Inhibition by Theanine of Binding of $[^3H]$AMPA, $[^3H]$Kainate, and $[^3H]$MDL 105,519 to Glutamate Receptors

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In an investigation of the mechanisms of the neuroprotective effects of theanine ($\gamma$-glutamylthylamid) in brain ischemia, inhibition by theanine of the binding of $[^3H]$(RS)-$\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), $[^3H]$kainate, and $[^3H]$(E)-3-(2-phenyl-2-carboxyethyl)-4,6-dichloro-1-H-indole-2-carboxylic acid (MDL 105,519) to glutamate receptors was studied in terms of its possible inhibiting effects on the three receptor subtypes (AMPA, kainate, and NMDA glycine), with rat cortical neurons. Theanine bound the three receptors, but its IC$_{50}$ of theanine was 80- to 30,000-fold less than that of $\lambda$-glutamic acid.

Key words: theanine; glutamate receptor; neuroprotection; tea

The glutamate-calcium theory has almost been accepted as an explanation of the mechanisms of ischemic neuronal death, in which glutamic acid, an excitatory neurotransmitter, is discharged in excess into the extracellular space by transient ischemia, and acts to excite toxicity. Theanine is an analog of the glutamic acid found in leaves of high-grade Japanese green tea (Camellia sinensis), and we have examined the effects of theanine ($\gamma$-glutamylthylamid) on glutamate receptors, and the possibility of its protecting against ischemic neuronal death. We earlier found a neuroprotective effect of theanine on the neuronal death of hippocampal CA1 neurons in transient forebrain ischemia of gerbils to which theanine was injected into the lateral ventricle before ischemia. In this study, the binding effects of theanine on three receptor subtypes, for (RS)-$\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate, and $N$-methyl-$\beta$-aspartate (NMDA) receptors, were studied to confirm the mechanism of theanine neuroprotection in a radioligand binding assay.

The inhibiting effects of theanine on glutamate receptor binding of AMPA, kainate, and the NMDA glycine site antagonist (E)-3-(2-phenyl-2-carboxyethyl)-4,6-dichloro-1-H-indole-2-carboxylic acid (MDL 105,519) in cerebral cortices of Wistar rat (MDS Pharma Services-Taiwan Pharmacology Laboratories, Taipei) were compared with those of $\lambda$-glutamic acid. The animal care protocol was approved by the Experimental Animal Care Committee of MDS Pharma Services-Taiwan.

The binding of $[^3H]$AMPA to AMPA receptors of glutamate-gated ion channels was measured by the methods of Honore et al. and Olsen et al. $[^3H]$AMPA was purchased from New England Nuclear (Boston, MA). AMPA and $\lambda$-glutamic acid were purchased from RBI (Natick, MA). KSCN from Merck (Hohenbaun, Germany), and Tris-base and Tris-HCI were purchased from Sigma (St. Louis, MO). Cerebral cortical membranes of male Wistar-derived rats weighing 150 to 200 g were prepared in modified Tris-HCI containing 200 mM KSCN (pH 7.4) buffer by standard techniques. A 5-mg sample of membrane was incubated with 5 nM $[^3H]$AMPA for 90 min at 4°C. Nonspecific binding was estimated in the presence of 1 mM $\lambda$-glutamic acid. Membranes were filtered and washed three times and the filters were counted to measure specifically bound $[^3H]$AMPA.

The binding of $[^3H]$kainate to kainate receptors of glutamate-gated ion channels was measured by the method of London and Coyle. $[^3H]$Kainate was purchased from New England Nuclear. Whole brain (except cerebellum) membranes of male Wistar-derived rats weighing 150 to 200 g were prepared in Tris-HCI (pH 7.4) buffer by standard techniques. A 10-mg portion of membrane was incubated with 5 nM $[^3H]$kainate for 60 min at 4°C. Nonspecific binding was estimated in the presence of 1 mM $\lambda$-glutamic acid. Membranes were filtered and washed three times and the filters were counted to measure specifically bound $[^3H]$kainate.

The binding of $[^3H]$MDL-105,519 to NMDA strychnine-insensitive glycine receptors of glutamate-gated ion channels was measured by the method of Siegel et al. $[^3H]$MDL-105,519 was purchased from Amersham (Buckinghamshire, UK), MDL-105,519 from RBI, and HEPES from Sigma. Cerebral cortical membranes of male Wistar derived rats weighing 150 to 200 g were prepared in HEPES (pH 7.7) buffer by standard techniques. A 2.5-mg portion of mem-

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brane was incubated with 0.33 nM $[^{3}H]$MDL-105,519 glycine for 30 min at 4°C. Nonspecific binding was estimated in the presence of 10 $\mu$M MDL-105,519. Membranes were filtered and washed 3 times and the filters were counted to measure specifically bound $[^{3}H]$MDL-105,519.

The binding activity of theanine on three subtypes of glutamate receptors was compared with 1-glutamatic acid (RBI, G-100). Under these conditions, three radioligand binding assays of $[^{3}H]$AMP, $[^{3}H]$kainate, and $[^{3}H]$MDL105,519 with theanine (Tokyo Kasei Kogyo, Tokyo, Japan) and 1-glutamatic acid were done, and the inhibition curves were plotted from the results. IC$_{50}$ values were calculated by nonlinear least-squares regression analysis with Microsoft Excel 2000 and Data Analysis Toolbox (MDL Information System Inc., San Leandro, CA). The $K_{i}$ values were calculated by the equation of Cheng and Prusoff$^{39}$ with the observed IC$_{50}$ of the tested compound, the concentration of the radioligand used in the assay, and the historical values for the $K_{d}$ of the ligand (obtained experimentally at MDS Pharma Services). The Hill coefficient, nH, defining the slope of the competitive binding curve was calculated with Data Analysis Toolbox. When Hill coefficients were significantly different from 1.0, the binding displacement may not follow the laws of mass action with a single binding size. Where IC$_{50}$, $K_{i}$, or nH data are presented without the SEM, data are not quantitative, and the values presented should be interpreted with caution. For all receptor binding assays, the percent inhibition was calculated as follows:

Inhibition (%) = \[
\frac{\text{cpm of compound} - \text{mean cpm of nonspecifically bound}}{\text{mean cpm of total bound} - \text{mean cpm of nonspecific bound}} \times 100
\]

IC$_{50}$ and nH were calculated with the Data Analysis Toolbox and nH may be described as follows.

nH: Hill coefficient (slope of the Hill plot).

$K_{i}$ is the equilibrium dissociation constant of the competitive inhibitor and was calculated as follows:

\[IC_{50} / (1 + [\text{Radioligand}] / K_{d})\]

Inhibition curves of glutamate receptor subtypes AMPA, kainate, and NMDA glycine were drawn, and the IC$_{50}$ values were calculated (Table 1). The IC$_{50}$ values of theanine were 24.6 ± 0.9 $\mu$M for AMPA, 41.5 ± 7.6 $\mu$M for kainate, and 347 ± 47 $\mu$M for NMDA glycine. The IC$_{50}$ values of 1-glutamatic acid were 0.311 ± 0.018 $\mu$M for AMPA, 0.537 ± 0.076 $\mu$M for kainate, and 0.011 ± 0.002 $\mu$M for NMDA glycine. Thus, theanine bounded with AMPA, kainate, and NMDA receptors, and its binding activity on AMPA and kainate receptors was 10-fold higher than on NMDA glycine receptors. The binding activity of 1-glutamatic acid with the NMDA glycine receptor was 30- to 50-fold that of AMPA and kainate receptors. The binding activity of theanine on the glutamate receptors subtypes was less than that of 1-glutamatic acid. The ionic charge of glutamic acid may be important to binding glutamate receptors because theanine is changed to glutamylethylamide by the formation of a complex of 1-glutamatic acid and ethylamine.

Though the binding activities of theanine on AMPA and kainate receptors were less than those on 1-glutamatic acid, these IC$_{50}$ values were on the order of 10$^{-5}$ M, and probably would be useful for neuroprotection in pharmacological uses. The AMPA receptor ordinarily is high permeable to Na$^+$, but not to Ca$^{2+}$. However, the subunit composition of the AMPA receptor may have changed so as to become permeable to Ca$^{2+}$. GluR2 gene expression is lowered, and Ca$^{2+}$ flows into cells in the hippocampal CA1 region that shows delayed neuronal death after transient ischemia.$^{9,10}$ These results suggested that theanine also participated in the neuroprotective effect by binding to AMPA receptors. Nellgård and Wieloch$^{14}$ reported that the antagonists that affect the AMPA receptor have a neuroprotective effect even when given after ischemia, so theanine administration after ischemia might be useful for neuroprotection.

Pyramidal neurons in the hippocampal CA3 region are susceptible to kainate toxicity,$^{11}$ and the binding activity of radiolabelled kainate is the highest at hippocampal CA3 in the brain.$^{13}$ Pyramidal neurons in hippocampal CA3 region die after intraperitoneal administration of 8 mg/kg kainate in rats, but this neuronal death is suppressed by intraventricular ad-
ministration of theanine before kainate treatment. These results suggested that theanine might participate in the neuroprotective effect by binding to the kainate receptor.

The binding of theanine with the NMDA receptors was 30,000-fold less than that of L-glutamic acid in this study. The IC₅₀ of the binding concentration of theanine on NMDA receptors was 10⁻⁴ M, one order of magnitude lower than on AMPA/kainate receptors. However, Nozawa et al. Reported that the intracellular Ca²⁺ concentration is increased temporarily by exposure to 800 μM theanine in cultured rat cortical neurons. This elevation in the Ca²⁺ concentration was inhibited by exposure to 50 μM D-2-amino-5-phosphonopentanoate (D-APV), a competitive NMDA antagonist with a specific ligand. Yokogoshi et al. Reported that theanine administration in the brain striatum increases dopamine release, but that theanine-induced dopamine release is inhibited with treatment first by D-APV. Maruyama and Takeda suggested that theanine is a competitive antagonist on glutamate receptors, but that its binding activity is not strong. Cotman et al. found that the NMDA receptors have a high density in the hippocampal CA1 region of the brain. We already reported that ischemia-induced delayed neuronal death in the hippocampal CA1 region is prevented in a dose-dependent way by treatment with 250 to 500 μM theanine through the lateral ventricle in the gerbil. These results suggested that there were antagonistic effects of theanine on the NMDA receptor providing neuroprotective action, although the binding activity of theanine on NMDA receptors was one of order of magnitude lower than on AMPA/kainate receptors.

Theanine therefore may participate in neuroprotection through its mechanisms of binding to glutamate receptor subtypes (AMPA, kainate, and NMDA receptors), and by blocking the binding to the glutamate receptor of L-glutamic acid discharged in excess into the extracellular space by cerebral ischemia, leading to neuronal death. However, this was suggested as one of the mechanisms, because the binding activity of theanine on glutamate receptors was lower than that of L-glutamic acid. Theanine is a natural glutamate analog, so it is may not only affect glutamate receptors but also may affect other mechanisms such as those with glutamate transporters, because glutamic acid discharged in excess into extracellular space is removed by glutamate transporters. These findings suggest that the inhibiting effect of theanine on neuronal death caused by transient brain ischemia is mild, and that the theanine in green tea may useful for prevention of neuronal death.

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References


