.

We are grateful to Dr. Shigetada Nakanishi and Dr. Masayoshi Mishina for generously providing pN60NMDAR1 and pBKANMDAε1. We thank Derek King for photography. This work was funded by the Science and Engineering Research Council (U.K.).

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Received 18 June 1993/10 August 1993; accepted 23 August 1993