Molecular Characterization of N-Methyl-d-aspartate Receptors Expressed in Mammalian Cells Yields Evidence for the Coexistence of Three Subunit Types within a Discrete Receptor Molecule*

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The N-methyl-d-aspartate R1 (NMDA R1), NMDA R2A, and NMDA R2C subunits were expressed transiently in double or triple combinations in human embryonic kidney (HEK) 293 cells. The biochemical and pharmacological properties of the cloned receptors were compared with those of adult mouse forebrain and cerebellum. Under conditions established for maximal expression, co-transfection of the NMDA R1 and R2C subunits yielded a protein detected immunologically with a molecular size of 780,000–850,000 daltons. No cell death was observed in the transfected cells, and the $K_{D}$ for $[3^H]MK801$ binding to the NMDA R1/R2C receptor was $346 \pm 158$ nm. This was in contrast to a value of $K_{D} = 22 \pm 9$ nm found for native cerebellar receptors. Co-transfection with NMDA R1/R2A/R2C subunits with a DNA ratio, 1:3:3, resulted in the expression of a protein with a size similar to the NMDA R1/R2C combination, but the affinity of $[3^H]MK801$ was now $22 \pm 5$ nm, and the percentage cell death post-transfection was 89 ± 17%. Immunoprecipitation assays of detergent-solubilized transfected cells with NMDA R1 subunit-specific antibodies co-precipitated the NMDA R2A and NMDA R2C subunits in 1/2A and 1/2C transfections, respectively. Similarly, immunoprecipitations with either NMDA R1 or NMDA R2C subunit-specific antibodies co-precipitated the NMDA R2A subunit in the R1/2A/2C triple transfections. These results show that the three NMDA receptor subunit types can co-assemble following their co-expression in mammalian cells with a pharmacological profile that is similar to that found for adult cerebellar NMDA receptors.

The N-methyl-d-aspartate (NMDA) pharmacological sub-class of glutamate receptor is a fast-acting ligand-gated cation channel with a high permeability for Ca$^{2+}$. Several genes encoding NMDA receptor subunits have been identified (1–5). These genes fall into two classes which are defined on the basis of the amino acid sequence homology of the deduced primary structures of the proteins they encode. These are the NMDA R1 and the NMDA R2 subunits. Diversity of the NMDA R1 subunit is created by extensive alternative splicing of the NMDA R1 subunit gene to yield eight isoforms (6), whereas heterogeneity of the NMDA R2 subunit is from the existence of multiple, related genes, NMDA R2A-R2D, (e.g. Refs. 2–5). Expression studies, predominantly utilizing the Xenopus oocyte translation system, have shown that although NMDA R1 can form functional homomeric glutamate-gated channels (e.g. Ref. 1), greatly enhanced glutamate-gated conductances are obtained by the co-expression of the NMDA R1 and an NMDA R2 subunit (2–5). In situ hybridization with subunit-specific probes corroborates the functional expression studies showing that the NMDA R1 mRNA is ubiquitously expressed whereas the NMDA R2 mRNA distributions show distinct but overlapping patterns (3–5). Thus, it has been suggested that native NMDA receptors may comprise an NMDA R1 subunit assembled with a subunit of one type of the NMDA R2 class, e.g. an NMDA R1/R2A receptor where the subunit ratios are not known. However, two recent reports have shown evidence for the co-assembly of three NMDA receptor subunit types in one protein. Wafford et al. (7) showed the preferential assembly of the NMDA R1, R2A, and R2C subunits following co-expression in Xenopus oocytes. The resulting recombinant protein differed in glycine sensitivity from both NMDA R1/R2A and NMDA R1/R2C receptors (7). Moreover, Sheng et al. (8) reported results which were consistent with the partial coexistence of NMDA R1, 2A, and 2B subunits in native receptors of adult rat forebrain.

We have established conditions for the optimal transient expression of cloned NMDA R1/R2A receptors in human embryonic kidney (HEK) 293 cells with minimal cell death, thus permitting their detailed biochemical and pharmacological characterization (9). It was of interest to compare the biochemical properties of other cloned NMDA receptor subtypes with those of the wild-type. We focused firstly on cerebellar NMDA receptors because of the restricted, high levels of expression of the NMDA R2C mRNA in cerebellum (3–5). In initial studies, we observed that co-expression of the NMDA R1 and NMDA R2C subunits did not yield a receptor with properties characteristic of adult cerebellar NMDA receptors. Further investigation provided both biochemical and pharmacological evidence for the co-association of the NMDA R1, NMDA R2A, and NMDA R2C subunits whose properties were reflective of native cerebellar NMDA receptors. We report these results in this paper.

EXPERIMENTAL PROCEDURES

Materials—($^{3}H$)-5-Methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine (MK801; 28.8 Ci/mmol) was from Du Pont (UK) Ltd. (Stevenage, Herts., UK). The peptide NMDA R1-(929-938), amino acid sequence LQLCSRHRES, was from Multiple Antigen Peptide (MAP), NMDA R1-(929-938), amino acid sequence LQLCSRHRES, was from Multiple Peptide Systems (San Diego, CA). The peptides Cys NMDA NCAR 2C (20–34), NMDA NCAR 2C (20–34), and the Multiple Antigen Peptide (MAP), NMDA 2A (1435–1445), and the Multiple Antigen Peptide (MAP), NMDA 2A (1435–1445)AA, (YKMPSPSIESDVAA)-MAP were from Peptide and Protein Research (University of Exeter, Devon, UK). Rabbit immunoglobulin, horseradish-linked whole antibody, and the enhanced chemiluminescence (ECL) detection system were from Amersham International (Bucks, UK). N-Glycosidase F was from Boehringer Mannheim (Lewes, East Sussex, UK).
Antibody Production and Characterization by Immunoblotting—A final primary antibody concentration of 2-10 pg/ml was used. The specificities of all immunoblots were demonstrated by the secondary antibodies with the peptide used previously (9). 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 x 10^4 per 250 ml/75 cm² flask. Cells were transfected using the calcium phosphate method and subsequently grown in the presence of 200 μg AP5 unless stated otherwise. For single and double DNA transfections, 1:10 DNA ratio was used. The DNA transfected was the DNA in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 4 h at 37 °C in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, and either 0.1% (w/v) SDS/1% (v/v) Triton X-100 or 0.2% (w/v) SDS/0.8% (v/v) Triton X-100. Solubilized proteins were collected by either centrifugation for 30 min at 4 °C or by passage through a 0.2-μm filter. Immunoprecipitation assays were carried out as described above. Control samples used affinity-purified nonimmune Ig.}

Expression of NMDA R1/NMDA R2C Heteromeric Receptors in HEK 293 Cells—We have previously reported that the maximum expression of NMDA R1/NMDA R2A heteromeric receptors in HEK 293 cells, determined by both [3H]MK801 binding activity and immunoblotting with subunit-specific antibodies, was dependent upon the ratio of the respective DNAs used and the presence of AP5 in the cell culture media post-transfection (9). Thus, in initial transfection studies for the expression of NMDA R1/NMDA R2C heteromeric receptors, the respective plasmid ratios were varied and the cells were screened for binding activity and immunoreactivity. The DNA ratios employed were NMDA R1/NMDA R2C:4, 1:1, 1:2, 1:3, 1:4. In all cases, NMDA R1 and NMDA R2C subunits were detected by immunoblotting. Both specificity anti-NMDA R2C antibodies recognized a single polypeptide in the transfected cells with M_r = 145,000 ± 2,900 (n = 9) (Fig. 1). Incubation of the respective antibodies with the peptide used for immunization prior to immunoblotting blocked the signals (Fig. 1). N-Deglycysolation of the transfected cell homogenates resulted in a decrease of the immunoreactive band to M_r = 133,000 ± 2,500 (Fig. 1). This value is consistent with that predicted for the mature core peptide from the cDNA sequence, i.e., 133,514 (3, 4). Single point [3H]MK801 binding assays revealed that maximum activity was expressed for a DNA ratio NMDA R1/NMDA R2C, 1:10, which gave a 2-fold increase in binding compared to the expression of NMDA R1 alone (n = 3, results not shown). A full saturation curve for the binding of [3H]MK801 to the cloned NMDA R1/NMDA R2C receptor was thus attempted using the 1:10 DNA ratio. The specific binding, however, was not saturable. The dissociation constant for the binding of [3H]MK801 to cloned NMDA R1/NMDA R2C receptor was determined to be K_d = 158 nM (n = 3) (Table I). The dissociation constant for the binding of [3H]MK801 to adult mouse cerebellum was, K_d = 22 ± 9 nM (n = 3) (Table I).

Expression and Pharmacological Characterization of NMDA R1/NMDA R2A/NMDA R2C Heteromeric Receptors in HEK

UK). MK801 maleate was from Research Biochemicals Inc. (Natick, MA; 5-2-amino-5-phosphonopentanoic acid (AP5), 5,7-dichlorokynurenine acid, and tenofovir were from Toronto Research (Bristol, UK). The Promega Cytotox 96™ cytotoxicity assay system was from Promega Biotech. Immunoprecipitation was from Life Technologies, Inc. The plasmid, pCIS was a gift from Dr. C. Gorman (Genetech). All other reagents and materials were either as reported previously (9, 10) or from commercial sources.
Assembly of Three Subunit Types in Cerebellar NMDA Receptors

293 Cells—Since both the dissociation constant for the binding of \(^{[3]H}\)MK801 to NMDA R1/NMDA R2C cloned receptors was significantly different from the value found for adult cerebellum (Table I) and a recent report had suggested the preferential co-assembly of the NMDA R1/NMDA R2A and the NMDA R2C subunits following expression in Xenopus oocytes (7), triple transfections of HEK 293 cells were carried out. The ratios of DNAs used for HEK 293 cell triple transfections were varied, and the dissociation constants for the binding of \(^{[3]H}\)MK801 to these various expressed receptors were determined. In all cases, the results were best fit by the binding of \(^{[3]H}\)MK801 to a single site. The \(K_d\) values are summarized in Table I together with values for the native receptor. For all transfection experiments, the expression of the respective subunits was verified by immunoblotting with subunit-specific antibodies (results not shown). From Table I, it can be seen that in agreement with previous work (19), the \(K_d\) for the binding of \(^{[3]H}\)MK801 to the cerebellar NMDA receptor is 4-fold lower than to the receptor of mouse forebrain. The \(K_d\) value for the binding of \(^{[3]H}\)MK801 to the NMDA R1/2A/2C cloned receptor for DNA transfection ratios of 1:3:3 agrees with that for the native cerebellar NMDA receptor (Table I). A DNA ratio of 1:3:10 results in a low affinity receptor similar to that found for NMDA R1/2C cloned receptors.

The pharmacological specificity of the NMDA R1/2A/2C, 1:3:3, cloned receptor was further studied and compared to that for the NMDA R1/2A receptor and native forebrain and cerebellar receptors. The characterization of the cloned NMDA R1/2C was not possible because of the low signal/noise ratio for \(^{[3]H}\)MK801 binding. The results are summarized in Table II and Fig. 2.

In the comparison between the pharmacological specificity of the forebrain and the NMDA R1/2A receptor, there is good agreement between the respective inhibitory constants for six drugs tested with the exception of Mg\(^{2+}\) which showed an approximate 14-fold lower affinity for the recombinant receptor. The correlation coefficients for the comparison of the rank order of potency between the forebrain receptor and NMDA R1/2A was \(r = 0.98\) including Mg\(^{2+}\) and \(r = 0.995\) excluding the value for Mg\(^{2+}\). For the comparison between the cerebellar receptor and NMDA R1/2A/2C, for six compounds tested, the correlation coefficient was \(r = 0.992\) (Fig. 2).

The Percentage Cell Death following NMDA Receptor Subtype Expression in HEK 293 Cells—It was earlier observed that the expression of the NMDA R1/2A receptor in HEK 293 cells resulted in cell death which was prevented by the inclusion of 200 \(\mu M\) AP5 in the cell culture media post-transfection (9). In contrast, we observed that the co-expression of the NMDA R1 and NMDA R2C receptor subunits in the same cell line apparently did not result in significant cell death. The percentage cell death was thus quantified for the NMDA R1/2C combination and for the triple transfected cells. The results are summarized in Table III. The percentage cell death of all combinations was calculated with respect to the expression of the NMDA R1/2A subtype which we have previously reported resulted in 100% death of transfected cells (15). From Table III, it can be seen that indeed there is no detectable cell death when the NMDA R1 and R2C subunits are co-expressed; thus, all subsequent experiments using this double combination did not require the presence of 200 \(\mu M\) AP5 post-transfection. In contrast, for the co-expression of the NMDA R1/2A/2C subunits in DNA ratios of 1:3:3 and 1:10:3, there is no significant difference in percentage cell death compared to the 100% for the NMDA R1/2A combination. For the DNA ratio 1:3:10 that yields a recombinant receptor with a low affinity for \(^{[3]H}\)MK801, the percentage cell death is reduced by approximately 50% (Table III). Note that for all combinations studied, the number of receptors expressed per mg of protein were in a similar range (Table I).

The Molecular Size of Recombinant NMDA Receptors—Because of the differences found for the various cloned receptors in both their affinity for the ligand, \(^{[3]H}\)MK801, and the percentage cell viability following transfection, it was important to establish that indeed, the receptor subunits were co-assembling to form heteromeric proteins. The respective sizes of the expressed receptors were determined by native poly-

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TABLE I

The dissociation constants for the binding of MK801 to native and recombinant NMDA receptors

<table>
<thead>
<tr>
<th>Sample</th>
<th>(K_d) (nM)</th>
<th>(B_{max}) (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse forebrain</td>
<td>5.4 ± 0.2</td>
<td>1809 ± 157</td>
</tr>
<tr>
<td>Mouse cerebellum</td>
<td>22.0 ± 9.0</td>
<td>552 ± 215</td>
</tr>
<tr>
<td>NMDA R1*</td>
<td>8.8 ± 2.5</td>
<td>113 ± 37</td>
</tr>
<tr>
<td>NMDA R1/2A*</td>
<td>7.0 ± 2.4*</td>
<td>407 ± 1477</td>
</tr>
<tr>
<td>(1:3) + AP5</td>
<td>346.0 ± 158*</td>
<td>1065 ± 405</td>
</tr>
<tr>
<td>(1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDA R1/2A/2C</td>
<td>13.0 ± 6.0</td>
<td>962 ± 77</td>
</tr>
<tr>
<td>(1:10:3) + AP5</td>
<td>22.0 ± 5.0*</td>
<td>486 ± 190</td>
</tr>
<tr>
<td>(1:3) + AP5</td>
<td>146.0 ± 137</td>
<td>243 ± 816</td>
</tr>
</tbody>
</table>

*Results from Ref. 10.

**Values in brackets show the range of \(B_{max}\) values.**

The \(K_d\) was determined by displacement with unlabeled MK801 using \(^{[3]H}\)MK801 at a final concentration of 30 nM.

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FIG. 1. Characterization of the anti-NMDA R2C subunit-specific antibodies by immunoblotting. Cell homogenates were prepared from HEK 293 cells transfected by the calcium phosphate method with pC15NMDA R1, pC15NMDA R2A, and NMDA R2C with a total of 20 \(\mu g\) of DNA. Immunoblotting was carried out in 6% SDS-PAGE under reducing conditions with 60 \(\mu g\) of protein applied per gel lane, transfer to nitrocellulose, and using the ECL detection system all as described under “Experimental Procedures.” Affinity-purified antibodies were employed at final concentrations of 2-10 pg/ml. Lanes all contain 60 pg of protein from HEK 293 cell homogenates co-transfected with the NMDA R1, R2A, and R2C clones and the antibodies used for immunoblotting with subunit-specific antibodies (results not shown). From Table I, it can be seen that in agreement with previous work (19), the \(K_d\) for the binding of \(^{[3]H}\)MK801 to the cerebellar NMDA receptor is 4-fold lower than to the receptor of mouse forebrain. The \(K_d\) value for the binding of \(^{[3]H}\)MK801 to the NMDA R1/2A/2C cloned receptor for DNA transfection ratios of 1:3:3 agrees with that for the native cerebellar NMDA receptor (Table I). A DNA ratio of 1:3:10 results in a low affinity receptor similar to that found for NMDA R1/2C cloned receptors.
Assembly of Three Subunit Types in Cerebellar NMDA Receptors

TABLE II
Comparison of the pharmacological specificity of [3H]MK801 binding to NMDA R1/2A/2C recombinant and native NMDA receptors

<table>
<thead>
<tr>
<th>Sample</th>
<th>MK801 inhibition constant (nM)</th>
<th>Tenocyclidine inhibition constant (nM)</th>
<th>Ketamine inhibition constant (nM)</th>
<th>Mg2+ inhibition constant (nM)</th>
<th>Ca2+ inhibition constant (nM)</th>
<th>Zn2+ inhibition constant (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse forebrain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDA R1/RMDA R2A</td>
<td>5.4 ± 0.2</td>
<td>75 ± 30</td>
<td>1700 ± 300</td>
<td>0.9 ± 0.3</td>
<td>26 ± 3</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>Mouse cerebellum</td>
<td>7.0 ± 2.4</td>
<td>64 ± 32</td>
<td>2500 ± 530</td>
<td>19 ± 7</td>
<td>121 ± 24</td>
<td>0.53 ± 0.01</td>
</tr>
<tr>
<td>NMDA R1/RMDA R2A/RMDA R2C</td>
<td>22.3 ± 9.0</td>
<td>1600 ± 144</td>
<td>5728 ± 320</td>
<td>4.4 ± 0.9</td>
<td>16.4 ± 7.5</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td>NMDA R1/RMDA R2A/RMDA R2C</td>
<td>22.2 ± 4.9</td>
<td>1600 ± 306</td>
<td>7099 ± 925</td>
<td>6.3 ± 3.3</td>
<td>7.5 ± 4.3</td>
<td>1.14 ± 0.09</td>
</tr>
</tbody>
</table>

* Dissociation constant.

The degree of significance between the inhibition constants was p < 0.01.

DNA ratios used for transfection were 1:3:3 for NMDA R1/2A/2C, respectively.

Fig. 2. Correlation of the rank order of pharmacological specificity between recombinant and native adult NMDA receptors. A shows the correlation of the affinity constants for the inhibition of [3H]MK801 binding for a series of compounds to NMDA receptors of adult mouse forebrain and NMDA R1/R2A closed receptors. B shows the correlation of the affinity constants for the inhibition of [3H]MK801 binding for a series of compounds to native NMDA receptors of adult mouse cerebellum and NMDA R1/2A/2C 1.3:3:3:3 cloned receptors.

TABLE III
The percentage cell death following the transient expression of NMDA receptor subtypes in HEK 293 cells

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Percentage cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA R1/2A (1:3)</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>NMDA R1/2C (1:10)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:10:3)</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:3:3)</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:3:10)</td>
<td>55 ± 17</td>
</tr>
</tbody>
</table>

determined per transfection and the results above show the means ± S.D. for n = 3 separate transfections.

either the NMDA R1/R2A or the NMDA R1/R2C clones. Cell homogenates were prepared, and immunoprecipitation studies were carried out. The results shown in Fig. 4. It can be seen that for the NMDA R1/R2A transfected cells, immunoprecipitation with the anti-NMDA R1 antibody resulted in the detection of the NMDA R2A subunit in the pellet. Similarly, for NMDA R1/R2C transfected cells, following immunoprecipitation with either anti-NMDA R1 or anti-NMDA R2C antibodies, NMDA R2C subunit was detected in the immune pellet. (Immunoprecipitation experiments using the anti-NMDA R2A antibody were not carried out because of antibody availability.) Control experiments using immunoprecipitation with nonimmune Ig did not detect NMDA receptor subunits in the pellets. These results therefore confirm the findings of the PAGE experiments that the two subunit types co-assemble. (Note that the strong immunoreactive bands with M, = 50,000 found with all samples is the immunoglobulin from the precipitation with the primary antibody. Further it is difficult to quantify the signals obtained in the immunoblots because of the differences in the antibody affinities; particularly the anti-NMDA R1 antibody has potent avidity).

HEK 293 cells were then transfected with all three clones, soluble extracts were prepared, and immunoprecipitation assays were carried out with a subunit-specific antibody. The immune pellets were subsequently assayed for the presence of the other two NMDA receptor subunits by immunoblotting using now a different specificity antibody. The results are shown in Fig. 5. It can be seen that following immunoprecipitation with anti-NMDA R1 antibodies, NMDA R1, NMDA R2A, and NMDA R2C subunits were all detected in the immune precipitates. Importantly, immunoprecipitation with anti-NMDA R2C antibodies also showed the presence of the NMDA R1, NMDA R2A, and NMDA R2C polypeptides in the pellets. For both sets of experiments, no NMDA receptor subunits were detected following immunoprecipitation with nonimmune Ig (Fig. 5). Stringent conditions were required to solubilize the cloned polypeptides for electrophoresis and immunoblotting using the subunit-specific antibodies. The results are shown in Fig. 3 for the NMDA R1/2A, NMDA R1/2A/2C, 1:3:3 and 1:3:10 receptors. For each combination, immunoblotting with the relevant antibodies showed the presence of a single, diffuse immunoreactive species with a molecular size in the range 780,000–850,000 daltons that was not present in the nontransfected 293 cells.

Demonstration of the Coexistence of the NMDA R1, NMDA R2A, and NMDA R2C Subunits by Immunoprecipitation.—A further series of experiments was carried out which were designed to substantiate the results from both the native PAGE and radioligand binding studies of the double and triple transfections. In the first study, HEK 293 cells were transfected with acrylamide gel electrophoresis and immunoblotting using the subunit-specific antibodies. The results are shown in Fig. 3 for the NMDA R1/2A, NMDA R1/2A/2C, 1:3:3 and 1:3:10 receptors. For each combination, immunoblotting with the relevant antibodies showed the presence of a single, diffuse immunoreactive species with a molecular size in the range 780,000–850,000 daltons that was not present in the nontransfected 293 cells.
Assembly of Three Subunit Types in Cerebellar NMDA Receptors

Fig. 3. Molecular size determination of NMDA receptors expressed in HEK 293 cells by native PAGE followed by immunoblotting. HEK 293 cells were transfected with either pCI5NMDA R1/R2A or pCI5NMDA R1/R2A/R2C with a DNA ratio 1:3:3 and 1:3:10. Cell homogenates were collected 24 or 40 h post-transfection, and native PAGE followed by immunoblotting was carried out as described under "Experimental Procedures." For each sample, 60 μg of protein was applied per gel lane. Lanes are: 1, 4, and 7, nontransfected cells; 2, 3, 5, and 6, HEK 293 cells transfected with NMDA R1/R2A; 8 and 10, HEK 293 cells transfected with NMDA R1/R2A/R2C with DNA ratio 1:3:3; and 9, HEK 293 cells transfected with NMDA R1/R2A/R2C with DNA ratio 1:3:10. R1 (1, 2, and 5), R2A (3, 4, and 6), and R2C (7–10) are the specificity antibodies used for immunoblotting. For lanes 5, 6, and 10, antibody was preincubated overnight at 4 °C with the peptide used for initial antibody production. The positions of protein standards (×10^5) are shown on the left. The filled arrowhead denotes the immunoreactive band detected by each specificity antibody.

Fig. 4. Immunoprecipitation of NMDA receptors from HEK 293 cells transfected with pCI5NMDA R1/pCI5NMDA R2A and pCI5NMDA R1/pCI5NMDA R2C double combinations. HEK 293 cells were co-transfected with either pCI5NMDA R1/R2A or pCI5NMDA R1/R2C. Cells were collected 24 or 40 h post-transfection for NMDA R1/R2A and NMDA R1/R2C, respectively, and solubilized with extraction buffer as described under "Experimental Procedures." Immunoprecipitation assays were carried out, and the pellets were subjected to immunoblotting with different specificity anti-NMDA receptor antibodies. A shows the results for the pCI5NMDA R1/R2A-transfected cells where the lanes are: 1 and 4, HEK 293 cell-solubilized extracts; 2 and 5, pellets obtained with anti-NMDA R1 (929–938) immunoprecipitation; 3 and 6, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1 is blotting with anti-NMDA R1 (929–938) antibodies, and R2A is blotting with anti-NMDA R2A (1435–1445) antibodies. B shows the results for the pCI5NMDA R1/R2C-transfected cells where the lanes are: 1 and 5, HEK 293 cell-solubilized extracts; 2 and 6, pellets obtained with anti-NMDA R1 (929–938) immunoprecipitation; 3 and 7, pellets obtained with anti-NMDA R2C (1227–1237) immunoprecipitation; and 4 and 8, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1 is blotting with anti-NMDA R1 (929–938) antibodies, R2A is blotting with anti-NMDA R2A (1435–1445), and R2C is blotting with anti-NMDA R2C (1227–1237) antibodies. For both, the positions of prestained protein standards (×10^5) are shown on the left.

NMDA receptors from the HEK 293 cell membranes with sufficient yield for these experiments. Therefore, control experiments were carried out to show that the co-precipitation of all three NMDA receptor subunits was not an artifact of the extraction procedure. The strategy here followed that used by Wenthold et al. (17). HEK 293 cells were transfected with either the NMDA R1, the NMDA R2A, or the NMDA R2C clone alone. Following harvesting, the three cell populations were pooled, and receptor extraction and immunoprecipitation were carried out as above. The results are shown in Fig. 6. Now, immunoprecipitation with an anti-NMDA R1 antibody did not result in the co-precipitation of either the NMDA R2A or the NMDA R2C polypeptides (Fig. 6). Conversely, immunoprecipitation with the anti-NMDA R2C antibody did not pellet either the NMDA R1 or the NMDA R2A subunits (results not shown).

DISCUSSION

Recently, insights into the functional properties of fast-acting neurotransmitter receptors have been made possible by a comparison between the respective recombinant proteins and those found in situ in the brain. The study of cloned NMDA receptors has to date focused largely on the use of the Xenopus oocyte expression system followed by electrophysiological characterization. Several groups have shown that the co-expression of
FIG. 5. Immunoprecipitation of NMDA receptors from HEK 293 cells co-transfected with pCISNMDA R1/pCISNMDA R2A/pCISNMDA R2C. HEK 293 cells were co-transfected with pCISNMDA R1/R2A and R2C. Cell homogenates were collected 40 h post-transfection, and solubilized extracts were prepared as described under "Experimental Procedures." Immunoprecipitation assays were carried out, and the resultant immune pellets were subjected to analysis by immunoblotting. Lanes are: 1, 4, and 7, pellets obtained with anti-NMDA R1 (929–938) immunoprecipitation; 2, 5, and 8, pellets obtained with anti-NMDA R2C (1227–1237) immunoprecipitation; 3, 6, and 9, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1, R2A, and R2C are immunoblotting with anti-NMDA R1 (929–938), anti-NMDA R2A (1435–1445), and anti-NMDA R2C (1227–1237) antibodies, respectively. The positions of protein standards (× 10^6) are shown on the left.

FIG. 6. Demonstration that the co-immunoprecipitation of three subunits in triple-transfected HEK 293 cells is not an artifact of receptor aggregation. HEK 293 cells were transfected with either pCISNMDA R1, pCISNMDA R2A, or pCISNMDA R2C alone or as a triple combination. Cell homogenates were collected 24 h post-transfection; the singly transfected cells were combined with a cell protein ratio of NMDA R1/2A/2C 1:3:3. Solubilized extracts were prepared, immunoprecipitation assays were carried out, and the immune pellets were analyzed by immunoblotting. Lanes are: 1, 3, and 5, pellets obtained from triple subunit combinations after immunoprecipitation with anti-NMDA R1 (929–938) antibodies; 2, 4, and 6, pellets obtained from single-subunit transfections after precipitation with anti-NMDA R1 (929–938) antibodies. R1, R2A, and R2C are immunoblotting with anti-NMDA R1 (929–938), anti-NMDA R2A (1435–1445), and anti-NMDA R2C (1227–1337) antibodies, respectively. The positions of protein standards (× 10^6) are shown on the left.

NMDA R1 with an NMDA R2 subunit is required for robust channel activity, thus suggesting that native receptors are, in the main, heteromeric (e.g., Refs. 2–5). Indeed, we have previously shown a 10–20-fold increase in [3H]MK801 binding sites for NMDA R1/R2A cloned receptors compared to NMDA R1 expressed alone (9). Further, the NMDA R1/NMDA R2 variant receptors have subtly different properties which include sensitivity to both glutamate and glycine, MK801 antagonism, voltage-dependent Mg^2+ block, and polyamine modulation (summarized in Ref. 20). Analysis at the single-channel level of NMDA R1/R2A and NMDA R1/R2C revealed channels with distinct properties (21, 22). For both these putative subtypes, a quantitative resemblance to native NMDA receptors was found. In the results reported herein, we have extended our characterization to additional putative cloned NMDA receptor subtypes again expressed transiently in mammalian cells. This included, importantly for a multisubunit protein, an investigation of the HEK 293 cell transfection conditions as well as both biochemical and immunological characterization of the respective expressed and native receptors.

For the NMDA R1 and NMDA R2A double transfection experiments, we showed that the two subunits co-assemble to form a heteromeric receptor with a molecular size of 780,000–850,000 daltons. This is within the range determined by cross-linking and gel filtration studies for native membrane-bound and solubilized NMDA receptors of rat brain by Brose et al. (23). The pharmacological specificity of the cloned NMDA R1/R2 receptor was similar to that found in adult mouse and rat brain. A notable difference was a 14-fold lower affinity for Mg^2+ inhibition of [3H]MK801 binding to the cloned receptor. Lynch et al. (24) recently reported a more detailed pharmacological profile of NMDA R1/NMDA R2A receptors expressed in HEK 293 cells. In their study, differences found between native and cloned receptors included a lack of spermidine stimulation of [3H]MK801 binding and a reduced enhancement of [3H]MK801 binding by Mg^2+. We have made similar observations for the absence of the stimulatory spermidine modulatory site. These findings concur with those for the effects of ifenprodil, a putative polyamine antagonist, on NMDA R1/R2A receptors expressed in Xenopus oocytes (25). With regard to the Mg^2+ effects on MK801 binding, although there are differences between the results herein and those of Ref. 24, these may be explained by the use of different radioligands and assay conditions (26). The pertinent point is that differences in Mg^2+ effects between cloned and native receptors are found in both reports. Further investigations of the Mg^2+ sensitivity of NMDA R1/R2A/R2B cloned receptors may resolve these observed inconsistencies (see below).

In initial experiments with the NMDA R1/R2C combination, respective DNA ratios were varied to optimize maximal transient expression which was assayed for by both [3H]MK801 binding activity and immunoblotting. Under all DNA ratios tested, NMDA R1 and R2C subunits were detected with subunit-specific antibodies. The molecular weight of the NMDA R2C subunit agreed with that predicted from the cDNA sequence and that in brain and after expression in S9 insect cells (27). Furthermore, the molecular size of the cloned protein determined by native gel electrophoresis (results not shown) was in the range found for native receptors. Immunoprecipitation studies further substantiated the co-association of both the NMDA R1 and NMDA R2C subunits in the transfected cells (Fig. 4). However, the affinity of the recombinant receptor for [3H]MK801 was >10-fold lower than found for native receptors (Table I). This reduced affinity is in agreement with electrophysiological studies where a decrease in MK801 sensitivity between NMDA R1/R2A and NMDA R1/R2C receptors was found (28). Triple subunit HEK 293 cell transfections, now including the NMDA R2A subunit at a defined DNA ratio, yielded a receptor with a single high affinity site for [3H]MK801. The determined K_d value was significantly different from that found for forebrain NMDA receptors but correlated with the K_d for cerebellar NMDA receptors (Tables I and II and Fig. 2). Immunoprecipitation assays and native PAGE further substantiated the co-assembly of all three subunit types (Figs. 3–6). It is noted, however, that each of these experimental paradigms does not show definitively the coexistence of the three subunits in one receptor. Double immunoprecipitations or the serial use of different specificity immunoaffectivity columns will be required for this (see below). But overall, the radioligand binding results, immunoprecipita-
tion studies, and native PAGE are most consistent with the co-assembly of the NMDA R1, R2A, and R2C subunits to form receptors resembling those of the NMDA receptors of adult cerebellum.

An interesting observation was the fact that despite a similar range of [3H]MK801 binding sites being expressed in the NMDA R1/R2C double transfection experiments, cell death was not found. This may be explained by a decreased sensitivity to l-glutamate activation and/or a decrease in the Ca²⁺ permeability of this expressed receptor subtype compared to NMDA R1A/R2B combinations. However, electrophysiological studies have shown that, in fact, NMDA R1A/R2C receptors have increased glutamate sensitivity compared to NMDA R1A/R2B (e.g. Ref. 4) and the two receptors reportedly have similar Ca²⁺ permeabilities (e.g. Ref. 29). Single-channel studies showed that the NMDA R1/R2C receptors had lower conductance states than the native system, the three subunits coexisting in an as yet unknown ratio. This co-association is consistent with the GABA, receptors present in the adult mouse cerebellum.

In summary, we have described the characterization of NMDA receptor subtypes expressed in mammalian cells. In particular, we have provided biochemical evidence for the co-assembly of three different NMDA receptor subunit types, NMDA R1, NMDA R2A, and NMDA R2C, following their transient co-expression in HEK 293 cells. A comparison of the pharmacological specificity between these cloned receptors with NMDA receptors expressed in the adult mouse cerebellum shows a high degree of correlation (r = 0.992), thus suggesting that in the native system, the three subunits coexist in an as yet unknown ratio. This co-association is consistent with in situ hybridization studies which show the co-localization of NMDA R1, NMDA R2A, and NMDA R2C mRNAs in adult cerebellar granule cells (29). The results substantiate the observations of Wafford et al. (7) which suggested a preferential co-assembly of the NMDA R1A/R2B subunits expressed in Xenopus oocytes. Furthermore, they are in agreement with those of Sheng et al. (8) where the co-association of NMDA R1, R2A, and 2B subunits in native receptors was inferred from immunoprecipitation studies with subunit-selective antibodies.

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