ELSEVIER

Biochimica et Biophysica Acta 1370 (1998) 192–198

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Transport mechanisms of a glycoside, *p*-nitrophenyl-β-D-glucopyranoside, across rat small intestinal brush-border membranes

Toshimasa Ohnishi, Sohei Higashi, Takashi Mizuma, Shoji Awazu *

Department of Biopharmaceutics, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

Received 27 October 1997; accepted 3 November 1997

Abstract

We examined the mechanism of *p*-nitrophenyl-β-D-glucopyranoside (*p*-NP-β-D-Glc) transport in brush-border membrane vesicles from rat small intestine. The initial uptake rate showed an overshoot phenomenon in the presence of an inwardly directed sodium-ion concentration gradient. The overshoot disappeared when the sodium-ion concentration gradient was replaced with a potassium ion concentration gradient. D-Glucose and *p*-NP-β-D-Glc analogues inhibited the uptake, whereas uridine, leucine and disaccharide did not. Data on the concentration dependence of *p*-NP-β-D-Glc uptake indicated that two carrier-mediated systems are involved. The uptake via the high-affinity site required an inwardly directed sodium-ion concentration gradient, while the uptake via the low-affinity site proceeded such a gradient. D-Glucose competitively inhibited the initial uptake of *p*-NP-β-D-Glc via the high-affinity site with a K_i value of 301 μM. The *p*-NP-β-D-Glc is transported in the small intestine via both the same carrier-mediated transport system that takes up D-glucose and a distinct low-affinity carrier-mediated transport system. © 1998 Elsevier Science B.V.

Keywords: β-glucoside transport; Brush-border membrane vesicle; Small intestine; (Rat)

1. Introduction

We have already suggested that glycosides such as *p*-nitrophenyl-β-D-glucopyranoside (*p*-NP-β-D-Glc) [1,2], *p*-nitrophenyl-β-D-galactopyranoside [1], sugar-coupled tyrosyl–glycyl–glycine [3] and 2-napthyl-β-D-glucopyranoside (2-NA-β-D-Glc) [4] are taken up via a carrier-mediated transport system, based on studies using rat everted sac techniques. Wang et al. [5] reported that D-glucose significantly inhibited the transport of N^4-D-glucopyranosyl-sulfamethazine in a rat everted sac model, and Hirayama et al. [6] reported that [^{3}H]D-glucose transport was significantly inhibited by methylazoxymethanol-β-D-glucopyranoside in rabbit intestinal brush border membrane vesicles (BBMVs). These reports suggested that these glucosides may be transported via the carrier-mediated glucose transporter in the small intestine. However, Lostao et al. reported that *p*-NP-β-D-Glc acted as a blocker or a non-transported inhibitor of the expressing carrier-mediated glucose transporter in *Xenopus leavis* oocytes expressing rabbit sodium/glucose co-transporter (SGLT1) mRNA

* Corresponding author. Fax: +81-426-76-3142; E-mail: awazu@ps.toyaku.ac.jp

PII S0005-2736(97)00262-9

[7]. Therefore, it is necessary to examine whether glycosides are actually transported by the carrier-mediated transport systems in the small intestine.

In the present study, we examined the uptake of *p*-NP-β-D-Glc in rat small intestinal BBMVs and we confirmed that *p*-NP-β-D-Glc shares a common carrier-mediated transport system with D-glucose. *p*-NP-β-D-Glc was selected as a model substrate, because it showed the largest transportability among the glycosides we previously examined [1,2].

2. Materials and methods

2.1. Chemicals

p-NP-β-D-Glc was purchased from Nacalai Tesque (Kyoto, Japan); *p*-aminophenyl-β-D-glucopyranoside (*p*-AP-β-D-Glc) and 2-NA-β-D-Glc from Sigma Chemical (St. Louis, USA); [^{3}H]D-glucose from Dupont New England Nuclear (Boston, MA). All other chemicals were commercial products of reagent grade.

2.2. Preparation of membrane vesicles

BBMVs were prepared by the method of Kessler et al. [8] and Tsuji et al. [9] with some modifications. Briefly, male Wistar rats (200–220 g) were killed by decapitation. The intestines were immediately removed and placed in ice-cold saline. They were washed with ice-cold saline, and everted. The mucosa was scraped off and homogenized in ice-cold 2 mM Tris–HEPES buffer (pH 7.1) containing 50 mM mannitol; the volume (ml) of buffer was 30 times the weight of the scraped mucosa (g). Homogenization was carried out with a Waring blender for 5 min at 15,000 rpm. A 1 M $CaCl_2$ solution was added to the homogenate to give a final concentration of 10 mM and the mixture was stirred in an ice bath for 15 min. It was centrifuged at $3000 \times g$ for 15 min at 4°C and the supernatant was centrifuged at $27{,}000 \times g$ for 30 min at 4°C. The resultant pellet was resuspended in a washing medium, through a 25 gauge needle. The final pellet of BBMVs was suspended in 10 mM Tris–HEPES buffer (pH 7.5) adjusted to 280 ± 5 mOsm with mannitol (buffer B). The protein concentration was 20.6 ± 0.94 mg protein ml^{-1} (mean ± S.E., $n = 6$).

2.3. Transport experiment

The uptake of *p*-NP-β-D-Glc by membrane vesicles was measured by the rapid filtration method described by Hopfer et al. [10] and Tsuji et al. [9]. Briefly, membrane vesicles suspended in buffer B were kept on ice until use. The uptake was started by adding 90 μl of incubation medium containing *p*-NP-β-D-Glc or [^{3}H]D-glucose to 10 μl of membrane vesicle suspension (206 ± 9.4 μg of protein). The mixtures was incubated at the appropriate temperature and the transport reaction was stopped at the desired time by adding 1 ml of ice-cold stop solution, 100 mM NaCl, 10 mM Tris–HEPES buffer pH 7.5, adjusted to 290 ± 5 mOsm with mannitol. The diluted samples were applied immediately to a Millipore filter (HAWP, 0.45 μm pore size, Millipore, Bedford, MA) and rapidly washed twice with 4 ml of ice-cold stop solution. Non-specific binding to the membranes and filters was determined by dilution of the vesicle suspension with 1 ml of ice-cold stop solution before addition of *p*-NP-β-D-Glc. Since no non-specific binding of *p*-NP-β-D-Glc or [^{3}H]D-glucose was detected, observed uptake amounts were not corrected. The detailed conditions for each experiment are given in the figure legends.

2.4. Assay method

The *p*-NP-β-D-Glc trapped on the Millipore filter was extracted with 500 μl of distilled water, and the amount present was determined by HPLC on a reverse-phase column (TSK-gel ODS-80Ts, 5 μm, 4.6 i.d. × 150 mm, Toso, Japan) using a 32% methanol/0.05% phosphate mobile phase at a flow rate of 1.0 ml min^{-1}, with a UV detector set at 302 nm. [^{3}H]D-glucose radioactivity was determined with a liquid scintillation counter.

2.5. Data analysis

The non-linear least-squares regression analysis program MULTI was used for data fitting to Eq. (1).

which describes one saturable (carrier-mediated) process and one non-saturable process, or Eq. (2). which describes two saturable process and a non-saturable process:

$$J = J_{max}[C]/(K_t + [C]) + k_d[C] \quad (1)$$

$$J = J_{max,1}[C]/(K_{t,1} + [C]) + J_{max,2}[C]/(K_{t,2} + [C]) + k_d[C] \quad (2)$$

where J_{max} is the maximum uptake rate for a carrier-mediated process; C is the concentration of substrate; K_t is the half-saturation concentration (Michaelis constant); and k_d is the non-saturable uptake rate constant, which was regarded as being the same as k_d at 4°C.

2.6. Statistical analysis

The data were statistically analyzed using the Student–Newman–Keuls test after one-way ANOVA. The criterion of significance was taken as $p < 0.05$.

3. Results

3.1. Purity of the membrane fraction and transport activity

The purity of the membrane fraction obtained by our procedure was evaluated by assaying the activity of a specific marker enzyme. The specific activity of alkaline phosphatase measured in the final BBMVs fraction showed a 9.1 ± 0.4 fold enhancement (mean $\pm$ S.E. $n = 8$) over the crude homogenate. This is in good agreement with the data obtained by Tsuji et al. [9]. Transport activity of the BBMVs was determined by measuring the transport of D-[^{3}H]glucose at 22°C. The uptake amount at 30 s was almost 8 times that at the steady state (at 60 min). This transient overshoot uptake of D-glucose in the presence of an inwardly directed sodium-ion concentration gradient is in good agreement with the data obtained by Kessler et al. [11]. This result indicated that the BBMVs prepared in this study were suitable for the *p*-NP-β-D-Glc transport study.

3.2. Sodium-ion concentration gradient dependence of p-NP-β-D-Glc uptake

The time courses of *p*-NP-β-D-Glc uptake at 27°C and 4°C in the BBMVs in the presence or the absence of a sodium-ion concentration gradient are shown in Fig. 1. The *p*-NP-β-D-Glc uptake was significantly accelerated in the presence of an inwardly directed sodium-ion concentration gradient (in/out = 0/100 mM) and showed an overshoot phenomenon with the maximum uptake at 60 s at 27°C. The overshoot phenomenon disappeared in the absence of the sodium-ion concentration gradient. At 4°C, the uptake rate was remarkably reduced. The uptake amounts at 120 s both in the presence and absence of the sodium-ion concentration gradient were very close to the plateau level which was observed at 3600 s, at 4°C. The uptake amount at 15 s after the reaction started was used in order to calculate the initial uptake rate, because the uptake was considered to proceed linearly for at least the first 15 s at 27°C.

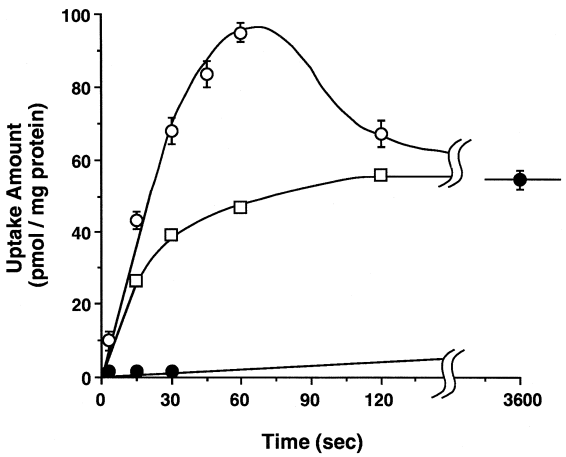

Fig. 1. The time courses of *p*-NP-β-D-Glc uptake by rat small intestinal brush-border membrane vesicles at 27°C (open symbols) and 4°C (closed symbols). Uptake of *p*-NP-β-D-Glc was measured by incubating BBMVs in 10 mM Tris–HEPES buffer pH 7.5 containing 100 mM NaCl (circle) or 100 mM KCl (square). Substrate concentration was 50 μM. Each point represents the mean ± S.E. of three to four experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol.

3.3. Effect of medium osmolarity on p-NP-β-D-Glc uptake

To distinguish between the transport of *p*-NP-β-D-Glc into the intravesicular space and binding to BBMVs, the initial uptake rates were measured in media with various values of osmolarity in the range of 300 to 1410 mOsm, adjusted with sucrose. The rates are plotted against the reciprocal values of osmolarity in Fig. 2. The intercept on the vertical axis was not significantly different from zero. These results suggested that *p*-NP-β-D-Glc was transported into the intravesicular space without significant binding to the membrane.

3.4. Effect of various compounds on p-NP-β-D-Glc and [^{3}H]D-glucose uptake

The inhibitory effects of various compounds on the initial uptakes of *p*-NP-β-D-Glc (Table 1) and [^{3}H]D-glucose (Table 2) were examined. The uptake of *p*-NP-β-D-Glc into BBMVs was inhibited by *p*-NP-β-D-Glc analogues such as 10 mM *p*-aminophenyl-β-D-glucopyranoside (*p*-AP-β-D-Glc), 1 mM 2-NA-β-D-Glc and 1 mM glucose, whereas 1 mM fructose, 1 mM sucrose, 1 mM uridine and 1 mM leucine had no effect (Table 1). The uptake of [^{3}H]D-glucose into BBMVs was inhibited by 1 mM D-glucose and 10 mM *p*-NP-β-D-Glc (Table 2).

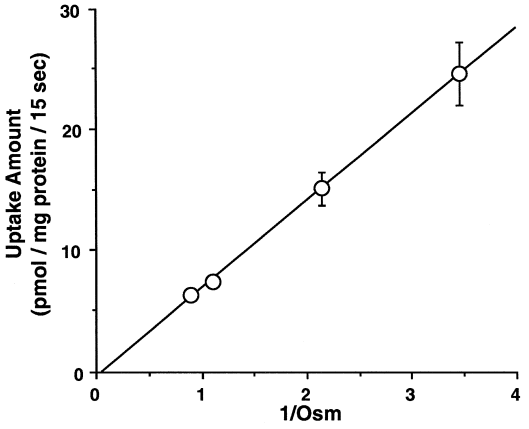

Fig. 2. Effect of extravesicular osmolarity on *p*-NP-β-D-Glc uptake. Vesicles were suspended in a buffer containing varying concentrations of sucrose to adjust the extravesicular osmolarity. Vesicles were incubated in each medium for 10 min at 4°C, then the initial *p*-NP-β-D-Glc uptake was determined at 15 s at 27°C. The uptake was plotted as a function of the reciprocal of the extravesicular osmolarity. Substrate concentration was 50 μM. Sodium-ion concentration gradient is in/out = 0/100 mM. Each point represents the mean ± S.E. of three to four experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol. The regression line was calculated by the least-squares method ($Y = 7.24\ X = -0.524,\ R = 0.999$).

Table 1
Inhibitory effect on transport of *p*-nitrophenyl-β-D-glucopyranoside (50 μM) into rat jejunal BBMVs

	Uptake rate (*p*mol mg^{-1} protein 15 s^{-1})
Control	47.0 ± 2.1
+ 10 mM *p*-AP-β-D-Gluc	21.3 ± 1.0 *
+ 1 mM 2-NA-β-D-Gluc	34.2 ± 1.7 *
+ 1 mM glucose	30.5 ± 1.0 *
+ 1 mM fructose	43.6 ± 2.5
+ 1 mM uridine	44.2 ± 1.3
+ 1 mM leucine	45.2 ± 2.4
+ 1 mM sucrose	43.3 ± 1.5
Sodium replacement	29.1 ± 1.2 *

Incubation temperature: 27°C.
Each value represents the mean ± S.E. of three to four experiments.
* $p < 0.05$.

3.5. Concentration dependence of p-NP-β-D-Glc uptake and inhibitory effect of D-glucose

When the initial uptake of *p*-NP-β-D-Glc was measured as a function of substrate concentration over the range from 50 μ M to 50 mM in the

Table 2
Inhibitory effect on transport of [^{3}H]D-glucose into rat jejunal BBMVs

	Uptake rate (*p*mol mg^{-1} protein 5 s^{-1})
Control	1.93 ± 0.10
+ 10 mM *p*-NP-β-D-Gluc	0.594 ± 0.03 *
+ 1 mM glucose	0.275 ± 0.01 *
4°C	0.338 ± 0.01 *

Incubation temperature: 22°C.
Each value represents the mean ± S.E. of four experiments.
* $p < 0.05$.

presence of an inwardly directed sodium-ion concentration gradient, saturable uptake was observed at 27°C (Fig. 3A), but not at 4°C. The Eadie–Hofstee plot, after subtraction of the non-saturable uptake at 4°C, clearly showed the involvement of at least two transport processes (Fig. 3B). The data were fitted to a model consisting of two saturable transport processes together with a non-saturable process (Eq. (2)) by use of the program MULTI. The AIC value from Eq. (2) was less than the AIC value from Eq. (1). The fitting to Eq. (2) gave $K_{t,1} = 0.8 \pm 0.8$ mM. $J_{max,1} = 0.4 \pm 0.3$ nmol mg^{-1} protein 15 s^{-1}, $K_{t,2} = 31.4 \pm$

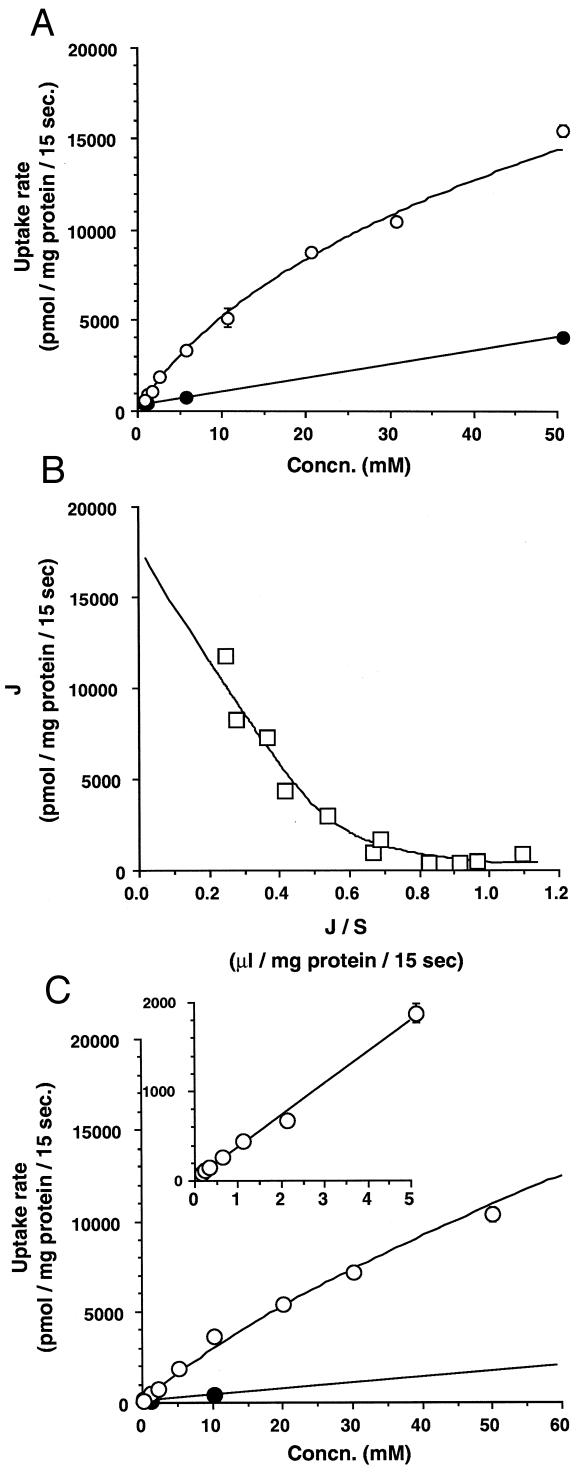

Fig. 3. Concentration dependence (circles, A) and Eadie–Hofstee plot (squares, B) of the sodium-ion concentration gradient-dependent uptake of *p*-NP-*β*-D-Glc and sodium-ion concentration gradient-independent uptake of *p*-NP-*β*-D-Glc (C) by rat small intestinal brush-border membrane vesicles. The initial uptake of *p*-NP-*β*-D-Glc was measured at 27°C (open circles) and 4°C (closed circles) for 15 s. The solid line shows the uptake rate simulated using the MULTI-fitted parameters. Sodium-ion or potassium-ion concentration gradient is in/out = 0/100 mM. Each point represents the mean ± S.E. of three to four experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol.

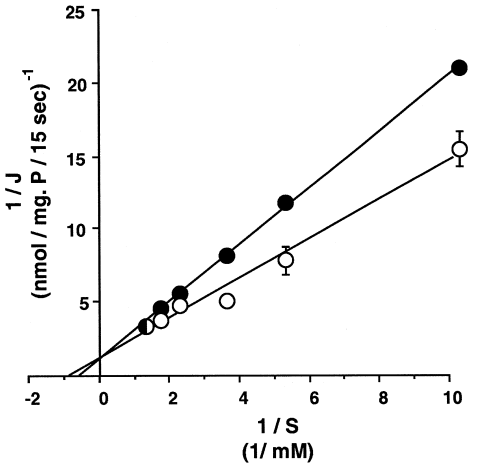

Fig. 4. Lineweaver–Burk plot of *p*-NP-*β*-D-Glc uptake by rat small intestinal brush-border membrane vesicles in the absence (open circles) and in the presence of 200 μM unlabeled D-glucose (closed circles). These data were corrected by subtraction of the uptake value in the presence of a potassium-ion concentration gradient. The initial uptake rate of *p*-NP-*β*-D-Glc was measured at 27°C for 15 s. D-Glucose was added simultaneously at the initiation of *p*-NP-*β*-D-Glc uptake. Sodium-ion or potassium-ion concentration gradient is in/out = 0/100 mM. Each point represents the mean of three to four experiments. The inhibition constant (K_i) of D-glucose was calculated to be 301 μM.

1.1 mM, $J_{max,2} = 16.4 \pm 0.7$ nmol mg^{-1} protein 15 s^{-1}. On the other hand, when the sodium-ion concentration gradient was replaced with a potassium-ion concentration gradient, the uptake increased linearly in the substrate concentration range from 50 μM to 5 mM (Fig. 3C, insert). This indicated that the saturable process via the high-affinity site had disappeared. In this case, all the data were fitted to a single saturable transport process together with a non-saturable process (Eq. (1)) by use of the program MULTI to give $K_{t,3} = 31.0 \pm 2.0$ mM, $J_{max,3} = 9.3 \pm 1.9$ nmol mg^{-1} protein 15 s^{-1}.

Lineweaver–Burk analysis, of the uptake values after the subtraction of those in the presence of the potassium-ion concentration gradient showed that D-glucose competitively inhibited the initial uptake of *p*-NP-β-D-Glc between 0.1 mM and 1.0 mM, where the high-affinity transport site was operative (Fig. 4). The inhibition constant (K_i) of D-glucose was calculated to be 301 μM.

3.6. Effect of various compounds on p-NP-β-D-Glc uptake via the low-affinity site

The inhibitory effects of various compounds on the initial uptake of *p*-NP-β-D-Glc via the low-affinity site were examined at a substrate concentration of 5 mM, which was assumed to saturate the high-affinity site but not the low-affinity site, in the presence of an inwardly directed 100 mM sodium-ion concentration gradient. As shown in Table 3, 100 mM glucose and 50 mM *p*-AP-β-D-Glc inhibited the uptake of *p*-NP-β-D-Glc, whereas 100 mM fructose, 100 mM uridine, 100 mM leucine and 100 mM cellobiose did not.

Table 3
Inhibitory effect on transport of *p*-nitrophenyl-β-D-glucopyranoside (5 mM) into rat jejunal BBMVs

	Uptake rate (*p*mol mg^{-1} protein 15 s^{-1})
Control	2656 ± 274
+ 50 mM *p*-AP-β-D-Gluc	1541 ± 111 *
+ 100 mM glucose	1772 ± 98 *
+ 100 mM fructose	2862 ± 320
+ 100 mM uridine	2402 ± 213
+ 100 mM leucine	2511 ± 114
+ 100 mM celobiose	3098 ± 104
Sodium replacement	1807 ± 105 *

Incubation temperature: 27°C.
Each value represents the mean ± S.E. of three to four experiments.
* $p < 0.05$.

4. Discussion

In the present study, we have examined whether *p*-NP-β-D-Glc shares a carrier-mediated transport system with D-glucose in rat intestinal BBMVs. The uptake of *p*-NP-β-D-Glc exhibited an overshoot phenomenon in the presence of inwardly directed sodium-ion concentration gradient. In addition, the uptake was inhibited by analogs, was concentration-dependent, and was inhibited competitively by D-glucose. The inhibition constant (K_i) of D-glucose was calculated to be 301 μM, which is close to the K_m value of D-glucose uptake (180 μM, 11). These results imply that *p*-NP-β-D-Glc is transported by SGLT1. This conclusion is consistent with our previous findings, using everted sac techniques (1–4), that glycosides are transported by a phlorizin-sensitive, sodium-ion concentration gradient dependent transport system in rat small intestine.

The kinetic study of the concentration-dependent uptake in the presence of a sodium-ion concentration gradient showed that rat intestinal BBMVs have at least two transporters for *p*-NP-β-D-Glc, one which high affinity and one with low affinity. The low-affinity carrier-mediated transport system ($K_m = 31.4 \pm 1.1$ mM, $J_{max} = 16.4 \pm 0.7$ nmol mg^{-1} protein 15 s^{-1}) appears not to require a sodium-ion concentration gradient, because its kinetic parameters determined in the presence of such a gradient coincided well with those of the saturable uptake in the absence of an inwardly directed sodium-ion concentration gradient ($K_m = 31.0 \pm 2.0$ mM, $J_{max} = 9.3 \pm 1.9$ nmol mg^{-1} protein 15 s^{-1}). Under the latter condition, [^{3}H]D-glucose uptake disappeared (data not shown). This result suggested that the low-affinity carrier system is different from SGLT1. This low-affinity carrier-mediated transport system may be specific for the glycoside, because the inhibitory effect of *p*-AP-β-D-Glc on *p*-NP-β-D-Glc uptake was greater than that of D-glucose (Table 3), and various other compounds were not inhibited (Table 3).

Our result is different from that of Lostao et al. [7], who classified *p*-NP-β-D-Glc as a non-transported inhibitor for rabbit SGLT1. The reason for this difference is unclear, though it may be related to the sodium-ion concentration gradient-independent carrier-mediated transport system.

In conclusion, we have demonstrated that *p*-NP-β-D-Glc is transported at the rat intestinal brush-border membrane not only via the same carrier-mediated transport system that takes up D-glucose, but also via a sodium-ion concentration gradient-independent, low-affinity carrier-mediated transport system. Further study will be necessary to characterize the latter transporter.

Acknowledgements

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (08772174) from the Ministry of Education, Science, Sports and Culture.

References

[1] T. Mizuma, K. Ohta, M. Hayashi, S. Awazu, Biochem. Pharmacol. 43 (1992) 2037–2039.
[2] T. Mizuma, K. Ohta, M. Hayashi, S. Awazu, Biochem. Pharmacol. 45 (1992) 1520–1523.
[3] T. Mizuma, N. Sakai, S. Awazu, Biochem. Biophys. Res. Commun. 203 (1994) 1412–1416.
[4] T. Mizuma, K. Ohta, S. Awazu, Biochim. Biophys. Acta 1200 (1994) 117–122.
[5] Y. Wang, R. Grigg, A. McCormack, H. Symondes, C. Bowmer, Biochem. Pharmacol. 46 (1993) 1864–1866.
[6] B. Hirayama, A. Hazama, D.F. Loo, E.M. Wright, G.E. Kisby, Biochim. Biophys. Acta 1193 (1994) 151–154.
[7] M.P. Lostao, B.A. Hirayama, D.D.F. Loo, E.M. Wright, Membrane Biol. 142 (1994) 161–170.
[8] M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller, G. Semenza, Biochim. Biophys. Acta 506 (1978) 136–154.
[9] A. Tsuji, T. Terasaki, I. Tamai, H. Hirooka, J. Pharmacol. Exp. Ther. 241 (1987) 594–601.
[10] U. Hopfer, K. Nristine, J. Perrotto, J. Isselebacher, J. Biol. Chem. 248 (1973) 25–32.
[11] M. Kessler, V. Tannenbaum, C. Tannenbaum, Biochim. Biophys. Acta 509 (1978) 348–359.