Immunochemical Characterization of Brain Synaptic Membrane Glutamate-binding Proteins*

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Two glutamate-binding proteins (71 and 63 kDa) were previously purified from synaptic plasma membranes (Chen, J.-W., Cunningham, M.D., Galton, V., and Michaelis, E. K. (1988) J. Biol. Chem. 263, 417–426). These proteins may play a role in glutamate neurotransmission in brain. Polyclonal antibodies were raised against the denatured glutamate-binding proteins in rabbits, including sets of antibodies against each of the binding proteins. The antibodies reacted specifically against both 71- and 63-kDa proteins. The antibodies recognized the denatured form of the proteins in Western blots and the native state of the proteins in enzyme-linked immunosorbent assays and in immunoaffinity chromatography and extraction procedures. All antibodies labeled most strongly the 71-kDa protein in Western blots, but extracted both proteins from solubilized synaptic membrane preparations. These findings indicate that the two proteins are closely related immunologically but that reactivity on Western blots differs between these two proteins. ImmunoeXtraction of the 71- and 63-kDa proteins led to a ~60% decrease in L-[3H]glutamate-binding activity associated with synaptic membrane proteins. Of the brain subcellular fractions examined, the isolated synaptic plasma membranes had the strongest reaction in enzyme-linked immunosorbent assays toward the anti-glutamate-binding protein antiserum. Electron microscopy combined with gold particle immunochemistry revealed the sites labeled by the antibodies as entities present either on the surface or within the post-synaptic membranes and the associated densities of brain nerve ending particles (synaptosomes). Immunochemical procedures of gold labeling with silver enhancement of labeled sites revealed selective neuronal labeling in brain regions enriched in glutamate neurotransmitter pathways such as the hippocampus. Labeling was along dendrites and around cell bodies of pyramidal neurons. Based on the pattern of histochemical labeling, the distribution of immune reactivity in synaptic membranes, and the extractions of a major component of membrane glutamate-recognizing protein by the antibodies, the glutamate-binding proteins must play a role in glutamate neurotransmission.

The amino acids L-glutamic and L-aspartic acid are the most prevalent excitatory neurotransmitters in the mammalian central nervous system (1–3). Several aspects of the function of these amino acids as chemical neurotransmitters have been explored, including their release from nerve endings upon depolarization of neurons, their interaction with specific neuronal membrane receptors, and the termination of their activity in the synaptic region through neuronal reuptake. The neuronal receptors for L-glutamic acid that form chemically activated ion channels in membranes have been classified into three major categories. The nomenclature used to define these three classes of excitatory amino acid receptors is derived from the agonists that activate the receptors: N-methyl-D-aspartate (NMDA), quisqualate, and kainate (4–6). A pharmacologically distinct type of glutamate receptor has been identified as that which leads to activation of phospholipase C following interaction with L-glutamic acid and some of its analogues (7, 8).

It is not known whether the classes of L-glutamate receptors defined pharmacologically represent distinct membrane protein complexes. In recent years, several laboratories have focused their efforts on the identification of proteins from brain tissue that may function as glutamate receptors. The approach used initially was to isolated proteins that bind L-glutamate, L-aspartate, or analogs of these excitatory amino acids. Proteins that bind L-glutamic, L-aspartic, quisqualic, and ibotenic acids have been purified from rat brain (9, 10). Those that bind the NMDA analog 3-(+)-2-carboxypiperazin-4-ylpropyl-1-phosphonate were also isolated from rat brain (11), while those that bind kainic acid were purified from frog brain (12) and chick brain (13). Very recently, the cDNA for the kainate-sensitive glutamate receptor-ion channel protein was cloned from rat brain cDNA (14). The cloning of the cDNA was not based on isolation of kainate-interacting proteins but on screening of cDNA libraries constructed from rat brain mRNA for expression of a kainate-activated ion channel in frog oocytes. The kainate receptor is a 100-kDa protein that has limited homology to the previously purified ~48-kDa kainic acid-binding proteins from frog and chick brain cerebellum (12, 13, 15, 16). The function of the frog and chick brain kainic acid-binding proteins has not yet been established.

There is evidence to suggest that the NMDA and quisqualate subtypes of glutamate receptors differ in their protein constitution from the kainate receptor-ion channel protein. Quisqualate and NMDA did not activate the kainate receptor expressed in frog oocytes (14). In addition, L-[3H]glutamate-binding...
binding proteins of both rat brain synaptic plasma membranes (17) and insect neuronal membranes (18) have been estimated to have a molecular size approximately equal to 75 kDa based on radiation-induced inactivation. The rat brain glutamate-binding sites have an estimated size of 75 ± 15 (S.E.) and those of insect neuronal membranes of 78 ± 8 kDa (17, 18). Furthermore, spider toxins that block both insect and mammalian glutamate receptors (19-21) have very recently been used to isolate proteins from rat brain membranes of molecular size ranging between 66 and 80 kDa (22). Therefore, proteins with molecular size of 70-80 kDa may be the predominant glutamate recognition proteins in neuronal membranes, whereas the 100-kDa kainate receptor may represent a limited subpopulation of glutamate receptors.

We have previously reported on the purification of two glutamate-binding proteins (71 and 63 kDa) from rat brain synaptic plasma membranes (9). These proteins had ligand binding characteristics that approximated those detected in intact granule cells from rat brain cerebellum (23), in synaptic membranes from chick brain (24), and in plasma membranes of neuronal tissue from insects (25). In the preparations mentioned above, including the isolated glutamate-binding proteins from synaptic plasma membranes, the recognition sites for L-[3H]glutamic acid had a preferential order for ligand binding affinities that was: L-glutamate > L-aspartate > ibotenate > quisquulate (9, 10, 23-25). Neither NMDA nor kainate receptor ligands exhibited substantial competition for these glutamate recognition sites. We suggested previously that the binding proteins that we had isolated may represent an L-glutamate/L-aspartate-quisquulate-type receptor but proof for such function would have to await the functional reconstitution of these proteins. We have evidence, from both ion flux measurements into liposomes that contain the purified 71- and 63-kDa proteins and planar lipid bilayer membranes that have the same proteins incorporated in them, that these glutamate-binding proteins have the characteristics of cation-conducting channels activated by L-glutamate, quisquulate, and ibotenate.2

The 71- and 63-kDa glutamate-binding proteins isolated from synaptic membranes had no enzymatic activity and differed in their ligand binding characteristics from membrane transport carriers for L-glutamate and L-aspartate. Of the two proteins, the 71-kDa protein reacted most strongly with concanavalin A-biotin (9). We suggested (9) that the 63-kDa protein may represent either a deglycosylated or proteolytic product of the 71-kDa protein or, alternatively, a subunit of the membrane glutamate-binding protein complex. We have also observed that these two proteins comigrate in sucrose density centrifugation experiments apparently both as monomers, with an estimated native molecular size of 75 kDa, and as dimers, either homodimers or heterodimers, with an estimated molecular size of 139 kDa.3 Because of their apparent existence as monomers in solution following purification, it is likely that each protein is capable of recognizing L-glutamate in the derivatized affinity matrix, thus producing the conditions for the copurification of these two proteins.

Since these two proteins may represent physiologically important recognition sites for L-glutamic acid in neuronal membranes, it is necessary to define more precisely the chemical characteristics of each protein and to trace their distribution in brain. In other studies, we described new chromatographic procedures for the separation of the two proteins.3 In the present study we have probed the immunochromatographic relationships between the 71- and 63-kDa proteins and have characterized the immunochromatographic specificity of polyclonal antibodies raised against the denatured form of these proteins. In addition, we have employed these polyclonal antibodies to determine by immunochromatographic and electron microscopic procedures whether they recognize glutamate-binding entities in synaptic plasma membranes and to visualize by light microscopy the location of these proteins in brain slices.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats (Crl:CD) were obtained from Charles River l-glutamic acid dehydrogenase (EC 1.4.1.3, bovine liver), L-glutamic acid decarboxylase (EC 4.1.1.15, Escherichia coli), glutaminase (EC 5.5.1.2, pork kidney), γ-glutamyl transpeptidase (EC 2.3.2.2, bovine kidney), L-glutamine synthetase (EC 6.3.1.2, sheep brain), p-nitrophenyl phosphate, gelatin (bovine skin, 225 bloom), diithiothreitol (DTT), Tween 20, and Triton X-100 were obtained from Sigma. Freund’s complete adjuvant was from GIBCO. Protein A-colloidal gold conjugate was from EY Laboratories. Carboxylfluorohance was obtained from Research Products Incorporated. Reticulocyte-free lysate was from Amersham Corp. All other chemicals used were from the same sources described previously (9).

**Purification of the Synaptic Membrane 71- and 63-kDa Glutamate-binding Proteins**—The isolation of synaptic plasma membranes from whole brain homogenates and the solubilization of membrane proteins with Triton X-100 were performed as previously described (9), including the presence of six protease inhibitors in all steps of membrane isolation and solubilization. The glutamate-binding protein fraction used for immunization of rabbits was purified from solubilized synaptic membrane proteins by affinity batch chromatography through L-glutamate-derivatized glass fiber followed by ion-exchange chromatography on DEAE-Sephadex as described previously (9). In the preparations used for immunization, the glutamate-binding protein fraction eluted from the glass fiber matrix was rechromatographed on the same matrix in order to obtain a higher degree of purification before proceeding to the ion-exchange chromatographic purification of the proteins. Purity of these protein preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by the determination of enrichment of L-glutamate-binding activity (9).

**Raising Antibodies against Denatured 71- and 63-kDa Proteins**—The proteins isolated by the procedure described above were essentially homogeneous preparations of the 71-kDa, primarily, and 63-kDa proteins. They were dialyzed against a solution of 5 mM DTT, 0.1% SDS for 24 h and then emulsified in Freund’s complete adjuvant (1:1 v/v). 100 µg of protein was injected subcutaneously into the backs of two rabbits. Rooster injections containing 20–60 µg of protein in incomplete adjuvant (1:1) were given at 5, 7, and 9 weeks. Two weeks after the final booster injection, sera were obtained from each animal. Preimmune sera were collected by the same procedure from each rabbit prior to the initiation of the immunization schedule. The serum was separated from whole blood by clotting and low speed centrifugation and stored in small aliquots at -20°C until used.

In an effort to raise antibodies that specifically recognize either the 71- or 63-kDa proteins, two other rabbits were immunized with glutamate-binding proteins that were prepared by the procedures described previously (9). The nitrocellulose sheets were stained with Coomassie Blue and the bands corresponding to the 71- and 63-kDa protein species were cut out and eluted from the nitrocellulose membranes of 78-80 kDa (17, 18). Therefore, synaptic plasma membranes and to visualize by light microscopy the location of these proteins in brain slices.

**Preparation of Synaptic Membrane Glutamate-binding Proteins**—The isolation of synaptic plasma membranes from whole brain homogenates and the solubilization of membrane proteins with Triton X-100 were performed as previously described (9), including the presence of six protease inhibitors in all steps of membrane isolation and solubilization. The glutamate-binding protein fraction used for immunization of rabbits was purified from solubilized synaptic membrane proteins by affinity batch chromatography through L-glutamate-derivatized glass fiber followed by ion-exchange chromatography on DEAE-Sephadex as described previously (9). In the preparations used for immunization, the glutamate-binding protein fraction eluted from the glass fiber matrix was rechromatographed on the same matrix in order to obtain a higher degree of purification before proceeding to the ion-exchange chromatographic purification of the proteins. Purity of these protein preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by the determination of enrichment of L-glutamate-binding activity (9).

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Preparation of IgGs from Immune and Preimmune Sera—Serum aliquots were diluted 1:4 with PBS and saturated ammonium sulfate was added to a final concentration of 40%. The mixture was incubated for 4 h at 0 °C, centrifuged for 10 min at 20,000 g, the pellet resuspended in 2.5 ml of 70 mM sodium phosphate buffer, pH 6.3, and dialyzed extensively against the same buffer at 4 °C. The dialysate was applied to a 5-ml column of DEAE-Sephadex which had been equilibrated with the sodium phosphate buffer and the peak at the void volume was collected. The purified IgG fractions were used immediately following purification.

Enzyme-linked Immunosassays (ELISA)—All proteins used in these assays were first dialyzed against 0.1% SDS, 5 mM DTT in H2O at 23 °C. This was followed by dialysis against deionized water at 4 °C for 2 h. The samples were then frozen and thawed twice at −20 °C. The enzyme-linked immunosassays were performed as described by Roy and Michaelis (28) with some modifications. Polyclonal, 200 µl of a solution containing 10 µg/ml in phosphate-buffered saline (PBS), was added to each well of round-bottom, 96-well microtiter plates. After a 30-min incubation, the poly-L-lysine was removed and 200 µl of the appropriate concentration of isolated protein or tissue fraction was added. Following a 4-h incubation at 23 °C, 50 µl of 0.25% (w/v) glutaraldehyde was added. The wells were emptied after a 15-min incubation and washed three times with 0.05% Tween 20 in PBS. Unreacted glutaraldehyde was blocked with a solution of 0.1% (w/v) gelatin, 100 mM glycine in PBS. The plates were washed twice with PBS, either preimmune serum was added to each well at the concentrations described in the figure legends and incubated overnight at 23 °C. The antibody dilutions were made in PBS buffer that contained 0.1% gelatin. The wells were rinsed with Tween 20/PBS and 200 µl of a 1:1000 dilution of anti-rabbit IgG-alkaline phosphatase conjugate in PBS was added to each well and incubated for 2 h. All wells were rinsed 12 times and 200 µl of p-nitrophenyl phosphate (2 mg/ml) in diethanolamine buffer was added. The plates were developed in the dark for 45 min and absorbance measured at 410 nm.

Polyacrylamide Gel Electrophoresis and Immunoblot Staining—SDS-PAGE was performed as described previously (9). Gels were either stained with silver nitrate or electrotransferred onto nitrocellulose. Immunoblotting of proteins to nitrocellulose filters was achieved with the colloidal gold preparation Aurodye according to the specifications of the manufacturer.

Immunochromatography of transferred proteins was performed according to the procedure of Blake and colleagues (29) using alkaline phosphatase conjugates of goat anti-rabbit antibodies (1:1000 dilution) and 5-bromo-4-chloro-3-indolyl phosphate together with nitroblue-tetrazolium (50). The sections were rinsed in 0.125% gelatin in PBS and incubated with 3% (w/v) gelatin in PBS for 1 h at 23 °C to block the remaining protein-binding sites. The filters were subsequently incubated with anti glutamate-binding protein antiserum (1:1000 dilution) in 1% gelatin-PBS buffer for 4 h at 30 °C.

Protein Determination and L-[3H]Glutamate-binding Assays—All proteins were dialyzed extensively against 40 mM Tris-SC0 buffer, pH 7.4, at 4 °C prior to the conduct of ligand binding assays. The final concentration of L-[3H]glutamate used in ligand binding assays was 100 nM and nonspecific glutamate binding was defined as the binding determined in the presence of 100 µM unlabeled L-glutamate. Ligand binding assays and analyses of ligand binding data were performed as described previously (9). Protein concentration of samples was estimated by the fluorescence procedure of Neuhoff and colleagues (30).

Immunofluorochromatography of Solubilized Synaptic Membrane Proteins—Protein of IgG from immune and preimmune sera was prepared as described above and linked to Reactigel according to the specifications of the manufacturer. Synaptic membranes were solubilized with Triton X-100 in the presence of protease inhibitors as described previously (9). Equal amounts of solubilized synaptic membrane proteins in 1% Triton X-100, 200 mM KCl, 10 mM potassium phosphate, pH 7.4, 0.5% Triton X-100, 0.5% Tween 20 in PBS were applied to the Reactigel matrix, the columns were extensively washed to A280 < 0.003. The fractions eluted during the protein loading and wash steps were collected and analyzed for ligand-binding activity. The proteins bound to the IgG-derivatized matrix were eluted by dialysis against a 1.0 M propionic acid solution, pH 3, collected in tubes, and immediately buffer exchanged by the addition of Tris base. These proteins were analyzed by SDS-PAGE.

Brain Polysome Preparation, Translation, and Immunoprecipitation of Proteins—Brain membrane-bound and free polysomes were prepared according to the procedures described by Heikila et al. (31). The A230/A280 ratio of free polysomes ranged between 1.6 and 1.8 and that of membrane-bound polysomes between 1.3 and 1.6. Cell-free translation of 10–25 A280 units of polysomes was conducted at 30 °C for 1 h in a reticulocyte lysate assay system that consisted of 12 µl of rabbit reticulocyte lysate, 2 µl of [35S]methionine (27 µCi), variable volumes of polysomes, and 1.6–6 µl of H2O to a final volume of 20 µl.

When immunoprecipitation of translated proteins was being attempted, the mixture of reticulocyte lysate, L-[35S]methionine, and brain polysomes was scaled upward 8–9 times that described above. Following in vitro translation, SDS was added to aliquots of the translation mixture to a final concentration of 2% (w/v). The mixture was incubated at 30 °C for 30 min, samples were diluted 4-fold with ice-cold 50 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 0.5% Tween 20, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 0.5 mM benzamidine in 5 mM Tris-Cl buffer, pH 7.4. To this mixture were added 20 µl of preimmune serum and following a 20-min incubation at 23 °C, 100 µl of protein A-Trisacryl suspension. The suspension was rotated end to end for 15 min at 23 °C and centrifuged in a Beckman Microfuge for 4 min at maximum speed. To the supernatant was added 50 µl of anti-71-kDa protein antiserum and 200 µl of protein A-Trisacryl, the mixture rotated at 4 °C for 45 h and the protein A-Trisacryl pellet collected by centrifugation. The pellets from both preimmune and immune serum treatment steps were washed five times with 0.6 ml Tris-Cl buffer, pH 7.4, containing 0.5 M NaCl, 0.1% SDS, 0.5% Triton X-100, 0.5% PMSF, 0.5 mM benzamidine. The washed pellets were resuspended in 70 µl of 5 mM Tris-Cl, pH 6.0, 2% glycerol, 10 mM DTT, 0.2% mercaptoethanol, 5% SDS, and subjected to SDS-PAGE. The gels were soaked in Fluorochrome, dried, and processed for fluorography at −76 °C for 7–21 days.

Immunohistochemical Labeling of Nerve Ending Particles—Nerve ending particles (synaptosomes) were isolated by density gradient centrifugation in Ficoll gradients (32). The isolated particles were rinsed twice in Tris-buffered saline (TBS) (0.1 M Tris-Cl, 0.15 M NaCl, pH 7.4) and resuspended in the same buffer that contained immune or preimmune serum at 1:5,000 dilution. The nerve ending particles were incubated with the serum for 3 h at 4 °C, rinsed twice in TBS, and incubated for 1 h at 4 °C with protein A-10-nm colloidal gold particle complexes. The complexes were centrifuged at 525 nm equal to 0.05. The synaptosomes were subsequently rinsed twice in TBS, fixed for 15 min in 1% glutaraldehyde in TBS, and rinsed twice in 0.1 M sodium-potassium phosphate buffer, pH 7.4. They were resuspended in 2% OsO4 in phosphate buffer, incubated for 15 min, rinsed three times with phosphate buffer, and incubated overnight at 4 °C in this buffer. They were subsequently dehydrated through 70% ethanol twice, embedded in LR white at 62 °C for 22 h, and thin-sectioned on a Sorval MT2B ultramicrotome. Sections were examined by transmission electron microscopy either without prior staining or following staining with uranyl acetate and lead citrate under Low Microscopy.

Procedures for Immunocytochemistry under Light Microscopy—The brains used for immunocytochemical studies were obtained following in situ fixation by the perfusion technique of Johnson (33). Each animal was anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and transcardiac perfusion was initiated through a 14-gauge needle placed in the left ventricle. Heparin (100 units/ml) in normal saline (100 ml/250 g body weight) was perfused at a rate of 5 ml/min. This was followed by perfusion with fixative solution (250 ml/250 g body weight) which contained 2% formalin in 0.1 M phosphate buffer, pH 7.2. Following perfusion with the fixative, the brain was removed, sliced coronally into five sections, and transferred to modified Karnovsky's fixative for 1 h, at room temperature. Sections (40–50 µm) for immunocytochemistry were prepared on a continuous Vibratome, floated into PBS, and stored at 4 °C until they were processed for immunocytochemistry. The sections were placed in water, then soaked in Lugol's iodine, rinsed in water, cleared in 5% thiosulfate, and finally rinsed again in water and PBS. This red step increases contrast in the tissue. Following rinsing in PBS, the sections were incubated in 0.1% Triton X-100 for 15 min, and rinsed twice in TBS. The sections were then incubated in 0.1% Triton X-100 for 1 h, in the presence of primary antibody. This was followed by a 2-h incubation in secondary antibody, rinsed in TBS, and incubated in 0.1% Triton X-100 for 1 h, in the presence of sample (protein A-colloidal gold complex). This was followed by two rinses in TBS and incubation with diaminobenzidine (DAB) solution for 20 min (44). After rinsing, sections were dehydrated and cleared through a graded ethanol series and xylene. The sections were then mounted on glass slides, and stored under a coverslip and nail polish.
labeling was performed after rinsing of the sections with deionized water. The silver-enhancement kit reagents were used according to the manufacturer's instructions. The sections were mounted in glycerol and examined and photographed on an Olympus 1000 microscope.

RESULTS

Specificity of Antibodies Raised against Synaptic Membrane Glutamate-binding Proteins—Immunization of rabbits with highly purified, reduced, and denatured glutamate-binding proteins resulted in raising antibodies that reacted specifically with purified glutamate-binding proteins. The sensitivity of immune reactivity was evaluated by the ELISA procedure. The preimmune serum tested under identical conditions to those used for the immune serum did not react with the purified glutamate-binding protein fraction. The reaction of the immune serum with glutamate-binding proteins could be titrated over a range of serum dilutions between 1:20 and 1:1500. The reactivity present in the immune serum toward glutamate-binding proteins was associated with the IgG fraction, as purified IgG exhibited concentration-dependent and saturable reactivity toward a set amount (100 ng/well) of the isolated glutamate-binding proteins.

The selectivity of the anti-glutamate-binding protein antibodies for the antigen against which they were raised is shown in Fig. 1. The antibodies raised against the glutamate-binding protein had high specific reactivity against a purified glutamate-binding protein fraction and low reactivity toward any of the glutamate-metabolizing enzymes tested. The observations made with the ELISA technique were also confirmed by Western blot analysis using highly purified preparations of the major protein subunit obtained from each enzyme. None of the enzyme proteins were labeled by our anti-glutamate-binding protein antibodies (data not shown).

The antibodies raised against the glutamate-binding proteins labeled a 66–71-kDa protein band very strongly and a 60–63-kDa protein band weakly in Western blots of both solubilized synaptic membrane preparations and partially purified binding protein fractions (Fig. 2). Many of the preparations of purified glutamate-binding proteins consisted mostly of the 66–70-kDa protein species. The 60–63-kDa protein might be a breakdown product generated during purification (9).

Immunochemical Distribution of the Glutamate-binding Proteins in Brain Subcellular Fractions and Liver Homogenates—The distribution of anti-glutamate-binding protein-like immune reactivity in various brain subcellular fractions is shown in Fig. 3. Different antiserum dilutions ranging from 1:50 to 1:200 were tested by the ELISA procedure against the same amount of each of the brain subfractions obtained during the isolation of synaptic membranes. There was progressive enrichment in specific immune reactivity in each membrane fraction from the crude cell and nuclear fraction (P1), to the Ficoll gradient-isolated synaptosomes, to the synaptic plasma membranes (Fig. 3). The myelin-enriched and the mitochondrial fraction obtained following Ficoll-sucrose density gradient centrifugation had low immune reactivity. The differences among brain subcellular fractions in immune reactivity with the antisera were detectable with serum dilutions up to 1:800 (data not shown).

The same procedure was used to determine the selectivity of immune reaction with liver proteins. The immunoreactivity of the antibodies against antigens present in liver homogenates was very low when compared with that exhibited against antigens in brain homogenates (data not shown). In this study, high concentrations of homogenate proteins (800–3200 ng/well) were employed in order to reach detectable levels of immune reactivity with the liver homogenate.

Immunochemical Relationships between the ~70- and ~63-kDa Proteins—Since the antibodies used in these studies were
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Fig. 3. Comparative immune reaction of anti-glutamate-binding protein antibodies with various brain subcellular fractions. Fractions obtained during the isolation of synaptic membranes from rat brain were bound to each well of the assay plates (100 ng/well) using the glutaraldehyde reaction procedure described under "Experimental Procedures" and incubated with varied concentrations of either preimmune or immune serum. Immune reactivity was determined according to the procedures described in Fig. 1. The values shown in this figure represent the net absorbance at 410 nm obtained by subtracting the value measured following reaction of each fraction with preimmune serum from that measured for the same fraction following incubation with the immune serum. Each value is the mean of triplicate determinations of reactions with immune and preimmune serum.

Raised against a purified glutamate-binding protein fraction that contained both the ~70- and ~63-kDa proteins, albeit a fraction that was very highly enriched in the ~70-kDa protein, it was necessary to explore further the immunochemical relationship between these two proteins. Antibodies were raised against each of the major protein bands in the isolated synaptic membrane glutamate-binding protein fractions, i.e. the ~70- and ~63-kDa proteins, according to the methods described under "Experimental Procedures." The antibodies were raised against SDS-denatured peptides that were electrophoresed onto nitrocellulose filters. The sera from the rabbit immunized with the ~70-kDa protein had high immune reactivity toward the 66-70-kDa protein and lesser but detectable reactivity toward the 60-63-kDa protein as determined by Western blot analysis (Fig. 4). High amounts of purified binding proteins (2-7 μg/lane) and low dilutions of antiserum (1:300) were used in this study in order to reveal all possible cross-reactions. Preadsorption of the antiserum with increasing amounts of partially purified glutamate-binding protein decreases, and at high concentrations of the binding proteins eliminated, the immune reactivity of the antiserum toward the ~70- and ~63-kDa proteins (Fig. 4A). Labeling of the ~63-kDa protein was blocked at much lower concentrations of binding protein preadsorbed to the anti-71-kDa antibodies than was the labeling of the ~70-kDa protein.

The pattern of immune labeling in Fig. 4A was indicative of recognition by the anti-71-kDa protein antibodies of both the ~63- and ~70-kDa species, and of diffuse protein bands of intermediate molecular mass between those two. Both proteins migrated as rather diffuse bands, especially when large amounts of protein were loaded on each gel lane (Fig. 4A). The heavy labeling of the 66-70-kDa protein and weak staining of proteins with molecular mass ranging between 63 and 71 kDa was characteristic of all fractions obtained during the purification of the glutamate-binding proteins (Fig. 4B).

This pattern of immune labeling of synaptic membranes and purified glutamate-binding proteins was not unique for the antibodies raised against the 71-kDa protein. The antibodies raised against the 63-kDa protein also labeled most strongly the 66-70-kDa species and relatively weakly the proteins whose Mr, ranged between 63,000 and 66,000 (Fig. 5A). The ~70-kDa synaptic membrane protein that is labeled by all sets of antibodies raised against different preparations of glutamate-binding proteins was also labeled very strongly following interaction with concanavalin A-biotin (Fig. 5B).

On the other hand, the ~63-kDa protein was labeled poorly by all sets of antibodies and by concanavalin A.

Imunoaffinity Purification of Glutamate-binding Proteins from Synaptic Plasma Membranes—Since all antibodies raised interacted most strongly with the ~70-kDa protein in Western blots, we examined the reactivity of the antibodies with synaptic membrane proteins in their native state by performing immunoaffinity purification experiments. Both the antibodies raised against the purified glutamate-binding protein fraction and those raised specifically against the 71-kDa protein species were linked to Reactigel matrices. These IgG-derivatized matrices were used to extract by immunoaffinity chromatography the protein complexes in solubilized synaptic plasma membranes that interacted with the antibodies. As is shown in Fig. 6, such complexes of proteins were isolated and found to contain only the 71- and 63-kDa protein species.
The identity of the proteins isolated by immunoaffinity chromatography was established in two ways. First, both the 63-kDa and 71-kDa proteins reacted with anti-glutamate-binding antibodies in Western blots (data not shown). Second, immunoaffinity chromatography on either an anti-glutamate-binding protein antibody column or an anti-71-kDa protein column quantitatively extracted most (77-89%) of the glutamate-binding activity of the solubilized synaptic membranes (Table I). Since chromatography of synaptic membrane proteins through a column derivatized with preimmune IgG brought about the loss of approximately 25% of glutamate-binding activity (Table I), then the specific immunoextraction of glutamate-binding proteins on columns derivatized with immune IgG must be 50-70% of such proteins.

Immunextraction of the glutamate-binding proteins from solubilized synaptic plasma membranes was also attempted using the protein A-Trisacryl procedure described under "Experimental Procedures" for the isolation of these proteins from cell-free translation systems. The specific glutamate-binding activity of the solubilized synaptic membrane extract was 0.026 (±0.003, S.E., n = 6) pmol/0.1 ml and of the soluble extract obtained following treatment with preimmune serum and protein A-Trisacryl was 0.025 (±0.005, S.E., n = 6) pmol/0.1 ml. The extract following treatment with the anti-71-kDa serum plus protein A-Trisacryl had binding activity that was equal to 0.012 (±0.002, S.E., n = 6) pmol/0.1 ml. These results indicate that treatment of solubilized synaptic membranes with preimmune serum extracted an insignificant amount of glutamate-binding activity, whereas immunoprecipitation with the anti-71-kDa protein antibodies eliminated 55% of the specific glutamate-binding activity in synaptic membranes. These results obtained with the protein A-Trisacryl immunoprecipitation correspond well with those described above for the IgG-derivatized Reactigel chromatography.

Inhibition of the Glutamate-binding Activity of Purified Binding Proteins by the Anti-glutamate-binding Protein Antibodies—Partially purified IgG (ammonium sulfate precipitate that was extensively dialyzed) from immune serum, but not that from preimmune serum, produced a significant inhibition of glutamate-binding activity of the isolated 71- and 63-kDa protein complex. Incubation for 4 h with the IgG fraction from the preimmune serum produced less than 20% inhibition when compared with control samples incubated in buffer only. On the other hand, incubation with immune IgG produced 82.1% (±9.8, S.E., n = 5, two different protein preparations) inhibition of the specific L-glutamate binding to the isolated proteins when compared with the binding activity of the proteins treated with preimmune IgG. The
average specific L-[3H]glutamate binding to the proteins in the presence of 100 nM L-[3H]glutamate and of preimmune IgG was 59.5 pmol/mg protein. The purified proteins used in these assays were freshly isolated (3-4 days old) and dialyzed. As described previously by Chen et al. (9), the specific binding activity of freshly isolated proteins is relatively low when compared with the activity reached within 10-14 days of storage at 4 °C. We selected to use freshly isolated proteins in order to make sure that no structural changes occurred prior to the treatment with IgG and conduct of the binding assays.

**Immunoaffinity Extraction of 35S-Labeled Proteins from Cell-free Translation of Brain Polysomes**—A cell-free translation system was used to determine whether the anti-glutamate-binding protein antibodies would interact with newly synthesized, non-glycosylated proteins formed by translation of brain polysome mRNA and to examine the molecular size of these in vitro synthesized proteins. Cell-free translation of rat brain polysome mRNA with rabbit reticulocyte lysate led to a linear increase in protein-associated radioactivity with increasing amounts of polysomes added up to 20 units of A260 nm and to the synthesis of many proteins that were not detected when reticulocyte lysate without polysomes was used. The translation activity measured in the presence of 20-25 units of either free or membrane-bound polysomes was 31 to 38 times that of the protein-associated radioactivity in translation mixtures with reticulocyte lysate only. When the labeled proteins synthesized by translation of membrane-bound polysomes were immunoprecipitated first with preimmune serum and protein A-Trisacryl to remove proteins that would attach nonspecifically to the matrix, then with the specific anti-71-kDa immune serum and protein A-Trisacryl, a 66-kDa protein band of 35S-labeled protein was repeatedly isolated in the fraction precipitated with the immune serum (Fig. 7). Extraction of the translation mixture with preimmune serum did not produce such selective enrichment of specific protein species. Weakly reactive bands of molecular size between 45 and 66 kDa were usually observed and may have been early stages of in vitro synthesized proteins (Fig. 7).

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**Immunohistochemical Labeling of Nerve Ending Particles**—Nerve ending particles were isolated from whole brain homogenates and processed for electron microscopic examination and immunocytochemistry as described under “Experimental Procedures.” Nerve ending particles were recognized by the characteristic appearance of multiple synaptic vesicles within the terminal region and the usual presence of intraterminal mitochondria (Fig. 8). In many nerve ending particles there was also preservation of the postsynaptic membrane with the characteristic densities associated with most brain synapses (Fig. 8). Incubation of nerve ending particles with preimmune serum or the IgG from the serum produced low levels of labeling following exposure to protein A/colloidal gold particle conjugates (Fig. 8A). Gold particles that accumulated over synaptosomes that were incubated with non-immune serum were distributed with approximately equal frequencies over presynaptic terminal areas and postsynaptic membranes and associated densities. On the other hand, incubation with anti-glutamate-binding protein serum or the IgG from this serum led to high levels of labeling of many nerve ending particles and especially of the associated postsynaptic densities (Fig. 8, B–E). Gold particle accumulation that signified antibody recognition of glutamate-binding protein-like immune reactivity was most frequently observed at the synaptic cleft region and, in particular, on the postsynaptic membrane (Fig. 8, D–E). Such labeling of the postsynaptic membrane was more clearly visible in unstained than in stained synaptosomes because the highly stained nature of postsynaptic densities obscured the gold-particle association with these membrane sites.

**Immunohistochemical Labeling of Neurons in Brain Slices**—A combination of autoradiographic techniques has been used to identify glutamate uptake sites that are localized at glutamate-releasing nerve endings (36, 37) and sites postsynaptic to glutamate-releasing nerve terminals labeled by glutamate

![Fig. 7. Fluorography of translation products of membrane-bound polysomes from rat brain and of translation products immunoprecipitated by specific anti-71-kDa antiserum. Cell-free translation of 121 units of membrane-bound polysomes was performed with reticulocyte lysate. The translation products were either immunoprecipitated with anti-71-kDa antiserum and subjected to SDS-PAGE and fluorography as described under “Experimental Procedures” (lane 1) or subjected to electrophoresis and fluorography without prior immunoprecipitation (lane 2).](attachment:image.png)

**Fig. 8. Immunogold labeling of sites in brain synaptosomes reacted with anti-glutamate-binding protein and preimmune serum.** Synaptosomes were isolated by Ficoll-sucrose density centrifugation, incubated with 1:5,000 dilution of either preimmune (A) or immune (B–E) serum and protein A/10-nm gold particles prior to fixation. Details of methods are described under “Experimental Procedures.” Some of the synaptosome pellets were stained with 2% uranyl acetate/lead citrate following fixation (D and E), whereas others (A–C) were left unstained in order to visualize gold particles more easily. Nerve endings contained many synaptic vesicles (SV). Postsynaptic membranes characterized by postsynaptic densities (PSD) were frequently attached to the nerve endings. A highly magnified image of a postsynaptic density is shown in B. Arrowheads on the micrographs point to sites labeled by the protein A/gold particles. The bar is equal to 100-nm length on all the micrographs except for B where it is 60 nm.
receptor ligands (38-41). These studies have demonstrated the very high enrichment of glutamate neurotransmission in the region of the hippocampus and the dentate gyrus. For this reason, we selected this region to explore the type of immunohistochemical labeling produced by the anti-glutamate-binding protein antibodies.

Colloidal gold labeling of glutamate-binding protein-like immune reactivity was used because of the discrete labeling that results from this procedure. However, in studies with light microscopy, such labeling was too weak to be detectable unless silver enhancement of the deposited gold particle label was employed. Using the techniques described under “Experimental Procedures” for protein A-gold particle labeling that was followed by silver particle deposition, we obtained selective labeling of neurons, not glial cells, in many brain regions. High levels of specific immune reactivity were observed in the hippocampus, dentate gyrus, the inner layers of cerebral cortex (Fig. 9, A and B), cerebellar cortex, and deep cerebellar nuclei. Examples of selective labeling of neurons and neuronal processes in the CA1 region of the hippocampus by the anti-serum to glutamate-binding proteins are shown in Fig. 9, C-E.

Incubation of the sections with preimmune serum did not label any neurons or neuronal processes (Fig. 9A); however, incubation with identically diluted immune serum (1:10,000 dilution) produced selective labeling of pyramidal cells in hippocampus, granule cells in dentate gyrus and the dendrites of both types of cells (Fig. 9B). Labeling of hippocampal neurons was not uniform as shown by the fact that pyramidal neurons of the CA1 region were most heavily labeled (Fig. 9B) and that even neurons of the CA1 region were not uniformly labeled (Fig. 9, B and C). The CA1 pyramidal cells near the subiculum area were more heavily labeled than neurons in adjacent CA2 regions (Fig. 9, B and C). The area of the basilar dendrites (SO) was generally less heavily labeled. Frequently, specifically labeled apical dendrites could be traced from their point of origin at the pyramidal cell layer to their location in the stratum radiatum (Fig. 9, D and E, open arrows). Some neuronal cell bodies were not labeled at all, whereas others were heavily labeled (Fig. 9E).

**DISCUSSION**

As was pointed out earlier, the 71- and 63-kDa glutamate-binding proteins may represent important physiological sites for glutamate activity in the central nervous system. We have evidence that these glutamate-binding proteins are part of a glutamate receptor complex on brain neuronal membranes, a receptor that is linked to a cation channel. An immunological and immunohistochemical approach was selected to determine the relationships between the 71- and 63-kDa proteins and to provide evidence that these proteins are located on neurons, and especially in membranes from the synaptic region of neurons in the brain.

Preparations of highly purified glutamate-binding proteins were immunogenic and the antibodies formed following immunization of rabbits with reduced and denatured binding proteins were very selective for these proteins as compared with antibody reaction with glutamate-metabolizing enzymes. Sera obtained prior to immunization of the rabbits with these proteins did not contain immune reactivity toward the glutamate-binding proteins. Since nanogram quantities of the binding proteins could be detected by use of the ELISA procedure, this method was used to characterize the distribution of the proteins in various subcellular fractions. The highest reactivity detected was associated with the fraction enriched in synaptic plasma membranes, although this enrichment was less than that determined for L-[3H]glutamate-binding entities (e.g. Ref. 9). One explanation for this apparent discrepancy between enrichment of ligand binding and immune reactivity in synaptic membranes is the possibility that the antibodies used have low levels of cross-reactivity with other proteins, such as the glutamate-metabolizing enzymes. Such cross-reactivity would increase the total immune reactivity of fractions such as the brain homogenate, where the enzyme proteins are far more abundant than the membrane-attached binding proteins.

When the antibodies were used to label proteins that had been electrophoretically separated by SDS-PAGE, both the 66–70- and 60–63-kDa proteins were labeled. The ~70-kDa protein was consistently labeled more strongly than the ~63-kDa protein. Antibodies were also raised to each of the two glutamate-binding proteins in the purified fraction, i.e. the 71- and 63-kDa proteins. Both types of antisera reacted in Western blots most strongly with the ~70-kDa protein. The protein bands at ~70 and ~63 kDa labeled by the antibodies were somewhat diffuse, which would indicate that the antibodies recognized a broad area of different molecular size proteins between 60 and approximately 73 kDa. These electrophoretic patterns of labeled proteins might be due to microheterogeneity of these membrane proteins, possibly because of differences in glycosylation.

Since we have consistently observed that the ~63-kDa protein in synaptic membranes is recognized by all antibodies raised, we assume that it is immunologically closely related.
to the ~70-kDa protein. Also, since the 63-kDa protein is very poorly stained by concanavalin A-biotin complexes, it is likely that this protein is either nonglycosylated or does not contain many mannosyl residues. We cannot offer specific reasons for the decreased reactivity on Western blots of the 63-kDa protein with all antibodies, including those that were raised specifically against this protein. It is possible that when this protein is electrotransferred to nitrocellulose sheets antibodies do not bind to it strongly because of its low level of glycosylation. An alternative explanation is that the ~70-kDa protein is more strongly immunogenic than the 63-kDa protein. Thus, even a small degree of contamination by the 71-kDa species of the 63-kDa protein samples that were used for immunization, may have produced antibodies that were most reactive with the 71-kDa protein. Finally, it is very likely that the 69-kDa protein is a proteolytic product of the 71-kDa protein and that all antibodies recognize both proteins, although they react more strongly with the 71-kDa protein in immunoblots. It should be noted, however, that all antibodies recognize and bind to the native 63-kDa protein. This was demonstrated in the immunoaffinity chromatography experiments.

The data obtained from immunoaffinity chromatography purification experiments clearly indicate that the antibodies raised can extract both the 71- and 63-kDa protein species, even though these same antibodies may be labeling only weakly the 63-kDa protein in electrotransfer experiments. The more important observation of the immunoaffinity chromatography experiments is not only that this extraction procedure removes these two proteins from solubilized synaptic plasma membranes, but that this removal leads to an approximately 60% decrease in the glutamate-binding activity of the solubilized synaptic membrane proteins. This provides strong evidence that the 71- and 63-kDa proteins are a major group of glutamate-binding entities associated with solubilized synaptic membrane proteins. The results obtained with these immunoaffinity chromatography experiments support the observations made previously by means of radiation inactivation experiments that a group of glutamate-binding proteins of approximately 75-kDa molecular size is predominant (17,18). We have recently observed that treatment of synaptic membranes with the antibodies to glutamate-binding proteins brought about inhibition of 50–60% of L-[H]glutamate-binding sites.4

If the 71- and 63-kDa proteins are a major class of glutamate-binding proteins in synaptic membranes from rodent brain, then they may be components of a predominant population of glutamate receptors. Fagg and Matus (42) have demonstrated that approximately 55% of L-[H]glutamate-binding entities in postsynaptic densities are related to the NMDA-type receptor and the remaining glutamate-binding entities are associated with quisqualate and kainate receptors. Thus, it is somewhat surprising that antibodies raised against proteins that do not interact with NMDA immunoprecipitated approximately 60% of all glutamate-binding entities in synaptic membranes. This may indicate that immunologically and structurally related proteins are part of more than one sub-type of glutamate-binding or receptor complexes in these membranes. In a recent report on the isolation of proteins that are thought to form the complex of the NMDA receptor, a protein band with estimated molecular size equal to 67 kDa was identified (43). It remains to be determined whether this 67-kDa protein is immunologically related to the 71-kDa glutamate-binding protein isolated in our laboratory.

In the in vitro mRNA translation studies using membrane-bound polysomes from whole brain homogenates, the major protein species immunoprecipitated by the anti-71-kDa antibodies was one of 66-kDa molecular size. The protein synthesized by brain polysomes in a cell-free translation system and immunoprecipitated by the antibodies is assumed to be the nonglycosylated precursor of the 71-kDa glutamate-binding protein. These studies demonstrate that glycosylation is not an absolute requirement for antibody recognition and that these antibodies can be used to isolate mRNA associated with the polysomes for future studies of expression of these proteins.

The distribution of immunohistochemical reactivity in subcellular fractions from brain tissue described under "Results" and the extraction by immunoaffinity procedures of a substantial portion of synaptic membrane glutamate-binding activity are indications that the 71- and 63-kDa proteins play a role in glutamate neurotransmitter activity in the synaptic region. Further support for this conclusion was obtained by the immunohistochemical studies. At the electron microscopic level, immune labeling of isolated, but intact, nerve ending particles was consistently observed in association with the synaptic region and, in particular, the postsynaptic membrane. There was low labeling of plasma membranes outside of the synaptic region or of presynaptic elements, such as mitochondria, synaptic vesicles, and presynaptic plasma membranes.

At the light microscopic level, antibody labeling was very specific for neuronal elements in the hippocampus, as well as other regions, especially the dendrites of neurons which are known to receive rich excitatory innervation from glutamate-releasing axon terminals (36–41). Excitatory innervation of the CA1 neurons of the hippocampus from the dentate gyrus via the mossy fibers and of the CA1 neurons via both the Schaeffer collaterals from the CA3 region and the perforant path from the entorhinal cortex is thought to be accomplished through glutamate-releasing nerve terminals (44). These nerve fibers form synapses on both apical and basilar dendrites of CA1 neurons, and on apical dendrites of CA3 neurons. The labeling of neuronal bodies is probably due to similar innervation at or near the cell body region of CA1 pyramidal neurons. In some studies that have examined the distribution of glutamate receptors by fluorographic procedures, there is fairly strong labeling in the layer of pyramidal neurons (e.g., Ref. 26), although such labeling is usually lower than that detected for the dendritic region. It is important to note also that in our studies labeling in the pyramidal cell layer was frequently around neuronal cell bodies rather than in cell bodies. A further characterization of the exact sites of neuronal labeling is currently being pursued at the electron microscopic level.

In conclusion, we believe that the antibodies that we have raised against a class of synaptic membrane glutamate-binding proteins are useful in the development of new immunooaffinity chromatography purification procedures, in the characterization of the distribution of these proteins in brain tissue at the light and electron microscopic level, in the exploration of the role of these glutamate-binding proteins in synaptic activity related to glutamate neurotransmission, and in the determination of the synthesis and insertion of these proteins into neuronal plasma membranes. We are currently using these antibodies in studies designed to probe these various levels of presence and activity of the glutamate-binding proteins. In addition, we have recently employed these antibodies in screening cDNA libraries that express brain proteins and have identified a few clones that produce strongly immunoreactive proteins. The approach of screening with

4 M. J. Eaton and E. K. Michaelis, unpublished observations.
antibodies and with cDNA probes synthesized on the basis of amino acid sequences from the 71-kDa protein will yield new information about the structure and function of these proteins.

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