Brain Microvessels Take up Large Neutral Amino Acids in Exchange for Glutamine

COOPERATIVE ROLE OF Na⁺-DEPENDENT AND Na⁺-INDEPENDENT SYSTEMS

(Received for publication, August 11, 1982)

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Some regulatory aspects of neutral amino acid transport were investigated in isolated brain microvessels, an in vitro model of the blood-brain barrier. Preloading of the microvessels with glutamine stimulated the subsequent uptake of other neutral amino acids by way of the Na⁺-independent L system, but had no effect on the uptake of either basic or acidic amino acids. Moreover, this stimulation was abolished when the loading step was carried out in the absence of Na⁺ ions or in the presence of a high concentration of α-methylaminoisobutyric acid, indicating that the microvessels were able to concentrate glutamine via the A system of amino acid transport.

Since the presence of the A system of neutral amino acid transport has not been detected in studies of blood-brain transport performed in vivo, the A system is probably associated with the antiluminal side of brain microvessels. Our results indicate, therefore, that the concentrative Na⁺-dependent A system and the exchanging Na⁺-independent L system can cooperate in the uptake of the large neutral hydrophobic amino acids. Such a cooperation may be relevant in the pathogenesis of some neurological disturbances such as hepatic encephalopathy, in which brain glutamine concentration is unusually high.

The “blood-brain barrier” which regulates the restricted movements of sugars, amino acids, and other solutes between the blood and the brain has been tentatively localized in the cell membranes of brain capillaries (1–3). A polar distribution of the Na⁺ transport systems has been suggested by in vitro and in vivo experiments. Thus, blood-borne NAAs appear to cross the blood-brain barrier only by using the Na⁺-independent L system, while another Na⁺ transport system, the Na⁺-dependent A system, seems only to be present on the antiluminal side of the microvessels (4). The involvement of increased L system activity in pathological conditions such as hyperammonemia and portal systemic shunting has recently been suggested (5, 6). It has also been hypothesized that the increased L system transport activity occurs through an increase of intracellular Gln content in the brain microvessels (6, 7).

The present paper demonstrates that increased intracellular Gln concentration affects the uptake by isolated brain microvessels of different categories of amino acids.

MATERIALS AND METHODS

The following materials were obtained from New England Nuclear: L-[1-¹⁴C]leucine, 335 or 50 mCi/mmol; L-[1-¹³C]lysine, 503 mCi/mmol; MeAIB and BCH, 4,96 and 53 mCi/mmol, respectively; [U-¹³C]proline, 503 mCi/mmol; [4,5-²H]leucine, 33,7 Ci/mmol; [1-¹³C]glucose, 4,63 Ci/mmol; and Aquasol-2. HEPES, unlabeled amino acids, and all other chemicals were obtained from Sigma, from Merck (Darmstadt, West Germany), or from Fluka AG. Chemische Fabrik (Buchs, Switzerland).

Isolation of Brain Microvessels—Microvessels were isolated from the gray matter of fresh bovine brain as described by Hjelle et al. (8) with minor modifications (7). Briefly, the gray matter was homogenized by hand in a buffer (1:1, w/v) containing 122 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 3 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 0.4 mM KH₂PO₄, and 10 mM HEPES, pH 7.4, and aerated with 95% O₂ + 5% CO₂. The homogenate was poured on a nylon sieve (86-μm pore size) and washed with a spray of ice-cold buffer. The material retained on the sieve was homogenized and washed again. Isolated vessels were immediately collected in the same buffer in a plastic tube and kept on ice until use. For some of the experiments, after the isolation step, microvessels were resuspended in Na⁺-free buffer in which NaCl and NaHCO₃ had been replaced, respectively, by choline chloride and KHCO₃.

Enzyme Assays—The microvessels were subjected to homogenization in a Potter-Elvehjem motor-driven apparatus in the buffer appropriate to the subsequent assay. Alkaline phosphatase was measured using p-nitrophenylphosphate as substrate in a reaction mixture containing 50 mM MgCl₂, 5 mM CaCl₂, 100 mM KCl, 5 mM p-nitrophenylphosphate, 100 mM Tris-HCl, pH 9. The reaction was initiated by addition of 0.1 ml of microvessel homogenate in a final volume of 1 ml. The mixture was then incubated at 37°C for 20 min and the reaction stopped by addition of 2 ml of 1 N NaOH and by cooling in an ice bath. The insoluble material was then removed by centrifugation for 10 min at 3000 × g, and the absorbance at 420 nm determined for each sample and converted to micromoles. γ-Glutamyl transpeptidase activity was determined using γ-glutamyl-p-nitroanilide as substrate, according to Orlovsky and Meister (10). γ'-Nucleotidase activity was determined in a reaction mixture containing 40 mM Veronal buffer, pH 7.5, 50 mM NaCl, and 1 mM γ'-AMP in a final volume of 2 ml. The mixture was then incubated at 37°C in a shaking bath and the reaction stopped by addition of 2 ml of 20% trichloroacetic acid. Insoluble material was removed by centrifugation at 5 min at 3000 × g and aliquots of the supernatant used for P, determination (11).
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The assay was carried out in the presence or absence of Ni\(^{2+}\) ions (0.1 mM Ni(CI)) which selectively inhibit 5'-nucleotidase activity without affecting nonspecific phosphatases. Glutamine synthetase activity was measured according to Wellner and Meister (12).

Purity of the Microvessel Preparations—The isolated microvessel preparations appeared to consist essentially of branching capillary segments with some small arterioles and venules. Neither phase-contrast light microscopy nor scanning electron microscopy (after fixation in 2.5% glutaraldehyde and shadowing with gold) showed any contamination by nervous or glial cells (Fig. 1).

The integrity and viability of the cells in the microvessel preparations were tested by the trypan blue exclusion method (8) which, at variance with the findings reported by Williams et al. (13), indicated almost 100% cell viability.

A consistently negligible uptake of \([^{14}C]sucrose\) was observed. If 1 \(\mu\)Ci/ml was added to microvessel suspensions containing about 1 mg/ml of protein, less than 50 pCi/mg of protein became protein-bound within the first minute. The subsequent increase over the entire 30-min time course was less than 100 pCi/mg of protein.

The microvessel preparations were found to be enriched, with respect to the gray matter, in \(\gamma\)-glutamyl transpeptidase and in alkaline phosphatase (Table I). Detectable activities of 5'-nucleotidase and of Gln synthetase were also present; these enzymatic activities were not likely due to contamination by other cells, since the enzyme activities were still detectable following extensive treatment with collagenase\(^{2}\) as described by Williams et al. (13).

\(^{2}\) P. Cardelli-Cangiano, M. A. Patrizi, F. Barberini, C. Cangiano, and R. Strom, unpublished data.

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Gray matter</th>
<th>Isolated microvessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Nucleotidase</td>
<td>50 ± 3</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>18 ± 7</td>
<td>208 ± 22</td>
</tr>
<tr>
<td>( \gamma )-Glutamyl transpeptidase</td>
<td>6 ± 0.5</td>
<td>190 ± 36</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>22 ± 0.1</td>
<td>2.6 ± 0.9</td>
</tr>
</tbody>
</table>

Table I

Comparison of enzymatic activities in isolated brain microvessels and in gray matter

Brain microvessels were obtained as outlined under "Materials and Methods." Enzymatic activities are expressed as means ± S.D. For the number of preparations shown in parentheses, enzyme levels were determined in triplicate on each preparation by the procedures indicated under "Materials and Methods.”

Kinetic Analysis—The initial (2 min) rate of uptake, when plotted...
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FIG. 2. Time course of Leu uptake within the first 3 min. The data (means ± S.D.) were obtained from three different experiments performed in triplicate. The shown linear regression represents the Leu uptake obtained after subtraction from the values observed at 37 °C (i.e., nonspecific binding). At 0 °C the rates of Leu uptake measured at the same fixed intervals reported in the figure were (from 10 to 180 s) 8.1 ± 0.6, 9.4 ± 0.5, 10 ± 0.4, 11.3 ± 0.3, 15 ± 0.6, and 16.5 ± 0.8 pmol mg of protein^{-1}, respectively.

FIG. 3. A Dixon plot (1/v versus (1)) showing the MeAIB-inhibiting (top) and the BCH-inhibiting (bottom) effect on Gln uptake. The initial 2-min uptake of Gln at 0.03 mM (○) and 0.1 mM (□) was tested in fresh isolated brain microvessels. All the experiments were carried out in the presence of Na^+ assuming that MeAIB and BCH compete, respectively, for the A and L system-mediated Gln uptake. The results represent the averages of three determinations. The observed K_i values were 175 and 275 μM for MeAIB and BCH, respectively.

FIG. 4. Relationship between intracellular Gln levels and Tyr uptake by brain microvessels. Top, 2-min Tyr uptake by brain microvessels measured at fixed time intervals after preloading with Gln. After 20 min of preloading with (open bars) or without (solid bars) 20 mM cold Gln, the microvessels were washed and rapidly resuspended in a warm (37 °C) Gln-free buffer. [1^4C]Tyrosine (1 μCi/ml) was added at the different time intervals indicated on the abscissa, starting from the resuspension of microvessels in the Gln-free buffer. Bottom, Gln levels in the microvessels (○) and in the medium (□) measured at different times after the resuspension of the Gln-preloaded microvessels in the Gln-free buffer.

as a function of amino acid concentration, showed the presence of a saturable component superimposed on a nonsaturable one. As previously described (7), the latter was calculated from the slope of the linear portion of the uptake curve between amino acid concentrations of 0.2 and 2.0 mM and then subtracted from the total uptake curve in order to estimate the steady state kinetic parameters of saturable transport. To this purpose the data are plotted in the S/v versus S "Hanes plot," which allows straightforward statistical analysis, according to Wilkinson (15). The data were subjected to nonlinear regression analysis to obtain the optimal estimate of the kinetic parameters and to evaluate the standard error impending on them.

Amino Acid Analysis—In order to measure the intracellular Gln concentration, the isolated microvessels were transferred after the preincubation step to warm (37 °C) Gln-free buffer and at fixed intervals thereafter filtered through a 44-μm pore nylon sieve on a vacuum manifold. The retained microvessels were then lysed in 1 ml of cold distilled water, using a glass homogenizer with a motor-driven Teflon pestle. After protein determination on a 100-μl aliquot of homogenate, 100 μl of 40% (w/v) sulfosalicylic acid containing norleucine as internal standard were added to the remaining homogenate. After centrifugation the supernatant was subjected to amino acid analysis in a Beckman 121 MB automatic amino acid analyzer using lithium citrate buffers, which allow the separate determination of Glu, Gln, and Asn.

RESULTS

Gln Uptake by Isolated Brain Capillaries—Kinetic analysis of Gln transport by isolated brain microvessels was performed either in the presence of Na^+ ions or in Na^+-free buffer. Under both experimental conditions, V_max had similar values of 900 ± 20 and 877 ± 15 nmol mg of protein^{-1}·min^{-1}, respectively, whereas the Na^+-independent component was characterized
by a lower affinity as compared to that observed in Na+-containing buffer ($K_m = 426 \pm 18$ versus $146 \pm 10 \mu M$). Fig. 3 shows that both MeAIB and BCH, ordinarily specific substrates of A and L systems, respectively, are competitive inhibitors of Gln uptake, showing $K_i$ values of 175 and 275 $\mu M$, respectively. Gln appears therefore to be transported by the A system as well as by the L system.

**Effect of Increased Intracellular Gln on NAA Uptake**—Incubation of the microvessels with Gln, followed by removal of the Gln-containing medium, resulted in an over 2-fold increase in the rate of subsequent Tyr uptake. Subsequently the rate of Tyr uptake declined to control values within approximately 15 min after removal of the microvessels from the Gln-containing medium, with a time course similar to that of the net escape of Gln from the same microvessels (Fig. 4). Kinetic analysis of the initial rate of uptake showed that Gln preloading affected only the maximal influx ($V_{max}$) of the saturable component of uptake (Fig. 5) since neither the $K_m$ for Tyr nor the nonsaturable component was changed. Preloading with Gln exerted similar effects on the uptake of other NAAs such as Leu and Trp, while the uptake of MeAIB and of Lys remained unaffected (Table II). The relation between the Gln efflux and the increased uptake of NAA was confirmed by the demonstration of an overshoot in the uptake of the NAA (Fig. 6) as to be expected in the presence of a transstimulation phenomenon (10). This relation was confirmed by reciprocal stimulation of the rate of $[^3H]$Gln net escape observed when increasing concentrations of other NAAs were added to the suspension medium (Fig. 7).

![Fig. 5. Hanes plot (S/[1] versus S) of the saturable component of Tyr uptake by both preloaded (●) and control (○) microvessels. Isolated microvessels were assayed for Tyr uptake (2 min at 37 °C) between the concentrations ranging from 25 to 1500 $\mu M$. After the subtraction of the nonsaturable component, which was comparable under the two experimental conditions, the obtained saturation curves were used to estimate the $V_{max}$ and $K_m$ values. Data shown are the averages of three different experiments ± S.D.](http://www.jbc.org/)

![Fig. 6. Overshoot effect in the time course of $[^{14}C]$Leu uptake by Gln-preloaded microvessels (●) and by microvessels preincubated in Gln-free buffer (○). After 20 min of preloading with 20 mM Gln, the microvessels were washed and resuspended in warm (37 °C) Gln-free buffer. Immediately after the resuspension, the labeled Leu was added and the time course uptake followed for 30 min. The data in the figure represent the averages ± S.D. obtained from three different experiments, each performed in triplicate.](http://www.jbc.org/)

**TABLE II**

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Radioactive amino acid</th>
<th>Uptake</th>
<th>10 s</th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
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</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>MeAIB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Buffer + Gln</td>
<td>MeAIB</td>
<td>8.6 ± 0.1</td>
<td>34.4 ± 3.8</td>
<td>42.5 ± 4.1</td>
<td>54.5 ± 5.3</td>
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<tr>
<td>Buffer</td>
<td>Lys</td>
<td>7.4 ± 0.8</td>
<td>28.4 ± 4.1</td>
<td>46.3 ± 6.3</td>
<td>56.5 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Buffer + Gln</td>
<td>Lys</td>
<td>2.5 ± 1.1</td>
<td>8.5 ± 0.3</td>
<td>18.5 ± 0.4</td>
<td>27.5 ± 1.3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2.7 ± 0.1</td>
<td>8.6 ± 0.3</td>
<td>18.9 ± 1.8</td>
<td>27.3 ± 2.5</td>
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</tbody>
</table>

**Effect of incubation of the isolated microvessels in 20 mM Gln on the subsequent uptake of MeAIB and Lys**—Isolated microvessels were incubated with or without 20 mM Gln in Na+-containing buffer, washed with cold Gln-free buffer, and resuspended in warm (37 °C) Gln-free buffer containing Na+. Radiolabeled amino acid was added, the microvessels suspension mixed, and portion of the suspension was quickly withdrawn and washed as described under “Materials and Methods” (10-s sample). Other samples were withdrawn at the indicated times. Uptake data are expressed as the average ± S.D. for three determinations.
Identification of the Transport System Involved in Glun-mediated Stimulation of NAA Uptake—It was found that Glun-mediated stimulation of NAA uptake was abolished if the preloading step was performed in Na+-free medium (Fig. 8, top). This finding underlines the importance of the Na+-dependent system in concentrating Gln inside microvessel endothelial cells. On the other hand, the presence of BCH, together with Gln during the preloading step in a Na+-containing buffer, caused little or no interference with the subsequent Gln-dependent stimulation of NAA uptake (Fig. 8, bottom). Conversely, the presence of a high concentration of MeAIB together with Gln during the preloading step completely abolished the stimulation by Gln of NAA uptake.

In other studies, microvessels were incubated in the presence of either MeAIB, Ala, Cys, or Ser (20 mM each) and the subsequent uptake of [14C]Leu was measured (Table III). Neither MeAIB nor Ala, Cys, and Ser were found to exert any stimulatory effect on subsequent NAA uptake. Incubation with Met (20 mM), however, strongly stimulated the subsequent uptake of [14C]Leu, while incubation with BCH had negligible stimulatory effect.

**DISCUSSION**

Our results show that in brain microvessels a relation exists between the L system-mediated uptake of NAA and the A system-mediated increase of intracellular Gln concentration. Kinetic analysis of Gln uptake revealed that Gln can enter brain microvessel endothelial cells using two different transport systems: a Na+-dependent (most likely the A system) and a Na+-independent one (most likely the L system).

Gln can thus be considered as a dual affinity NAA, while other NAAs such as Trp, Tyr, and Leu are known to utilize predominantly the L system (16–19). High intracellular concentration of Gln within brain microvessels stimulated uptake of the other NAAs. This effect appeared to be proportional to the internal Gln concentration (Fig. 4) and was due to a stimulation of the rate of transport (Vmax) by the L system rather than to a change of the Km of uptake. Furthermore, the transport activity of the A system as indicated by MeAIB uptake remained unaffected. The presence of a transstimulation effect of Gln on the uptake of NAA and the increased influx of labeled intracellular Gln in the presence of high extracellular Leu suggest that a high intracellular Gln concentration acts on the uptake of NAA essentially by an L system-mediated mechanism. The ability of Gln to be taken up by the concentrative A system and also to be transported by the exchanging activity of the L system is probably essential for the induction of this effect.

The two Gln carrier systems appeared therefore to act in close cooperation. Significantly higher levels of Gln have indeed been detected in brain microvessels isolated from rats with a portocaval shunt (7), or in vivo, after incubation of the microvessels suspensions with NH4+ ions (20). High Gln concentrations have indeed been found in the cerebrospinal fluid of patients with hepatic encephalopathy (i.e. neurological disturbances associated with severe liver disease) (21) as well as in animals subjected to surgical portocaval anastomosis (22–24). The asymmetric distribution of the NAA transport systems found in the blood-brain barrier (i.e. a system present only on the antiluminal side of brain microvessels), together with the experimental findings mentioned above, allow us to hypothesize that the increased rate of NAA transport across the blood-brain barrier is due to a cooperation between the A and the L systems, the latter being actually responsible for NAA uptake.

These results, by assigning a precise role to Gln-NAA exchange in regulating the rate of NAA uptake by brain microvessels, support the hypothesis of an involvement of brain Gln synthesis in the pathogenesis of hepatic encephalopathy (6, 20, 21).

**Acknowledgments**—The skilled technical assistance of Vincenzo Peresempio and Laura Edwards is gratefully acknowledged.

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Glutamine Uptake and Exchange in Brain Microvessels

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