Glutamine transport by basolateral plasma-membrane vesicles prepared from rabbit intestine

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INTRODUCTION

L-Glutamine, a major energy substrate for intestinal epithelial cells, can be extracted from intraluminal contents across the brush-border membrane and from arterial blood via the basolateral membrane. The purpose of the present study was to characterize glutamine transport by the basolateral membrane of rabbit epithelial cells. Transport of glutamine by isolated basolateral-membrane vesicles was mediated by both Na⁺-dependent and Na⁺-independent carriers. Tests were performed to distinguish glutamine uptake by likely transport agencies, including Systems A, ASC, N, IMINO, NBB, L and ASC. The Na⁺-dependent glutamine uptake was strongly inhibited by an excess of 2-(methylamino)isobutyric acid (MeAIB), and glutamine was equally effective in inhibiting MeAIB transport. The reciprocal inhibition analysis, as well as a sensitivity to increased H⁺ concentration, indicates that Na⁺-dependent glutamine transport across the basolateral membrane is mediated by System A. The saturable Na⁺-independent glutamine transport was markedly inhibited by 2-aminoisobicyclo-[2,2,1]-heptane-2-carboxylic acid (‘BCH’) and insensitive to changes in assay pH, suggesting uptake via System L rather than System ASC. The presence of a Na⁺-dependent carrier to mediate active transport of glutamine across the basolateral membrane is probably essential to ensure a continuous supply of this vital substrate to the enterocyte in the post-absorptive state.

Abbreviations used: BBMV, brush-border plasma-membrane vesicles; BLMV, basolateral plasma-membrane vesicles; MeAIB, 2-(methylamino)isobutyric acid; GGT, γ-glutamyl transpeptidase; BCH, 2-aminoisobicyclo-[2,2,1]-heptane-2-carboxylic acid.

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MATERIALS AND METHODS

Membrane (BLMV) preparation

Male New Zealand White rabbits weighing 2.5–3.5 kg were fasted overnight. A lethal injection of sodium pentobarbital (65 mg/kg) was given after sedation with fentanyl and droperidol. The entire small intestine was removed and immediately placed in ice-cold 1 mM-Tris/Hepes (pH 7.5)/0.9 % NaCl. The jejunal segment, defined as the proximal two-fifths of the mobile small intestine, was opened longitudinally and rinsed with the above saline buffer. The mucosal layer was then removed by scraping with a glass slide, and BLMV were prepared by a modification of the method described by Orsenigo et al. [21]. Mucosal scrapings from two rabbits were diluted in 60 ml of a buffer containing 250 mM-sucrose, 0.1 mM-phenylmethylsulfonyl fluoride and 10 mM-triethanolamine chloride, pH 7.6, (Solution 1) and then homogenized for 1 min with a Polytron homogenizer (Brinkmann, Westbury, NY, U.S.A.). This homogenate was centrifuged in a Sorvall SS-34 rotor at 4 °C for 10 min at 2500 g to remove cellular debris and connective tissue. The resultant supernatant was centrifuged for 20 min at 22000 g, and the white fluffy upper layer of the pellet was resuspended in 20 ml of Solution 1. The remaining small brown pellicle was discarded. The resuspended fraction, a crude plasma-membrane preparation, was homogenized with 15 strokes in a Potter–Elvehjem homogenizer before being diluted with Percoll in Solution 1 to produce a final concentration of 15% (v/v) Percoll. The solution was centrifuged for 45 min at 48000 g in a Beckman 60Ti rotor. A distinct band of membrane was detectable near the middle of the gradient, which was removed with the aid of a syringe and 18-gauge needle. After dilution in Solution 1, the mixture was centrifuged for 20 min at 48000 g. The supernatant was discarded and the membrane pellet diluted to 20 ml with a buffer containing 250 mM-sorbitol and 10 mM-Tris/Hepes, pH 7.5 (Solution 2). CaCl₂ was added to a final concentration of 10 mM, and the solution incubated at 4 °C for 20 min with stirring. The mixture was then centrifuged for 10 min at 3000 g, and the pellet resuspended in 20 ml of Solution 2. This solution was centrifuged at 48000 g for 20 min, and the resulting BLMV-enriched fraction was resuspended in a small volume of Solution 2. Samples of the BLMV were taken for protein determination, and the remainder was divided into batches and stored frozen in liquid nitrogen until use. Each batch was only thawed once, just before use.

Marker enzymes

The relative purity of freshly prepared BLMV was assayed by using Na⁺/K⁺-ATPase and γ-glutamyl transferase (GGT) as markers for the basolateral membrane and brush-border membrane preparations respectively. The Na⁺/K⁺-ATPase activity was assayed as described by Kilberg & Christensen [22]. For determination of P, the method of Fiske & Subbarow [23] was used. GGT activity was assayed by the method of Meister et al. [24].

Amino acid transport assay

Transport assays were initiated by adding 20 μl of BLMV (20–60 μg of membrane protein) which had been incubated at 37 °C for 2 min to 20 μl of 100 μM-[³H]glutamine in K⁺ or Na⁺ uptake buffer (10 mM-Hepes, pH 7.5, 1 mM-MgCl₂ and 200 mM of either KSCN or NaSCN), also previously warmed to 37 °C. The mixture was vortex-mixed and incubated at 37 °C for the appropriate time period, as indicated in each Figure. Uptake was terminated by addition of 1 ml of ice-cold phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.5), and the suspension was immediately passed over a nitrocellulose filter (0.45 μm pore size) under a 193 kPa (28 lb/in²) vacuum. The filter was washed with 2 x 5 ml of phosphate-buffered saline, placed in a scintillation vial, and then 5 ml of scintillation mixture (Beckman, Ready Protein Plus) was added. Control incubations contained no membrane vesicles, but were filtered, and the filters were washed as usual to determine blank values. Radioactivity trapped on the filters was measured by scintillation spectrometry in a Beckman LS 3801 liquid-scintillation system. Each of the experiments reported was repeated with different membrane preparations, and, although there were differences in absolute transport velocities between membrane preparations, qualitatively, the findings reported were reproducible.

Protein determination

The protein content of BLMV samples was measured after precipitation of the protein with ice-cold trichloroacetic acid (final concn. 10%, w/v). After centrifugation at 10000 g for 20 min, the protein pellets were resuspended in 0.2 mM-NaOH containing 0.2% SDS. A copper reagent [25] containing 0.58 mM copper–disodium EDTA, 189 mM-NaCO₃, 100 mM-NaOH and 1% SDS was added at a ratio of 630 μl of reagent for each 100 μl of protein solution. After incubation for 10 min at room temperature, 60 μl of Folin/Ciocalteu phenol reagent (diluted with water 1:1 just before use) was added to each sample. The A250 was determined after a 30 min incubation at room temperature. BSA was used as the protein standard.

Materials

Male New Zealand White rabbits were obtained from a colony maintained by the University of Florida Animal Resource Facility, 1-[³H]Glutamine, 1-[³H]leucine and 1-[³H]leucine were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. [³H]MeAIB was purchased from American Radiolabelled Chemicals., St. Louis, MO, U.S.A. Nicotinellus filters (0.45 μm pore size, Type GN-6) were obtained from Gelman Sciences, Ann Arbor, MI, U.S.A. All other reagents or chemicals were purchased from Sigma Chemical Corp., St. Louis, MO, U.S.A.

RESULTS AND DISCUSSION

Na⁺/K⁺-ATPase activity was used as a specific marker for basolateral membrane, and the rabbit BLMV used in the studies reported here were enriched 12-fold (homogenate = 6.5 and BLMV = 79 μmol of ATP hydrolysed/h per mg of protein). This magnitude of enrichment is nearly identical with that seen by Ghishan et al. [16] and Orsenigo et al. [15] for the preparation of rat intestinal BLMV. GGT activity was assayed to estimate the extent of brush-border membrane contamination. The GGT activity was found to be enriched 1.3-fold (homogenate = 3.3 and BLMV = 4.32 μmol/h per mg of protein), indicating some contamination of the BLMV. The impact of this contamination is addressed below.

Uptake of glutamine by rabbit BLMV in the presence of an artificially created Na⁺ gradient was greater than that in KSCN (Fig. 1). Glutamine transport in KSCN was maximal at 3 min, and the accumulation remained constant thereafter. The Na⁺-dependent transport of glutamine exhibited a classic overshoot pattern, with a peak occurring at 1 min, followed by a slow decay such that the rate began to approach zero. These data indicate the presence of one or more Na⁺-dependent glutamine-transport systems in our BLMV.

A number of different amino acid transport systems are thought to mediate Na⁺-dependent uptake of glutamine in other cells or tissues. Given that the rabbit BLMV preparation showed a slight contamination by brush-border membrane, it was necessary also to consider the possibility that transport systems for glutamine on the brush-border membrane were responsible...
for the Na\(^{+}\)-dependent uptake of glutamine in our experiments. Stevens et al. [19] have described several Na\(^{+}\)-dependent transport activities in rabbit brush-border membranes: the neutral brush-border (NBB), INIMO, and PHE systems. The NBB system transports most neutral L-amino acids, but does not utilize MeAIB as a substrate [13]. An activity analogous to System A does not appear to be expressed on the brush-border surface. Imino acids such as proline, and to a limited extent MeAIB, are transported via the INIMO system [20]. The PHE system is highly specific for phenylalanine and methionine [13].

Based on the system specificities discussed above, selective amino acids were tested for their ability to inhibit Na\(^{+}\)-dependent glutamine transport by the BLMV (Table I). Strong inhibition by MeAIB suggested that glutamine may be transported via System A, given that Systems ASC [26,27], N [4] and NBB [13] do not transport N-methylated amino acid analogues effectively. Some Na\(^{+}\)-dependent transport of MeAIB in brush-border membranes can be mediated by the INIMO system [20]. Stevens & Wright [20] noted that proline was an ideal substrate for the INIMO system, whereas glycine was totally ineffective as an inhibitor. Considering that glycine is transported to some extent via System A in most cells [28], but not by the brush-border INIMO system, its effect on glutamine transport was used to test INIMO activity. As illustrated in Table 1, glutamine uptake was nearly abolished by glycine, and this inhibition was unchanged on addition of excess proline to the uptake assay.

Further evidence for the role of System A in Na\(^{+}\)-dependent glutamine transport by intestinal BLMV is that the transport of MeAIB was totally inhibited by glutamine (50 \(\mu\)M-[\(^{3}\)H]MeAIB uptake in the absence of inhibitor = 11.1, uptake in the presence of 10 \(\mu\)M-glutamine = 0.2 pmol/30 s per mg of protein). Furthermore, the complete inhibition of 50 \(\mu\)M-MeAIB uptake by 10 \(\mu\)M-glycine (11.1 versus 0.1 pmol/30 s per mg of protein) argues against significant contamination by the brush-border INIMO system [13,20]. The data demonstrate that glycine, MeAIB and glutamine fulfill the criteria of the 'qualitative ABC Test', and thus are transported by a common carrier [29]. Sensitivity to increasing H\(^{+}\) concentration has been helpful in distinguishing System A activity from that of the relatively pH-insensitive System ASC in a number of cell types [30,31]. As illustrated in Fig. 2, the Na\(^{+}\)-dependent transport of glutamine by BLMV was markedly decreased when the assay pH was below

![Fig. 1. Time course for transport of glutamine by intestinal BLMV](image)

The uptake of 50 \(\mu\)M-[\(^{3}\)H]glutamine was measured at 37 \(^\circ\)C in either KSCN (▲) or NaSCN (■) uptake buffer. The rate of Na\(^{+}\)-dependent transport (●) was calculated by subtracting the rate observed in the absence of Na\(^{+}\) from that observed in its presence. The data are presented as means ± S.D. of assays in triplicate; where not shown, S.D. bars are contained within the symbols.

![Fig. 2. pH-sensitivity of Na\(^{+}\)-dependent glutamine transport by intestinal BLMV](image)

The uptake of 50 \(\mu\)M-[\(^{3}\)H]glutamine was measured for 30 s at 37 \(^\circ\)C in KSCN (▲) or NaSCN (■) transport buffer which had been adjusted to an appropriate pH so that dilution with the membrane stock solution produced the final assay pH as shown. The rate of Na\(^{+}\)-dependent transport (●) was calculated by subtracting the uptake observed in the absence of Na\(^{+}\) from that observed in its presence. The data are the means ± s.d. of at least three determinations; where not shown, S.D. bars are contained within the symbols.

### Table 1. Inhibition analysis of Na\(^{+}\)-dependent and Na\(^{-}\)-independent transport of glutamine by intestinal BLMV

The uptake of 50 \(\mu\)M-[\(^{3}\)H]glutamine was determined in the absence or presence of 10 \(\mu\)M unlabelled amino acid. Transport was measured for 30 s at 37 \(^\circ\)C. When necessary, uptake mixtures were balanced appropriately with regard to osmolarity with choline chloride. The data are given as means ± S.D. of assays in triplicate.

<table>
<thead>
<tr>
<th>Inhibitor (10 mM)</th>
<th>Na(^{+})-dependent [(^{3})H]glutamine transport (pmol/30 s per mg of protein)</th>
<th>Inhibitor (10 mM)</th>
<th>Na(^{-})-independent [(^{3})H]glutamine transport (pmol/30 s per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>25.3 ± 2.1</td>
<td>None</td>
<td>30.8 ± 1.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.3 ± 3.4</td>
<td>Glutamate</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Glycine + proline</td>
<td>1.8 ± 4.4</td>
<td>Alanine</td>
<td>11.3 ± 1.6</td>
</tr>
<tr>
<td>Asparaginic acid</td>
<td>2.0 ± 2.6</td>
<td>Asparagine</td>
<td>5.5 ± 1.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.1 ± 2.9</td>
<td>BCH</td>
<td>8.5 ± 1.9</td>
</tr>
<tr>
<td>MeAIB</td>
<td>4.6 ± 2.7</td>
<td>Phenylalanine</td>
<td>12.1 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidine</td>
<td>11.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Threonine</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tryptophan</td>
<td>11.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valine</td>
<td>16.0 ± 0.1</td>
</tr>
</tbody>
</table>

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6.5. Although the exact value of pH required to inhibit BLMV glutamine transport completely may be lower than that for System A in other cells, the data demonstrate that Na⁺-dependent glutamine uptake is clearly pH-sensitive, thus lending support to the assignment of System A.

The time course for saturable Na⁺-independent transport of glutamine illustrated that the maximal accumulated glutamine occurred by 30 s, and remained fairly constant thereafter (Fig. 3). A large non-saturable component was present at each time point, which accounted for most of the glutamine uptake by BLMV. The magnitude and possible role of non-saturable transport in vivo is unclear. Studies were undertaken to identify the saturable Na⁺-dependent system(s) responsible for glutamine uptake by the BLMV. The System L carrier exhibits broad specificity and is responsible for transport of nearly all neutral amino acids, but has greatest affinity for amino acids possessing aromatic or branched side chains [32,33]. It has been characterized through the use of the non-metabolizable branched-chain analogue 2-amino-2-cyclohexyl-2-heptanone-2-carboxylic acid (BCH) [32-34].

Another Na⁺-independent system for neutral amino acids has been identified which is distinct from System L [33,34]. It has been designated System asc, the name reflecting its Na⁺-independence as well as a substrate-specificity similar to that of the Na⁺-dependent System ASC [35]. Threonine has been used effectively as a substrate for this carrier, but valine, alanine and serine also are transported by this system [33,34].

The inhibitory effects of several amino acids on Na⁺-independent glutamine uptake by rabbit BLMV were tested, and they were effective inhibitors of glutamine transport (Table 1). The data suggest uptake via a single system with broad specificity, such as System L, or uptake via both Systems asc and L. Given the degree of inhibition in the presence of BCH, a poor substrate for System asc, it appeared likely that System L played the primary role in glutamine transport by BLMV. However, owing to the strong attenuation of glutamine uptake by threonine (Table 1), the concurrent presence of System asc could not be excluded. Studies were undertaken to determine the inhibitory ability of glutamine, leucine, BCH and threonine on the transport of a classic System L substrate, leucine, as well as on threonine, the proposed selective substrate for System asc [33,34]. The saturable Na⁺-independent transport of leucine was totally inhibited by the System L-specific analogue BCH, and glutamine and threonine were equally effective (Table 2). Conversely, threonine uptake was similarly affected by glutamine, leucine and BCH, with 85% or greater inhibition by each of these amino acids. The reciprocal inhibition of glutamine, leucine and threonine depicted in Tables 1 and 2 satisfies qualitatively the tenets of the ‘ABC test’, thus indicating transport via a single, common, carrier [29]. It is unlikely that the carrier is System asc, given the highly effective inhibition of threonine uptake by typical System L substrates such as leucine and BCH [33,34].

System asc has been shown to be highly sensitive to lowering the assay pH [34], whereas System L transport is stable or slightly stimulated by increasing concentration of H⁺ [4]. Carrier-mediated Na⁺-independent transport of 50 μM [³H]glutamine by BLMV was slightly greater at lower pH values when measured over the pH range 6.0–7.5 (pH 6.0 = 32.4, pH 6.5 = 31.1, pH 7.0 = 24.5 and pH 7.5 = 27.3 pmol/30 s per mg of protein). This pattern is most consistent with the known characteristics of System L, rather than those of System asc. These results extend previous reports by demonstrating that System L mediates Na⁺-independent glutamine uptake.

Conclusions

We have demonstrated that System A is responsible for Na⁺-dependent transport of glutamine across the BLM of rabbit jejunal enterocytes, and that System L is responsible for the Na⁺-independent uptake. Table 3 is a summary of the criteria that we have used for characterization of glutamine transport. Five potential transport systems were considered, but three were excluded after demonstration of strong MeAIB-dependent inhibition of glutamine uptake and reciprocal inhibition of MeAIB transport by glutamine. MeAIB tolerance could only be characteristic of either System A or the IMINO system. The possibility that Na⁺-dependent glutamine transport was mediated by the IMINO system as the result of brush-border membrane contamination of BLMV was eliminated with the observation that glycine, a substrate not tolerated by the IMINO system [20], produced strong inhibition of glutamine uptake. The pH-sensitivity of glutamine transport provided further evidence for System A activity. Evaluating each of the carrier properties listed in Table 3 has eliminated a role for Systems ASC, N, NBB and IMINO and indicates that Na⁺-dependent glutamine uptake is mediated by System A. A similar experimental approach has been used to determine the route of Na⁺-independent glutamine transport (Table 3). The marked inhibition of glutamine uptake by BCH and an insensitivity to changes in H⁺ concentration

![Fig. 3. Time course for Na⁺-independent uptake of glutamine by intestinal BLMV at 37 °C](image-url)
Table 3. Summary of the differentiating characteristics of potential glutamine-transport systems in rabbit intestine basolateral plasma membranes

<table>
<thead>
<tr>
<th>Characteristics of glutamine transport</th>
<th>Potential systems</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Na⁺-dependent</td>
<td>Yes</td>
</tr>
<tr>
<td>MeAIB-inhibitable</td>
<td>Yes</td>
</tr>
<tr>
<td>Glycine-inhibitable</td>
<td>Yes</td>
</tr>
<tr>
<td>pH-sensitivity</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Potential systems

<table>
<thead>
<tr>
<th>Na⁺-independent</th>
<th>L</th>
<th>asc</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCH-inhibitable</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>pH-insensitivity</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

indicate that System L mediates Na⁺-independent glutamine uptake across the basolateral membrane of rabbit jejunal enterocytes.

The existence of a Na⁺-dependent transporter in the basolateral membrane could be viewed as inconsistent with conventional theories of trans-epithelial transport [36]. Traditionally, it has been thought that Na⁺-dependent transport of nutrients occurs primarily at the brush-border membrane and that completion of trans-epithelial transport results when absorbed substrates efflux across the basolateral membrane to the blood through Na⁺-independent carriers. However, the reports of Na⁺-dependent glutamine uptake by BLMV are not surprising, given the metabolic importance of glutamine to the enterocyte [1,2]. The enterocyte depends on a constant supply of glutamine, but receives luminal glutamine only in the immediate post-prandial period. As first suggested by others [15], given the metabolic requirement for glutamine by this cell, it may be imperative that a Na⁺-dependent active transport mechanism exists for post-absorptive accumulation of glutamine from the blood.

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