Improvement of Intestinal Absorption of Peptide Drugs by Glycosylation: Transport of Tetrapeptide by the Sodium Ion-Dependent D-Glucose Transporter

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Abstract □ A tetrapeptide (Gly-Gly-Tyr-Arg, GGYR), which is not transported by di- or tripeptide transporters, was glycosylated with *p*-(succinylamido)phenyl α- or β-D-glucopyranoside (α,β-SAPG) to investigate whether these glycosylated molecules are transported by the Na^+-dependent D-glucose transporter. Their uptake into brush-border membrane vesicles (BBMVs) and transport through the intestinal membrane were examined using the rapid filtration technique and the everted sac method. It was observed that glycosylation at the α-amino position of GGYR increased resistance to aminopeptidase activity and inhibited its degradation. When α- and β-SAPG-GGYR were incubated with BBMVs, overshoot uptake was observed about 2 min after the start of incubation in the presence of an inward Na^+ gradient. This uptake remained unaffected by the addition of GGYR while it was significantly inhibited when Na^+ was replaced with K^+ or α- and β-SAPG-GGYR were incubated with BBMVs at 4 °C. Uptake was also markedly inhibited either with 1 mM phloridzin or 10 mM D-glucose. These findings suggested that the Na^+-dependent glucose transporter (SGLT-1) played an important role in the uptake of both α- and β-SAPG-GGYR into BBMVs. A comparison of α- with β-SAPG-GGYR revealed that the amount of β-SAPG-GGYR taken up was greater than that of α-SAPG-GGYR. From the everted sac method data, it was shown that the elimination clearance from the mucosal side, CL_{el}, and permeation clearance to the serosal side, CL_p, were 15.82 ± 6.83 and 0.83 ± 0.06 μL/min/cm for α-SAPG-GGYR and 44.52 ± 3.61 and 3.50 ± 0.81 μL/min/cm for β-SAPG-GGYR, respectively, and that α-SAPG-GGYR was more resistant to enzymatic degradation than β-SAPG-GGYR. Permeation of both α- and β-SAPG-GGYR was inhibited in the presence of D-glucose and in the absence of a Na^+ gradient, suggesting that both α- and β-SAPG-GGYR were transported by the Na^+-dependent D-glucose transporter. The permeation clearance transported by the Na^+-dependent D-glucose transporter, $(CL_p)_{Na+}$, of β-SAPG-GGYR was about 5 times greater than that for α-SAPG-GGYR. This result may be ascribable to the fact that the β-form of glucose has higher affinity to SGLT-1 than the α-form. The results of the present study encourage further investigations on improvements in intestinal absorption of peptide drugs by glycosylation.

1. Introduction

Development of peptide drugs as therapeutic agents has proved very difficult because of their poor membrane penetrability and extreme susceptibility to enzymatic degradation in the gut lumen and enterocytes. To improve the systemic availability of peptides, it is necessary to enhance their absorption and to increase their resistance to enzymatic degradation. Many approaches have been suggested to circumvent these barriers, such as coadministration of peptidase inhibitors,[1−4] coadministration of absorption enhancers,[5,6] and modifications of the peptide structure.[7−9]

It has been shown recently that there are specific carrier-mediated transport systems in the intestine which facilitate absorption of amino acids, oligopeptides, monosaccharides, monocarboxylic acids, phosphate, bile acids, and several water-soluble vitamins.[10] Strategies to improve the bioavailability of poorly absorbed drugs have been designed by modifying drugs so that they are absorbed by specialized intestinal transporters.

The role and function of glucose transporters in the general control of glucose homeostasis are currently being elucidated. Five human facilitative-diffusion hexose transporters (GLUT-1 to GLUT-5) in different tissues have been characterized by molecular cloning.[11,12] A low-affinity transporter GLUT-2 is known to be present in the basolateral membrane of intestinal epithelial cells. There are Na^+-dependent glucose transporters, referred to as SGLT-1, present at a high level in human intestine and also at a somewhat lower level in rat, which actively transport glucose across the apical brush-border of epithelial cells.[13]

In the previous study we synthesized glycosylated insulin, *p*-(succinylamido)phenyl-α-D-glucopyranoside (SAPG)-substituted insulin (SAPG-INS) and utilized SGLT-1 to improve the intestinal absorption of insulin.[14] It was observed that SAPG-INS was less susceptible to enzymatic degradation than native insulin and had an increased affinity for brush-border membrane vesicles (BBMVs) prepared from rat small intestine. It remained doubtful, however, whether insulin was taken up into BBMVs by this transporter because of its large molecular size.

In the present study we synthesized glycosylated tetrapeptides, α- and β-SAPG-Gly-Gly-Tyr-Arg (α,β-SAPG-GGYR), to investigate whether glycosylated peptide is transported by the Na^+-dependent D-glucose transporter. A tetrapeptide is considered to be the smallest peptide which is not transported by di- or tripeptide transporters. We selected GGYR because of its ease of radioiodination of tyrosine and commercial availability. We also examined their uptake into BBMVs and transport through the intestinal membrane by the rapid filtration technique and the everted sac method.

2. Materials and Methods

2.1. Materials—Gly-Gly-Tyr-Arg (GGYR), amastatin, chymostatin, and phosphoramidon were purchased from Peptide Institute Inc. (Osaka, Japan). $Na^{125}I$ was purchased from Amersham Japan (Tokyo, Japan). Lactoper-

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oxidase and phloridzin were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyphosphinothioyl chloride (Mpt-Cl) was purchased from Tokyo Kasei Co. (Tokyo, Japan). *p*-Nitrophenyl α-D-glucopyranoside (α-NPG), *p*-nitrophenyl *β*-D-glucopyranoside (*β*-NPG), palladium carbon, and dansyl chloride were purchased from Nakalai Tesque Inc. (Kyoto, Japan). All other chemicals were analytical grade and were used without further purification. Deionized redistilled water was used to prepare all solutions.

2.2. Synthesis of SAPG and SAPG-GGYR—The synthesis of SAPG was performed according to the method of Jeong et al.[15] Briefly, the nitrophenyl group on α-NPG was reduced to an aminophenyl group with ammonium formate in the presence of palladium carbon yielding *p*-aminophenyl α-D-glucopyranoside (α-APG). APG was then allowed to react with succinic anhydride at room temperature for 4 h to produce SAPG. The coupling of SAPG to GGYR was performed using a Mpt (dimethylphosphinothionyl) mixed anhydride method according to the work of Ueki et al.[16] Briefly, GGYR (170 mg, 321 μmol) was dissolved in a 1:1 (v/v) mixture of water and dimethyl formamide (DMF, 36 mL total volume) which was cooled in an ice bath after adjustment of the pH to 9.5 with 0.1 N NaOH. One hundred and twenty milligrams of SAPG was dissolved in 1.08 mL of DMF containing 80 μL (320 μmol) of tri-*n*-butylamine, then 34 μL (320 μmol) of Mpt-Cl was added at 0 °C and the solution was stirred for 15 min. Next 160 μL (640 μmol) of tri-*n*-butylamine was added. This SAPG solution was added dropwise to the GGYR solution in an ice bath and the pH of mixed solution was adjusted to 9.5 with 0.1 N NaOH. After the solution was allowed to stand at room temperature for 1 h, it was stirred overnight while the pH was maintained at 9.5.

2.3. Purification of SAPG-GGYR—The organic reagents and solvents were separated from glycosylated GGYR using a Sephadex LH-20 gel column (1.0 × 15 cm). The crude mixture (5 mL) was applied to the column and eluted with 100 mM phosphate buffer at a flow rate of 18 mL/h. The UV absorption of each fraction (3 mL) was measured at 254 nm, and the fractions showing increased absorption, indicating the presence of peptide, were collected and pooled. The glycosylated GGYR was further purified by a DEAE anion-exchange chromatography using a Con Sep LC 100 with a Mem Sep 1010 cartridge (Millipore, Bedford, MA), which was eluted with a solution of 20 mM Tris-HCl buffer (pH 8.5) at a rate of 3 mL/min using a linear gradient to 1 M NaCl. Fractions comprising the first peptide peak that eluted were pooled and dialyzed using a Microasilyser S1 with a Acipex Cartridge AC 110−10 (Asahi Chemical Industry Co. Ltd., Tokyo, Japan). The solution obtained was lyophilized to give a white-yellow powder.

2.4. Determination of SAPG-GGYR—*HPLC Analysis*— Samples were analyzed using a Waters 600E system (484 UV/Vis detector, 741 Data Module, Millipore Corp., Waters Chromatography Division, Milford, MA) on an ASAHI PAK GS-320 column (7.6 × 500 mm, Asahi Chemical Industry, Co. Ltd., Tokyo, Japan) or on a Carbonex TCAS column (4.6 × 100 mm, Tonen Co. Ltd., Tokyo, Japan) at a flow rate 1.0 or 0.4 mL/min. The mobile phase consisted of 50 mM ammonium acetate/acetonitile (80:20% v/v).

Analysis of the Position of SAPG Substitution—The position of SAPG-substitution on GGYR was estimated according to the method of Gray[17] with some modifications. Briefly, peptide (1 mg) was dissolved in 10 μL of 0.2 M sodium hydrogen carbonate solution and dried in a vacuum. This was redissolved in 10 μL of distilled water and the pH of the solution was adjusted to 8.5−9.0. Then 10 μL of dansyl chloride solution (2.5 mg/mL in acetone) was added and the solution was incubated at 37 °C. After 1 h the solution was dried in a vacuum and 100 μL of 6 N HCl was added. The tube was sealed by heating in a fine oxygen-gas flame. Hydrolysis was carried out at 105 °C for 18 h. After this period, the tube was centrifuged at 3000 rpm for 5 min. The tube was then opened and HCl was removed under vacuum. After dissolving of the hydrolyzed products in ammonium acetate−acetonitile solution, they were analyzed on the HPLC system described above. As a reference, a sample of the peptide was hydrolyzed before dansylation and the composition of amino acids was analyzed. Four dansyl amino acids (*O*-DNS-L-Tyr, DNS-Gly, DNS-L-Arg, and *N*,*O*-diDNS-L-Tyr) were detected when the lyophilized product was dansylated after hydrolysis while no amino acid was detected when it was hydrolyzed after dansylation. The retention times of *O*-DNS-L-Tyr, DNS-Gly, DNS-L-Arg, and *N*,*O*-diDNS-L-Tyr were 20.1, 26.5, 33.6, and 34.6 min, respectively (mean value of two experiments). These findings suggested that the lyophilized product was the compound substituted at the Gly terminal position.

Determination of the Sugar/Peptide Ratio—The number of sugar groups on the glycosylated peptide was determined using the phenol/sulfuric acid method[18] and a Micro BCA protein assay reagent kit (Pierce, Rockford, IL). Briefly, the glycosylated peptide (1 mg) was dissolved in 1 mL of a 5% aqueous solution of phenol. To this solution was added 5 mL of concentrated H_2SO_4, followed by mixing. After this mixture was allowed to stand for 30 min its absorbance was measured at 490 nm. The concentration of sugar was determined by comparison with a standard curve for SAPG over the range from 0 to 0.1 mg/mL. The peptide concentration was determined according to the standard protocol of the Micro BCA protein assay reagent. From the observed concentrations of sugar and peptide, the sugar/peptide ratio of the glycosylated peptide was shown to be 1.

Measurement of Molecular Weight and Polarity—One milligram of the purified glycosylated peptide was dissolved in glycerol and a secondary ion mass spectrogram (SIMS) was obtained using a Hitachi M-83A mass spectrometer. SAPG-substituted GGYR was identified by a molecular ion peak, MH^+, at 805. The polarity of α- or *β*-SAPG-GGYR was measured using a digital polarimeter (DIP-360, JASCO Corporation, Tokyo Japan) at room temperature. The $[\alpha]_D$ values were 140.74° and 24.99° for α-SAPG and α-SAPG-GGYR, respectively, and −48.00° and −8.33° for *β*-SAPG and *β*-SAPG-GGYR, respectively. Samples were also analyzed by HPLC on a Carbonex TCAS column at 0 °C. The mobile phase consisted of 50 mM ammonium acetate /acetonitile (80:20) at a flow rate of 0.4 mL/min. Two main peaks were observed: one at a retention time of 3.07 min for α-SAPG-GGYR and one at a retention time of 5.31 min for *β*-SAPG-GGYR. The purity of α- and *β*-SAPG-GGYR were 96.5 and 96.0%, respectively, as determined from HPLC chromatograms (mean value of two experiments).

2.5. In Vitro Experiment—*Preparation of Brush Border Membrane Vesicles (BBMVs)*—BBMVs were prepared by Mg^{2+} precipitation according to the methods of Tiruppathi et al.[19] and Takuwa et al.[20] Briefly, male Wistar rats (body weight 200 ± 20 g) which had been fasted for 18−24 h were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg) and then sacrificed by exsanguination through the carotid arteries. The abdomen was opened by a median incision and the small intestine (from the duodenum to ileum) was excised. The portion of the intestine was cut into small pieces (7−8 cm) and rinsed with ice-cold 0.9% saline and then cut open longitudinally. The mucosa was softly scraped off with a glass slide and was homogenized

with an Excel Auto Homogenizer (Model OX-7, Nihonseiki Co. Ltd., Tokyo Japan) at 12000 rpm for 90 s in 20 vol (v/w) of 6 mM Tris−NaOH buffer solution (pH 7.5) containing 150 mM mannitol and 2.5 mM EGTA. To this homogenate was added 1 M $MgCl_2$ solution to a final concentration of 10 mM, and the mixture was allowed to stand for 15 min. After centrifugation at 3000*g* for 10 min, the supernatant fraction was centrifuged at 42000*g* for 30 min. The resultant pellet was suspended in 10 mL of uptake buffer (16 mM Tris−10 mM HEPES−HCl containing 15 mM KCl and 270 mM mannitol, pH 7.5) and the mixture was centrifuged again at 42000*g* for 30 min. The second pellet was resuspended in 1 mL of uptake buffer and the solution was passed slowly through a syringe fitted with a 25-gauge needle to make the vesicle size uniform. The final protein concentration of the brush-border membrane preparation, which was determined by a Micro BCA protein assay, was adjusted to 10 mg/mL. The purity of BBMVs was assessed by assaying the activity of the marker enzyme alkaline phosphatase (ALP) using a Alkaline Phospha-K Test Wako (Wako Pure Chemical Industries, Ltd., Osaka Japan). The ALP activity was about 10 times higher in BBMVs compared to the homogenate.

Uptake of D*-Glucose into BBMVs*—A radiolabeled D-glucose solution containing 185 kBq/mL [^{3}H]-D-glucose, 1 mM unlabeled D-glucose, and 100 mM NaCl in uptake buffer solution was prepared. A 100 μL portion of this solution and 20 μL of BBMVs suspension were incubated in each test tube at 25 °C for 5 min. The reaction was started by adding the labeled D-glucose solution to the BBMVs suspension. After an appropriate incubation time, 3 mL of ice-cold stop buffer solution (10 mM Tris−11 mM HEPES−HCl containing 153 mM KCl, pH7.5) was added. BBMVs were collected on nitrocellulose filters (0.45 μm) and washed three times with 4 mL of ice-cold stop buffer solution. The filter was added to 10 mL of Scintisol EX-H (Dojindo Laboratories Co. Ltd., Kumamoto Japan), and vesicle-associated radioactivity was determined after solubilization of the filter in a liquid scintillation counter (ASC-3600, Aloka Co. Ltd., Tokyo Japan). The blank value was determined by mixing the labeled D-glucose solution with the BBMVs suspension followed by immediate filtration and washing with the stop buffer solution. This value was subtracted from the data.

Enzymatic Degradation of α*- and* β*-SAPG-GGYR and GGYR*—A 50 μL portion of BBMVs suspension (5 mg of protein/mL) was mixed with 150 μL of 5 mM α- or β-SAPG-GGYR or GGYR solution and the mixture was incubated at 37 °C. At set time, 250 μL of acetonitrile and 50 μL of internal standard (200 μM tryptophan) were added, and the mixture was centrifuged at 12000 rpm for 15 min. The supernatant was filtered through a membrane filter (0.45 μm). An aliquot of this supernatant was analyzed using a Waters 600E HPLC system on a TSK-gel ODS-80T$_M$ column (4.5 × 150 mm, Tosoh, Co. Ltd., Tokyo, Japan) at a flow rate of 1 mL/min. The mobile phase consisted of 50 mM ammonium acetate/methanol (90:10% v/v).

Iodination of α*- and* β*-SAPG-GGYR and GGYR*—α- and β-SAPG-GGYR and GGYR were iodinated according to the method of Hamlin et at.[21] Briefly, 2.5 mg of α- or β-SAPG-GGYR or GGYR was dissolved in 600 μL of 0.1 M sodium citrate buffer (pH 5.2). To this solution were added 2 μL of 0.05 M H_2O_2 , 2 μL of KI/Na^{125}I mixture solution (0.05 M KI:3.7 GBq/mL Na^{125}I = 3:1 by vol), and 2 μL of lactoperoxidase solution in 125 mM EDTA (final concentration of lactoperoxidase 9.25 μM) successively with stirring every 10 min. The mixture was applied to a Sephadex A-25 column (1.6 × 5 cm), eluted with 0.1 M sodium citrate buffer (pH 5.2), and then fractionated into labeled and unlabeled GGYR. The radioactivity of each 0.6 mL fraction was determined in an autowell γ-counter (Aloka, ARC-300). Concentration measurements of peptide solutions were performed using a Hitachi model U-3000 recording spectrophotometer, and the specific radioactivity was calculated.

Uptake of α*- and* β*-SAPG-GGYR and GGYR into BBMVs*—Uptake or adsorption of α- and β-SAPG-GGYR and GGYR into BBMVs was measured at 37 °C by a rapid filtration technique according to the method of McCarthy et al.[22] The ^{125}I-labeled peptide solution (195 μL) was mixed with an equal volume of 32 mM Tris−20 mM Hepes-HCl buffer solution containing 30 mM KCl, 200 mM NaCl, and 540 mM mannitol (pH 7.5) in the uptake experiment with an inward Na^+ gradient or 32 mM Tris−20 mM Hepes-HCl buffer solution containing 230 mM KCl and 540 mM mannitol (pH 7.5) in the uptake experiment with an inward K^+ gradient. This mixture (390 μL) and the BBMVs suspension (130 μL) were incubated separately at 37 °C (or 4 °C) for 5 min then mixed and incubated at 37 °C (or 4 °C). The osmolarity of this mixed solution was 502 mOsm, which was measured using a osmometer (Osmette, Precision Systems, Inc.). Hyperosmolar and hypoosmolar buffers (350, 635, and 773 mOsm) were prepared by adjusting the amount of D-mannitol added to the solution. At set time intervals after mixing, the vesicles (65 μL) were sampled from a plastic test tube and sucked through a prewashed nitrocellulose filter with a pore size of 0.45 μm (Advantec Toyo, Tokyo, Japan). The filter was washed three times with 4 mL of ice-cold stop buffer solution. The filter was transferred to an RIA tube and radioactivity was measured in an auto-well γ-counter (Aloka, ARC-300). The blank value was determined by mixing the peptide with the vesicle suspension followed by immediate filtration and washing with the stop buffer solution. This value was subtracted from the uptake data.

Everted Gut-Sac Technique—The permeability of α- and β-SAPG-GGYR and GGYR was studied using the everted gut-sac technique.[23] Male Wistar rats (weighing 200 ± 20 g), which had been fasted for 18−24 h, were anesthetized by an ip injection of 50 mg/kg sodium pentobarbital. After making a median incision in the abdomen, the small intestine was cut at two positions i.e., 2 and 9 cm from Treitz's ligaments. The entire length of the small intestine was carefully removed and placed in Krebs Ringer buffer solution (KRBS, pH 7.4) which was bubbled with 95% O_2: 5% CO_2 and then rinsed. After 15 min, the end of the segment was ligated and the segment was everted on a glass rod and rinsed. The other end of the segment was cannulated with a Teflon tube to form a sampling port. A 2.0 g glass weight was tied to the end of the sac to prevent changes in its internal volume. The sac was filled with 1 mL of KRBS and placed in a test tube containing 10 mL of test solution (or 10 mL of KRBS containing 3 mM fluorescein isothiocyanate−dextrans 4000 as a leakage marker) that was continuously bubbled with 95% O_2:5% CO_2. Every 20 min samples (100 μL) were drawn from the serosal or mucosal side of the sac, and 110 μL acetonitrile and 10 μL of internal standard were added. The volumes removed were replaced with fresh KRBS. The mixture was then centrifuged at 12000 rpm for 15 min and the supernatant was filtered through a membrane filter (0.45 μm). An aliquot of this supernatant was analyzed in the same way as those described in the paragraph "Enzymatic Degradation of α- and β-SAPG-GGYR and GGYR".

Estimation of Elimination Clearance (CL_{el}) and Permeation Clearance (CL_p)—The concentration of glycosylated GGYR on the mucosal side decreased so rapidly that we could not consider it constant. The elimination clearance (CL_{el}) of intact glycosylated GGYR or GGYR per unit length of small intestinal gut was calculated, therefore, according

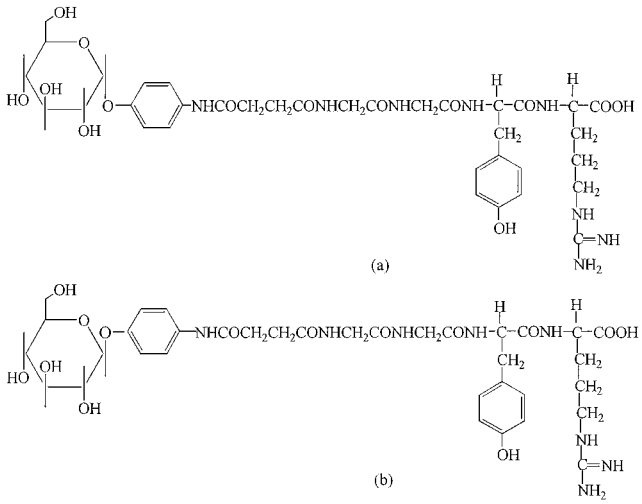

Figure 1—Structures of α-SAPG-GGYR (a) and β-SAPG-GGYR (b).

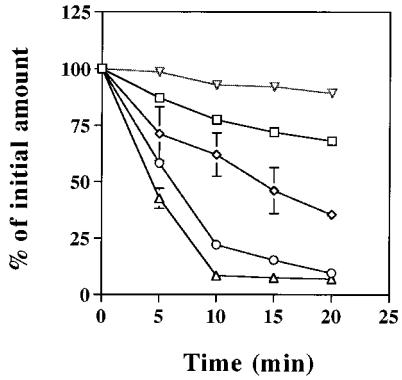

Figure 2—Degradation of GGYR in the presence of BBMVs. A 50 μL sample of BBMVs suspension (5 mg of proten/mL) was mixed with 150 μL of 5 mM GGYR solution and the mixture was incubated at 37 °C. At set time, 250 μL of acetonitrile and 50 μL of internal standard (tryptophan) were added, and the mixture was centrifuged at 12000 rpm for 15 min. The supernatant was analyzed by HPLC after the filtration with a membrane filter (0.45 μm). The results are given as means ± SE ($n = 4$). Key: (△) control; (□) amastatin (33.3 μM); (◇) phosphoramidon (33.3 μM); (○) chymostatin (33.3 μM); (▽) amastatin + phosphoramidon + chymostatin (33.3 μM each).

to the method of Taki et al.,[24] using an area under the curve (AUC) of mucosal concentration vs time plot from time 0 to 80 min and the eliminated amount as follows:

$$CL_{el} = (\text{eliminated amount in the mucosal side solution})/AUC_{0-80} \quad (1)$$

Similarly, the permeation clearance CL_p of intact glycosylated GGYR or GGYR was calculated using eq 2.

$$CL_p = (\text{amount that permeated to the serosal side solution})/AUC_{0-80} \quad (2)$$

3. Results and Discussion

3.1. Enzymatic Degradation of α- and β-SAPG-GGYR—The glycosylated GGYRs used in this study were confirmed as α- and β-SAPG-GGYR by the analytical methods described in Materials and Methods. The structures of these compounds are shown in Figure 1.

The degradation of GGYR in the presence of BBMVs is shown in Figure 2. GGYR was degraded so rapidly that only 7% of the initial amount remained 10 min after the start of incubation. When GGYR was incubated with BBMVs in the presence of 33.3 μM amastatin, a known potent aminopeptidase inhibitor, about 80% of intact GGYR remained 10 min after the start of incubation. It was reported[1] that 20 μM amastatin inhibited hydrolysis of Leu-enkephalin, YGGFL, by over 90%. When GGYR was incubated with 33.3 μM phosphoramidon or 33.3 μM chymostatin, about 60% and 20% of the initial amount remained 10 min after the start of incubation, respectively. Phosphoramidon is a potent inhibitor of brush-border endopeptidase, and chymostatin is an inhibitor of chymotrypsin which will very likely cleave GGYR between Y and R. In addition, more than 90% of the initial amount remained 20 min after the start of incubation when GGYR was incubated with BBMVs in the presence of 33.3 μM each of amastatin, phosphoramidon, and chymostatin. It was reported that the brush-border membrane of intestinal mucosal cells contains various endo- and exopeptidases such as aminopeptidases, dipeptidylaminopeptidase, carboxypeptidase, and angiotensin-converting enzyme (dipeptidyl carboxypeptidase).[25] The effective inhibition of GGYR degradation by amastatin and phosphoramidon indicated that GGYR was mainly degraded by aminopeptidase and partially degraded by endopeptidase. The degradation of α- and β-SAPG-GGYR is shown in Figure 3. The remaining amounts of α- and β-SAPG-GGYR were 90.8 and 93.5%, respectively, 10 min after incubation started, while the remaining amount of GGYR in the presence of 33.3 μM amastatin was 77.5%. These findings indicated that glycosylation at the α-amino position of GGYR increased its resistance to aminopeptidase degradation. These results also indicated that glycosylation might increase resistance not only to the degradation of aminopeptidase but also, to some extent, to the degradation of endopeptidase.

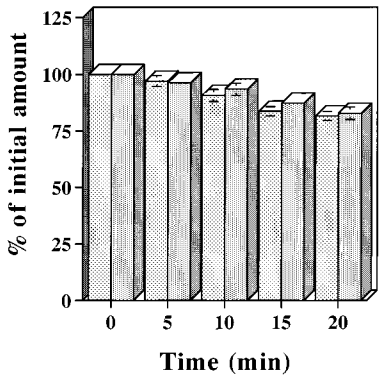

Figure 3—Degradation of α- and β-SAPG-GGYR in the presence of BBMVs. A 50 μL sample of BBMVs suspension (5 mg of protein/mL) was mixed with 150 μL of 5 mM α- or β-SAPG-GGYR solution and the mixture was incubated at 37 °C. At set time, 250 μL of acetonitrile and 50 μL of internal standard (tryptophan) were added, and the mixture was centrifuged at 12000 rpm for 15 min. The supernatant was analyzed by HPLC after the filtration with a membrane filter (0.45 μm). The results are given as means ± SE ($n = 4$). Key: (light gray) α-SAPG-GGYR; (dark gray) β-SAPG-GGYR.

3.2. Uptake of D-Glucose and α- and β-SAPG-GGYR—We studied the uptake of D-glucose into BBMVs by the rapid filtration method as a control experiment to check the function of a Na^+-dependent D-glucose transporter. Overshoot uptake was observed about 1 min after incubation started in the presence of an inward gradient of Na^+ at 37 °C (data not shown). We considered from this finding that BBMVs with Na^+-dependent D-glucose transporters were properly prepared. The mechanism of transport of glycosylated peptide into BBMVs was then studied. Since the uptake values represent the sum of transport into the intravesicular space and the nonspecific binding of the peptide to the external membrane surface, the amount of

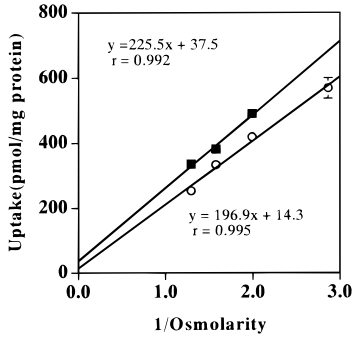

Figure 4—Effect of osmolarity on the uptake of α- and β-SAPG-GGYR by BBMVs. The ^{125}I-labeled peptide solution (195 μL) was mixed with an equal volume of 32 mM Tris–20 mM Hepes-HCl buffer solution containing 30 mM KCl, 200 mM NaCl, and 540 mM mannitol (pH 7.5). This mixture (390 μL) and the BBMVs suspension (130 μL) were mixed and incubated at 37 °C. The osmolarity of this mixed solution was 502 mOsm. Hyperosmolar and hypoosmolar buffers (350, 635, and 773 mOsm) were prepared by adjusting the amount of D-mannitol added to the solution. The sampling and measurement of radioactivity were described in the experimental section. The results are given as means ± SE (n = 4). Key: (○) α-SAPG-GGYR; (■) β-SAPG-GGYR.

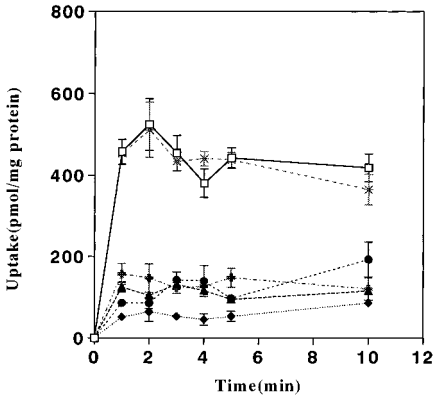

Figure 5—Time-course changes in α-SAPG-GGYR uptake by BBMVs. The ^{125}I-labeled peptide solution (195 μL) was mixed with an equal volume of 32 mM Tris–20 mM Hepes-HCl buffer solution containing 30 mM KCl, 200 mM NaCl, and 540 mM mannitol (pH 7.5) in the uptake experiment with an inward Na^+ gradient or 32 mM Tris–20 mM Hepes-HCl buffer solution containing 230 mM KCl and 540 mM mannitol (pH 7.5) in the uptake experiment with an inward K^+ gradient. This mixture (390 μL) and the BBMVs suspension (130 μL) were mixed (the mixed solution contained 1 mM phloridzin or 7.5 mM GGYR or 10 mM glucose in some experiments) and incubated at 37 °C (or 4 °C). The sampling and measurement of the radioactivity was described in the experimental section. The results are given as means ± SE (n = 4). Key: (□) in the presence of a Na^+ gradient at 37 °C; (◆) in the presence of a Na^+ gradient at 4 °C; (●) in the presence of a K^+ gradient at 37 °C; (▲) in the presence of a Na^+ gradient and 1mM phloridzin at 37 °C; (*) in the presence of a Na^+ gradient and 7.5 mM GGYR at 37 °C; (+) in the presence of a Na^+ gradient and 10 mM glucose at 37 °C.

nonspecific binding was first analyzed by determining the uptake of the peptide as a function of the reciprocal of osmolarity.[26] Figure 4 shows the dependence of α- and β-SAPG-GGYR transport on extravesicular medium osmolarity. Extrapolation of the straight lines for α- and β-SAPG-GGYR to infinite osmolarity gave values of about 15 and 38 pmol/mg of peptide, respectively which represent the amount of nonspecific surface binding. These values represented about 3.6 and 7.5% of each uptake value at 502 mOsm. It was shown from these values that the observed uptake was mainly intravesicular and surface binding was not significant.

Time-course changes in the uptake of α-SAPG-GGYR by BBMVs are shown in Figure 5. As described above, the glycosylation of GGYR inhibited enzymatic degradation as did a mixture of aminopeptidase and endopeptidase inhibitors. We therefore incubated α- or β-SAPG-GGYR with BBMVs without these inhibitors, and overshoot uptake was observed after about 2 min in the presence of an inward Na^+ gradient. This uptake remained unaffected by the addition of 7.5 mM GGYR. Conversely, uptake was significantly inhibited when Na^+ was replaced with K^+ or when BBMVs were incubated with α-SAPG-GGYR at 4 °C. The transport of α-SAPG-GGYR was markedly inhibited either with 1 mM phloridzin or with 10 mM D-glucose. These findings indicated that the Na^+-dependent glucose transporter played an important role in the uptake of α-SAPG-GGYR into BBMVs.

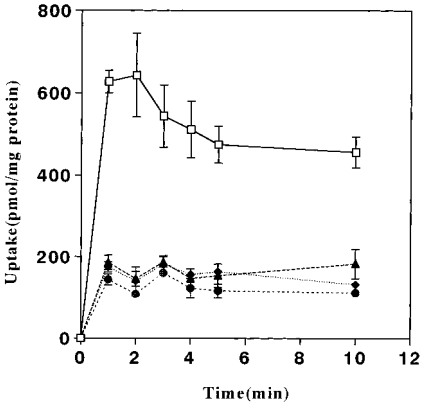

Figure 6—Time-course changes in β-SAPG-GGYR uptake by BBMVs. The experimental conditions were the same as those described in the legend to Figure 5 except that no experiments using GGYR and glucose were performed. The results are given as means ± SE (n = 4). Key: (□) in the presence of a Na^+ gradient at 37 °C; (◆) in the presence of a Na^+ gradient at 4 °C; (●) in the presence of a K^+ gradient at 37 °C; (▲) in the presence of a Na^+ gradient and 1mM phloridzin at 37 °C.

Time-course changes in uptake of β-SAPG-GGYR by BBMVs are shown in Figure 6. Overshoot uptake was observed about 2 min after incubation started in the presence of an inward gradient of Na^+ at 37 °C. The uptake was significantly inhibited when Na^+ was replaced with K^+, when BBMVs were incubated with β-SAPG-GGYR at 4 °C or in the presence of 1 mM phloridzin. These findings showed that β-SAPG-GGYR was also taken up by the Na^+-dependent glucose transporter. To clarify the difference between α- and β-SAPG-GGYR, time-course changes in uptake were compared (Figure 5 and 6) together with the uptake of GGYR in the presence of 33.3 μM each of amastatin, chymostatin, and phosphoramidon (data not shown). The uptake of β-SAPG-GGYR by BBMVs was significantly greater than that of α-SAPG-GGYR at 1 min after the incubation, and GGYR was taken up or associated with BBMVs by about 50% of the value seen for α-SAPG-GGYR, but no overshoot was observed (data not shown). Two types of glucose transporter have been reported so far, namely, facilitated-diffusion transporter and Na^+-coupled active transporter. The former are referred to as GLUT-1 to GLUT-5 and the latter is referred as SGLT-1. SGLT-1 can transport both glucose and galactose and is present at a high level in the intestine.[27] The structual requirements for substrates to interact with SGLT-1 are considered to be (1) the substrate must have a D-pyranose ring configuration, (2) it must possess a C1 chair form, and (3) the hydroxyl group in the glucose molecule at carbon 2 must be in the equatorial position.[28–30] As both α- and β-SAPG-GGYR satisfy these conditions, it is feasible that they may interact with SGLT-1 and be actively taken up by BBMVs or absorbed from rat intestine. It is well-known that phloridzin, which has a β-configuration of glucose, inhibits the transport of α-D-glucosides. This means that the β-form has higher affinity for SGLT-1 than the α-form of glucose. Mizuma et al.[31] studied the intestinal absorption of ano-

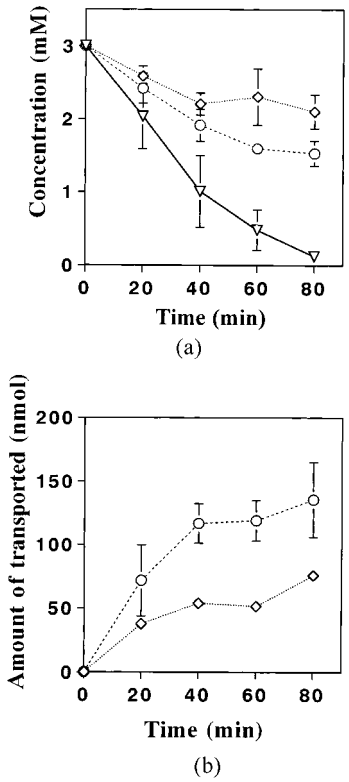

Figure 7—Time-course changes in the elimination of α- and β-SAPG-GGYR on the mucosal side (a) and appearance of intact constituents on the serosal side (b). The sac was filled with 1 mL of KRBS and was placed in a test tube containing 10 mL of 3 mM GGYR or α- or β-SAPG-GGYR solution. Every 20 min, samples (100 μL) were drawn from the serosal or mucosal side of the sac, and 110 μL of acetonitrile and 10 μL of internal standard were added. The volumes removed were replaced with fresh KRBS. The mixture was then centrifuged at 12000 rpm for 15 min and the supernatant was filtered through a membrane filter (0.45 μm). The HPLC analysis was described in the experimental section. The results are given as means ± SE ($n = 4$). Key (▽) GGYR; (◇) α-SAPG-GGYR; (○) β-SAPG-GGYR.

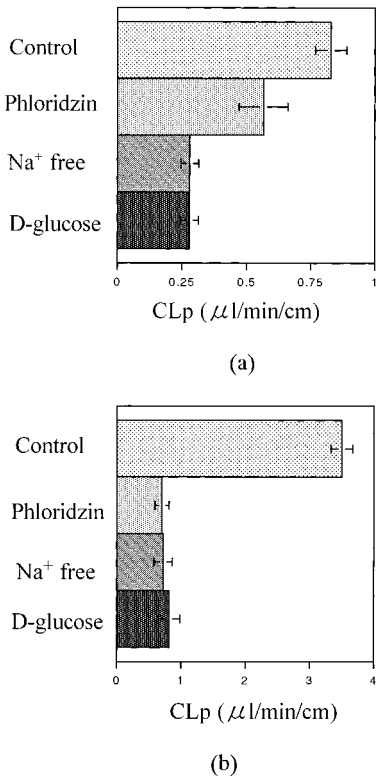

Figure 8—Permeation clearance (CL_p) of α-SAPG-GGYR (a) and β-SAPG-GGYR (b). The amount of intact α- or β-SAPG-GGYR that permeated to the serosal side solution during 80 min in the presence of 10 mM of phloridzin, 10 mM of D-glucose and in the absence of Na^+ was determined in the same way as those described in the legen to Figure 7, and the permeation clearance (CL_p) was calculated according to eq 2. The results are given as means ± SE ($n = 4$).

mers of glucosides and showed that the affinity of the β-anomer for SGLT-1 was higher than that of the α-anomer of glucosides. Our finding described above was in accordance with this result.

3.3. Permeation of α- and β-SAPG-GGYR through Intestinal Everted Sac—To study further the mechanism of transport of glycosylated GGYR across the intestinal tract, the everted sac technique was employed. Time-course changes in elimination of glycosylated GGYR on the mucosal side and the appearance of intact constituents on the serosal side are shown in Figure 7a,b. As GGYR was eliminated quickly from the mucosal side and no intact GGYR was detected on the serosal side, it is evident that GGYR was degraded enzymatically on the mucosal side. Both α- and β-SAPG-GGYR were eliminated relatively slowly on the mucosal side and intact α- and β-SAPG-GGYR permeated to the serosal side. These findings showed that glycosylation of GGYR increased its stability to enzymatic degradation and facilitated its transport through the intestinal membrane. The amount of β-SAPG-GGYR eliminated from the mucosal side was greater than that of α-SAPG-GGYR and the amount of β-SAPG-GGYR that permeated to the serosal side was about twice that of α-SAPG-GGYR. The fractions of α- and β-SAPG-GGYR across the everted sac as compared to the amount introduced at the mucosal side were about 0.25 and 0.45%, respectively. To express elimination and permeation more quantitatively, we estimated the elimination clearance (CL_{el}) and permeation clearance (CL_p) according to eqs 1 and 2. The CL_{el} and CL_p values were 15.8 ± 6.83 and 0.83 ± 0.06 μL/min/cm for α-SAPG-GGYR, and 44.5 ± 3.61 and 3.50 ± 0.81 μL/min/cm for β-SAPG-GGYR, respectively. The amount of intact constituent eliminated from the mucosal side is considered to be the sum of the amount that degraded on the mucosal side and the amount that permeated to the serosal side. Although we did not calculate the total amount that permeated including metabolites which were degraded enzymatically during transport through the intestinal membrane and on the serosa, degradation clearance (CL_d) for β-SAPG-GGYR was greater than that for α-SAPG-GGYR (data not shown). These findings suggested that α-SAPG-GGYR was more stable to enzymatic degradation than β-SAPG-GGYR. In addition, a considerable amount of metabolite GGYR was detected on the serosal side solution both for α- and β-SAPG-GGYR. The amount of GGYR 80 min after the start of the permeation experiment was about 100 and 200 nmol for α- and β-SAPG-GGYR (data not shown). Since GGYR itself did not permeate to the serosal side because of enzymatic degradation and very low permeability, it was considered that α- or β-SAPG-GGYR was bioactivated to the parent peptide during transport through the intestinal membrane of the serosa. Bioactivation following the absorption step is one important issue in the use of the prodrug principle for improving epithelial delivery of peptides.

We then studied the contribution of the Na^+-dependent D-glucose transporter to the absorption of α- and β-SAPG-GGYR. Permeation clearances of α- and β-SAPG-GGYR in the presence of 10 mM of phloridzin, 10 mM D-glucose and in the absence of a Na^+ gradient are shown in Figure 8a,b. Permeation of α-SAPG-GGYR was not markedly inhibited in the presence of phloridzin ($CL_p = 0.55$ μL/min/cm), while in the presence of D-glucose and in the absence

of Na^+ it was significantly inhibited (CL_p = 0.28, 0.285 μL/min/cm). In the case of β-SAPG-GGYR, CL_p was significantly decreased in the presence of phloridzin and D-glucose and in the absence of Na^+. These findings showed that both α- and β-SAPG-GGYR were transported by the Na^+-dependent D-glucose transporter. However, the finding that permeation of α-SAPG-GGYR was slightly inhibited by phloridzin suggested the possibility that this α-anomer was actively transported by the Na^+-dependent D-glucose transporter with low affinity for β-D-glucose. Ohnishi et al. have reported that there are two kinds of transporter which have high and low affinity for β-D-glucoside.[32]

To clarify the difference in absorption between α- and β-SAPG-GGYR, we calculated the permeation clearance transported by the Na^+-dependent D-glucose transporter $(CL_p)_{Na+}$ by subtracting the CL_p value in the absence of Na^+ from that in the presence of Na^+. The $(CL_p)_{Na+}$ was 2.8 μL/min/cm for β-SAPG-GGYR and was about 5 times greater than that for α-SAPG-GGYR (0.5 μL/min/cm). Since the β-form has higher affinity for SGLT-1 than the α-form of glucose, as described above, a large amount of β-SAPG-GGYR might be transported to the serosal side.

4. Conclusion

It was shown from the study using BBMVs and the everted sac method that glycosylation at the α-amino position of GGYR increased its resistance to aminopeptidase and inhibited its degradation and that the Na^+-dependent glucose transporter played an important role in the intestinal absorption of both α- and β-SAPG-GGYR. It was also indicated that the amount of β-SAPG-GGYR transported was greater than that of α-SAPG-GGYR. The finding that a tetrapeptide was transported by the Na^+-dependent glucose transporter may be useful in the improvement of intestinal absorption of peptide drugs which show poor membrane penetrability.

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