A study of the oligomeric state of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-preferring glutamate receptors in the synaptic junctions of porcine brain

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The number of the subunits in an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-preferring L-glutamate receptor in the synaptic junctions of porcine brain was investigated in this study. Upon incubation of the synaptic junctions with three cross-linking reagents, dimethyl adipimidate (DMA), dimethyl suberimidate (DMS) and *N*-succinimidyl-(4-azidophenyl)-1,3′-dithiopropionate (SADP), AMPA receptor subunits in higher-molecular-mass aggregates were detected by immunoblotting. These aggregates migrated as proteins of approx. 200, 300 and 400 kDa. The number and identity of the subunits in a solubilized AMPA receptor were also investigated here. Two samples, W1 and W2, enriched in AMPA receptors were prepared from synaptic junctions by a combination of detergent-solubilization, anion-exchange chromatography and wheatgerm agglutinin affinity chromatography. Hydrodynamic behaviour analyses revealed that the majority of the AMPA receptors in either one of these samples were asymmetrical detergent-surrounded particles with a protein mass around 350 kDa. SDS/PAGE analysis revealed that the majority of AMPA receptors in the W1 sample were comprised of dimers of 106 kDa subunits which were covalently linked by disulphide bonds. Cross-linking these receptors with SADP yielded a new band of approx. 400 kDa. The results obtained here, either from the studies of AMPA receptors embedding in synaptic junctions or from those of detergent-solubilized and partially purified receptors, suggest that AMPA receptors contain a basic core structure comprising of four 106 kDa subunits.

INTRODUCTION

Glutamate is the principal excitatory neurotransmitter in the mammalian nervous system. Glutamate receptors are currently classified into three groups of ionotropic receptors, i.e. *N*-methyl-D-aspartate (NMDA)-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-, kainate-preferring receptors and a family of metabotropic receptors [1–3]. Numerous studies have demonstrated that glutamate receptors are not only essential for conveying excitatory information across synapses; they are also involved in brain development and many higher functions of the brain, e.g. learning and memory [4,5].

The primary function of the AMPA-preferring glutamate receptors, also known as the AMPA receptor, is to mediate fast synaptic transmission along excitatory pathways in the central nervous system of mammals. Four cDNAs, i.e. GluR1–4, coding for rat AMPA receptor subunits have been isolated [6,7]. Co-expression studies of recombinant subunits in oocytes or transfected cells [8,9], as well as biochemical studies using antibodies against rat GluR1–4 subunits [10,11], have suggested that AMPA receptors are hetero-oligomers. Consistently, studies employing a combination of patch–clamp recordings and amplification by means of the PCR have revealed that most of the neurons in the rat brain contain mRNAs of more than one kind of AMPA receptor subunits [12,13]. Henley et al. [14] and Honore and Nielsen [15] have proposed that a synaptic AMPA receptor may include an allosteric modulatory subunit of 108 or 112 kDa in addition to the subunits with ligand-binding sites. The size of native AMPA receptors has been estimated in several reports. The AMPA receptors have been solubilized from rat brain membranes by Triton X-100 [16], by CHAPS [17], or by Triton X-100 plus digitonin [18]. By gel-filtration chromatography, the molecular mass of the Triton X-100-solubilized [^{3}H]AMPA-binding sites has been estimated to be 425 kDa, whereas those of the CHAPS-solubilized binding sites has been estimated to be 610 kDa. The molecular mass of the Triton X-100/digitonin-solubilized rat brain AMPA receptors, as identified by their [^{3}H]kainate-binding activity, has been estimated to be 650 kDa [18]. Furthermore, Wenthold et al. [10] have reported that treating rat brain synaptic membranes with a cross-linking reagent, dithiobis(succinimidyl propionate), results in new protein bands of 325, 470 and 590 kDa recognized by antibodies against the rat AMPA receptors in immunoblotting analyses. Two species of L-[^{3}H]glutamate-binding sites, with pharmacological properties closely resembling those of a synaptic AMPA receptor, have been solubilized from the synaptic junctions of porcine brain by Triton X-114 and KCl [19,20]. The protein masses of these putative receptors have been estimated on the basis of their hydrodynamic properties to be 368 and 560 kDa [20]. Assuming that a native AMPA receptor is composed of subunits with molecular masses around 100 kDa, the aforementioned results indicate that the number of the subunits in a native AMPA receptor could be 3, 4, 5 or 6.

The use of bifunctional cross-linkers can provide information about the number and arrangement of subunits in oligomeric proteins. In this study, using three different cross-linking reagents, the AMPA receptor subunits in the isolated synaptic junctions are covalently linked into higher-molecular-mass species, which yield important information concerning the number and size of the subunits in a native AMPA receptor. The protein mass of AMPA receptors solubilized and partially purified from porcine brain has also been determined on the basis of their hydrodynamic

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DMS, dimethyl suberimidate; DMA, dimethyl adipimidate; DSP, dithiobis(succinimidyl propionate); HRP, horseradish peroxidase; nACh, nicotinic acetylcholine; NMDA, *N*-methyl-D-aspartate; SADP, *N*-succinimidyl-(4-azidophenyl)-1,3′-dithiopropionate; WGA, wheatgerm agglutinin.

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properties and confirmed by chemical cross-linking experiments. The protein compositions of these receptors have also been analysed by SDS/PAGE. The results obtained in this study are consistent with the hypothesis that synaptic junctional AMPA receptors, either in a 'solubilized' form or a 'native' form, are tetrameric assemblies consisting of 106 kDa subunits.

METHODS AND MATERIALS

Materials

L-[^{3}H]Glutamic acid (50–60 Ci/mmol) and [^{3}H]AMPA (56.6 Ci/mmol) were purchased from Du Pont–NEN. Antibodies to rat GluR2 and GluR3 subunits [anti-(rat GluR2/3) antibodies] and to rat GluR1 subunit [anti-(rat GluR1) antibody] were obtained from Chemicon (Temecula, CA, U.S.A.). Hepes, Triton X-114, cross-linked glycogen phosphorylases *b*, sucrose, wheat-germ agglutinin (WGA) and Trizma were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose CL-6B was purchased from Pharmacia Biotech (Uppsula, Sweden). All of the cross-linking reagents used in this study were purchased from Pierce Co (U.S.A.). DMSO and other chemicals were obtained from Merck-Shuchardt Chemical Co (Germany). 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 μm pore size). Synaptic junctions were isolated from fresh porcine brain tissues according to the procedure described previously [19].

Chemical cross-linking experiments

Three bifunctional cross-linking reagents, DMA (dimethyl adipimidate), DMS (dimethyl suberimidate) and SADP [*N*-succinimidyl-(4-azidophenyl)-1,3′-dithiopropionate], were used in this study. The functional groups of DMA and DMS are imido esters which form covalent linkages with neighbouring primary amines under alkaline conditions. In addition, the functional groups of DMS and DMA are separated by a linker 11 Å and 8.6 Å in length, respectively. SADP contains two different functional groups, a 4-azidophenyl and an *N*-succinimidyl group; these groups are separated by a 13.9 Å linker which contains a cleavable disulphide bond. The *N*-succinimidyl group of SADP reacts effectively with neighbouring primary amines under a neutral pH. The 4-azidophenyl group of SADP is activated by UV light, and the activated functional group, in turn, interacts non-specifically with neighbouring amino acid residues.

Prior to performing cross-linking reactions, synaptic junctions were dialysed against a buffer solution containing 100 mM NaCl and 50 mM Hepes at pH 7.4 (for the cross-linking reactions with SADP) or a buffer solution containing 100 mM NaCl and 50 mM Hepes at pH 8.2 (for the cross-linking reactions with DMA and DMS) at 4 °C overnight. Solubilized receptor samples were dialysed against the same buffer solutions containing Triton X-114 (1 %, v/v) at 4 °C overnight. All of the reactions were performed at 4 °C. For the cross-linking reactions with DMS and DMA, 16 μl of freshly prepared DMS or DMA solutions (in the dialysing buffer solutions for synaptic junctions) at concentrations of 6, 20, 60 and 90 mM were added to synaptic junctions (200 μl, 0.5 mg of protein/ml) within 1 min of dissolving DMA or DMS crystals in the buffer solution. Aliquots (12 μl) of freshly prepared DMS or DMA solutions were added to the reaction mixtures at 20 min and 40 min later. Reactions were stopped 80 min after the final addition of reagents by adding Tris (final concentration 100 mM). For cross-linking synaptic junctions with SADP, 4 μl SADP stock solutions, at concentrations of 0.1, 0.5, 2.5, 5 and 10 mM (freshly dissolved in DMSO), were added to synaptic junctions (90 μl, 1 mg of protein/ml). The resultant mixtures were incubated at 4 °C in the dark. Aliquots (3 μl) of SADP stock solutions were added to the reaction mixtures 30 and 60 min later. Reactions were stopped 1 h after the final addition of SADP by adding Tris (final concentration 100 mM). Reaction mixtures were then subjected to flash photolysis. For cross-linking the solubilized sample, 2 μl of SADP stock solutions at concentrations of 2.5 and 5 mM were added to the solubilized sample (45 μl). The resultant reaction mixtures were incubated at 4 °C in the dark. Aliquots (2 μl) of SADP stock solutions were added to the reaction mixture 30 min later, and 1 μl of SADP stock solutions were added 60 min later. Reactions were stopped 1 h after the final addition of SADP and subjected to flash photolysis. Photolysis was performed by exposing reaction mixtures to irradiation with flashes of light (Softlite 1400 M, Tocad Company Limited, Japan; flash duration 1/5000 s) while the samples were kept on ice.

Immunoblotting analysis

Proteins were first separated by SDS/PAGE according to the procedure of Laemmli [21] or the method of Weber and Osborn [22] and then transferred to a PVDF (polyvinyldiene difluoride) membrane. The resulting membrane was blocked with Tris-buffered saline (TBS: 100 mM Tris at pH 7.4 and 100 mM NaCl) containing BSA (3 %, w/v) and non-dairy creamer (3 %, w/v) at room temperature for 1 h. Subsequently, the membrane was incubated with primary antibody in TBS at 4 °C overnight and then with secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 1 h. HRP on the membrane was then detected by an enhanced chemiluminescence (ECL) kit (Amersham) following the protocol provided by the manufacturer. Stripping the Western blots was performed by washing the membranes with a solution containing 100 mM 2-mercaptoethanol/62.5 mM Tris hydrochloride at pH 6.7 and 2 % SDS at 50 °C for 1 h as suggested by Amersham. ELISA was performed according to the procedures of Katnik et al. [23]. Protein concentration was determined by the bicinchoninic acid method [24]. Proteins on SDS/PAGE gels were detected by the silver staining method [25].

Partial purification of AMPA receptors

AMPA receptors were solubilized and partially purified from synaptic junctions isolated from porcine brain according to the procedures described in [26]. Briefly, AMPA receptors were solubilized by Triton X-114 and KCl, and the solubilized sample was applied to a column of DEAE-Sepharose CL-6B (2.5 cm × 32 cm). AMPA receptors were eluted from the column in two major peaks by a gradient of 0 to 0.5 M NaCl. The fractions under the two major peaks were pooled and then circulated overnight at 4 °C on a WGA affinity column (bed volume 10 ml). AMPA receptors were eluted from the column by a solution containing 50 mM Tris/acetate at pH 7.4, 0.2 % Triton X-114 and 0.4 M *N*-acetylglucosamine. The resultant samples were designated as the W1 and W2 samples. The W1 and W2 samples were enriched in L-[^{3}H]glutamate-binding activities (B_{max} values > 500 pmol/mg of protein), and AMPA receptors in the W1 and W2 samples could be further purified by sedimentation through continuous sucrose-density gradients to more than 80 % homogeneity. However, the sample isolated from the W2 sample also contained a labile protein of 110 kDa and a 120 kDa protein recognized by antibodies to rat kainate-sensitive receptor subunits, GluR6 and GluR7 [26].

Characterization of the hydrodynamic properties of detergent-solubilized receptors

This was performed by the methods of Clarke [27], Clarke and Smigel [28] and Wu and Chang [20]. The velocities of receptor–detergent complexes travelling in a centrifugal field through a continuous sucrose-density gradient are approximately constant and independent of the angular acceleration [29]. The sedimentation coefficients of receptor–detergent complexes at T (°C) and in a medium m, $S_{T,m}$, can be expressed by eqn. (1)

$$S_{T,m} = v/(\omega^2 r_{avg}) \quad (1)$$

where v is the average migration velocity (cm/s), ω is the angular velocity (rad/s), and r_{avg} is distance (cm) from the rotational axial to the halfway of migration of the particle. Assuming that the receptors bind the same amount of detergent molecules in H_2O and ^2H_2O, the partial specific volumes ($\bar{v}$) of a receptor–detergent complex were evaluated by eqn. (2):

$$S_{5,H_2O}/S_{5,^2H_2O} = (\eta^{5C}{}_{^2H_2O}/\eta^{5C}{}_{H_2O})(1-\rho^{5C}{}_{H_2O}\bar{v})(1-\rho^{5C}{}_{^2H_2O}\bar{v}) \quad (2)$$

where $\eta^{5C}{}_{H_2O}$, $\eta^{5C}{}_{^2H_2O}$, $\rho^{5C}{}_{H_2O}$ and $\rho^{5C}{}_{^2H_2O}$ are viscosities and densities at r_{avg} in H_2O- or ^2H_2O-based sucrose-density gradients. Viscosities were determined with a viscometer (LVIDV-11, Brookfield; sample size 0.5 ml, setting 60 rev./min) with pure water as the reference. $S_{20,w}$, the sedimentation coefficient at 20 °C in pure water, of a receptor–detergent complex was then calculated by eqn. (3).

$$S_{20,w} = (S_{T,m})(\eta_{T,m})(1-\rho_{20,w}\bar{v})/(\eta_{20,w})/(1-\rho_{T,m}\bar{v}) \quad (3)$$

By using the values of Stoke's radius (a), which was determined by gel-filtration chromatography, $S_{20,w}$ and $\bar{v}$, the molecular mass, M, of a receptor–detergent complex was estimated by eqn. (4).

$$M = (6\pi\eta_{20,w}Na)(S_{20,w})/(1-\rho_{20,w}\bar{v}) \quad (4)$$

In eqn. (4), N is the Avogadro constant. Next, the contributions of protein and detergent to the molecular mass estimated for a receptor–detergent complex were evaluated by the following equations:

$$M_c = M_p(1+\alpha) \quad (5)$$

$$\bar{v}_c = \bar{v}_p[1/(1+\alpha)] + \bar{v}_d[\alpha/(1+\alpha)] \quad (6)$$

where M_c is the molecular mass of a receptor–detergent complex, M_p is the molecular mass of the receptor in the complex, $\bar{v}_c$ is the partial specific volume of the detergent–receptor complex, $\bar{v}_p$ is the partial specific volume of protein, $\bar{v}_d$ is the partial specific volume of detergent, and α is the weight ratio between detergent and protein of the receptor–detergent complex. The accuracy of the experimental procedure described here in estimating protein masses was tested. The molecular masses of catalase and thyroglobulin were estimated by this experimental procedure to be 3.5 % and 11.6 %, respectively, lower than the molecular masses of these proteins, 232 and 669 kDa (T.-Y. Wu and Y.-C. Chang, unpublished work). Although these values were in the range of variations when this procedure was used to estimate the molecular masses of other proteins [28], these underestimations were possibly due to the sucrose gradient (6–26 %) used here [30]. As a result, the mass of an unknown protein (between 232 and 669 kDa) is probably 3.5–11.6 % higher than the mass estimated by the above procedure.

The frictional coefficient of a detergent–receptor complex was evaluated by eqn. (7) [31]

$$f/f_0 = a[4\pi N/3M/(\bar{v}+\delta/\rho_{20,w})]^{1/3} \quad (7)$$

where f/f_0 is the ratio of the friction of the binding sites over that of a spherical molecule with similar molecular mass, hydration and partial specific volume. δ is the solvation factor which is, in most cases, 0.2 g of solvent/g of protein [31].

Sucrose-density-gradient centrifugation

An SW-28 rotor (r_{av} 11.82 cm, Beckman) was used here. Sucrose gradients [6–26 % (w/v) sucrose in 50 mM Tris/acetate at pH 7.4 plus 0.1 % (v/v) Triton X-114] with a total volume of 28 ml were prepared by a gradient maker (Jule, U.S.A.). Sucrose concentrations were determined by a refractometer (Atago Co., Japan). Gradients were maintained at 5 °C for 4–6 h before use. Next, soluble receptor samples (2 ml) were applied on top of the gradient. Gradients were then centrifuged at 5 °C for various lengths of time. After centrifugation, sample fractions were removed from the bottom of each centrifuge tube. The migration distance of a binding site was the distance between the middle point of the applied sample and the middle point of the fraction of peak binding activity.

Gel-filtration chromatography

The Stoke's radius of AMPA receptors was determined by gel-filtration chromatography. Gel-filtration chromatography was carried out at 4 °C with a Sepharose CL-6B column (2.5 cm × 42 cm) which had been pre-equilibrated with 50 mM Tris/acetate at pH 7.4 plus Triton X-114 (0.2 %, v/v). Thyroglobulin, apoferritin, catalase and alcohol dehydrogenase were used as standard proteins. The first two proteins were detected by the Bradford method [32], and the latter two proteins were detected by their enzymic activities according to the procedures described in the Worthington Enzyme Manual [33].

Radioligand binding assay

L-[^{3}H]Glutamate bound to receptors was quantified by the poly(ethylene glycol)–immunoglobulin precipitation method as described previously [19]. [^{3}H]AMPA binding was assayed by a similar method except for including KSCN (0.1 M) in reaction mixtures. L-[^{3}H]Glutamate and [^{3}H]AMPA concentrations in assaying mixtures were 115 and 20 nM, respectively. Non-specific L-[^{3}H]glutamate and [^{3}H]AMPA binding activities were determined in the presence of unlabelled L-glutamate (0.5 mM) and AMPA (0.5 mM), respectively.

RESULTS

Chemical cross-linking experiments of synaptic junctions

Bifunctional cross-linking reagents were used here to link synaptic AMPA receptor subunits with proteins situated next to them. Three different cross-linking reagents were used in this study to ensure that the observed cross-linking pattern was not reagent-specific. Synaptic junctions were incubated at pH 8.2 and 4 °C in the presence of different concentrations of DMS or DMA. In an earlier study [26], we found that porcine AMPA receptors were comprised of 106 kDa glycoproteins which were recognized by anti-(rat GluR2/3) and anti-GluR1 antibodies. Therefore, to investigate the cross-linked products containing AMPA receptor subunits, DMA- or DMS-cross-linked synaptic junctions were subjected to Western blot analysis with anti-(rat GluR2/3) antibodies. Regularly spaced bands corresponding to the monomer and oligomers up to the tetramer of subunits with a molecular mass around 100 kDa were clearly observed above the background (Figure 1A and inset of Figure 1C). Densitometric scans of these Western blot results indicated that the background included a rather broad peak stretching from the dimeric band to the middle between the tetrameric band and gel front (a rep-

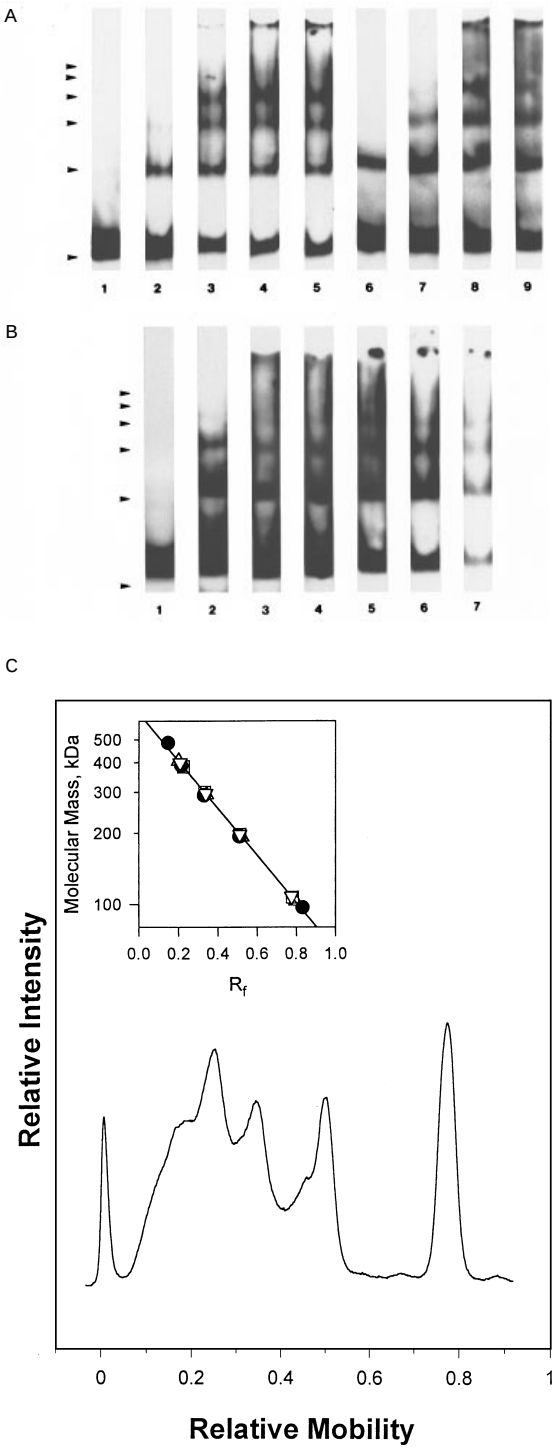

Figure 1 Chemical cross-linking of AMPA receptor oligomers by DMS, DMA and SADP

(**A**) Immunoblot of DMA- or DMS-cross-linked synaptic junctions. Samples (19 μg of protein) were separated by an SDS/3.5%-polyacrylamide gel by the method of Weber and Osborn [22] under reducing conditions, transferred to a PVDF membrane and treated with anti-(rat GluR2/3) antibodies. Samples were treated as follows: lane 1, synaptic junctions prior to the cross-linking reaction; lanes 2–5, synaptic junctions cross-linked with 1, 5, 10 and 15 mM DMA; lanes 6–9, synaptic junctions cross-linked with 1, 5, 10 and 15 mM DMS. (**B**) Immunoblot of SADP-cross-linked synaptic junctions. Samples were as follows: lane 1, synaptic junctions (13.5 μg of protein) prior to the cross-linking reaction and analysed under reducing conditions; lane 2, synaptic junctions (13.5 μg of protein) prior to the cross-linking reaction and analysed under non-reducing conditions; lanes 3–7, synaptic junctions (13.5 μg of protein) cross-linked with 0.01, 0.05, 0.25, 0.5 and 1 mM SADP and analysed under non-reducing conditions. The arrowheads on the left side of the immunoblots indicate the positions of molecular mass markers, from bottom to top: 97.4, 194.8, 292, 389.9, 487 and 584.4 kDa. (**C**) A densitometric scan of the Western blot of DMA-cross-linked synaptic junctions (lane 5 of **A**). Inset, the calibration of the PAGE, with the logarithm of the molecular masses of the cross-linked glycogen phosphorylase *b* (●) plotted against the relative mobility (R_f). The correlation coefficient is 0.996. The peak positions of DMA (15 mM)-, DMS (15 mM)- and SADP (0.5 mM)-linked aggregates were obtained by densitometric scanning of lanes 5 and 9 of (**A**) and lane 6 of (**B**), respectively, and are shown as □, △ and ▽, respectively.

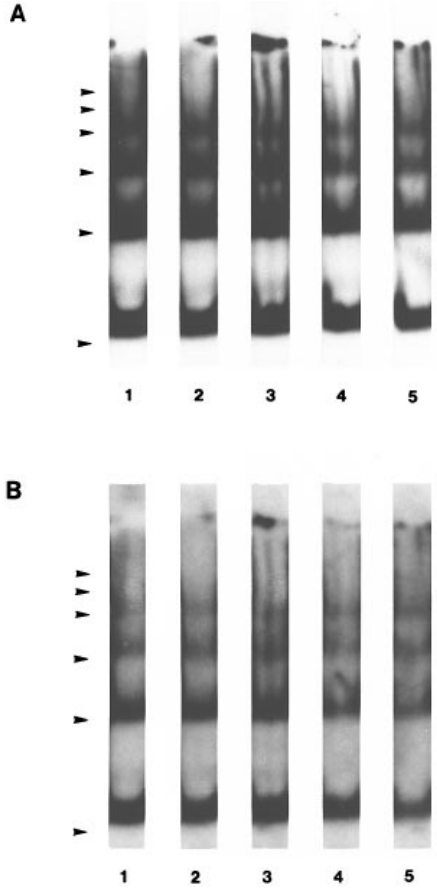

Figure 2 Chemical cross-linking of AMPA receptor oligomers by SADP under varied photolytic conditions

(**A**) Immunoblot of SADP-cross-linked synaptic junctions probed with anti-(rat GluR2/3) antibodies. Following the incubation in the dark at 4 °C for 1 h, a reaction mixture containing synaptic junctions (1 mg of protein/ml) and 0.5 mM SADP was either not exposed to flash photolysis (lane 1) or exposed to one (lane 2), five (lane 3), ten (lane 4) or 15 (lane 5) flashes of light. The resultant reaction mixtures (13.5 μg of protein) were then separated by SDS/3.5%-PAGE by the method of Weber and Osborn [22], transferred to a PVDF membrane and treated with anti-(rat GluR2/3) antibodies. (**B**) Immunoblot of SADP-cross-linked synaptic junctions probed by anti-(rat GluR1) antibodies. The Western blot as shown in (**A**) was stripped and then probed with anti-(rat GluR1) antibody.

resentative scan is shown in Figure 1C). The extent of cross-linking increased with DMA or DMS concentration. A plateau was reached at about 5 mM DMA and 10 mM DMS, above which the extent of cross-linking appeared to be similar. The immunoreactive material near the front of the gel was probably the aggregates of cross-linked junctional proteins. When the protein concentration in the DMS-cross-linking reaction mixtures was decreased from 0.5 mg/ml to 0.2 mg/ml, the cross-

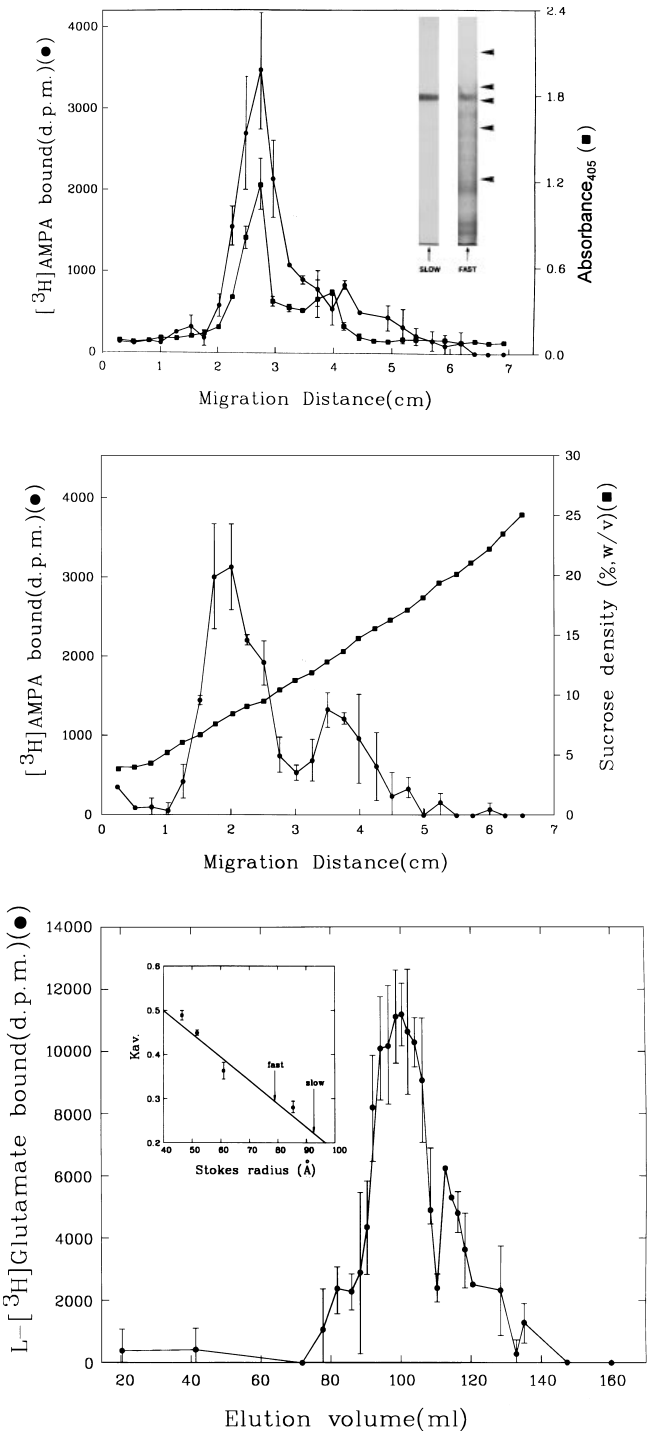

Figure 3 Hydrodynamic studies of the AMPA receptors in the W1 sample

Upper panel: sucrose-density-gradient centrifugation in a water-based gradient. The W1 sample (2 ml) was applied to a water-based continuous sucrose gradient, and the gradient was centrifuged at 83 000 ***g*** and 5 °C for 30 h. Sample fractions of 1 ml were removed from the bottom of the gradient. A 200 μl portion of each fraction was removed for assaying [^{3}H]AMPA binding activity (●). A 50 μl portion of each fraction was removed for ELISA assay (■) using anti-(rat GluR2/3) antibodies (0.5 μg/ml) as the primary antibody. Inset, SDS/PAGE analysis of the AMPA receptors in the W1 sample. Aliquots (100 μl) of the fraction at the migration distance of 2.8 cm (labelled as slow) and 500 μl of the fraction at the migration distance of 3.9 cm (labelled as fast) were separated by an SDS/9%-polyacrylamide gel by the method of Laemmli [21] and detected by the silver staining method. Arrowheads on the right side mark the position of the molecular mass markers from bottom to top: 44, 67, 97, 114 and 200 kDa. Middle panel: sucrose-density-gradient centrifugation in a ^2H_2O-based gradient. The W1 sample (2 ml) was applied to a ^2H_2O-based continuous sucrose gradient. The gradient was centrifuged at 83 000 ***g*** and 5 °C for 40 h. Sample fractions of 1 ml were removed from the bottom of the gradient. A 200 μl portion of each fraction was removed for assaying [^{3}H]AMPA binding activity (●). Sucrose density, (■). Lower panel: gel-filtration chromatography. The W1 sample (5 ml) was applied to a Sepharose CL-6B (1.8 cm × 112 cm) chromatography column and eluted with buffer A plus Triton X-114 (0.2%); 2 ml fractions were collected. Samples (200 μl) were removed and assayed for L-[^{3}H]glutamate binding activity in the absence or presence of unlabelled L-glutamate (0.5 mM). Each value represents the mean (±S.D) of three determinations of specific binding. The results are taken from a single experiment of a total of two experiments. Inset, the calibration of the column, with the partition coefficients K_{av} plotted against the Stoke's radius. The correlation coefficient is 0.969. Each point represents the mean ± S. D. of three experiments. The markers (●), with Stoke's radius in parentheses, are thyroglobulin, apoferritin, catalase, and alcohol dehydrogenase. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume of receptors, V_0 = void volume and V_t = total gel bed volume. Arrows indicated the elution volumes of the major (labelled as slow) and minor (labelled as fast) peaks of [^{3}H]AMPA binding activity.

linking pattern of AMPA receptor subunits remained the same (results not shown).

Synaptic junctions were also cross-linked with different concentrations of several hetero-bifunctional reagents. Treatments with *N*-hydroxysuccinimidyl-4-azidosalicylic acid, sulphosuccinimidyl-4-(*p*-azidophenyl)butyrate and sulphosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate induced extensive cross-linking reactions among synaptic junctional proteins. Immunoblotting analysis with anti-(rat GluR2/3) antibodies revealed that only three bands corresponding to monomers, dimers and trimers of AMPA receptor subunits were clearly observed; meanwhile those larger than 350 kDa were indistinguishable from a dark background (results not shown). In contrast, SADP-cross-linked bands were clearly found above the background of blots and yielded useful information. Because SADP itself contained a cleavable disulphide bond, the immunoblotting analysis of SADP-cross-linked aggregates was performed under non-reducing conditions. The results indicated, prior to SADP treatment, that there were three major bands and a faint band detected by anti-(rat GluR2/3) antibodies (lane 2 of Figure 1B). These bands migrated as monomers, dimers, trimers and tetramers of 106 kDa subunits. These higher-molecular-mass bands were dissociated into the monomer band of 106 kDa when the analysis was performed under reducing conditions (lane 1 of Figure 1B), implying that these higher-molecular-mass bands were held together by disulphide bonds. As the concentration of SADP in the reaction mixture increased, more 106 kDa monomers were linked into the higher-molecular-mass species (Figure 1B and inset of Figure 1C). However, when SADP concentration was higher than 0.5 mM, the overall intensity of the Western blot decreased significantly, although the presence of four bands was still evident. A similar observation was reported previously in a study wherein a similar cross-linking reagent, *N*-sulphosuccinimidyl-(4-azidophenyl)-1,3′-dithiopropionate was used to cross-link detergent-solubilized synaptophysin [34]. A possible explanation is that after treatment with high concentrations of SADP, polypeptides are heavily modified by these reagents, thereby leading to a reduction in their binding with antibodies. The immunoreactive material observed in the front of the gel might be aggregates of cross-linked junctional proteins. Analysis of the SADP-linked synaptic junctions, which were exposed to different numbers of flashes of light, indicated that the cross-links among subunits were formed even before the exposure to flashes of light (lane 1 of Figures 2A and 2B). It is possible that the bound SADP molecules, whose linker contains a disulphide bond, undergo a disulphide exchange with free thiol groups of the AMPA receptor subunits and those of the neighbouring proteins [35,36], thereby covalently linking two proteins together even prior to irradiation with UV light. Again, only four

Table 1 Molecular size of Triton X-114-solubilized AMPA-preferring glutamate receptors

Stoke's radii, sedimentation coefficients and partial specific volumes were means ± S.D. of two to five independent experiments (*n*, in parentheses). The means of these data were used in the calculation of the remaining data shown in this Table.

	W1 sample		W2 sample	
	Minor receptor species	Major receptor species	Minor receptor species	Major receptor species
$S_{20,w}$ (S)	18.1 ± 0.6 (5)	11.3 ± 0.5 (5)	18.3 ± 0.6 (3)	11.3 ± 0.1 (5)
ν (cm^3/g)	0.80 ± 0.10 (5)	0.82 ± 0.12 (5)	0.80 ± 0.07 (4)	0.81 ± 0.09 (5)
Stoke's radius (*a*) (nm)	7.8 ± 0.1 (2)	9.3 ± 0.1 (2)	8.0 ± 0.3 (2)	9.3 ± 0.3 (2)
Molecular masses (kDa)				
Receptor–detergent complex	800	650	830	620
Receptor protein	480	320	500	340
Frictional coefficient	1.14	1.45	1.16	1.48

immunoreactive bands corresponding to monomers, dimers, trimers and tetramers of subunits with a mass around 100 kDa were observed in these experiments, and this cross-linking pattern was similar over a 4-fold range of protein concentration (0.5–2.0 mg/ml) (results not shown). The Western blot of Figure 2(A) was also re-probed with anti-(rat GluR1) antibodies (Figure 2B). The resultant immunostaining pattern closely resembled that probed with anti-(rat GluR2/3) antibodies (Figure 2A). These results, together with those obtained from DMS- and DMA-cross-linking experiments, suggested that in synaptic junctions three proteins, with masses around 100 kDa, are situated within the reaction distances of DMA, DMS and SADP (8.6 Å, 11.0 Å and 13.9 Å, respectively) of an AMPA receptor subunit. Therefore, an AMPA receptor in the synaptic junctions of porcine brain is likely to be an assembly of four subunits with molecular masses around 100 kDa.

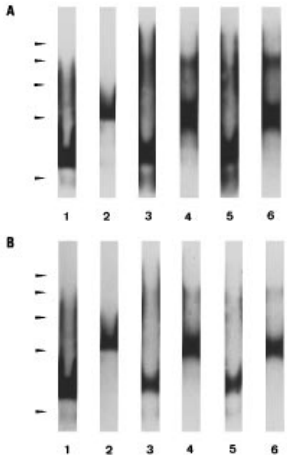

Figure 4 Chemical cross-linking of the isolated AMPA receptor oligomers with SADP

(**A**) Immunoblot of SADP-cross-linked AMPA receptor oligomers probed by anti-(rat GluR2/3) antibodies. AMPA receptor oligomers in the peak fraction (1 μg of protein/ml) obtained in the experiment as shown in Figure 3(A) were cross-linked with SADP, subjected to SDS/PAGE analysis by the method of Weber and Osborn [22], transferred to a PVDF membrane, and treated with anti-(rat GluR2/3) antibodies. Samples are as follows: lanes 1 and 2, AMPA receptors (0.016 μg of protein) before the cross-linking reaction; lanes 3 and 4, AMPA receptors (0.016 μg of protein) cross-linked with 0.25 mM SADP; lanes 5 and 6, AMPA receptors (0.016 μg of protein) cross-linked with 0.5 mM SADP. Immunoblotting analyses of lanes 1, 3 and 5 were performed under reducing conditions while those of lanes 2, 4 and 6 were performed under non-reducing conditions. Arrows on the left side of the blot indicate the position of the molecular mass markers from bottom to top: 97.4, 194.8, 292, 389.9 and 487 kDa. (**B**) Immunoblot of SADP-cross-linked solubilized AMPA receptor oligomers probed by anti-(rat GluR1) antibodies. The Western blot as shown in (**A**) was stripped and then immunostained with anti-(rat GluR1) antibody.

Hydrodynamic study of solubilized AMPA receptors

The size of detergent-solubilized AMPA receptors in the W1 sample was determined by their hydrodynamic properties. When the W1 sample was analysed by sucrose-density-gradient centrifugation in a H_2O-based gradient, an asymmetrical peak of [^{3}H]AMPA binding activity appeared (Figure 3, upper panel). When the fractions obtained in this experiment were assayed by an ELISA method using anti-(rat GluR2/3) antibodies as the primary antibody, this broad peak was resolved into a major and a minor peak. When the sample was analysed in a ^2H_2O-based gradient, the presence of two peaks was evident (Figure 3, middle panel). The average migration velocities of these AMPA receptors in H_2O- and ^2H_2O-based gradients were then calculated. Next, the W1 sample was analysed by gel-filtration chromatography, and a major and a minor species of receptor were also found. The Stoke's radii of these receptor species in the W1 sample were estimated from the calibration curve shown in the inset of Figure 3(lower panel). The hydrodynamic properties of these receptors were calculated by eqns. (1)–(4) and are shown in Table 1. The molecular masses of the major and minor receptor–detergent complexes in the W1 sample were calculated to be 650 and 800 kDa, respectively. We then used eqns. (5) and (6) to calculate the masses of the protein parts of these complexes. The partial specific volume of Triton-series detergents, 0.908, was used here for υ_d [37]. Although the partial specific volume of glutamate receptor proteins was unknown, an average value of 0.73 was used here, as originally suggested by Clarke [27] and used in other studies [38–41] for various membrane proteins. The molecular masses of the protein parts of the major and minor species in the W1 sample were calculated to be 320 and 480 kDa, respectively. The frictional coefficients of the two receptor species found in the W1 sample were greater than one, indicating that they were asymmetrical macromolecules (Table 1).

The protein compositions of the AMPA receptors in the W1 sample

SDS/PAGE analysis revealed that the major receptor species of the W1 sample consisted of a single protein band of 106 kDa (inset of Figure 3, upper panel) with a purity of 85%, as estimated by a densitometric scan of the silver-stained gel (results

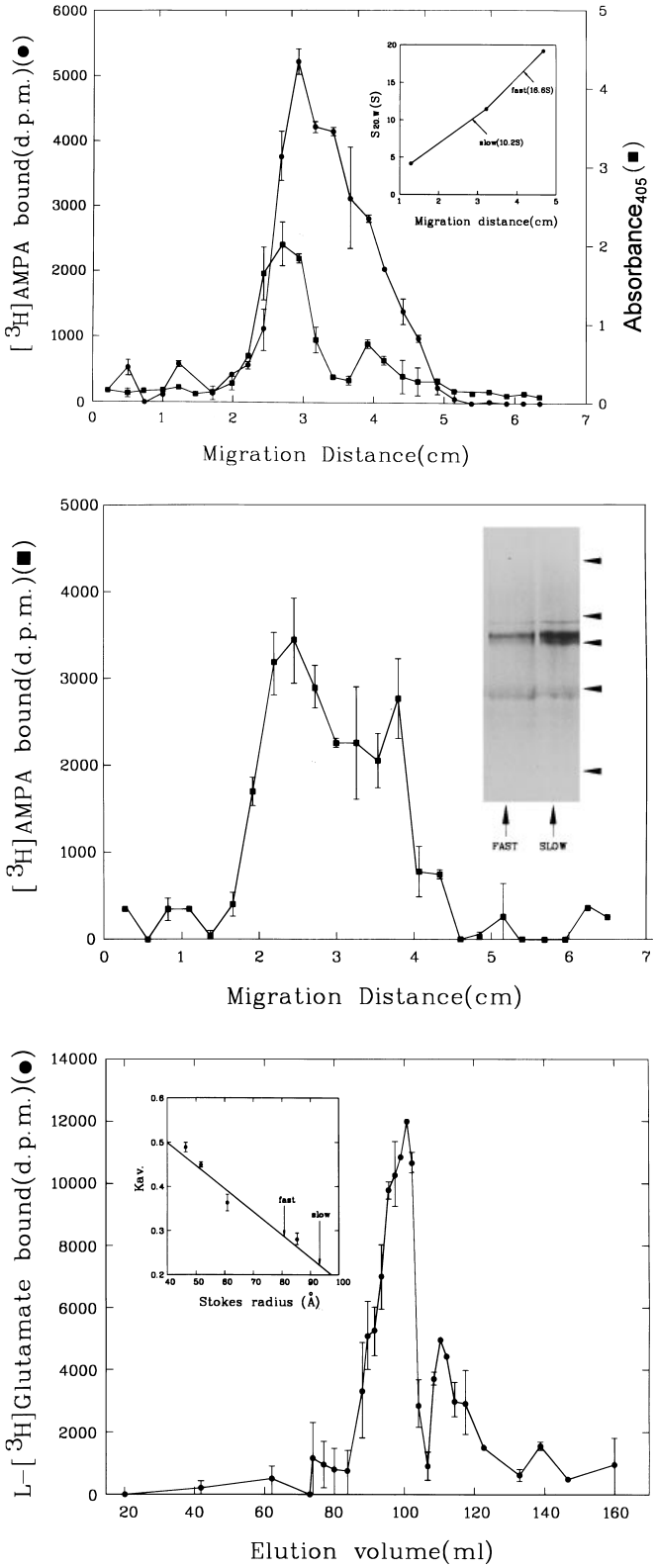

Figure 5 Hydrodynamic studies of the AMPA receptors in the W2 sample

Upper panel: sucrose-density-gradient centrifugation in a water-based gradient. The W2 sample (2 ml) was applied to a water-based continuous sucrose gradient, and the gradient was centrifuged at 83 000 ***g*** and 5 °C for 30 h. Sample fractions of 1 ml were removed from the bottom of the gradient. A 200 μl portion of each fraction was removed for assaying [^{3}H]AMPA binding activity (●). A 50 μl portion of each fraction was removed for ELISA assay (■) using anti-(rat GluR2/3) antibodies (0.5 μg/ml) as the primary antibody. Inset, calibration of the gradient, with the sedimentation coefficient plotted against the migration distance. The markers (●), with sedimentation coefficients in parentheses, are thyroglobulin (19S), catalase (11.3 S) and BSA (4.3 S). Middle panel: sucrose-density-gradient centrifugation in a ^2H_2O-based gradient. The W2 sample (2 ml) was applied to a ^2H_2O-based continuous sucrose gradient. The gradient was centrifuged at 83 000 ***g*** and 5 °C for 40 h. Sample fractions were removed from the bottom of the gradient. A 200 μl portion of each fraction was removed for assaying [^{3}H]AMPA binding activity (■). Inset, SDS/PAGE analysis of the AMPA receptors in the W2 sample. Samples (100 μl) of the fraction at the migration distance of 2.5 cm (labelled as slow) and 100 μl of the fraction at the migration distance of 3.8 cm (labelled as fast) were separated by an SDS/9%-polyacrylamide gel by the method of Laemmli [21] and detected by the silver staining method. Arrowheads on the right side mark the position of the molecular mass markers from bottom to top: 44, 67, 97, 114 and 200 kDa. Lower panel: gel-filtration chromatography. The W2 sample (5 ml) was applied to a Sepharose CL-6B (1.8 cm × 112 cm) chromatography column and eluted with buffer A plus Triton X-114 (0.2%); 2 ml fractions were collected. Samples (200 μl) were removed and assayed for L-[^{3}H]glutamate binding activity in the absence or presence of unlabelled L-glutamate (0.5 mM). Each value represents the mean (±S.D) of three determinations of specific binding. Inset, the calibration of the column, with the partition coefficients K_{av} plotted against the Stoke's radius. The correlation coefficient is 0.969. Each point represents the mean ± S. D. of three experiments. The markers (●), with Stoke's radius in parentheses, are thyroglobulin, apoferritin, catalase, and alcohol dehydrogenase. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume of receptors, V_0 = void volume and V_t = total gel bed volume. Arrows indicated the elution volumes of the major (labelled as slow) and minor (labelled as fast) peaks of [^{3}H]AMPA binding activity. The results are taken from a single experiment of a total of two experiments.

not shown). The protein mass of the major receptor species was estimated to be 320 kDa by its hydrodynamic properties (Table 1). Considering the underestimation of the protein mass by a factor between 3.5 and 11.6% in a hydrodynamic analysis (see the Materials and methods section), the major species of receptors in the W1 sample appeared to contain three or four 106 kDa subunits. SDS/PAGE analysis of the minor receptor species revealed the presence of a major 106 kDa protein and some minor proteins (inset of Figure 3, upper panel). The protein mass of these receptors was estimated to be 509 kDa by their hydrodynamic properties (Table 1).

Chemical cross-linking of the AMPA receptors in the W1 sample

To determine whether the number of the subunits of the major receptor species in the W1 sample was three or four, the peak fraction of Figure 3(upper panel) was incubated with 0.25 or 0.5 mM SADP on ice for 1 h, followed by exposure to flashes of light. Immunoblotting analysis with either anti-(rat GluR2/3) or anti-(rat GluR1) antibodies revealed that prior to cross-linking reaction the 106 kDa subunits of the major receptors were linked into dimers by disulphide bonds (lanes 1 and 2 of Figures 4A and 4B). A proportion of these dimers was further cross-linked into tetramers by SADP (lanes 4 and 6 of Figures 4A and 4B). These results, together with those obtained with hydrodynamic studies, indicated that the majority of AMPA receptors in the W1 sample are tetramers of 106 kDa subunits. Similar chemical cross-linking reactions were not performed with the sample containing the minor receptor species because of the low content of AMPA receptors in this sample.

Estimation of the mass of a second kind of AMPA receptor solubilized from synaptic junctions

We have already demonstrated that the majority of the AMPA receptors in the W1 sample are tetramers of 106 kDa subunits. The molecular masses of the AMPA receptors in the W2 sample were also estimated on the basis of their hydrodynamic properties (Table 1). The W2 sample also contained two species of receptors which could be separated by gel-filtration chromatography or sucrose-density-gradient centrifugation analysis (Figure 5). The hydrodynamic properties of these receptor species resembled closely those of the major and minor receptor species in the W1

sample. The protein masses of these receptors in the W2 sample were estimated to be 340 kDa and 500 kDa respectively. Consistent with our previous observation [26], SDS/PAGE analysis revealed that these two receptors were primarily comprised of 106 kDa protein while a minor, unidentified, yet labile protein of 110 kDa was also present in these samples. Although it was unclear if this 110 kDa protein also participated in the assembly of the AMPA receptor oligomers in the W2 sample, the similarities of the hydrodynamic properties of the major AMPA receptors in the W1 and W2 samples suggested that the major receptor species in the W2 sample was also a tetramer. The protein mass of the minor receptor species was estimated to be 500 kDa by its hydrodynamic properties (Table 1). Considering the underestimation of the protein mass by a factor between 3.5 and 11.6% in a hydrodynamic analysis (see the Materials and methods and Results sections), the minor receptor species appeared to contain either five or six 106 subunits.

DISCUSSION

In this study, we examined the oligomeric state of the AMPA receptor either situated in synaptic junctions (the 'native' state) or solubilized from synaptic junctions by detergent and partially purified by a combination of anion-exchanging chromatography and WGA-affinity chromatography (the 'solubilized' state). Analysis of DMA-, DMS- and SADP-cross-linked synaptic junctions revealed a general cross-linking pattern of AMPA receptor subunits which suggests that AMPA receptors in synaptic junctions are tetrameric. Consistently, a combination of hydrodynamic studies, SDS/PAGE analysis and chemical cross-linking experiments indicated that the majority of the partially purified Triton X-114-solubilized AMPA receptors were assemblies comprising four 106 kDa subunits.

The W1 and W2 samples contain two species of AMPA receptor: a major tetrameric species and a minor species with a protein mass of 500–600 kDa. These two species of AMPA receptors were reported to be present in comparable amounts in the crude Triton X-114 extract of porcine brain synaptic junctions [20]. Different values of the size of rat brain AMPA receptors solubilized by Triton X-100, CHAPS, or Triton X-100 plus digitonin were also reported by other laboratories [16–18]. Interestingly, the size of the CHAPS-solubilized rat AMPA receptors (610 kDa, sedimentation coefficient = 18.0 S, Stoke's radius = 8.2 nm) [17] and Triton X-100/digitonin-solubilized rat AMPA receptors (650 kDa, Stoke's radius = 8.27 nm) [18] are almost identical to the size of the 500–600 kDa AMPA receptors in the Triton X-114 extract of porcine synaptic junctions (protein mass = 562 kDa, sedimentation coefficient = 18.5 ± 0.4 S, Stoke's radius = 8.3 ± 0.1 nm) [20]. On the other hand, the mass of the Triton X-100-solubilized rat AMPA receptors, 425 kDa, [16] is similar to that of the tetrameric AMPA receptor species found in the Triton X-114 extract of porcine synaptic junctions (protein mass = 352 kDa) [20]. The above comparison suggests that the size of AMPA receptors in detergent extracts of isolated brain membranes is dependent on the reagents, such as CHAPS, Triton X-114, Triton X-100, digitonin, buffering reagents, salts and glycerol, used in the solubilization step. As a result, the 425 kDa species predominates in Triton X-100-solubilized rat brain preparations while the 610 kDa-species predominates in CHAPS-solubilized and Triton X-100/digitonin-solubilized rat brain preparations; both species are present in Triton X-114-solubilized porcine brain preparation. We hence propose that during the detergent solubilization step, aggregates with a protein mass around 500–600 kDa are formed by an association between a tetrameric AMPA receptor and other proteins, which may be dissociated AMPA receptor subunits or some unidentified proteins. The ratio between the 500–600 kDa aggregates and tetramers in the resultant extract may vary depending on reagents used in the solubilization step. However, non-specifically associated proteins in the 500–600 kDa aggregates are removed, due to their relatively weaker association with AMPA receptor subunits, by further purification with chromatography and sedimentation methods. As a result, the tetrameric receptors become the predominant species in the W1 and W2 samples.

In this study, it was found that antibodies to rat AMPA receptor subunits recognized three higher-molecular-mass aggregates in DMA-, DMS- or SADP-cross-linked synaptic junctions isolated from porcine brain, and the molecular masses of these aggregates were estimated to be around 200, 300 and 400 kDa. In a similar experiment, Wenthold et al. [10] reported that chemically cross-linking rat brain synaptic membranes with another cross-linker, dithiobis(succinimidylpropionate) (DSP), also yielded three higher-molecular-mass aggregates recognized by anti-(rat AMPA receptor) antibodies. However, the molecular masses of these DSP-cross-linked aggregates were estimated to be 325, 470 and 590 kDa. The different results described above are possibly attributed to the different electrophoresis systems, cross-linking reagents, molecular mass standards and membrane preparations used in these studies.

On the basis of amino acid sequences, neurotransmitter-gated ion channels can be further classified into nicotinic acetylcholine (nACh) receptor and glutamate receptor subfamilies [42–44]. The former subfamily includes nACh, γ-aminobutyric acid, glycine and 5-hydroxytryptamine ($5HT_3$) (serotonin) receptors. Members of the second subfamily include three broad groups of ionotropic glutamate receptors, i.e. AMPA receptors, kainate receptors and NMDA receptors. Although all of the aforementioned receptors mediate fast synaptic transmission, significant differences in the size, amino acid sequence and number of putative transmembrane segments exist between the members of the two subfamilies [6,45–48]. In addition to these differences, the results obtained in this study further suggest that the quaternary structure of the AMPA receptor is tetrameric, whereas all of the receptors belonging to the nACh receptor subfamily are pentameric. In support of our conclusion that AMPA receptors are tetrameric is the recent finding that the pore structure of AMPA receptors closely resembles those of voltage-gated Shaker potassium channels and cyclic-nucleotide-gated channels [44,49,50]. The quaternary structure of Shaker potassium channels is known to be tetrameric [51], while cyclic-nucleotide-gated channels are also proposed to be tetrameric [52].

There are some differences in the structures of the AMPA receptors in the 'solubilized' and 'native' states. When the Western blot analysis of synaptic junctions was performed in the absence of 2-mercaptoethanol, it was found that a significant proportion of AMPA receptor subunits were covalently linked into oligomers up to the tetramer (Figure 1B). On the other hand, the same analysis of the major AMPA receptor in the W1 sample revealed that all of the AMPA receptor subunits were linked by disulphide bonds into dimers (Figure 4). Because the formation of an intramolecular disulphide bond is dependent on the conformation of a protein, the different patterns of inter-subunit disulphide bonds of the 'native' and 'solubilized' AMPA receptors probably indicate some differences in their conformations. Nevertheless, these conformational differences do not appear to affect the overall quaternary structure, a tetramer, of these receptors. Furthermore, these observations are consistent with earlier observations that membrane environments influence the properties of rat AMPA receptors [53] and those of other receptors as well [54–56]. Further studies are required to elucidate

the functional consequences of this conformational change of AMPA receptors.

The properties of synaptic AMPA receptors have been shown to be under the influences of allosteric modulatory proteins [14,15], protein phosphorylation [57–60] and synaptic activity [61]. Each of these modulatory effects involves physical contacts of a synaptic AMPA receptor with a modulatory protein, a kinase, an anchoring or a structural protein. In this study, immunoblotting analysis of chemically cross-linked synaptic junctions revealed, in addition to the four major immunoreactive bands corresponding to the monomer and covalently linked dimer, trimer and tetramer, a broad background stretching from the dimeric band to the middle between the tetrameric band and gel front (Figure 1C). This broad, immunoreactive background is nearly absent in the immunoblotting analysis of the partially purified W1 sample (Figure 4). This broad background is thus likely to represent the cross-linked products of AMPA receptor subunits with their surrounding structural or modulatory proteins. Since the basic principle governing the chemical cross-linking reactions of a particular protein is the statistical distribution of neighbouring molecules, the cross-linking patterns obtained in this study indicate that the associative interactions between these structural or modulatory proteins with AMPA receptor subunits should be weaker or less frequent than the associative reactions among subunits in a native AMPA receptor. Therefore, a tetrameric assembly of AMPA receptor subunits may represent a core structure of a physiologically active AMPA receptor.

In summary, the results obtained with chemical cross-linking studies of synaptic junctional AMPA receptors and those obtained with a combination of hydrodynamic and chemical cross-linking studies of Triton X-114-solubilized and partially purified AMPA receptors are consistent with the hypothesis that a synaptic AMPA receptor consists of a core structure comprising of four subunits. Our observation, however, does not exclude the possibility that a physiologically active synaptic AMPA receptor may contain some other proteins such as modulatory proteins, kinases, or structural proteins in association with the tetrameric core structure for its proper channel properties and localization.

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REFERENCES

1 Monaghan, D. T., Bridge, R. J. and Cotman, C. W. (1989) Annu. Rev. Pharmacol. Toxicol. **29**, 365–402
2 Mayer, M. L. and Westbrook, G. L. (1987) Prog. Neurobiol. **28**, 197–276
3 Nakanishi, S. (1994) Neuron **13**, 1031–1037
4 Constantine-Paton, M., Cline, H. T. and Debski, E. (1990) Annu. Rev. Neurosci. **13**, 29–164
5 Bliss, T. V. P. and Collingridge, G. L. (1993) Nature (London) **361**, 31–39
6 Hollmann, M. and Heinemann, S. (1994) Annu. Rev. Neurosci. **17**, 31–108
7 Seeburg, P. H. (1993) Trends Neurosci. **16**, 359–365
8 Burnashev, N., Monyer, H., Seeburg, P. H. and Sakmann, B. (1992) Neuron **8**, 189–196
9 Nakanishi, N., Shneider, N. A. and Axel, R. (1990) Neuron **5**, 569–581
10 Wenthold, R. J., Yokotani, N., Doi, K. and Wada, K. (1992) J. Biol. Chem. **287**, 501–507
11 Brose, N., Huntley, G. W., Stern-Bach, V., Sharma, G., Morrison, J. H. and Heinemann, S. F. (1994) J. Biol. Chem. **269**, 16780–16784
12 Bochet, P., Audinat, E., Lambolez, B., Crepel, F., Rossier, J., Iino, M., Tsuzuki, K. and Ozawa, S. (1994) Neuron **12**, 383–388
13 Geiger, J. R. P., Melcher, T., Joh, D.-S., Sakmann, B., Seeburg, P. H., Jonas, P. and Monyer, H. (1995) Neuron **15**, 193–204
14 Henley, M. H., Nielsen, M. and Barnard, E. A. (1992) J. Neurochem. **58**, 2030–2036
15 Honore, T. and Nielsen, M. (1985) Neurosci. Lett. **54**, 27–32
16 Hunter, C., Wheaton, K. D. and Wenthold, R. J. (1990) J. Neurochem. **54**, 118–125
17 Blackstone, C. D., Moss, S. J., Martin, L. J., Levey, A. I., Price, D. L. and Huganir, R. L. (1992) J. Neurochem. **58**, 1118–1126
18 Hampson, D. R., Huie, D. and Wenthold, R. J. (1987) J. Neurochem. **49**, 1209–1215
19 Chang, Y.-C., Lin, Y.-H., Lee, Y.-H. and Leng, C.-H. (1991) J. Neurochem. **57**, 1921–1926
20 Wu, T.-Y. and Chang, Y.-C. (1994) Biochem. J. **300**, 365–371
21 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22 Weber, K. and Osborn, M. (1969) J. Biol. Chem. **244**, 4406–4412
23 Katnik, I., Podgorska, M. and Dobryszycka, W. (1987) J. Immunol. Methods **102**, 279–282
24 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
25 Wray, W., Boulikas, T., Wray, V. P. and Hancock. R. (1981) Anal. Biochem. **118**, 198–203
26 Chang, Y.-C., Wu, T.-Z., Li, B.-F., Gao, L.-H., Liu, C.-I, and Wu, C.-L. (1996) Biochem. J. **319**, 49–57
27 Clarke, S. (1975) J. Biol. Chem. **250**, 5459–5469
28 Clarke, S. and Smigel, M. D. (1989) Methods Enzymol. **172**, 696–709
29 Martin, G. R. and Ames, B. N. (1961) J. Biol. Chem. **236**, 1372–1379
30 Osterman, L. A. (1984) in Methods of Protein and Nucleic Acid Research, vol. 1, Springer-Verlag, Berlin and New York, Chap. 5
31 Tanford, C. (1961) in Physical Chemistry of Macromolecules, John Wiley and Sons, New York, Chap. 6
32 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
33 Worthington Enzyme Manual (1972) Worthington Biochemical Incorporation, Freehold, NJ, U.S.A.
34 Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W. W. and Betz, H. (1988) Science **242**, 1050–1053
35 Liu, T. Y. (1977) in The Proteins, vol. 3, 3rd edn. (Neurath, H. and Hill, R. L. eds.), Academic Press, New York, p. 239
36 Wong, S. S. (1993) in Chemistry of Protein Conjungation and Crosslinking, CRC Press, Inc., Boca, Raton, Ann Arbor, Boston and London, p. 215
37 Haga, T., Haga, K. and Hulme, E. C. (1990) in Receptor Biochemistry, a Practical Approach (Hulme, E. C., ed.), pp. IRL Press, Oxford University Press, Oxford, New York and Tokyo, p. 10
38 Reynold, J. A. and Karlin, A. (1978) Biochemistry **17**, 2035–2038
39 Mamalaki, C., Stephenson, F. A. and Barnard, E. A. (1988) J. Neurochem. **52**, 124–134
40 Baron, M., Wisher, M. H., Thamm, P. M., Saunders, D. J., Brondenburg, D. and Sonksen, P. H. (1981) Biochemistry **20**, 4156–4161
41 Peterson, G. L., Rosenbaum, L. C., Broderich, D. J. and Schimerlik, M. J. (1986) Biochemistry **25**, 3189–3202
42 Unwin, N. (1993) Cell **72**/Neuron **10** (Suppl.), 31–41
43 Galzi, J.-L. and Changeux, J.-P. (1994) Curr. Opin. Struct. Biol. **4**, 554–565
44 Wo, Z. G. and Oswald, R. E. (1995) Trends Neurosci. **18**, 161–168
45 Hollmann, M., Maron, C. and Heinemann, S. (1994) Neuron **13**, 1331–1343
46 Stern-Bach, Y., Bettler, B., Hartley, M., Shephard, P. O., O'Hara, P. J. and Heinemann, S. (1994) Neuron **13**, 1345–1357
47 Bennett, J. A. and Dingledine, R. (1995) Neuron **14**, 373–384
48 Unwin, N. (1993) J. Mol. Biol. **229**, 1101–1124
49 Sutcliffe, M. J., Wo, Z. G. and Oswald, R. E. (1996) Biophys. J. **70**, 1575–1589
50 Wood, M. W., VanDongen, H. M. A. and VanDongen, A. M. J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4882–4886
51 Li, M., Unwin, N., Stauffer, K. A., Jan, Y.-N. and Jan, L. Y. (1994) Curr. Biol. **4**, 110–115
52 Zagotta, W. N. and Siegelbaum, S. A. (1996) Annu. Rev. Neurosci. **19**, 235–263
53 Hall, R. A., Kessler, M. and Lynch, G. (1992) J. Neurochem. **59**, 1997–2004
54 Florio, V. A. and Sternweis, P. C. (1985) J. Biol. Chem. **260**, 3477–3483
55 Luedtke, P. R. and Mokinoff, P. B. (1987) Biochem. Pharmacol. **36**, 3255–3264
56 Stephenson, F. A., Watkins, A. E. and Olsen, R. W. (1982) Eur. J. Biochem. **123**, 291–298
57 Nakazawa, K., Milawa, S., Hashikawa, T. and Ito, M. (1995) Neuron **15**, 697–709
58 McGlade-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D. A. and Soderling, T. R. (1993) Nature (London) **362**, 640–642
59 Raymond, L. A., Blackstone, C. D. and Huganir, R. L. (1993) Trends Neurosci. **16**, 147–153
60 Tan, S.-E., Wenthold, R. J. and Soderling, T. R. (1994) J. Neurosci. **14**, 1123–1129
61 Kullmann, D. M. and Siegelbaum, S. A. (1995) Neuron **15**, 997–1002

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