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The enhancing effect of soybean-derived sterylglucoside and β-sitosterol β-D-glucoside on nasal absorption in rabbits

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Abstract

The aim of this study was to elucidate the efficiency of soybean-derived sterylglucoside (SG) and its main component β-sitosterol β-D-glucoside (Sit-G), as nasal absorption enhancers. Nasal administration of verapamil with SG and Sit-G showed the higher bioavailabilities (60.4 and 90.7%, respectively) than that with lactose (39.8%). It was clear that SG and Sit-G promoted the absorption of verapamil through nasal mucosa. To elucidate the mechanism, we measured the calcein leakage from liposomes by incubation with SG, Sit-G, oleic acid, soybean-derived sterol, and β-sitosterol to investigate transcellular absorption and measured the changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) by Sit-G to analyze paracellular absorption. The large amount of calcein leakage induced by enhancers was consistent with an enhancement of bioavailability of verapamil and insulin following nasal administration (oleic acid < SG < Sit-G). Moreover, Sit-G increased $[Ca^{2+}]i$ in the medium containing Ca^{2+}, but not in Ca^{2+} free medium. This result suggested that Sit-G increases the fluidity of the mucosal membrane and facilitates Ca^{2+} influx from extracellular sources. In conclusion, a possible explanation for SG and Sit-G to promote drug absorption, is that they may affect both paracellular pathway and transcellular pathways caused by pertubation of lipid.

Keywords: Nasal administration; β-Sitosterol β-glucoside (Sit-G); Soybean derived glucoside (SG); Intracellular calcium; Enhancer; Verapamil

1. Introduction

The nasal mucosa is a potential delivery site for the systemic administration of peptide and protein drugs because of rich vascularization, avoidance of the hepatic first pass effect, and ease of administration. The permeation routes for nasal admin-

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istration of drugs include transcellular and paracellular pathways. Most nasally administered drugs have small molecular weights and are lipophilic and partition rapidly from the luminal fluid into the cell (transcellular pathway). But peptide and protein drugs with high molecular weights do not partition to a large extent into the cell membranes, and consequently are excluded from transcellular transport (Fix, 1987; Madara and Trier, 1987). These drugs are absorbed through paracellular pathway (Gumbnier, 1987) which occupies only a very small surface compared to the transcellular route (Madara, 1989).

For these reasons, the bioavailability of peptide and protein drugs following intranasal administration is very low. Nevertheless, the bioavailability of these drugs can be improved by co-administration of absorption enhancers, such as bile salt (Wüthrich et al., 1994), cyclodextrin (Abe et al., 1995) and saponin (Pillion et al., 1995). Many of these compounds, however, are harmful to nasal epithelium membranes and interfere with nasal mucociliary movement (Merkus et al., 1991; Marttin et al., 1995). Therefore, the search for safe and effective absorption enhancers is a major research in both academia and industry.

Recently, we reported that soybean-derived sterylglucoside (SG) and its sterol (SS) which consist of 49.9% β-sitosterol β-D-glucoside (Sit-G) and β-sitosterol (Sit) as main components, respectively, promoted insulin absorption following nasal administration of insulin suspension (Maitani et al., 1995) and powder dosage forms in rabbits (Yamamoto et al., 1998). Yamamoto et al. (1998) reported SG and SS caused no damage to nasal mucosa in successive intranasal administration for 5 days. However, this effect may exert only insulin absorption through nasal mucosa by making the special interaction with insulin and SG. Additionally, the enhancement mechanism of these compounds is not clear.

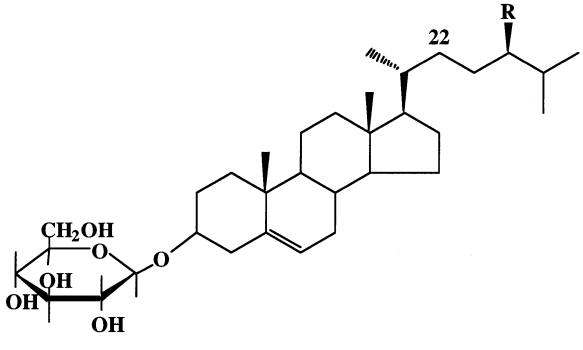

Fig. 1. Chemical structure and components of soybean-derived sterylglucoside (SG) β-Sitosterol β-D-glucoside (Sit-G); R = C_2H_5 (49.9%), campesterol β-D-glucoside; R = CH_3 (29.1%), stigmasterol β-D-glucoside; R = C_2H_5 and Δ^{22} (13.8%), brassicasterol β-D-glucoside; R = CH_3 and Δ^{22} (7.2%).

In this paper, we examined the enhancing activity of SG and Sit-G on nasal absorption of verapamil as model drug employing powder dosage forms in vivo in rabbits. The bioavailability of verapamil following nasal administration was about 40% (Arnold et al., 1985) and this fluorescent drug can be measured to low concentration. Therefore, we considered that verapamil was suitable as model drug to investigate the enhancement action of SG and Sit-G. Moreover, in this paper, the mechanism of enhancement action of SG and Sit-G on the paracellular and transcellular pathway was studied by the measurement of calcein leakage from liposomes with entrapped calcein to estimate paracellular enhancement and intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) in nasal mucosa excised from rabbits to estimate transcellular enhancement.

2. Materials and methods

2.1. Material

SG (M.W. 573.32) and SS (M.W. 409.17) were kindly supplied by Ryukakusan (Tokyo, Japan). SG is a mixture of the glucosides of Sit (49.9%) (M.W. 414.69), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) as shown in Fig. 1. SS was obtained by hydrolysis of the glucose bonds of SG components, i.e. SS is the aglycon of SG. Sit was purchased from Tama Biochemical (Tokyo, Japan). Sit-G (M.W. 578.84) was purchased from Essential Sterolin Products (Netherlands), and the purity of Sit-G was identified and determined to be more than 96% by ^{13}C-NMR and thin layer chromatography (data

not shown). Verapamil hydrochloride, cremophor EL, dimethyl sulphoxide (DMSO), and Dulbecco's phosphate-buffered saline (D-PBS) were purchased from Sigma Chemical (St Louis, MO). Oleic acid (M.W. 282.5) and calcein were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Fura PE3-AM was purchased from Wako Pure Chemical Industries (Osaka, Japan). Egg phosphatidylcholine and cholesterol were purchased from NOF (Tokyo, Japan). All other chemicals were obtained from commercial sources and were of analytical reagent grade.

2.2. Preparation of the verapamil powder dosage forms

Verapamil hydrochloride (75 mg) was dissolved in 50 ml purified water, and then each of 225 mg lactose, SG or Sit-G was added in this solution. Verapamil hydrochloride suspension was freeze-dried and the resulting powder was passed through a 200-mesh sieve. The physical form of verapamil hydrochloride in all powder formulations measured by X-ray diffraction apparatus (RINT 1400, Rigaku, Tokyo, Japan) was amorphous (data not shown).

2.3. Animal experiments and determination of verapamil

The prepared powder dosage forms were administered to male Japanese white rabbits weighing 2.5–3.0 kg purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). A 10 cm polyethylene tube with a diameter of 1.05 mm (Natsume Seisakusho, Tokyo, Japan) was fitted to a syringe and inserted into the nose of a rabbit (Yamamoto et al., 1998). About 10 mg of verapamil powder dosage forms was loaded into a syringe and administered to the left side of the nasal cavity at a dose of 0.75 mg/kg. For intravenous administration, verapamil hydrochloride solution (7.5 mg/ml per kg) in saline was injected into the ear vein of rabbits at a dose of 0.75 mg/kg. At each point, 0.3 ml blood sample was collected from the ear vein with a heparinized syringe. Plasma was separated by centrifugation at 13 000 rpm for 3 min. To 100 μl samples, 1 ml acetonitorile containing 140 ng/ml butyl *p*-aminobenzoate was added as an internal standard. Samples were mixed with a vortex mixer and centrifuged at 13 000 rpm for 5 min. The organic phase was taken and transferred to a disposable tube. This solution was evaporated at 50°C under a gentle stream of nitrogen and the residues were dissolved in 200 μl of HPLC mobile phase.

A volume of 50 μl of this solution was injected into an HPLC equipped with a fluorescent detector. The instrumentation consisted of a Shimadzu LC 10AL liquid chromatography, SIL 10A autoinjector, CR-6A chromatopac, SCL 10A system controller and RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). The detector setting was 275 nm for excitation and 315 nm for emission (Christrup et al., 1990). A 3.9×300 mm column packed with Nova Pak C18 (Waters, Milford, USA) was employed. The mobile phase was acetonitrile: 0.13 M KH_2PO_4 solution (40:60) pumped at a rate of 1.0 ml/min (Christrup et al., 1990).

2.4. Analysis of pharmacokinetic data

The total area under verapamil concentration-curve (AUC) from time 0 to infinity was estimated from the sum of successive trapezoids between each data point, plus an estimation of the tail area from the last concentration-time point to infinity. The mean residence time (MRT) values were determined using MULTI (Yamaoka et al., 1981). Bioavailability was defined on the basis of AUC according to the following equation:

$$\text{Bioavailability } (\%) = AUC_{nasal}/AUC_{i.v.} \times 100 \quad (1)$$

where AUC_{nasal} and $AUC_{i.v.}$ represent AUC after nasal administration and intravenous administration of 0.75 mg/kg verapamil, respectively.

2.5. Preparation of liposomes

Liposomes with entrapped calcein were prepared with 70 μmol egg phosphatidylcholine and 30 μmol cholesterol according to a reverse-phase evaporation method (Szoka and Papahadjopou-

los, 1978; Qi et al., 1995). Briefly, the lipid mixture was dissolved in organic solvent and then mixed with 4 ml 1/10 diluted phosphate-buffered saline (pH 7.31; 1/10 PBS) containing 20 mM calcein. The mixture was sonicated and organic solvents were evaporated to form a liposome suspension. Liposomes were successively extruded through 0.4 and 0.2 μm pore polycarbonate membranes (Nuclepore, USA) at about 60°C. Free calcein was separated by gel filtration of liposome suspension through a Sephadex G-50 column with 1/10 PBS.

2.6. Calcein leakage from liposomes by enhancer

Each enhancer (SG, Sit-G, SS, Sit, oleic acid) was dissolved in methanol-1/10 PBS at various concentrations (0.05, 0.2, 0.5 mM). Then a liposome-1/10 PBS suspension was added to this solution. This solution was incubated at 37°C for 60 min. For control, liposomes were incubated without enhancer in methanol-1/10 PBS. The calcein leakage was assessed by fluorescent spectrometer (Hitachi F-4010, Tokyo, Japan) at 490 nm for excitation and 520 nm for emission (Qi et al., 1995). At each time point, 100 μl of sample was collected and added to 2 ml 1/10 PBS. Calcein leakage was determined from the following equation:

$$\text{Calcein leakage (\%)} = \{(Ft - F_0)/(2F_\infty - Ft)\} \times 100 \quad (2)$$

where F_0 and Ft are the fluorescence intensities of the calcein before and at time t after incubation, respectively, and F_∞ represents the fluorescence intensity when 100 μl 10% Triton X-100 was added to 100 μl sample to completely collapse liposomes with entrapped calcein.

2.7. Measurement of $[Ca^{2+}]i$

Fura PE3-AM was loaded into nasal mucosa excised from nasal turbinate of rabbit by the method of Suenaga and Kamata (1998). Briefly, fura PE3-AM was dissolved in DMSO and mixed with cremophor EL. Nasal mucosa was exposed to 1 μM fura PE3AM in the presence of 0.04% cremophor EL in the dark for 5 h at room temperature. The tissue was rinsed with D-PBS with or without 1.4 mM Ca^{2+} and placed in a bath containing 8 ml D-PBS with or without Ca^{2+} at 37°C. The tissue was held vertically and equilibrated for 30 min. Then, DMSO was added to a final concentration of about 1% (v/v). After 10 min, 2 ml of 1% DMSO dissolved Sit-G (200 μM) was added to the bath followed by 50 μl $CaCl_2$ solution (200 mM) in saline, and finally 4 ml ionomycin solution (26 μM) was added at each time to check fura PE3 adsorption, but inside the cells. $[Ca^{2+}]i$ was measured as the change in fluorescence intensity of fura PE3 bound to calcium with a dual-wavelength fluorometric calcium analyzer, CAF 110 (Japan Spectroscopic, Tokyo, Japan) at 340 nm and 380 nm for excitation and at 500 nm for emission (Suenaga and Kamata, 1998).

2.8. Statistical analysis

Each value was expressed as the mean ± S.D. For group comparisons, the one-way layout analysis of variance (ANOVA) with duplication was applied. Significant differences in the mean values were evaluated by Student's unpaired t-test. A P value of less than 0.05 was considered to be significant.

3. Results

3.1. Effects of SG and Sit-G on absorption of verapamil

SS and Sit could not be used for verapamil powder dosage form in intranasal administration since they could not be freeze-dried.

Fig. 2 shows the plasma concentration profile of verapamil after nasal administration of verapamil powder dosage forms with lactose, SG, and Sit-G at a dose of 0.75 mg/kg to rabbits. The maximum concentration (Cmax) of verapamil was reached at about 7 min after intranasal administration, and the powder dosage with Sit-G indicated the highest plasma concentration of verapamil in those with lactose and SG.

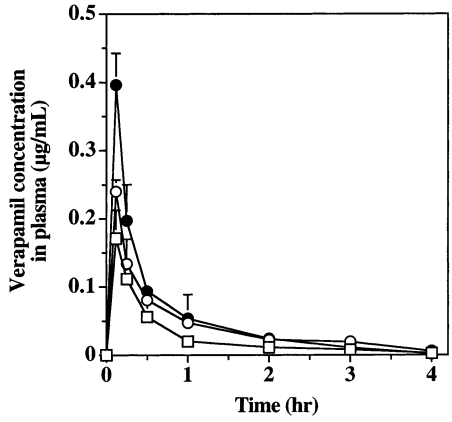

Fig. 2. Plasma concentration of verapamil following nasal administration of verapamil powder with lactose (□), SG (○), or Sit-G (●) in rabbits at a dose of 0.75 mg/kg. Each value represents the mean ± S.D. ($n = 5$).

Table 1 shows pharmacokinetic parameters after nasal administration of verapamil powder dosage forms and intravenous administration of verapamil hydrochloride solution in saline at a dose of 0.75 mg/kg to rabbits. All SG and Sit-G parameters were significantly higher than that of lactose ($P < 0.05$) except for MRT. Especially, the bioavailability increased to 60.4 and 90.7% by using SG and Sit-G, respectively, whereas it was 39.8% by lactose. The MRT of nasal administration was nearly identical to the i.v. value ($P > 0.05$).

3.2. Calcein leakage from liposomes by enhancers

To determine the effect of enhancers on transcellular absorption, we measured the calcein leakage from liposomes in incubation with enhancers.

Fig. 3 shows calcein leakage from liposomes with enhancers by incubation at 37°C for 30 and 60 min. There is about 9.1 and 15.7% of calcein leakage without enhancer (control) by incubation at 30 and 60 min, respectively. Calcein leakage was increased with increasing concentrations of enhancers and incubation times except at 0.05 mM Sit for 60 min. The calcein leakage by SG and Sit-G significantly increased in the above their solubility, as shown as a hatched line in Fig. 3. On the other hand, absorption by SS and Sit was saturated at above 0.2 mM.

Fig. 4 shows calcein leakage from liposomes by oleic acid (A) and various enhancers (B). Calcein leakage from liposomes increased in a concentration-dependent manner up to 1 mM of oleic acid as with SS and Sit at above 0.2 mM. SG and Sit-G induced greater calcein leakage than the other enhancers (Fig. 4(B)). Table 2 summarizes calcein leakage by incubation with enhancers for 60 min.

Table 1
Pharmacokinetic parameters of verapamil following intravenous and nasal administration of verapamil powder dosage form with lactose, SG, or Sit-G at a dose of 0.75 mg/kg verapamil in rabbits[a]

	Cmax (μg/ml)	MRT[b] (h)	AUC (μg/h per ml)	Bionvailability[c] (%)
Intravenous				
Solution	–	1.02 ± 0.12	290.1 ± 91.2	100
Nasal administration				
Lactose	171.3 ± 40.2]**]***	89 ± 0.16	115.1 ± 19.5]**]***	39.8 ± 6.7]**]***
SG	240.4 ± 20.1]***	1.00 ± 0.10	175.0 ± 20.2]**	60.4 ± 7.0]*
Sit-G	396.3 ± 50.2	0.95 ± 0.27	262.7 ± 43.2	90.7 ± 19.6

[a] Each value represents the mean ± S.D. ($n = 5$).
[b] MRT is the mean resistance time, that is the average time of a molecules residues in body.
[c] Bioavailability = $(AUC_{nasal}/AUC_{i.v.}) \times 100$.
* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$.

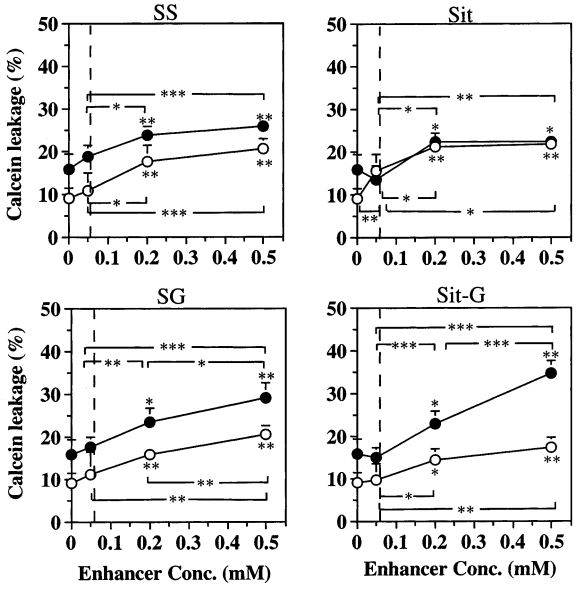

Fig. 3. Effect of SG, Sit-G, SS, and Sit on calcein leakage from liposomes with entrapped calcein in methanol-l/10 PBS (1:1) after incubation at 37°C for 30 (○) or 60 min (●). Total lipid concentration was 1.238 μmol/ml. Hatched line indicates the solubility of each enhancer in methanol-1/10 PBS (1: 1). Each value represents the mean ± S.D. ($n = 6$). Unless otherwise indicated $*P < 0.01$, $**P < 0.001$ versus control.

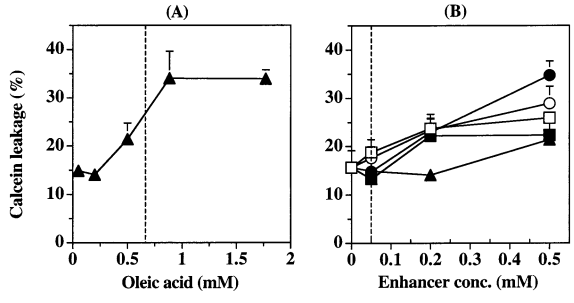

Fig. 4. Effect of various enhancers on calcein leakage from liposomes with entrapped calcein in incubation in methanol-1/10 PBS (1:1) at 37°C for 60 min. (A) Oleic acid, (B) ▲, oleic acid; □, SS; ■, Sit; ○, SG; ●, Sit-G. Hatched line indicates the solubility of each enhancer in a mixture solution of methanol-1/10PBS (1:1). Each value represents the mean ± S.D. ($n = 3$–6). The total lipid concentration was 1.238 μmol/ml.

3.3. Change of $[Ca^{2+}]i$ by Sit-G

The efficiency of Sit-G as an enhancer for verapamil and insulin (Ando et al., 1998) was higher than that of SG. We considered that the drug absorption was facilitated through nasal mucosa by SG as the same mechanism as by Sit-G. Therefore, the effects on the paracellular pathway by Sit-G was investigated by measuring the changes in $[Ca^{2+}]i$.

Fig. 5 shows changes in $[Ca^{2+}]i$ of excised nasal mucosa in Ca^{2+} free D-PBS (A) and D-PBS containing 1.4 mM Ca^{2+} (B) after the addition of Sit-G. As shown in Fig. 5 (A), the addition of $CaCl_2$ rapidly increased $[Ca^{2+}]i$ after treatment with Sit-G, although the addition of Sit-G induced no change $[Ca^{2+}]i$ in Ca^{2+} free D-PBS. $[Ca^{2+}]i$ was increased by the addition of ionomycin and secondary $CaCl_2$, respectively, to reach an equilibrium with intra and extracellular Ca^{2+} (Fig. 5 (A)). On the other hand, Sit-G increased $[Ca^{2+}]i$ in D-PBS containing Ca^{2+} (Fig. 5 (B)). The addition of DMSO did not affect $[Ca^{2+}]i$ levels in D-PBS containing Ca^{2+} (Fig. 5 (B)), though the addition of DMSO increased $[Ca^{2+}]i$ in Ca^{2+} free D-PBS (Fig. 5 (A)). After treatment with ionomycin, the addition of $CaCl_2$ led to an increase in $[Ca^{2+}]i$ (Fig. 5(A)).

4. Discussion

SG and SS have very low solubility in water (about 53 μg/ml) and they can be used as a suspension and as a powder formulation for enhancer and excipient (Ando et al., 1998; Yamamoto et al., 1998). We have reported that SG and Sit-G facilitated insulin absorption through nasal mucosa from insulin powder (Yamamoto et al., 1998) and suspension dosage form (Ando et al., 1998). In the rabbits, insulin powder dosage formulation with 1% SG resulted in 26.3% bioavailability of insulin and such powder formulations are superior in providing improved enhancement of insulin transport across the nasal mucosa as compared to their suspension (Ando et al., 1998; Yamamoto et al., 1998).

In this study, the efficiency of SG and Sit-G as enhancers was estimated by intranasal administration of verapamil powder dosage forms. Cmax and bioavailability were significantly increased by

SG compared with lactose (Fig. 2 and Table 1) ($P < 0.01$). The bioavailability of verapamil with Sit-G increased about 1.5-fold compared to that of SG (Table 1). These results show that SG and Sit-G promote both insulin and verapamil absorption through nasal mucosa, indicating they function without special interaction via an insulin exclusive absorption pathway. Furthermore, the efficiency of Sit-G as an enhancer was significantly higher than SG ($P < 0.05$), which agrees with our previous results for insulin (Ando et al., 1998).

The use of bioadhesive polymer such as chitosan prolonged MRT, which facilitated the drug absorption through mucosa (Sugimoto et al., 1998). In our data, MRT values following nasal administration with SG and Sit-G were almost same as that following i.v., whereas bioavailability and Cmax were significantly increased by SG and Sit-G (Table 1). These results suggest that SG and Sit-G induce significantly faster absorption of verapamil from a powder dosage form as i.v. injection.

Liposomes with entrapped calcein were used as a model for nasal mucosa to examine the effect of enhancers on transcellular absorption, especially to estimate the influence of enhancer to lipid. Calcein leakage from liposomes in the presence of the enhancers is an indirect method of looking at transcellular permeation. However, the culture of nasal epithelium is difficult since it consists of three kinds of cells. Therefore, we cannot directly observe the transcellular permeation of drug using cell culture. Liposomes are often used for biomembrane model though they do not have receptor (Custodio et al., 1993). As shown in Fig.

Table 2
The effect of various enhancers on calcein leakage from liposomes with entrapped calcein after incubation at 37°C for 60 min[a]

Enhancer concentration (mM)	Calcein leakage (%)		
	0.05[b]	0.2	0.5[c]
SG	17.6 ± 2.3	23.3 ± 3.3	28.9 ± 3.5
Sit-G	14.8 ± 2.4	22.9 ± 2.8	34.7 ± 2.9
SS	18.8 ± 2.7	23.7 ± 2.2	25.9 ± 1.7
Sit	13.3 ± 3.4	22.2 ± 0.2	22.3 ± 0.9
Oleic acid	14.9 ± 1.8	14.1 ± 0.8	21.4 ± 3.4

[a] Each value represents the mean ± S.D. ($n = 3$–6).
[b] SG, Sit-G, SS, and Sit were solubilized in methanol-l/10 PBS (1:1).
[c] Oleic acid was solubilized in methanol-1/10 PBS (1:1). Lipid concentration in methanol and PBS (1:1) was 1.238 μmol/ml.
* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$.

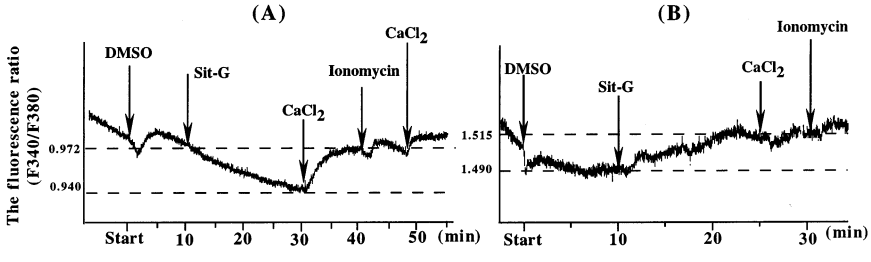

Fig. 5. The effect of 50 μM Sit-G on intracellular calcium concentrations in excised nasal mucosa from rabbits in Ca^{2+} free D-PBS (A), and in D-PBS containing Ca^{2+} (B). The arrows indicate the time of addition of l% DMSO (2 ml), 200 μM Sit-G (50 μl), 200 mM $CaCl_2$ (50 μl), and 26 μM ionomycin (4 ml). Ordinate axis represents the change of fluorescent intensity ratio of fura PE3 and this change corresponds to that of the intracellular calcium levels.

Table 3
Comparison between bioavailability and calcein leakage from liposomes with entrapped calcein by SG, Sit-G, and oleic acid[a]

	Calcein leakage[b] (%)	Bioavailablity (%)	
		Insulin[c]	Verapamil[d]
SG	28.9 ± 3.5]*	6.7 ± 1.8]*	60.4 ± 6.7]*
Sit-G	34.7 ± 2.9]***]*	11.3 ± 1.6]**	90.7 ± 19.6]
Oleic acid	21.4 ± 3.4]	3.2 ± 1.4]	–[e]

[a] Each value represents the mean ± S.D. ($n = 3$–6).
[b] The enhancer concentration was 0.5 mM and the incubation was at 37°C for 60 min.
[c] Pharmacological bioavailability of intranasally administered insulin-PBS solution (10 IU/kg) containing 1.0% SG and Sit-G, and 10% oleic acid from Ando et al. (1998).
[d] Bioavailability following intranasal administration of verapamil powder containing SG or Sit-G (1:3, weight) at a dose of 0.75 mg/kg.
[e] No data.
* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$.

3 and Table 2, calcein leakage induced by enhancers increased significantly compared with control (methanol-1/10 PBS without enhancer) and was SG: 28.9%, Sit-G: 34.7%, SS: 25.9%, Sit: 22.3%, and oleic acid: 21.4% in 0.5 mM enhancer. The value by Sit-G was significantly higher than those by other enhancers ($P < 0.05$). These values may reflect the order of efficiency of enhancer (oleic acid < Sit < SS < SG < Sit-G). SS and Sit appeared to stabilize the liposomes above about 16 mol% (0.2 mM) within 30 min. We have reported that insulin permeation was promoted through an artificial membrane modified with lipid by SG, and suggested that SG affected lipids in mucosal membrane (Ando et al., 1998). Also, we have reported that the destabilizing effect of SS or SG on liposomes was greater in the following order: SS < cholesterol < SG, because SS may be closely packed in phospholipid layer, but the glucose group of SG may disturb the optical packing state (Muramatsu et al., 1994; Qi et al., 1995). The differences in enhancer efficacy for Sit-G and SG may be due to the character of the sterol side-chain and its role in determining sterol-sterol and sterol-phospholipid interactions as in analogues of cholesterol having different side-chain structure (Schuler et al., 1990; Slotte et al., 1994).

Oleic acid has been used extensively as an enhancer in drug delivery from rectal (Suzuki et al., 1998) and buccal (Tsutsumi et al., 1998) mucosa, and has been shown to alter membrane permeability by increasing the motional freedom or fluidity of the membrane phospholipids (Wang et al., 1994). Oleic acid may be incorporated into egg-phosphatidylcholine up to lipid to oleic acid (1: 1 molar ratio) (Fig. 4 (A)). Wang et al. (1994) reported that oleic acid at high concentration (0.1 mM) is capable of nonspecific disruption of the alveolar membrane. However, our data suggested that oleic acid might cause little or no disruption but does increase the fluidity of the liposomal membrane, since it resulted in below 40% calcein leakage.

Table 3 shows calcein leakage from liposomes, and pharmacological bioavailability following nasal administration of insulin suspension (Yamamoto, 1992; Ando et al., 1998) and verapamil powder with enhancers. Insulin suspension, i.e., insulin-PBS solution containing 1% SG and Sit-G (18 mM), and 10% oleic acid (354 mM) was administered to the nasal cavity at a dose of 10 IU/kg in rabbits (Yamamoto 1992; Ando et al., 1998). Both pharmacological bioavailability of insulin and calcein leakage in 0.5 mM enhancer were increased in the order of oleic aid < SG < Sit-G. These data suggest that the enhancers dis-

turb the transcellular pathway of the lipid layer in nasal mucosa.

Paracellular absorption of drugs is restricted by tight junctions. Moreover, it was reported that the opening of tight junction was related to increased $[Ca^{2+}]i$ (Tomita et al., 1996). To estimate the effect of SG and Sit-G on paracellular absorption, especially tight junctions, enhancer-induced changes in $[Ca^{2+}]i$ were compared to disodium ethylenediamine-tetraacetate (EDTA), sodium caprate and oleic acid induced absorption (Wang et al., 1994; Tomita et al., 1996).

Tomita et al. (1996) suggested that EDTA and sodium caprate open tight junctions since the former stimulated protein kinase C by chelation of extracellular Ca^{2+}, and the latter stimulated the contraction of the actomyosin rings by increasing of $[Ca^{2+}]i$ in Caco-2 cells. The solubility of SG and Sit-G is very low in water so that the extraction of extracellular Ca^{2+} could not be determined in this study.

As shown in Fig. 5 (A), $[Ca^{2+}]i$ was not changed by the addition of Sit-G, but after treatment with Sit-G, addition of $CaCl_2$ caused increases in $[Ca^{2+}]i$. On the other hand, the addition of Sit-G resulted in increases in $[Ca^{2+}]i$ in D-PBS containing Ca^{2+}. These results suggest that the mechanism by which Sit-G increases $[Ca^{2+}]i$ is different from the mechanism used by sodium caprate, which increases $[Ca^{2+}]i$ in medium irrespective of Ca^{2+} (Tomita et al., 1996). Wang et al. (1994) reported that below 0.05 mM oleic acid causes increases in $[Ca^{2+}]i$ in alveolar cells without measurable membrane damage, although it failed to activate the elevation of $[Ca^{2+}]i$ in Ca^{2+} free medium. They suggested that Ca^{2+} flux from extracellular sources by oleic acid induced membrane alteration is responsible for increases in $[Ca^{2+}]i$ (Wang et al., 1994).

Our results on changes in $[Ca^{2+}]i$ and in calcein leakage suggest that the mechanisms for increasing $[Ca^{2+}]i$ may be similar for Sit-G and oleic acid. Sit-G may increase the motional freedom of lipids in the mucosal membrane, and facilitate Ca^{2+} influx from extracellular sources, causing increases in $[Ca^{2+}]i$ and the opening of tight junctions. Another explanation is that Sit-G may act as an ionophore and make holes as reverse micelles (Sakai et al., 1998). Presently, we are measuring transepithelial electrical resistance of the nasal mucosa to reveal whether SitG causes opening of tight junctions.

5. Conclusions

Nasal administration of verapamil powder with SG or Sit-G resulted in high bioavailability. SG and Sit-G facilitated verapamil and insulin absorption, and Sit-G was a more effective enhancer than SG. We also investigated SG and Sit-G enhancement of transcellular absorption using liposomes, and paracellular absorption by measuring changes in $[Ca^{2+}]i$ induced by Sit-G. The high levels of calcein leakage induced by enhancers are consistent with the high bioavailability of verapamil and insulin following nasal administration (oleic acid $<$ SG $<$ Sit-G). Moreover, Sit-G increased $[Ca^{2+}]i$ in Ca^{2+} containing, but not in Ca^{2+} free medium. This suggests that Sit-G may increase the fluidity of mucosal membrane and also may facilitate Ca^{2+} influx from extracellular sources. Another explanation is that Sit-G may act as ionophores and make holes as reverse micelles. Thus SG may enhance both paracellular and transcellular absorption of drugs.

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References

Abe, K., Irie, T., Uekama, K., 1995. Enhanced nasal delivery of luteinizing hormone releasing agonist buserelin by oleic acid solubilized and stabilized in hydroxypropyl-β-cyclodextrin. Chem. Pharm. Bull. 43, 2232–2237.

Ando, T., Maitani, Y., Yamamoto, T., Takayama, K., Nagai, T., 1998. Nasal insulin delivery in rabbits using soybean-

derived sterylglucoside and sterol mixtures as novel enhancers in suspension dosage forms. Bio. Pharm. Bull. 21, 862–865.

Arnold, T.H., Tackett, R.L., Vallner, J.J., 1985. Pharmacokinetics of acute intranasal administration of verapamil: comparison with i.v. and oral administration. Biopharm. Drug Dispos. 6, 447–454.

Christrup, L.L., Bonde, J., Rasmussen, S.N., Rassing, M.R., 1990. Relative bioavailability of (±)-verapamil hydrochloride administered in tablets and chewing gum. Acta Pharm. Nord. 2, 371–376.

Custodio, J.B., Almeida, L.M., Madeira, V.M., 1993. The anticancer drug tomoxifen induces changes in the physical properties of model and native membranes. Biochim. Biophys. Acta 1150, 123–129.

Fix, J.A., 1987. Absorption enhancing systems for the GI system. J. Control. Rel. 6, 151–156.

Gumbnier, B., 1987. Structure, biochemistry, and assembly of epithelial tight junctions. Am. J. Physiol. 253, 749–758.

Madara, J.L., Trier, J.S., 1987. Functional morphology of the mucosa of the small intestine. In: Johnson, L.R. (Ed.), Physiology of the Gastrointestinal Tract, 2nd ed. Raven Press, New York, pp. 1251–1266.

Madara, J.L., 1989. Loosening tight junctions. Lessons from the intestine. J. Clin. Invest. 83, 1089–1094.

Maitani, Y., Yamamoto, T., Takayama, K., Nagai, T., 1995. The effect of soybeanderived sterol and its glucoside as an enhancer of nasal administration of insulin in rabbits in vitro and in vivo. Int. J. Pharm. 117, 129–137.

Marttin, E., Verhoef, J.C., Romeijn, S.G., Merkus, F.W.H.M., 1995. Effects of absorption enhancers on rat nasal epithelium in vivo: release of marker compounds in the nasal cavity. Pharm. Res. 12, 1151–1157.

Merkus, F.W.H.M., Verhoef, J.C., Romeijn, S.G., Schipper, N.G.M., 1991. Absorption enhancing effect of cyclodextrins on intranasally administered insulin in rats. Pharm. Res. 8, 588–592.

Muramatsu, K., Maitani, Y., Machida, Y., Nagai, T., 1994. Effect of soybean-derived sterol and its glucoside mixtures on the stability of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylcholine/cholesterol liposomes. Int. J. Pharm. 107, 1–8.

Pillion, D.J., Recchia, J., Wang, P., Marciani, D.J., Kensil, C.R., 1995. DS-I, modified Quillaja Saponin, enhances ocular and nasal absorption of insulin. J. Pharm. Sci. 84, 1276–1279.

Qi, X.R., Maitani, Y., Takayama, K., Nagai, T., 1995. Effect of soybean-derived sterols on the in vitro stability and the blood circulation of liposomes in mice. Int. J. Pharm. 114, 33–41.

Sakai, M., Imai, T., Ohtake, H., Azuma, H., Otagiri, M., 1998. Effect of absorption enhancers on cytoskeletal actin filaments in Caco-2 cell monolayers. Life Sci. 63, 44–54.

Schuler, I., Duportail, G., Glasser, N., Benveniste, P., Hartmann, M.A., 1990. Soybean phosphatidylcholine vesicles containing plant sterol: a fluorescence anisotropy study. Biochim. Biophys. Acta 1028, 82–88.

Slotte, J.P., Jungner, M., Vicheze, C., Bittman, R., 1994. Effect of sterol side-chain structure on sterol-phosphatidylcholine interaction in monolayers and small unilamellar vesicles. Biochim. Biophys. Acta 1190, 435–443.

Sugimoto, A., Yoshida, M., Yata, T., Higaki, K., Kimura, T., 1998. Evaluation of poly(vinyl alchol)-gel spheres containing chitosan as dosage form to control gastrointestinal transit time of drugs. Biol. Pharm. Bull. 21, 1202–1206.

Suenaga, H., Kamata, K., 1998. Lysophosphatidylcholine potentiates contractile responses by enhancing vasoconstrictor-induced increase in cytosolic free Ca^{2+} in rat aorta. Eur. J. Pharmacol. 361, 217–226.

Suzuki, A., Morishita, M., Kajita, M., et al., 1998. Enhanced colonic and rectal absorption of insulin using multiple emulsion containing eicosapentaenoic acid and docosahexaenoic acid. J. Pharm. Sci. 87, 1196–1202.

Szoka, F., Papahadjopoulos, D., 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA 75, 4194–4198.

Tomita, M., Hayashi, M., Awazu, S., 1996. Absorption-enhancing mechanism of EDTA, caprate, and decananoylcarnitine in caco-2 cells. J. Pharm. Sci. 85, 608–611.

Tsutsumi, K., Obata, Y., Takayama, K., Loftsson, T., Nagai, T., 1998. Effect of the codliver oil extract on the buccal permeation of ionized and nonionized forms of ergotamine using the keratinized epithelial-free membrane of hamster cheek pouch mucosa. Int. J. Pharm. 174, 151–156.

Yamamoto, T. 1992. Absorption of insulin through nasal mucosa from ophthalmic oily suspensions and the absorption promoting effect of sterols in rabbits. Master Thesis, Hoshi University.

Yamamoto, T., Maitani, Y., Takayama, K., Nagai, T., 1998. High absorbency and subchronic morphologic effects on the nasal epithelium of nasal insulin powder dosage form with a soybean-derived sterylglucoside mixture. Bio. Pharm. Bull. 21, 866–870.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (MULTI) for microcomputers. J. Pharma. Dyn. 4, 879–885.

Wang, L.Y., Ma, K.H. J., Pan, W.F., et al., 1994. Alveolar permeability enhancement by oleic acid and related fatty acid: evidence for calcium-dependent mechanism. Pharm. Res. 11, 513–517.

Wüthrich, P., Martenet, M., Buri, P., 1994. Effect of formulation additives upon the intranasal bioavailability of peptide drug: tetracosactide ($ACTH_{1-24}$). Pharm. Res. 111, 278–282.