Role of Oxoproline in the Regulation of Neutral Amino Acid Transport across the Blood-Brain Barrier*

(Received for publication, December 19, 1995, and in revised form, May 7, 1996)

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Regulation of neutral amino acid transport was studied using isolated plasma membrane vesicles derived from the bovine blood-brain barrier. Neutral amino acids cross the blood-brain barrier by facilitative transport system L1, which may allow both desirable and undesirable amino acids to enter the brain. The sodium-dependent amino acid systems A and B0,+ are located exclusively on abluminal membranes, in a position to pump unwanted amino acids out. γ-Glutamyl transpeptidase, the first enzyme of the γ-glutamyl cycle, is an integral protein of the luminal membrane of the blood-brain barrier. We demonstrate that oxoproline, an intracellular product of the γ-glutamyl cycle, stimulates the sodium-dependent systems A and B0,+ by 70 and 20%, respectively. Study of system A showed that 2 mM oxoproline increased the affinity for its specific substrate N-methylaminoisobutyrate by 50%. This relationship between the activity of the γ-glutamyl cycle and system A transport may provide a short-term regulatory mechanism by which the entry of potentially deleterious amino acids (i.e., neurotransmitters or their precursors) may be retarded and their removal from brain accelerated.

The endothelial cells of cerebral capillaries are joined by tight junctions forming the blood-brain barrier (BBB). Thus, hydrophilic nutrients, such as amino acids, require the presence of carriers in the respective luminal and abluminal membranes to reach the brain. Using isolated plasma membrane vesicles from cerebral endothelial cells it has been shown that system L1, characterized by affinity for a broad spectrum of neutral amino acids (especially large neutral amino acids) (1), is equally distributed between luminal and abluminal membranes (2). Therefore, system L1 is in a position to facilitate neutral amino acid movement between blood and brain. Two sodium-dependent transport systems, A and B0,+ are also present (2, 3), but they exist only in abluminal membranes (2). The sodium-dependent systems are, therefore, in a position to pump amino acids out of the brain extracellular fluid. Although system A preferentially transports small neutral amino acids (1), and system B0,+ favors the transport of neutral and basic amino acids (4), there is a considerable overlap in the spectrum of amino acids that can be transported by each of the systems: L1, A, and B0,+ (5). This overlap in transport activity coupled with the asymmetrical distribution of the amino acid transporters at the BBB provides a potential means for the regulation of amino acid delivery to the brain.

γ-Glutamyl transpeptidase (GGT) is located on the luminal membrane of cerebral endothelial cells (6). A role for GGT in amino acid transport was suggested by Meister and co-workers (7–9), as part of the γ-glutamyl cycle. However, the idea that the cycle is involved directly in amino acid translocation into cells is controversial, having received support (10–17) and criticism (18–22).

Studies using mammary glands from lactating rats and placenta of pregnant rats showed that oxoproline, an intermediate of the γ-glutamyl cycle, serves as an intracellular signal to stimulate amino acid transport (23, 24). To determine whether such a regulatory mechanism is present in the BBB, amino acid transport was measured in isolated luminal- and abluminal-enriched plasma membrane vesicles derived from bovine cerebral capillary endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—L-(ring-2,6-d3)Phenylalanine (46 Ci/mmol), N-[3-14C]-methylaminoisobutyric acid (MeAIB, 56.3 mCi/mmol), L-(43C(U))alanine (150.9 mCi/mmol), and [14C(U)]succrose (475 mCi/mmol) were purchased from DuPont NEN. Collagenase type IA and acivicin were obtained from Sigma. Protein assay was purchased from Bio-Rad.

Solutions—The intravesicular solution used in all transport experiments was storage buffer (290 mM mannitol and 10 mM HEPES, buffered with Tris to pH 7.4) unless otherwise stated. The extravesicular solution (reaction medium) was storage buffer supplemented with 100 mM KCl or 100 mM NaCl and the appropriate radiolabeled substrate, as indicated in the figure legends.

Isolation of Endothelial Cell Membranes—Brain microvessels were isolated, and luminal and abluminal plasma membrane vesicles were prepared by a modification (3) of the procedure described by Betz et al. (25). The membranes were layered on a discontinuous Ficoll gradient (0, 3, 10, and 20% Ficoll) and centrifuged at 162,500 × g for 2.5 h at 4 °C. Five fractions containing the membrane vesicles were collected at different Ficoll interphases. The vesicles were then diluted in storage buffer, and aliquots were stored at −80 °C until used.

Preparation of Vesicles for Transport Experiments—Storage buffer with or without oxoproline (or glutamate) was added to the frozen membrane vesicles. Vesicles opened by the freezing process reseal during thawing and incorporate the external medium (26–28). The vesicles were then allowed a 2-h period for volume equilibration. Acivicin (0.25 mM), an inhibitor of GGT (29), was added for the final 0.5 h of volume equilibration as a precaution against transpeptidation of amino acid substrates during the course of the experiment (e.g., γ-glutamyl amino acids that may form through reaction with glutathione).

The vesicles were then centrifuged at 37,500 × g for 25 min at 4 °C. The supernatant was removed and the pellet resuspended in storage

* This work was supported in part by Grant NS31017 from the NINDS, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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¶ Supported by Dirección General de Investigaciones Científicas y Técnicas Grant PM 91-0198, Spain, and Fondo de Investigaciones Sanitarias Grant FIS 94/1573, Spain.

The abbreviations used are: BBB, blood-brain barrier; GGT, γ-glu tamyl transpeptidase; MeAIB, N-(methylamino)isobutyric acid; PSA, permeability-surface area.
buffer at a protein concentration between 2.5 and 5 µg/ml.

The reaction was started by adding 10 µl of reaction medium (see above) to 10 µl of the membrane suspension. After incubation at 37 °C for the times indicated, the reaction was stopped by diluting with 1 ml of stopping solution (145 mM NaCl and 10 mM HEPES/Tris, pH 7.4) at 4 °C, followed by rapid filtration over a 0.45-μm Gelman Metrical filter. The filters were rinsed four times with 1 ml of stopping solution, and the retained radioactivity was counted by liquid scintillation spectrometry. Control samples containing the same amount of radioactivity, but without membranes, were filtered as described above to correct for retention of radiolabeled material by the filter. Vesicle integrity was examined by measuring diffusional uptake of 10 µM [14C]sucrose. The PSA values were 0.21 and 0.13 mV for luminal and abluminal enriched membranes, respectively (3). These values were considerably less than the permeability of system Gly for MeAIB is poor with a affinity of system Gly for MeAIB is poor with a

Experimental Design—The synthetic amino acid analog MeAIB is especially useful because it is carried principally by the sodium-dependent transport system A (1, 30–32) with a Kₘ between 300 and 700 µM (2, 33). The only other transport system that has been shown to have any affinity for MeAIB is the sodium-dependent system Gly which transports glycine, sarcosine, and some imino acids (30). However, the affinity of system Gly for MeAIB is poor with a Kₘ of magnitude higher (12 mM) (34). We determined the Kₘ in abluminal BBB membranes to be 560 µM (see “Results” and Table II) as expected for system A. There was evidence for a second component that accounted for only 3% of the total rate of transport and was indistinguishable from diffusion (data not shown). We concluded that MeAIB was carried principally, if not exclusively, by the system A transporter.

In addition to system A, there is another sodium-dependent transport system in abluminal membranes of the BBB (2). This system, known as B⁺⁺, transports neutral and basic amino acids but has no affinity for MeAIB (30). System B⁺⁺ also transports 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid (BCH), which is not transported by system A (4, 30, 32, 35). To determine the transport activity of system B⁺⁺, the uptake of alanine, a substrate of both systems A and B⁺⁺, was measured in the presence of 5 mM MeAIB. This concentration is sufficient to block 90% of the activity associated with system A.

The facilitative system L1 has a strong affinity for large neutral amino acids but has neither a dependence on a sodium gradient nor an affinity for MeAIB (1, 36). Therefore, phenylalanine was used as the substrate to measure system L1 in the absence of a sodium gradient.

Protein Determination—Protein concentrations were determined using the Bio-Rad protein microassay, with bovine serum albumin as the standard (37).

Calculations—In transport studies where uptake was measured as a function of time, the data were fitted to a single exponential equation: uptake = A(1 − e⁻ᵏᵗ). A double exponential equation, uptake = A₁(1 − e⁻ᵏ₁ᵗ) − A₂(1 − e⁻ᵏ₂ᵗ), was used when an inwardly directed sodium gradient caused a transient outwardly directed amino acid gradient. The initial rates of transport were calculated as the product of the slope of the least-squares line in the absence of a sodium gradient, or A₁, Kₛ − A₂Kₛ in the presence of a sodium gradient.

The PSA represents the sum of specific and nonspecific transport components (Vₛ + Sₖ + Kₛ + Kₛ). The PSA values, expressed as µmol min⁻¹ mg⁻¹ of protein, were obtained by measuring the initial rate of uptake of radiolabeled substrate transport was determined as the product of the substrate concentration and PSA. A best fit analysis was employed to determine the kinetic parameters using the Michaelis-Menten equation and included a term for nonspecific diffusion, (Vₛ[S] + Kₛ[S]).

RESULTS

Properties of Membrane Vesicles—Five membrane fractions were obtained at the interfaces of a discontinuous Ficoll gradient (Fig. 1). Fractions 1 (0/5% interface, enriched in GGT), and 3 (10/15% interface, enriched in system A activity), were taken as luminal and abluminal enriched membranes, respectively (3).

The integrity of membrane vesicles was assayed by measuring the PSA of [14C]sucrose. The PSA values were 0.21 and 0.13 µmol min⁻¹ mg⁻¹ of protein for luminal and abluminal vesicles, respectively. Loading of abluminal vesicles with 2 mM oxoproline gave a similar value, 0.11 µmol min⁻¹ mg⁻¹ of protein⁻¹. These values were considerably less than the permeability measured for substrates (see below), indicating that nonspecific diffusion was a minor component of uptake at short incubation times.

Oxoproline Stimulation of System A in Abluminal-enriched Vesicles—In the presence of an inwardly directed sodium gradient, MeAIB was initially concentrated in the vesicles (overshoot) and then gradually declined to an equilibrium value following dissipation of the sodium gradient (Fig. 2A). The overshoot effect was greater in vesicles preloaded with 2 mM oxoproline. No overshoot was observed when potassium was substituted for sodium, whether or not oxoproline was present (Fig. 2B).

Oxoproline also stimulated the initial rate of MeAIB transport by system A (Fig. 3). This effect was concentration-dependent and maximal at about 2 mM. Therefore, 2 mM oxoproline was used for the remaining experiments.

The magnitude of the stimulatory effect of oxoproline on the initial rate of system A transport was shown by a comparison of the PSA (Table I). In the absence of a sodium gradient, oxoproline did not stimulate transport. The presence of a sodium gradient, oxoproline stimulated the initial rate of MeAIB transport by about 70%.

Oxoproline Stimulation of System B⁺⁺ in Abluminal-enriched Vesicles—The sodium-dependent transport of 100 µM [14C]alanine was measured over 15 s in the presence and absence of MeAIB to determine the activity of system B⁺⁺ transport activity. MeAIB-insensitive alanine transport in the presence and absence of oxoproline was 367 ± 5 and 307 ± 6 pmol min⁻¹ mg⁻¹ of protein⁻¹, respectively, indicating that oxoproline stimulated system B⁺⁺ transport by about 20%.

Kinetic Constants in Abluminal-enriched Membranes—Kinetic constants were measured to characterize further the effect of oxoproline on system A transport because it was stimulated to a greater degree than system B⁺⁺. The presence of oxoproline decreased the Kₛ by 50%, with no change in the Vₘₙₚ (Table II). Control vesicles loaded with glutamate, a metabolite of oxoproline, showed no stimulation of transport.

Kinetic Study in Luminal-enriched Membranes—Phenylalanine was used as a representative substrate to measure system L1 transport activity. No effect of oxoproline was observed. The Kₛ of the L1 system for phenylalanine in control and oxoproline-loaded vesicles was 12.0 ± 0.6 and 11.7 ± 0.9 µM, respectively. The Vₘₚ in the control and loaded vesicles was 19130
DISCUSSION

The principal findings were as follows. 1) Oxoproline accelerated the initial rate of substrate transport by the two sodium-dependent amino acid carriers known to be at the abluminal membrane of the BBB (systems A and B\textsuperscript{0,+}). 2) System A, which was stimulated to the greatest degree, manifested an increased affinity for its substrate. 3) There was no effect of oxoproline on the facilitated transport of phenylalanine. These observations suggest that oxoproline, produced as part of the γ-glutamyl cycle (7–9), may be an important link in the short term control of sodium-dependent amino acid transport by the BBB.

System L1 is distributed symmetrically between the luminal and abluminal membrane domains of cerebral endothelial cells (2) and serves to facilitate the diffusion of neutral amino acids between blood and brain. Although L1 is characterized by a high affinity for large neutral amino acids, it has a measurable affinity for almost all neutral amino acids (38). There is also a nonsaturable component that may allow an additional influx of neutral amino acids, accounting for about 5% of carrier-mediated transport (38). The combined effect of these two pathways could provide a supply of both essential and nonessential amino acids, some of which may be neurotransmitters or modulators of neurotransmission.

Two sodium-dependent amino acid transport systems (A and B\textsuperscript{0,+}) are present exclusively on the abluminal membrane of the BBB (2). The capacity of these transporters is an order of magnitude greater than the facilitative transporter L1 (2). Because the electrochemical gradient for sodium is oriented to flow from the extracellular fluid into the endothelial cells, these sodium-dependent transport systems are in a position to export amino acids from the brain extracellular fluid. Thus, amino acids that pass both endothelial cell membranes and enter the
basement membrane space could be actively, and selectively, pumped back across the abluminal membrane. This asymmetrical distribution of sodium-dependent carriers has the potential, therefore, to restrict the availability of amino acids to the brain.

The γ-glutamyl cycle has been shown to influence amino acid transport in diverse tissues. The first reaction of the cycle occurs extracellularly and is catalyzed by GGT. (8). Glutathione is exported across the luminal membrane, and transpeptidation occurs in the presence of extracellular amino acids. The γ-glutamyl amino acids that result enter cells by a transport system that is not shared by free amino acids. Intracellularly, γ-glutamyl amino acids are substrates of γ-glutamyl cyclotransferase, which converts the γ-glutamyl amino acids into oxoproline and the corresponding free amino acids. The subsequent conversion of oxoproline to glutamate by oxoprolinase is the rate-limiting step of the γ-glutamyl cycle (39).

Irrespective of whether the γ-glutamyl cycle directly mediates significant amino acid transport, it does seem to influence amino acid transport systems. This has been shown in mammalian glands of lactating rats and placenta of pregnant rats in which the activity of the γ-glutamyl cycle was correlated with active amino acid transport (23, 24). Specifically, it appears that oxoproline, produced intracellularly as an intermediary metabolite of the γ-glutamyl cycle, acts as a signal to activate the translocation of amino acids into these tissues.

The presence of GGT in the BBB has been an enigma. GGT activity is high in tissues that actively transport amino acids (8), such as the brush border of the proximal convoluted tubules of the kidney (42), the lactating mammary gland (43), and the apical portion of the intestinal epithelium (44). The BBB differs from these tissues in that it is not associated with active amino acid uptake from plasma. Although brain requires essential amino acids for its function and growth, their supply is not much greater than the demand, and it is difficult to detect arteriogenous differences of amino acids across the brain (45, 46). It has, therefore, been puzzling why brain capillaries have such high GGT activity.

Our data support the hypothesis that the γ-glutamyl cycle and GGT serve to monitor the availability of amino acids to the brain and constitute the first step in a control mechanism that influences the accessibility and content of brain amino acids (Fig. 4). The question arises whether the oxoproline concentrations that exist in vivo are sufficient to stimulate sodium-dependent transport. Although we are unaware of measurements of oxoproline in cerebral microvessels, the concentrations in normal human plasma and various tissue extracts are between 20 and 50 μM (39, 40) and as high as 6 mM in plasma and cerebrospinal fluid in pathological conditions (41). Stimulation of sodium-dependent transport of MeAIB was a linear function of the oxoproline concentration up to 2 mM, a range that does not seem unreasonable.

The transpeptidation activity of GGT is a function of the plasma concentration and spectrum of amino acids (47), both of which may vary considerably, depending on nutritional status. This provides a feedback mechanism in which the γ-glutamyl amino acids produced by GGT enter cerebral capillary endothelial cells and are converted to oxoproline, which in turn activates the A system at the abluminal membrane. Since the A system is oriented to remove amino acids from the brain in an energy-dependent fashion, its up-regulation could provide a control mechanism to guard against elevations of amino acids in brain when their availability is excessive. This is of particular interest with regard to smaller nonessential amino acids for which system A has a relatively high affinity. Thus, this process may serve to modulate entry of amino acids that serve as neurotransmitters or their precursors.

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