Carrier-Mediated Transport Systems for Glucose in Mucosal Cells of the Human Oral Cavity

YUJIRO OYAMA, HITOSHI YAMANO, AKIKO OHKUMA, KEN-ICHI OGAWARA, KAZUTAKA HIGAKI, AND TOSHIKIRO KIMURA*

Contribution from *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1 Tsushima-naka, Okayama 700-8530, Japan.*

Received July 22, 1998. Final revised manuscript received January 8, 1999.
Accepted for publication May 27, 1999.

Abstract □ The in vitro uptake study was performed using the isolated cells of human oral mucosa, buccal and the dorsum of the tongue, to investigate the mechanisms of glucose uptake. The uptake of D-glucose was much larger in cells of the dorsum of the tongue than in buccal cells and was inhibited more extensively by 2-deoxy-D-glucose, a substrate of facilitative glucose transporters, than by α-methyl-D-glucoside, a specific substrate of SGLT1, suggesting the larger contribution of a facilitative transporter than Na^+/glucose cotransporter. Furthermore, from the results of inhibition studies by several sugar analogues including maltose and D-mannose, GLUT1 and/or GLUT3 were suggested to take part in the glucose uptake by oral mucosa. Therefore, we have attempted to confirm the expression of glucose transporters on the oral mucosa by employing Western blotting. As a result, it was suggested that SGLT1, GLUT1, GLUT2, and GLUT3 are expressed in the epithelial cells of human oral mucosa.

Introduction

Oral mucosa has been used as a site for drug delivery, because the drug thus administered reaches systemic circulation circumventing both intra-alimentary canal and hepatic first-pass eliminations.[1−3] Generally, the mechanism of drug absorption from the oral cavity is passive diffusion.[4,5] On the other hand, not only some nutrients, D-glucose,[6] amino acid,[7] glutathione,[8] thiamine,[9] and nicotinic acid,[10] but also an aminocephalosporin antibiotic cefadroxil[11] were reported to be absorbed by specialized transport mechanisms from the human oral cavity, using a buccal absorption test method.[4] Recently, we developed perfusion cells, which can be applied on five regions in the human oral cavity, the dorsum of the tongue, ventral surface of the tongue, labial mucosa, floor of mouth, and buccal mucosa,[12] and could show the presence of the specialized transport system for D-glucose in the dorsum of the human tongue.[13] D-Glucose is transported into the enterocytes across the brush border membrane by a Na^+/glucose cotransporter (SGLT1), and then the hexose is transported out across the basolateral membrane by a facilitative sugar transporter (GLUT2).[14,15] Furthermore, some glucose transporters have been found in mammalian tissues.[16] However, details of sugar transporters present in oral epithelial cells have not been clarified.

In the present study, the mechanism of D-glucose uptake by epithelial cells isolated from human buccal mucosa and the dorsum of the tongue was examined to clarify the glucose transport system in the human oral cavity.

* Corresponding author. Tel (+81)-86-251-7948. Fax (+81)-86-251-7926. E-mail: kimura@pheasant.pharm.okayama-u.ac.jp.

Materials and Methods

Materials—^{3}H-D-Glucose, ^{14}C-L-glucose, and ^{14}C-2-deoxy-D-glucose were purchased from Amersham International (Buckinghamshire, England). A liquid scintillator, Clear-Sol I, was purchased from Nacalai Tesque (Kyoto, Japan). D-Glucose (Ishizu Pharmaceutical Co., Osaka, Japan), L-glucose (Tokyo Kasei Kogyo Co., Tokyo, Japan), 2-deoxy-D-glucose, and fluorescein isothiocyanate dextran (FITC-dextran, MW 35600, Sigma Chemical Co., St. Louis, MO) were used as supplied. Iatro-Chrom GLU-L_Q and Glucose-Test Wako were purchased from Iatron Laboratories, Inc. (Tokyo) and Wako Pure Chemical Industries (Osaka), respectively. Rabbit anti-SGLT-1, rabbit anti-GLUT-1, rabbit anti-GLUT-2, and rabbit anti-GLUT-3 were obtained from Chemicon International (Temecula, CA). Prestained molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE) used in this study were phosphorylase (102 kDa), bovine serum albumin (BSA) (78 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (34.2 kDa), soybean trypsin inhibitor (28.3 kDa), and lysozyme (19.9 kDa) (Bio-Rad Laboratories, Hercules, CA). All other chemicals were regent grade and commercially available.

Subjects—Ten healthy volunteers, aged 22−25 years, participated in this study. Written informed consent, in accordance with the principles of the Declaration of Helsinki, was obtained from each volunteer prior to the study.

Preparation of Oral Mucosal Cells—Epithelial cells were isolated from the buccal mucosa or the dorsum of the tongue of volunteers using a mouthwash technique.[17] Briefly, mucosal cells were obtained on the morning of assay. Subjects swirled high-purity water around their mouth for a short period of time in conjunction with molar scraping action for buccal mucosa or the dorsum of the tongue. This procedure was repeated several times. The pooled expectorate containing mucosal cells was filtered through three layers of 250 μm nylon mesh, centrifuged at 3000 rpm for 10 min, and gently resuspended in the incubation buffer. The composition of the incubation buffer was 100 mM mannitol, 80 mM NaCl, 20 mM Tris-HCl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mg/mL bovine serum albumin (BSA). The cell suspension was centrifuged again at 3000 rpm for 10 min and resuspended in the incubation buffer at the concentration of 1×10^6 cells/mL.

Uptake Experiments—The uptake of the substrate was determined by a rapid filtration technique.[18] Uptake studies were initiated by the addition of 0.4 mL of a buffer solution containing a labeled substrate to 0.1 mL of the cell suspension, which was preincubated at 37 °C for 30 min. At the desired times, 4 mL of ice-cold stop solution (100 mM mannitol, 100 mM $MgCl_2$, 8 mM HEPES, 4 mM Tris and 1 mM NaCl, pH 7.4) was added to the mixture. The resulting mixtures were immediately filtered through prewetted 0.45 μm filters (Fuji Photo Film, Tokyo). The filters were quickly rinsed with 4 mL of the ice-cold stop solution three times and transferred into a counting vial for the determination. Background value or nonspecific adsorption to the filter was determined by using the incubation buffer without mucosal cells. This value was subtracted from the uptake data.

Absorption Experiments (Buccal Absorption Test)—The experiments were carried out according to the method of Kurosaki et al.[11] which was originated by Beckett and Triggs.[4] Briefly, a 2-deoxy-D-glucose solution (10 mL) containing an unabsorbable marker, FITC-dextran (1 μM), was placed in the mouth. The

10.1021/js980298f CCC: $18.00
Published on Web 07/03/1999
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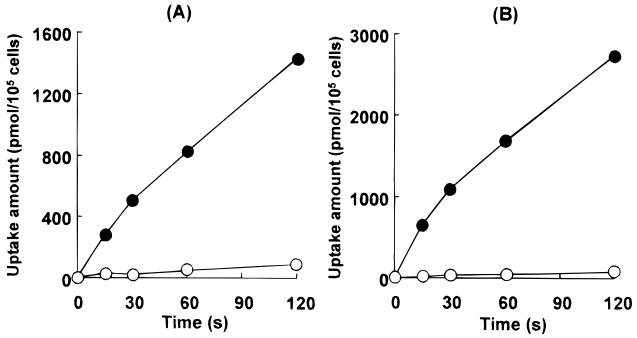

Figure 1—Uptake of D- and L-glucose by isolated cells of human buccal mucosa (A) and dorsum of tongue (B). Initial concentration of glucose was 0.1 mM. Key: ●, D-glucose; ○, L-glucose. Results are expressed as the mean with a vertical bar showing the SE of four experiments. The bars are hidden behind symbols.

solution in the mouth was vigorously agitated using the cheeks and tongue for 5 min, and then the solution was expelled as completely as possible. The subject quickly rinsed his mouth three times with the same buffer solution (total volume, 20 mL) and expelled the rinsing. The expelled solutions were combined and were adjusted to 50 mL with the buffer solution. From the values of the initial and final concentrations of 2-deoxy-D-glucose and the marker, percentage of 2-deoxy-D-glucose absorbed during a period of 5 min was calculated.

Analytical Methods—For uptake studies, radioactivity was measured by a Beckman LS-232 liquid scintillation counter. For absorption studies, D-glucose and 2-deoxy-D-glucose were determined using commercially available assay kits, Iatro-Chrom GLU-L_Q (glucose oxidase method). L-Glucose was determined using commercially available assay kits, Glucose-Test Wako (*o*-toluidine-boric acid method). FITC-dextran was determined spectrofluorometrically (ex 495 nm, em 514 nm).

SDS–Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS–PAGE was performed using a 12% polyacrylamide gel for protein separation. Cells suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 1 μM aprotinin were disrupted by Ultrasonic Generator US300 (Nissei, Tokyo) for 30 min, and the suspension was centrifuged at 200*g* for 30 min. The supernatant fraction was centrifuged at 27000*g* for 1 h. The precipitate was resuspended in 10 mM Tris-HCl buffer (pH 7.4) containing 2% SDS, 2% Triton X-100, and 2 mM EDTA to dissolve membrane proteins. The protein samples were loaded on top of a 4.5% stacking gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes. Remaining active sites on the nitrocellulose membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline for 1 h at room temperature on a flat bed shaker. Membranes were incubated overnight at 4 °C with rabbit anti-human antibodies against SGLT1, GLUT1, GLUT2, or GLUT3 under constant agitation, and then incubated further for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Horseradish peroxidase activity was revealed using ECL Western blotting detection reagents (Amersham International).

Statistical Analysis—Statistical significance was evaluated using Student's *t*-test or analysis of variance. Results are expressed as mean ± SE of more than three experiments.

Results

Isolated Mucosal Cells—The photomicroscopic observation of the mucosal cells stained with hematoxylin–eosin revealed that cells isolated from the dorsum of the tongue contain cornified cells, but cells from buccal mucosa do not. The specific character of both sites was observed. The viability of the cells was maintained as 80–90% even 3 h after the isolation.

Uptake of Glucose by Isolated Mucosal Cells—Figure 1 shows the time course of glucose uptake by the cells isolated from buccal mucosa (A) and the dorsum of the tongue (B). As is evident from the figure, the uptake

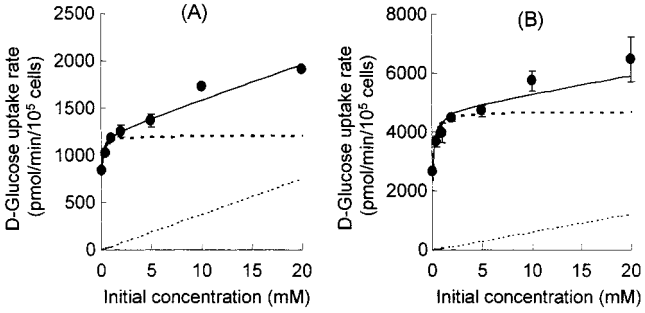

Figure 2—Concentration dependency of D-glucose uptake by isolated cells of human buccal mucosa (A) and dorsum of tongue (B). Results are expressed as the mean with a vertical bar showing the SE of three experiments. —, fitting curve; ••••, uptake via carrier-mediated system; ----, uptake via passive diffusion (uptake rate of L-glucose).

of D-glucose was much greater than that of L-glucose in both cells, and stereospecificity was observed. Furthermore, the uptake by cells from the dorsum of the tongue was greater than that from buccal mucosa.

Figure 2 shows the concentration dependency of the initial rate (in 1 min) of D-glucose uptake by the cells isolated from buccal mucosa (A) and the dorsum of the tongue (B). The kinetic parameters for D-glucose uptake were calculated by fitting these data to the eq 1.

$$v = V_{max}C/(K_m + C) + k_dC \qquad (1)$$

where v is the initial uptake rate at the concentration C, V_{max} and K_m are the maximum uptake rate and Michaelis constant for the specialized transport system, respectively, and k_d is the first-order rate constant for the uptake by simple diffusion. The initial uptake rate constants for L-glucose were used as k_d values: they were 37.7 ± 6.7 nL/min/10^5 cells for cells of buccal mucosa and 61.8 ± 7.8 nL/min/10^5 cells for cells from the dorsum of the tongue. The kinetic parameters for D-glucose uptake thus obtained were as follows: V_{max} and K_m values for cells of buccal mucosa are 1207.4 ± 124.2 pmol/min/10^5 cells and 51.2 ± 47.8 μM, respectively; on the other hand, those for cells from the dorsum of the tongue are 4696.3 ± 132.0 pmol/min/10^5 cells and 97.7 ± 19.8 μM, respectively. The statistical analysis by ANOVA clarified that V_{max} and k_d values in the two cells are significantly different while K_m values are not.

To investigate the characteristics of the glucose transport system, the effect of some transport inhibitors and specific conditions on the uptake by isolated oral mucosal cells were examined, and the results are shown in Table 1. In both mucosal cells, D-glucose uptake at the concentration of 0.05 mM was markedly reduced in the ice-cold condition or by 0.5 mM *p*-chloromercuribenzoic acid (PCMB), suggesting that the uptake is a carrier-mediated process. Since D-glucose uptake was significantly reduced in the Na^+-free condition, it is suggested that a part of this uptake system is Na^+-dependent. The significant inhibition by 1 mM 2,4-dinitrophenol and the slight inhibition, but not statistically significant, by 1 mM phlorizin were observed. These results suggest that a Na^+-dependent D-glucose transport system is operating, at least partly, in the uptake process. To further clarify the carrier system, the effect of various sugars (50 mM) on D-glucose uptake (1 mM) was examined, and the results are also shown in Table 1. α-Methyl-D-glucoside, a specific substrate of SGLT1, significantly inhibited the D-glucose uptake in both mucosal cells. The uptake of D-glucose was inhibited more extensively by substrates of facilitative transporters, 2-deoxy-D-glucose, D-mannose, and maltose. Figure 3 shows the comparison of the inhibitory effects on D-glucose uptake by isolated mucosal cells between α-methyl-D-glucoside and 2-deoxy-

Table 1—Effect of Special Conditions, Transport Inhibitors, and Sugar Analogues on D-Glucose Uptake by Cells Isolated from Human Buccal Mucosa and Dorsum of Tongue[a]

condition or additive	uptake rate (% of control)	
	buccal	dorsum of tongue
control	100.0 ± 4.1	100.0 ± 1.7
ice-cold	6.9 ± 1.4***	3.7 ± 0.5***
Na^+-free	77.4 ± 4.3**	87.3 ± 2.2***
with 0.5 mM *p*-chloromercuribenzoic acid	45.8 ± 0.6***	47.2 ± 2.6***
with 1 mM 2,4-dinitrophenol	78.5 ± 2.1**	80.3 ± 2.3***
with 1 mM phlorizin	86.2 ± 2.5	72.4 ± 2.4***
with 50 mM mannitol	100.0 ± 2.7	100.3 ± 2.9
with 50 mM α-methyl-D-glucoside	40.3 ± 1.4***	34.3 ± 2.3***
with 50 mM 2-deoxy-D-glucose	7.4 ± 0.8***	7.0 ± 0.5***
with 50 mM D-mannose	11.1 ± 0.2***	22.8 ± 3.8***
with 50 mM maltose	6.0 ± 0.9***	5.6 ± 0.4***

[a] Concentration of D-glucose was 0.05 mM. Control values for buccal cells and cells of dorsum of the tongue were 121.3 ± 5.0 and 250.2 ± 4.3 pmol/min/10^5 cells, respectively. Results are expressed as the mean± SE of 3–8 experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared with each control value.

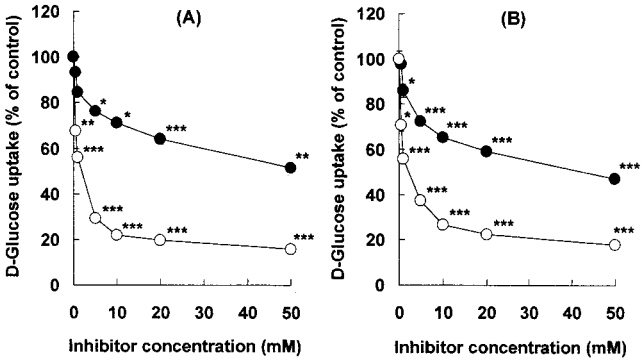

Figure 3—Effect of specific sugars on D-glucose uptake by isolated cells of human buccal mucosa (A) and dorsum of tongue (B). Initial concentration of D-glucose was 0.1 mM. Key: ●, with α-methyl-D-glucoside; ○, with 2-deoxy-D-glucose. Results are expressed as the mean with a vertical bar showing the SE of four experiments. The bars are hidden behind symbols. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$, compared with the control.

D-glucose. As is evident from the figure, the inhibitory effect of 2-deoxy-D-glucose is greater than that of α-methyl-D-glucoside, suggesting that the contribution of the facilitative glucose transporters to D-glucose uptake by oral mucosal cells is larger than that of active Na^+/glucose cotransporter.

To further analyze the facilitative glucose transport system, similar uptake studies by isolated cells from buccal mucosa and the dorsum of the tongue were performed for 2-deoxy-D-glucose. Figure 4 shows the concentration dependency of the initial rate of 2-deoxy-D-glucose. Kinetic parameters for 2-deoxy-D-glucose were similarly calculated using the eq 1 shown above, and the results are as follows: V_{max}, K_m, and k_d values for cells of buccal mucosa are 417.9 ± 167.8 pmol/min/10^5 cells, 0.555 ± 0.705 mM, and 29.3 ± 11.2 nL/min/10^5 cells, respectively; on the other hand, those for cells from the dorsum of the tongue are 965.0 ± 115.7 pmol/min/10^5 cells, 0.619 ± 0.263 mM, and 69.1 ± 4.6 nL/min/10^5 cells, respectively. The k_d values thus obtained were similar to the initial uptake rate constants of L-glucose, which were shown above, in both cells. The statistical analysis by ANOVA clarified that both V_{max} and k_d values are significantly different between the two cells, while the difference of K_m value was not statistically significant. Table 2 shows the effect of ice-cold conditions, PCMB, or other sugars on 2-deoxy-D-glucose uptake by oral

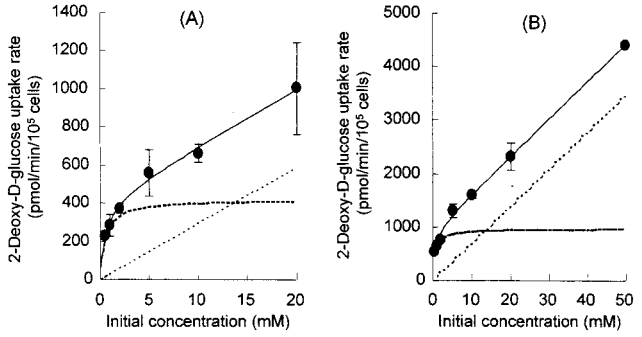

Figure 4—Concentration dependency of 2-deoxy-D-glucose uptake by isolated cells of human buccal mucosa (A) and dorsum of tongue (B). Results are expressed as the mean with a vertical bar showing the SE of three experiments. —, fitting curve; ••••, uptake via carrier-mediated system; ----, uptake via passive diffusion.

Table 2—Effect of Special Conditions, Transport Inhibitors, and Sugar Analogues on 2-Deoxy-D-glucose Uptake by Cells Isolated from Human Buccal Mucosa and Dorsum of Tongue

condition or additive	uptake rate (% of control)	
	buccal	dorsum of tongue
control	100.0 ± 1.0	100.0 ± 1.1
ice-cold	2.6 ± 0.3***	3.8 ± 1.0***
with 0.5 mM *p*-chloromercuribenzoic acid	2.3 ± 0.5***	4.3 ± 0.7***
with 10 mM mannitol	94.8 ± 2.9	97.5 ± 3.0
with 10 mM 2-deoxy-D-glucose	1.6 ± 1.2***	5.2 ± 1.2***
with 10 mM D-mannose	11.1 ± 1.5***	26.2 ± 1.8***
with 10 mM maltose	16.8 ± 1.0***	66.5 ± 0.7***
with 10 mM D-allose	35.5 ± 2.9***	43.1 ± 4.2***

[a] Concentration of 2-deoxy-D-glucose was 0.1 mM. Control values for buccal cells and cells of dorsum of tongue were 152.5 ± 1.8 and 211.8 ± 2.2 pmol/min/10^5 cells, respectively. Results are expressed as the mean± SE of three experiments. *** $p < 0.001$, compared with each control value.

mucosal cells. In both mucosal cells, 2-deoxy-D-glucose uptake at the concentration of 0.1 mM was markedly reduced in ice-cold conditions or by 0.5 mM PCMB. D-Mannose, maltose, and D-allose, sugars transported by the facilitative transporters, inhibited the uptake of 2-deoxy-D-glucose, suggesting that this sugar can be transported by multiple facilitative transporters.

2-Deoxy-D-Glucose Absorption from Oral Cavity—Oral mucosal epithelium is a stratified squamous epithelium with the polarity. In our previous study, we showed that D-glucose is absorbed from oral cavity by carrier-mediated mechanism, a part of which is Na^+-dependent.[13] To investigate whether the facilitative transport system operates on the apical surface of the mucosal cell or not, the absorption of 2-deoxy-D-glucose was examined by buccal absorption test. At the concentration of 1 mM, the absorption rates for 2-deoxy-D-glucose, D-glucose, and L-glucose were 630.9 ± 122.0, 3026.1 ± 91.3, and 198.9 ± 28.4 nmol/5 min, respectively. Although the absorption rate of 2-deoxy-D-glucose is lower than that of D-glucose, the inhibition by D-allose was observed: the absorption rate of 2-deoxy-D-glucose at 0.5 mM (443.2 ± 56.4 nmol/5 min) was inhibited to 267.7 ± 30.6 nmol/5 min ($p < 0.05$) by 5 mM D-allose. Although it was difficult to determine the kinetic parameters for 2-deoxy-D-glucose due to very low absorption (%) of this sugar at the high initial concentration, the expression of the facilitative transporter for D-glucose on the apical surface of the oral mucosal cells was suggested.

Western Blot Analysis of Glucose Transporters Present in Oral Mucosal Membrane—To identify glucose transporters present in oral mucosal membrane, Western blot analysis of the membrane protein was per-

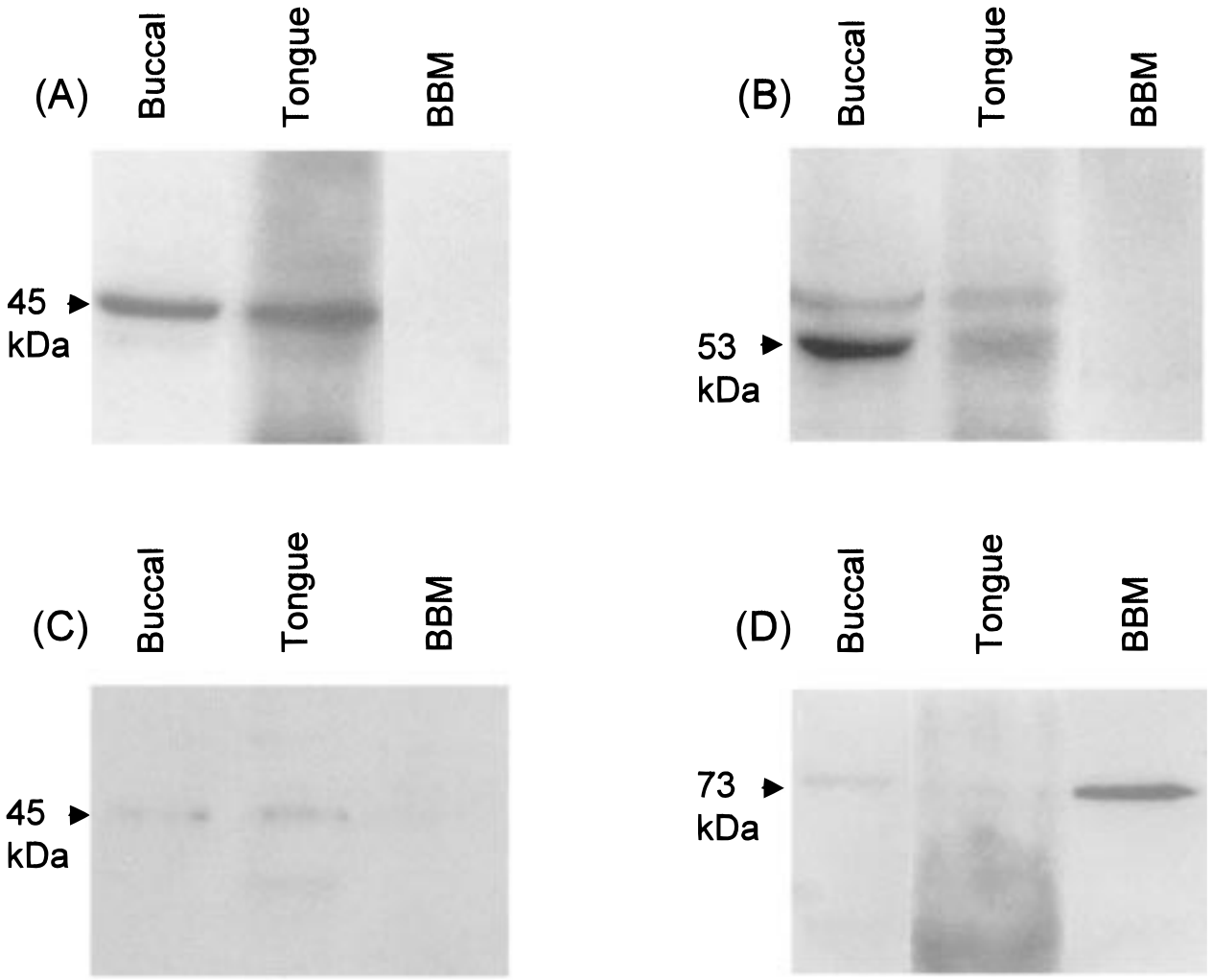

Figure 5—Western blot analysis of glucose transporters in isolated cells of human buccal mucosa, dorsum of the tongue, and brush border membranes of rat small intestine. Protein samples (20–50 μg) were electrophoresed through a SDS–PAGE gel, transferred to a nitrocellulose membrane and incubated with rabbit anti-human antibodies against GLUT1 (A), GLUT2 (B), GLUT3 (C), and SGLT1 (D). The membranes were incubated further with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. Horseradish peroxidase activity was revealed using ECL Western blotting detection reagents.

formed. The brush border membrane prepared from rat small intestine (BBM) was used as the positive control of SGLT1. The results are shown in Figure 5. Although there are some unidentified bands, not only the band for GLUT1 (45kDa[19]) but also the bands for GLUT2 (53kDa[15]), GLUT3 (45kDa[20]) and SGLT1 (73kDa[15]) are found in both buccal mucosal cells and cells of the dorsum of the tongue.

Discussion

D-Glucose is one of the nutrients that can be absorbed from oral cavity by the carrier-mediated mechanism.[6,13] In our previous study, we showed using perfusion cells for human oral mucosa that the carrier-mediated absorption of D-glucose is predominant in the dorsum of tongue.[13] However, details of glucose transporters present in the oral mucosal cells were remaining to be clarified. In the present study, oral mucosal cells were isolated from human buccal mucosa and the dorsum of tongue using a mouth-wash technique[16] and glucose transport systems present in these cells were examined. Lee et al. reported that buccal cells thus obtained containing superficial, intermediate and prickle cells, but not basal cells, are osmotically very stable and did not undergo any discernible swelling.[17] We confirmed that the viability of oral mucosal cells thus prepared was maintained as 80−90% over 3 h after the isolation.

As shown in Figures 1 and 2, stereospecific uptake of D-glucose was observed in both buccal mucosal cells and cells of the dorsum of the tongue. In the perfusion cell study described previously, we could not find the specialized transport mechanism for D-glucose in the buccal mucosa.[13] However, this isolated cell study could clarify that the specialized transport system for D-glucose is present not only in the dorsum of the tongue but also in the buccal epithelium. This discrepancy may be caused by difference in the surface area; that is, the large surface area of isolated mucosal cells could enable them to take up D-glucose more efficiently via the specialized transport mechanism. The result of the inhibition study (Table 1) suggested that, in addition to Na^+/glucose cotransporter, the facilitative glucose transporter is expressed in the oral epithelial cells. Furthermore, it was shown that the contribution of the facilitative transport mechanism to overall D-glucose transport in the oral mucosal cells is more predominant than Na^+-dependent active transport mechanism. The facilitative transport system present in the oral mucosal cells was suggested by the 2-deoxy-D-glucose uptake study (Figure 4, Table 2). D-Mannose, maltose, and D-allose inhibited the uptake of 2-deoxy-D-glucose. D-Mannose is transported by GLUTs1−4.[21,22] Maltose is an inhibitor of glucose transport by GLUT3, but not by GLUT2 or 4.[22] On the other hand, D-allose is an effective inhibitor

of glucose transport by GLUT2, but not by GLUT1, 3, or 4.[22] Thus, multiple facilitative transporters are suggested to be present in the oral mucosal cells. In addition, it was clarified by the buccal absorption test of 2-deoxy-D-glucose that the facilitative transport system is expressed on the apical surface of the stratified squamous epithelium. It has been reported that GLUT1, but not GLUT4, is expressed in human oral mucosa.[23,24] Finally, the result of the Western blot analysis suggested that GLUT1, GLUT2, GLUT3, and SGLT1 are expressed in the oral epithelial membrane (Figure 5). Further studies are necessary to elucidate how the active and facilitative transporters are operating in the stratified squamous epithelium of oral cavity.

As to the kinetic parameters for D-glucose uptake by isolated oral mucosal cells, V_{max} value for the dorsum of the tongue was 2.5 times larger than that for buccal mucosa, while K_m values for both cells were not significantly different. Although these values are pooled parameters of parallel Michaelis−Menten equations, the larger V_{max} value in the dorsum of the tongue suggests that transporters for D-glucose are more abundant in this site. The parameters obtained by 2-deoxy-D-glucose uptake experiments are also pooled values for multiple facilitative glucose transporters, but the larger transport capacity of the facilitative transport system in the dorsum of tongue than in the buccal is suggested, while K_m values in both cells were not significantly different. The reduction of D-glucose uptake rate in the Na^+-free medium and the inhibition of D-glucose uptake by α-methyl-D-glucoside were similar in degree in both mucosal cells, suggesting that Na^+/glucose cotransporter is also expressed more predominantly in the dorsum of tongue than in the buccal.

In conclusion, the in vitro uptake study using the isolated cells of human oral mucosa, buccal and the dorsum of the tongue, revealed that not only the Na^+/glucose cotransporter but also facilitative glucose transporters are expressed in the oral mucosal cells and that the transport system is more abundant in the dorsum of tongue than in the buccal. The glucose transporters were confirmed by the Western blot analysis.

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Acknowledgments

The authors are grateful to Professor T. Tsuchiya and Dr. T. Kuroda, Gene Research Center, Okayama University, for their helpful guidance with respect to the Western blot analysis. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture in Japan.

JS980298F