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THE ENHANCED D-GLUCOSE UPTAKE IN BRUSH BORDER MEMBRANE VESICLES BY 2,6-DI-O-METHYL-β-CYCLODEXTRIN

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D-glucose uptake in jejunal brush-border membrane vesicles (BBMV) isolated from rats, investigated by a filtration method was found to be significantly elevated in 2 mM 2,6-di-O-methyl-β-cyclodextrin (DM-CD) treated BBMV compared to control BBMV. In the presence of an inwardly directed NaCl gradient, D-glucose uptake in control vesicles at 15s was seven-fold over that of the equilibrium level at 30 min. As for the DM-CD-treated BBMV in the presence of the NaCl gradient, the overshoot magnitude of the D-glucose uptake was much higher than that in the control BBMV. The D-glucose uptake in control and DM-CD-treated BBMV could be described by an equation giving sum of a saturated term displaying Michaelis-Menten kinetics and a passive diffusion term. By using the equation, the calculation of stereospecific Na^+-dependent D-glucose uptake in control BBMV gave an apparent Km and Vmax values of 0.63 (mM) and 0.87 (*nmole/mg protein/15s*), and the DM-CD-treated BBMV showed 0.44 (mM) and 1.79 (*nmole/mg protein/15s*), respectively. There was a significant increase in Vmax value in the DM-CD-treated BBMV compared to the control, but the Km and passive diffusion term showed no significant differences. The membrane fluidity of the lipid layer and cholesterol content of BBMV were remarkably changed by DM-CD-treatment, indicating that the microenviromental conditions around the glucose carrier in the BBMV may be altered by DM-CD-treatment.

Методом фильтрации выявлено, что поглощение D-глюкозы в мембранах крайных ворсинок у везикул (МКВВ) тощей кишки, выделенных из крыс, значительно повышается при их обработке 2мМ раствором 2,6-ди-О-метил-β -циклодекстрина (ДМ-ЦиД), чем в контрольных необработанных вариантах. При наличии внутренне контролируемого градиента NaCl поглощение D-глюкозы в контрольных везикуллах было 7-раз больше за 15 сек, чем это показание при уровне равновесия за 30 мин. При градиенте NaCl в ДМ-ЦиД обработанных МКВВ вариантах величина связывания D-глюкозы намного выше, чем в контрольных МКВВ вариантах. Поглощение D-глюкозы в контрольных и ДМ-ЦиД обработанных МКВВ вариантах можно описать уравнением, показывающим сумму насыщенного периода по кинетике Михаэлиса-Ментена и пассивного диффузионного периода. С помощью уравнения рассчитаны значения Km и Vmax для Na^+-зависимого связывания D-глюкозы, что составляет 0,63(мМ) и 0,87 (нмоль/мг белка/15сек) в контрольных и 0,44(мМ) и 1,79 (нмоль/мг белка/15сек) в ДМ-ЦиД обработанных МКВВ вариантах, соответственно. Наблюдается значительное повышение значения Vmax в ДМ-ЦиД в обработанных МКВВ вариантах по сравнению с контрольными вариантами, но значения Km и периода пассивной диффузии отличаются незначительными разницами. Текучесть липидного слоя и содержание холестерола в МКВВ заметно менялись при ДМ-ЦиД обработке, указывая, что условия микросреды вокруг носителя глюкозы в МКВВ могут быть изменены при обработке ДМ-ЦиД.

Ֆիլտրացիայի մեթոդով բացահայտվել է, որ կապումը D-գլյուկոզի կլանումը առնետից անջատված բարակ աղիքի վեզիկուլների սահմանային թավիկների մեմբրաններում (ՎՍԹՄ), բավականին բարձրանում է դրանց 2,6-դի-O-մեթիլ- β -ցիկլոդեքստրինի (ԴՄ-ՑիԴ) 2մՄ լուծույթով մշակելիս, քան ստուգիչ չմշակված նմուշներում: NaCl-ի ներքին կարգավորող գրադիենտի առկայությամբ D-գլյուկոզի կլանումը ստուգիչ վեզիկուլներում եղել է 7 անգամ ավելի 15 վրկ ընթացքում, քան այդ

ցուցանիշը հավասարության մակարդակի դեպքում 30 րոպեի ընթացքում: NaCl-ի գրադիենտի դեպքում ԴՄ-ՑիԴ-ով մշակված ՎՍՄԹ-ի նմուշներում D-գլյուկոզի կլանման արժեքը բավականին բարձր է, քան ՎՍՄԹ-ի ստուգիչ նմուշներում: D-Գլյուկոզի կլանումը ստուգիչ և ԴՄ-ՑիԴ-ով մշակված ՎՍՄԹ-ի նմուշներում կարելի է նկարագրել հավասարմամբ, որը ցույց է տալիս Միխաելիս-Մենտենի կինետիկայի հագեցման ժամանակահատվածի և պասսիվ դիֆուզիայի ժամանակահատվածի գումարը: Հավասարման միջոցով հաշվարկել են Na+-ից կախված D-գլյուկոզի կլանման Km և Vmax արժեքները, որը կազմում է 0,63 (մՄ) և 0,87 (նմոլ/մգ սպիտակուց/15վրկ) և 0.44(մՄ) և 1,79 (նմոլ/ մգ սպիտակուց/15վրկ) ՎՍԹՄ-ի ստուգիչ և ԴՄ-ՑիԴ-ով մշակված նմուշներում համապատասխանաբար: Դիտվում է Vmax արժեքի բավականին բարձրացում ԴՄ-ՑիԴ-ով մշակված ՎՍԹՄ-ի նմուշներում, համեմատած ստուգիչ նմուշների հետ, բայց Km-ի և պասսիվ դիֆուզիայի ժամանակահատվածի արժեքները զանազանվում են չնչին տարբերությամբ: Լիպիդային շերտի հոսունությունը և խոլեստերոլի քանակը ՎՍԹՄ-ում նկատելիորեն փոփոխվում են ԴՄ-ՑիԴ-ով մշակելիս, որը ցույց է տալիս, որ միկրոմիջավայրի պայմանները ՎՍԹՄ-ում գլյուկոզի կրիչի շուրջը կարող են փոփոխվել ԴՄ-ՑիԴ-ով մշակելիս:

Introduction

D-glucose is actively cotransported with sodium across the intestinal brush-border membranes (BBM) via carrier- mediated process. It has been demonstrated that alterations in the lipid composition and /or physical state of the plasma membranes of intestinal cells can influence the activity of several important enzymes and transport systems located in these membranes [1]. The intestinal uptake of D-glucose was shown to be increased by the manipulation of dietary fatty acids [2] and by enzyme-inducing agents, [3] whereas it was decreased by drugs [4,5] and by some kind of disease [6,7]. Further, the increased D-glucose uptake was modified by manipulation of dietary macronutrients [8]. Thus, there seems to be association between alterations in membrane fluidity and changes in transport functions, although this association appears to vary according to membrane components [9-11].

Cyclodextrins (CD), a cyclic oligosaccharide consisting of 1,4-linked glucose units, form inclusion complexes with hydrophobic drugs. α, β and γ-CD have different internal cavity diameters, each showing a different formation constant with different-sized guest molecules. CD-membrane interactions have been reported: CD induced morphological changes and hemolytic activity in human erythrocytes [12] and enhanced the leakage of calcein from liposomes [13]. Although, the effects of CD on artificial membranes or red cell membranes are thought to be due to an interaction with membrane lipid components, the direct action of CD on biological membranes at the absorption site of drugs has not yet been completely clarified. We have reported that the membrane permeability for drug penetrated by a passive diffusion across the intestinal membrane was remarkably enhanced by β-CD [14, 15] That was due to the interaction between the membrane lipid components and CD.

The present study was undertaken to investigate whether 2,6-di-O-methyl-β-cyclodextrin (DM-CD) enhances the carrier mediated D-glucose uptake by BBMV DM-CD, the methylated form of natural β-CD, is more soluble in water than the parent compound. We found that the uptake of D-glucose in BBMV treated with DM-CD was significantly increased compared with uptake in control BBMV. Therefore, the perturbation of lipid layer of BBMV induced by DM-CD caused the enhancement of D-glucose uptake via carrier mediated process.

Experimental

Materials. All reagents were of the highest purity available. 2,6-di-O-methyl-β-cyclodextrin (DM-CD), tris(hydroxymethyl)-aminomethane (Tris) and N-2-hydroxyethylpiperadine-N'-2 - ethanesulfonic acid (Hepes) were purchased from Wako Pure chem. Ind. ^{3}H labelled D-glucose was obtained from the Radiochemical Center, Amersham.

Preparation of BBMV from Rat Small Intestine. BBMV were prepared from Wistar rats (230-250g) according to the method of Kesseler et al. with some modifications [16]. Segments of small intestine (50cm), except for the ileum, were used for the preparation of the vesicles. The activity of alkaline phosphatase [17], sucrase [18] used as marker enzymes of the BBMV and Na-K ATPase [19] as a marker enzyme of the basolateral membrane were determined. Alkaline phosphatase and sucrase were enriched during purification process. Na-K ATPase activity was not detected in the BBMV preparation. The protein concentration was determined by the method of Lowry et. al [20] using bovine serum albumin as a standard.

DM-CD Treatment of BBMV. The BBMV suspension was incubated with 2 mM DM-CD in pH 7.5 100 mM mannitol and 10mM Hepes-Tris buffer at 37°Cfor 10 min and then centrifuged at 27,000 g for 20 min. The pellet was resuspended in the same buffer and the protein content was determined. Control BBMV suspension was incubated in pH 7.5 100 mM mannitol and 10 mM Hepes-Tris buffer at 37°C for 10 min and then treated in the same manner as the DM-CD treatment.

Glucose Uptake Experiment. BBMV suspension (50 ml) in 100 mM mannitol and 10 mM Hepes-Tris buffer (pH 7.5) was added to a 50 ml of transport buffer (100 mM NaCl, 100 mM mannitol, and 10 mM Hepes-Tris, at pH 7.5) with 0.1-10 mM D-glucose tracer labelled with ^{3}H. The mixture was incubated at 25°C and the reaction was stopped with 2ml ice-cold 200mM NaCl and 10mM Hepes-Tris buffer (pH 7.5). We calculated the uptake from radioactivity trapped on the filter by dissolving the filter overnight in Creosol (Nacalai Tesque Ind.,) and determining the radioactivity with a liquid scintillation counter (LSC 3500, Aloka).

Measurement of Fluidity. The lipid fluidity of the BBMV was determined by the measurement of fluorescence polarization, using 2-(9-anthroyloxy)stearic acid (2-As; excitation, 390 nm; emission, 452 nm), 1,6-diphenyl-hexatriene (DPH; excitation, 380 nm; emission, 455 nm) and N-(1-anilinonaphtyl-4)maleimide (ANM; excitation, 355 nm; emission, 448 nm) as fluorescent probes. The 2-AS and DPH were dissolved in tetrahydrofuran, each to a concentration of 1 mM; ANM was dissolved in the same buffer solution. An aliquot of fluorescent probe solution was added to the BBMV suspensions, followed by incubation for 30 min at 37°C. The BBMV suspensions were separated from unlabelled fluorescent probes by washing with buffer and centrifuging. This procedure was repeated 3 time at 4°C. Fluorescence polarization was determined with fluorescence spectrophotometer (Hitachi, F3010) at 25°C. The fluorescence polarization (P) was calculated with the following equation:

$$P = \frac{Ivv - G * Ivh}{Ivv + G * Ivh} \qquad G = \frac{Ihv}{Ihh}$$

where Ivv, Ivh, Ihv and Ihh were the fluorescence intensities oriented in a parallel manner (Ivv) and in a perpendicular manner(Ihh).

Measurement of Cholesterol and Phospholipid in BBMV. Total phospholipid and cholesterol in the BBMV were extracted according to the method of Folch et al [21]. The lipids extracted with mixed solvent (chloroform-methanol/2:1) were washed with 0.1mM KCl, and concentrated by evaporating under N_2 stream. Cholesterol and total phospholipid in the BBMV were determined by the method of Zlatkis and Zak [22] and Bartlett [23], respectively.

Results

The D-glucose uptake of BBMV was determined in the presence of an initial 100 mM NaCl gradient (inside<outside). As shown in Figure 1, the values for initial rate and overshoot magnitude of D-glucose uptake in the vesicles was increased significantly by the treatment with DM-CD compared with values of control vesicles. The equilibrium values of D-glucose uptake by both control and treated membrane vesicles were similar, suggesting that the intravascular volume of BBMV was not changed by the DM-CD treatment. Further the initial uptake rates of D-glucose in the BBMV in the presence of an initial 100 mM KCl gradient showed no significant difference between BBMV treated with DM-CD and the controls.

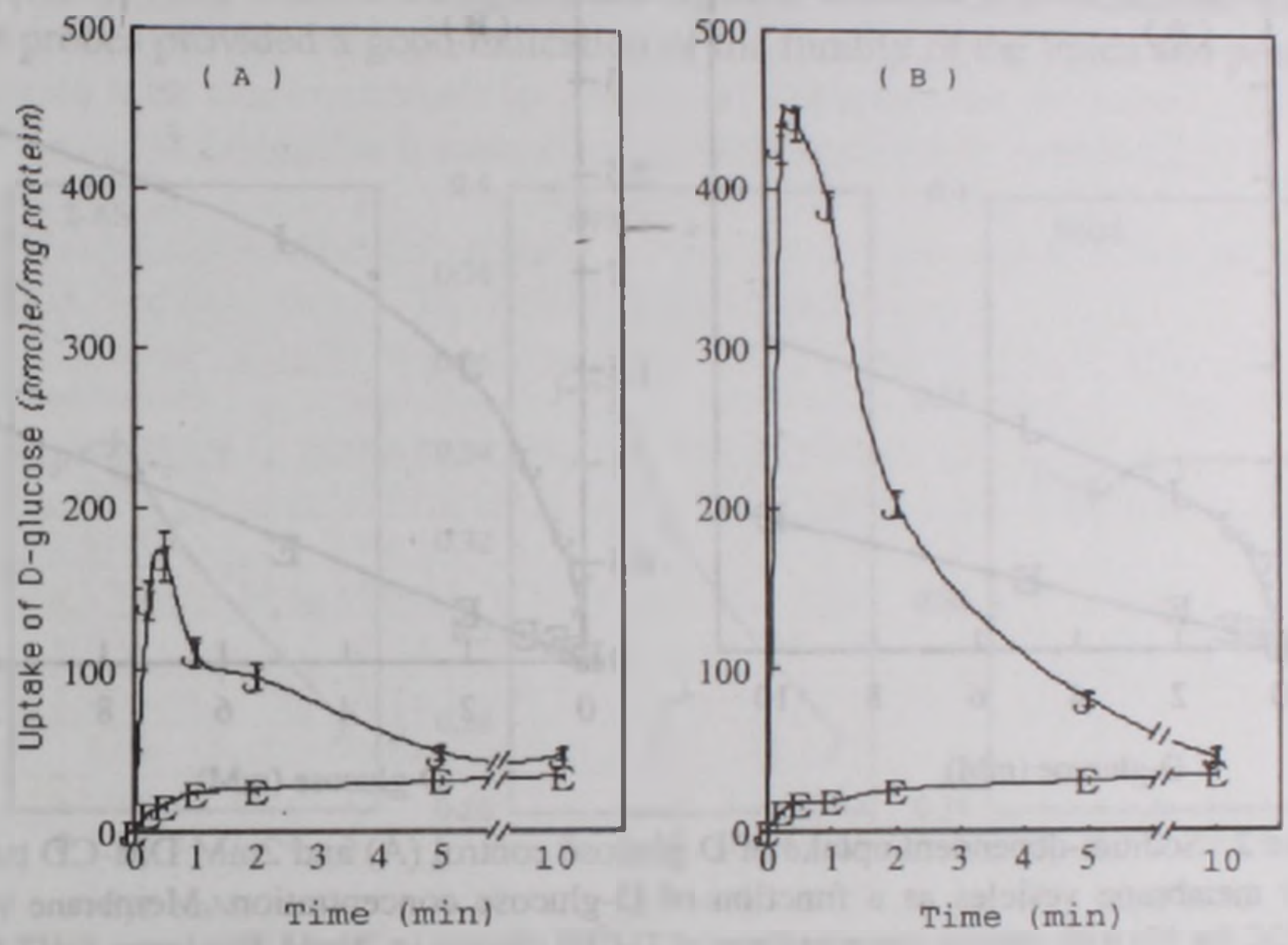

Figure 1. Time course of D-glucoseuptake by brush border menbrane vesicles; control (A) and 2mM DM-CD treatment (B). Control and 2mM DM-CD-treated membrane vesicles were incubated at 25C in 20mM Tris-hepes (pH7.4) containing 100mM mannitol in thepresence of either 100mM NaCl (J) or 100mM KCl (E). Each point represents the mean of five experiments performed in duplicate determinations.

Figure 2 shows the initial uptake of D-glucose as a function of the initial concentration in the presence of NaCl or KCl gradient. The relationship between the concentration and the rate of uptake was non-linear in the presence of NaCl gradient and linear in the presence of KCl gradient. The uptake of D-glucose in the presence of NaCl gradient was assumed to be described by the sum of a saturated term displaying Michaelis-Menten kinetics plus a second non-saturable term, since the D-glucose uptake rate (V) can be expressed by the following equation:

$$V = \frac{V_{max} * (D-glucose)}{K_m + (D-glucose)} + P * (D-glucose)$$

where Vmax is the maximum velocity of Na^+-dependent uptake, Km is the D-glucose concentration at which the Na^+-dependent uptake is half maximal, and P is the permeability coefficient. The non-linear least squares fitting of the experimental data gave the parameters listed in Table 1. In the control experiment, the apparent Km and Vmax were 0.63 (mM) and 0.87 (nmoles/mg protein/15s), whereas the Km of the 2mM DM-CD treated BBMV was not significantly different from that of the control. However, the Vmax of the DM-CD-treated BBMV was two-fold that of the control. The passive permeability of the DM-CD-treated BBMV was slightly, but not significantly increased compared with that of the control.

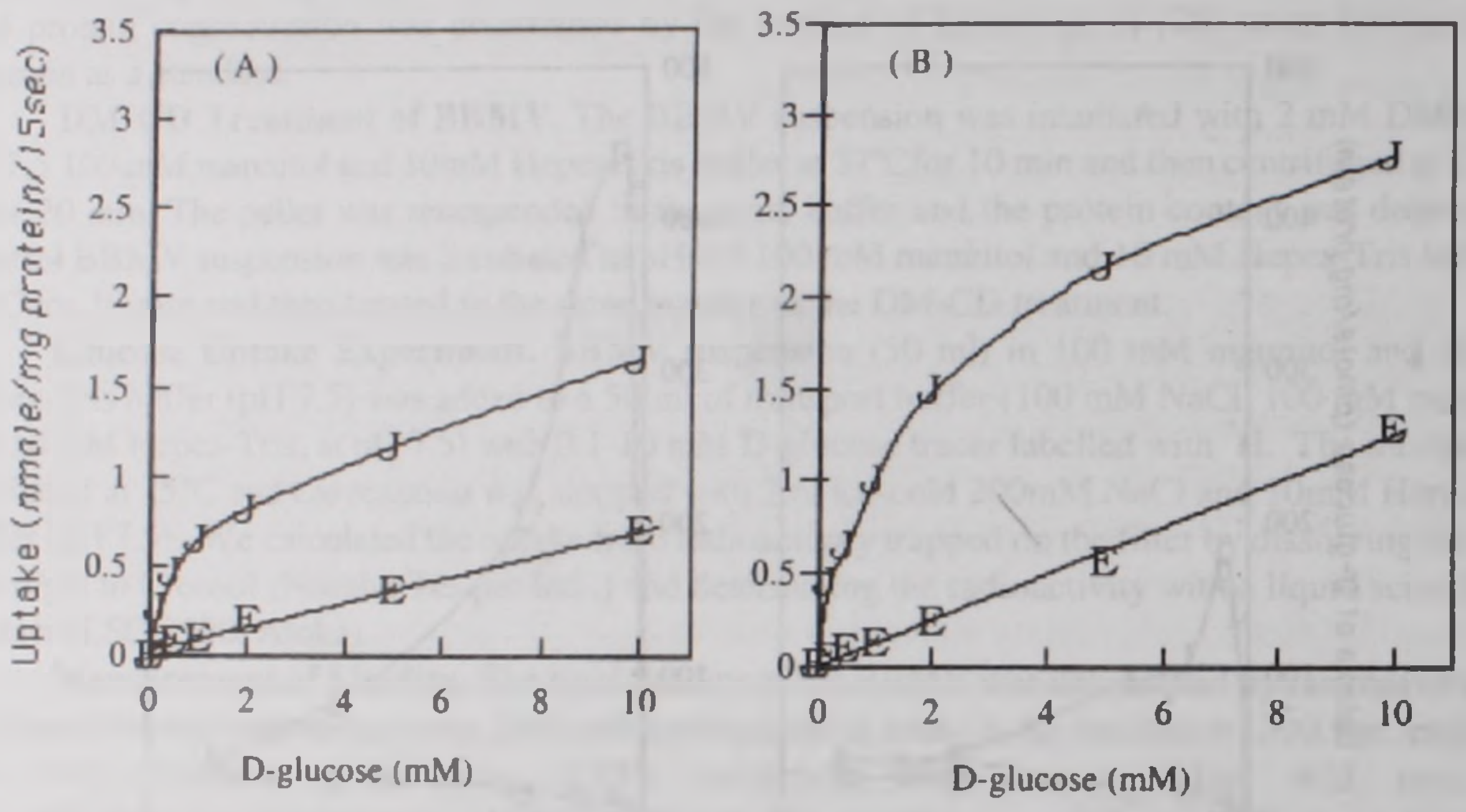

Figure 2. Sodium-dependent uptake of D-glucose control (A) and 2mM DM-CD treatment (B) brush border membrane vesicles as a function of D-glucose concentration. Membrane vesicles were incubated at 25C for 15s with various concentrations of D-[^{3}H] glucose in 20mM Tris-hepes (pH7.4) containing 100mM mannitol in the presence of either 100mM NaCl (J) or 100mM KCl (E). Each point represents the mean of five experiments performed in duplicate determinations.

Table 1. Kinetic Parameters for the Uptake of D-Glucose in Brush Border Membrane Vesicles.

	Michalis-Menten kinetic parameters		First order rate constant
	Km (mM)	Vmax (*nmole/mg protein/15s*)	(*μl / mg protein/15s*)
control	0.631 ± 0.071	0.872 ± 0.045	0.080 ± 0.005
DM-CD	0.431 ± 0.066	1.786 ± 0.110*	0.103 ± 0.013

Each point represents the mean ± S.E. of five experiments performed in threee determinations.
* Significantly different from control, $p < 0.001$

To obtain information on the mechanisms of the enhancing effect of DM-CD on the D-glucose uptake was measured membrane fluidity of BBMV fluorescence spectrophotometrically. The fluorescence polarization of 2-AS, DPH and ANM measured in control and DM-CD-treated BBMV is shown in Fig.3. 2-AS and DPH were used as probes for monitoring the degree of fluidity of the lipid bilayer. 2-AS probably localizes in the bilayer closer to the aqueous interface [24], and DPH has been found to localize in the hydrophobic interior of the BBMV [25]. ANM was used for monitoring the fluidity of the protein. The fluorescence polarization measurements of the BBMV were carried out at 25°C after pretreatment with DM-CD. 2 mM DM-CD enhanced the uptake of D-glucose in the BBMV, and also remarkably altered the fluorescence polarization of 2-AS and DPH. The fluorescence polarization of ANM, which binds covalently and preferentially to sulfhydryl groups of proteins [26], showed no significant changes with DM-CD treatment. Thus, use of fluorescent probes provided a good indication of the fluidity of the lipids and proteins in the BBMV.

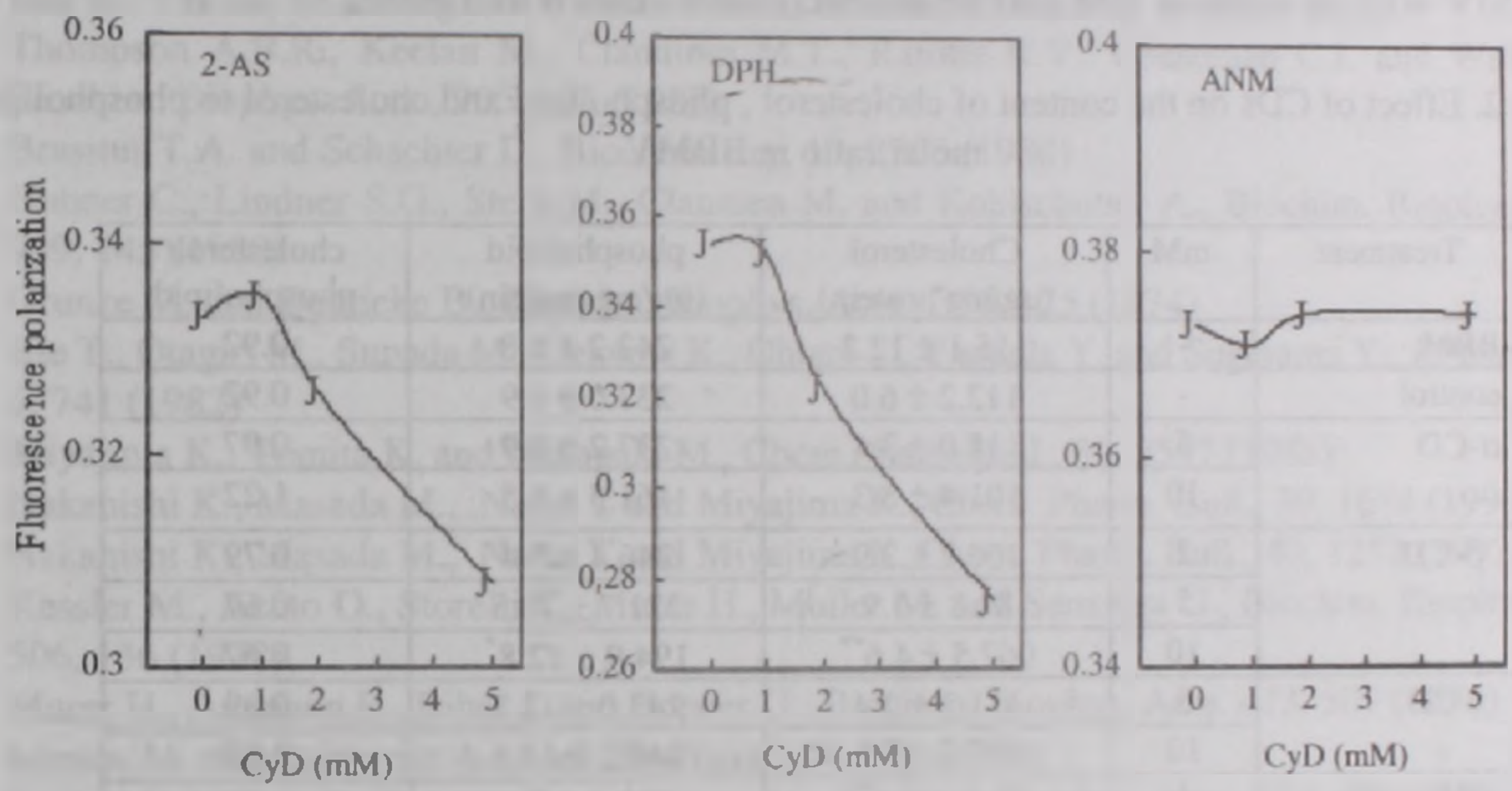

Figure 3. Effect of DM-CD on the fluoresecence polarization of 2-AS-, DPH-, and ANM-labeled bruch border membrane vehicles. Each point represents the mean of four experiments performed in duplicate determinations.

Discussion

Values for osmotically active space and the non-specific binding for D-glucose in the DM-CD-treated BBMV were similar to those values in control BBMV (Fig. 1). There was no significant difference in the passive transport properties of control and DM-CD-treated BBMV, in terms of the time-dependence of D-glucose influx in KCl solution. Thus, we regarded the integrity and membrane barrier function of DM-CD-treated BBMV as not so much altered.

The Vmax of the 2 mM DM-CD-treated BBMV differed significantly from that of control membranes ($p<0.001$); however the Km showed no difference in control and DM-CD-treated membranes. The increased absorption of D-glucose from the intestine in diabetes mellitus is well known. Tuji et al. reported an increased Vmax for glucose uptake in streptozotocin-induced diabetic rats, without any effect on Km value, suggesting that number

of glucose transporters was increased [27]. Tompson also reported similar phenomena in intact intestinal tissue and diabetic rats [28]. Thus, the increased Vmax of DM-CD-treated BBMV would appear to reflect changes in the number of D-glucose transporters. However, the enhanced D-glucose uptake in BBMV treated with DM-CD in this study can not be explained by an increase in the number of carriers, as we show below.

In this study, fluorescence polarization measurements with 2-AS and DPH as probes indicated a marked increase in the membrane fluidity of the aqueous interface and the hydrophobic interior of the BBMV. However, fluorescence polarization of ANM-labelled BBMV did not induce by DM-CD treatment, indicating that DM-CD may not interact with protein of the BBMV.

An increase in membrane cholesterol content reduced membrane fluidity [30], whereas conversely, a decrease in cholesterol content increased membrane fluidity [31]. The activity of some enzymes in the membrane was affected by alterations in the cholesterol content of the membrane [1]. Therefore, we measured the content of cholesterol and total phospholipid in the BBMV with or without DM-CD treatment, that is shown in Table 2.

Table 2. Effect of CDs on the content of cholesterol , phospholipid and cholesterol to phospholipid molar ratio in BBMV

Treatment	mM	Cholesterol (μg/mg protein)	phospholipid (μg/mg protein)	cholesterol/ phospholipid
BBM	-	115.1 ± 12.2	242.2 ± 6.8	0.92
control	-	112.2 ± 6.0	237.0 ± 6.9	0.92
α-CD	5	118.0 ± 3.4	237.2 ± 6.9	0.97
	10	101.4 ± 8.3	161.5 ± 5.5	1.22
β-CD	2	100.2 ± 2.9	246.4 ± 5.4	0.79
	5	81.8 ± 3.9	221.7 ± 10.5	0.67
	10	067.5 ± 4.6**	194.9 ± 12.8*	0.67
γ-CD	5	111.2 ± 2.4	243.0 ± 12.3	0.89
	10	107.2 ± 5.6	240.2 ± 13.6	0.87
DM-β-CD	1	86.8 ± 3.3**	240.6 ± 5.1	0.70
	2	74.9±3.7**	206.9 ± 21.6	0.70
	5	26.9 ±2.5**	34.6±10.4**	0.40

Each point represents the mean ± S.E. of five experiments performed in threee determinations. Significant difference from control, * $p < 0.05$, ** $p < 0.01$

DM-CD caused the liberation of cholesterol depending on the concentration, whereas did not cause phospholipid from BBMV (2mM DM-CD). The lowering of the cholesterol content of the BBMV by DM-CD induced the increase of membrane lipid fluidity, leading to an increased membrane permeability. We have reported that β-CD interacted with the cholesterol of intestinal membrane, resulting in withdrawal the lipid molecules by forming inclusion complexes [15]. We found here that D-glucose uptake in the BBMV was remarkably increased by the short-term treatment with DM-CD. DM-CD, as well as β -CD, has also been shown to interact with lipid components of the BBMV and to induce the membrane fluidity; this action may influence the transport properties of BBMV by altering membrane cholesterol content. The enhanced mobility of membrane lipids may result in perturbation of boundary lipids near protein molecules in the membrane. Thus, the enhanced D-glucose uptake by the BBMV treated with DM-CD may be due to alterations of membrane

components of the BBMV, resulting in increased membrane fluidity. The complexity of the situation in the treated BBMV requires additional studies before a definitive conclusion can be reached. Further investigations are currently underway to clarify the mechanism underlying the enhanced D-glucose uptake of DM-CD-treated BBMV.

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