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Hydrodynamic and pharmacological characterization of putative α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate-sensitive L-glutamate receptors solubilized from pig brain

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L-[^{3}H]Glutamate binding sites with characteristics resembling that of membrane-bound α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-subtype L-glutamate receptors have been solubilized from pig brain synaptic junctions by Triton X-114. Binding of [^{3}H]AMPA to these soluble sites in the presence of KSCN results in a curvilinear Scatchard plot that can be resolved into a high-affinity component and a low-affinity component. These Triton-X-114-solubilized sites can be further separated into two species of binding sites by gel-filtration chromatography or sucrose-density-gradient centrifugation. The pharmacological profiles of these two species of binding site are almost identical, and the rank orders of potency for glutamatergic drugs in displacing L-[^{3}H]glutamate binding to these sites are quisqualate > 6,7-dinitroquinoxaline-2,3-dione > 6-cyano-7-nitroquinoxaline-2,3-dione > AMPA > L-glutamate > kainate ⩾ *N*-methyl-D-aspartate = L-2-amino-4-phosphonobutyrate. Both sites are found to bind [^{3}H]AMPA, and in the presence of KSCN the binding activities are significantly enhanced. Analysis of the hydrodynamic behaviour of these binding sites by sucrose-density-gradient centrifugation in H_2O- and ^2H_2O-based solvents and gel-filtration chromatography has revealed that one of these sites (Stokes radius 8.3 nm, sedimentation coefficient 18.5 S) consists of 562 kDa protein and 281 kDa detergent, and the other site (Stokes radius 9.6 nm, sedimentation coefficient 13.4 S) consists of 352 kDa protein and 569 kDa detergent. Frictional coefficients of these sites indicate that these receptor–detergent complexes are asymmetrical in structure, consistent with large transmembrane proteins.

INTRODUCTION

L-Glutamate is generally considered as the major excitatory neurotransmitter in the mammalian central nervous system. The neuronal activities of L-glutamate are mediated by at least five subtypes of receptors, including *N*-methyl-D-aspartate (NMDA)-, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate-, L-2-amino-4-phosphonobutyrate (L-APB)-, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid- and kainate-preferring subtype receptors (Monaghan et al., 1989). Numerous studies have already demonstrated that L-glutamate receptors not only mediate synaptic transmission along excitatory pathways, but also participate in the development of synaptic connections and in various brain functions, such as learning and memory (Mayer and Westbrook, 1987; Constantine-Paton et al., 1990).

A major physiological function of AMPA/kainate-subtype L-glutamate receptors is mediating fast synaptic transmission. Recently, cDNA clones encoding kainate-sensitive and AMPA-selective channel subunits have been obtained. The sizes of the polypeptides encoded by these cDNAs were found to be around 100 kDa (Hollmann et al., 1989; Keinanen et al., 1990; Boulter et al., 1990; Sakimura et al., 1990; Bettler et al., 1990, 1992; Nakanishi et al., 1990; Egebjerg et al., 1991). The co-expression of different cDNAs encoding these subunits results in channels with unique electrophysiological characteristics (Nakanishi et al., 1990; Burnashev et al., 1992), suggesting that native AMPA/kainate-subtype L-glutamate receptor is likely to be composed of hetero-oligomers. The complex structure of this receptor has also been implicated by the finding that it exists in two interchangeable states with different affinities for [^{3}H]AMPA, and the relaxation constant between these states is affected by chaotropic ions (Honore and Drejer, 1988; Hall et al., 1992). Few studies of the structural properties of AMPA/kainate-subtype receptor have been reported. Target-size analysis has suggested that the functional size of this receptor is 52 kDa (Honore and Nielsen, 1985). Gel-filtration analysis of this receptor solubilized from chick, pig and rat brain has indicated that the detergent–receptor complex may have a molecular mass ranging from 373 to 1020 kDa (Hampson and Wenthold, 1988; Hunter et al., 1990; Chang et al., 1991; Blackstone et al., 1992; Wenthold et al., 1992). Sucrose-density-gradient centrifugation analysis has shown that the sedimentation coefficient of detergent-solubilized L-glutamate receptors is between 11 and 18 S (Henley and Barnard, 1989; Blackstone et al., 1992). These estimates, however, are very likely to be lower than the real sedimentation coefficient, due to the lower partial specific volumes of protein standards used in these studies than those of detergent–receptor complexes (Martin and Ames, 1961).

L-Glutamate binding sites were first solubilized and partially purified from rat brain (Michaelis, 1975). Many preparations containing solubilized L-glutamate binding sites have also been reported (Michaelis et al., 1983; Kuonen and Roberts, 1987; Chen et al., 1988; Chang et al., 1990). Recently, kainate-, AMPA- and quisqualate-sensitive L-glutamate binding sites have been solubilized and partially purified from pig brain synaptic junctions in our laboratory (Chang et al., 1991). These soluble binding sites are intrinsic membrane glycoproteins and have a pharmacological profile resembling that of membrane-bound AMPA/kainate-subtype L-glutamate receptors. In the present study, we found that these Triton-X-114-solubilized L-[^{3}H]glutamate binding sites also bind [^{3}H]AMPA, and the binding activity

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; L-APB, L-2-amino-4-phosphonobutyrate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitroquinoxaline-2,3-dione.

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is enhanced by including KSCN in assay mixtures. These soluble L-[^{3}H]glutamate binding sites are further resolved into two species of binding sites with distinct sizes and sedimentation coefficients. The pharmacological properties of these sites have been characterized. The molecular masses of these solubilized receptors have also been evaluated according to their hydrodynamic properties.

MATERIALS AND METHODS

Materials

L-[^{3}H]Glutamic acid (50–60 Ci/mmol) and [^{3}H]AMPA (56.6 Ci/mmol) were purchased from Du Pont–NEN. Phenylmethanesulphonyl fluoride, Hepes, sucrose and Trizma were obtained from Sigma Chemical Co. Quisqualic acid, kainic acid, AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX) and L-APB were purchased from Research Biochemicals (U.S.A.). Sepharose CL-6B was purchased from Pharmacia. Other chemicals were purchased 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 μm pore size).

Solubilization of synaptic junctions

Synaptic junctions were prepared from the cerebral and hippocampal portions of fresh pig brains as described previously (Chang et al., 1991). Isolated synaptic junctions were solubilized by incubating with Triton X-114 (1 %, v/v) and KCl (0.15 M) on ice for 1 h. After being clarified by centrifugation (150000 g at 4 °C for 1 h), solubilized preparations were 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 two changes of dialysis buffer. The dialysed soluble preparation was used as starting material in further experiments. Protein concentrations of synaptic junctions were determined by the method of Bradford (1976) after samples had been incubated in 0.1 M NaOH at room temperature for 30 min. Triton-X-114-solubilized samples were quantified by the bicinchoninic acid method (Smith et al., 1985).

Sucrose-density-gradient centrifugation

This was performed with a SW-28 rotor ($r_{av.}$ 11.82 cm; Beckman). Sucrose gradients [5–30 % (w/v) sucrose in buffer A plus 0.1 % Triton X-114] with a total volume of 25 ml were prepared with a gradient maker (Jule, U.S.A.). Sucrose concentrations were determined with a refractometer (Atago Co., Japan). Gradients were kept at 5 °C for 4–6 h before use. Solubilized synaptic junctional preparations were applied on top of the gradient. Gradients were then centrifuged at 5 °C for various lengths of time. After centrifugation, sample was 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 peak fraction of binding sites. Viscosities were determined with a viscometer (LVIDV-11, Brookfield; sample size 0.5 ml, setting 60 rev./min) with pure water as the reference.

Gel-filtration chromatography

This was carried out at 4 °C with a Sepharose CL-6B column (2.5 cm × 42 cm) which had been pre-equilibrated with buffer A plus Triton X-114 at concentrations ranging from 0.1 to 1 % (v/v). It was found that changes in the detergent concentration used in the elution buffer (0.1, 0.2 or 1.0 %, v/v) did not affect the elution volumes (variation was within 2 %) of solubilized L-[^{3}H]glutamate binding sites.

Radioligand-binding assay

L-[^{3}H]Glutamate binding to the soluble samples was quantified by the poly(ethylene glycol)–immunoglobulin precipitation method as described previously (Chang et al., 1991). [^{3}H]AMPA binding to the soluble samples was determined by a filtration method (Bruns et al., 1983). Filtration assays were performed by the following procedures. Reaction mixtures (final volume 210 μl) containing soluble binding sites, [^{3}H]AMPA (final concn. 20 nM) and other reagents in buffer A plus 1 % Triton X-114 were incubated at 4 °C for 1 h. After incubation, 200 μl of the reaction mixture was removed and filtered through a GF/B glass-fibre filter membrane mounted on a cell-harvester (Hoeffer 425) under vacuum. Before use, the GF/B membrane had been soaked in polyethyleneimine solution (0.33 %, v/v) for at least 1 h. After samples had completely passed through the membrane, 10 ml of ice-cold washing solution (50 mM Tris/acetate, pH 7.4) was evenly applied to the membrane to remove residual radioligand bound to the membrane. Membranes were then transferred to scintillation vials, mixed with 10 ml of scintillation fluor (Ultrafluor; National Diagnostics), and counted for radioactivity with a liquid-scintillation counter (1600TR, Packard) 12 h later. Nonspecific binding of L-[^{3}H]glutamate and [^{3}H]AMPA was determined in the presence of 0.5 mM unlabelled L-glutamate. Data for L-[^{3}H]glutamate and [^{3}H]AMPA binding to solubilized preparations were analysed by the software Kinetics, EBDA, Ligand and Lowry from Elsevier–Biosoft. For pharmacological characterization of the slow and fast sites, fractions containing these sites were pooled from four to six gradients. Eight concentrations of each glutamatergic drug were tested, and assays were performed in triplicate.

Determination of hydrodynamic properties

Hydrodynamic properties of Triton-X-114-solubilized L-[^{3}H]glutamate binding sites were investigated by the methods of Clarke (1975) and Clarke and Smigel (1989). The velocity of a macromolecule travelling in a centrifugal field through a sucrose density gradient is approximately constant and is independent of the angular acceleration (Martin and Ames, 1961). The sedimentation coefficient of a macromolecule 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 point of the migration of the particle. Assuming that these binding sites bind the same amount of detergent in H_2O and ^2H_2O, the partial specific volumes ($\bar{v}$) of detergent-binding-site complexes were evaluated by eqn. (2):

$$s_{5,H_2O}/s_{5,D_2O} = (\eta^{5\,°C}{}_{D_2O}/\eta^{5\,°C}{}_{H_2O})(1-\rho^{5\,°C}{}_{H_2O}\bar{v})/(1-\rho^{5\,°C}{}_{D_2O}\bar{v}) \quad (2)$$

where $\eta^{5\,°C}{}_{H_2O}$, $\eta^{5\,°C}{}_{D_2O}$, $\rho^{5\,°C}{}_{H_2O}$ and $\rho^{5\,°C}{}_{D_2O}$ are viscosities and densities at $r_{avg.}$ in H_2O- or ^2H_2O-based sucrose density gradients. $s_{20,w}$, the sedimentation coefficient at 20 °C in pure water, can then be calculated by eqn. (3):

$$s_{20,w} = (s_{T,m})(\eta^{T}{}_{m})(1-\rho^{20\,°C}{}_{w}\bar{v})/(\eta^{20\,°C}{}_{w})(1-\rho^{T}{}_{m}\bar{v}) \quad (3)$$

By using the values of Stokes radius (a), $s_{20,w}$ and v as described above, the molecular mass M, of binding-site complexes can be estimated by eqn. (4):

$$M = (6\pi\eta^{20\,°C}{}_{w}Na)(s_{20,w})/(1-\rho^{20\,°C}{}_{w}\bar{v}) \quad (4)$$

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

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

$$\bar{v}_c = \bar{v}_p\left(\frac{1}{1+\alpha}\right)+\bar{v}_d\left(\frac{\alpha}{1+\alpha}\right) \quad (6)$$

where M_c is the molecular mass of the detergent–receptor 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 detergent/protein weight ratio of the detergent–receptor complex. The frictional coefficients of binding sites were evaluated by eqn. (7) (Tanford, 1961):

$$f/f_0 = a[4\pi N/3M(v+\delta/\rho^{20}{}_w)]^{\frac{1}{3}} \quad (7)$$

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 (Tanford, 1961).

Table 1 Effects of KSCN on [³H]AMPA binding activity

Data represent means ± S.D. of three independent experiments: *$P < 0.05$ (Student t test) versus the ratio for the solubilized synaptic junctions. Assay mixtures containing 20 nM [³H]AMPA and different samples in the absence or presence of 0.1 M KSCN were incubated at 4 °C for 1 h before filtration through polyethyleneimine-soaked glass-fibre membranes.

	[³H]AMPA binding activity (pmol/mg of protein)		Ratio
	−KSCN	+KSCN	+KSCN/−KSCN
Solubilized synaptic junctions	0.15 ± 0.05	0.51 ± 0.01	3.5 ± 0.1
Fast binding site	0.87 ± 0.28	3.11 ± 1.01	3.7 ± 0.1
Slow binding site	0.08 ± 0.03	0.17 ± 0.05	2.2 ± 0.1*

RESULTS

[³H]AMPA binding

[³H]AMPA binding to Triton-X-114-solubilized synaptic junctions was quantified by the filtration assay using polyethyleneimine-soaked glass-fibre filters. Inclusion of KSCN in assay mixtures resulted in a 3.5 ± 0.1-fold increase in [³H]AMPA bound to the solubilized preparation (Table 1). In the absence of KSCN, a single class of binding sites was observed with K_d of 0.67 ± 0.07 μM and $B_{max.}$ of 1.88 ± 0.15 pmol/mg of protein (means ± S.D., $n = 3$). The Hill coefficient was 0.98 ± 0.04 ($n = 3$). In the presence of KSCN, a curvilinear Scatchard plot of [³H]AMPA binding was observed (results not shown). A two-site model provided a significantly better fit to this curve than did a one-site model (F test, $P = 0.01$). The K_d and $B_{max.}$ values of the high-affinity site were respectively 9.9 ± 0.5 nM and

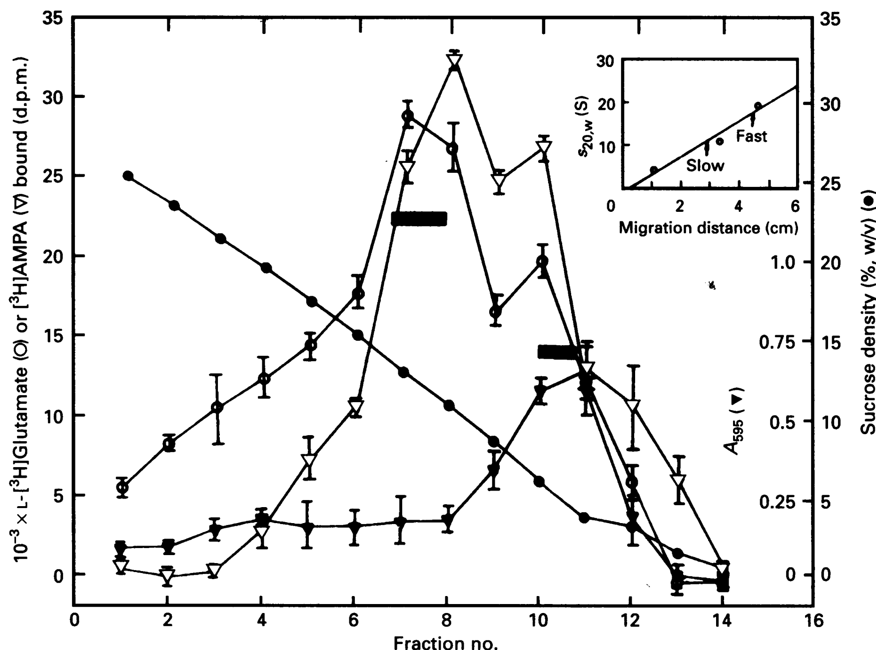

Figure 1 Sucrose-density-gradient centrifugation analysis of L-[³H]glutamate and [³H]AMPA binding sites solubilized from synaptic junctions

Solubilized preparation (5 ml) was applied to the gradient containing Triton X-114 (0.1%). Centrifugation (83000 g) was carried out for 24 h at 5 °C in a SW-28 rotor. Then 2 ml fractions were collected from the bottom. Fractions under the horizontal bars were pooled and used in the experiments of Figures 3 and 4. A 200 μl portion of each fraction was removed and incubated with L-[³H]glutamate (252 nM) for assaying L-glutamate binding activity (○) in the absence or presence of unlabelled L-glutamate (0.5 mM). The resulting specific binding data were shown here. A 10 μl sample was removed for protein determination (▼). Sucrose density of each fraction was determined with a refractometer (●). The calibration curve (inset) was obtained by using standards including thyroglobulin (19 S), catalase (11.3 S) and BSA (4.3 S). The apparent sedimentation coefficients of the fast and slow sites were calculated to be 16.3 and 10.9 S respectively. Each value represents the mean (± S.D.) of three determinations. The results are taken from a single representative experiment of a total of three experiments. Another gradient was centrifuged and fractionated under the same conditions as described above. A 200 μl portion of each fraction was removed and assayed for [³H]AMPA binding activity (▽). Each value represents the mean (± S.D.) of three determinations.

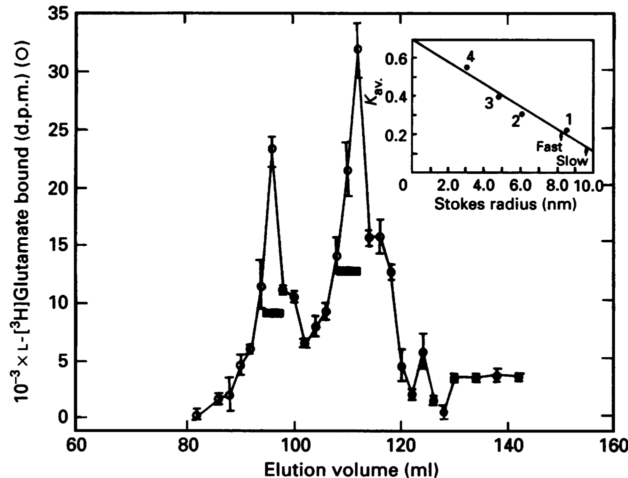

Figure 2 Gel-filtration chromatography analysis of L-[³H]glutamate binding sites solubilized from synaptic junctions

Solubilized preparation (5 ml) was applied to a column of Sepharose CL-6B (2.4 cm × 42 cm) and eluted with buffer A plus Triton X-114 (0.1%); 2 ml fractions were collected. Samples (200 μl) were removed and assayed for L-[³H]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 representative experiment of a total of two experiments. The calibration (inset) was obtained using thyroglobulin, catalase, alcohol dehydrogenase and ovalbumin. The Stokes radii of two major peaks were calculated to be 8.3 and 9.6 nm. $K_{av.} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume of binding sites, V_0 = void volume and V_t = total gel bed volume. Fractions under the horizontal bars were pooled and used in the experiments of Figure 3.

0.46 ± 0.07 pmol/mg of protein, and the K_d and $B_{max.}$ values of the low-affinity site were respectively 1.53 ± 0.93 μM and 2.19 ± 0.66 pmol/mg of protein (means ± S.D., $n = 3$).

Analysis of soluble binding sites by sucrose-density-gradient centrifugation and gel-filtration chromatography

Two species of L-[³H]glutamate binding sites were found after sucrose-density-gradient centrifugation of the solubilized preparation (Figure 1). A similar profile for [³H]AMPA binding activity was also observed. The binding activity recovered in fractions under these two peaks (as shown under the horizontal bars in Figure 1) was $30 \pm 8\%$ ($n = 4$) of the total activity applied. The apparent sedimentation coefficients of these binding sites were estimated from a standard curve (inset in Figure 1) to be 16.3 and 10.9 S, designated as the fast and slow sites respectively. Most of the proteins in the sample were found in fractions near those containing the slow site. Similarly to the results of a previous study (Chang et al., 1991), L-[³H]glutamate binding sites in the solubilized preparation were also resolved into two peaks by gel-filtration chromatography (Figure 2). The binding activity recovered in fractions under these two peaks was $59 \pm 6\%$ ($n = 2$) of the total activity applied to the column. According to the calibration curve shown in the inset of Figure 2, the Stokes radii of these binding sites were estimated to be 8.3 ± 0.1 and 9.6 ± 0.1 nm ($n = 2$), so the sites were designated as the 8.3 nm and 9.6 nm sites respectively. The 8.3 and 9.6 nm sites (Figure 2) were found to correspond respectively to the fast and slow sites (Figure 1) on the basis of the following observations. First, sucrose-density-gradient centrifugation of the 8.3 nm site resulted in two peaks of binding sites, with the major peak co-migrating with the fast site. On the other hand, sucrose-density-gradient centrifugation of the 9.6 nm site resulted in two peaks of binding sites, with the major peak co-migrating with the slow site (Figures 3a and 3b). Second, gel-filtration chromatography of the fast and slow binding sites as obtained in Figure 1 revealed

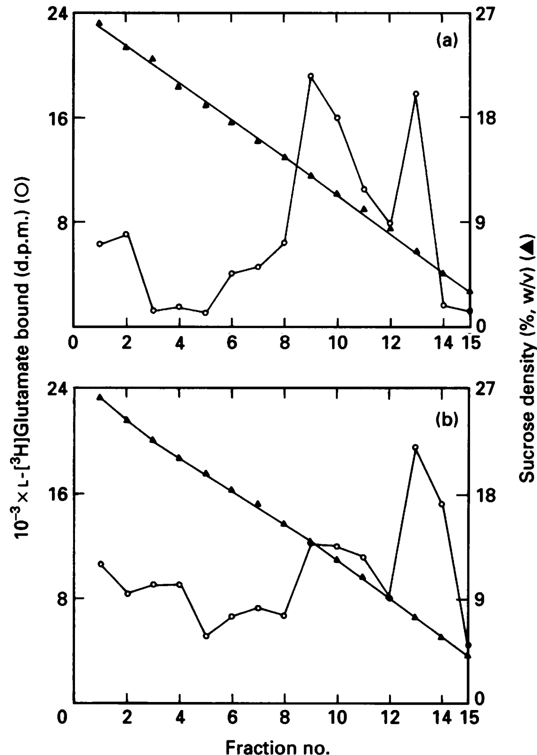

Figure 3 Sucrose-density-gradient centrifugation analysis of the 8.3 nm site (a) and the 9.6 nm site (b)

A 4 ml portion of pooled sample containing the 9.6 and 8.3 nm sites as obtained in the gel-filtration chromatography experiment of Figure 2 was applied to sucrose density gradients. The centrifugation (83000 g) was carried out for 24 h at 5 °C; 2 ml fractions were collected from the bottom, and 200 μl of each fraction was removed for assaying L-[³H]glutamate binding activity (○). ▲, Sucrose density.

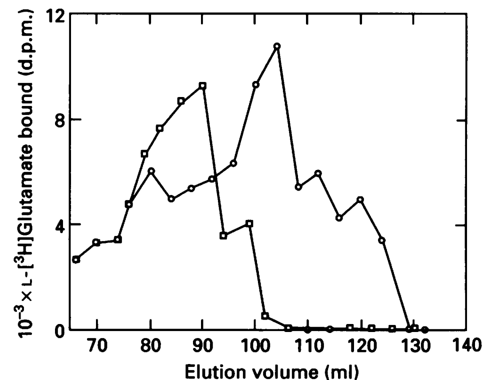

Figure 4 Gel-filtration chromatography analysis of the fast site (○) and the slow site (□) as obtained by sucrose-density-gradient centrifugation of the Triton X-114-solubilized preparation from Figure 1

Samples (5 ml) were applied to a Sepharose CL-6B column (2.5 cm × 42 cm) and eluted by buffer A plus Triton X-114 (0.1%); 2 ml fractions were collected. A 200 μl portion of each fraction was assayed for L-[³H]glutamate binding activity.

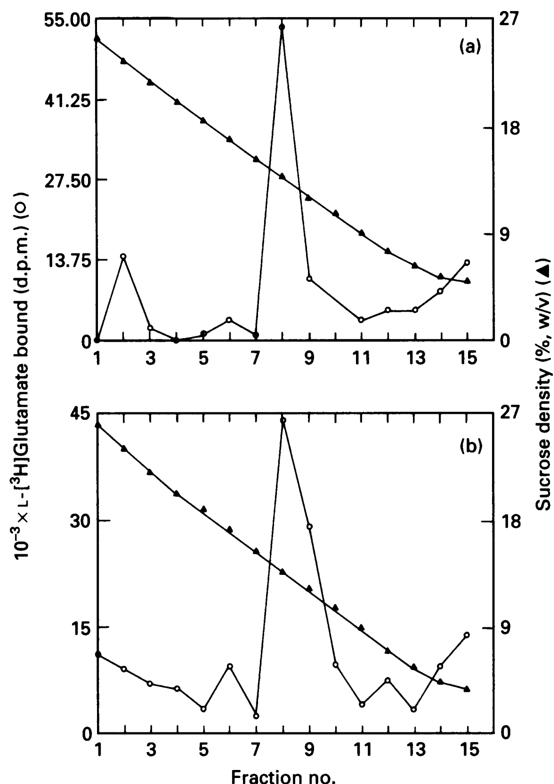

Figure 5 Sucrose-density-gradient centrifugation analysis of the fast site (a) and the slow site (b) as obtained by sucrose-density-gradient centrifugation of Triton X-114-solubilized preparation from Figure 1

Samples (3 ml) were added to sucrose density gradients, and centrifugation (83000 g) was carried out for 24 h at 5 °C; 2 ml fractions were collected from the bottom, and 200 μl of each fraction was removed for assaying L-[^{3}H]glutamate binding activity (○). ▲, Sucrose density.

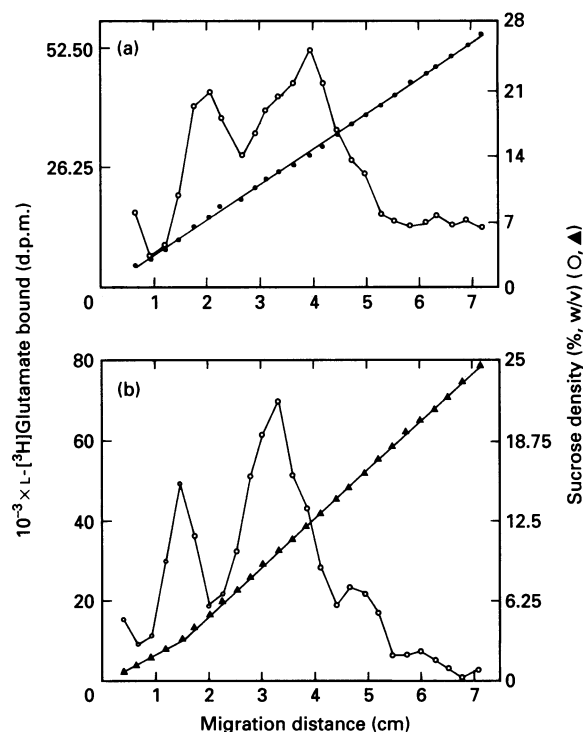

Figure 6 Sucrose-density-gradient centrifugation analyses of L-[^{3}H]glutamate binding sites in solubilized synaptic junctions in H_2O-based (a) and ^2H_2O-based (b) gradients

A 4 ml portion of solubilized preparation was applied to the gradient, and centrifugation (83000 g) was carried out for 24 h in a H_2O-based gradient or for 45 h in a ^2H_2O-based gradient; 1 ml fractions were collected from the bottom. A 200 μl portion of each fraction was removed for assaying L-[^{3}H]glutamate binding activity (○). ●, ▲, Sucrose density. The results are taken from a single representative experiment of a total of three experiments.

Table 2 Inhibition constants (K_i values) of various drugs on L-[^{3}H]glutamate binding

IC_{50} values were obtained from three experiments. In each experiment at least eight concentrations (between 0.01 and 100 μM) of ligand were examined. K_i values (means ± S.D.) were calculated from these IC_{50} values by using the equation of Cheng and Prusoff (1973).

	K_i (μM)	
Ligand	Fast site	Slow site
Quisqualate	0.08 ± 0.01	0.08 ± 0.01
DNQX	0.21 ± 0.08	0.14 ± 0.01
CNQX	0.28 ± 0.10	0.21 ± 0.02
AMPA	0.36 ± 0.02	0.33 ± 0.03
L-Glutamate	0.69 ± 0.17	0.92 ± 0.18
Kainate	1.71 ± 0.28	2.22 ± 0.42
NMDA	> 100	> 100
L-APB	> 100	> 100

that the apparent sizes of the fast and slow sites respectively resembled those of the 8.3 and 9.6 nm sites (Figure 4). When the fast and slow sites (Figure 1) as separated by sucrose-density-gradient centrifugation were again analysed by the same technique, a single peak of binding site was found in either sample (Figures 5a and 5b), which migrated with a velocity identical with that of the fast site as shown in Figure 1.

Pharmacological characterization of soluble binding sites

The pharmacological properties of the fast and slow L-glutamate binding sites as separated by sucrose-density-gradient centrifugation were studied (Table 2). The K_d and $B_{max.}$ values of L-[^{3}H]glutamate binding to the fast site were 0.90 ± 0.23 μM and 49.7 ± 6.1 pmol/mg of protein respectively, and the Hill coefficient was 1.03 ± 0.12 (means ± S.D., $n = 3$). The K_d and $B_{max.}$ values of L-[^{3}H]glutamate binding to the slow site were 0.90 ± 0.16 μM and 13.3 ± 2.5 pmol/mg of protein respectively, and the Hill coefficient was 0.96 ± 0.04 (means ± S.D., $n = 3$). L-[^{3}H]Glutamate bound to fast and slow sites could be displaced by agonists and antagonists for non-NMDA receptors. The rank orders of potencies of both sites were: quisqualate > DNQX > CNQX > AMPA > L-glutamate > kainate ≫ NMDA = APB. The K_i values of these ligands for the fast and slow binding sites were shown in Table 2. Similarly to that of Triton-X-114-solubilized synaptic junctional preparations, [^{3}H]AMPA binding activities of the fast and slow sites were found to increase 3.7 ± 0.1 ($n = 3$)- and 2.2 ± 0.1 ($n = 3$)-fold respectively, when KSCN (0.1 M) was included in assay mixtures (Table 1).

Study of the hydrodynamic properties

Figure 6(a) shows the profile of L-[^{3}H]glutamate binding activity after centrifugation for 24 h at 83000 g (r_{av} 11.82 cm) at 5 °C in a H_2O-based sucrose density gradient. When the centrifugation was carried out for 20 and 24 h, the v values of the fast site were

Table 3 Physical properties of Triton-X-114-solubilized L-[^{3}H]glutamate binding sites

Stokes radii, sedimentation coefficients and partial specific volumes were means ± S.D. of two or three independent experiments (n, in parentheses). The means of these data were used in the calculation of the remaining data shown in this Table.

	Fast site	Slow site
Stokes radius (nm)	8.3 ± 0.1 (n = 2)	9.6 ± 0.1 (n = 2)
Sedimentation coefficient (S)	18.5 ± 0.4 (n = 3)	13.4 ± 0.5 (n = 3)
Partial specific volume (cm^3/g)	0.79 ± 0.06 (n = 3)	0.84 ± 0.11 (n = 3)
Molecular mass (kDa)		
Protein–detergent complex	843	921
Protein component	562	352
Detergent/protein (g/g)	0.5	1.62
Frictional coefficient	1.20	1.33

0.16 and 0.17 cm/h respectively, and those for the slow site were 0.11 and 0.11 cm/h. This result showed that the average migration velocities of these binding sites in a sucrose density gradient were essentially constant, as predicted by Martin and Ames (1961). Figure 6(b) shows the profile of L-glutamate binding sites after centrifugation for 45 h in a ^2H_2O-based sucrose density gradient, and the v values of the fast and slow sites were calculated to be 0.074 and 0.030 cm/h respectively. By eqn. (1), the sedimentation coefficients, s_{5,H_2O}, of the fast and slow sites in a H_2O-based sucrose density gradient were calculated to be 6.25 ± 0.20 and 4.51 ± 0.21 S (n = 3) respectively. The sedimentation coefficients, s_{5,D_2O}, of the fast and slow sites in a ^2H_2O-based sucrose density gradient are 3.20 ± 0.10 and 1.44 ± 0.17 S (n = 3) respectively. The partial specific volumes of the fast and slow sites were thus calculated by eqn. (2) to be 0.79 ± 0.06 and 0.84 ± 0.11 cm^3/g (n = 3) respectively. The $s_{20,w}$ values of the fast and slow sites were calculated by eqn. (3) to be 18.5 ± 0.4 and 13.4 ± 0.5 S (n = 3) respectively. The molecular masses, M, of the fast and slow binding sites were estimated by eqn. (4) to be 843 and 921 kDa respectively. When the average partial specific volume of proteins, 0.73 cm^3/g (the average value between 0.71 and 0.75 cm^3/g), was used for $\bar{v}_p$ and the partial specific volume of Triton-series detergents, 0.908 cm^3/g, was used here for $\bar{v}_d$ (Haga et al., 1990) in eqns. (5) and (6), the α values of the fast and slow sites were calculated to be 0.50 and 1.62 respectively. The M_p values of the fast and slow sites were then calculated to be 562 and 352 kDa respectively. The fast and slow sites thus contain 281 and 569 kDa of detergent respectively. From eqn. (7), the frictional coefficients of the fast and slow sites are 1.20 and 1.33 respectively. The hydrodynamic parameters and molecular masses of the fast and slow L-[^{3}H]glutamate binding sites are listed in Table 3.

DISCUSSION

L-Glutamate binding sites solubilized from pig brain synaptic junctions resemble the membrane-bound AMPA/kainate-subtype L-glutamate receptors in the following aspects. First, the pharmacological profiles of various glutamatergic drugs in displacing L-[^{3}H]glutamate binding to the soluble sites and synaptic receptors are almost identical (Chang et al., 1991). Second, as for the synaptic receptors, the addition of KSCN to assay mixtures results in an enhancement of [^{3}H]AMPA binding to the soluble binding sites and produces a curvilinear Scatchard plot of [^{3}H]AMPA binding which can be resolved into a high-affinity component and a low-affinity component (Honore and Drejer, 1988; Hall et al., 1992). Third, the soluble binding sites are intrinsic membrane glycoproteins recognized by wheat-germ agglutinin (Chang et al., 1991). The same lectin has been reported to block the desensitization of AMPA/kainate receptors (Zorumski et al., 1990; Vyklicky et al., 1991). Finally, these soluble binding sites are prepared from purified synaptic junctions where non-NMDA receptors are concentrated (Foster et al., 1981; Jones and Baughman, 1991). As a result, it is concluded that Triton-X-114-solubilized L-[^{3}H]glutamate binding sites obtained in this study are most likely to represent the soluble form of AMPA/kainate-sensitive L-glutamate receptors of pig brain.

Two species of binding sites with different sizes and sedimentation coefficients have been found in Triton-X-114-solubilized preparations. The sedimentation coefficients of these sites have been evaluated by two methods. The first method involves the use of a calibration curve constructed for three proteins and their sedimentation coefficients (Figure 1), and the coefficients thus obtained are called apparent sedimentation coefficients. The second method involves the measurements of the migration velocities of these sites under a centrifugal field along a H_2O- or ^2H_2O-based sucrose density gradient, and the coefficients thus obtained represent the real sedimentation coefficients of these binding sites. Consistent with the prediction of Martin and Ames (1961), the real sedimentation coefficients of Triton-X-114-solubilized L-glutamate receptors are higher than their apparent sedimentation coefficients.

The Stokes radius and apparent sedimentation coefficient of the fast site, 8.3 nm and 16.3 S, found in this study are similar to those of kainate- or AMPA-binding sites solubilized from other sources reported previously. The apparent sedimentation coefficient of [^{3}H]AMPA binding sites solubilized from chick brain has been reported to be 18 S (Henley and Barnard, 1989). The Stokes radius of the quisqualate-sensitive [^{3}H]kainate binding site solubilized from rat brain is found to be 8.27 nm (Hampson et al., 1987). In a more recent report, the Stokes radius of [^{3}H]AMPA binding sites solubilized by Chaps from rat brain has been estimated to be 8.1 nm, and the apparent sedimentation coefficient of these binding sites is 18.0 S (Blackstone et al., 1992). These Chaps-solubilized rat [^{3}H]AMPA binding sites appear to contain L-glutamate receptor subunits encoded by cDNA GluR 1. The close resemblance between the fast sites obtained in this study and the AMPA- and kainate-binding sites reported previously in their Stokes radii, apparent sedimentation coefficients and pharmacological properties suggests that AMPA/kainate-subtype receptors solubilized from chick, rat or pig brain share common structural features. Based on the hydrodynamic behaviour of the major soluble L-glutamate binding sites, the fast sites, obtained here, the molecular mass of protein component of this binding site is calculated to be 562 kDa and that of the surrounding detergents is 281 kDa. The friction coefficient of this detergent–receptor complex is estimated to be 1.20, which suggests that the detergent–receptor complex behaves as a particle with the shape of either a prolate or an oblate ellipsoid with an axial ratio between 4 and 5. Although the accuracy of these axial-ratio estimates is affected by the actual degree of hydration of this complex, the result is consistent with the idea that this L-glutamate receptor is a large transmembrane protein.

In addition to the fast L-glutamate binding sites described above, a minor binding site with a Stokes radius of 9.6 nm and sedimentation coefficient of 13.4 S is also found. This binding site consists of 65% detergent and 35% protein. The frictional coefficient of the slow site is 1.33, suggesting that this detergent–

receptor complex behaves as a prolate or an oblate ellipsoid with an axial ratio between 5 and 7. Despite the similarities between the slow and fast L-glutamate binding sites in their affinities for L-[^{3}H]glutamate, pharmacological profiles for various glutamatergic drugs and KSCN-induced activation of [^{3}H]AMPA binding, these two sites differ significantly in molecular masses. Interestingly, the slow site can be converted into the fast site by repeated sucrose-density-gradient centrifugation, indicating that these two sites share some common structural determinants. Assuming that the 562 kDa protein component of the fast site is an oligomer of different subunits, as suggested for L-glutamate receptors (Nakanishi et al., 1990; Burnashev et al., 1992), the 352 kDa protein component of the slow site is likely to represent a part of this oligomeric structure. In the solubilized preparation, the structure of the 352 kDa receptor may be stabilized by some unidentified factors such as residual lipids in the preparation, or an unique detergent structure with a mass of 569 kDa, or proteins. Further purification of the solubilized preparation by sucrose-density-gradient centrifugation (as shown in this study), or by anion-exchange chromatography and wheat-germ agglutinin affinity chromatography (Chang et al., 1991) may remove many components from the binding-site complexes, including those stabilizing the 352 kDa form of the receptor. As a result, a part of the 352 kDa binding site may re-associate to form the 562 kDa oligomeric structure.

The results obtained here suggest that Triton-X-114-solubilized AMPA/kainate-sensitive L-glutamate receptors stay in a major and a minor form with molecular masses of 562 and 352 kDa respectively. That native non-NMDA L-glutamate receptors having structures with different sizes has also been suggested by a recent study by Wenthold et al. (1992). These authors have found that the purification of synaptic membrane samples, either chemically cross-linked or not, by immunoaffinity chromatography specific for non-NMDA receptors results in multiple proteins with molecular masses of 108, 325, 470 and 590 kDa. Among these proteins, the 325 and 590 kDa proteins may be the rat brain equivalents of the protein components in the slow and fast sites as obtained here.

The sizes of different subunits of AMPA/kainate-sensitive L-glutamate receptors were deduced from their cDNAs as around 100 kDa. The major form of AMPA/kainate-sensitive L-glutamate binding sites found here, the fast sites, may represent a pentamer of these subunits. Such a structure has been suggested, based on the fact that ionotropic L-glutamate receptor subunits share structural features with other neurotransmitter-gated ion channels, such as γ-aminobutyric acid, nicotinic acetylcholine and glycine receptors (Swope et al., 1992; Nakanishi, 1992). These channels are thought to be hetero-oligomeric complexes consisting of five subunits, each of which contains four membrane-spanning segments. The minor form of AMPA/kainate-sensitive L-glutamate binding sites, the slow sites, may represent a trimer of the subunits of AMPA/kainate-sensitive L-glutamate receptors. The presence of the slow sites in the detergent-solubilized preparations may be resulted from the partial dissociation of the pentameric fast sites. Alternatively, the slow sites may represent a particular form of L-glutamate receptor in neurons, similar to the trimeric form of acetylcholine receptor found during the process of subunit oligomerization (Green and Claudio, 1993). Furthermore, the solubilized L-glutamate binding sites obtained here may also include other polypeptides such as the modulatory subunit, as proposed by studies using radiation-inactivation techniques (Henley et al., 1992; Honore and Nielsen, 1985). This will be answered when these solubilized putative AMPA/kainate-sensitive receptors are further purified and characterized in the future.

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