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HIGH-AFFINITY GLUTAMIC ACID BINDING TO BRAIN SYNAPTIC MEMBRANES

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SUMMARY

Rat brain homogenate preparations exhibited two types of glutamate binding, one a high-affinity ($K_1 = 0.2\ \mu M$) and the other a low-affinity type ($K_2 = 4.4\ \mu M$). The high-affinity binding was primarily associated with the plasma membrane subcellular fractions and in particular with the synaptic membrane subfraction. This L-glutamate binding was found to be strongly stereospecific for the L-form and was almost totally reversible. The synaptic membrane glutamate binding was partially inhibited by neuro-excitatory and neuro-inhibitory amino acids but was not affected by amino acids lacking in neuropharmacologic activity. The membrane-associated L-glutamate binding system could be solubilized by Triton X-100 without loss of its high-affinity binding activity. The chemical nature of this glutamate binding component was found to be that of a glycolipoprotein. It is proposed that this glutamate binding system represents the physiologic receptor on neuronal membranes of this amino acid.

INTRODUCTION

It is well known that L-glutamic acid exhibits strong neuro-excitatory activity upon microiontophoretic application to mammalian central nervous system neurons [1, 2]. This neuronal excitation is apparently produced by interaction with receptors on the external surface of nerve cell membranes as L-glutamate has no effect when injected intracellularly [3]. However, since L-glutamate has been found to excite almost all neurons in the mammalian central nervous system [4], it has been suggested that the receptor site interacting with glutamate may be a non-specific part of all nerve cell membranes [5].

In the present study an attempt has been made to examine the presence of a glutamate receptor in the rat brain, to determine its possible localization on neuronal membranes, and to evaluate the specificity of its interaction with glutamate and other neuroactive amino acids. Receptor activity has been defined in this study as

the binding affinity of the tissue preparations for L-glutamate if such binding exhibits characteristics that are similar to the physiologic and pharmacologic properties of glutamate excitation in the central nervous system.

MATERIALS AND METHODS

Chemicals

L-glutamic acid, L-aspartic acid, D-glutamic acid, and γ-aminobutyric acid were obtained from Nutritional Biochemicals, Cleveland, Ohio; all other amino acids, as well as trypsin, phospholipase C, glutamate dehydrogenase, glutamate decarboxylase, and concanavalin A were purchased from Sigma Chemical Co., St. Louis, Mo. The source of L-[^{14}C]glutamic acid (200 Ci/mole) and γ-amino-[^{14}C]butyric acid (80 Ci/mole) was New England Nuclear Co., Boston, Mass., whereas L-[^{3}H]glutamic acid (20 Ci/mmole) was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio. All other chemicals used were of analytical reagent grade.

Preparation of tissue samples

Whole brains from adult male Sprague–Dawley rats were finely minced and homogenized in 0.32 M sucrose containing 12.5 μmoles Ca^{2+}/g wet brain tissue. The centrifugation scheme of Cotman and Mathews [6] was employed to isolate the following subfractions: (1) a mixed glial–neuronal plasma membrane subfraction, (2) a synaptic membrane subfraction, and (3) a mitochondrial subfraction. The mitochondrial fraction was further separated from co-precipitated nerve ending particles by a method similar to that of Cotman and Taylor [7]. The degree of purity of the various membrane subfractions and the mitochondrial subfraction was monitored both by electron micrographs and by determination of the activity of marker enzymes associated with each fraction. The electron micrographs of the nerve ending particle subfraction prior to osmotic rupture showed it to be enriched in pinched off nerve endings as previously reported [6]. Marker enzyme determinations ($(Na^+–K^+)$-ATPase, succinate dehydrogenase, and glutamate dehydrogenase) further confirmed the high degree of homogeneity of the various subfractions. Other brain subcellular fractions isolated included the synaptic vesicles, the microsomal membranes, and the cell soluble fraction obtained by the centrifugation scheme of Rodríguez de Lores Arnaiz et al. [8], and a nuclear subfraction separated according to the method of Løvtrup-Rein and McEwen [9].

Triton X-100 solubilization of the synaptic membranes was accomplished in the following steps: (1) stirring an aliquot of membrane suspension (5–8 mg protein per ml) in the presence of 0.5% (v/v) Triton X-100, (2) raising the pH of the suspension to 9.5 by the addition of 0.3 M NaOH while continuing to stir for 1 min, (3) bringing the pH back to 7.4 by the addition of 0.1 M HCl, and (4) further diluting the solution with phosphate buffer to a final Triton X-100 concentration of 0.25 % (v/v). The dorsal root ganglion preparation used was obtained by homogenizing 6–8 dorsal root ganglia in a manner similar to that employed for the brain tissue. All procedures were conducted at 0–4 °C.

Binding assay

L-glutamic acid binding to tissue suspensions and other proteins was measured by equilibrium dialysis using Visking dialysis tubing. Each dialysis sac contained

0.2 ml of tissue suspension (0.15 mg protein/ml) together with isotopically diluted ligand(s). Complete equilibration of the sac contents against 4.0 ml of a sodium phosphate buffer solution (pH 7.5, ionic strength 0.05) was achieved by overnight incubation at 4 °C in stoppered culture tubes. Duplicate samples from each dialysis sac and the outside buffer were counted either in a Low-Beta II counter (Beckman Instruments) or mixed in vials with 5 ml of a scintillation fluid [10] and counted in a Packard Tricarb liquid scintillation counter.

Protein concentrations were determined according to the method of Lowry et al. [11], using bovine serum albumin as the standard.

The points drawn on the inverse plots and Dixon [12] plots were line-fitted by means of the least squares method.

RESULTS

Brain homogenate and subcellular binding of glutamate

The binding of L-glutamic acid to whole brain homogenate preparations (Curve A, Fig. 1) reveals the apparent existence of three glutamate-binding processes. The first is dominant in the ligand concentration range of 0.1–1.0 μM, whereas the second process becomes apparent in the range of 1.5–10 μM L-glutamate. At ligand concentrations higher than 8 μM, the glutamate binding isotherm assumes a linear, non-saturable character which was thought to represent non-specific adsorption to or passive diffusion into brain subcellular structures. After this linear process was extrapolated to zero concentrations and subtracted from the experimental data according to the method of Bogdanski et al. [13], the glutamate binding isotherm exhibited typical

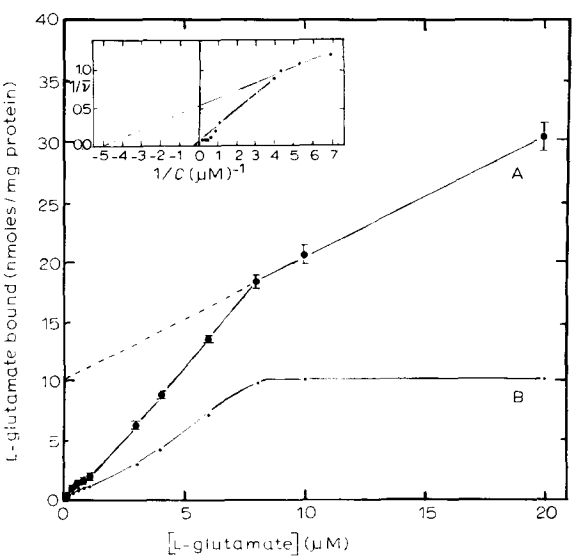
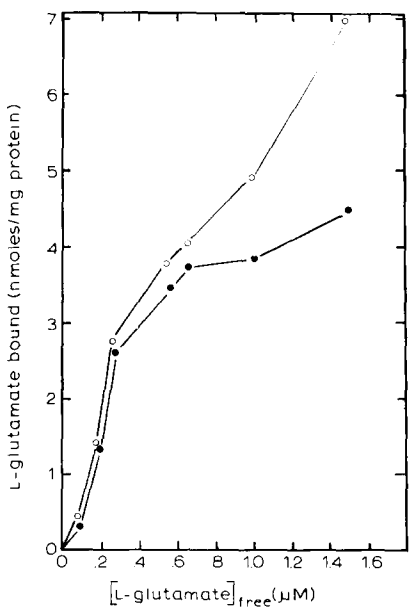


Fig. 1. Binding of L-glutamate to rat brain homogenate. Each point in binding Isotherm A represents the mean of 5–8 experimental determinations ±S.E. Curve B represents the corrected data after subtraction of the non-saturable binding process (see text). Insert contains the inverse plot of the corrected data. $\bar{v}$, nmoles L-glutamate bound/mg protein; C, free L-glutamate concentration, μM.

saturation kinetics (Curve B, Fig. 1). Plotting of these corrected data according to the method of Klotz [14] (insert, Fig. 1) confirmed the existence of two sets of binding sites with the following estimated dissociation constants and maximum binding capacities: $K_1 = 0.2\ \mu M$ and $n_1 = 1.8$ nmoles L-glutamate bound/mg protein for the high-affinity sites; $K_2 = 4.4\ \mu M$ and $n_2 = 8.8$ nmoles L-glutamate bound/mg protein for the low-affinity sites.

This glutamate binding appears to be a property of central nervous tissue because, unlike the brain homogenate preparation, the dorsal root ganglion homogenates exhibited no glutamate binding activity over the same range of concentrations in three different experiments. Similarly, negative results were obtained when binding to bovine serum albumin, egg albumin, bovine liver glutamate dehydrogenase, and bacterial glutamate decarboxylase was measured under identical conditions.

In the next series of experiments, an attempt was made to determine the subcellular site of the glutamate interaction observed in brain homogenates. The ligand concentration range selected for measurement of glutamic acid binding to the various subcellular fractions was 0.1–1.2 μM, since this range reflected high-affinity binding in homogenates. All subfractions were pre-incubated with $4.2 \cdot 10^{-3}$ M D-glutamate for 30 min and the binding of L-glutamate was assayed in the presence of $2 \cdot 10^{-4}$ M D-glutamate. It was found that the D-enantiomer did not interfere with the high-affinity binding of the L-form by synaptic membranes but, on the contrary, it seemed to decrease any interference by the linear, non-saturable process previously described (Fig. 2). Fig. 3 reveals that the synaptic membrane preparation and the mixed neu-



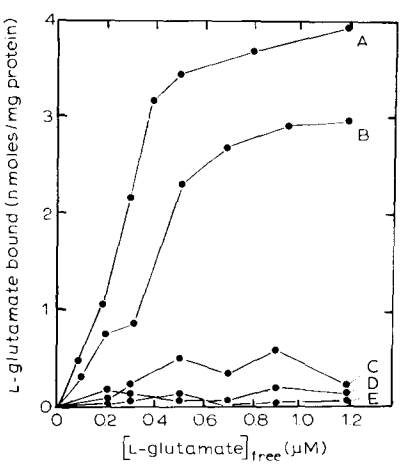


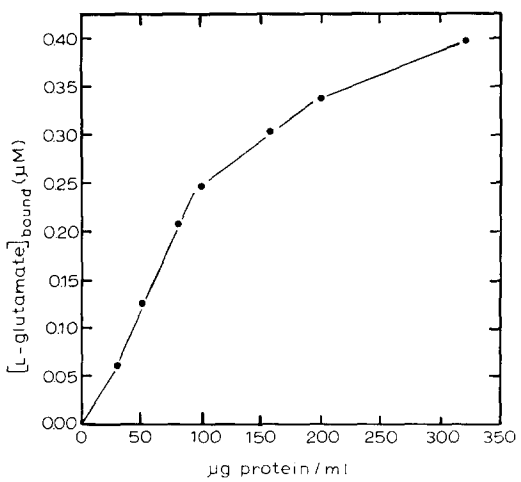
Fig. 2. L-glutamate binding to a synaptic membrane preparation in the absence (○) and in the presence (●) of D-glutamate.

Fig. 3. Binding of L-glutamate to various cellular subfractions: A, synaptic membranes; B, mixed neuronal-glial membranes; C, mitochondria; D, microsomal membranes; E, nuclei.

ronal–glial membrane fraction exhibited a higher binding capacity than all the other subfractions. The synaptic vesicle and cytosol fractions showed no binding at all and were not included in this figure. Due to the importance of the synaptic structures in neurotransmission and the greater homogeneity of the synaptic membrane preparation, this subfraction was selected for further study of the characteristics of the binding system.

Characteristics of L-*glutamate binding to synaptic membranes*

The binding of L-glutamic acid to the synaptic membranes was found to vary in direct relation to the protein concentration of the membrane suspension (Fig. 4). The measured bound radioactivity did not represent incorporation of the labeled glutamate into proteins since less than 10% of the bound radioactivity at the end of equilibration was associated with the washed trichloroacetic acid precipitates of tissue protein. Also, addition of an excess of non-labeled L-glutamate (to 0.1 mM) after equilibration in the presence of 0.2 μM L-glutamate caused greater than 99% release of the bound radioactivity. Furthermore, there was no evidence that the labeled glutamate had been metabolically altered during equilibrium dialysis since the only radioactivity above background corresponded to the glutamic acid spot on paper and thin-layer chromatograms of the contents of the dialysis sacs.



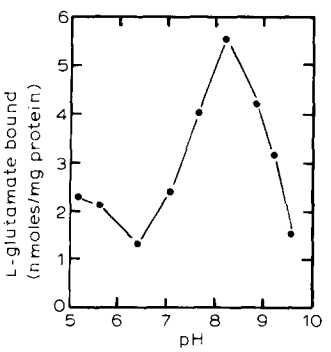
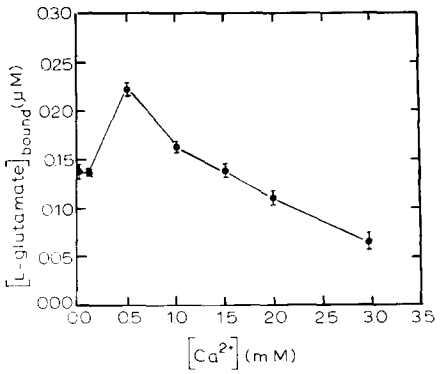


Fig. 4. Binding of 0.2 μM L-glutamate to increasing concentrations of the synaptic membrane preparation expressed as μg of protein per ml of solution.

Fig. 5. Effect of pH on the binding of 0.5 μM L-glutamate to the synaptic membranes. For pH values between 5.2 and 6.6, the buffer used was 0.05 M citrate–phosphate; for the pH range 7.6–9.5, a 0.05-M borate buffer was employed.

The interaction of L-glutamic acid with the synaptic membranes was found to be sensitive to changes in the pH of the medium (Fig. 5) with maximum binding obtained at pH 8.2. It also exhibited sensitivity to the presence of Ca^{2+} (Fig. 6) demonstrating a biphasic response with increased glutamate binding at 0.2–0.5 mM Ca^{2+} concentrations followed by decreased binding at higher Ca^{2+} concentrations. On



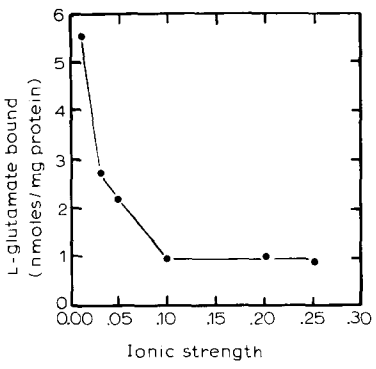


Fig. 6. The effect of Ca^{2+} on L-glutamate (0.2 μM) binding to synaptic membranes. Equilibrium assay was developed in a 0.05-M Tris–HCl buffer, pH 7.4, with increasing amounts of Ca^{2+} added. Each point is the mean of four determinations from two experiments $\pm$ S.E.

Fig. 7. Effect of ionic strength on the binding of L-glutamic acid (0.7 μM) to a synaptic membrane suspension. Equilibrium dialysis was developed in sodium phosphate buffer, pH 7.4, whose ionic strength was increased by the addition of varying amounts of NaCl.

the other hand Mg^{2+}, over the same range of concentrations, did not affect glutamate binding. Of the monovalent cations, only the effect of Na^+ was examined primarily because of the dependence of the brain synaptosome glutamate transport systems on this cation [15]. The binding of glutamate as measured in Tris–HCl buffer (0.05 M, pH 7.4) in the presence and absence of 40 mM NaCl showed a small decrease when Na^+ was present. This effect of Na^+ was probably due to the inverse relationship of glutamate binding to the ionic strength of the medium as shown in Fig. 7. Thus glutamate binding to the synaptic membranes does not appear to be directly dependent on the presence of Na^+.

The interaction of L-glutamic acid with the synaptic membranes resembles its

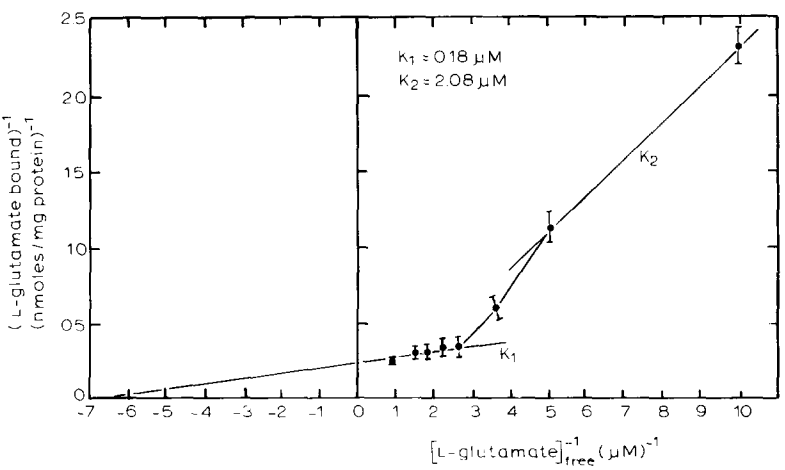


Fig. 8. Inverse plot of the binding of L-glutamic acid to brain synaptic membranes. Each point represents the mean of four determinations $\pm$S.E.

interaction with the whole brain homogenate as evidenced by the similarity of their dissociation constants. Analysis of the glutamate binding to the synaptic membranes by the method of Klotz [14] (Fig. 8) demonstrates again the existence of two types of binding sites, a high-affinity and a low-affinity set. The maximum binding capacity of the synaptic membrane high-affinity sites was estimated to be 4.44 nmoles L-glutamate bound/mg protein, which suggests that these membrane preparations are enriched in the glutamate binding components. The two types of binding sites associated with both the membrane and the brain homogenate preparations did not bind glutamate independently but appeared to interact in a positively cooperative manner yielding Hill coefficients in the range of 1.6–2.6.

Examination of the effects of other amino acids on the glutamate binding system revealed patterns of interaction qualitatively similar to some of the known properties of the glutamate–physiologic receptor interaction (Table I). Both the excit-

TABLE I

BLOCKADE OF L-GLUTAMATE (0.5 μM) BINDING TO SYNAPTIC MEMBRANES BY VARIOUS AMINO ACIDS

Binding assays were conducted in the presence of an excess of D-glutamate. The results are averages of duplicate determinations from five experiments.

Competitive ligand (1.0 μM)	% Inhibition
Excitatory	
L-aspartic acid	65.8 ± 8.8
D, L-homocysteic acid	61.6 ± 10.6
Cysteine sulfinic acid	44.5 ± 6.2
Inhibitory	
Glycine	34.0 ± 4.3
γ-Aminobutyric acid	86.3 ± 11.2
β-Alanine	23.3 ± 4.1
Antagonistic	
Diethyl ester of glutamate	24.1 ± 3.8
Non-neuroactive	
N-acetyl-L-aspartate	− 6.5 ± 2.1*
Amino oxyacetic acid	− 7.0 ± 4.8*
Glutamine	2.2 ± 1.4

* − sign denotes an increase in binding

atory and the inhibitory amino acids examined interfered with glutamate binding as did the partial glutamate antagonist diethylester of glutamate [16]. Amino acids that do not affect the neuro-excitatory activity of glutamate had no effect on the binding of L-glutamic acid. The inhibition of glutamate binding produced by L-aspartate appears to be competitive in nature ($K_i = 0.98$ μM from Dixon plot of Fig. 9) whereas γ-aminobutyric acid blocked in a non-competitive manner ($K_i = 0.94$ μM). The non-competitive interaction of glutamate and γ-aminobutyric acid was also demonstrated by double-isotope studies measuring the binding of L-[^{3}H]glutamate to membranes that had bound γ-amino[^{14}C]butyric acid (Fig. 10). Glutamate binding displaced only

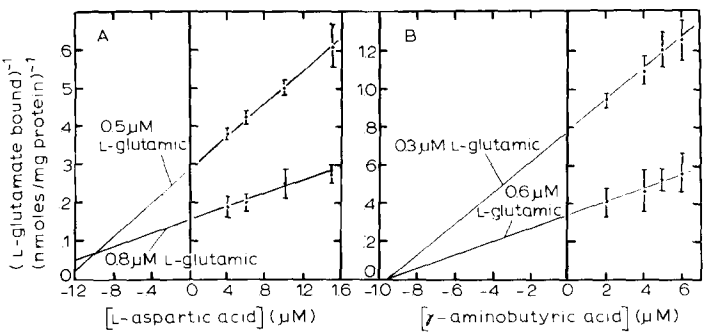


Fig. 9. Dixon plots of the inhibition by L-aspartate (A) and γ-aminobutyric acid (B) of the binding of L-glutamic acid to brain synaptic membranes. Each point represents the mean of three experiments ±S.E.

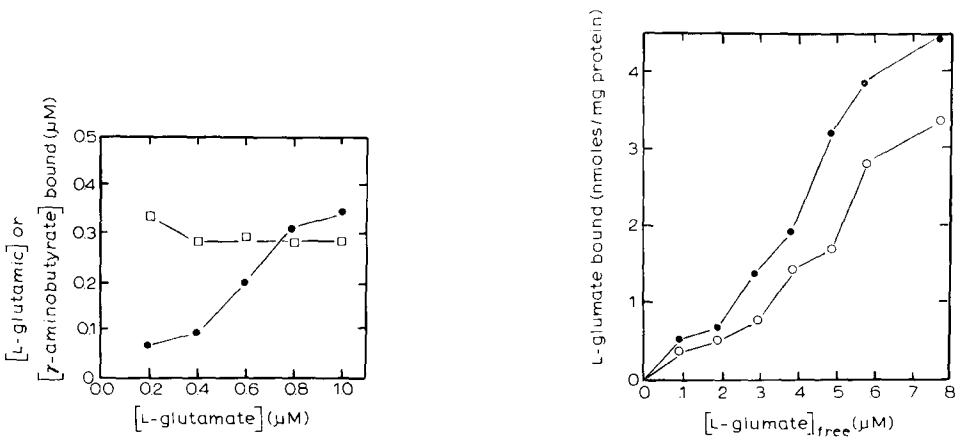


Fig. 10. Binding of L-[^{3}H]glutamate to brain synaptic membranes that were preincubated with γ-amino[^{14}C]butyric acid (0.8 μM) for 1 h at 4 °C. Amount of L-glutamate bound (●); amount of γ-aminobutyric acid bound (□).

Fig. 11. Isotherm of L-glutamate binding to a Triton X-100-solubilized synaptic membrane preparation (●) and the corresponding membrane particulate suspension (○).

a small fraction of the bound γ-aminobutyric acid. Glycine, like γ-aminobutyric acid, acts as a non-competitive inhibitor with a weaker inhibitory activity (K_i = 4.5 μM).

Chemical nature of the glutamate binding system

Treatment of the synaptic membranes with either sodium deoxycholate or Triton X-100 was effective in solubilizing the glutamate binding component. The Triton X-100 solubilization procedure has consistently given a greater than 90% recovery of membrane proteins in the clear supernatant after a 3-h centrifugation at 100 000 × *g*. L-glutamate binding activity of this supernatant has been found to be equal to or slightly higher than that of the synaptic membranes from which the soluble extract was obtained (Fig. 11). Triton X-100 alone in buffer did not show any bind-

TABLE II

EFFECT OF VARIOUS ENZYMES AND CONCANAVALIN A ON L-GLUTAMATE (0.2 μM) BINDING

The synaptic membrane particulate and the Triton X-100-solubilized preparations were incubated with each of the enzymes at 23 °C for 1 h and with concanavalin A at 0 °C for 30 min. The results are averages of duplicate determinations from two experiments.

Treatment	Average % inhibition of binding to	
	Particulate	Soluble extract
Trypsin (0.5 mg/ml)	20	37
Pronase (0.5 mg/ml)	20	88
β-Glucosidase (0.5 mg/ml)	-*	52
Phospholipase C (0.2 units/ml)	56	76
Concanavalin A (0.1 mg/ml)	80	100
Concanavalin A (0.02 mg/ml)	52	100

* Not determined

ing affinity for glutamate nor has there been any precipitate formation of the solubilized membrane preparation at the end of equilibration. Glutamate binding to the solubilized preparation exhibits many similarities to the intact membrane–glutamate interaction as manifested by its dissociation constants (K_1 = 0.18–0.21 μM, K_2 = 0.8–1.2 μM), by its cooperative nature (Hill coefficient = 2.6–3.0), and by its partial inhibition by the same neuro-excitatory and neuro-inhibitory amino acids found to block binding to the intact membranes. Finally, both the membrane-attached and the solubilized glutamate binding component are sensitive to various enzymatic treatments and to exposure to concanavalin A (Table II). The effects of these chemical treatments on the glutamate binding entity suggest that its chemical nature is that of a membrane glycoprotein with closely interacting phospholipids.

DISCUSSION

L-Glutamic acid was found to bind to rat brain homogenate and brain plasma membrane preparations with a high degree of affinity and specificity. Unlike the membrane subfractions that were enriched in glutamate binding activity, the other subcellular components showed little or no interaction with this amino acid. This membrane-associated glutamate binding activity seen in the brain cannot necessarily be assumed to be a general property of all nervous tissue cells since dorsal root ganglion cells did not exhibit such binding. These sensory afferent neurons have a full complement of glutamate metabolizing enzymes and have been postulated to use glutamic acid as their neurotransmitter [17]. However, they have no synaptic terminals on them [18] and are known to be unresponsive to iontophoretic applications of glutamate [19]. This seems to suggest that glutamate binding to neuronal membranes is a property of neurons that can be synaptically activated.

The demonstrated stereospecificity for L-glutamic acid binding to brain membrane preparations in the presence of an excess of D-glutamate corresponds well with the electrophysiologic findings of the strong brain excitatory activity obtained

with the L-enantiomer but not the D-form [2]. Further correspondence with the physiologic receptor was exhibited by the effects of various neuro-active amino acids on glutamate binding. The competitive inhibition of glutamate binding produced by L-aspartate is in agreement with the observations of similar competitive inhibition by aspartic acid of glutamate activity in the locust neuromuscular junction [20] and is in agreement with the theoretical model of a common excitatory amino acid receptor in the mammalian central nervous system [1]. On the other hand, the inhibitory amino acids γ-aminobutyric acid, glycine, and β-alanine [1, 2] appear to bind to a different site and possibly exercise their inhibition of both glutamate-induced excitation in the brain [21] and of glutamate binding through allosteric interaction with the excitatory binding site.

The inhibition of glutamate binding by micromolar concentrations of γ-aminobutyric acid and glycine also helps to distinguish this binding process from the glutamate transport system of brain synaptosomes which is not affected by these amino acids up to concentrations of 10^{-3} M [22]. Another distinguishing characteristic between these two processes is the observation that glutamate binding activity does not depend on the presence of Na^+ whereas the glutamate transport system does [15].

When the pattern of inhibition of glutamate binding by the amino acids tested is considered together with the absence of glutamic acid binding to pure glutamate metabolizing enzyme preparations or to subfractions enriched with such enzymes, e.g. mitochondria, it appears unlikely that this binding represents interaction with brain enzyme systems. In addition, the preservation of the membrane glutamate binding activity following solubilization with Triton X-100 negates the possibility that this binding process represents uptake or entrapment of glutamate into membrane vesicles.

All of these considerations suggest that the high-affinity glutamate binding of the brain plasma membranes may represent the interaction of glutamic acid with its physiologic receptor on neuronal membranes. Since the binding capacity of the synaptic membrane fragments was found to be the highest of any of the cellular subfractions, it would not be unreasonable to conclude that the population of glutamate receptors is highest in the synaptic region but not necessarily exclusively localized in that region. This would go along with the possibility that L-glutamic acid may function as a neurotransmitter or a modulator of synaptic activity in the mammalian central nervous system.

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