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Purification and Characterization of a High-Molecular-Weight Endogenous Glutamate-Binding Inhibitor in Porcine Brain

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Endogenous glutamate modulator
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Abstract
A high-molecular-weight glutamate-binding inhibitor (HGBI) from porcine brain extract was purified to homogeneity. The results of this purification process show that glutamate receptor activity can be regulated by a high-molecular-weight protein, which inhibits [^{3}H]*L*-glutamate binding to excitatory amino acid (EAA) receptors. The purified HGBI appears to be a protein with a molecular weight of approximately 70 kD. The purified HGBI is negatively charged, suggesting that it may contain acidic amino acids, and most likely, *L*-glutamate- and *L*-aspartate-enriched regions, responsible for its surface charge as well as for its binding to glutamate receptors. Inhibition of [^{3}H]*L*-glutamate binding by the purified HGBI is reversible, and appears to change the binding kinetics. This endogenous ligand for glutamate receptors has unique characteristics separating it from all the other ligands found so far in the EAA receptor system. This HGBI represents a new class of modulator for the EAA receptor, thus further investigation of the function and structure of the HGBI should provide new understanding of the mechanisms of EAA-mediated neurotransmission.

Glutamate is the main excitatory amino acid (EAA) transmitter in the vertebrate central nervous system (CNS). The elucidation of EAA receptor classes has evolved over the past 30 years [for a review see ref. 20]. After the initial suggestion of a single receptor type, evidence accumulated to suggest the presence of a multiple-receptor-subtype system. Several lines of investigation indicate that at least five EAA receptors exist, all with distinct functions. Three of them are defined by the depolarizing actions of their selective agonists (N-methyl-*D*-aspartate, NMDA; kainate, KA; and quisqualate, or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, AMPA) and their blockade by selective antagonists [9, 30]. The activation of these three ionotropic receptors leads directly to the opening of ion channels (Na^+, K^+ and Ca^{2+}). A fourth receptor, the *L*-2-amino-4-phosphonobutyrate (AP4) receptor, appears to represent an inhibitory autoreceptor [7, 18]. The fifth, the metabotropic EAA receptor, activated by trans-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD) and quisqualate, modifies phospholipase C (PLC)-linked inositol phosphate (IP) metabolism [17, 18, 21, 27].

The presence of multiple EAA receptor subtypes also implies the presence of endogenous ligands (EL) besides

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L-glutamate (*L*-glu) and L-aspartate (*L*-asp) as ligands for these receptors. This is analogous to the opioid system, in which the presence of multiple opioid receptors has led to the discovery of met-enkephalin and leu-enkephalin as the ligands for the δ receptor, β-endorphin for the ε receptor, and dynorphins for the κ receptor [5, 13, 34]. For the EAA system, some endogenous molecules such as quinolinic acid, *L*-homocysteic acid, methyltetrahydrofolate and glutamate/aspartate-containing peptides, e.g. N-acetyl-aspartyl glutamate (NAAG), have been isolated and proposed as possible EL for EAA receptors [4, 23, 35]. NAAG has been proposed as a putative neurotransmitter for the EAA system since it is present in the mammalian CNS at relatively high concentrations, and a high-affinity NAAG-binding site has been reported [1, 35]. Quinolinic acid has also been implicated as a possible etiological agent of Huntington's disease [4]. Endogenous modulators for EAA receptors, such as glycine, polyamines, Mg^{2+} and Zn^{2+} for the NMDA receptor [14, 22, 25], and kynurenic acid for the KA receptor [6], have been extensively studied and found to modulate their respective EAA receptor subtypes allosterically or synergistically.

The action of some endogenous ligands on EAA receptors demonstrated by [^{3}H]*L*-glu binding is mostly determined by competitive interaction. In some cases, modulators allosterically interacting with the EAA-agonist-binding sites can also affect glutamate receptor binding. Therefore, the [^{3}H]*L*-glu binding assay which has been employed for pharmacological characterization of EAA receptors for almost two decades [3, 11, 19, 33] will allow us to identify more endogenous EAA receptor ligands in the CNS. In some preliminary studies, two endogenous ligands, which exhibit distinct chemical properties different from other EAA receptor ligands, have been found in the mammalian brain [31, 32]. It is thus important to further purify and characterize these new ligands to understand their functions. The information obtained from these studies will not only shed light on regulation mechanism of EAA transmission, but also provide clues to the possible etiology of certain degenerative neurological disorders.

In preliminary studies performed in this laboratory, we found that a fraction of porcine brain extract, with a molecular weight 30 kD, contained glutamate-binding inhibitors [31, 32]. This finding is analogous to the study of the diazepam-binding inhibitor, a 10-kD protein which binds to benzodiazepine-binding sites to modulate $GABA_A$ receptor activity [2, 12, 24, 26]. Since study of the diazepam-binding inhibitor has led to the elucidation of a regulatory mechanism for the $GABA_A$ receptor, it will be valuable to characterize the high-molecular-weight glutamate-binding inhibitors (HGBI) for further understanding modulation mechanisms of EAA receptors. In this study, the results included show (1) the presence of endogenous HGBI that inhibit binding of [^{3}H]*L*-glu to the glutamate-binding sites, (2) purification of one of these HGBI using column chromatographies and gel electrophoresis and (3) physical and pharmacological characterizations of the purified HGBI.

Materials and Methods

Materials

Whole fresh porcine brains were obtained from a local slaughter house. [^{3}H]*L*-glu was obtained from American Radiolabel Chemicals (St. Louis, Mo., USA). DE-52 was from Whatman (Clifton, N.J., USA). Acrylamide, N,N'-methylene-bis-acrylamide, tetramethylethylenediamine, ammonium persulphate, and protein assay solutions were obtained from Bio-Rad (Richmond, Calif., USA). Coomassie brilliant blue R-250 was obtained from Eastman Kodak (Rochester, N.Y., USA). Trypsin inhibitors, phenylmethanesulfonyl fluoride (PMSF), leupeptin, and sodium dodecyl sulfate (SDS) were obtained from Sigma (St. Louis, Mo., USA). Molecular-weight standard markers were obtained from Pharmacia (Piscataway, N.J., USA). All the other chemicals and reagents were obtained through regular commercial sources.

Membrane Preparation for [^{3}H]L-glu-Binding Assays

The membrane preparation started with homogenization of fresh porcine brains in 0.32 *M* sucrose (10 × volume at 4 °C). The homogenate was centrifugated at 1,000 *g* for 15 min, and the supernatant was further centrifuged at 100,000 *g* for 1 h at 4 °C. The resulting pellets were suspended in 50 m*M* Tris-citrate (0.5 × volume, pH 7.2) and frozen at −80 °C. This membrane preparation was subjected to freezing and thawing once more, and washed twice in 5 m*M* Tris-HCl (20 × volume, pH 7.2) to remove endogenous glutamate and aspartate. These washed membranes (W2 membrane) were stored at −20 °C in 50 m*M* Tris-HCl (pH 7.2). Immediately before the binding assay, W2 membranes were quickly washed (5 min) twice in 50 m*M* Tris-HCl containing 0.1% Triton X-100 (4 × volume, pH 7.2), followed by three washes in 5 m*M* Tris-HCl (4 × volume, pH 7.2). These extensively washed membranes were then subjected to [^{3}H]*L*-glu-binding assays.

[^{3}H]L-glu-Binding Assay

Glutamate-receptor-binding assays were conducted as previously described for benzodiazepine-receptor-binding assays with some modifications [15]. Each binding assay consisted of a 750-μl sample of membrane (1 mg/ml protein concentration) in 50 m*M* Tris-HCl buffer (pH 7.2), 100 μl [^{3}H]*L*-glu (3.11 n*M*), various volumes of glutamate-binding inhibitor (GBI) samples, and complementary volumes of 50 m*M* Tris-HCl buffer (pH 7.2), making a final incubation volume of 1 ml. For total binding (TB), the binding mixtures were incubated for 45 min at 4 °C in the presence of [^{3}H]*L*-glu (3.11 n*M*). Nonspecific binding (NB) and ligand binding (LB) were obtained by including 100 μl of 10 m*M* *L*-glu or various volumes of the GBI in the binding mixture 15 min prior to the [^{3}H]*L*-glu incubation. The

incubation was terminated by centrifugation (6 min, 17,000 *g*). The pellet obtained was rinsed twice with 1 ml double-distilled water, before dilution in 450 µl buffer and transferred for radioactivity counting. Specific binding (SB) was obtained by subtracting NB from TB. Inhibition of [^{3}H]*L*-glu binding by the GBI was calculated as [(TB–LB)/SB] × 100, with a 50% inhibition representing 1 arbitrary unit of GBI activity. To assess the effect of the GBI on a dose-response curve for glutamate binding, a sample of the GBI with approximately 1 arbitrary unit was incubated with various concentrations of [^{3}H]*L*-glu. The binding data obtained from these dose-response assays were analyzed using computer radioligand-binding-analysis programs [16].

[^{3}H]L-glu Binding of the GBI

[^{3}H]*L*-glu-binding activity of the GBI was conduced as described above. GBI samples from a DE-52 column were incubated with 3 n*M* [^{3}H]*L*-glu for 45 min at 4 °C, followed by adding 300 µl of 1 mg/ml γ-globulin for 15 min to facilitate protein precipitation. The reaction was stopped by adding 300 µl of 50% polyethylene glycol (PEG) 8000 to precipitate the GBI, followed by filtration through a Whatman GF-B filter paper with vacuum suction. Filters were washed three times with 5 ml 8% PEG before being transferred to counting vials for radioactivity counting. The nonspecific binding of the GBI was performed by including 1 m*M* *L*-glu in the binding mixture during the incubation.

Extraction of GBI

Whole porcine brains were blended in 10 × volumes of ice-cold double-distilled water containing trypsin inhibitor (10 µg/ml), PMSF (1 m*M*), 1 m*M* 2-aminoethylisothiouronium bromide hydrobromide (AET), and leupeptin (0.1 mg/ml), followed by centrifugation at 100,000 *g* at 4 °C for 1 h. The supernatant was collected and filtered through an Amicon Hollow Fiber concentration unit with a 30 kD cutoff filter cartridge (HP 30). The material retained by the concentrator (F1) served as the source of GBI for further purification.

DE-52 Ion Exchange Chromatography

Concentrated F1 was loaded onto a DE-52 anion exchange column (2.5 × 100 cm) equilibrated with 50 m*M* Tris-HCl buffer (pH 7.2). After the sample was loaded, the column was reequilibrated with 50 m*M* Tris-HCl, followed by a step increase in buffer concentration up to 100 m*M*, then up to 300 m*M* Tris-HCl (pH 7.2).

High-Performance Liquid Chromatography (HPLC) with DEAE Anionic Exchange and GF-250 Gel Filtration Column

A DEAE column (Beckman) and a gel filtration GF 250 column (Zorbax, DuPont) were used for the GBI purification. A UV detector and pump were controlled by a Beckman HPLC-programmed GOLD system. A GBI sample from the preparative DE-52 (pool 3; see results and fig. 1) was filtered through a centricon 100 (Amicon), followed by concentration of the filtrate obtained in a centricon 30, to obtain pool 3a. This concentrated pool 3a was injected onto a HPLC DEAE column, followed by a GF 250. Absorbance of the eluate was monitored at 280 nm, and fractions collected were concentrated in a Savant SpeedVac, dialyzed and assayed for GBI activity.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was performed as previously described [28] with minor modifications. For nondenaturing (ND) PAGE, slab gels (0.75 mm thick) consisting 7.5% polyacrylamide in 187 m*M* Tris

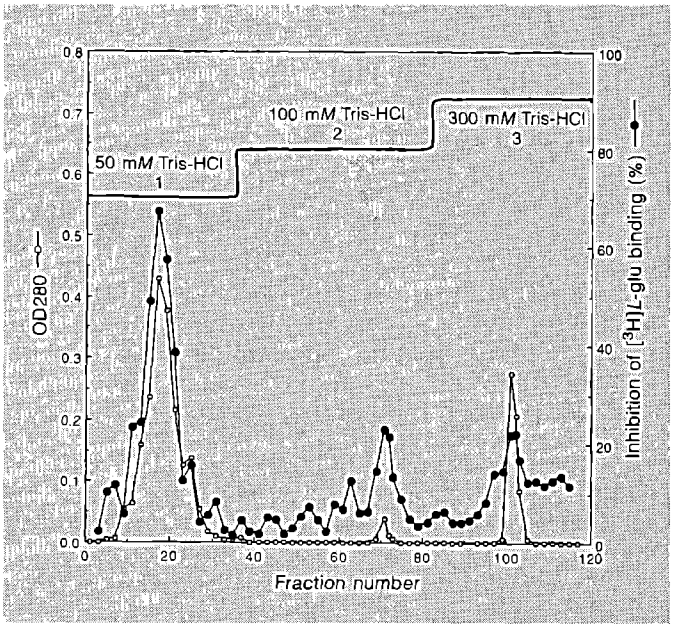


Fig. 1. Purification of GBI on a preparative DE-52 column. A 500-ml sample of the high-speed supernatant from porcine brain extract (approximately 40 g brain) was concentrated in a HP 30 to 50 ml. The concentrated extract was then loaded onto a DE-52 column (1.75 × 35 cm) equilibrated with 50 m*M* Tris-HCl (pH 7.2). The column was washed twice with 50 m*M* Tris-HCl (pH 7.2; fractions 1–40, pool 1), followed by two washes with 100 m*M* Tris-HCl (pH 7.2; fractions 41–80, pool 2), and two washes with 300 m*M* Tris-HCl (pH 7.2; fractions 81–120, pool 3). The elution profile was monitored by a UV detector at 280 nm wavelength (○). A 100-µl sample from each fraction was used in [^{3}H]*L*-glu-binding assays to determine GBI activity (●).

(pH 8.4) and 1 mg/ml ammonium persulfate were used. Denaturing PAGE (SDS-PAGE) consisted of 7.5% polyacrylamide gels with 1% SDS in 187 m*M* Tris (pH 8.4) and 1 mg/ml ammonium persulfate and a sample buffer with 0.5% β-mercaptoethanol. GBI samples from various steps of purification were prepared in electrophoresis running buffer (25 m*M* Tris, 192 m*M* glycine, pH 8.4) containing 10% glycerol, 0.5% β-mercaptoethanol, and 1 m*M* AET for ND-PAGE. Samples for SDS-PAGE were prepared in running buffer (25 m*M* Tris-HCl, 192 m*M* glycine and 0.1% SDS, pH 8.4) containing 10% glycerol, 0.5% β-mercaptoethanol and 1% SDS. Both gel electrophoreses were performed at constant voltage (250 V for 3–4 h; 4 °C for ND-PAGE, 16–18 °C for SDS-PAGE). Standard molecular-weight markers were loaded along with the GBI samples for size determination. For Coomassie blue staining of proteins, gels were stained for 8 h in 0.05% Coomassie brilliant blue R-250, 40% methanol and 7% acetic acid, followed by 1 h destaining in 50% methanol, 10% acetic acid, and 2 h destaining in 7% acetic acid and 5% methanol.

HGBI in [^{3}H]L-glu-Binding Assay with Protease Digestion

Purified HGBI samples were treated with trypsin (2 mg/ml) for 40 min at 37 °C. Digestion was stopped by adding trypsin inhibitor

Table 1. Purification of HGBI from porcine brains (800 g)

Sample	Total activity[a]	Material mg protein	Specific activity[b]	Yield %	Fold purification[c]
F1	11,510.0	2,016.0	5.71		
DE-52 pool 3	1,960.0	311.0	6.30		
Pool 3a	148.0	108.7	1.36	100.0	1.00
HPLC-DE-1	92.0	16.2	5.68	62.2	4.18
HPLC-DE-2	54.2	4.3	12.60	36.6	9.26
HPLC-GF250	17.71	1.1	16.01	11.9	11.80

[a] One activity unit is the amount of GBI that inhibits 50% of [^{3}H]*L*-glu binding.

[b] Specific activity is defined as activity/mg protein.

[c] GBI activity in pool 3a from DE-52 was used as a reference: 100% yield and 1.0-fold purification. Preparations obtained prior to this step were contaminated by *L*-glu and other inhibitors which had significant glutamate-binding activity, and hence are not included for the measurement of the degree of purification of the HGBI.

Table 2. Purification of GBI on a preparative DE-52 column

Sample in DE-52	Total protein mg	Total GBI activity, U	Specific GBI activity U/mg protein
Pool 1	1040	7800	7.51
Pool 2	665	1750	2.63
Pool 3	311	1960	6.30

8 liters of high-speed supernatant from porcine brain extract (8,607 g brain) were concentrated in HP 30 to 400 ml. The concentrated sample was loaded onto a preparative DE-52 column (2.5 × 100 cm) equilibrated with 50 m*M* Tris-HCl, pH 7.2. Conditions for pools 1, 2 and 3 were the same as for DE-52 in figure 1.

Table 3. [^{3}H]*L*-glu-binding activity and inhibition of [^{3}H]*L*-glu binding of GBI pools from preparative DE-52

GBI sample	Specific binding of [^{3}H]*L*-glu pmol/mg protein	GBI activity U/mg protein[a]
1a (30–100 kD)	0.553	26.40
1b (>100 kD)	0.019	3.55
3a (30–100 kD)	0.004	1.36
3b (>100 kD)	0.085	4.35

[a] One unit activity represents the amount of GBI which gives 50% inhibition of [^{3}H]*L*-glu binding.

(final concentration 0.1 mg/ml) in the mixture for 30 min at 4°C. This final mixture was added into the binding mixture for [^{3}H]*L*-glu-binding assays. Two parallel experiments were performed as controls. One control was a HGBI sample incubated with binding buffer under the same conditions as described above, except that trypsin and trypsin inhibitor were excluded. The other control was buffer only incubated with trypsin and trypsin inhibitor, which showed no effect on [^{3}H]*L*-glu binding.

Results

Purification of GBI by Column Chromatography

The purification steps of the GBI from porcine brain are summarized in table 1. Five steps were used to purify the GBI to homogeneity from 100 brains. This represented an approximately 12-fold purification, the overall yield being 11% of the GBI sample obtained from pool 3a of the preparative DE-52 column.

The concentrated high-speed supernatant was loaded onto a 2.5 × 35 cm DE-52 column equilibrated in 50 m*M* Tris-HCl. The column was then washed with two bed volumes of the equilibration buffer (pool 1, fractions 1–40; fig. 1), followed by the same volume of 100 m*M* Tris-HCl (pool 2, fractions 41–80) and 300 m*M* Tris-HCl elution (pool 3; fractions 81–120). A larger preparation was loaded onto a 2.5 × 100 cm DE-52 column, and the elution data are summarized in table 2. It was found that pool 1 and pool 3 had higher specific activity than pool 2. Furthermore, pool 1 also contained high [^{3}H]*L*-glu-binding activity (table 3). The other two pools were also tested for glutamate-binding activity, and it was found that substances with molecular weights between 30 and 100 kD in pool 3 (3a) containing negligible glutamate-binding activity. Therefore, pool 3a was chosen for further purification.

Fractions from pool 3a were concentrated using a centricon 30 and 100 (see Materials and Methods), and purified by two steps of DEAE HPLC column chromatography. The first DEAE HPLC column was eluted with a gradient consisting of Tris-HCl from 50 to 500 m*M*. The GBI activity was eluted at 255 m*M* Tris-HCl. The active fractions from five DEAE HPLC columns were combined and concentrated using a centricon 10, then applied to a second DEAE HPLC column. The column was eluted as before except that a gradient of 100–300 m*M* Tris-HCl (pH 7.2) was used. GBI activity was eluted at 200 m*M* Tris-HCl. The active fractions from six of these DEAE HPLC columns were combined and concentrated using a centricon 10, then applied to a gel filtration HPLC column.

The molecular weight of the GBI was estimated from a GF 250 HPLC column, which was calibrated with molecular-weight standards. The GBI was estimated to have a molecular weight around 70 to 80 kD, and hence is a high-molecular-weight GBI, referred to as the HGBI. Active fractions along with the adjacent fractions from the GF 250 HPLC column were concentrated for further characterization.

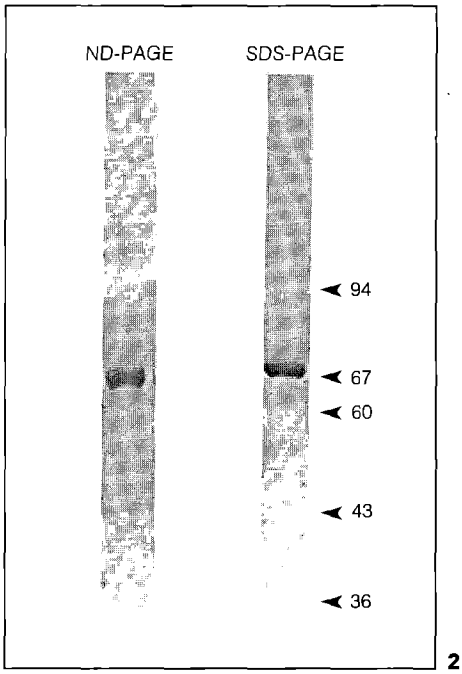
Identification of HGBI on Gel Electrophoresis

The HGBI obtained after the GF 250 gel filtration column appeared to be homogeneous, as judged from native and SDS-PAGE, in which a single protein band was obtained (fig. 2). The most active sample from the GF 250 was loaded for 7.5% PAGE and 7.5% SDS-PAGE. A single protein band corresponding to a molecular weight of 70 kD was found in SDS-PAGE (fig. 2), suggesting that the HGBI is a single-polypeptide-chain protein. In addition, it was found that the 70-kD protein band was only shown in gels containing the active fraction.

The most active HGBI fractions from each step of purification were loaded using the same total amount of protein (2.5 μg) for 7.5% SDS-PAGE, and stained for protein pattern comparisons. As shown in figure 3, a 70-kD protein band was seen in a preparative DE-52 sample with light density (far-right lane). This 70-kD protein band was stained more heavily in the DEAE HPLC samples (next two lanes), and most enriched in the GF 250 HPLC sample (two left lanes). From these results, it is concluded that the HGBI appears to be a protein with a molecular weight of 70 kD, in both native and denatured form.

Stability of the HGBI

The purified HGBI appeared to be unstable, since a loss of activity was observed following heat treatment (95°C for 1 h), or acid-base treatment at pH 1.8 and 11.2. In order to verify that the HGBI is a protein, an HGBI sample was subjected to trypsin treatment (see Methods). It was found that the HGBI treated with trypsin showed much higher activity (83% inhibition of [^{3}H]*L*-glu binding) than a sample without trypsin (45% inhibition). Since a background control with a mixture of trypsin and trypsin inhibitor used in the experiment had no effect on [^{3}H]*L*-glu binding, it is certain that the increase in HGBI activity was mainly due to trypsin digestion. The HGBI activity was also sensitive to oxidant treatment. Oxidized glutathione (1 m*M*) treatment for 40 min at room temperature produced a decrease in HGBI activity from 82 to 21% inhibition of [^{3}H]*L*-glu binding.



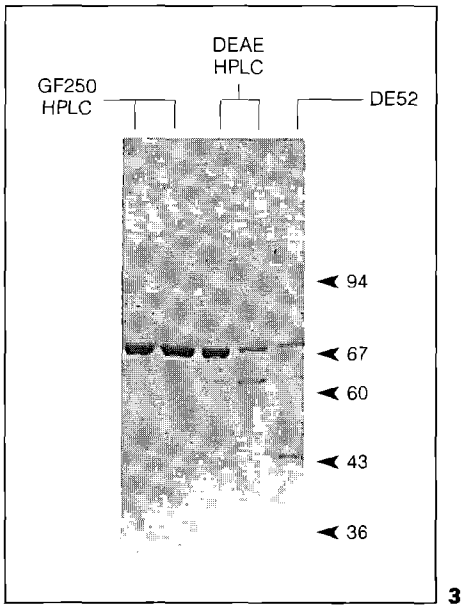


Fig. 2. Analysis of the HGBI on ND- and SDS-PAGE. The fraction with the highest HGBI activity obtained from the GF 250 column was loaded for 7.5% ND (left lane) and a SDS-PAGE (right lane). Molecular weight markers (in kD) are also indicated.

Fig. 3. Analysis of the HGBI at various purification stages in SDS-PAGE. The same amount of protein (2.5 μg) from peak fractions of HGBI samples as loaded for 7.5% SDS-PAGE and stained with Coomassie blue for protein. From the right, the sample in the first lane was obtained from the preparative DE-52 column; the samples in the next two lanes were obtained from the second DEAE HPLC column, and the samples in the last two lanes were obtained from the GF 250 HPLC column. Molecular-weight markers (in kD) are also indicated.

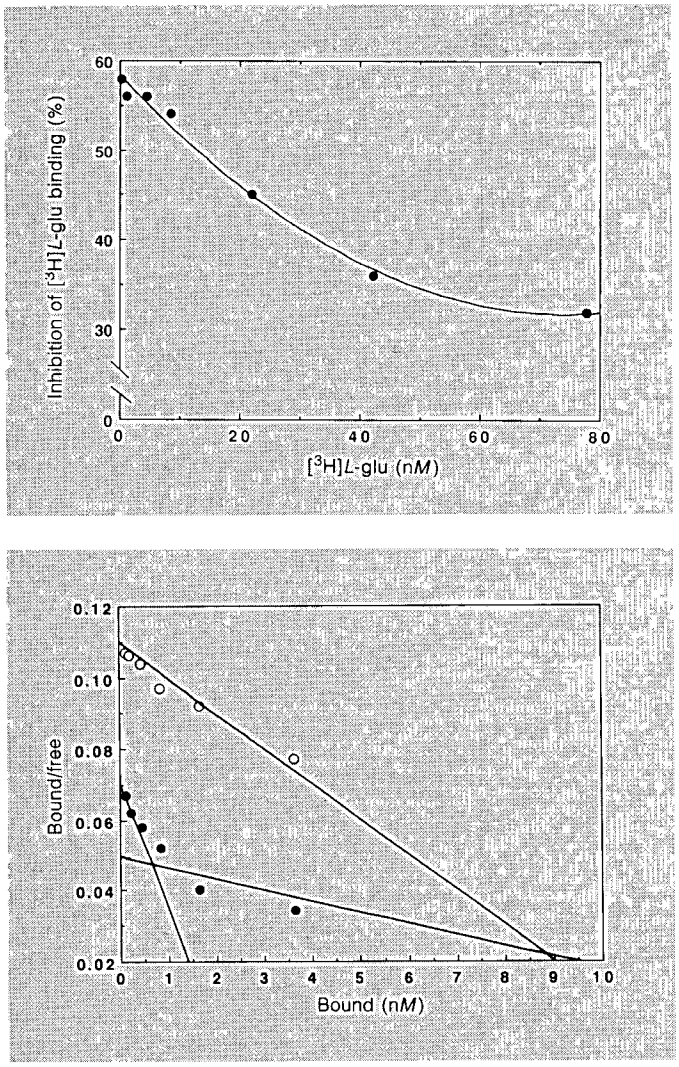


Fig. 4. Actions of the HGBI on [^{3}H]*L*-glu-binding kinetics. **a** HGBI activity as a function of [^{3}H]*L*-glu concentration. [^{3}H]*L*-glu-binding assays were performed as described in Materials and Methods, except that various concentrations of [^{3}H]*L*-glu (0.6–78 n*M*) were included in the assays, which were incubated with a fixed concentration of the HGBI. TB and NB for each concentration of [^{3}H]*L*-glu were obtained to determine inhibition of glutamate binding by the HGBI at each concentration. **b** Effect of the HGBI on a dose-response curve [^{3}H]*L*-glu binding. [^{3}H]*L*-glu-binding assays were performed as described above, without (○) or with (●) samples of the HGBI. Data obtained from binding assays were analyzed using the EBDA program [16] and plotted as shown.

Reversibility of HGBI Inhibition on [^{3}H]L-glu Binding

Samples of partially purified HGBI from the first DEAE HPLC chromatography (1.16 U activity, 58% inhibition of [^{3}H]*L*-glu binding) were incubated with various concentrations of [^{3}H]*L*-glu (0.6–78 n*M*) in [^{3}H]*L*-glu-binding assays. The HGBI activity was found to decrease from 58 to 32% inhibition as the [^{3}H]*L*-glu concentration reached 78 n*M* (fig. 4a).

Effect of the HGBI on a Dose-Response Curve of [^{3}H]L-glu Binding

Samples of the HGBI obtained from the first DEAE HPLC column (1.2 U activity) were incubated with various concentrations of [^{3}H]*L*-glu in glutamate-binding assays (total volume 0.5 ml). A Scatchard plot was obtained for the dose-response curves with and without the HGBI. As shown in figure 4b, in the absence of the HGBI, the dose-response curve gave a linear Scatchard plot (best-fitted by a one-site model) with a dissociation constant (K_d) of 127 n*M* and a B_{max} of 9.0 pmol/mg protein. In the presence of the HGBI, the Scatchard plot changed to a two-side model, with K_d of 47 and 475 n*M* and B_{max} of 1.5 and 9.2 pmol/mg protein for the high- and low-affinity components, respectively.

Discussion

In the present study, the purification and characterization of a HGBI isolated from porcine brain extract was obtained. This endogenous ligand for glutamate receptors has a unique characteristic different from all the other ligands found in the EAA receptor system so far. Three main observations have emerged from the experimental results: (1) glutamate receptor activity can be regulated by a high-molecular-weight protein, which inhibits [^{3}H]*L*-glu binding to EAA receptor; (2) a HGBI, which may contain glutamate-enriched peptides, was purified to homogeneity with a molecular weight estimated at 70 kD, and (3) inhibition of [^{3}H]*L*-glu binding by the purified HGBI is reversible, and appears to change the binding kinetics from a one-site to a two-site model.

During preparative DE-52 purification, the GBI activity was found not only in both concentrations of buffer used for elution (100 and 300 m*M* Tris-HCl), but also in the void volume (pool 1). This observation suggests that there might be more than one HGBI in the brain. However, it should be noted that substances such as glutamate-binding proteins could bind to [^{3}H]*L*-glu, and thereby decrease the concentration of radiolabeled ligands in the receptor-binding assays. Therefore, pool 3a, which has high specific GBI activity and negligible glutamate-binding activity was chosen as the starting material for further purification. Since pool 3a, instead of crude brain extract, was used as a reference for obtaining estimates of purifica-

tion of the HGBI, a 12-fold purification seems reasonable. Although the multiplicity of the HGBI is still unclear, it is conceivable that several HGBI are involved in the modulation of EAA receptor activity.

The HGBI purified from pool 3a has chemical properties similar to that exhibited by a protein, such as heat and acid-base instability. This is further strengthened by the observation from gel electrophoresis, in which a protein band correlates well with the HGBI activity, and also by the result showing that the HGBI is sensitive to trypsin digestion. It is interesting that the trypsin-digested HGBI was found to be more active than the native protein in inhibiting [^{3}H]*L*-glu binding. This result could be due to the fact that the trypsin treatment may produce glutamate or glutamate-containing peptides which have structural similarity to glutamate and hence are more potent than the native protein in inhibiting glutamate receptor binding. This is similar to the diazepam-binding inhibitor system in which fragments obtained by trypsin digestion are more potent than the native peptide in inhibiting the binding of diazepam to the benzodiazepine-binding site [10, 12]. Although the nature of the HGBI remains unclear, it seems that it is a negatively charged molecule. This is based upon the observation that the purified HGBI can be eluted only by a high salt concentration in anion exchange columns. This property could result from exposure of some acidic amino acids, such as *L*-glu and *L*-asp, on the surface of the HGBI molecule. Although interpreting competitive inhibition in receptor-binding assays is complex [29], these results strengthen the idea that the purified HGBI could be a competitive inhibitor for glutamate receptors, possessing an acidic-amino-acid-enriched surface for binding to agonist-binding sites.

That the HGBI was purified to homogeneity is suggested by the single protein band observed in PAGE analysis. Compared to the protein profile of the pool 3a sample on SDS-PAGE, the purified HGBI obtained from the GF 250 column seems to be one of the four major proteins seen in the pool 3a profile. As pool 3a contained about one-seventh of the total protein (approximately 2,100 mg) extracted from ten porcine brains, it is estimated that each porcine brain (approximately 80 g) contained approximately 5–7 mg of the HGBI.

Since most of the EL for glutamate receptors are small molecules (such as amino acids and metal ions), the discovery of a HGBI may suggest a new mechanism of EAA neurotransmission. An analogous example is the diazepam-binding inhibitor for the benzodiazepine receptor. The diazepam-binding inhibitor is a 10-kD protein which was found to be a competitive inhibitor for [^{3}H]diazepam binding to the benzodiazepine receptor [12]. In vivo studies have shown that the diazepam-binding inhibitor acted as a partial agonist-antagonist for allosteric modulation of $GABA_A$ receptor activity, and its posttranslational processing products were found to inhibit diazepam binding [10, 12]. Some of these phenomena are very similar to those seen with the HGBI, a protein ligand with its trypsin-digested products inhibiting glutamate binding, and also assumed to be a competitive glutamate receptor ligand. It should be noted that the appearance of competitive inhibition could also result from a negative allosteric modulation of the radioligand-binding sites [8, 29]. In addition, the HGBI may be structurally too large to be a competitive ligand for the glutamate-binding site. Therefore, the possibility of the HGBI being a noncompetitive inhibitor must also be considered.

In summary, a HGBI from porcine brain extract was purified to homogeneity. The purified HGBI appears to be a protein with a molecular weight of approximately 70 kD. The HGBI is highly negatively charged, suggesting that it may contain acidic amino acids and, most likely, *L*-glu- and *L*-asp-enriched regions, responsible for its surface charge as well as for its binding to glutamate receptors. Inhibition of [^{3}H]*L*-glu binding by the HGBI is reversible, and appears to be a competitive inhibition. Since this HGBI represents a new class of modulator for the EAA receptor, further investigation of the function and structure of the HGBI will undoubtedly shed new light on the understanding of EAA-mediated neurotransmission.

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