Reassessment of [³H]Glutamate Binding to Human Brain Membrane Preparations

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ABSTRACT— We studied [³H]glutamate binding to human brain membrane preparations. We found that the binding assay of [³H]glutamate should be done at 4°C using glass-fiber filters pretreated with polyethyleneimine, sterile incubation buffer, and membrane preparations pretreated with a detergent. Under these assay conditions, we have characterized the three subtypes of glutamate receptors: N-methyl-D-aspartate-, quisqualate- and kainate-sensitive binding sites. Scatchard analysis revealed that the N-methyl-D-aspartate-sensitive [³H]glutamate binding sites consisted of a single component with a dissociation constant (K_d) of 28.2 ± 2.8 nM and total concentration (B_{max}) of 36.2 ± 3.6 fmol/mg protein ($N = 8$). These data were much different from those presented in previous studies.

L-Glutamate (GLU) is a candidate of major excitatory neurotransmitters in the mammalian central nervous system. From ligand binding experiments, glutamate binding sites in the brain are now divided into three subtypes according to differential affinities to exogenous agonists: N-methyl-D-aspartate (NMDA)-, quisqualate (QA)- and kainate (KA)-sensitive binding sites. [³H]glutamate is a useful ligand because it is able to interact with all three subtypes of the acidic amino acid receptors. However, there have been inconsistencies and lack of reproducibility in experiments using [³H]glutamate as a ligand (1–4). Recently, Ogita and Yoneda (5, 6) pointed out methodological artifacts and problems in the [³H]glutamate receptor binding assay and showed an apparent dissociation constant of 70 nM, which is more than ten-fold lower than

the values obtained from the previous reports in rat brain preparations (1–4) by using their improved method.

The binding characteristics to human post-mortem brain membrane preparations are also controversial and inconclusive (7, 8). Apparent dissociation constant values of 844 nM to 1520 nM to [³H]glutamate binding in the human temporal cortical membrane preparation have been reported (7), and these are more than ten- to twenty-fold higher than the value obtained from rat brain preparations by Ogita and Yoneda (5, 6).

We decided to find the best assay conditions for [³H]glutamate binding to human brain membrane preparations. Ogita and Yoneda (5, 6) have proposed five points that would facilitate the development of optimum assay conditions for the binding experiments of [³H]glutamate: 1) purification of [³H]glutamate by column chromatography, 2) treatment of

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the glass-fiber filters with polyethyleneimine (PEI), 3) sterilization of the incubation buffer, 4) treatment of the membrane preparations with a detergent, and 5) the incubation temperature. In this report, we presented the significance of these factors in [^3H]glutamate binding assays for human brain membrane preparations.

MATERIALS AND METHODS

Autopsy materials

Brain tissues were obtained at autopsy from eight subjects (mean age, 78 years; mean post-mortem delay, 8 hours) with no clinical or morphological evidence of brain pathology. None of them had been treated with neuroleptics or antidepressants. The causes of death were cardiac insufficiency or a terminal respiratory condition. Immediately after autopsy, the brains were divided into halves sagittally; one half was used in the biochemical binding assays and the other half was examined histopathologically. The half to be used for the biochemical binding assays was stored at -80°C until thawing for homogenization and the binding assay. Sixteen hours prior to dissection, the frozen brains were transferred to a -20°C cold box to allow sections to be easily and uniformly cut. They were cut coronally by a meat slicer in a 4°C room into approximately 3-mm-thick slices. The brain region was punched out from successive frozen slices using precooled needles. All brains appeared normal on histological examination. The brain region examined was the frontal cortex (Brodmann area 10).

Membrane preparations

The tissue samples were homogenized in 10 vol. of 50 mM Tris-acetate buffer (pH 7.4) using a Teflon-glass Potter-Elvehjem homogenizer (700 rpm, 10 strokes), and the homogenate was centrifuged at $1,000 \times g$ for 10 min. The supernatant was collected and centrifuged at $50,000 \times g$ for 20 min. The obtained pellet was resuspended in 30 vol. of 50 mM Tris-acetate buffer (pH 7.4), and stored at

-20°C for more than 18 hours. On the day of the experiment, after thawing at room temperature, the membrane suspension was incubated either in the presence or absence of 0.08% Triton X-100 at 37°C for 30 min according to the method of Ogita and Yoneda (5) with a slight modification. After incubation, the suspension was centrifuged at $50,000 \times g$ for 20 min, and the pellet was washed three times by resuspension in the buffer, followed by centrifugation. The final pellet was resuspended in the buffer and stored at -80°C . Protein concentrations of the membrane preparations were determined by the method of Lowry et al. (9).

Binding assays

The membrane preparations (containing 100 μg protein) were incubated with [^3H]glutamate in 50 mM Tris-acetate buffer (pH 7.4) in a final volume of 250 μl at 4°C for 30 min. The reaction was terminated by adding 3 ml of ice-cold buffer and filtered under reduced pressure through Whatman GF/C glass-fiber filters which were presoaked in 0.1% PEI for at least 5 hours according to the method of Bruns et al. (10) with a slight modification. The filters were washed three times with 3 ml ice-cold buffer and counted for radioactivity in 5 ml of scintillation fluid at the counting efficiency of 51–54%. Before each experiment, the buffer solution used for the incubation was boiled at 100°C and then filtered through a millipore filter (Millex-GV 0.22 μm). Specific binding was calculated as the difference of the bindings in the absence and presence of 100 μM GLU (GLU-sensitive binding sites), 100 μM NMDA (NMDA-sensitive binding sites), 100 μM QA (QA-sensitive binding sites) or 100 μM KA (KA-sensitive binding sites).

Drugs used

[^3H]Glutamate (specific activity, 54.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). N-methyl-D-aspartate (NMDA), quisqualate (QA), DL-2-amino-5-phosphonovaleric acid (APV) and

DL-2-amino-4-phosphonobutyric acid (APB) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glutamate (GLU) and kainate (KA) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade or of the purest grade commercially available.

RESULTS

Here, we will report the significance of five factors. 1) The purity of $[^3\text{H}]$ glutamate: We could not find any substantial contamination in the commercially available $[^3\text{H}]$ glutamate as determined by column chromatography. When the purchased $[^3\text{H}]$ glutamate (25.8 MBq; New England Nuclear, Boston, MA, U.S.A.) was applied to a 5-ml column containing a slurry of Dowex cation exchange resin (50W-X8 mesh in the H^+ -form) and eluted by 15 ml of distilled water and 30 ml of 0.5 M HCl, all radioactivity was recovered in fractions eluted by HCl (data not shown). 2) $[^3\text{H}]$ Glutamate binding to glass-fiber filters: $[^3\text{H}]$ Glutamate bound to Whatman GF/C glass-fiber filters dose-dependently. This binding was eliminated by preceding treatment of filters with 0.1% PEI (data not shown). 3) Sterilization of the incubation buffer: The binding data of $[^3\text{H}]$ glutamate were apparently more stable when the incubation buffer had been boiled and then filtered through a millipore filter before use (Fig. 1). 4) Treatment of the membrane preparations with a detergent: We obtained no substantial $[^3\text{H}]$ glutamate binding to untreated brain membrane preparations. The binding to membrane preparations pretreated with 0.08% Triton X-100 for 30 min was saturable and allowed us to assess the binding parameters (data not shown). 5) The reaction temperature: The binding was more stable under the incubation temperature of 4°C than 37°C (data not shown).

From the data presented above, we concluded that the binding assay of $[^3\text{H}]$ glutamate should be performed at 4°C , using glass-fiber filters treated with PEI, sterilized incubation buffer and membrane preparations pretreated

Fig. 1. Time-dependent binding of $[^3\text{H}]$ glutamate to human cortical membrane preparations pretreated with Triton X-100. Membrane preparations (100 μg of protein) were incubated in buffer containing 40 nM $[^3\text{H}]$ glutamate for up to 45 min. The incubation buffer was either untreated (B) or used after boiling at 100°C for 30 min and then filtered through millipore filters (A). $\bullet$: Total binding, $\circ$: Non-specific binding in presence of 100 μM glutamate, $\star$: Specific binding, difference of total and non-specific binding.

with a detergent.

Under these assay conditions, $[^3\text{H}]$ glutamate binding was dose-dependently inhibited by exogenous glutamate analogues including APV and APB. The efficiency for the inhibition of the binding was in the order of $\text{GLU} > \text{QA} > \text{KA} > \text{NMDA} = \text{APV} > \text{APB}$ (Fig. 2). Specific $[^3\text{H}]$ glutamate binding reached an equilibrium within 15 min at 4°C . These bindings

Fig. 2. Inhibition of specific [^3H]glutamate binding to human brain membrane preparations. Membrane preparations (100 μg of protein) were incubated with 40 nM [^3H]glutamate at 4°C for 30 min, in the presence of increasing concentrations of various glutamate analogues: ●: GLU, ○: QA, *: KA, ■: NMDA, △: APV, ▲: APB. The results are the mean $\pm$ S.E.M. of data from 4–8 experiments.

were reversible and Scatchard analysis of saturation isotherms revealed that GLU-, QA-, KA- or NMDA-sensitive [^3H]glutamate binding was saturable and that each of them consisted of a single component (Fig. 3, Table 1). In the case of NMDA-sensitive [^3H]glutamate

Table 1. Specific [^3H]glutamate binding to human brain membrane preparations

Sensitive site	K_d (nM)	$B_{\max}$ (fmol/mg protein)
Glutamate	26.4 ± 2.9	56.0 ± 7.9
NMDA	28.2 ± 2.8	36.2 ± 3.6
Quisqualate	26.8 ± 4.0	57.2 ± 8.7
Kainate	16.4 ± 2.6	27.7 ± 3.9

Values were determined from Scatchard plots and represented the mean $\pm$ S.E.M. of 6–8 separate experiments.

binding, the rate constant of association (K_{+1}) was $2.133 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, and the rate constant of dissociation (K_{-1}) was 0.0673 min^{-1} . The dissociation constant calculated from the equation $K_d = K_{-1}/K_{+1}$ was 31.6 nM, which agreed well with that estimated from Scatchard analysis.

DISCUSSION

The binding parameters obtained from Scatchard analysis were much different from those presented in the previous study. The K_d value was 1/20 and the $B_{\max}$ value was 1/500 the values reported by Cross et al. (8); and the K_d value was 1/30 and the $B_{\max}$ value

Fig. 3. Saturation isotherms (A) and Scatchard plots (B) of NMDA-sensitive [^3H]glutamate binding to human brain membrane preparations (100 μg of protein) pretreated with Triton X-100, at 4°C for 30 min, over the concentration range from 4 to 60 nM.

was 1/20 the values reported by Cowburn et al. (7). There may be several reasons for the discrepancies in the results of glutamate binding by different laboratories. Our present result suggests that differences in assay conditions and buffers may produce different results. In addition, B_{max} values of these glutamate subtypes in human brains were about 1/20 of those found in rat brain, although the K_d values were equivalent (5). Moreover, 100 μ M KA inhibited [³H]glutamate binding by 50% in this experiment and by 18–30% in the previous reports using the rat brain (11, 12). These findings suggest a species difference.

The role of excitotoxicity of glutamate in neuronal damages caused by ischemia/hypoxia now seems to be established. It has been hypothesized that the excitotoxicity of the amino acid may also be involved in the pathogenesis of neurodegenerative diseases of unknown etiology, including Alzheimer's disease. However, there are some discrepancies in the changes in NMDA, QA and KA-sensitive glutamate receptor binding in Alzheimer's disease reported by different laboratories (13). Although the severity of a disease might determine the degree of receptor changes measured, our present study indicates the importance of the assay and buffer conditions and, we have proposed, an appropriate method to identify possible changes in the glutamate receptor subtypes in Alzheimer postmortem brains.

The most well-characterized class of glutamate receptors is the NMDA receptor (14), owing in large part to the existence of compounds that specifically block NMDA binding sites (15). In contrast, elucidation of the non-NMDA receptors, the QA and KA receptors, has been hampered by the lack of such compounds. QA-sensitive [³H]glutamate binding most likely represents binding to the physiologically defined QA receptor on post synaptic neuronal membranes (1, 16). Therefore, the [³H]glutamate binding site that was displaced by 100 μ M QA was termed the QA-sensitive binding site in this experiment. It has been reported that the QA inhibition was not specific

for the QA subtype of glutamate receptors, and QA binds with high affinity to the QA and KA receptor subtypes and with low affinity to the NMDA subtype (17–19). However, it has also been reported that KA and QA bind the same receptor and activate the receptor in cultured neuronal cells (20, 21). As the detection of glutamate receptor subtypes is controversial, further studies for glutamate receptor subtypes are required. Recently, [³H] α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) has been used as a specific radioligand available for quisqualate receptors (22, 23), and [³H]KA has been used to purify the KA receptors (24, 25). These [³H]AMPA and [³H]KA might be useful ligands to further clarify the properties of the glutamate receptors in the human brain.

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