Evaluation of L-Glutamate Clearance Capacity of Cultured Rat Cortical Astrocytes

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Astrocytes have a function in the uptake of excitatory neurotransmitter L-glutamate *via* the glutamate transporter. To evaluate the L-glutamate clearance capacity of astrocytes, we developed a colorimetric method for the determination of L-glutamate concentration and measured changes in extracellular L-glutamate concentration in rat cortical astrocyte cultures. When L-glutamate (50—200 μM) was added to astrocyte cultures and incubated for 1—8 h, the extracellular L-glutamate concentration declined with time. When L-glutamate was mixed with astrocyte culture supernatants only, no significant change in L-glutamate concentration was observed, ruling out the possibility that L-glutamate is spontaneously or enzymatically degraded in the extracellular space. Alternatively, the time-dependent decline of extracellular L-glutamate concentration was blocked by the presence of glutamate uptake inhibitors, indicating that the glutamate uptake system of astrocytes plays a major role in the clearance of extracellular L-glutamate.

Key words L-glutamate; colorimetric measurement; astrocyte; glutamate transporter; culture

L-Glutamate functions as a major excitatory neurotransmitter in the central nervous system, but excessive stimulation by L-glutamate is known to cause excitoxic damage to neurons, which may underlie the abnormal neuronal degeneration observed following hypoxia, ischemia, hypoglycemia and seizures.[1—3] Astrocytes have a function in the uptake of extracellular L-glutamate *via* the glutamate transporter, and may serve to protect neurons from L-glutamate toxicity. Indeed, it has recently been reported that familial amyotrophic lateral sclerosis, a neurodegenerative disease, is caused by the impairment of a glutamate uptake system of astrocytes.[4] Thus, drugs which can promote L-glutamate uptake by astrocytes may be useful in the treatment of some neurodegenerative diseases.

To evaluate the capacity of astrocytes to clear extracellular L-glutamate, it is necessary to measure changes in extracellular L-glutamate concentration. Chromatographic analysis is a widely used method for the quantitation of L-glutamate concentration, but requires time and specific apparatus. Nicholls *et al.* have developed a simple fluorometric method which measures the fluorescence of NADPH produced by the reaction of L-glutamate and glutamate dehydrogenase (E.C. 1.4.1.3).[5] In the present study, we modified the method by Nicholls *et al.* and developed a colorimetric method using the redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Our method is very convenient and economical, and can be used in many laboratories. In this paper, we describe the details of our colorimetric method for the measurement of L-glutamate concentration and its application to evaluate the L-glutamate clearance capacity of cultured astrocytes.

MATERIALS AND METHODS

Chemicals MTT, β-nicotinamide adenine dinucleotide (NAD), 1-methoxyphenazine methosulfate (MPMS), DL-*threo*-β-hyroxyaspartate (THA), L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), dihydrokainate and quisqualate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Glutamate, glutamate dehydrogenase from beef liver (EC 1.4.1.3) and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Determination of L-Glutamate Concentration The principle of our colorimetric method is illustrated in Fig. 1. Accompanying the reaction of L-glutamate with glutamate dehydrogenase, NAD should be converted into NADH, which would in turn reduce MTT (yellow) to MTT formazan (purple) by the support of MPMS, an electron coupling agent.[6] One ml of the substrate mixture was prepared as follows: 20 U glutamate dehydrogenase and 2.5 mg NAD were dissolved in 0.9 ml of 0.2 M Tris–HCl buffer (pH 8.2) with 0.1% (v/v) Triton X-100, then 0.1 ml of MTT stock solution (2.5 mg/ml in phosphate-buffered saline, pH 7.4) and 1 μl of MPMS stock solution (100 mM in phosphate-buffered saline, pH 7.4) were added. Fifty μl of culture supernatant was transferred to another 96-well culture plate, then mixed with 50 μl of the substrate mixture. The reaction proceeded for 10 min at 37 °C and was stopped by adding 100 μl of a solu-

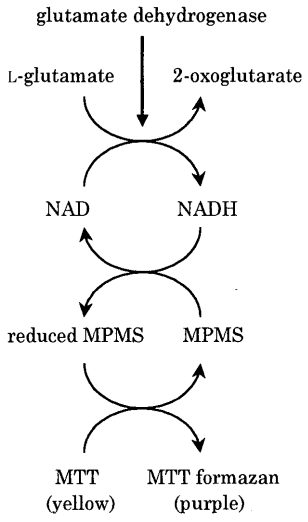

Fig. 1. Principle of Colorimetric Measurement of L-Glutamate Concentration

Accompanying the reaction of L-glutamate and glutamate dehydrogenase, NAD is converted into NADH, which in turn reduces MTT to MTT formazan by the support of MPMS, an electron coupling agent.

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tion containing 50% dimethylformamide and 20% sodium dodecyl sulfate (DMF/SDS, pH 4.7), which was originally developed for extracting intracellular MTT formazan in the MTT reduction assay.[7] Other purposes of this solution are to increase the solubility of MTT formazan and to eliminate background absorbance from the phenol red present in the culture medium. In addition, it is known that at a pH higher than 5.5, MTT is spontaneously converted into MTT formazan, while at a pH lower than 4, MTT formazan is converted into MTT again.[7] MTT formazan was very stable in DMF/SDS (pH 4.7), and there was no change in the color for several days. The absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm. The standard curve was constructed in each assay using cell-free culture medium containing known concentrations of L-glutamate. The concentration of L-glutamate in samples was estimated from the standard curve.

Cell Culture Primary cultures of astrocytes were prepared from the cerebral cortices of 2-d-old neonates of Wistar rats, as described previously.[8,9] Briefly, dissociated cortical cells were suspended in modified Eagle's medium containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% fetal bovine serum, and plated on uncoated 25 cm^2 flasks at a density of 600000 cells/cm^2. A monolayer of type I astrocytes was obtained 12—14 d after the plating. Non-astrocytes such as microglia were detached from the flasks by shaking, and were removed by changing the medium. Astrocytes were dissociated by trypsinization and reseeded on uncoated 48-well plates at a density of 20000 cells/cm^2. After the cells became confluent (approximately 9—10 d later), the culture medium was switched to serum-free medium, and experiments were initiated 24 h later.

Data Analysis Each assay was done in duplicate, and the same experiments were repeated with separate cultures obtained from different litters. The data are represented as the mean±S.E.M. of the values obtained from separate cultures.

RESULTS

First of all, we checked the sensitivity, linearity and specificity of our method for the quantitation of L-glutamate concentration. A medium containing 6.25—1000 μM L-glutamate was mixed with the substrate mixture containing glutamate dehydrogenase, NAD, MTT, MPMS and Triton X-100. The reaction proceeded for 10 min at 37 °C, and the absorbance was measured after the addition of DMF/SDS. As shown in Fig. 2, MTT formazan was produced in proportion to L-glutamate concentration. The standard curve was linear for 6.25—200 μM L-glutamate, but reached a plateau at >600 μM L-glutamate. This assay condition will be optimal for measuring 1—200 μM L-glutamate. When D-glutamate or L-aspartate (10 μM—10 mM) was mixed with the substrate mixture, no MTT formazan was produced (data not shown). These results confirm that the L-glutamate concentration can be quantitatively and specifically determined by our method.

Next, using the above method, we measured changes in extracellular L-glutamate concentration in astrocyte cultures. L-Glutamate (25—200 μM) was exogenously added to astrocyte cultures, then the cell culture supernatants were collected

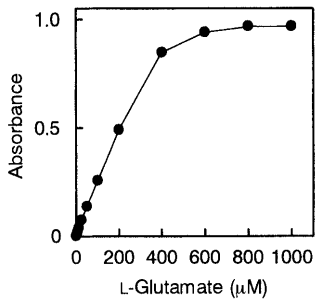

Fig. 2. A Standard Curve for the Determination of L-Glutamate Concentration

Fifty μl of the substrate mixture was mixed with 50 μl of the culture medium containing 6.25, 12.5, 25, 50, 100, 200, 400, 600, 800 or 1000 μM L-glutamate, and the reaction proceeded at 37 °C for 10 min. Following the addition of DMF/SDS, the absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm. An example from one experiment is shown here.

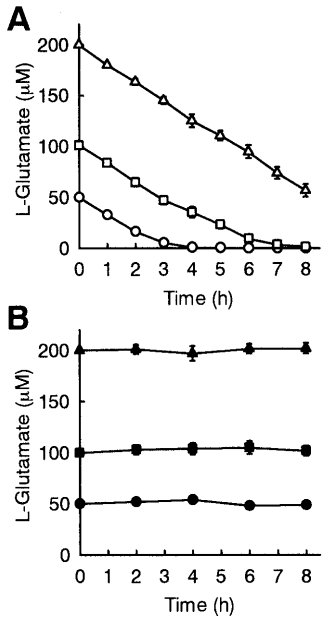

Fig. 3. Time- and Concentration-Dependent Decline in Extracellular L-Glutamate Concentration in Rat Cortical Astrocyte Cultures

(A) L-Glutamate (○, 50 μM; □, 100 μM; △, 200 μM) was added to astrocyte cultures at time 0. Following incubation for 1—8 h, the culture supernatant was collected for the determination of L-glutamate concentration. (B) Fifty μl of astrocyte culture supernant was transferred to another 96-well culture plate, and L-glutamate (●, 50 μM; ■, 100 μM; ▲, 200 μM) was added. Following incubation for 1—8 h, L-glutamate concentration was determined. All data are represented as the means±S.E.M. of 5 separate observations.

1—8 h later. As shown in Fig. 3A, the extracellular L-glutamate concentration declined with time. Fifty μM L-glutamate was decreased to below 1 μM within 4 h. One hundred μM L-glutamate was decreased to below 1 μM within 8 h. Two hundred μM L-glutamate was decreased to approximately 30% 8 h later.

Why did extracellular L-glutamate concentration decline with time? Two possibilities were considered: 1) L-glutamate is spontaneously or enzymatically degraded in the extracellular medium, or 2) extracellular L-glutamate is removed by the glutamate uptake system of astrocytes. To test the first possibility, 50 μl of the astrocyte culture supernatant was transferred to another culture plate, then L-glutamate was added to it. However, in this case, the L-glutamate concentration was not changed at least up to 8 h after the addition of L-glutamate (Fig. 3B), indicating that the presence of astrocytes is

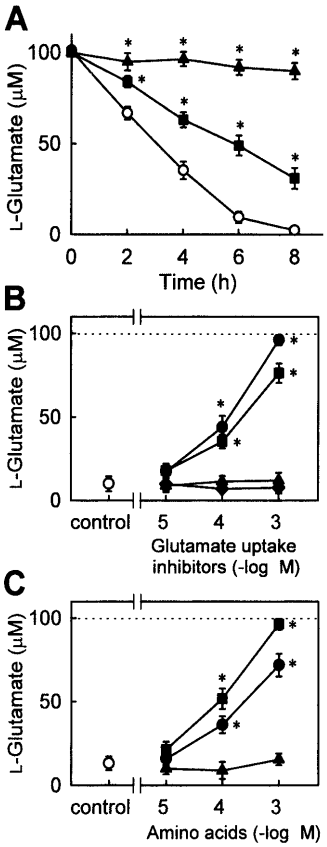

Fig. 4. Effects of Glutamate Uptake Inhibitors and Amino Acids on the Clearance of Extracellular L-Glutamate in Rat Cortical Astrocyte Cultures

(A) Effect of THA on time-course. L-Glutamate (100 μM) was added alone (○) or together with THA (■, 100 μM; ▲, 1000 μM) at time 0. Following incubation for 1—8 h, the culture supernatant was collected for the determination of L-glutamate concentration. (B) Concentration-dependent effects of THA, PDC, dihydrokainate and quisqualate. L-Glutamate (100 μM) was added alone (○) or together with THA (●), PDC (■), dihydrokainate (▲) or quisqualate (◆), and the concentration of L-glutamate remaining in the culture supernatant was determined 6 h later. (C) Concentration-dependent effects of D-aspartate (●), L-aspartate (■) and D-glutamate (▲). Experimental procedure was as in B. All data are represented as the means±S.E.M. of 5 separate observations. * $p<0.01$ *vs.* L-glutamate alone (○), Duncan's multiple range test.

required for the time-dependent decline in extracellular L-glutamate. To test the second possibility, the effects of four glutamate uptake inhibitors, THA, PDC, dihydrokainate and quisqualate, were investigated. As shown in Fig. 4A and B, the time-dependent decline in extracellular L-glutamate concentration was significantly blocked by THA or PDC, but not by dihydrokainate or quisqualate. In addition, glutamate transporters are known to transport D- and L-aspartate as well as L-glutamate, but poorly transport D-glutamate.[10] If extracellular L-glutamate was removed by the glutamate uptake system, the decline of L-glutamate concentration should be blocked by D- and L-aspartate, but not by D-glutamate. We thus compared the effects of stereoisomers of glutamate and aspartate. Indeed, the decline of extracellular L-glutamate concentration was significantly blocked by D- and L-aspartate, but not by D-glutamate (Fig. 4C).

DISCUSSION

Our colorimetric method quantitatively and specifically measures the concentration of L-glutamate. Our method is very convenient and economical, and can be used in many laboratories. Although the assay condition described in this paper is optimal for measuring 1—200 μM L-glutamate, the sensitivity can be adjusted by changing the reaction time or the concentration of substrates (glutamate dehydrogenase, NAD or MPMS).

The glutamate uptake activity has been conventionally evaluated by measuring the amount of [^{3}H]glutamate uptake into the cells. However, this measurement does not indicate to what degree the glutamate uptake contributes to the clearance of extracellular L-glutamate. To evaluate the glutamate clearance capacity of the cells, it is necessary to measure changes in extracellular L-glutamate concentration. We applied our colorimetric method to astrocyte cultures, and measured changes in extracellular L-glutamate concentrations. Exogenously added L-glutamate gradually disappeared from the culture medium with time. L-Glutamate concentration was unchanged when L-glutamate was mixed with culture supernatants only, ruling out the possibility that L-glutamate is spontaneously or enzymatically degraded in the extracellular medium. The presence of astrocytes is required for the clearance of extracellular L-glutamate.

It has been reported that astrocytes possess three different types of glutamate uptake systems: Na^+-dependent uptake, Cl^--dependent uptake and Ca^{2+}-dependent uptake.[11] The Na^+-dependent uptake system transports D- and L-aspartate as well as L-glutamate, and is inhibited by THA. In contrast, the Cl^-- and the Ca^{2+}-dependent uptake systems do not handle D- and L-aspartate as substrates, and is strongly inhibited by quisqualate. The glutamate clearance observed in the present study was inhibited by D- and L-aspartate or THA, but was not affected by quisqualate. These results suggest that the Na^+-dependent glutamate uptake system, but not the Cl^-- and the Ca^{2+}-dependent systems, contributes to the clearance of extracellular L-glutamate.

Five subtypes of Na^+-dependent glutamate transporters have been identified in the nervous system, *i.e.*, EAAC1, GLT-1, GLAST, EAAT4 and EAAT5.[12—17] It is known that GLT-1 and GLAST are expressed in astrocytes. THA and PDC block both GLT-1- and GLAST-mediated glutamate transport, while dihydrokainate selectively inhibits GLT-1.[18] In the present study, the glutamate clearance was blocked by THA and PDC, but not by dihydrokainate, suggesting that GLAST, but not GLT-1, play a major role.

In a previous study, we confirmed that our cultured astrocytes express a high level of GLAST, but GLT-1 expression was too low to be detected by Western blot analysis.[19] Although it remains unclear why GLT-1 was not detected in our cultures, the expression of GLT-1 may be influenced by experimental conditions. Swanson *et al.* have recently reported that GLT-1 is not expressed in pure astrocyte cultures, but that its expression is enhanced in the presence of neurons.[20] Considering that our cultures consist of high-purity (>98%) astrocytes, the low expression of GLT-1 may be due to the lack of neuronal regulation.

In conclusion, we developed a colorimetric method for the quantitation of L-glutamate concentration and demonstrated that the glutamate uptake system of astrocytes plays a major role in the clearance of extracellular L-glutamate. Our evaluation method should be useful in the search for drugs that can promote or inhibit glutamate clearance by astrocytes.

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