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Biochimica et Biophysica Acta 1370 (1998) 252–258

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Mechanism of the inhibitory effect of imipramine on the Na^+-dependent transport of L-glutamic acid in rat intestinal brush-border membrane

Mitsuru Sugawara, Masaya Kato, Michiya Kobayashi, Ken Iseki, Katsumi Miyazaki *

Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Sapporo 060, Japan

Received 14 October 1997; accepted 18 November 1997

Abstract

The mechanism of the inhibitory effect of imipramine, a lipophilic organic cation on the Na^+-dependent transport of L-glutamic acid across intestinal brush-border membrane was investigated. The uptake of L-glutamic acid by intestinal brush-border membrane vesicles was dependent on the concentration of Na^+. Fitting of the uptake data in the presence of various concentrations of Na^+ using Hill equation yielded a Hill coefficient of 2.18. This result suggest that the carrier system of L-glutamic acid has at least two sites for Na^+-binding. By the analysis of double reciprocal plot and Dixon-type plot, it was found that imipramine inhibits the transport of L-glutamic acid by interacting competitively with the binding sites of Na^+, but not inhibit L-glutamic acid binding site. Moreover, the effect of imipramine on the transport of L-alanine and D-glucose which are co-transported with only one Na^+ molecule was also suggestive of interaction with the Na^+-binding sites on the carrier. These results indicate that the mechanism of the inhibitory effect of imipramine on the Na^+-dependent carrier systems is common for all systems regardless of the stoichiometry or substrates. © 1998 Elsevier Science B.V.

Keywords: Na^+-dependent transport; L-glutamic acid; Lipophilic cation; Imipramine; Brush-border membrane; Intestine; (Rat)

1. Introduction

Na^+-dependent carrier-mediated transport systems exist widely in various organs, and play an important role in the disposition of nutrients and sometimes drugs. It was reported that these Na^+-dependent carrier-mediated transport systems can be inhibited by various lipophilic organic cations [1–4]. A typical compound is harmaline which inhibits the transport of sugar and neutral amino acid by interacting competitively with the Na^+-binding site on the carrier protein [1,3,4]. Imipramine, a tricyclic antidepressant, also inhibits the Na^+-dependent transporters of sugar and serotonin by a similar mechanism [1,2,5,6]. However, in the literature there are some discrepancies in interpreting the manner of inhibition with respect to the competition between substrate and these cationic

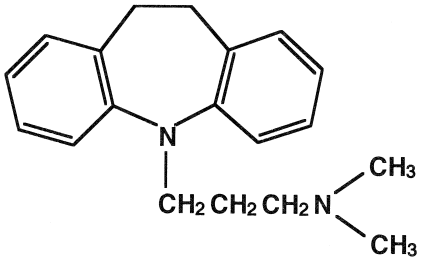

Fig. 1. Chemical formula of imipramine.

* Corresponding author. Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Sapporo 060, Japan. E-mail: katsumim@med.hokudai.ac.jp

PII S0005-2736(97)00270-8

inhibitors. Young et al. [4] reported that harmaline non-competitively inhibits the transport of alanine mediated by amino acid transport system ASC in the human erythrocytes. It was also reported that the uptake of serotonin by human and rat platelets [6] and rat enterocytes [2] can be inhibited by imipramine with a non-competitive manner. On the other hand, a competitive-type of inhibition was reported for the inhibitory effect of harmaline and imipramine on the uptake of methyl α-D-glucose by everted rings of rat small intestine [1] and for the effect of imipramine on the uptake of serotonin by human platelet. Furthermore, the analysis of inhibition manner of organic cation was carried out only for transporters with a transport stoichiometry of substrate and Na^+ of 1:1, and there is no information on co-transporters with more than two Na^+.

In the present study, we investigate the effect of imipramine (Fig. 1) on the uptake of L-glutamic acid, which is transported with two Na^+ molecule, by rat intestinal brush-border membrane vesicles in order to clarify the type of inhibitory effect with respect to the competition on the substrate binding site and the Na^+-binding sites. Moreover, we examine the effect of imipramine on the uptake of L-alanine and D-glucose to compare the type of inhibition of these transporters.

2. Materials and methods

2.1. Chemicals

L-[2,3-^{3}H]Alanine (2.00 TBq/mmol), D-[U-^{14}C]glucose (10.8 GBq/mmol), and L-[G-^{3}H]glutamic acid (2.07 TBq/mmol) were obtained from Amersham International. D-Glucose and L-glutamic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alanine and Imipramine hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan) and Sigma Chemical (St. Louis, MO, USA), respectively. All other chemicals were of the highest grade available.

2.2. Preparation of intestinal brush-border membrane vesicles

Male Wistar rats (200–250 g) were used for the isolation of membrane vesicles and all the procedures were carried out at 4°C or ice-cold conditions. The small intestinal brush-border membrane vesicles were prepared according to Mg^{2+}/EGTA precipitation methods [7,8] with some modifications. The scraped intestinal mucosa was homogenized with a Warring blender (Nihon Seiki, Japan) at 16 000 rpm for 4 min in an appropriate volume (40 ml/rat) of a homogenizing buffer composed of 50 mM D-Mannitol, 2.5 mM EGTA, and 2 mM Tris (pH 7.1 adjusted by addition of NaOH). Then, $MgCl_2$ was added to a final concentration of 10 mM. After 20 min, the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant was then centrifuged at $17\,000 \times g$ for 30 min. Then the pellet was suspended and homogenized by a Dounce-type homogenizer (10 strokes) in 40 ml of the same homogenizing buffer. $MgCl_2$ was added again to a final concentration of 10 mM and the first two steps of centrifugation were repeated once more. The resulting pellet was washed with an experimental buffer composed of 100 mM D-Mannitol, 100 mM KCl, and 20 mM Hepes/Tris (pH 7.5) by homogenizing in 40 ml of the experimental buffer using Dounce-type homogenizer and centrifugation at $27\,000 \times g$ for 30 min. The final pellet was resuspended in the experimental buffer using a Dounce-type homogenizer to give a final protein concentration of 10–15 mg/ml. Enrichment of the brush-border membrane fraction was 9–11-fold compared to the initial homogenate from assessment of the specific activity of the membrane enzyme marker, alkaline phosphatase.

2.3. Uptake experiments

The uptake of substrates into membrane vesicles was performed at 25°C (D-glucose) or 37°C (L-alanine and L-glutamic acid) by a rapid filtration technique according to our previous reports [9,10] with minor modification. Briefly, the reaction was initiated by mixing of 20 μl of membrane vesicle suspension with 100 μl of the transport buffer (20 mM Hepes/Tris (pH 7.5), 100 mM D-mannitol, and suitable concentrations of KCl and NaCl (indicated in figure legend)) containing 12 KBq/ml of labeled and suitable concentrations of non-labeled substrate (to adjust substrate concentration) with or without various concentrations of imipramine. Then at the stated time, the reaction was terminated by diluting the reaction mixture with 4 ml of the ice-cold stop buffer (150 mM NaCl, 20 mM Hepes/Tris, pH 7.5) fol-

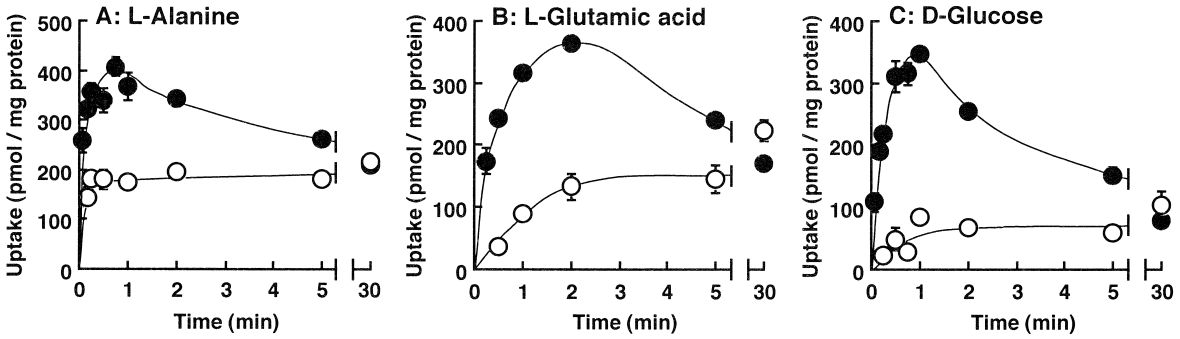

Fig. 2. Time-course of the uptake of L-alanine (A), L-glutamic acid (B) and D-glucose (C) in the presence (●) or absence (○) of inward Na^+ gradient. Membrane vesicles (20 μl) were incubated with 100 μl of a transport buffer containing 240 μM substrate and either 100 mM NaCl or 100 mM KCl. Each point represents the mean ± S.E.M. of 3–6 measurements.

lowed by filtration through a Millipore filter (HAWP, 0.45 μm, 2.5 cm diameter). The filter was then washed once with 4 ml of ice-cold stop buffer.

2.4. Analytical procedure

The determination of labeled substrates was carried out by liquid scintillation counting. Protein was measured by the method of Lowry et al. [11] with bovine serum albumin as the standard.

3. Results

3.1. Functional integrity of the intestinal brush-border membrane vesicles

Fig. 2 shows the time-course of the uptake of L-[^{3}H]alanine, D-[^{14}C]glucose, and L-[^{3}H]glutamic acid in the presence or absence of inward Na^+-gradient. The initial uptake rate of all substrates was stimulated and an overshoot phenomenon was observed in the presence of a Na^+-gradient. The transport activity was comparable to other reports about the uptake of these substrates [12–14]. These results reveal a functional integrity of the membrane preparations.

3.2. Effect of imipramine on the Na^+-dependent transport

Table 1 shows the effect of imipramine on the initial uptake of substrates in the presence or absence of a Na^+-gradient. Imipramine inhibited the uptake in the presence of a Na^+-gradient with a concentration dependent manner, whereas the uptake in the absence of Na^+-gradient was not affected. Moreover, the equilibrium (30 min) uptake in the presence of a Na^+-gradient was also not affected (not shown). These results suggest that the inhibitory effect of imipramine is exclusive for the Na^+-dependent carrier-mediated component. Therefore, in the subsequent kinetical analyses, we normalized the uptake values by subtracting the uptake in the absence of Na^+-gradient from the total uptake in the presence of Na^+-gradient in order to precisely evaluate the net effect induced by imipramine on the Na^+-dependent transport component.

3.3. Type of the inhibitory effect of imipramine on the transport of L-glutamic acid

Fig. 3 shows a double reciprocal (3A) and a Dixon-type (3B) plots of the inhibitory effect of imipramine on the transport of L-glutamic acid with respect to competition for L-glutamic acid binding site. The manner of inhibition is in agreement with a noncompetitive rather than competitive type with a K_I value of 0.81 (mM).

Then we examined the effect of imipramine on the transport of L-glutamic acid with respect to the inhibition of Na^+-binding. It has been reported that the stoichiometry of the Na^+-dependent transport of L-glutamic acid is 2:1 (Na^+:L-glutamic acid) [15,16]. We examined the dependency of the transport of L-glutamic acid on the Na^+ concentration at 200 μM L-glutamic acid, and analyzed it using the Hill equation:

$$v = \mathrm{Vm}[\mathrm{Na}^+]^n / \left(K_{\mathrm{Na}}^n + [\mathrm{Na}^+]^n\right) \quad (1)$$

Table 1
Effect of imipramine on the uptake of L-alanine, L-glutamic acid, and D-glucose in the presence or absence of a Na^+ gradient

Substrate (incubation time)	Imipramine concn. (mM)	Uptake (pmol/mg protein)	
		In the presence of Na^+ gradient	In the absence of Na^+ gradient
Alanine (15 s)	0	365.4 ± 31.8	186.9 ± 13.4
	0.25	N.D.	162.7 ± 13.3
	0.5	277.0 ± 16.9 *	165.9 ± 17.8
	1	231.1 ± 24.0 *	N.D.
	2	131.3 ± 11.0 *	N.D.
Glutamic acid (30 s)	0	230.1 ± 19.1	47.3 ± 10.5
	0.5	174.7 ± 27.3 *	45.4 ± 10.9
	1	154.9 ± 41.2 *	46.3 ± 9.9
	2	115.3 ± 43.1 *	46.4 ± 11.9
Glucose (15 s)	0	228.2 ± 28.1	63.9 ± 13.0
	0.125	N.D.	57.6 ± 17.8
	0.25	N.D.	67.9 ± 8.0
	0.5	134.2 ± 24.2 *	60.8 ± 13.4
	0.75	N.D.	44.1 ± 20.2
	1	95.8 ± 29.4 *	N.D.
	2	69.1 ± 19.9 *	N.D.

Membrane vesicles (20 μM) were incubated with 100 μM of a transport buffer containing 240 μM substrate, various concentrations of imipramine and either 100 mM NaCl or 100 mM KCl. Each value represents the mean ± S.D. of 3-17 measurements.
* $p < 0.001$ significantly different from the value without imipramine. N.D.: not determined.

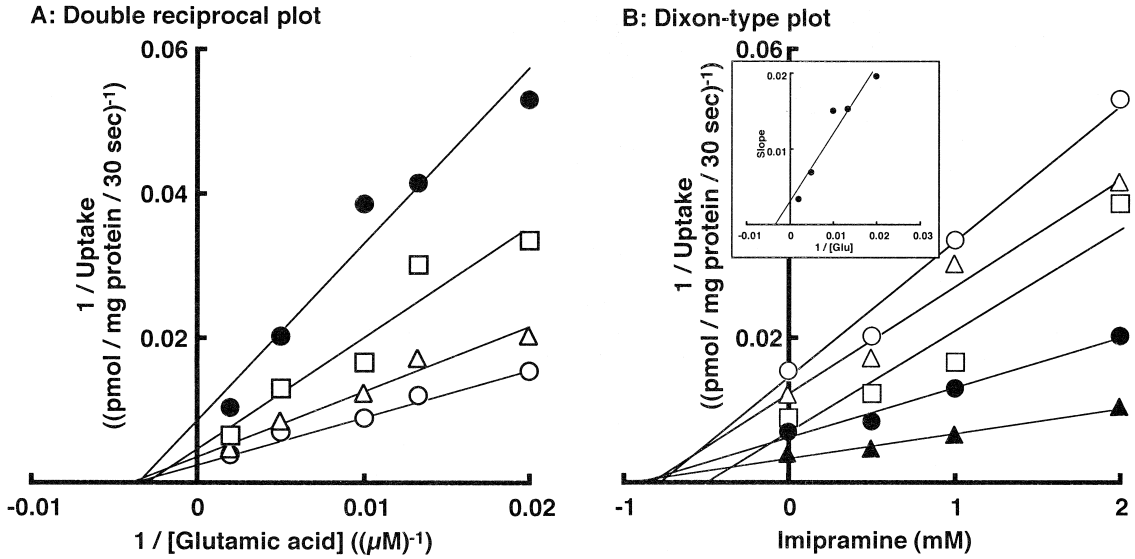

Fig. 3. Analysis of the inhibitory effect of imipramine on L-glutamic acid uptake with respect to the inhibition of L-glutamic acid binding to the carrier. Membrane vesicles (20 μl) were incubated with 100 μl of transport buffer containing 100 mM NaCl and various concentrations of L-glutamic acid and imipramine. Each point represents the mean value of 3–6 measurements. A; Final concentrations (mM) of imipramine were 0 (○), 0.5 (△), 1 (□) and 2 (●). B; Final concentrations (μM) of L-glutamic acid were 50 (○), 75 (△), 100 (□), 200 (●) and 500 (▲). (Inset: replot of the slopes of Dixon-type plot)

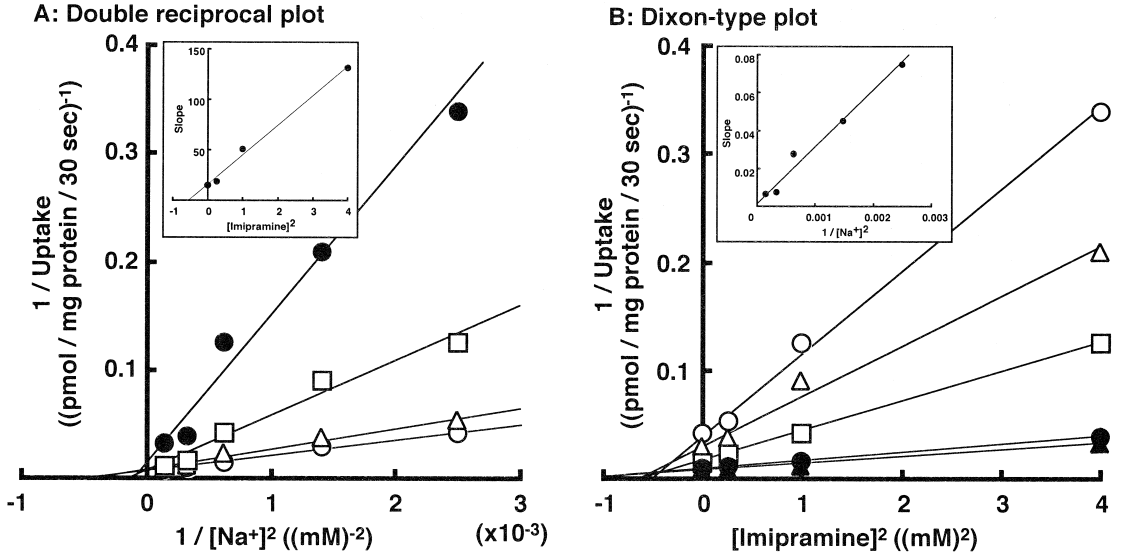

Fig. 4. Analysis of the inhibitory effect of imipramine on L-glutamic acid uptake with respect to the inhibition of Na^+ binding to the carrier. Membrane vesicles (20 μl) were incubated with 100 μl of a transport buffer containing 240 μM L-glutamic acid and various concentrations of Na^+ and imipramine. Each point represents the mean value of three measurements. A: Final concentrations (mM) of imipramine were 0 (○), 0.5 (△), 1 (□) and 2 (●). (Inset: replot of the slopes of the double reciprocal plot); B: Final concentrations (mM) of Na^+ were 20 (○), 27 (△), 40 (□), 55 (●) and 83 (▲). (Inset: replot of the slopes of Dixon-type plot)

where n, and K_{Na} are the Hill and affinity coefficients of the Na^+-binding to the carrier, respectively.

Fitting of the uptake data of L-glutamic acid in the presence of various concentrations of Na^+ using Eq. (1) yields the following values: $n = 2.18$, $K_{Na} = 50$ (mM), and $Vm = 184$ (pmol/ mg protein/30 s). Because the Hill coefficient is 2.18, it is suggested that this carrier system has at least two sites for Na^+-binding.

For the sake of simplicity, we referred to the report of Prezioso and Scalea [16] and analyzed the data depending on the following supposition: (1) L-glutamic acid binding to the carrier moiety is permissible only after both of the Na^+-binding sites are occupied; (2) Na^+ does not bind to the carrier moiety which is already occupied by imipramine; (3) the number of the carrier moieties that occupied only one Na^+-site by Na^+ or imipramine are negligible because of the cooperativity. Therefore, the transport kinetics of L-glutamic acid can be resembled by the following equation:

$$v = Vm[Glu][Na^+]^2 / \left\{ K_{Na1} \cdot K_{Na2} \cdot K_{Glu} + K_{Glu}[Na^+]^2 + [Glu][Na^+]^2 \right\} \quad (2)$$

Then, the equation of the relation between the transport rate and Na^+ concentration and imipramine concentration is:

$$v = Vm[Glu][Na^+]^2 / \left\{ K_{Na1} \cdot K_{Na2} \cdot K_{Glu} \left(1 + [I]^2 / K_{I1} \cdot K_{I2}\right) + K_{Glu}[Na^+]^2 + [Glu] \times [Na^+]^2 \right\} \quad (3)$$

where K_{Na1}, K_{Na2}, K_{Glu} are the affinity constants of the binding of Na^+ to the first and the second binding sites of carrier, and the affinity constant of the binding of L-glutamic acid, respectively. K_{I1} and K_{I2} are the affinity constants of imipramine to the first and the second binding sites of the carrier. [Glu] and $[I]$ are the concentrations of L-glutamic acid and Imipramine, respectively. From Eq. (3), the reciprocal of v is proportional to $[I]^2$ and the reciprocal of $[Na^+]^2$. Fig. 4 shows the plot of $1/v$ vs. $1/[Na^+]^2$ and $1/v$ vs. $[I]^2$. As shown in the inset of Fig. 4, the inhibition of L-glutamic acid transport is competitive with respect to the binding of Na^+. $K_{I1} \cdot K_{I2}$ value calculated from the x-intercept of the replot of the slopes obtained from $1/v$ versus $1/[Na^+]^2$ is 0.53 (mM^2).

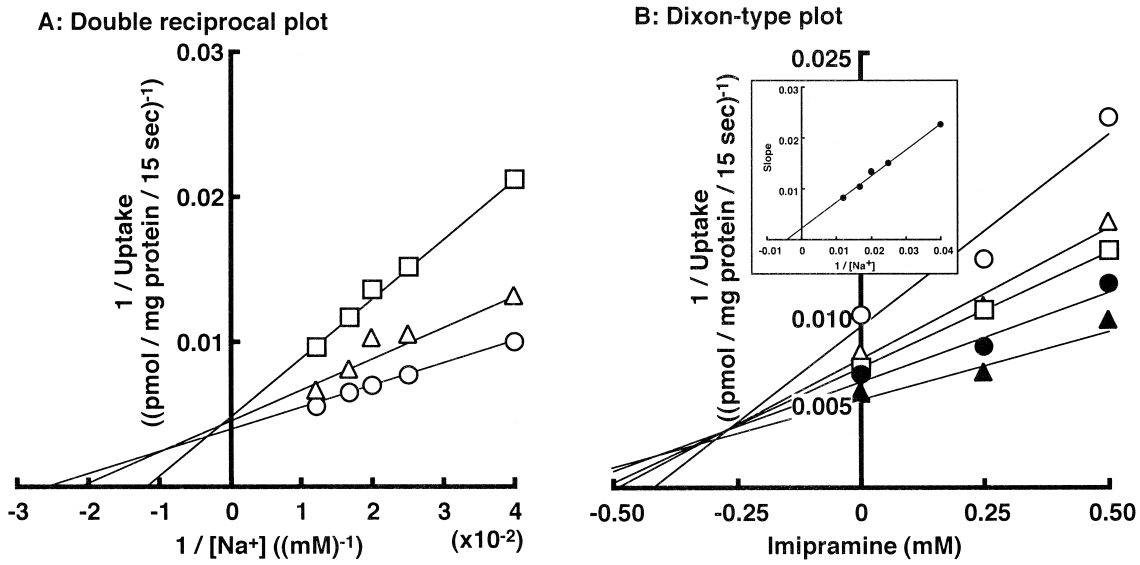

Fig. 5. Analysis of the inhibitory effect of imipramine on L-alanine uptake with respect to the inhibition of Na^+ binding to the carrier. Membrane vesicles (20 μl) were incubated with 100 μl of a transport buffer containing 240 μM L-alanine and various concentrations of Na^+ and imipramine. Each point represents the mean value of 3–4 measurements. A: Final concentrations (mM) of imipramine were 0 (○), 0.25 (△) and 0.5 (□). B: Final concentrations (mM) of Na^+ were 25 (○), 40 (△), 50 (□), 60 (●) and 83 (▲). (Inset: replot of the slopes of Dixon-type plot)

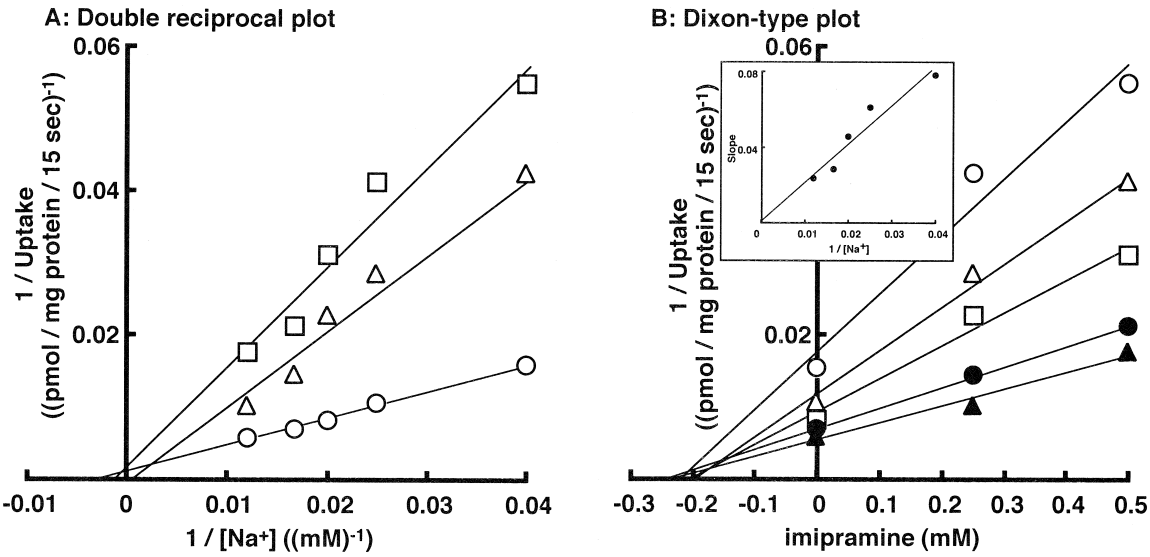

Fig. 6. Analysis of the inhibitory effect of imipramine on D-glucose uptake with respect to the inhibition of Na^+ binding to the carrier. Membrane vesicles (20 μl) were incubated with 100 μl of a transport buffer containing 240 μM D-glucose and various concentrations of Na^+ and imipramine. Each point represents the mean value of 3–7 measurements. A: Final concentrations (mM) of imipramine were 0 (○), 0.25 (△) and 0.5 (□). B: Final concentrations (mM) of Na^+ were 25 (○), 40 (△), 50 (□), 60 (●) and 83 (▲). (Inset: replot of the slopes of Dixon-type plot)

3.4. Type of the inhibitory effect of imipramine on the transport of L-alanine and D-glucose

Plots of inhibitory effect of imipramine on the uptake of L-alanine and D-glucose in the presence of various concentrations of Na^+ are shown in Figs. 5 and 6, respectively. Double reciprocal plot and Dixon plot showed that the inhibition of the uptake of both substrates was due to a competitive or partially competitive inhibition of Na^+-binding by imipramine. Moreover, the replots of the slopes obtained from Dixon plot indicate that the inhibitory effect exerted on the D-glucose transport is rather a pure competitive type whereas that exerted on the L-alanine transport is rather a mixed one although the y intercept is very close to the origin. The K_I values for L-alanine and D-glucose transport were 0.26 (mM) and 0.17 (mM), respectively.

4. Discussion

This is the first study to analyze the effect of an organic cation on the transport of L-glutamic acid. L-Glutamic acid is an acidic amino acid transported by Na^+ co-transport system with a stoichiometry of substrate: $Na^+ = 1{:}2$. Therefore, it is interesting to compare the inhibitory effects with those obtained with L-alanine, a neutral amino acid, and D-glucose, which have a stoichiometry value with Na^+ of 1:1.

On the basis of some assumptions mentioned above (Section 3.3), we introduced Eq. (3) which shows the kinetics of the inhibition when the transport ratio of substrate and Na^+ is 1:2, and inhibition is occurred on Na^+-binding sites. We used this equation for the analysis of mechanism of inhibitory effect by imipramine on L-glutamic acid transport. As shown in Fig. 4, imipramine competitively inhibit the binding of Na^+, but it does not compete with L-glutamic acid (Fig. 3). On the other hand, it is possible that imipramine inhibit the transport of L-glutamic acid by bind to only one of two Na^+-binding sites. However, when we analyze the uptake data using the equation under this assumption, linear line was not obtained by Dixon type plot ($1/v$ vs. $[I]$). Therefore, it would be appropriate that imipramine bind to both two Na^+-binding sites. These results are similar to the results with L-alanine and D-glucose (Figs. 4 and 5), and suggest that the manner of the inhibitory effect of imipramine on the Na^+-dependent carrier systems is common for all systems regardless of the stoichiometry or substrates. Although the sequential order of the inhibitory events for L-glutamic acid transport can not be elucidated from the results of this study, a model that imipramine binds to both Na^+-binding sites before the binding of L-glutamic acid can be inferred [16] from the graphical fitting using Eq. (3) (Fig. 4).

Acknowledgements

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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