Amino acid assignment to one of three blood-brain barrier amino acid carriers

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ENTRY OF AMINO ACIDS and other metabolites into brain from blood takes place through the blood-brain barrier (BBB), despite the exclusion of most small polar solutes in blood. This transport through the brain capillary endothelial cell, the presumed site of the BBB, must almost entirely be due to special transport mechanisms in the capillary cell plasma membranes. BBB transport; brain uptake; tritiated water; carboxylic acids; neutral amino acids, basic amino acids, acidic amino acids; [113mIn]EDTA; carotid injection

METHODS

The general methodology has been described previously (4) and is only summarized here. Wistar rats, 275–350 g, of either sex and on routine laboratory diet were anesthetized by intraperitoneal pentobarbital. The right common carotid artery was surgically exposed and cannulated with a 27-gauge (0.38 mm OD) needle. A 0.2-ml bolus of Ringer solution buffered to pH 7.55 with 4 mM HEPES buffer (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, Calbiochem, La Jolla, Calif.) containing approximately 0.25 μCi of 14C-labeled amino acid, 1 μCi 3HOH and 50 μCi [113mIn]EDTA was rapidly injected. In competition studies the injected solution also contained unlabeled substances in the 0.05–10 mM concentration range to measure inhibition of the brain uptake of the respective 14C-labeled amino acids. In all instances the pH of the injected solution was 7.55. The 3HOH served as a highly diffusible internal standard, whereas the [113mIn]EDTA was a nondiffusible internal standard correcting for incomplete washout of the bolus from the brain blood compartment. The [113mIn]EDTA stock solution was prepared using 1.0 ml of 0.04 N HCl eluate (ca. 1 mCi/ml) from a 113Sn generator (New England Nuclear Corp., Billerica, Mass.) to which 0.008 ml EDTA solution (150 mg/ml) was added. After mixing, 0.035 ml sodium bicarbonate solution (3.75 g/50 ml) was added.

The rat was decapitated 15 s after carotid injection, and the right half of the brain rostral to the midbrain was subjected to routine liquid scintillation counting of 14C and 3H. After routine solubilization and mixing with an organic scintillator, the specimen was counted for 113mIn. The liquid scintillation counting of 113mIn conversion electrons in the presence of 14C and 3H has been previously described (8) and was applied here with some modification. The 113mIn conversion electrons (370–390 keV) are counted in a pulse-height window set to include about 10% of the 3H disintegrations as a lower threshold and to infinity as an upper limit (Fig. 1). This channel setting causes the conversion electron scintillations to be counted with a constant high efficiency through a wide range of quench, thus obviating the quench correction calculation previously required (8). An aliquot of the injected fluid vehicle or mix was similarly counted. After a 24-h delay to allow decay of the 113mIn (t1/2 = 100 min), the specimen was recounted and...
BBB AMINO ACID TRANSPORT SYSTEMS

The difference between the two indices gives the net uptake index relative to the percentage of the 3HOH uptake. Similarly, the ratio in the injected vehicle when multiplied by 100 serves to count becomes nearly independent of quench through a wide range of quenching. By using a very low pulse-height threshold, ob- served lirSrn In count becomes nearly independent of quench through a wide range of quenching. *l:smIn is counted immediately after prepara-
tion of sample and again 24 h later, when lXzjmIn has decayed (t d = 10q min). Residual counts in llemIn window, largely 14C, are subtracted from previous day's count to obtain net 113mIn counts. :'H and 14C are routinely as though 113mIn had never been present.

any residual counts appearing in the 113mIn window were subtracted from the previous count to obtain the net 113mIn count. This window setting counts most of the 14C and a small fraction of 3H in the specimen (Fig. 1).

The 14C/3H ratio in the tissue divided by the same ratio in the injected vehicle when multiplied by 100 provides the brain uptake index (BUI) of the test substance as percentage of 3HOH uptake. Similarly, the 113mIn/3H ratio in the vehicle provides the 113mIn uptake index relative to the percentage of the 3HOH uptake. The difference between the two indices gives the net brain uptake index for the test substance.

net BUI = \[
\frac{\text{tissue } 14C/\text{tissue } 3H}{\text{mix } 14C/\text{mix } 3H} \times 100
\]

That 3HOH is nearly completely cleared during a single brain passage is supported by the failure to see a "bolus" effect in jugular blood after arterial injection (9) or by the external monitoring of annihilation gamma emissions from brain after carotid injection of 18O water (6). At abnormally high blood flow rates, labeled water clearance is measurably incomplete (6).

The studies can be classified into two subgroups. Those amino acids having uncompeted uptakes greater than about 10% were each injected in 4 mM concentrations, and the effect of this unlabeled competitor on the uptake of [14C]leucine and [14C]arginine was measured. The labeled leucine and arginine we considered as representative neutral and basic amino acids. The amino acids so studied subsequently are referred to as high-uptake amino acids and include phenylalanine, leucine, tyrosine, isoleucine, methionine, tryptophane, valine, DOPA, and cysteine. No indium correction was used in this group of acids.

A second group of amino acids having uncompeted uptakes (BUIs) of less than 10% were also studied, but inhibition of uptake was assessed by competing unlabeled 4 mM phenylalanine and 10 mM arginine against the 14C labeled amino acid under study. These low-up-
take amino acids included threonine, glutamine, asparagine, serine, alanine, proline, glycine, and glutamic and aspartic acids. Indium correction was used because the background of the method without indium precluded sensitive indication of inhibition. These acids subsequently are referred to as low-uptake amino acids. Histidine was included in this group, even though it is a fairly high-uptake acid, because our preliminary studies (4) suggested both neutral and basic carrier affinities and we wanted to clarify this point since our current studies indicated such duality would be unique among the amino acids studied.

RESULTS

The high- and low-uptake amino acids tested and the respective injected concentrations are indicated in Tables 1 and 2, respectively. The injected concentrations in the uncompeted labeled amino acids are unequal because of the range of specific activities provided by the radiochemical suppliers (Amersham/Searle Corp., Arlington Heights, Ill. and New England Nuclear Corp.). Each BUI listed is an average of three animals or is otherwise stated.

High-uptake neutral and basic amino acids. Table 1 indicates all of the high-uptake amino acids are clearly grouped into those having neutral or basic carrier affinities. The neutral carrier system transported phenylalanine, leucine, tyrosine, isoleucine, methionine, tryptophane, valine, DOPA, and cysteine. Affinity for a basic amino acid carrier system was demonstrated for arginine, ornithine, and lysine.

<table>
<thead>
<tr>
<th>TABLE 1. Inhibition of l-[14C]leucine or l-[14C]arginine uptake by adding 4 mM unlabeled high-uptake amino acids to injected solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unlabeled L-Amino Acid</strong></td>
</tr>
<tr>
<td>Added, 4 mM</td>
</tr>
<tr>
<td>None added (uncom-</td>
</tr>
<tr>
<td>peted control)</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Tryptophane</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>DOPA</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Ornithine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
</tbody>
</table>

* BUI, significance (t test) of inhibition relative to uncompeted BUI. For each value n = 3 animals. No indium corrections.
Acid at the BBB is thus established. The charge effect of the imidazolium group (pK 6.00) at the carrier site appears to be negligible, thus creating a neutral molecule at physiological pH.

Low-uptake neutral amino acids. Each labeled amino acid was competed against 10 mM concentration of the same unlabeled amino acid to demonstrate self-inhibition. In turn, competition of unlabeled 4 mM L-phenylalanine and 10 mM L-arginine was investigated to establish affinity to neutral or basic amino acid carrier sites. Table 2 demonstrates self-inhibition and cross-inhibition by 4 mM L-phenylalanine of all neutral amino acids tested with the exception of glycine and L-alanine. Self-inhibition of the uptakes of L-alanine and glycine were not demonstrable. They are near the threshold of sensitivity of the method, and these data suggest these two amino acids do not measurably penetrate the BBB by a selective carrier system. The 10 mM L-arginine did not inhibit the uptake of any of the neutral acids tested, thus demonstrating that the neutral amino acids are transported by an independent neutral amino acid carrier system.

The transport of L-glutamine and L-asparagine by the neutral amino acid carrier system is demonstrated.

Although most of the amino acids studied here were those having unmeasurably low uptakes by the previous technique, L-histidine was included in this study, despite its high uptake, because of its unusual charge characteristics. It is commonly considered as a basic amino acid, being isoelectric at pH 7.59. Previous investigation of its brain uptake using carotid injection and a diffusible internal standard (4) indicated it was transported by the neutral carrier, but some lesser affinity for the basic carrier was also evident. This apparent affinity for the basic carrier was based on a single animal. In the present, more extensive studies, L-phenylalanine cross-inhibited L-histidine while L-arginine did not. A further delineation of L-histidine properties in this system was elucidated by studying its ability to inhibit the uptake of L-aspartic acid, an acidic amino acid. In the presence of 10 mM L-histidine, the BUI of L-aspartic was unchanged (BUI 2.39 ± 0.82, n = 3, P < 0.05), indicating no affinity for the acidic carrier system. The biological behavior of L-histidine as a neutral amino acid at the BBB is thus established. The charge effect of the imidazolium group (pK 6.00) at the carrier site appears to be negligible, thus creating a neutral molecule at physiological pH.

TABLE 2. Uncompeted uptake of $^{14}$C-labeled low uptake L-amino acids and effect of addition of unlabeled neutral and basic L-amino acids

<table>
<thead>
<tr>
<th>Neutral</th>
<th></th>
<th></th>
<th>10 mM Self-Inhibition</th>
<th>4 mM L-Phenylalanine Inhibition</th>
<th>10 mM L-Arginine Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acid</td>
<td>Injected Conc</td>
<td>BUI</td>
<td>n</td>
<td>BUI</td>
<td>n</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>.008</td>
<td>6.73 ± 1.01</td>
<td>3</td>
<td>1.25 ± .07</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Glutamine</td>
<td>.005</td>
<td>4.79 ± .49f</td>
<td>3</td>
<td>2.42 ± .27</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>.0125</td>
<td>3.73 ± .27</td>
<td>3</td>
<td>1.66 ± .20</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>L-Serine</td>
<td>.0166</td>
<td>3.61 ± .37</td>
<td>6</td>
<td>1.30 ± .10</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>.0166</td>
<td>3.33 ± .35</td>
<td>5</td>
<td>1.71 ± .40</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>L-Proline</td>
<td>.0100</td>
<td>1.31 ± 1.6</td>
<td>6</td>
<td>0.96 ± 1.7</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>.0250</td>
<td>0.77 ± .18</td>
<td>3</td>
<td>0.52 ± .12</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>.0500</td>
<td>25.49 ± 3.33</td>
<td>3</td>
<td>2.89 ± .13</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

In this table and Tables 3 and 4, P represents the significance of inhibition relative to uncompeted BUI (Brain Uptake Index), expressed according to Dunnett's method for experiments making multiple comparisons with a control (1). Indium correction was used. n, number of animals. * L-[14C]aspartic vs. 10 mM histidine 2.39 ± .82, n = 3, P > .05. t L-[14C]glutamine was self-competed using 4 mM unlabeled L-glutamine and 4 mM aspartic acid, rather than using 10 mM.

Acidic

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Injected Conc</th>
<th>BUI</th>
<th>n</th>
<th>BUI</th>
<th>n</th>
<th>P</th>
<th>BUI</th>
<th>n</th>
<th>P</th>
<th>BUI</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Aspartic acid*</td>
<td>.008</td>
<td>2.44 ± .34</td>
<td>3</td>
<td>0.09 ± .05</td>
<td>&lt;.01</td>
<td>2.18 ± .51</td>
<td>&gt;.05</td>
<td>2.00 ± .23</td>
<td>4</td>
<td>&gt;.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>.0100</td>
<td>2.51 ± .16</td>
<td>3</td>
<td>0.33 ± .08</td>
<td>&lt;.01</td>
<td>2.14 ± .24</td>
<td>&gt;.05</td>
<td>2.18 ± .44</td>
<td>6</td>
<td>&gt;.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inhibition tests injecting unlabeled neutral and basic L-amino acids

DISCUSSION

Amino acids are lipophobic in living systems by virtue of their dissociation at tissue pH and by the polar groups present in the un-ionized state. Their passage through cell membranes, and thus through the BBB, would be greatly constrained were it not for the presence of selective BBB transport mechanisms. These
mechanisms currently are presumed to be functions of specific affinity sites on carrier proteins studied through the plasma membranes of brain capillary endothelial cells. These sites are quite specific for the various amino acids, the present study indicating that all of the amino acids exhibiting a measurable uptake, with the exception of glycine, alanine, and proline, can be assigned to one of the carrier systems and each has their affinities for a particular carrier, but assignment to one carrier or another seems based on proton lability and thus on net molecular charge at physiological pH. When, for example, glutamic acid is aminated to glutamine, its BBB penetration not only increases substantially, but is now via the neutral carrier system rather than the acidic carrier. Asparagine similarly switches affinity when aminated from aspartic acid.

The measurement of brain uptake of test substances during a single passage following carotid injection has been expressed previously as a percentage of uptake of a simultaneously injected highly diffusible internal standard of reference, \(^3\)HOH. Using only this diffusible internal reference, a method background of about 2% was established using nondiffusible test substances assumed not to penetrate the BBB measurably during a single brain passage (4). By measuring the isotope content of arterial blood following decapitation, this background has been shown (unpublished observations) to be almost entirely (>80%) due to incomplete brain blood compartment washout during the 15 s between injection and decapitation with only a minor contribution from systemically recirculating radiisotope.

The present method elaborates upon the previous technique by including a second, nondiffusible internal standard of reference (\(^{113}\)InEDTA) in the injected mixture. This nondiffusible reference corrects for the failure of complete brain blood compartment washout of the injected bolus and reduces the background of the method to near zero. This allows the useful measurement of brain uptake to test substances only 1–2% cleared by brain in one passage. The correction introduced is 1–2.5%, and this variance probably largely represents variable amounts of blood in the brain specimens and variably complete radioisotope washout in different animals. It allows the delay time between injection and decapitation to be reduced to 5 s (although 15 s was used here), since blood compartment washout is nearly complete by that time, the brain exchange having been in the first 1–2 s. Any slight variations in washout will be corrected by the \(^{113}\)InEDTA. The use of \(^{113}\)InEDTA is highly practical, since \(^{113}\)In is generator produced and accordingly inexpensive, it chelates very readily, has a 100-min \(t_{1/2}\), and can thus be ignored after the first 24 h. In addition, it counts efficiently in both liquid scintillation and sodium iodide crystal well counting systems.

The present data, together with previous studies in this laboratory (4), demonstrate neutral BBB carrier transport of phenylalanine, tyrosine, leucine, isoleucine, methionine, tryptophane, histidine, DOPA, valine, cysteine, threonine, lysine, glutamine, asparagine, and serine. Affinity for the basic amino acid carrier was demonstrated for arginine, ornithine, and lysine. Affinity for an independent acidic amino acid carrier is shown here for aspartic and glutamic acids.

The demonstration of carrier-mediated entry of acidic amino acids into brain is compatible with the observation that systemic radioactive glutamate readily enters brain when introduced systemically (2), but artificial elevation of plasma unlabeled glutamate results in no elevation of brain glutamate (2). These observations are consistent with a low-capacity BBB carrier system which saturates at low concentrations. A previous study (10) of brain amino acids during a single micrcirculatory passage did not demonstrate the existence of an acidic amino acid carrier. The method

<table>
<thead>
<tr>
<th>TABLE 3. Uptake of U-C-labeled l- acidic amino acids in presence of unlabeled stereoisomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled Stereoisomers</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>L-Aspartic acid (1 mM)</td>
</tr>
<tr>
<td>L-Aspartic acid (1 mM)</td>
</tr>
<tr>
<td>L-Glutamic acid (1 mM)</td>
</tr>
<tr>
<td>L-Aspartic acid (1 mM)</td>
</tr>
<tr>
<td>L-Aspartic acid (1.05 mM)</td>
</tr>
<tr>
<td>L-Aspartic acid (0.05 mM)</td>
</tr>
<tr>
<td>L-Glutamic acid (0.05 mM)</td>
</tr>
<tr>
<td>L-Glutamic acid (0.005 mM)</td>
</tr>
</tbody>
</table>

Probability (P) values listed define significant differences in BU1 listed above, in comparison to uncorrected BU1 given in Table 1.

<table>
<thead>
<tr>
<th>TABLE 4. Uptake of U-C-labeled aspartic acid in presence of carboxylic acids, dicarboxylic acid and their amides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled Carboxylic Acids</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Fumaric acid (4 mM)</td>
</tr>
<tr>
<td>Succinic acid (4 mM)</td>
</tr>
<tr>
<td>Pyruvic acid (10 mM)</td>
</tr>
<tr>
<td>Pyruvic acid (2 mM)</td>
</tr>
<tr>
<td>Pyruvic acid (2 mM)</td>
</tr>
<tr>
<td>L-Lactic acid (10 mM)</td>
</tr>
<tr>
<td>L-Lactic acid (10 mM)</td>
</tr>
<tr>
<td>D-Lactic acid (10 mM)</td>
</tr>
<tr>
<td>L-Acetamide (1 mM)</td>
</tr>
<tr>
<td>L-Glutamine (1 mM)</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. P values compare uncorrected BU1 of L-Asp (Table 1) with these given here.
used compared the jugular venous content of test substance relative to a nondiffusible reference substance after simultaneous arterial injection. Since the difference between test substance and reference substance becomes vanishingly small as extraction approaches zero, the method is not suited to measuring extractions of a few percent as observed here for the acidic amino acids.

When integrating data presented here with previous studies of brain transport of amino acids (3), care must be taken to differentiate the relatively few studies in vivo of BBB transport of amino acids from the larger number of in vitro studies of brain cellular uptake (2) determined in tissue slices.

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