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Selective association of *N*-methyl aspartate and quisqualate types of L-glutamate receptor with brain postsynaptic densities

(excitatory amino acids/receptor classes/synapse/subcellular fractionation/radioligand binding)

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ABSTRACT **Recognition sites for the excitatory neurotransmitter, L-glutamate, were studied in synaptic plasma membranes and postsynaptic densities (PSDs) isolated from rat brains. The results demonstrate (*i*) that L-glutamate binding sites may be resolved into three distinct subtypes (categories A1, A2, and A4), each corresponding to an electrophysiologically identified receptor class, and (*ii*) that the *N*-methyl aspartate (A1) and quisqualate (A2) receptor types are selectively associated with PSDs. L-[^{3}H]Glutamate bound to an apparently homogeneous population of sites in PSDs with a K_d of 3.39×10^{-7} M and a B_{max} (maximum number of binding sites) of 6.1 pmol/mg of protein. Inhibition studies demonstrated that these sites could be resolved into two distinct subtypes. *N*-Methyl aspartate maximally inhibited 58% of PSD-located L-glutamate binding sites with a K_i of 7.2×10^{-6} M (the A1 site), and quisqualate inhibited 42% with a K_i of 1.1×10^{-6} M (the A2 site); the effects of both substances were additive. Experiments with a range of acidic amino acid analogues indicated that the ligand selectivities of these two binding sites conformed to those of the *N*-methyl D-aspartate and quisqualate receptor classes defined electrophysiologically. The Cl^--dependent population of L-glutamate binding sites (the A4 site), which predominates in synaptic membranes, was absent from isolated PSDs.**

Excitatory synaptic mechanisms in the brain are thought to involve L-glutamate as a neurotransmitter (1, 2). The neuronal receptors for this amino acid have been studied electrophysiologically and have been subdivided into four classes by pharmacological analysis. The most selective compounds for these receptor classes are *N*-methyl D-aspartate (*N*MeDAsp), quisqualate, kainate and L-2-amino-4-phosphonobutyrate (L-APB), while the natural transmitter, L-glutamate, acts at each of these sites (1, 3, 4).

One strategy for analyzing the biochemical properties of neurotransmitter receptors has been to investigate those sites in isolated brain membranes that are labeled by selective receptor-active ligands (5). Using this approach, we recently demonstrated two distinct populations of Na^+-independent L-[^{3}H]glutamate binding sites in synaptic plasma membranes (SPMs) (6–8). One of these is dependent on Cl^-, is stimulated by Ca^{2+}, and pharmacologically resembles the L-APB-sensitive class of excitatory amino acid receptor (the A4 site; nomenclature according to ref. 4), which has been demonstrated electrophysiologically at specific synapses in the brain and spinal cord (9–12). The Cl^-/Ca^{2+}-independent binding sites in SPMs have been less well characterized but are probably a mixed population, since they display some properties expected of the *N*MeDAsp receptor class and are also sensitive to inhibition by quisqualate (7, 13).

The existence of these two types of L-glutamate binding site raises several questions. Do they represent distinct molecules or are they modified forms of the same molecule? Are they all involved in postsynaptic function or are they receptors with junctional, extrajunctional, or presynaptic roles? Earlier binding studies do not address these questions, since the SPM fractions routinely used comprise a variety of synaptic and extrasynaptic membrane structures (14, 15). Previously, we showed that of two γ-aminobutyrate binding sites present in brain SPMs, only one is associated with the postsynaptic density (PSD) (16). The PSD is a proteinaceous organelle associated with the postjunctional membrane and is thought to provide a framework for the attachment of transmitter receptors and other elements participating in the postsynaptic neuronal response (14, 15, 17, 18). It is an especially prominent component of excitatory (type I) synapses (17). In the present study, we have examined the binding of L-[^{3}H]-glutamate to isolated PSDs as a means of (*i*) better defining binding site heterogeneity and (*ii*) determining the nature of those transmitter recognition sites that initiate the excitatory response in the postsynaptic neuron. Our data indicate that the Cl^--dependent type of L-glutamate binding site (class A4), which is the major population present in SPMs, is absent from the PSD. Those sites present in PSDs may be subdivided into two populations which, as in the case of the electrophysiologically derived receptor classification scheme, are differentially sensitive to N-methyl DL-aspartate (*N*MeAsp) and quisqualate (sites A1 and A2, respectively).

MATERIALS AND METHODS

Chemicals. L-[G-^{3}H]Glutamic acid of specific radioactivity of 29–45 Ci/mmol (1 Ci = 37 GBq) was purchased from Amersham. Triton X-100 (gas chromatographic grade) and the L and D isomers of glutamic and aspartic acids were obtained from Merck; *N*MeAsp, quinolinic, and kainic acids, from Sigma; quisqualic, ibotenic, DL-2-amino-5-phosphonovaleric (DL-APV), and DL-2-amino-7-phosphonoheptanoic (DL-APH) acids, from Cambridge Research Biochemicals (Harston, U.K.); and DL-2-amino-3-phosphonopropionic acid and DL-APB, from Calbiochem (La Jolla, CA). DL-2-amino-6-phosphonohexanoic acid was kindly provided by J. F. Collins (London).

Subcellular Fractionation. SPMs and PSDs were isolated from fresh brains (excluding brainstem) of adult male rats (strain RAI, 180–250 g; Ciba–Geigy). The SPM fraction was prepared by the method of Jones and Matus (19), and this was treated with either 0.5% or 1.0% Triton X-100, followed by centrifugation through 1.0 M sucrose, to yield PSDs (18, 20) (the results obtained with either concentration of Triton X-100 were indistinguishable and were pooled). Fractions were washed four times by resuspension and cen-

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Abbreviations: PSD, postsynaptic density; SPM, synaptic plasma membrane; APB, 2-amino-4-phosphonobutyrate; APH, 2-amino-7-phosphonoheptanoate; APV, 2-amino-5-phosphonovalerate; *N*MeAsp, *N*-methyl DL-aspartate; *N*MeDAsp, *N*-methyl D-aspartate.

trifugation in 0.5 mM Hepes/KOH buffer (pH 7.2) and finally were resuspended in a small volume of the same buffer for protein determination (21). The morphology and composition of SPMs and PSDs previously has been documented in detail (14, 15, 17–20).

L-[^{3}H]Glutamate Binding Assay. The specific Na^+-independent binding of L-[^{3}H]glutamic acid to SPMs and PSDs was determined by using a centrifugation procedure (6, 22). Aliquots of SPM (200 μg of protein) or PSD (50–100 μg of protein) were incubated in triplicate in a final volume of 1.0 ml with 50 nM L-[^{3}H]glutamate (except for K_d determinations; see Fig. 1) and other substances as indicated in the *Results*. Unless stated otherwise, the assay buffer was 50 mM Tris acetate (pH 7.0). After 30 min at 32°C, tubes were centrifuged for 3 min in an Eppendorf microcentrifuge, and the supernatant was aspirated. Radioactivity in the pellets was determined by liquid scintillation spectrometry (45% counting efficiency) after dissolution in 2% $NaDodSO_4$ and addition of 8 ml of scintillant. Specific binding (defined as that which was displaceable by 0.5 mM unlabeled L-glutamate) comprised 70 ± 2% of total binding (n = 20). Results are presented as means ± SEM.

RESULTS

Kinetics of L-Glutamate Binding to PSDs. The binding of L-[^{3}H]glutamate to PSDs was saturable, and Scatchard analyses (Fig. 1) revealed an apparently homogeneous population of binding sites with a K_d of 3.39 ± 0.89 × 10^{-7} M and a B_{max} (maximum number of binding sites) of 6.1 ± 0.4 pmol/mg of protein (n = 5). Hill coefficients were close to unity (1.09 ± 0.02, n = 5; Fig. 1 *Inset*), indicating an absence of cooperativity.

Absence of Cl^--Dependent L-Glutamate Binding Sites from PSDs. Previous studies have shown that the major population of L-glutamate binding sites in SPMs (the A4 site) is dependent on Cl^- (8, 23), is down-regulated by 1–5 mM Na^+ (24), and is selectively inhibited by the phosphonic acid analogue APB (6). Therefore, these characteristics were utilized to determine whether these sites were present in PSDs. Figs. 2 and 3 illustrate that there are striking differences in the glutamate binding properties of these two subcellular fractions. Thus, Cl^- in the range of 1–40 mM exerted little effect on the binding of L-glutamate to PSDs, in marked contrast to the almost 4-fold stimulation of binding observed in SPMs (Fig. 2*A*). Similarly, the inhibitory effect of 1–5 mM Na^+, which is a feature associated with Cl^--dependent L-glutamate binding sites in SPMs, was not apparent in PSDs (Fig. 2*B*). Hence, PSDs do not appear to contain the Cl^--dependent class of L-glutamate binding sites. [An additional point to note in passing is that the L-glutamate binding sites that are dependent on higher concentrations of Na^+ and are thought to reflect binding to membrane transport sites (4) also were not present in the PSD preparations (Fig. 2*B*).]

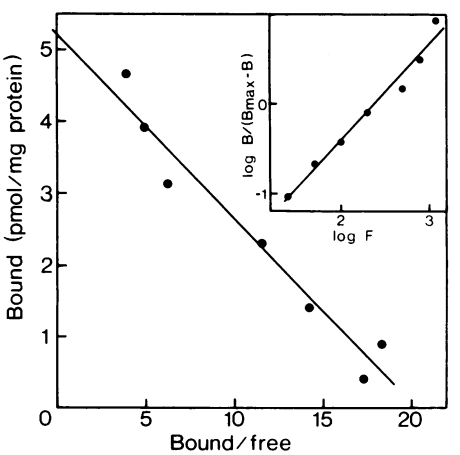

FIG. 1. Scatchard plot describing the specific binding of L-[^{3}H]-glutamate (25–1200 nM) to PSDs isolated from the rat brain. (*Inset*) Hill plot of the same data, in which F is the free concentration (nM) of L-[^{3}H]glutamate and B the amount bound (pmol/mg of protein). Values are means of triplicate determinations and were fitted by linear regression analysis. Five such experiments were performed.

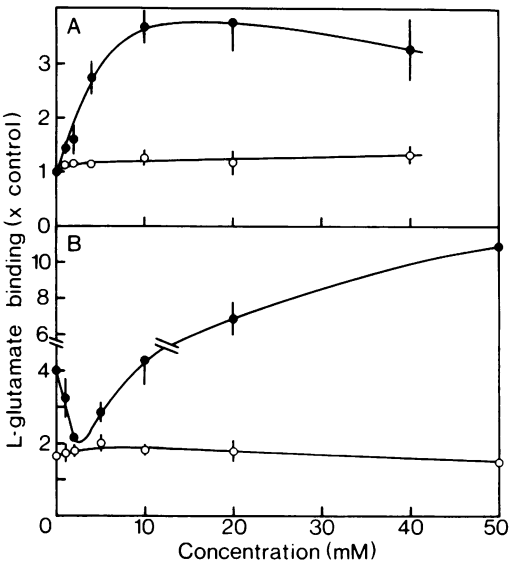

FIG. 2. Effect of Cl^- (*A*) and Na^+ (*B*) on the specific binding of L-[^{3}H]glutamate to SPMs (●) and PSDs (○). The effect of Cl^- (as Tris chloride) was examined in 50 mM Tris acetate buffer and of Na^+ (acetate salt), in 50 mM Tris chloride buffer (to expose Cl^--dependent binding sites; see refs. 6–8). Values are expressed relative to the basal binding measured in Tris acetate buffer alone (=1) and are means ± SEM of data from three or four independent experiments.

The absence of this population of binding sites from PSDs was confirmed by using the selective ligand APB. In SPMs, DL-APB inhibits L-glutamate binding to Cl^--dependent sites with a K_i of ≈1.0 × 10^{-5} M, whereas millimolar concentrations are required to inhibit Cl^--independent binding (6, 13). These observations were verified in the present study (Fig. 3). In PSDs, however, only low-affinity inhibition by APB was apparent both in the presence and absence of Cl^- (Fig. 3), and Scatchard analyses of these data yielded K_i values (Tris chloride buffer, 2.0 ± 0.9 × 10^{-3} M; Tris acetate buffer, 3.3 ± 0.3 × 10^{-3} M) close to those reported for Cl^--

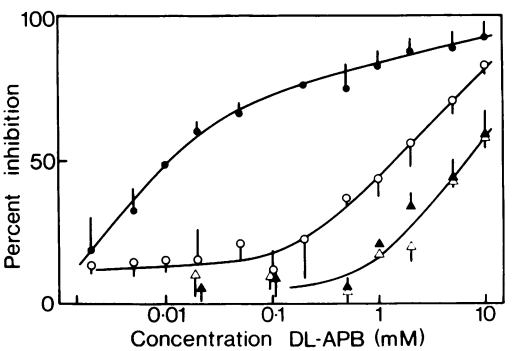

FIG. 3. Semilogarithmic plots describing the inhibition of L-[^{3}H]-glutamate binding to SPMs (●,○) and PSDs (▲,△) by DL-APB. Assays were conducted with 50 mM Tris acetate buffer (○,△) or 50 mM Tris chloride buffer (●,▲) to expose Cl^--dependent binding sites (6–8). Values are means ± SEM of data from three or four independent experiments.

independent binding sites in SPMs (13). In sum, these data indicate that the Cl^--dependent, APB-sensitive population of L-glutamate binding sites is absent from PSDs.

Evidence for Two Distinct L-Glutamate Binding Sites in PSDs. The nature of those L-glutamate binding sites present in PSDs was assessed by determining the inhibitory potencies of a number of compounds with demonstrated activity at excitatory amino acid receptor sites (see refs. 1 and 3). Scatchard analyses of the data (Fig. 4) indicated that L-glutamate itself was the most potent inhibitor examined, with a K_i (2.0×10^{-7} nM; Table 1) similar to the K_d value determined for the binding of radiolabeled L-glutamate (3.39×10^{-7} M; see above). D-Glutamate showed a lower affinity by a factor of almost 50, whereas no stereospecificity was apparent for aspartate (Table 1). The selective receptor agonists *N*MeAsp and quisqualate, the *N*MeDAsp-receptor-preferring agonist ibotenate, and the *N*MeDAsp-receptor-antagonists APV and APH all displayed K_i values in the low 10^{-6} M range. Kainic and quinolinic acids and the other phosphonic acid derivatives examined were less potent.

The Scatchard analyses (see Fig. 4) also revealed that *N*MeAsp and quisqualate each maximally inhibited only a proportion of the sites labeled by L-[^{3}H]glutamate. In every individual experiment in which both substances were examined in parallel, the sum of their B_{max} values totalled close to 100% [B_{max} values, as a percentage of total L-glutamate binding: for *N*MeAsp, 59, 68, 52, 45, 68 (mean 58%); and, respectively, for quisqualate, 47, 31, 49, 51, 34 (mean 42%)], and this suggested that PSDs comprise two distinct populations of L-glutamate binding sites. If this hypothesis were true, the inhibitory effects of *N*MeAsp and quisqualate should be additive, and this was indeed verified experimentally. Thus, 100 μM *N*MeAsp and 10 μM quisqualate [concentrations that, based on their K_i values (Table 1), would be predicted to give near-maximal inhibitions of their individual sites] separately inhibited L-glutamate binding to PSDs by 51 ± 2% and 39 ± 3%, respectively, and, when added together, by 82 ± 5% (n = 3). Hence, these data demonstrate that L-glutamate binding sites in PSDs may be subdivided into two populations on the basis of their differential sensitivities to *N*MeAsp and quisqualate.

Selectivities of *N*MeAsp- and Quisqualate-Sensitive L-Glutamate Binding Sites. A series of experiments was conducted to determine whether the various acidic amino acid analogues used (Table 1) were acting primarily at the *N*MeAsp-sensitive or quisqualate-sensitive components of L-glutamate binding in PSDs and, hence, whether the specificities

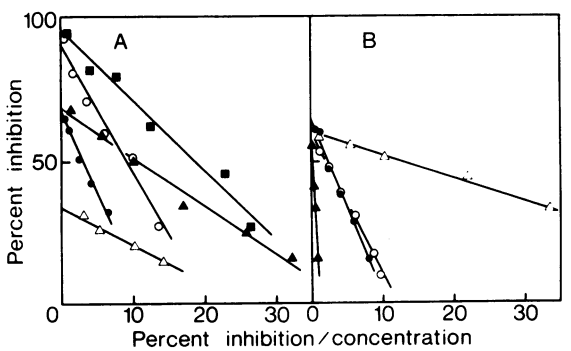

FIG. 4. Scatchard plots of the inhibition of L-[^{3}H]glutamate binding to PSDs by acidic amino acid analogues. Data from two experiments are shown. (*A*) First experiment. ●, *N*MeAsp; ○, ibotenate; ▲, L-aspartate; △, quisqualate; ■, L-glutamate (for glutamate, the abscissa scale is ×0.1). (*B*) Second experiment. ●, *N*MeAsp; ○, DL-APH; ▲, quinolinate; △, DL-APV. Values are means of triplicate determinations and were fitted by linear regression analysis. The K_i values calculated from such plots and the number of experiments for each analogue are given in Table 1.

Table 1. Inhibition of L-glutamate binding to postsynaptic densities by acidic amino acid analogues

Analogue	Concentration range, μM	$K_i \times 10^6$, M
*N*MeAsp	1–100	7.2 ± 1.2 (12)
Quisqualate	0.5–10	1.1 ± 0.3 (6)
Kainate	10–500	29 ± 10 (3)
L-Glutamate	0.1–10	0.20 ± 0.04 (6)
D-Glutamate	2–50	9.7 ± 3.7 (4)
L-Aspartate	0.5–50	2.1 ± 0.8 (5)
D-Aspartate	0.5–50	1.6 ± 0.7 (3)
Ibotenate	2–200	7.1 ± 3.0 (7)
Quinolinate	20–200	91 ± 24 (3)
DL-APP	50	*9 ± 8 (4)
DL-APB	20–10,000	3300 ± 300 (3)
DL-APV	0.5–50	1.0 ± 0.2 (3)
DL-APHX	50	*26 ± 5 (4)
DL-APH	0.5–50	4.6 ± 1.3 (3)

Analogues were tested over the concentration range indicated, and K_i values were calculated (see ref. 6) from the relationship $K_i' = K_i(1 + F/K_d)$, where K_i' is the apparent K_i value determined from Scatchard plots (Fig. 4), F is the free molar concentration of L-[^{3}H]glutamate (50×10^{-9} M), and K_d is its equilibrium dissociation constant (3.39×10^{-7} M, see text). Values are means ± SEM, with the number of independent experiments shown in parentheses. APP, 2-amino-3-phosphonopropionic acid, APHX, 2-amino-6-phosphonohexanoic acid.
*Percentage inhibition at 50 μM.

of these sites conformed to the *N*MeDAsp and quisqualate receptor classes described electrophysiologically. This was achieved by examining the inhibitory activity of each analogue at a concentration close to its K_i value (Table 1), in conjunction with sufficient *N*MeAsp or quisqualate to saturate the majority of their respective sites (see previous section). This approach revealed marked differences in specificity between these two subpopulations of L-glutamate binding sites (Table 2). Thus, quisqualate itself and kainate acted almost exclusively at the quisqualate-sensitive group of sites (their inhibitory actions were nearly abolished by 10 μM quisqualate). In contrast, *N*MeAsp, DL-APV, and the D-isomers of aspartate and glutamate displayed an essentially absolute preference for the *N*MeAsp-sensitive population. L-aspartate and ibotenate also inhibited principally the *N*Me-

Table 2. Ligand specificities of *N*MeAsp-sensitive and quisqualate-sensitive L-glutamate binding sites in PSDs

Analogue	Conc., μM	% inhibition in presence of: 100 μM *N*MeAsp	10 μM quisqualate	Site preference
*N*MeAsp	10	5 ± 2	55 ± 6	N >> Q
Quisqualate	2	59 ± 6	9 ± 3	Q >> N
Kainate	50	67 ± 11	14 ± 6	Q >> N
D-Glutamate	10	5 ± 8	35 ± 6	N >> Q
L-Aspartate	2	19 ± 2	51 ± 1	N > Q
D-Aspartate	2	3 ± 9	54 ± 8	N >> Q
Ibotenate	10	38 ± 5	64 ± 7	N > Q
DL-APV	5	−1 ± 9	41 ± 2	N >> Q

Analogues were tested at the concentration indicated, and values for percentage inhibition of L-[^{3}H]glutamate binding are with respect to the residual specific binding after inhibition either by 100 μM *N*MeAsp or by 10 μM quisqualate (to block their respective binding sites, see text). Values are means ± SEM of data from three or four independent experiments. Based on these data, the preference of each analogue for the *N*MeAsp-sensitive (N) or quisqualate-sensitive (Q) L-glutamate binding sites was determined. See text for additional details.

Asp-sensitive component of L-glutamate binding but were active to a minor extent at quisqualate-sensitive sites. This pattern of selectivity corresponds well to that derived from electrophysiological studies (1, 3).

Additional evidence that the *N*MeAsp-sensitive population of L-glutamate binding sites in PSDs may be equated with functionally defined *N*MEDAsp receptors was obtained by using a homologous series of α-amino-ω-phosphono carboxylic acids, which exhibit a characteristic profile of activity at this receptor type (11, 25). In agreement with the electrophysiological studies, the most potent inhibitors of L-glutamate binding were APV and APH (Table 1). Moreover, as judged from Scatchard plots (Fig. 4*B*) and additivity experiments (Table 2), these compounds acted selectively at the *N*MeAsp-sensitive component of L-glutamate binding. Another *N*MEDAsp receptor ligand, quinolinic acid (26), also inhibited L-glutamate binding to the *N*MeAsp-sensitive binding sites (Fig. 4*B*) but with lower affinity (Table 1).

DISCUSSION

Neuronal receptors for the excitatory transmitter, L-glutamate, have been subdivided into several categories by electrophysiological investigation (1, 3, 4). Radioligand binding techniques provide a means of directly examining the properties of these receptors in the absence of cellular influences and ultimately of characterizing them biochemically (5). Fig. 5 summarizes the subtypes of L-glutamate binding site described in this report, their relationship to the electrophysiologically defined receptor classes, and their distribution in SPMs and PSDs. Our data demonstrate (*i*) that L-glutamate binding sites may be resolved into three distinct populations, each of which corresponds to an electrophysiologically defined receptor, and (*ii*) that the *N*MeAsp (A1) and quisqualate (A2) receptor classes are selectively associated with brain PSDs.

The PSD is a prominent feature of excitatory (type I) synapses in the brain (17), where it is thought to provide a structural framework for transmitter receptors and other molecules involved in the postsynaptic neuronal response (14, 15). However, although its morphology and protein composition have been described in detail (14, 15, 17–20), little is known of its relationship to those molecules that mediate the synaptic response. The present findings indicate that two classes of excitatory amino acid receptor in the brain (the A1 and A2, or *N*MEDAsp and quisqualate sites, respectively) are closely linked to the PSD and, hence, suggest that these receptors directly subserve the excitatory response in the postsynaptic neuron. Such a role is supported by electrophysiological studies at a number of synapses in the central nervous system. Thus, recent investigations using receptor antagonists have shown that the *N*MEDAsp receptor type mediates synaptically evoked neuronal excitation in spinal segmental tracts (11, 27) and in the olfactory cortex (12), whereas the quisqualate receptor may be involved in transmission in hippocampal pathways (28, 29), at lateral olfactory tract terminals (12), and in the cochlear nucleus (30) (for additional pathways, see ref. 1). Our data do not indicate whether the *N*MeAsp and quisqualate populations of L-glutamate receptors are located at the same or separate PSDs, although the electrophysiological investigations cited above, coupled with recent autoradiographic experiments (31), are consistent with the hypothesis that these receptor types function at different synapses.

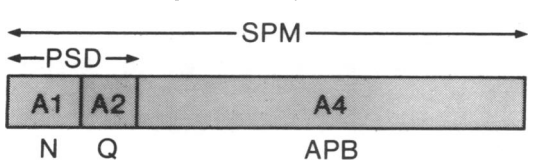

FIG. 5. Schematic diagram to illustrate the subtypes of L-[^{3}H]-glutamate binding sites and their subcellular distribution. Sites A1 and A2 (nomenclature according to ref. 4) are the *N*MeAsp-sensitive and quisqualate-sensitive binding sites described here and correspond to *N*MEDAsp (N) and quisqualate (Q) receptor classes as defined electrophysiologically; both are located in PSDs. Site A4 is the Cl^--dependent, APB-sensitive site (6–8); it is found only in SPMs and not in PSDs. Site A3 is defined as that labeled with nanomolar affinity by kainic acid (4) and was not detected in the present study.

That the L-glutamate binding sites identified in PSDs do indeed represent *N*MEDAsp and quisqualate receptors was evident from an analysis of their ligand selectivity. Thus, the *N*MeAsp-sensitive L-glutamate binding sites (the A1 site) exhibited a preference for agonists (*N*MeAsp, L- and D-aspartate, D-glutamate, ibotenate, and quinolinate) and antagonists (APV and APH) with demonstrated activity at *N*MEDAsp receptors (see refs. 1 and 3) but not for substances active at other classes of excitatory amino acid receptor (quisqualate, kainate, APB). Moreover, the K_i value for APV at these binding sites (1.0 μM) is in good agreement with that estimated electrophysiologically (1.4 μM; ref. 11). The quisqualate-sensitive (A2) sites, on the other hand, showed a preference for quisqualate itself, kainate (see ref. 32) and, in preliminary experiments (not shown), the quisqualate-receptor-agonist α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (kindly provided by P. Krogsgaard-Larsen). Interestingly, the proportions of *N*MeAsp and quisqualate sites detected in PSDs (58% and 42%, respectively) are similar to those previously observed for Cl^-/Ca^{2+}-independent binding sites in SPMs (7). This, coupled with their ion independence (this report) and stability to freeze/thaw cycles (unpublished data), suggests that the L-glutamate binding sites in PSDs and Cl^-/Ca^{2+}-independent binding sites in SPMs (7, 13) are identical populations.

Based on ionic and pharmacological criteria, our data demonstrate that the major population of L-glutamate binding sites found in SPMs (the A4, or APB-sensitive site) is absent from isolated PSDs. Another excitatory amino acid receptor class (A3), that characterized by high affinity for kainate (4), was also not detected. One explanation of these results is that these receptor types are located not at the PSD itself but on presynaptic or extrasynaptic membrane components that are removed during PSD isolation. In support of this proposal, Harris and Cotman recently have suggested that APB may act presynaptically at the perforant path-granule cell synapse in the dentate gyrus (33), and a number of studies indicate that acidic amino acid analogues, including kainate, may regulate the depolarization-induced release of excitatory amino acids *in vitro* (34–37). Evidence for extrasynaptic receptors is more limited, although in invertebrate species where glutamate mediates neuromuscular transmission, both depolarizing and hyperpolarizing L-glutamate receptors are found on the muscle surface (36). However an alternative explanation is that the APB and/or kainate receptor classes are more loosely bound to the PSD and, hence, are lost during its preparation. Kainate binding sites have been found in a "synaptic junction" fraction isolated from the rat brain by using low concentrations of Triton X-100 (39), and a recent report suggests that these sites may be solubilized by the higher detergent concentrations used to prepare PSDs (40).

Further investigation of the various L-glutamate receptor classes described here must include their biochemical characterization either as distinct molecular species or as related forms of the same molecule whose function is modulated posttranslationally or by other components of the synaptic environment. In this respect, the present study focuses our attention on the *N*MEDAsp- and quisqualate-selective sites as the molecular species most relevant to elucidating the postsynaptic actions of excitatory amino acid transmitters in the brain.

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