# Purification and biochemical characterization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate-sensitive L-glutamate receptors of pig brain

Yen-Chung CHANG*, Tzong-Yuan WU, Bee-Fen LI, Ling-Hao GAO, Chun-I LIU and Chia-Lin WU
Institute of Life Science, National Tsing Hua University, Hsinchu, Taiwan 30043, Republic of China

Two preparations of glutamate receptors were purified from the synaptic junctions of pig brain by a combination of detergent solubilization, anion-exchange chromatography, wheat-germ agglutinin affinity chromatography and sedimentation through sucrose gradients. These preparations were enriched in specific L-[$^3$H]glutamate binding activity (> 5000 pmol of glutamate binding sites/mg of protein), and the rank order of ligand affinity for binding to these preparations was: quisqualate > 6-cyano-7-nitroquinoxaline-2,3-dione > α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) > L-glutamate > kainate ≫ *N*-methyl-D-aspartate ≈ L-2-amino-4-phosphonobutyrate. SDS/PAGE analysis revealed that more than 80 % of the protein in either of these preparations appeared as a single protein band of 106 kDa. Two-dimensional gel electrophoresis further revealed that these 106 kDa proteins consisted of a series of acidic proteins which were recognized by antibodies against rat AMPA receptor subunits. These 106 kDa proteins were also recognized by wheat-germ agglutinin and concanavalin A; in addition, peptide N-glycosidase F treatment of these preparations decreased their size to 99 kDa. Our results suggest that the putative glutamate receptors isolated here are likely to belong to the AMPA subtype of glutamate receptors in pig brain. Using the purification procedure reported here, 5 μg of AMPA receptor proteins can be isolated from 250 g of pig brain tissue.

## INTRODUCTION

L-Glutamate is generally considered to be the principal excitatory neurotransmitter in the mammalian nervous system. The neuronal responses elicited by L-glutamate are mediated by three broad subtypes of ionotropic receptors, i.e. *N*-methyl-D-aspartate (NMDA)-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and kainate-preferring receptors, and by a family of metabotropic receptors [1,2]. L-Glutamate and its receptors are not only essential for conveying excitatory information across synapses but are also involved in brain development and many higher functions of the brain, e.g. learning and memory [3,4].

The AMPA-preferring glutamate receptor mediates fast excitatory synaptic transmission in the central nervous system of mammals. The cDNAs coding for AMPA receptor subunits, i.e. GluR1–4, have been obtained [5,6]. GluR1–4 subunits are polypeptides of around 900 residues, with approx. 70 % amino acid identity between them. Recent investigations have indicated that AMPA receptor subunits have a topology comprising three transmembrane segments [7–10], although a topology comprising four or five transmembrane segments has also been proposed [11,12]. The extracellular ligand binding domain and channel-lining segments of these subunits are proposed to resemble those of bacterial periplasmic amino acid binding proteins and of voltage-gated channels respectively [9,10]. In the brain, an intact AMPA receptor molecule is considered to be a hetero-oligomeric assembly [13–15]; in addition, its functions are under the influence of protein kinase activities and glycosylation [16–19].

Glutamate receptors have been purified from the central nervous system of *Xenopus laevis* [20] and from crab muscle [21]. In addition, three smaller glutamate receptors, also known as kainate binding proteins, have been purified from the brains of goldfish, frog and chick [22–24]. Many attempts have been made to purify different glutamate receptors from mammalian brain tissue. Glutamate binding proteins were solubilized from the brains of different animals [25–31], and three such proteins [25–27,30] were purified to apparent homogeneity. A spider toxin (Jorotoxin) binding protein was also purified from rat hippocampus [32]. However, because these proteins did not exhibit pharmacological properties similar to those of a synaptic glutamate receptor, the identities of these proteins remained to be determined. Ly and Michaelis [33] reported the isolation of a set of glutamate binding proteins which acted as ion channels with some properties similar to those of NMDA receptors when reconstituted into lipid membranes. AMPA receptors were solubilized and partially purified by workers in several laboratories [34–38]. A preparation containing high- and low-affinity [$^3$H]kainate binding sites, which might also include AMPA receptors, was solubilized from rat brain and partially purified by gel filtration chromatography and lectin affinity chromatography [35]. [$^3$H]AMPA binding sites were also solubilized from rat brain and partially purified by chromatography and sedimentation methods [36,37] or by immunoprecipitation with antibodies against AMPA receptor subunits [15,38].

Our previous work indicated that AMPA-, quisqualate- and kainate-sensitive L-[$^3$H]glutamate binding sites could be solubilized from pig brain synaptic junctions by Triton X-114 plus KCl; in addition, these soluble binding sites could be partially purified by conventional biochemical means [34]. In the present paper, we extend that work and report a procedure for purifying the AMPA/kainate-sensitive L-glutamate receptors from pig brain

Abbreviations used: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; WGA, wheat-germ agglutinin; conA, concanavalin A; PNGase F, peptide N-glycosidase F; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; L-APB, L-2-amino-4-phosphonobutyrate; 2D, two-dimensional; PVDF, poly(vinylidene difluoride); TBS, Tris-buffered saline; HRP, horseradish peroxidase; DAB, 3,3′-diaminobenzidine tetrachloride.

* To whom correspondence should be addressed.

to apparent homogeneity. The biochemical properties of the isolated receptors were also studied and are reported here.

## MATERIALS AND METHODS

### Materials

L-[$^3$H]Glutamic acid (50–60 Ci/mmol), [$^3$H]AMPA (56.6 Ci/mmol) and [$^3$H]kainic acid (58.0 Ci/mmol) were purchased from Du Pont–NEN. Subtype-specific antibodies to rat AMPA receptor subunits GluR1, GluR2/3 and GluR4, and to rat NMDA receptor subunits NR1 and NR2A/B, were obtained from Chemicon International Inc. (Temecula, CA, U.S.A.). Antibodies to rat kainate-selective glutamate receptor subunits GluR6 and GluR7 were obtained from Upstate Biotechnology Inc. (New York, NY, U.S.A.). PMSF, Hepes, sucrose, wheat-germ agglutinin (WGA), concanavalin A (conA), CNBr-activated Sepharose 4B, pepstatin A, peptide N-glycosidase F (PNGase F), *N*-octyl $\beta$-D-glucoside, *N*-acetylglucosamine, chemically cross-linked glycogen phosphorylases and Trizma were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Quisqualic acid, kainic acid, NMDA, AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and L-2-amino-4-phosphonobutyrate (L-APB) were obtained from Research Biochemicals. DEAE-Sepharose CL-6B was purchased from Pharmacia. Other chemicals were obtained from Merck-Shuchardt Chemical Co. Water used in this study was purified by a Mini-Q system (Millipore), and buffer solutions were sterilized either by autoclaving or by filtering through a filter membrane (0.22 $\mu$m pore size).

### Procedures for isolation and solubilization of synaptic junctions

Synaptic junctions were prepared from fresh pig brain as described previously [34]. Synaptic junctions (protein concentration 2.5 mg/ml) were solubilized by incubation with Triton X-114 (1 %, v/v) and KCl (0.15 M) on ice for 1 h. Following clarification by centrifugation (150000 ***g*** at 4 °C for 1 h), the resulting supernatant was dialysed against buffer A (50 mM Tris/acetate, 0.1 mM EDTA and 0.1 mM EGTA, pH 7.4) plus Triton X-114 (1 %) for 5 h with three changes of the dialysing buffer. The dialysed sample was used as the starting material in further experiments. Protein concentrations of synaptic junctions were determined by the Lowry method [39]. Protein concentrations of the solubilized preparations and of DEAE-chromatography-purified samples were determined by the bicinchoninic acid method [40]. Protein concentrations of samples purified by WGA affinity chromatography and sucrose-density-gradient centrifugation were determined by quantifying the total amount of amino acids after acid hydrolysis using an automated amino acid analyser (Waters).

### Anion-exchange chromatography and preparation of WGA affinity columns

Anion-exchange chromatography was carried out at 4 °C using a DEAE-Sepharose CL-6B column (2.5 cm × 32 cm) which had been pre-equilibrated with buffer A/Triton X-114 (1 %, v/v). WGA–Sepharose 4B was generated by coupling WGA to CNBr-activated Sepharose 4B according to the procedure of Cuatrecasas and Parikh [41]. Before use, the WGA-containing column was washed with 100 ml of buffer A/Triton X-114 (1 %).

### Sucrose-density-gradient centrifugation

An SW-28 rotor ($r_{av.}$ 11.82 cm; Beckman) was used to perform sucrose-density-gradient centrifugation according to the procedures described previously [42]. Sucrose gradients in buffer A/0.1 % Triton X-114 were prepared with a gradient maker (Jule) from two sucrose stock solutions containing 5 % and 30 % (w/v) sucrose. Gradients were maintained at 4 °C for 4–6 h before use. Samples were then applied on top of the gradients, and gradients were centrifuged at 5 °C for 30 h. Following centrifugation, sample fractions were removed from the bottom of the centrifuge tube.

### Electrophoresis analysis

SDS/PAGE was performed by the method of Laemmli [43] or the method of Weber and Osborn [44]. Two-dimensional (2D) SDS/PAGE was carried out with a mini-gel apparatus (Protean II; Bio-Rad) according to the procedures described by Dunbar et al. [45]. For separation in the first dimension, the pH gradient was formed of a mixture containing 3 vol. of Ampholyte with a pH range of 6–8 and 1 vol. of Ampholyte with a pH range of 3–10. 2D SDS/PAGE standards (Bio-Rad) were always included in the sample as internal standards. Proteins separated by one- or two-dimensional SDS/PAGE were detected either by Coomassie Blue or by the silver staining method [46].

### Immunoblotting analysis

Proteins were first separated by one- or two-dimensional SDS/PAGE and then transferred to a poly(vinylidene difluoride) (PVDF) membrane. The resultant membrane was incubated with Tris-buffered saline (TBS; 100 mM Tris, pH 7.4, 100 mM NaCl) containing BSA (3 %, w/v) and non-dairy creamer (3 %, w/v) at room temperature for 1 h. The membrane was then sequentially incubated with primary antibody in TBS at 4 °C overnight, then with a secondary antibody conjugated to horseradish peroxidase (HRP) at room temperature for 1 h, and finally with the substrate solution containing 3,3′-diaminobenzidine tetrachloride (DAB; 0.02 %, w/v) and $H_2O_2$ (1:10000, v/v) in citrate (100 mM) at pH 5.1. In some cases, an ECL kit (Amersham) was used for detection. For the Western blots containing proteins separated by 2D SDS/PAGE, membranes were first stained by the Protogold method (British BioCell Int.) for detection of internal standards, leaving receptor proteins unstained because of their being present in lower amounts. Membranes were next processed for immunostaining. Proteins on gels were quantified in a densitometer (Molecular Dynamics).

### Interactions with lectins and enzymic cleavage of polysaccharide moieties

Western blots containing S1 and S2 samples (see the Results section) were first blocked by TBS containing BSA (3 %) and non-dairy creamer (3 %), and then incubated with HRP-conjugated conA or HRP-conjugated WGA at 4 °C overnight. The resultant blots were washed with TBS three times and then incubated with the substrate solution containing DAB and $H_2O_2$. Deglycosylation reactions were performed following the procedures described by Blackstone et al. [37]. Samples were first denatured with 1 % SDS at 95 °C for 1 min. *N*-Octyl $\beta$-D-glucoside (final concentration 2 %, w/v) was added to dilute the SDS to 0.1 %. PNGase F (final concentration 0.6 unit/ml), PMSF (0.25 mM), EDTA (1 mM), EGTA (1 mM) and pepstatin A (40 $\mu$g/ml) in PBS were added to the sample solution. The reaction mixture was incubated at 37 °C for 20 h. A second dose of PNGase F (0.6 unit/ml) was then added to the mixture, which was incubated for another 6 h. Next, the sample was concentrated by lyophilization for SDS/PAGE and immunoblotting analyses.

### Radioligand binding assay

[$^3$H]Kainate and L-[$^3$H]glutamate binding to receptors was quantified by the poly(ethylene glycol)–immunoglobulin precipitation method as described previously [34]. [$^3$H]AMPA binding was assayed by a similar method, except that KSCN (0.1 M) was included in the reaction mixture. Non-specific L-[$^3$H]glutamate, [$^3$H]kainate and [$^3$H]AMPA binding sites were determined in the presence of unlabelled L-glutamate (0.5 mM), kainate (0.5 mM) and AMPA (0.5 mM) respectively. Data for L-[$^3$H]-glutamate binding were analysed using the programs Kinetics, EBDA, Ligand and Lowry from Elsevier–Biosoft.

## RESULTS

### Solubilization and purification of AMPA/kainate-sensitive glutamate receptors from pig brain

Synaptic junctions were enriched with both NMDA and non-NMDA receptors [34,47]. We reported previously that L-[$^3$H]-glutamate binding sites solubilized from pig brain synaptic junctions by Triton X-114 and KCl were sensitive to ligands of non-NMDA receptors, i.e. quisqualate, kainate and AMPA, but were not sensitive to NMDA or L-APB [34,42]. These results indicated that Triton X-114/KCl treatment released non-NMDA receptors, but not NMDA receptors, from synaptic junctions. In the present study, glutamate receptors in synaptic junctions, in samples solubilized from synaptic junctions by Triton X-114/KCl and in insoluble material after Triton X-114/KCl treatment, were analysed by immunoblotting with antibodies against rat AMPA, NMDA and kainate receptors. It was found that antibodies to rat GluR1 and GluR2/3 subunits specifically recognized a single protein band of 106 kDa, and that anti-(rat NR1) and anti-(rat NR2A/B) antibodies specifically recognized protein bands of 120 kDa and 170–180 kDa respectively in synaptic junctions (Figure 1). Anti-(rat GluR6/7) antibodies specifically detected a protein of approx. 120 kDa (Figure 1). These results were consistent with previous observations that antibodies to rat GluR1, GluR2/3, NR1, NR2A/B and GluR6/7 recognized rat brain proteins of 108, 108, 120, 172 and 118 kDa respectively in immunoblotting analyses [37,48–50]. Nevertheless, anti-(rat GluR4) antibody did not detect any synaptic junction proteins even at concentrations 10 times higher than those of antibodies against rat GluR1 or GluR2/3 used in immunoblotting experiments (results not shown). It is possible that in the present study the synaptic junctions were prepared from brain tissue containing relatively low levels of GluR4 subunits [5], or that pig brain AMPA receptor subunits, whose amino acid sequences are unknown, do not contain the epitope recognized by anti-(rat

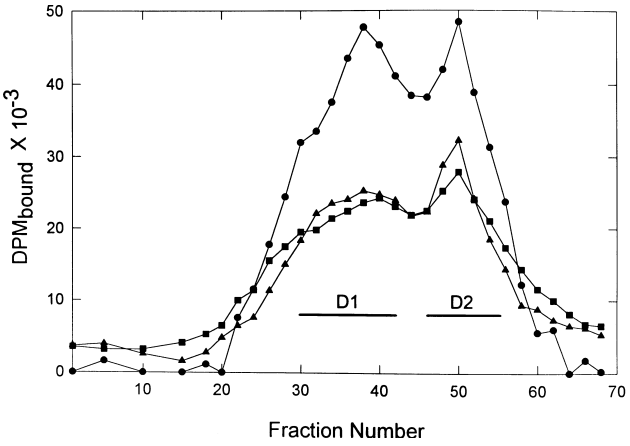


**Figure 2 DEAE-Sepharose CL-6B chromatography of a Triton X-114-solubilized synaptic junction preparation**

A solubilized preparation (70–80 ml) was applied to a DEAE-Sepharose CL-6B column (2.5 cm × 32 cm). The column was washed with 50 ml of buffer A containing Triton X-114 (1%, v/v) and subsequently with a 0–0.5 M linear NaCl gradient in the same solution (total volume 300 ml). Fractions of 3 ml were collected. Samples of 200 μl of each fraction were incubated with 230 nM L-[$^3$H]glutamate, 20 nM [$^3$H]AMPA or 8.6 nM [$^3$H]kainate at 4 °C for 1 h for assaying L-[$^3$H]glutamate (●), [$^3$H]AMPA (■) and 10 × [$^3$H]kainate (▲) binding [given as $10^{-3} \times {}^3$H bound (d.p.m.)]. Each point represents the mean of three determinations. The results are from a single experiment representative of six. Fractions under the horizontal lines were pooled and designated as samples D1 and D2.

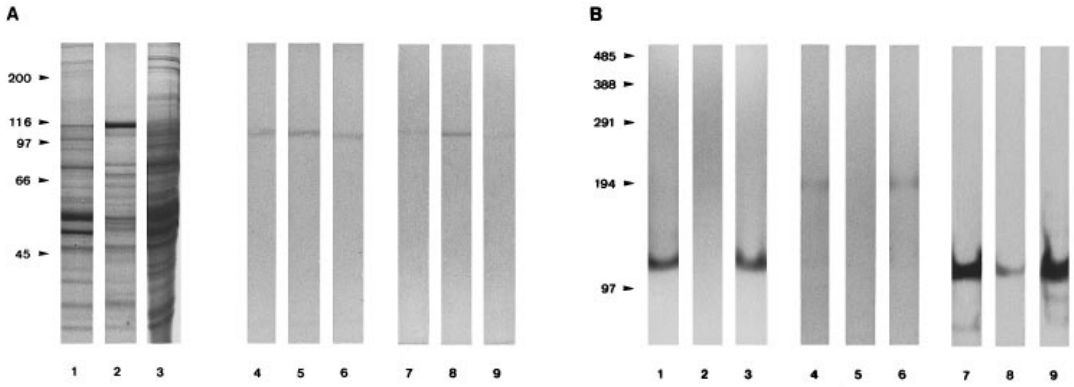


**Figure 1 Triton X-114 and KCl treatment selectively solubilizes AMPA receptors from synaptic junctions**

Synaptic junctions were incubated with 1% (v/v) Triton X-114 and 0.15 M KCl on ice for 1 h. The sample was then centrifuged at 150000 ***g*** at 4 °C for 1 h. (**A**) Synaptic junctions (10 μg of protein; lanes 1, 4 and 7), the resultant supernatant (10 μg of protein; lanes 2, 5 and 8) and the pellets (40 μg of protein; lanes 3, 6 and 9) were subjected to SDS/PAGE analysis (in 9% gels, by the method of Laemmli [43]). Proteins in the gel were detected by Coomassie Blue (lanes 1–3), or were transferred to a PVDF membrane and immunostained with anti-(rat GluR1) antibodies (lanes 4–6) or anti-(rat GluR2/3) antibodies (lanes 7–9). Indicated molecular size standards are 200, 116, 97, 66 and 45 kDa. (**B**) Synaptic junctions (10 μg of protein; lanes 1, 4 and 7), solubilized sample (5 μg of protein; lanes 2, 5 and 8) and pellets (10 μg of protein; lanes 3, 6 and 9) were subjected SDS/PAGE analysis (in 4.5% gels, by the method of Weber and Osborn [44]) and immunoblotted with anti-(rat NR1) (lanes 1–3), anti-(rat NR 2A/B) (lanes 4–6) or anti-(rat GluR6/7) antibodies. Indicated molecular mass standards are 485, 388, 291, 194 and 97 kDa. DAB and $H_2O_2$ were used in the immunoblotting analysis of lanes 1–6, and an ECL kit was used in the immunoblotting analysis of lanes 7–9.

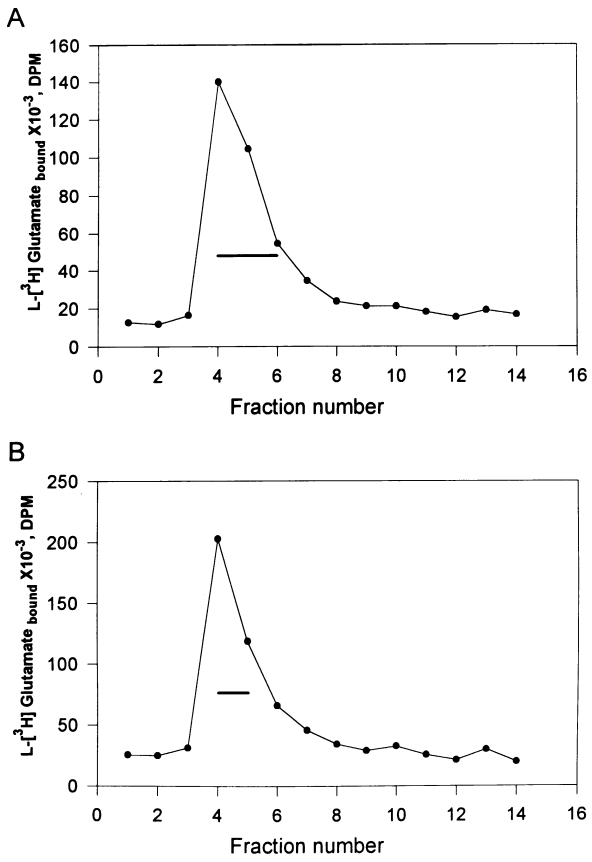


**Figure 3 WGA–Sepharose 4B chromatography of DEAE-Sepharose-CL-6B-purified samples**

Samples D1 (**A**) and D2 (**B**) were circulated on WGA–Sepharose CL-4B columns (bed volume 10 ml) at 4 °C overnight. The column was washed with 50 ml of buffer A containing Triton X-114 (1%, v/v). Subsequently, the column was washed with the same solution plus 0.4 M *N*-acetylglucosamine, and fractions of 2 ml were collected. Samples of 200 $\mu$l of each fraction were removed for assaying L-[$^3$H]glutamate binding (d.p.m.). Each point represents the mean of three determinations. The results are from a single experiment representative of six. Fractions under the horizontal lines in (**A**) and (**B**) were pooled and designated as samples W1 and W2 respectively.

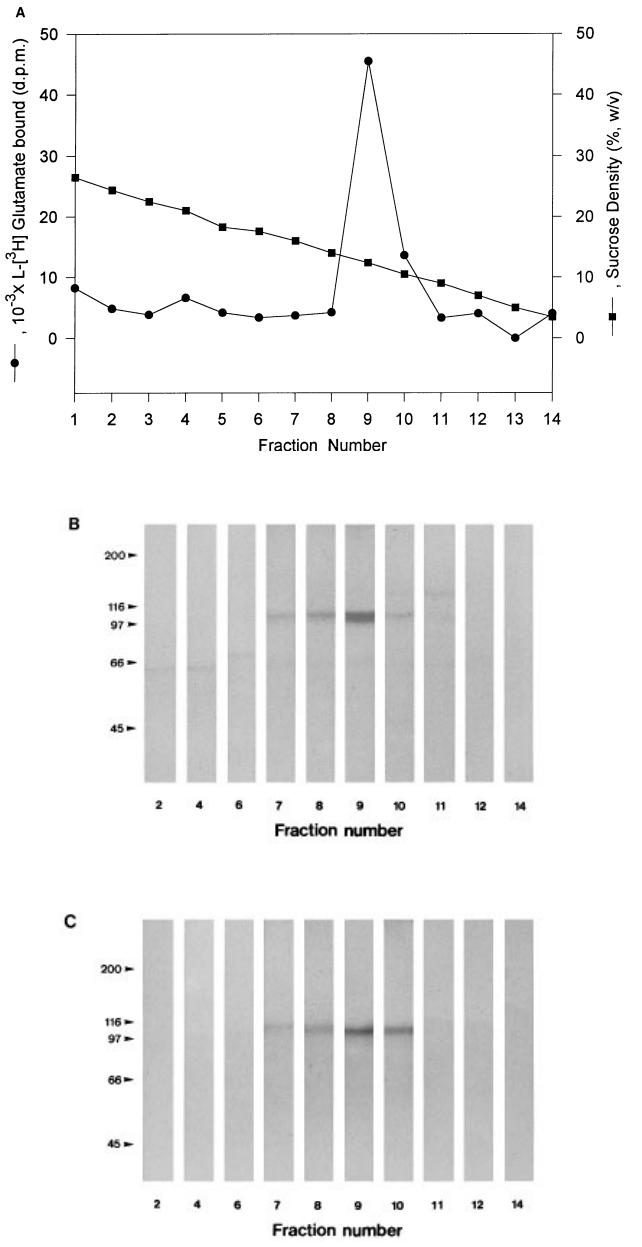


**Figure 4 Purification of AMPA receptors in WGA–Sepharose 4B column-purified sample W1 by sucrose-density-gradient centrifugation**

Sample W1 (4 ml) was applied to a continuous sucrose density gradient (4–26%) in buffer A containing Triton X-114 (0.1%, v/v). Gradients were then centrifuged at 83000 ***g*** and 5 °C in an SW-28 rotor for 30 h. Sample fractions of 2 ml were then collected from the bottom of the gradients. (**A**) Samples of 200 $\mu$l of each fraction were removed and incubated at 4 °C for 1 h with 230 nM L-[$^3$H]glutamate for assaying L-[$^3$H]glutamate binding (●). Each value represents the mean of three determinations. The sucrose density of each fraction (■) was determined using a refractometer. The migration velocity of the major binding site peak was $1.1 \pm 0.2$ mm/h ($n = 3$). (**B**) Samples of 100 $\mu$l of each fraction were concentrated and subjected to SDS/PAGE analysis (in 9% gels, by the method of Laemmli [43]). The resultant gel was stained by the silver staining method. (**C**) Samples of 2.5 $\mu$l of each fraction were subjected to SDS/PAGE analysis and immunoblotted with anti-(rat GluR2/3) antibodies. An ECL kit was used for detection. Indicated molecular size standards are 200, 116, 97, 66 and 45 kDa.

GluR4) antibody. It was found that, following treatment with Triton X-114 and KCl, a large proportion of the 106 kDa protein recognized by anti-(rat GluR1) and anti-(rat GluR2/3) antibodies was solubilized from synaptic junctions. In contrast, proteins recognized by anti-(rat NMDA receptor) antibodies were not solubilized by Triton X-114/KCl, and only a small portion of the 120 kDa proteins recognized by anti-(rat kainate receptor) antibodies was released from the synaptic junctions (Figure 1). These results were consistent with our previous observations [34,42].

Triton X-114/KCl-solubilized samples were applied to a column of DEAE-Sepharose CL-6B (Figure 2). The binding sites for L-[$^3$H]glutamate, [$^3$H]kainate and [$^3$H]AMPA were co-eluted in two major peaks by a gradient of 0–0.5 M NaCl (Figure 2). The fractions under the two major peaks were pooled and designated as the D1 and D2 samples. Next, each of the D1 and D2 samples was circulated overnight at 4 °C on a WGA affinity column (bed volume 10 ml). L-[$^3$H]Glutamate binding sites were eluted from the column by a solution containing 50 mM Tris/acetate, pH 7.4, 0.2% Triton X-114 and 0.4 M *N*-acetylglucosamine (Figure 3). Fractions under the peaks in Figure 3 were pooled and designated as the W1 and W2 samples. These samples were then applied to linear sucrose density gradients and centrifuged at 83000 ***g*** at 5 °C for 30 h. L-[$^3$H]Glutamate binding sites in the W1 sample were found to migrate in the gradient as a single peak (Figure 4A). SDS/PAGE analysis of the resulting

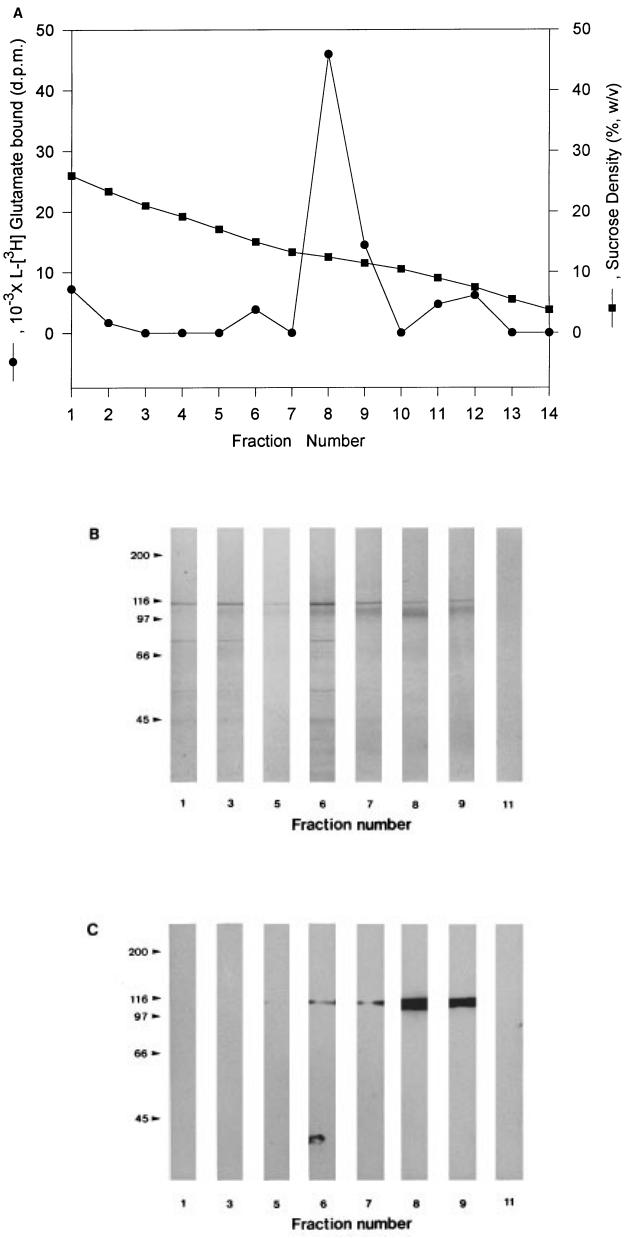


**Figure 5 Purification of AMPA receptors in WGA–Sepharose 4B-purified sample W2 by sucrose-density-gradient centrifugation**

Sample W2 (4 ml) was applied to a continuous sucrose density gradient (4–26%) in buffer A containing Triton X-114 (0.1%, v/v). Gradients were centrifuged at 83000 ***g*** and 5 °C for 30 h in an SW-28 rotor. Sample fractions of 2 ml were then collected from the bottom of gradients. (**A**) Samples of 200 μl of each fraction were removed and incubated at 4 °C for 1 h with 230 nM L-[$^3$H]glutamate for assaying L-[$^3$H]glutamate binding (●). Each value represents the mean of three determinations. The sucrose density of each fraction (■) was determined using a refractometer. The migration velocity of the major binding site peak was $1.2 \pm 0.1$ mm/h ($n = 3$). (**B**) Samples of 100 μl of each fraction were concentrated and subjected to SDS/PAGE analysis (in 9% gels, by the method of Laemmli [43]). The resultant gel was stained by the silver staining method. (**C**) Samples of 100 μl of each fraction were subjected to SDS/PAGE analysis and subsequently immunoblotted with anti-(rat GluR 2/3) antibodies. Western blots were developed with DAB and $H_2O_2$. Indicated molecular size standards are 200, 116, 97, 66 and 45 kDa.

fractions revealed enrichment of a 106 kDa protein in the fractions containing high L-[$^3$H]glutamate binding activity (Figure 4B). High GluR2/3 immunoreactivity, as indicated by the intensity of a 106 kDa band immunostained by anti-(rat GluR2/3) antibodies, was also found in these fractions (Figure 4C). Analysis of the fractions obtained on sucrose-density-gradient centrifugation analysis of the W2 sample yielded similar results. Thus L-[$^3$H]glutamate binding sites migrated as a single peak (Figure 5A), and an enrichment of a 106 kDa protein (Figure 5B) and a parallel enrichment in GluR2/3 immunoreactivity, as indicated by the intensity of a 106 kDa band immunostained by anti-(rat GluR2/3) antibodies, was noted in the fractions containing high L-[$^3$H]glutamate binding activity (Figure 5C). In addition, SDS/PAGE analysis also revealed the presence of a minor 110 kDa protein in the fractions containing high L-[$^3$H]glutamate binding activity (Figure 5B). This 110 kDa protein was not recognized by anti-(rat GluR2/3) antibodies (Figure 5C). Fractions under the peaks in Figures 4(A) and 5(A) were pooled and designated as S1 and S2 samples respectively. The amount of total protein, the total number of L-[$^3$H]glutamate binding sites and the $B_{max}$ value at each step in the purification process to give S1 and S2 are summarized in Table 1.

### SDS/PAGE and immunoblotting analyses of S1 and S2

SDS/PAGE analysis of samples S1 and S2 was performed (Figure 6A). Densitometric scans of lanes 1 and 2 of Figure 6(A) revealed that 85% and 84% of the protein in S1 and S2 appeared as a single 106 kDa band (Figure 6E). The 110 kDa protein in freshly prepared S2 sample became barely detectable by SDS/PAGE analysis after the sample was kept on ice for several days (Figure 6A), indicating that this 110 kDa protein is labile. The 106 kDa protein bands found in the S1 and S2 samples were recognized by anti-(rat GluR1) and anti-(rat GluR2/3) antibodies (Figures 6A), but not by anti-(rat GluR4) antibodies (results not shown). These results imply that the proteins of 106 kDa in the S1 and S2 samples are immunologically related to rat AMPA receptor subunits.

### The 106 kDa proteins are glycoproteins

Treatment of samples S1 and S2 with PNGase F decreased the size of the major 106 kDa proteins to 99 kDa; in addition, the resultant deglycosylated products were still recognized by anti-(rat GluR2/3) antibodies (Figure 6B). The results indicate that these 106 kDa proteins contain N-linked carbohydrate moieties with masses of 6–7 kDa. The 106 kDa proteins of samples S1 and S2 were recognized by WGA or conA conjugated with HRP (Figure 6C). A 138 kDa protein recognized by WGA and conA was also present in the S1 and S2 samples. This protein was barely detectable in one-dimensional SDS/PAGE (Figure 6A) and appeared as very faint spots (with isoelectric points below 5.0) in 2D SDS/PAGE analysis (Figure 7). These results indicate that these 138 kDa proteins are likely to be highly glycosylated and, therefore, not easily detectable by the silver staining method.

### Presence of kainate receptor subunits in S2 but not in S1

Since a small proportion of the 120 kDa proteins recognized by anti-(rat GluR6/7) antibodies was solubilized from the synaptic junction by Triton X-114 and KCl (Figure 1), we examined whether these proteins were present in the isolated S1 and S2 samples. Immunoblotting analyses revealed that kainate receptor subunits were enriched in the D2 sample and co-purified with the AMPA receptors in the S2 sample (Figure 6D). Nevertheless, the co-purified kainate receptor subunits were only detectable in the Western blot and not in the silver-stained gels (Figure 6A). Similar to the labile 110 kDa protein found in the S2 sample, the

**Table 1 Purification of AMPA- and kainate-sensitive glutamate receptors from pig brain**

Data are means ± S.D. from two preparations. For each of the samples obtained from a preparation, binding was examined with at least eight concentrations of L-glutamate, ranging from 0.15 to 100 $\mu$M. The dissociation constants ($K_d$) of L-glutamate of samples SJ, solubilized SJ, D1, D2, W1, W2, S1 and S2 were 0.65 ± 0.05, 1.10 ± 0.20, 0.80 ± 0.10, 1.35 ± 0.35, 0.60 ± 0.01, 1.45 ± 0.35, 0.79 ± 0.29 and 0.96 ± 0.45 $\mu$M respectively. Total binding sites is calculated as $B_{max}$ × total protein. Recovery of activity is the $B_{max}$ value of the sample expressed as a percentage of the $B_{max}$ of sample SJ.

| Sample | Total protein (mg) | $B_{max}$ (pmol/mg) | Total binding sites (pmol) | Recovery of activity (%) | Enrichment (fold) |
|---|---|---|---|---|---|
| SJ | 198.5 ± 50.0 | 19.9 ± 0.6 | 3954.1 ± 1002.2 | 100 | 1 |
| Solubilized SJ | 58.0 ± 10.0 | 25.7 ± 1.6 | 1492.3 ± 273.1 | 37.7 ± 11.8 | 1.3 |
| DEAE-Sepharose | | | | | |
| D1 | 12.2 ± 1.7 | 43.0 ± 3.2 | 524.5 ± 83.1 | 13.3 ± 4 | 2.2 |
| D2 | 20.0 ± 3.9 | 29.4 ± 0.8 | 588.0 ± 115.7 | 14.9 ± 4.8 | 1.5 |
| WGA–Sepharose CL-4B | | | | | |
| W1 | 0.39 ± 0.08 | 574.8 ± 45.5 | 224.2 ± 49.3 | 5.7 ± 1.9 | 28.9 |
| W2 | 0.62 ± 0.19 | 583.2 ± 76.9 | 361.6 ± 120.6 | 9.1 ± 3.8 | 29.3 |
| Sucrose-density-gradient centrifugation | | | | | |
| S1 | 0.0026 ± 0.0009 | 5387 ± 822 | 14.0 ± 5.3 | 0.35 ± 0.02 | 270.4 |
| S2 | 0.0029 ± 0.0002 | 6068 ± 1785 | 17.6 ± 5.3 | 0.45 ± 0.02 | 304.6 |

120 kDa kainate receptor subunits in the S2 sample were labile, and most were degraded into proteins of 60–70 kDa. In contrast, anti-(rat GluR6/7) antibodies did not detect any proteins in the S1 or D1 samples (Figure 6D).

### 2D SDS/PAGE analysis of S1 and S2

Samples S1 and S2 were further analysed by 2D SDS/PAGE (Figure 7). This analysis revealed that the 106 kDa proteins in both S1 and S2 consisted of a series of proteins with various isoelectric points, ranging between 5.0 and 6.6 (Figures 7C and 7F). The presence of these proteins was unlikely to be an artifact because the internal standards (2D SDS/PAGE standards; Bio-Rad) in the same gel were well separated. The relative proportions of these 106 kDa proteins in the S1 and S2 samples appeared to be similar. Western blotting analysis further indicated that each of the 106 kDa proteins in S1 and S2 was recognized by anti-(rat GluR1) and anti-(rat GluR2/3) antibodies (Figures 7A, 7B, 7D and 7E). 2D SDS/PAGE and immunoblotting analyses of S1 and S2 after deglycosylation with PNGase F, and of the W1 and W2 samples after dephosphorylation with type II alkaline phosphatase (5 $\mu$g/ml, 4 h at 37 °C), were also performed. The immunostaining patterns of the deglycosylated S1 and S2 samples were nearly identical, consisting of more than eight 99 kDa spots which were recognized by anti-(rat GluR2/3) antibodies and with different isoelectric points in the range 5.0–6.6 (results not shown). The immunostaining patterns of the dephosphorylated W1 and W2 samples were also similar, consisting of eight major 106 kDa spots which were recognized by anti-(rat GluR2/3) antibodies and with various isoelectric points ranging between 5.0 and 6.6 (results not shown). Taken together, these results indicate that the number of AMPA receptor subunits separated by isoelectric focusing was not significantly altered by deglycosylation or dephosphorylation, although the isoelectric points of the separated spots were slightly altered by these treatments.

### Analysis of the L-[$^3$H]glutamate binding activities of S1 and S2

Scatchard transformation of the L-[$^3$H]glutamate binding data (at least eight L-glutamate concentrations ranging between 0.1 and 100 $\mu$M were examined with samples purified from three different synaptic junction preparations) indicated that both S1 and S2 contained a single population of L-[$^3$H]glutamate binding sites. The $K_d$ and $B_{max}$ values for L-[$^3$H]glutamate binding in the S1 sample were 0.79 ± 0.29 $\mu$M and 5387 ± 822 pmol/mg of protein respectively, and the corresponding values for the S2 sample were 0.96 ± 0.45 $\mu$M and 6068 ± 1785 pmol/mg of protein respectively. The Hill coefficients for L-[$^3$H]glutamate binding to S1 and S2 were 1.0 ± 0.1 and 1.1 ± 0.2 respectively. Various glutamatergic drugs were found to displace L-[$^3$H]glutamate bound to S1 and S2. The inhibition constants were determined and are listed in Table 2. The rank order of their affinities was: quisqualate > CNQX > AMPA > L-glutamate > kainate ≫ NNMDA ≈ L-APB. This order of agonist potency is identical to that for homomeric cloned AMPA receptors: quisqualate > AMPA > L-glutamate > kainate [5].

## DISCUSSION

In this study, two samples enriched in putative AMPA/kainate-sensitive L-glutamate receptors, designated as samples S1 and S2, were isolated from pig brain. SDS/PAGE analysis revealed that both S1 and S2 contained a major protein band at 106 kDa. The 106 kDa proteins of these samples resembled rat AMPA receptor subunits in their apparent molecular sizes [15,37], in their sizes after PNGase F treatment [37,51], and in their immunoreactivities with anti-(rat AMPA) receptor antibodies. Furthermore, the pharmacological properties of the S1 and S2 samples closely resembled those of the reported AMPA receptor [5,52]. We thus conclude that the 106 kDa proteins in S1 and S2 samples represent AMPA receptor subunits in pig brain.

SDS/PAGE analysis of the S1 and S2 samples revealed that the major 106 kDa protein accounted for more than 80 % of the protein in these samples (Figure 6D). The purities of the S1 and S2 samples can also be calculated theoretically from the maximum binding site numbers (the $B_{max}$ values) of these samples (Table 1). Assuming that each 106 kDa AMPA receptor subunit contains a single L-glutamate binding site, 1 mg of protein of the S1 sample ($B_{max}$ = 5378 pmol/mg) is calculated to contain 0.57 mg of AMPA receptor subunits (5378 pmol × 106000 g/mol). By a similar calculation, 1 mg of protein of the S2 sample ($B_{max}$ = 6068 pmol/mg) is calculated to contain 0.64 mg of AMPA receptor subunits. In this study, the number of glutamate binding sites was quantified by the poly(ethylene glycol) precipitation method. Because it is uncertain whether all the binding sites are being measured, the theoretically calculated purities of the S1

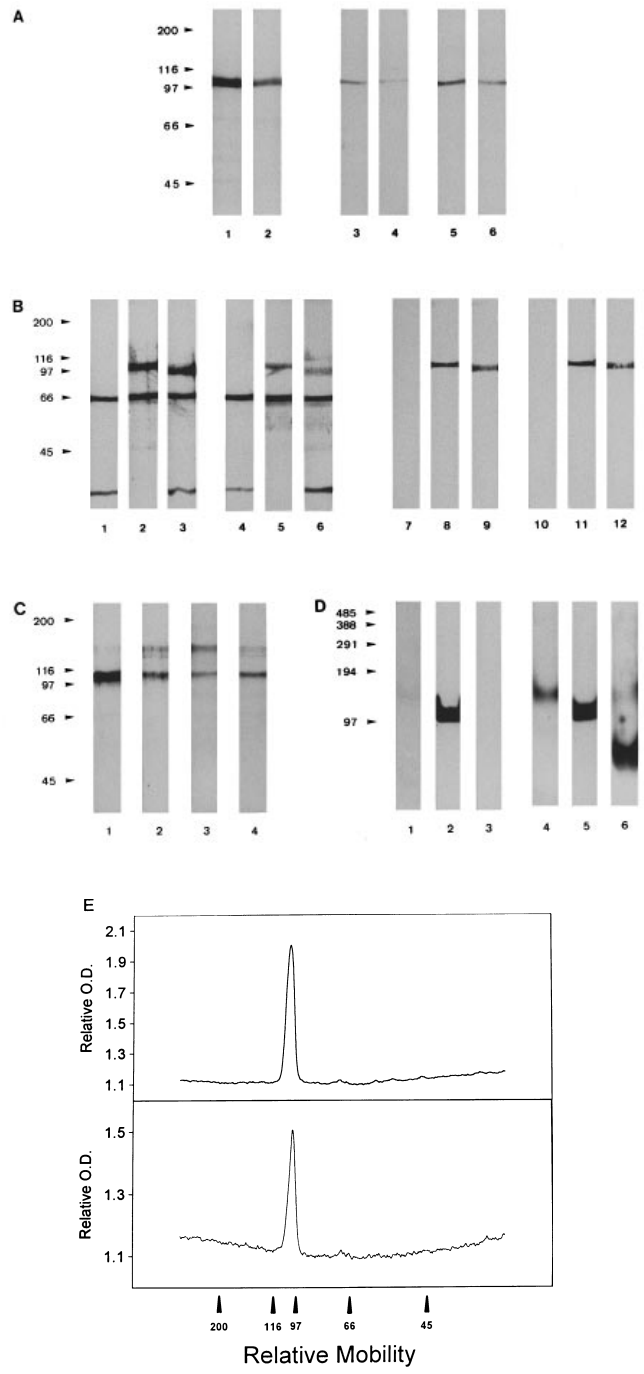


**Figure 6 Biochemical characterization of proteins in samples S1 and S2**

(**A**) SDS/PAGE and immunoblotting analysis of S1 and S2. Samples S1 (150 ng of protein; lanes 1, 3 and 5) and S2 (80 ng; lanes 2, 4 and 6) were subjected to SDS/PAGE analysis (in 9% gels). Proteins in the resultant gel were detected by the silver staining method (lanes 1 and 2). Proteins in the gel were also transferred to a PVDF membrane and immunostained with anti-(rat GluR1) antibodies (lanes 3 and 4) or anti-(rat GluR2/3) antibodies (lanes 5 and 6). (**B**) Enzymic deglycosylation of S1 and S2. Samples S1 (150 ng of protein) and S2 (120 ng of protein) were each incubated with or without PNGase F and then subjected to SDS/PAGE analysis (in 9% gels). Lane 1, reaction mixture without S1 sample; lane 2, S1 in reaction mixture without PNGase F; lane 3, S1 in reaction mixture with PNGase F; lane 4, reaction mixture without S2 sample; lane 5, S2 in reaction mixture without PNGase F; lane 6, S2 in reaction mixture with PNGase F. For lanes 1–6, proteins in the gel were detected by the silver staining method; lanes 7–12 show immunoblotting of proteins in lanes 1–6 respectively with anti-(rat GluR2/3) antibodies. The 66 kDa protein band found in lanes 1–6 was a contamination of the *N*-octyl glucoside used in this study, and the 34–35 kDa protein band found in lanes 1, 3, 4 and 6 was PNGase F. (**C**) The 106 kDa proteins in samples S1 and S2 are recognized by WGA and conA lectins. Samples S1 and S2 (150 ng of protein) were each subjected to SDS/PAGE analysis (in 9% gels) and then transferred to a PVDF membrane. The resultant membrane was incubated with WGA conjugated with HRP (lanes 1 and 2) or with conA conjugated with HRP (lanes 3 and 4). Unidentified proteins around 138 kDa were also recognized by conA and WGA. These proteins appeared as very faint yellowish bands in SDS/PAGE gels stained by the silver staining method. (**D**) Kainate receptor subunits are present in sample S2 but not S1. Samples D1 (1 μg of protein; lane 1), S1 (36 ng of protein; lanes 2 and 3), D2 (1 μg of protein; lane 4) and S2 (35 ng of protein; lanes 5 and 6) were subjected to SDS/PAGE analysis (in 4.5% polyacrylamide gels, by the method of Weber and Osborn [44]) and immunoblotted with anti-(rat GluR6/7) antibodies. (**E**) Densitometric scans of silver-stained SDS/PAGE gel lane 1 (upper trace) and lane 2 (lower trace) of (**A**). Relative absorbance (O.D.) is shown. Indicated molecular size standards in (**A**)–(**C**) are 200, 116, 97, 66 and 45 kDa, and in (**D**) are 485, 388, 291, 194 and 97 kDa..

and S2 samples, i.e. 57% and 64% respectively, may be underestimates. Based on the purities of S1 and S2, estimated either from theoretical calculations or from densitometric scans of silver-stained SDS/PAGE gels, it is clear that these samples are highly enriched in AMPA receptors. Therefore, by using a simple procedure as described here which only involves conventional biochemical methods, one can isolate 5 μg of AMPA receptor proteins from 250 g of pig brain tissue. It is worth noting that the procedure reported here closely resembles those developed for the purification of calcium channels from skeletal muscle [53] and sodium channels from *Electrophorus electricus* [54].

In the present study, we found that crude detergent extracts of pig brain synaptic junctions contain two species of AMPA receptors which can be physically separated by anion-exchange chromatography to give the D1 and D2 samples (Figure 2). This observation implies that these two kinds of AMPA receptors differ in their charges or in the charges of the protein(s) associated with them. The AMPA receptors purified from the D1 and D2 samples appeared to be mainly composed of subunits of 106 kDa, and the 106 kDa subunits in the S1 and S2 samples showed many similar properties, such as being recognized by anti-(rat GluR1) and anti-(rat GluR2/3) antibodies, containing N-linked saccharide moieties of 6–7 kDa, and containing saccharide moieties recognized by WGA and conA. Above all, the 106 kDa subunits in the S1 and S2 samples can be separated into more than eight protein spots by isoelectric focusing, and the resulting multiple spot patterns of these two samples are rather alike. Thus the observed heterogeneity of the AMPA receptors in the crude detergent extract is most likely due to the association of AMPA receptors with other proteins with different physical properties. Despite the charge difference, the quaternary structures of these two kinds of receptors appear to be similar, as these receptors migrate in sucrose density gradients at almost identical velocities ($1.1 \pm 0.2$ and $1.2 \pm 0.1$ mm/h) (Figures 4A and 5A). Furthermore, the migration velocities of these receptors are very similar to that (1.1 mm/h) of one of the two AMPA receptor species, as separated by gel-filtration chromatography or sucrose-density-gradient centrifugation, in the crude detergent extract [42]. The protein mass of this receptor has been estimated to be 368 kDa on the basis of its hydrodynamic properties. It is likely that the protein mass of the AMPA receptors purified in the present study is also around 368 kDa.

SDS/PAGE analysis (2D) shows that the major 106 kDa proteins in the S1 and S2 samples comprise more than eight acidic polypeptides with different isoelectric points (Figure 7). There are two possible origins of such a multiple-spot pattern: amino acid composition and post-translational modifications. Immunoblotting experiments revealed that each of these spots is recognized by anti-(rat GluR1) and anti-(rat GluR2/3) antibodies. Assuming that these antibodies can selectively recognize

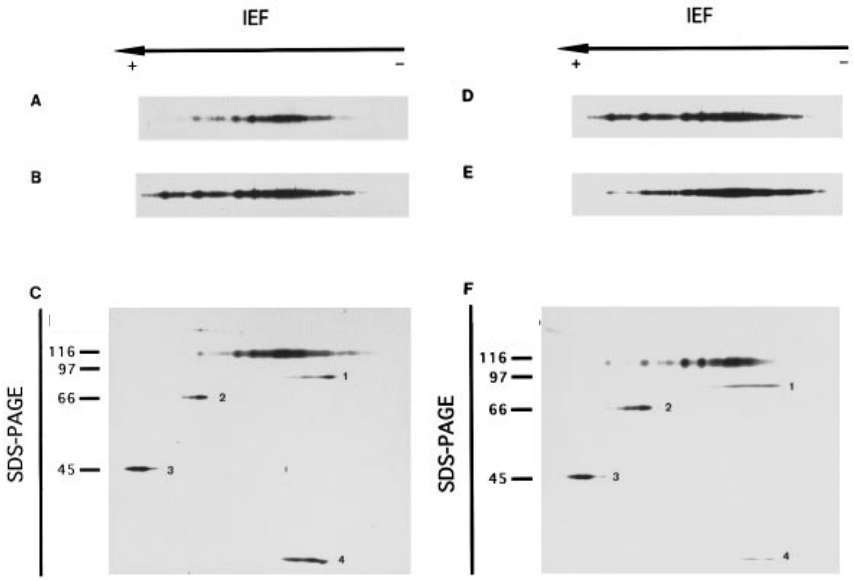


**Figure 7 2D SDS/PAGE and immunoblotting analyses of samples S1 and S2**

Samples S1 (300 ng of protein; **A**–**C**) and S2 (280 ng of protein; **D**–**F**) were separated by 2D SDS/PAGE. Proteins were transferred to a PVDF membrane and immunoblotted with anti-(rat GluR1) antibodies (**A** and **D**) or anti-(rat GluR2/3) antibodies (**B** and **E**). Proteins in gels were detected by the silver staining method (**C** and **F**). Panels (**C**) and (**F**) also show 2D SDS/PAGE standards (Bio-Rad), including hen egg white conalbumin (spots 1; pI 6.0, 6.3 and 6.6), BSA (spots 2; pI 5.4, 5.5 and 5.6), bovine muscle actin (spots 3; pI 5.0 and 5.1) and bovine carbonic anhydrase (spots 4; pI 5.9 and 6.0), separated along with sample S1 or S2. IEF, isoelectric focusing.

**Table 2 Inhibition constants of various drugs for L-[$^3$H]glutamate binding**

$IC_{50}$ values (i.e. the drug concentration at which half of the bound L-[$^3$H]glutamate was displaced) for different drugs were obtained from the data obtained with samples S1 and S2 from two preparations. At least eight concentrations (between 0.1 and 100 $\mu$M) of ligand were examined. $K_i$ values were calculated using the equation $K_i = IC_{50}/(1 + L/K_d)$, where $L$ is the concentration of L-[$^3$H]glutamate (150 nM) and $K_d$ is the dissociation constant of L-glutamate.

| | $K_i$ ($\mu$M) | |
|---|---|---|
| Ligand | S1 | S2 |
| Quisqualate | $0.08 \pm 0.02$ | $0.21 \pm 0.10$ |
| CNQX | $0.20 \pm 0.10$ | $0.31 \pm 0.27$ |
| AMPA | $0.28 \pm 0.11$ | $0.43 \pm 0.10$ |
| L-Glutamate ($K_d$) | $0.79 \pm 0.29$ | $0.96 \pm 0.45$ |
| Kainate | $2.00 \pm 0.15$ | $3.36 \pm 1.15$ |
| NMDA | $> 100$ | $> 100$ |
| L-APB | $> 100$ | $> 100$ |

different pig AMPA receptor subunits, the results of Figure 7 imply that each of these spots contains the epitopes recognized by these antibodies. Thus it appears that some of these spots may represent different variants of AMPA receptor subunits created by alternative splicing and RNA editing [5,6,55,56]. Because the possible numbers of variants of GluR2 and GluR3 subunits are rather large, post-transcriptional modifications may account for the presence of the multiple spots recognized by anti-(rat GluR2/3) antibodies in Figure 7. On the other hand, the pre-mRNA of GluR1 is only known to be alternatively spliced at the 'flip/flop' region. Even if a C-terminal alternative variant exists, and one has not been reported thus far, the number of possible GluR1 variants is not expected to exceed four. Thus post-transcriptional modifications alone cannot account for the presence of more than eight spots recognized by the anti-GluR1 antibody in Figures 7(A) and 7(D). In the present study we found that deglycosylation or dephosphorylation only resulted in changes in the isoelectric points of the protein spots as separated by isoelectric focusing, without significantly changing the number of these spots. Therefore post-translational modifications other than N-glycosylation and phosphorylation of AMPA receptor subunits have to be taken into consideration here. Although the specificities of anti-(rat GluR1) and anti-(rat GluR2/3) antibodies have been tested and established in several studies [48,57,58], the specificities of these antibodies in recognizing the cognate AMPA receptor subunits of pig brain remain to be established. Until this is done, the identities of each of the 106 kDa spots shown in Figure 7 cannot be definitely assigned.

The 106 kDa proteins in the S1 and S2 samples are glycoproteins. The N-linked carbohydrate moieties of these 106 kDa proteins are around 6–7 kDa in size, as indicated by PNGase F digestion experiments. The oligosaccharide moieties on the 106 kDa proteins of samples S1 and S2 are recognized by WGA and conA lectins. These results are in agreement with the characteristics of the oligosaccharide side chains of rat AMPA receptors [51]. The binding of receptor proteins to WGA is easily reversed by free *N*-acetylglucosamine; therefore a WGA affinity column is used as one of the purification steps described here. In contrast, the interaction between the solubilized receptors and conA is very strong, and the bound receptor cannot be released from the column by free sugar molecules (Y.-C. Chang, unpublished work). This strong interaction may be a result either of non-specific hydrophobic interactions or, more likely, of the multivalent interactions between the immobilized conA and the saccharide determinants on the receptor [59].

In summary, we report here a simple procedure for purifying AMPA receptors from pig brain. Samples containing 5 $\mu$g of

AMPA receptor protein and with a purity greater than 80% could be obtained from 250 g of pig brain tissue. The determination of the correlation between the AMPA receptors isolated here and physiologically active AMPA receptors awaits further study. However, the biochemical and pharmacological properties of the purified receptors closely resemble those of synaptic AMPA receptors, indicating that the AMPA receptors purified here do represent physiologically active AMPA receptors. The described purification procedure for AMPA receptors will facilitate future work on the biochemical and structural characterization of these receptors.

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