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Glutamine transport by rat basolateral membrane vesicles

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Glutamine, a neutral amino acid, is unlike most amino acids, has two amine moieties which underlies its importance as a nitrogen transporter and a carrier of ammonia from the periphery to visceral organs. The gastrointestinal tract utilizes glutamine as a respiratory substrate. The intestinal tract receives glutamine from the luminal side and from the arterial side through the basolateral membranes of the enterocyte. This study characterizes the transport of glutamine by basolateral membrane vesicles of the rat. Basolateral membranes were prepared by a well validated technique of separation on a percoll density gradient. Membrane preparations were enriched with Na^+/K^+-ATPase and showed no 'overshoot' phenomena with glucose under sodium-gradient conditions. Glutamine uptake represented transport into the intravesicular space as evident by an osmolality study. Glutamine uptake was temperature sensitive and driven by an inwardly directed sodium gradient as evident by transient accumulation of glutamine above the equilibrium values. Kinetics of glutamine uptake under both sodium and potassium gradients at glutamine concentrations between 0.01 and 0.6 mM showed saturable processes with V_{max} of 0.39 ± 0.008 and 0.34 ± 0.05 nmol/mg protein per 15 s for both sodium-dependent and sodium-independent processes, respectively. K_m values were 0.2 ± 0.01 and 0.55 ± 0.01 mM, respectively. pH optimum for glutamine uptake was 7.5. Imposition of negative membrane potential by valinomycin and anion substitution studies enhanced the sodium-dependent uptake of glutamine suggesting an electrogenic process, whereas the sodium-independent uptake was not enhanced suggesting an electroneutral process. Other neutral amino acids inhibited the initial uptake of glutamine under both sodium-dependent and sodium-independent conditions. We conclude that glutamine uptake by basolateral membranes occurs by carrier-mediated sodium-dependent and sodium-independent processes. Both processes exhibit saturation kinetics and are inhibited by neutral amino acids. The sodium-dependent pathway is electrogenic whereas the sodium-independent pathway is electroneutral.

Introduction

Glutamine is a nonessential neutral amino acid with an uncharged polar R group. The polarity of glutamine is contributed by its amide group. Glutamine serves as a nitrogen transporter and a carrier of ammonia from the periphery to visceral organs [1–3]. The carbon skeleton of glutamine is metabolized through the Krebs cycle to yield 30 mol of ATP per mol. Therefore, glutamine is as efficient a fuel as glucose which generates 36 mol of ATP per mol.

The studies of Windmueller et al., have demonstrated that the small intestine of the rat extracts 20 to 30 percent of plasma glutamine with each circulation. Approximately 64 percent of the glutamine carbon was oxidized to carbon dioxide suggesting that the intestine utilizes glutamine as its respiratory fuel supply. In contrast, 37% of glutamine nitrogen appears as ammonia [4–7]. Glutamine appears to be similarly metabolized whether it enters the mucosal cells across the brush border or across the basolateral cell membrane from the arterial blood [4–7].

The process by which glutamine enters the enterocyte across the basolateral membrane is now known. We have designed the current studies to determine the transport of glutamine across basolateral membranes of rat jejunum. Basolateral membranes were prepared by differential centrifugation and separation on a percoll density gradient.

Material and Methods

D-[^{3}H]Glucose (18.1 Ci/mmol), ^{45}Ca (14.6–26 mCi/mg) and L-[G-^{3}H]glutamine (39 mCi/mmol) were

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purchased from (Amersham/Searle, Des Plaine, IL). Unlabelled glutamine and other amino acids were purchased from Sigma Chemical Corporation, St. Louis, MO. Cellulose nitrate filters, 0.45 μm pore size were obtained from Sartorius filters Inc., Hayward, CA.

Preparation of basolateral membrane vesicles (BLMVs). Jejunal basolateral membrane vesicles were prepared using a modified centrifugation technique followed by separation on a Percoll gradient [8,9]. Two adolescent rat jejunums were used for each preparation. The intestinal segments were removed, flushed with ice-cold Ringer's lactate solution (Buffer I) and then filled with buffer solution (37°C) containing 1.5 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM trisodium citrate and 2 mM dithiothreitol (pH 7.4) (buffer II). The segments were then clamped and incubated for 15 minutes in a shaking water bath at 37°C. The clamps were then removed and the contents were emptied. The segments were then filled with ice-cold buffer containing 100 mM mannitol, 100 mM KCl, 24 mM Hepes-Tris buffer pH 7.4 (Buffer III) and gently palpated by fingers for 5 min to release epithelial cells. The contents were then drained into a beaker on ice and the volume was brought up to 250 ml in Buffer III. The cells were then centrifuged at $200 \times g$ for 5 min and the cell pellet was homogenized in 250 ml Buffer III in an Omni mixer for 3 min. The homogenate was then brought up to 300 ml with Buffer III and centrifuged at $2500 \times g$ for 20 min. The supernatant was then collected and centrifuged at $22000 \times g$ for 25 min. The supernatant was discarded and the resulting fluffy layer of the pellet was resuspended in 90 ml of Buffer III and homogenized in a glass Teflon homogenizer (20 strokes). The resultant homogenate was mixed with Percoll (Pharmacia) at a concentration of 12.5 percent and then centrifuged at $48000 \times g$ for 45 min. A distinct band of basolateral membranes was seen at the upper one third of the Percoll gradient. The band was aspirated by a needle and the pellet resuspended in the appropriate transport buffer. The resuspended pellet was then centrifuged at $48000 \times g$ for 20 minutes and finally suspended in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4).

Transport measurements. Uptake of substrates (D-glucose, calcium and glutamine) was measured by a rapid filtration technique [9,10]. All experiments were performed at 25°C. Transport was initiated by adding 20 μl of the final vesicle suspension to the desired incubation media containing labeled substrate. The composition of the incubation media for each individual experiment is described in the table legends of the Results section. At the desired time intervals, the reaction was stopped by the addition of ice-cold 'stop solution' consisting of 100 mM mannitol, 100 mM sodium chloride, 20 mM Hepes-Tris buffer (pH 7.4). The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size, Sartorius Filters, Inc., Hayward, CA) and kept under suction while being washed with ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA) using Bray's solution (New England Nuclear Corp., Boston, MA) as a liquid scintillant. Radioactivity remaining in the filters after pipetting incubation medium into the radioactive substrate in the absence of vesicles was used as background.

Statistical evaluation. The unpaired Student's *t*-test and ANOVA was used to evaluate mean values between conditions studied. A P value less than 0.05 was considered statistically significant.

Results

Glucose and calcium uptake by basolateral membranes

D-Glucose uptake showed no 'overshoot' phenomena or sodium dependency indicating no contamination with brush-border membranes. Calcium uptake in the presence of ATP showed 'overshoot' phenomena compared to uptake in the absence of ATP, suggesting the functional integrity of these membranes [9,11,12].

Glutamine uptake versus binding

To determine whether glutamine uptake represents transport into the intravesicular space or mere binding, two studies were conducted. First, an osmolality study was done in which the incubation media osmolality was varied and glutamine uptake was determined at 10 minutes. As seen in Fig. 1, glutamine uptake was into osmotically sensitive space as depicted by the formula $y = 0.046x - 0.004$, correlation coefficient = 0.93. Second, glutamine uptake was determined at 25°C and

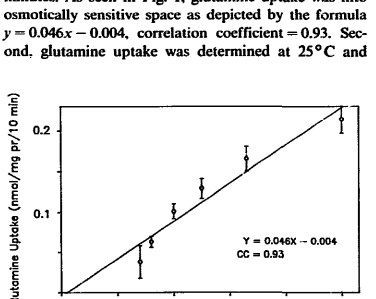

Fig. 1. BLM vesicles were prepared in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4). Reaction was started by the addition of 20 μl membrane vesicle to a different media containing 100 mM NaCl and increasing amount of mannitol to achieve osmolalities between 200–500 mosM, and 0.02 mM glutamine and tracer [^{3}H]glutamine. The reaction was stopped at 10 min. Values are means ± S.E. from three separate experiments.

TABLE I

Effect of temperature on glutamine uptake

BLM vesicles were prepared in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4). The reaction was started by the addition of 20 μl membrane vesicles to a media containing 100 mM NaCl, 100 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4), 0.02 mM glutamine and [^{3}H]glutamine. Transport studies were performed at 0°C and at 25°C. The reaction was stopped at 5, 30 and 60 s. Values are means ± S.E. from three separate experiments.

Time (s)	Glutamine uptake (nmol/mg protein)	
	0°C	25°C
5	0.0067 ± 0.0030	0.0210 ± 0.0022 *
30	0.0098 ± 0.0067	0.0400 ± 0.0067 *
60	0.0170 ± 0.0069	0.0430 ± 0.0130 *

* Mean values at 25°C are significantly different from the corresponding mean values at 0°C ($P < 0.001$).

0°C. Table I depicts glutamine uptake at 5, 30 and 60 s. As seen, glutamine uptake was significantly decreased at 0°C compared to 25°C, indicating temperature dependency which is a property of carrier-mediated processes [13].

In an effort to determine the degree of glutamine metabolized by basolateral membrane vesicles, we incubated basolateral membranes with 0.5 and 2.5 mM glutamine and determined the concentration of glutamine and glutamate after 20 min by previously validated high-pressure liquid chromatography method [14]. Ninety-five (95%) percent of the glutamine remained intact suggesting minimal metabolism to glutamate. Almost all of the metabolized glutamine was recovered as glutamate and ammonia.

Effect of Na^+ and K^+ gradients on glutamine uptake

Glutamine uptake was determined under inwardly directed sodium and potassium gradients. Glutamine uptake was stimulated by sodium gradient with distinct 'overshoot' phenomena compared to potassium gradients. Glutamine uptake appears linear up to 30 s. Equilibrium values were reached at 10 min (Fig. 2). Vesicles size was 1.4 μl/mg protein. These results indicate the Na^+ dependency of glutamine transport across the basolateral membrane.

Kinetics of glutamine uptake

Glutamine uptake was determined under initial rate conditions at glutamine concentrations between 0.01 and 0.6 mM under both sodium and potassium gradients. A saturable process was observed for both sodium-dependent and sodium-independent processes (Fig. 3). Kinetic parameters were calculated by a computerized model of Michaelis-Menten kinetics. K_m and V_{max} values for the sodium-dependent process were 0.2 ± 0.01 mM and 0.39 ± 0.008 nmol/mg protein per 15 s. Whereas the kinetic parameters for the sodium-independent process were 0.55 ± 0.01 mM and 0.34 ± 0.05 nmol/mg protein per 15 s for K_m and V_{max}, respectively.

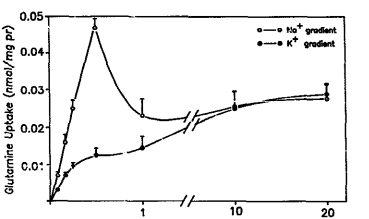

Fig. 2. BLM vesicles were prepared in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4). Reaction was started by the addition of 20 μl membrane vesicles to a media containing in final concentration, 100 mM NaCl, 100 mannitol, 20 mM Hepes-Tris buffer (pH 7.4), 0.02 mM glutamine and tracer [^{3}H]glutamine or 100 mM KCl, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4), 0.02 mM glutamine and tracer [^{3}H]glutamine. Reaction was stopped at 5, 10, 20, 30 s and 1, 10 and 20 min. Values are means ± S.E. from three separate experiments.

pH optimum of glutamine uptake

Glutamine uptake was determined under different pH conditions of the incubation media. Maximal uptake occurred at pH 7.5 while severe inhibition occurred at lower pH condition (Table II).

Effect of membrane potential on glutamine uptake

The effect of an imposed electrochemical membrane potential was studied using two methods: (1)

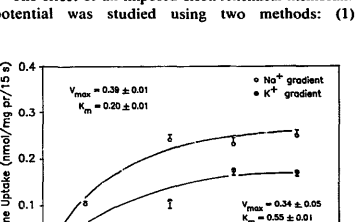

Fig. 3. BLM vesicles were prepared in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4). Reaction was started by the addition of 20 μl vesicles to a media containing either 100 mM NaCl, 100 mM mannitol and 20 mM Hepes-Tris or 100 mM KCl, 100 mM mannitol and 20 mM Hepes-Tris. Glutamine concentration in each incubation medium ranged between 0.01 and 0.6 mM and tracer [^{3}H]glutamine. The reaction was stopped at 15 s. K_m and V_{max} were derived from a computerized model of the Michaelis-Menten kinetics.

TABLE II

Effect of pH on glutamine uptake

BLM vesicles were prepared in 300 mM mannitol and 20 mM Hepes-Tris buffer (pH 7.4). The reaction was started by the addition of 20 μl vesicles to a media containing 100 mM NaCl, 100 mM mannitol and varying amounts of Tris-Mes to achieve pH values from 5.5 to 8.0, 0.02 mM glutamine and ^{3}H glutamine. The reaction was stopped at 15 s. Values are means ± S.E. from three separate experiments.

pH	Glutamine uptake (nmol/mg protein per 15 s)
6.0	0.0031 ± 0.0005
6.5	0.0091 ± 0.0008
7.0	0.0110 ± 0.0016
7.5	0.0280 ± 0.0052 *
8.0	0.0150 ± 0.0046

* Mean values at pH 7.5 are significantly different compared to the corresponding mean values at other pH conditions ($P < 0.05$–0.001).

valinomycin induced K^+ diffusion potential, and (2) anion substitution. In the first experiment, the membrane potential was induced by pre-incubating the vesicles with 50 mM KCl, 200 mM mannitol and 20 mM Hepes-Tris (pH 7.4) and the reaction was started by addition of the vesicles to media containing 50 mM NaCl, 200 mM mannitol and 20 mM Hepes-Tris (pH 7.4) and valinomycin 10 μg/mg protein. Uptake values were compared to those obtained under voltage clamp conditions (i.e., incubation of 200 mM mannitol and 50 mM KCl preloaded vesicles in 50 mM KCl 50 mM NaCl, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4) and valinomycin) ($[K_o^+] = [K_i^+]$). Because valinomycin mediates the electrogenic movement of K^+ down its concentration gradient, a negative membrane potential is generated [11]. Table III depicts that at 10 and 30 s,

TABLE III

Effect of membrane potential on glutamine uptake

BLM vesicles were prepared in 50 mM KCl, 200 mM mannitol and 20 mM Hepes-Tris (pH 7.4). The reaction was started by the addition of 20 μl vesicles to a media containing either 200 mM mannitol, 50 mM NaCl, 20 mM Hepes-Tris (pH 7.4) and valinomycin 10 μg/mg protein or 50 mM KCl, 50 mM NaCl, 100 mM mannitol, 20 mM Hepes-Tris (pH 7.4) and valinomycin 10 μg/mg protein (voltage clamp condition). The reaction was stopped at 10 s, 15 s and 30 s. Values are means ± S.E. from three separate experiments.

Time (s)	Glutamine uptake (nmol/mg protein)	
	Voltage clamp	negative membrane potential
10	0.0033 ± 0.0010	0.0170 ± 0.0058 *
15	0.0052 ± 0.0006	0.0210 ± 0.0070 *
30	0.0077 ± 0.0036	0.0270 ± 0.0055 *

* Mean values with negative membrane potential are significantly different compared to the corresponding mean values under voltage clamp conditions ($P < 0.001$).

TABLE IV

Effect of 4 mM neutral amino acids on labelled glutamine uptake

	Glutamine uptake (nmol/mg protein per 15 s)	
	Na^+-dependent	Na^+-independent
Control	0.07 ± 0.01	0.013 ± 0.001
Unlabelled glutamine	0.01 ± 0.001 *	0.009 ± 0.001 *
L-Serine	0.02 ± 0.001 *	0.009 ± 0.002 *
L-Asparagine	0.019 ± 0.002 *	0.009 ± 0.002 *
Glycine	0.019 ± 0.002 *	0.009 ± 0.001 *
Methionine	0.022 ± 0.001 *	0.01 ± 0.001 *
Leucine	0.014 ± 0.001 *	0.01 ± 0.001 *
Valine	0.0123 ± 0.001 *	0.011 ± 0.001 *

* $P < 0.05$ (Control for Na^+-dependent and Na^+-independent uptake compared to neutral amino acids). Addition of 40 mM concentration of amino acids resulted in a similar inhibition.

there was significant differences with negative membrane potential, indicating an electrogenic process of uptake. Values at equilibrium were similar. To further resolve the question of membrane potential, the effect of sodium salts with different anion permeabilities was tested on glutamine uptake. Sodium thiocyanate represents highly permeable anion compared to sodium sulphate and sodium chloride permeability is intermediate. Because sodium thiocyanate enters the vesicles faster, a negative membrane potential is generated earlier than with sodium sulphate. Glutamine uptake at 10 s was highest with the NaSCN followed by NaCl and Na_2SO_4 (0.02 ± 0.001, 0.015 ± 0.002 and 0.01 ± 0.001 nmol/mg protein, respectively) suggesting that Na^+-dependent glutamine uptake is electrogenic. Similar experiments of glutamine uptake were done with KSCN, KCl and potassium gluconate. No significant differences were observed, suggesting that K^+-dependent glutamine uptake is electroneutral (0.01 ± 0.001, 0.009 ± 0.001 and 0.01 ± 0.001 nmol/mg protein per 10 s, respectively).

Effect of neutral amino acids on glutamine uptake

Table IV depicts the effect of neutral amino acids on the sodium-dependent and sodium-independent glutamine uptake by basolateral membranes. As seen, unlabelled glutamine and other neutral amino acids inhibited significantly labeled glutamine uptake. The inhibition, however, was more severe with the Na^+-dependent process.

Discussion

Glutamine is consumed by replicating cells such as fibroblasts [15], lymphocytes [16], tumor cells [17] and intestinal epithelial cells [4–7]. These cells have low intracellular glutamine and high glutamine activity. The basolateral membrane of the enterocyte provides an

important pathway for glutamine transport from the arterial side. This pathway becomes essential during fasting state and catabolic states when glutamine is released from the skeletal muscles [18,19].

Our study characterizes for the first time, the details of the process of glutamine transport by basolateral membranes of the enterocyte of rats. We used a well validated technique for isolation of purified basolateral membranes [9,11,12]. Previous studies in our laboratory have demonstrated the biochemical and functional characteristics of these vesicles. Na^+/K^+-ATPase, a marker for basolateral membranes, was enriched 10–12-fold while markers for brush border and subcellular organelles were impoverished. D-glucose uptake showed no 'overshoot' phenomena in the presence of Na^+ gradient indicating no contamination with brush border membranes whereas Ca^{2+} uptake was stimulated by ATP indicating the presence of an active calcium pump which is known to be located at the basolateral membranes [9,11,12].

Glutamine uptake by basolateral membranes was into osmotically sensitive spaces as evident by osmolality study. Furthermore, the temperature dependency of the initial uptake suggests a carrier-mediated process [13]. Glutamine uptake was driven by the inwardly directed sodium gradient. This suggests that a secondary active process is involved i.e., glutamine is driven by the Na^+ gradient across the membrane. Similar mechanisms for glutamine uptake for liver plasma membranes [20] and renal brush-border membranes [21,22] have been reported. This process is pH dependent with marked decrease in glutamine uptake at lower pH. This pathway is shared by other neutral amino acids. These characteristics best fit system (A) as described by Christensen [23]. Furthermore, this system exhibits saturation kinetics with K_m of 0.2 mM which can function below physiological levels of plasma concentrations of glutamine (0.4–0.6 mM). The Na^+-dependent glutamine transport is electrogenic as evident by valinomycin and anion substitution studies.

The second process which appears to be operative at the basolateral membrane is the Na^+-independent pathway. This process is electroneutral and shared by other neutral amino acids. The kinetic parameters for this system exhibit a lower affinity (K_m 0.55 mM) compared to the Na^+-dependent process (K_m 0.2 mM) However, the V_{max} is similar. This system best fits the (L) system as described by Christensen [23]. In support of these findings, Bradford and McGivan demonstrated the presence of system A and L like systems in freshly isolated enterocytes. Moreover, these authors suggested the presence of an exchange system between extracellular glutamine and intracellular alanine [24].

Therefore, we believe that system A and L are operative at the basolateral membrane of the rat. Both systems exhibit saturation kinetics with different affinities to glutamine.

Acknowledgements

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