Differential Assembly of Coexpressed Glutamate Receptor Subunits in Neurons of Rat Cerebral Cortex*

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In the rat, subunits of the glutamate receptor family fall into three pharmacologically distinct groups: a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid preferring receptors (Glu R1-4), kainate preferring receptors (Glu R5-7, KA 1, KA 2), and N-methyl-D-aspartate preferring receptors (NMDA R1, NMDA R2A-2D). In the present study, we demonstrate immunocytochemically that the majority of neurons in rat cerebral cortex coexpress members of all three groups of glutamate receptor subunits, Glu R2/3, Glu R5/6/7, and NMDA R1. Using immunofluorescence purified or immunoprecipitated a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, kainate and N-methyl-D-aspartate receptors, we show that a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors containing Glu R1-4, kainate receptors containing Glu R6, Glu R7, and KA 2 and N-methyl-D-aspartate receptors containing NMDA R1 each form distinct protein complexes that do not share subunits. Our data indicate that a mechanism exists which allows for the specific assembly of selected glutamate receptor subunits into functionally and structurally distinct heteromeric receptors.

Homotopic glutamate receptors are thought to be the principal mediators of fast synaptic excitation in the vertebrate central nervous system. Triggered by the expression cloning of the first glutamate receptor (Glu R1) from rat brain (1), a large family of related glutamate receptor proteins has been identified in recent years. In the rat, members of this protein family fall into three pharmacologically distinct groups, one of which consists of two subgroups: 1) a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)1 preferring receptors (Glu R1-4), 2) kainate preferring receptors (low affinity kainate preferring Glu R5-7; high affinity kainate preferring KA 1 and KA 2), and 3) N-methyl-D-aspartate (NMDA) receptors (NMDA R1, NMDA R2A-2D). In addition, two "orphan receptors" with unknown function have been identified (for reviews see Refs. 2 and 3). Despite dramatic advances in the characterization of cloned glutamate receptors, little is known about the structure and subunit composition of native receptors in the vertebrate brain. Glutamate receptors are thought to be oligomeric glycoprotein complexes (4-6), most likely pentamers. Biochemical studies using subunit-specific antibodies (5) as well as coexpression studies of recombinant subunits, in which changes in the rectification properties and current amplitudes were used as distinguishing criteria (7-11), suggest that Glu R1-4 are able to form functional heteromeric receptor complexes with each other. Likewise, the low affinity kainate preferring subunits Glu R5 and Glu R6 are able to assemble with the high affinity kainate-binding subunit KA 2 to form heteromeric complexes that differ from the respective homomeric receptors in their current/voltage relationship, desensitization properties, agonist selectivity/potency, or current amplitude (12, 13). In contrast, assembly of the low affinity kainate preferring subunits Glu R5-7 with AMPA preferring subunits Glu R1-4 could not be demonstrated in recombinant coexpression experiments (14-17). Using similar criteria, the NMDA receptor subunit NMDA R1 was shown to form heteromeric receptors with NMDA R2A-2D (18-21).

In the present study, we examined the subunit composition of native glutamate receptors from rat cerebral cortex. We demonstrate by immunocytochemistry that the majority of cortical neurons coexpress members of all three groups of glutamate receptor subunits (Glu R2/3, Glu R5/6/7, and NMDA R1). Using immunofluorescence purified or immunoprecipitated AMPA, kainate, and NMDA receptors, we show that AMPA receptors, kainate receptors, and NMDA receptors each form distinct protein complexes that do not share subunits. Our data indicate that a mechanism exists which allows for the specific assembly of glutamate receptor subunits into functionally and structurally distinct heteromeric complexes.

MATERIALS AND METHODS

Antibodies—Two monoclonal antibodies directed against the NMDA R1 subunit (clones 51.4 and 54.2) were generated according to standard procedures (22, 23) using a fusion protein encoding glutathione S-transferase in frame with NMDA R1 residues 660-811 (representing the intracellular loop between putative transmembrane regions III and IV; residue numbers as in Ref. 24). Both antibodies recognize a protein of 116 kDa in Western blots of rat brain synaptic plasma membranes and human embryonic kidney 293 cells transfected with the NMDA R1 clone. No protein is detected in Western blots of untransfected 293 cells (Fig. 1, A and C). In addition, both antibodies labeled NMDA R1-transfected 293 cells in immunocytochemical experiments while untransfected cells remained unlabeled (Fig. 1, B, C, E, and F). These data indicate that the monoclonal antibodies are specific for NMDA R1. It is important to note that a total of eight splice variants of NMDA R1 have been reported. They are created by combinations of three different,
independently occurring NMDA R1 splicing events (25). However, changes in NMDA R1 primary structure due to these splicing events are restricted to the C- and N-terminal hydrophilic domains of the protein, while the antigen used for the generation of the monoclonal antibodies described is exactly shared by all NMDA R1 splice variants (25). Therefore, our antibodies should equally detect all known NMDA R1 splice variants. Indeed, in experiments examining the relative abundance of different NMDA R1 splice variants using specific polyclonal antibodies, we could demonstrate that neither a 63-base pair insertion into the C-terminal exon 22 (generating a novel C terminus) nor the use of an alternate splice acceptor site in the C-terminal exon 22 (generating a novel C terminus) altered NMDA R1 antigenicity as detected with the monoclonal antibodies described here. The same fusion protein as described above (NMDA R1 residues 660–811) was used for the generation of the affinity purified polyclonal rabbit antibody directed against NMDA R1 (6). Characteristics of the monoclonal antibody 3All have been published previously (26). Additional experiments indicate that the antibody is specific for Glu R2 on Western blots but recognizes Glu R2 (but not Glu R1 or Glu R3) in immunoprecipitations. The characteristics of the monoclonal antibody directed against Glu R5/6/7 (clone 4F5) have been published previously (27). Affinity purified polyclonal antibodies directed against Glu R1 (4), Glu R2/3, Glu R4 (28), and Glu R6/7 (29) were a gift of Dr. Robert Wenthold (Bethesda, MD). The batch of affinity purified polyclonal antibodies directed against Glu R2/3 that was used in the immunocytotoxicity experiments was obtained from Chemicon (Temecula, CA) (see also Ref. 31).

Immunocytotoxicity—Sprague-Dawley rats (300–350 g, n = 5) were anesthetized with a mixture of ketamine (44 mg/kg, inra-peritoneal) and xylazine (5 mg/kg, inra-peritoneal) and perfused transcardially with cold 4% paraformaldehyde. The brains were removed, postfixed for 4 h, and 50-μm sections were cut in the frontal plane on a Vibratome. Sections were incubated for 72 h in cold phosphate-buffered saline with combinations of the following monoclonal antibodies or polyclonal antisera: anti-NMDA R1 (54.1, mouse monoclonal IgG, 1:250); anti-Glu R2/5/6/7 (4F5, mouse monoclonal IgM, 1:500; Ref. 27); anti-Glu R2/3 (rabbit IgG, 1:200; Chemicon, Temecula, CA). All sections were then processed for double immunofluorescence using mouse μ or γ chain or rabbit IgG specific secondary and FITC- and Texas-red-conjugated tertiary antibodies of the appropriate specificity. Sections were viewed and photographed on a Zeiss Axioshot photomicroscope using the appropriate filter blocks for the selective visualization of FITC and Texas-red. Control experiments verified that there was no cross-reactivity of the secondary and tertiary antibodies used. Bleedthrough of the fluorophores was routinely monitored by examining the FITC or Texas-red fluorescence with the inappropriate filters and was non-existent under the conditions used.

Immunofluorinity Purification and Immunoprecipitation—Immunofluorinity purification of AMPA receptors containing Glu R2/Glu R4 (using 5 mg of monoclonal antibody 3All coupled to CNBr-activated Sepharose, Pharmacia LKB Biotechnology Inc.) and of NMDA receptors containing NMDA R1 (using 5 mg of monoclonal antibody 54.2 coupled to CNBr-activated Sepharose, Pharmacia) were performed as described (32). Starting material was a Triton X-100 extract of rat cortex synaptic plasma membranes (2 mg/ml protein concentration, 1% Triton X-100, 200 μM NaCl, 200 μM phenylmethylsulfonyl fluoride, 25 mM Tris-Cl, pH 7.4) which is known to contain only intact AMPA and NMDA receptor complexes (6).

Immunoprecipitation of kainate receptors from Triton X-100 solubilized rat cortex synaptic plasma membranes (4 mg/ml protein concentration, 1% Triton X-100, 200 mM NaCl, 200 μM phenylmethylsulfonyl fluoride, 25 mM Tris-Cl, pH 7.4) was performed using affinity purified
Expression and Assembly of Glutamate Receptors

RESULTS

Colocalization of NMDA, AMPA, and Kainate Receptors in Single Cortical Neurons—In order to determine whether individual neurons in rat cerebral cortex coexpress different groups of glutamate receptors, we performed double labeling immunofluorescent studies. Immunoreactivity for Glu R2/3, Glu R5/6/7, or NMDA R1 was distributed throughout rat cerebral cortex and was localized to neuronal somata and/or the proximal apical dendrites of pyramidal cells (Fig. 2). In general, Glu R subunit-immunolabeled cells were present in all cortical layers, with the greatest concentrations in layers II/III and V. In primary somatosensory cortex (S1), colocalization of immunoreactivities for Glu R2/3 and Glu R5/6/7 revealed that virtually all Glu R5/6/7-positive cells were also immunoreactive for Glu R2/3 (Fig. 2, A and B), although some variation was observed between cortical areas. Similarly, virtually all Glu R2/3-immunoreactive cells in S1 were also positive for NMDA R1 (Fig. 2, C and D). In contrast, many small NMDA R1 immunoreactive cells present in superficial layers of rat S1 were not Glu R5/6/7-immunoreactive (Fig. 2, E and F), although all Glu R5/6/7-immunoreactive cells in layer V were also immunoreactive for NMDA R1 (Fig. 2, E and F).

Immunopurification of AMPA and NMDA Receptors from Cortex—AMPA and NMDA receptors were purified in a single step using affinity chromatography with specific antibodies (monoclonal antibody 3A11 directed against Glu R2/Glu R4 for AMPA receptors, and monoclonal antibody 54.2 directed against NMDA R1 for NMDA receptors). In both cases, only 5% of the total receptor content in synaptic membranes could be solubilized (see also Ref. 6). With an optimal ratio between affinity column volume and column load, binding of NMDA R1 containing receptors to and elution from the affinity column was almost quantitative. In contrast to this, only 5–10% of solubilized Glu R2/Glu R4 containing receptors could be bound to and subsequently eluted from the affinity column (see below). Enrichment of the target proteins was estimated by quantitative Western blotting (ECL method). Typical enrichment factors for NMDA R1 were >3000, while Glu R2/Glu R4 immunoreactivity was typically enriched >1000-fold in the purified material as compared with the membrane extract. In both cases, absolute yields were generally very low, ranging from 100–200 μg of total eluted protein (in a volume of 1–2 ml) from 300 mg of rat cortex synaptic plasma membranes. The protein composition of affinity purified AMPA and NMDA receptors was analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining of the acrylamide gels (representative gels are shown in Fig. 3). In purified AMPA receptors, a broad protein band at 100–105 kDa was detected which presumably represents glutamate receptor subunits Glu R1–4 (see also Fig. 4A). Purified NMDA receptors contained a protein band of 116 kDa which

FIG. 2. Colocalization of Glu R2/3, Glu R5/6/7, and NMDA R1 in rat cortical neurons. Pairs of fluorescence micrographs taken through single sections of rat somatosensory cortex showing colocalization patterns of Glu R subunits. Double labeling for: Glu R5/6/7 (A) and Glu R2/3 (B), taken through layer V; NMDA R1 (C) and Glu R2/3 (D), taken through layer III; NMDA R1 (E) and Glu R5/6/7 (F), taken through layers III-V. Solid arrows in each pair denote cells double-immunofluorescent. Open arrows denote small, NMDA R1-positive cells in layer III (E) which were not Glu R5/6/7-immunofluorescent (F). Bar, 50 μm.

FIG. 3. Immunopurification of AMPA and NMDA receptors. Silver-stained gels of purified fractions. Arrowheads indicate position of Glu R1–4 (left) and NMDA R1 (right). Strongly staining bands in purified NMDA receptors below the 205-kDa marker may represent NMDA R2A-2D subunits. Asterisks denote IgG heavy and light chains bleeding from the columns.
FIG. 4. Western blot analysis of purified glutamate receptors. A, AMPA receptors; B, kainate receptors; C, NMDA receptors. Fractions from the purifications were analyzed with the indicated antibodies. Glutamate receptors migrate at 97,000 to 116,000 Da. Additional bands at ~55 and ~25 kDa in some E and P lanes (A (NMDA R1) and B (Glu R6/7, KA 2, Glu R1, Glu R2/3)) are IgG heavy chain (~55 kDa) and light chain (~25 kDa). They are stained because in the noted cases the antibodies used for purification and detection were from the same species, causing the secondary antibody in Western blotting experiments to detect the antibody used for purification. Antibodies to Glu R4 and Glu R6/7 show cross-reactive bands (A (Glu R4) and C (Glu R6/7)) that have been described previously (28, 29). The responsible proteins are not identified and do not appear in every preparation (compare e.g. A (Glu R4)) with C (Glu R4) or A (Glu R6/7) with C (Glu R6/7) and may therefore represent degradation products.

Western Blot Analysis of Immunoisolated AMPA, Kainate, and NMDA Receptors—The subunit composition of immunoisolated glutamate receptors was studied by Western blot analysis. The analysis of fractions obtained during a purification of AMPA receptors containing Glu R2/Glu R4 is shown in Fig. 4A. Purified receptors (fractions designated as E) contain Glu R1, Glu R2, Glu R2/3, and Glu R4, but not Glu R6/7, KA 2, or NMDA R1, as determined with subunit specific antibodies. These data indicate that Glu R1 and Glu R3 form heteromeric receptors with Glu R2 and/or Glu R4. Surprisingly, only a small fraction of Glu R2/Glu R4-containing receptors (5–10% of solubilized Glu R2/Glu R4 containing receptors) bound to the affinity column, even if the ratio between loaded material and column volume was greatly reduced (not shown), suggesting that the majority of solubilized Glu R2/Glu R4 containing AMPA receptors are not accessible to the immobilized antibodies.

Consistent with these findings, quantitative immunoprecipitation of Glu R6/7 led to the quantitative coprecipitation of KA 2, while Glu R1–4 and NMDA R1 did not copurify and remained in the supernatant (Fig. 4B). When NMDA R1-containing NMDA receptors were quantitatively depleted from a Triton X-100 extract of synaptic plasma membranes using immunoaffinity chromatography, none of the examined AMPA- and kainate-preferring receptor subunits (Glu R1–4, Glu R6/7, and KA 2) were found to copurify with NMDA R1 (Fig. 4C). In each case, copurification or coprecipitation of “foreign” receptor subunits with a given complex (e.g. NMDA R1 with Glu R1–4 or Glu R6/7 and KA 2, Glu R1–4 with NMDA R1 or Glu R6/7 and KA 2, Glu R6/7 and KA 2 with NMDA R1 or Glu R1–4) was only apparent on long exposures of the Western blots (>45 min, not shown). In these cases, the amount of copurifying material was always smaller than 1% of the amount of the respective subunit that did not copurify. Together, these data indicate that AMPA-, kainate-, and NMDA-preferring glutamate receptor subunits reside in distinct protein complexes.

DISCUSSION

Molecular and expression cloning have led to the detailed characterization of a family of mammalian glutamate receptor subunits that is comprised of more than 16 members (2, 3). This
large number of different receptor subunits allows, in principle, for a great diversity in glutamate receptor complexes, which are thought to be pentamers (4–6).

In the present study, we demonstrate that the majority of neurons in rat cerebral cortex coexpress members of all groups of glutamate receptors (Fig. 2). In order to examine the subunit composition of glutamate receptors coexpressed in these cells, we studied material that was immunoisolated from a Triton X-100 extract of rat cortex synaptic plasma membranes. We showed previously by size exclusion chromatography that under these solubilization conditions the receptor complexes remain intact (6, see also 4). Despite the great potential for diversity in subunit composition of native glutamate receptor complexes, rat cortical neurons coassemble only selected subunits with each other. With respect to receptor assembly, glutamate receptor subunits fall into three groups which correspond to the various subunit families as distinguished according to their sequence homology. Neuroglutamases coassemble the AMPA preferring subunits Glu R1-4 into one set of receptors and the kainate preferring subunits Glu R6 and 7 as well as Glu R8 (26), as obtained by NMDA R2A-2D. No significant overlap between these three groups was observed, the contamination of a particular receptor with a foreign subunit never exceeding 1% (Fig. 4). Surprisingly, only 5–10% of solubilized Glu R2/Glu R4 containing receptors bound to the affinity column (Fig. 4). However, NMDA R1 containing NMDA receptors as well as Glu R6 containing kainate receptors could be quantitatively removed from membrane extracts and eluted from the respective affinity matrix. Neither purified NMDA receptors nor purified kainate receptors contained Glu R1-4. This finding excludes the possibility that the Glu R2/Glu R4-containing receptors that did not bind to the Glu R2/Glu R4 affinity column coassemble with NMDA or kainate receptor subunits.

Our findings agree with a previous study on AMPA-prefering glutamate receptor subunits employing chemical cross-linking and immunoprecipitation of cross-linked material, which showed that Glu R1–4 are coassembled (5). In addition, conclusions correlate well with pharmacological data obtained in coexpression studies suggesting coassembly of recombinant Glu R1-4, of Glu R6 and Glu R8 with KA 2, and of the NMDA receptor subunits with each other (7–21). Conversely, it is unlikely that receptors comprised of both kainate-prefering and NMDA-prefering subunits are formed under normal conditions. Evidence for such “mixed” receptors has been obtained with Xenopus oocytes injected with rat brain or Xenopus brain poly(A)+ RNA (36), as well as in studies of purified glutamate receptors from Xenopus central nervous system (37).

The mechanism that leads to the assembly of only certain glutamate receptors is currently unknown. However, it is not restricted to rat cortical neurons. A pattern of assembly similar to the one described here was found in a glial cell line expressing various NMDA glutamate receptor subunits (26), as well as in HeLa cells transfected with combinations of Glu R1, Glu R3, and Glu R8 (see also Refs. 7–21 for physiological analyses of subunit combinations in other expression systems). This demonstrates that cells which normally do not express glutamate receptors are able to sort and assemble them appropriately when transfected with vectors coding for glutamate receptor subunits. It is therefore likely that a thermodynamic driving force rather than a (cell) specific sorting mechanism causes the apparent selectivity of the assembly process.

The immunopurification procedure described in the present study allowed us to specifically purify AMPA and NMDA receptors in amounts that are sufficient to perform protein sequencing of the receptor complexes. It is likely that sequence analysis of proteins copurifying with glutamate receptor complexes (see Fig. 2) will aid in our understanding of the composition and function of this class of transmitter receptor.

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