The Involvement of L-Type Amino Acid Transporters in Theanine Transport

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L-Theanine has favorable physiological effects in terms of human health, but the mechanisms that transport it to its target organs or cells are not completely defined. To identify the major transport mechanisms of L-theanine, we screened for candidate transporters of L-3H-theanine in several mammalian cell lines that intrinsically express multiple transporters with various specificities. All of the cells tested, T24, HepG2, COS1, 293A, Neuro2a, and HuH7, absorbed L-3H-theanine. Uptake was significantly inhibited by the addition of L-leucine and by a specific inhibitor of the system L transport system, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). L-3H-Theanine uptake occurred mostly independently of Na+. These results indicate that L-theanine was taken up via a system L-like transport system in all of the cells tested. Additionally, in experiments using cells stably expressing two system L isoforms, LAT1 and LAT2, we found that the two isoforms mediated theanine transport to similar extents. Taken together, our results indicate that L-theanine is transported mostly via the system L transport pathway and its isoforms.

Key words: L-theanine; L-type amino acid transporters; γ-glutamylmethylamide synthetase

L-Theanine (theanine; amino acid is L-isomer unless otherwise stated.) is a major free L-amino acid found in Japanese green tea leaves. It enhances the sweet taste of the tea. Numerous studies have reported that theanine reduces blood pressure in spontaneously hypertensive rats, relief from convulsions induced by caffeine, generation of α-electric waves in the human brain, effects on brain levels of norepinephrine, serotonin, 5-hydroxyindoleacetic acid, and dopamine, and protection of hepatocyte and nerve cells from oxidative stress induced by hydrogen peroxide, ethanol, and neurotoxicants. This suggests that theanine is good for human health.

Several studies have reported that theanine, as well as other amino acids, is taken up into cells via amino acid transporters on the plasma membrane, but the transport mechanisms that mediate theanine uptake into the cells are not completely clear. Both Yokogoshi et al. and Kakuda et al. have suggested that transport of theanine into the small intestine, across the blood-brain barrier, and into neurons might occur via several transport systems, such as Na+-dependent neutral amino acid systems B, A, ASC, and N and Na+-independent neutral amino acids system L transport pathway. These suggestions are based on findings that theanine inhibited the absorption of glutamine and large neutral amino acids, such as leucine and tryptophan, into organs. These transport systems show preferential affinity for amino acids as follows: system B, broad dipolar neutral amino acids; system L, large neutral amino acids such as leucine and tryptophan; system A, alanine and other small and polar neutral amino acids; system ASC, small neutral amino acids such as serine, alanine, and cysteine; system N, neutral amino acids such as glutamine, asparagine, and histidine. Although multiple transport systems for amino acids with overlapping substrate specificities are known to be present in mammalian cells, it is not known whether these transport system play major roles in theanine transport in the human body.

In addition, a recent cloning study revealed that each transport system consists of multiple isoforms. For example, system A consists of three highly homologous isoforms, SNAT1, SNAT2, and SNAT4 while system L has four isoforms, called LAT1, LAT2, LAT3, and LAT4. Isotypes belonging to the same system show similar but not equal substrate specificities toward amino acids in the transport pathway. Among these transport systems and transporter isoforms, the transport molecules of theanine have not been identified.

The aim of this study was to identify the main transport systems and transporter isoforms that convey theanine across the membrane in mammalian cells. To this end, initially we compared the 3H-theanine uptake ability of six cell lines derived from several mammalian organs. Based on the pharmacological profiles of the theanine uptake processes of the cells, we identified system L as the main theanine transport system. We directly measured the theanine uptake activity of the two
transporter isoforms of system L, which were exogenously expressed in mouse cells, and determined the kinetic parameters for the transport of theanine by these isoforms.

**Materials and Methods**

**Strain and culture conditions.** To identify the major mechanisms of theanine transport in mammalian cells, T24 (derived from human bladder carcinoma cells), HuH7 (from human hepatoma cells), HepG2 (from human hepatocellular carcinoma cells), 293A (from human embryonic kidney cells), Neuro2A (from mouse neuroblastoma cells), and COS1 (from African monkey kidney cells) cells were used. To determine the theanine kinetics of the system L isoforms, S2 cells exogenously expressing hLAT1 and hLAT2 (S2-hLAT1 and S2-hLAT2 respectively) were used. S2-hLAT1 and S2-hLAT2 cells were established previously by transfection of S2 cells with pcDNA3.1 plasmid vectors containing full-length cDNAs of hLAT1 and hLAT2 respectively. They showed high expression of hLAT1 and hLAT2, and had higher L-glutamate uptake activity than the S2 cells.

The exogenously introduced hLAT1 and hLAT2 levels were 20 to 1000-fold higher than the endogenous mLAT1 and mLAT2 levels in the S2-LAT1 cells and the S2-LAT2 cells.

COS1, T24, HuH7, HepG2, and Neuro2A cells were grown in DMEM medium supplemented with 10% FBS and penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO2. 293A was cultured under same conditions as the COS1 cells, apart from the addition of non-essential amino acids to the culture medium. S2-hLAT1, S2-hLAT2, and S2-mock (cell clones transfected with a control pcDNA3.1 plasmid vector without insert) were cultured in DMEM/Hams’s F-12 medium supplemented with 5% FBS, 10 mg/L of transferrin, 80 μg/L of insulin, 10 μg/L of epidermal growth factor, and 500 mg/L of G418 at 33°C/37°C. 1000-fold higher than the endogenous mLAT1 and mLAT2 levels in the S2-LAT1 cells and the S2-LAT2 cells.

**Enzyme preparation.** Recombinant γ-glutamylmethylethylamine syntheses (GMAS) produced by E. coli AD409 (DE3) harboring pET21aGM (an expression vector for the GMAS gene of Methylovorus mays no. 9) was prepared by a method described previously. One unit of GMAS was defined as the amount of enzyme forming 1 μmol of γ-glutamylhydroxamate per min at 30°C in a reaction mixture consisting of 50 mM sodium glutamate, 15 mM hydroxylamine, 7.5 mM ATP, 30 mM MgCl2, 100 mM imidazole buffer (pH 8.0), and recombinant GMAS.

**Synthesis and purification of 3H-theanine.** 3H-Theanine was synthesized enzymatically from L-glutamic acid, ethylamine, and ATP by recombinant GMAS. The reaction mixture (1.0 mL) for 3H-theanine synthesis was 1 μmol L-glutamic acid (24 MBq/mmol), 2 mM ethylamine·HCl, 2 mM ATP, 30 mM MgCl2, 100 mM imidazole buffer (pH 7.8), and 1.4 units/mL of GMAS. The reaction was carried out at 30°C over 5 h, and was terminated by heating at 70°C for 5 min. After heating, the reaction mixture was centrifuged at 12,000 g for 10 min at 4°C. The supernatant obtained by centrifugation was applied to a column (0.25 mL) of Dowex 50 that had been prepared as the H+ form. The column was washed with water and then with 2 mL ammonium hydroxide. The ammonium hydroxide eluate was evaporated, and the residue was dissolved in a small volume of water. The solution was loaded on a Dowex 1 column (CH3COO– form, 0.25 mL). The column was washed with water, and the unadsorbed fraction was concentrated to dryness. The dry fraction was dissolved in a small volume of water and used as 3H-theanine. 3H-Theanine gave a single spot corresponding to that of authentic theanine on a paper chromatogram without contamination of 3H-glutamic acid.

**Measurement of 3H-theanine, 3H-leucine, and 3H-glutamine uptake activity.** Cells cultured in 1.9 cm2 multi-well plates were washed twice with Hank’s Buffer with NaCl (Hank’s Na+-Containing Buffer: 125 mM NaCl, 48 mM KCl, 1.3 mM CaCl2, 1.2 mM MgCl2, 25 mM Heps, 5 mM Tris, and 5.6 mM Glucose, pH 7.4), followed by incubation in the same buffer at 37°C for 10 min. The cells were then incubated in various concentrations of 3H-theanine in the Hank’s Na+-Containing Buffer (Uptake Medium). The reaction was carried out at 37°C, and was terminated by gentle aspiration of the Uptake Medium. The cells were rinsed 3 times with ice-cold Hank’s Na+-Containing Buffer and then solubilized with 0.5 mL of 0.1 M NaOH. The radioactivity of the solubilized cell solution was measured by liquid scintillation counter using 3 mL of scintillation cocktail (Clear Sol 1). Each data point in the figures and tables represents the mean ± SD uptake activity (n = 4–6). Statistical analyses were done by Student’s unpaired t-test.

To measure Na+-independent amino acid uptake activity, Na+-Free Buffer and Uptake Medium were used. NaCl in the Hank’s Na+-Containing Buffer and the Uptake Medium was replaced with choline chloride at the same concentration. To evaluate the inhibitory effect of amino acids on 3H-theanine uptake, non-radio-labeled amino acids were added to the Uptake Medium as described in the legends of the relevant Figures 2, 3, and 4.

**Results**

**Uptake of 3H-theanine by cultured mammalian cell lines.** To compare theanine uptake ability among the mammalian cell lines, T24, HuH7, HepG2, 293A, Neuro2A, and COS1 cells were incubated with 3H-theanine at 4°C and at 37°C. As shown in Fig. 1, all the cultured cells accumulated 3H-theanine at 37°C with incubation time. At 4°C, marked accumulation of 3H-theanine was not detected. The accumulation of 3H-theanine occurred in a time- and temperature-dependent manner, indicating that 3H-theanine uptake occurred via carrier-mediated transport.

The level of 3H-theanine accumulation was highest in the T24 cells of all the cell lines studied. 3H-Theanine accumulation increased linearly with incubation time up to 12 min, and reached a concentration of 5 n mole/mg of protein. On the other hand, the level of 3H-theanine accumulation was lowest in the HuH7 cells. These differences in the ability of the cells to accumulate 3H-theanine might reflect variations in the amount, the activity, or the kinds of amino acid transporter mediating theanine transport in the six cell lines.

**Characterization of theanine transport in mammalian cell lines**

Initially, the theanine transport reaction of the T24 cells, which displayed the greatest theanine transport

![Fig. 1. Time-Courses of 3H-Theanine Accumulation in Six Cell Lines.](image-url)

The cells were incubated at 4 or 37°C in Uptake Medium containing 50 μM 3H-theanine and 125 mM Na+ (see “Materials and Methods”). Symbols: ○, at 37°C; □, at 4°C. Points represent mean ± SD (n = 4).
ability, was characterized by inhibition experiments in which the uptake of 50 μM [3H]-theanine was measured in the presence of an L-isomer amino acid at 500 μM. As shown in Fig. 2A, accumulation of [3H]-theanine in the T24 cells was strongly inhibited by leucine, phenylalanine, and tryptophan, which are preferential substrates of the system L transport pathway. The accumulation of [3H]-theanine was also inhibited by the addition of a conventional specific inhibitor of system L, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH). On the other hand, moderate inhibition was seen after the addition of glutamine, serine, alanine, valine, and histidine. Basic amino acids such as lysine and arginine, and acidic amino acids such as glutamic acid and aspartic acid, hardly inhibited [3H]-theanine uptake.

The requirement for Na+ in the uptake of theanine by T24 cells was evaluated by replacing NaCl in the Uptake Medium with choline chloride. As shown in Fig. 2B, omission of Na+ from the Uptake Medium slightly decreased the rate of [3H]-theanine uptake in the T24 cells, indicating that most uptake of theanine by T24 cells is mediated by an Na+-independent process. The results presented in Fig. 2A and B suggest that system t—that is, an Na+-independent, large, neutral amino acid transport system—might transport theanine into T24 cells.

On the basis of the results for the T24 cells, the transport of [3H]-theanine in other cell lines was characterized. The accumulation of [3H]-theanine in the 293A, HepG2, COS1, Neuro2a, and HuH7 cells was also significantly inhibited by the addition of leucine and BCH to the reaction mixture (Fig. 3A and B). Most theanine uptake did not require Na+ (Fig. 3C), as for the T24 cells.

Collectively, these findings suggest that theanine is taken up into mammalian cells mainly via the system L transport pathway. In fact, those cells showing high uptake of [3H]-theanine also showed high uptake of [3H]-leucine, which proceeds in an Na+-independent manner and was strongly inhibited by BCH addition (data not shown). Accordingly, the differences in the amounts of [3H]-theanine accumulation observed among the cell lines (Fig. 1) might reflect variations in the expression levels or the activity of system L transporters in these cell lines.

The small contribution of an Na+-dependent glutamine transporter to theanine uptake by the cells

As described previously, [H]-theanine might be transported via a transport system common to glutamine, such as system B, A, ASC, N, or L. However, the above results indicate that theanine was transported mostly into mammalian cells via the system L transport pathway. It is likely that at least some of the cell lines used in this study have glutamine transport systems in addition to system L. With the exception of system L, glutamine transport systems need Na+ for amino acid transport. Hence we investigated the presence of glutamine transport systems other than system L in all six cell lines by comparing the [3H]-glutamine uptake ability of these cell lines under Na+-containing and Na+-free conditions (Table 1).

In both the presence and the absence of Na+, the six cell lines took up [3H]-glutamine in a time- and temperature-dependent manner (data not shown). However, omission of Na+ from the [3H]-glutamine uptake solution significantly decreased the rate of [3H]-glutamine uptake, indicating that most [3H]-glutamine uptake under the Na+-containing condition proceeded in an Na+-dependent manner. This indicates that all six cell lines expressed Na+-dependent glutamine transport systems in addition to the Na+-independent glutamine transport system (system L), and that the Na+-dependent glutamine transport systems were the primary transport pathways for [3H]-glutamine absorption into the cells.

This requirement for Na+ in the uptake of [3H]-glutamine in all cell lines differed absolutely from the uptake of [3H]-theanine (compare Table 1 column 3 with Fig. 3C). As shown in Fig. 3C, uptake of [3H]-theanine proceeded mostly in an Na+-independent manner. This difference in Na+ requirements as between [3H]-glutamine and [3H]-theanine uptake led us to the conclusion that Na+-dependent glutamine transport systems, such as system B, A, N, and ASC, transport minimally [3H]-theanine into cells despite the expression of these transporters in the cells.

As shown in Fig. 3B, however, the accumulation of theanine in the Na+-containing buffer was not completely inhibited by the addition of BCH. Furthermore, replacement of NaCl with choline chloride slightly decreased the rate of theanine accumulation in the cells (Fig. 3C). These findings suggest that an Na+-dependent transport system distinct from system L transported some theanine across the membrane. Residual uptake of theanine in the presence of BCH was significantly inhibited by the addition of serine and alanine (a canonical substrate of system ASC[14,23]), but not by glycine or proline (system A[14,16]) (data not shown), suggesting that system ASC is minimally involved in the transport of theanine across the membrane into the cells.
Cell lines

Glutamine Uptake

Comparison of Na\(^+\)-Dependent and Na\(^+\)-Independent \(^{3}\)H-Glutamine Uptake

Table 1. Comparison of Na\(^+\)-Dependent and Na\(^+\)-Independent \(^{3}\)H-Glutamine Uptake

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>With Na(^+) (nmol/mg/min)</th>
<th>Without Na(^+) (nmol/mg/min)</th>
<th>Without Na(^+)/With Na(^+) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>2.988 ± 0.021</td>
<td>0.905 ± 0.035</td>
<td>30 ± 1.1**</td>
</tr>
<tr>
<td>293A</td>
<td>2.023 ± 0.226</td>
<td>0.172 ± 0.007</td>
<td>8.5 ± 9.1**</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.970 ± 0.038</td>
<td>0.233 ± 0.014</td>
<td>24 ± 0.1**</td>
</tr>
<tr>
<td>COS1</td>
<td>0.938 ± 0.005</td>
<td>0.370 ± 0.010</td>
<td>38 ± 1.0**</td>
</tr>
<tr>
<td>HuH7</td>
<td>0.745 ± 0.082</td>
<td>0.330 ± 0.005</td>
<td>44 ± 0.7**</td>
</tr>
<tr>
<td>Neuro2A</td>
<td>2.120 ± 0.123</td>
<td>0.510 ± 0.033</td>
<td>24 ± 1.5**</td>
</tr>
</tbody>
</table>

The rates of \(^{3}\)H-glutamine (50μM) uptake in six cell lines were measured in Uptake Medium with and without 125 mM Na\(^+\). The reaction was carried out at 37 °C for 5 min.

**a** The rate of \(^{3}\)H-glutamine uptake without Na\(^+\) is expressed as percentages of that with Na\(^+\). Values represent mean ± SD (n = 4–6). **p < 0.01 vs. the \(^{3}\)H-glutamine uptake rate measured with Na\(^+\).

Uptake of theanine was mediated by hLAT1 and hLAT2

System L activity is mediated by four isoforms of an L-type amino acid transporter, LAT1, LAT2, LAT3, and LAT4. Among these isoforms, mouse cell lines stably expressing hLAT1 (S2-hLAT1 cells) and hLAT2 (S2-hLAT2 cells) have been established. Using S2-hLAT1 and S2-hLAT2 cells, we examined to determine whether these transport isoforms can transport theanine.

As shown in Fig. 4A, both S2-LAT1 and S2-LAT2 cells took up \(^{3}\)H-theanine in a time- and temperature-dependent manner in Na\(^+\)-free Uptake Medium. The amount of theanine that accumulated in these cells was significantly higher than that in the S2-mock cells, control cells transfected with a pcDNA3.1 plasmid vector without insert DNA.

As Fig. 4B shows, \(^{3}\)H-theanine accumulation was inhibited by the addition of amino acids. Accumulation of theanine in the S2-LAT1 and S2-LAT2 cells was inhibited by the addition of hLAT1- and of hLAT2-prefering substrates: large neutral amino acids (leucine, phenylalanine, and tryptophan) and α-leucine for LAT1, \(^{3}\)H-theanine was strongly inhibited by a system L preferential amino acid, leucine, and a system L specific inhibitor, BCH. In addition, the uptake of \(^{3}\)H-theanine under Na\(^+\)-free conditions was almost equal to that under Na\(^+\) present conditions. Collectively, these findings suggest that \(^{3}\)H-theanine uptake was mediated by the system L transport pathway in these cell lines.

Discussion

In this study, we found that the system L transport pathway is mainly responsible for the uptake of theanine into cells of several organs, including the kidney and liver, and the neurons. Accumulation of \(^{3}\)H-theanine was strongly inhibited by a system L preferential amino acid, leucine, and a system L specific inhibitor, BCH. In addition, the uptake of \(^{3}\)H-theanine under Na\(^+\)-free conditions was almost equal to that under Na\(^+\)-present conditions. Collectively, these findings suggest that \(^{3}\)H-theanine uptake was mediated by the system L transport pathway.
system in these cell lines. As for the transporter isoforms comprising system L, we found that hLAT1 and hLAT2 transport theanine using mouse cell lines stably expressing hLAT1 (S2-hLAT1 cells) and hLAT2 (S2-hLAT2 cells). We also determined the kinetic parameters of theanine uptake. The affinities of these isoforms for theanine uptake are the functional theanine transport system that takes theanine to the target cells, where it fulfills various physiological functions. Because system L consists of four isoforms (LAT1, LAT2, LAT3, and LAT4), further investigation is necessary to evaluate the involvement of LAT3 and LAT4 in theanine uptake into cells in the human body.

Recent studies have reported that the expression of LAT2 in intestinal epithelial cells is limited to the basolateral membrane and does not include the apical membrane, and that LAT2 does not mediate the absorption of amino acids into intestinal epithelial cells from the gastrointestinal tract. What is the transport system that functions as a transporter of theanine into intestinal epithelial cells? Kitaoka et al. have reported that theanine absorption into the small intestine via an electrogenic Na+-dependent transport system might be mediated by system B. On the basis of a recent review, this transport system might be system B0 or system B0t. Uptake of theanine by the small intestine is reportedly weaker than that by hLAT1 or hLAT2. The $K_m$ value (21.4 mM) of the Na+-dependent transport system is approximately 10-fold higher than that of system L. On view of such a low affinity for theanine of the Na+-dependent transport system, the contribution of the Na+-dependent transport system to theanine uptake might be small under physiological conditions. As discussed by Kitaoka et al., another amino acid transport system might be involved in theanine uptake into intestinal epithelial cells. Further detailed investigation is necessary to understand the complete mechanism of theanine uptake into the small intestine.

Because theanine is a structural analog of glutamine, it might be transported by a transport system of glutamine, such as system A, ASC, or LAT. However, the contribution of system A, ASC, and LAT to theanine uptake is small, because most theanine uptake proceeds in an Na+-independent manner (Fig. 3C). This finding is consistent with a previous study of theanine absorption.
tion in the neurons and the astroglia of the rat brain: accumulation of \(^3\)H-theanine in rat synaptosomal fractions was significantly inhibited in the presence of large neutral amino acids, and it occurred mostly in an Na\(^+\)-independent manner, but that study did not identify the transporter that mediated theanine uptake in the neurons and the astroglia.

In conclusion, this study indicates that theanine is transported mainly by the system L transport pathway. This is consistent with previous studies of theanine absorption in the brain and neurons. Hence our finding reflect the mobilization of theanine around the human body. Among the transporter isoforms comprising system L, hLAT1 and hLAT2 mediated the uptake of theanine with similar affinity. The kinetic parameters may provide important information to understand the molecular mechanisms by which theanine induces its various physiological functions.

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