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Sequential ordered mechanism for the sodium-glutamate transport in intestinal brush border membrane vesicles

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Abstract

The glutamate/aspartate carrier localized in the brush-border membrane vesicles from enterocytes is known as a transport system catalyzing a sodium-substrate cotransport driven by the sodium gradient across the membrane. The kinetics of this transport system is studied by analogy with an enzymatic bi-substrate reaction. The results of this approach can be summarized as follows: (1) The dependence of the L-glutamate transport rate on the sodium concentration is sigmoidal, and the stoichiometry of the transport is 2 Na^+/1 glutamate/1 carrier molecule. (2) The mechanism is sequential ordered, with L-glutamate binding after both the sodium cations. In addition, there is a very high degree of cooperativity between the two sodium binding sites.

Keywords: Glutamate; Transport; Sodium ion cotransport; Kinetics; Brush-border membrane vesicle; (Rat intestine)

1. Introduction

A transport system specific for the amino acids L-glutamate and L-aspartate has been described in enterocytes and, later on, in brush-border membrane vesicles [1]. It is Na^+-dependent and apparently operates a cotransport Na^+-substrate, similarly to a number of systems identified in the apical membrane of intestine, kidney and other epithelial cells. A partial characterization of the transport system in rat enterocyte brush-border membrane vesicles indicates activation effects by K^+, when present inside the vesicles and by Cl^-, when present outside [2]. At present, a more precise characterization of the system is not available. In this paper we have used a kinetic approach to get an insight into the mechanism of the transport of L-glutamate across the vesicle membrane of rat enterocyte brush border, operated by the glutamate/aspartate carrier. In particular, we have tried to understand the role of the sodium ion in the transport process and its stoichiometry with respect to the substrate. In order to correctly interpret the experimental kinetic data, we have adapted the classical methods of the enzyme kinetics to the carrier-mediated transmembrane transport process, taking into account the differences between the latter and a true enzyme reaction.

2. Materials and methods

2.1. Chemicals

L-[^{3}H]Glutamate was obtained from Amersham Italy; all chemicals used were of analytical grade purity.

2.2. Preparation of membrane vesicles

Sprague–Dawley rats were killed by decapitation. The intestines were immediately removed and placed in ice-cold NaCl 0.9% solution. After washed twice with the same solution, they were everted and scraped. Brush-border membrane vesicles (BBMV) were prepared within 3 h after death of the animal. Five or six intestines were used for the BBMV preparation. The scraped mucosa was resuspended in 60 ml of ice-cold buffer containing 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5), then diluted 1:5 (v/v) with distilled water and homogenized with a mixer for 2 min at the maximum speed. Subsequently the BBMV were prepared according to the Mg^{2+}/EGTA method

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described by Hauser et al. [3], using a Sorvall RC 5B centrifuge equipped with an SS 34 rotor.

2.3. Enzyme assays

The purity of the BBMV preparation was monitored by measuring the specific activity of alkaline phosphatase (EC 3.1.3.1) as described earlier [4]. The specific activity of alkaline phosphatase measured in the homogenate was 250 ± 70 nmoles of substrate consumed per min per mg protein, measured at 37°C, while in the final BBMV pellet the specific activity was 10- to 12-times more than that measured in the homogenate.

2.4. Protein determination

Protein content was determined by the Bradford method [5] using the Bio-Rad kit (Bio-Rad, Richmond, CA, USA) and γ-globulin as a standard. The protein concentration of the vesicle preparation was measured before the uptake was started, and adjusted so that 90 μg of vesicle membranes per experimental point were used.

2.5. Uptake assays

Uptake assays were carried out using the rapid filtration technique as described elsewhere [6,7]. Briefly, 6 μl of membrane vesicle suspension (equivalent to 90 μg of membrane protein) were added to 24 μl of incubation medium. At the time indicated 1 ml of ice-cold isotonic stop solution of the following composition: 150 or 250 mM NaCl, 20 mM Hepes-Tris (pH 7.5), was added to the incubation test tube, mixed on a vortex mixer and pipetted onto a Millipore filter (45 μm pore size). Thereafter 5 ml of stop solution were pipetted onto the filter to wash the aspecific radioactivity bound. The radioactivity trapped on the filters was measured by standard liquid scintillation techniques. Membrane free incubation media, for each L-[^{3}H]glutamate concentration, were used as blanks: the counts of the samples were at least 4-times higher than those of the blanks. Each experiment was performed at least four times; only the results of a typical experiment are shown. Experiments were always performed in triplicate. Experimental scatter of triplicates was always less than 10% of the mean values. Uptake rates are generally expressed as pmol/mg protein per min. In all experiments the incubation time was 7 s, since in preliminarily performed control assays the L-glutamate uptake (measured both in the presence and in the absence of NaCl) was linear up to 15 s of incubation throughout the L-glutamate concentrations used. The kinetics of the uptake were analyzed by a non-linear regression procedure with the aid of a personal computer.

3. Results

3.1. Sodium L-glutamate cotransport

The glutamate/aspartate carrier located in the rat enterocyte BBMV has been defined as sodium dependent transport system involving Na^+-substrate cotransport [1]. As shown in Fig. 1, the initial rate of the L-[^{3}H]glutamate uptake by BBMV is higher in the presence of an inwardly directed gradient of Na^+, is lower when the sodium gradient is abolished. A still lower, but significant, transport activity remains even in the total absence of sodium, here referred to as sodium-independent transport. The dependence of the glutamate transport rate on the glutamate concentration has been studied in the concentration range 0.005–10 mM at constant external 100 mM [Na^+]. In the micromolar range, up to 0.1 mM glutamate, a linear correlation has been observed (not shown). At glutamate concentrations in the range 0.1–10 mM, as shown in Fig. 1A, a non-saturative curve is obtained. On the other hand, the sodium-independent transport rate appears to vary linearly, and the slope of the straight line is in the range of the permeability coefficients of transmembrane diffusion processes. The sodium-independent transport has therefore been subtracted from the respective one measured in the presence of sodium. The net sodium dependent uptake rates so obtained fit to a hyperbolic saturation curve. Fig. 1B shows an Eadie-Hofstee plot of the net sodium dependent uptake rate, from which a K_m of 7.3 mM for L-glutamate is calculated; other similar experiments gave K_m values ranging from 6 to 9 mM.

We have also measured the glutamate transport rate as a function of the Na^+ concentration, at constant glutamate concentration (Fig. 2A). In this case the dependence appears to be sigmoidal. The experimental data have been

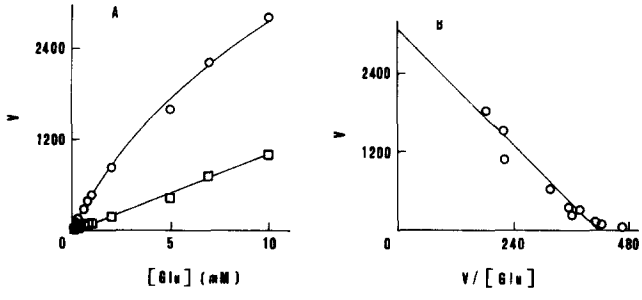


Fig. 1. Substrate concentration dependence of L-glutamate transport. (A) Membrane vesicles were suspended in 100 mM mannitol, 200 mM *N*-methylglucamine gluconate, 20 mM Hepes-Tris (pH 7.5). The uptake at 7 s was started by adding the membrane vesicles to media containing (final concentrations), 100 mM *N*-methylglucamine gluconate, 20 mM Hepes-Tris (pH 7.5), L-[^{3}H]glutamate, at the concentrations indicated, and 100 mM Na gluconate (○) or 100 mM *N*-methylglucamine gluconate (□). (B) Eadie–Hofstee plot of the data corrected for the sodium-independent component. Transport rate *V* is expressed as pmol/mg protein per min.

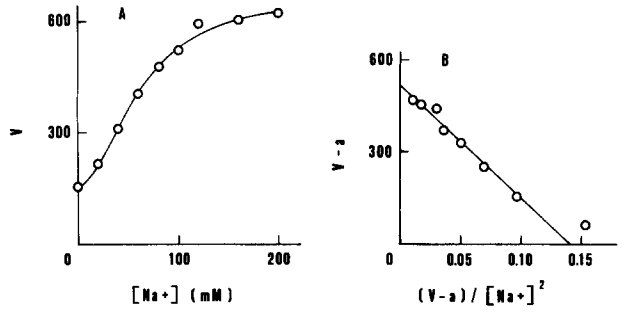


Fig. 2. Sodium concentration dependence of L-glutamate transport. (A) Membrane vesicles were suspended in 100 mM mannitol, 200 mM *N*-methylglucamine gluconate, 20 mM Hepes-Tris (pH 7.5). The uptake at 7 s was started by adding the membrane vesicles to media containing (final concentrations) 20 mM Hepes-Tris (pH 7.5), 2 Mm L-[^{3}H]glutamate, and Na gluconate at the concentrations indicated. At concentrations lower than 200 mM sodium, Na gluconate was isotonically replaced by *N*-methylglucamine gluconate. (B) Eadie–Hofstee type plot of the data corrected for the sodium-independent component. Transport rate V is expressed as pmol/mg protein per min.

analyzed, by a computer aided non-linear regression procedure, by fitting them in the general equation:

$$V = \left[V_m[S]^n/\left(K_{0.5}^n + [S]^n\right)\right] + a \qquad (1)$$

according to Hill [8,9]. In such equation n is the Hill number and a is the uptake rate experimentally determined at zero sodium concentration (156 ± 13 pmol/mg protein per min, mean value ± S.D.). The best fitting of the data provides the following parameters: $n = 2.02 \pm 0.22$, $K_{0.5} = 61 \pm 4$ mM, $V_m = 523 \pm 24$ pmol/mg protein per min. The corresponding curve is overimposed in Fig. 2A. It can be seen that the experimental points fit well to the theoretical curve. Fig. 2B shows an Eadie-Hofstee type plot, obtained by plotting the net sodium dependent uptake rate $(V-a)$ vs. $(V-a)/[Na^+]^n$. In this plot the correct value of n will yield a straight line with slope $K_{0.5}^n$. As can be seen in Fig. 2B such condition is satisfied for $n = 2$. The mean value of n as calculated from five independent experiments is 2.26 ± 0.40. We can therefore conclude that the Hill coefficient is close to 2, indicating that the number of sites for Na^+ on the carrier molecule is at least 2.

We have anyway to consider that the significance of the parameters obtained by experiments such as those in Figs. 1 and 2 is limited by the non-saturating concentration of the second substrate. In fact, we can look at transmembrane cotransport processes as enzymatic two-substrate reactions. In order to evaluate the true kinetic parameters and get more information about the transport mechanism, we have performed transport experiments where the concentrations of both substrates were changed. In Fig. 3 the results of a an experiment of this type are shown as double-reciprocal plots. Glutamate transport rate is reported as a function of the L-glutamate concentration at four fixed sodium concentrations in A and, vice versa, as a function of $[Na^+]^2$ at four L-glutamate concentrations in B. It is evident that in A the straight lines obtained at different sodium concentrations meet at a point on the axis of ordinates; in B, instead, straight lines are also obtained at each L-glutamate concentration, but they meet at a point on the left of the axis of ordinates. Therefore, the saturation rate with respect to L-glutamate is independent on the Na^+ concentration, while the saturation rate with respect to sodium varies with the L-glutamate concentration. According to the classical enzyme kinetics this pattern indicates a sequential ordered mechanism, with L-glutamate binding after sodium. On this base the equation that fits to the experimental data of Fig. 3 is:

$$V = V_m \frac{[Na^+]^2[Glu]}{2K_{Na}K_{Glu} + K_{Glu}[Na^+]^2 + [Na^+]^2[Glu]} \qquad (2)$$

This equation can assume the forms:

$$V_{Glu} = V_m \frac{[Glu]}{K_{Glu}\left(1 + 2K_{Na}/[Na^+]^2\right) + [Glu]} \qquad (3)$$

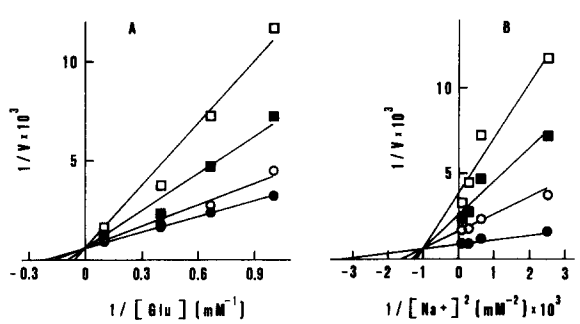


Fig. 3. Bi-substrate dependence of the L-[^{3}H]glutamate transport rate. Membrane vesicles were suspended in 100 mM mannitol, 200 mM *N*-methylglucamine gluconate, 20 mM Hepes-Tris (pH 7.5). The uptake at 7 s was started by adding the membrane vesicles to five series of media containing 0, 20, 40, 60 and 120 mM Na gluconate, respectively, and *N*-methylglucamine gluconate enough to reach 200 mM total salt concentration, as well 20 mM Hepes-Tris (pH 7.5) and L-[^{3}H]glutamate. In each series L-glutamate concentration varied as follows: 1, 1.5, 2.5, 10 mM (final concentrations). (A) Double-reciprocal plot of the L-glutamate transport rate versus L-glutamate concentration at 20 (□), 40 (■), 60 (○), 120 (●) mM sodium. (B) Double-reciprocal plot of the L-glutamate transport rate versus $[Na^+]^2$ at 1 (□), 1.5 (■), 2.5 (○), 10 (●) mM L-glutamate. The data shown have been corrected for the sodium-independent component. Transport rate V is expressed as pmol/mg protein per min.

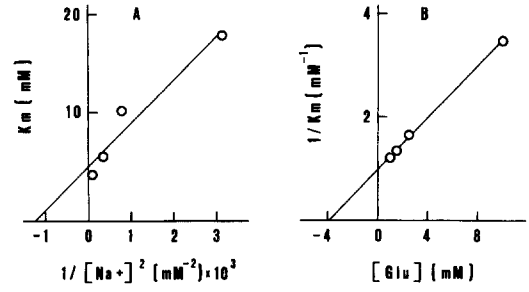


Fig. 4. Replot of data of Fig. 3. (A) Plot of the reciprocal of the x-axis intercepts of Fig. 3A vs. $1/[Na^+]^2$. (B) Plot of the x-axis intercepts of Fig. 3B vs. [Glu].

and

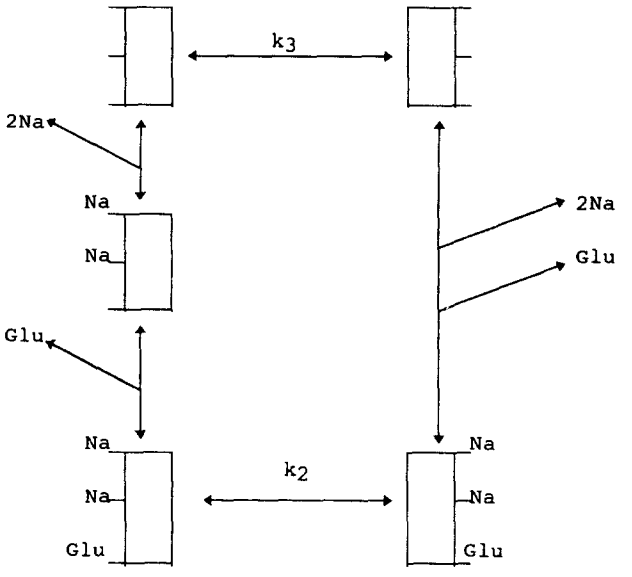
$$V_{Na} = \frac{V_m}{1 + \frac{K_{Glu}}{[Glu]}} \cdot \frac{[Na^+]^2}{\frac{2K_{Na}}{(1 + [Glu]/K_{Glu})} + [Na^+]^2} \quad (4)$$

that refer to Fig. 3A and Fig. 3B, respectively.

The intercepts with the abscissa of Fig. 3A and Fig. 3B have been then replotted to extrapolate the true K_m for the substrates (Fig. 4). From the experiment reported we obtain $K_{Glu} = 4.5$ mM and $K_{0.5}$ for Na = 21 mM, extrapolated from Fig. 4A and Fig. 4B, respectively.

4. Discussion

High-affinity systems for glutamate are commonly reported in various tissues [10–13], with K_m values in the micromolar range. Our study provides strong evidence for the existence, in rat intestinal brush-border membrane, of a low-affinity Na^+-dependent transport system. This system is similar to that described in chicken intestine [14], although in our case a high-affinity transport system could not be detected. The data presented provide clear indications about the stoichiometry and the kinetic mechanism of the L-glutamate transport process in enterocyte BBMV. A first indication is that the carrier molecule binds two sodium ions per L-glutamate molecule. This conclusion is based on the experiment of Fig. 2, which gives a Hill number of 2. Such result actually indicates that there are two or more cooperative binding sites for Na^+ on the carrier molecule. If the sodium binding sites were more than two, it would mean that some carrier molecules would bind one Na^+, some other two, some other three and so on: on an average, there would be two sodium ions bound per carrier molecule. This possibility can anyway be excluded, because in this case the velocity equation would be more complex than Eq. 2, and would result in lines not meeting at a single intersection point as we see in Fig. 4. On the other hand, if the binding sites for sodium are two, they exert the highest degree of cooperativity; i.e., the number of carrier molecules binding only one Na^+ are negligible with respect to those with both sodium sites occupied at the stationary state. The pattern of Fig. 3, with the V_m in A independent on Na^+ concentration and the V_m in B increasing with the L-glutamate concentration, strongly indicates a sequential binding mechanism with the order: Na^+ (low-affinity site), Na^+ (high-affinity site), L-glutamate. It has to be noted that different binding sequences, or any random mechanism, would lead to different patterns of the double-reciprocal plots. Furthermore, the simple velocity Eqs. 3 and 4, corresponding to straight lines convergent to a single point, also imply the condition of rapid equilibrium of the fast steps of the process. On the base of these indications the following scheme of kinetic mechanism is proposed:



The two translocation steps that imply a protein rearrangement inside the membrane are considered rate determining, while the carrier–substrate interactions are much faster and therefore considered at the equilibrium in the stationary state. According to the Haldane procedure [9], a rate equation can be obtained:

$$V = \frac{k_2[C_T]}{1 + k_2/k_3} \cdot \frac{Na^+]^2[Glu]}{\frac{2K_{Na}K_{Glu}}{1 + k_2/k_3} + \frac{K_{Glu}[Na^+]^2}{1 + k_2/k_3} + [Na^+]^2[Glu]} \quad (5)$$

where $[C_T]$ is the total amount of carrier, k_2 and k_3 are the kinetic constants of the rate determining steps, K_{Glu} is the true affinity constant of the Na-carrier complex for L-glutamate, K_{Na} is the product of the two affinity constants of the carrier for Na^+. It is easy to see that Eq. 5 is a more complete form of Eq. 2 and that the two equations would become identical if $k_2 \ll k_3$ (i.e., if the translocation step of the substrate-carrier complex were the only rate determining one). Therefore the experimental affinity constants calculated from Fig. 4 are not the true ones, unless $k_2 \ll k_3$. An evaluation of the k_2/k_3 ratio has still not been achieved, but a previously reported trans-stimulation effect of L-glutamate on the substrate uptake [1] seems to indicate that the latter is not likely.

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