Contribution of Hydrophobicity of Nonionic Detergents to Membrane Lipid Fluidity and Disopyramide Uptake by Rat Intestinal Brush-Border Membrane Vesicles

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The contribution of hydrophobicity of different types of detergents to disopyramide uptake by rat small intestinal brush-border membrane vesicles was studied in relation to their membrane lipid fluidity and the physicochemical parameters of the detergents, *i.e.*, hydrophile-lipophile balance (HLB). Span-, Tween-type detergents or glycerol esters at non-solubilizing concentrations (0.01–0.05% (w/v)) decreased the extent of maximum uptake of the drug in the presence of outward H^+ -gradient, but not in the absence of the gradient. The fluorescence anisotropy of the vesicles using diphenylhexatriene (DPH), as reflected by its incorporation into the membrane inner lipid layer, decreased with the addition of all detergents used. In contrast, that of the vesicles using trimethylammoniumphenyl phenylhexatriene (TMA-DPH), which reflected its incorporation into the membrane outer lipid layer, increased depending on the concentration of Tween-type detergents except for Tween 81 and Tween 85, glycerol esters (MO-500, MO-750, ML-500 and ML-750); it decreased with the addition of Span-type detergents, Tween 81 and glycerol ester (MO-310). Therefore, the membrane lipid fluidity change of the outer leaflet, rather than the inner lipophilic domain, of the membrane vesicles caused by the detergents was found to be dependent on the hydrophobicity, but not on the type of detergent. This seems to correlate with the inhibitory effects on the facilitated uptake of the drug by the membrane vesicles.

Key words hydrophobicity; membrane lipid fluidity; nonionic detergent; brush-border membrane; disopyramide; HLB number

The brush-border membrane vesicles from the intestine and kidney are widely used as useful model membrane systems in transport studies of drugs and eluates.¹⁾ Recent studies have indicated that membrane lipid fluidity may be involved in absorption processes of D-glucose,^{2–5)} L-leucine,^{6,7)} calcium,⁸⁾ vinblastine,⁹⁾ daunomycin⁹⁾ and sulfanilic acid.¹⁰⁾ These findings suggest that membrane lipid fluidity plays an important role in membrane transport mechanisms. For example, Dudeja *et al.* reported that Na^+ dependent D-glucose transport in rat intestinal brush-border membranes is enhanced by treatment with a membrane mobility agent, 2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl)octanate.²⁾ Wagner *et al.* reported that treatment of rat renal brush-border membranes with oleic acid results in a suppression of D-glucose transport and an increase of the membrane lipid fluidity.⁴⁾ From these findings, it is suggested that differences in the contribution of the membrane lipid fluidity to membrane transport systems may be due to the difference in chemical characteristics of the compounds used. No detailed or critical examination of the relationship between membrane lipid fluidity and drug uptake by nonionic detergents has been performed, however. Therefore, it is of interest to examine whether or not changes in the membrane lipid fluidity induced by membrane mobility agents are directly related to the transport mechanism of drugs.

In the present study, disopyramide, an H^+ -counter transport system reported by Takahashi *et al.*¹¹⁾ as a model drug and 17 kinds of nonionic detergents were used. Influence of these detergents on the efficiency of disopyramide uptake by brush-border membrane vesicles and their membrane lipid fluidity was examined.

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MATERIALS AND METHODS

Materials Disopyramide was obtained from Fukuju Seiyaku Co. (Toyama, Japan), and *p*-chlorodisopyramide from Roussel Uclaf (Romainville, France). 1,6-diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide (TMA-DPH) and pyrene were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Nonionic detergents used are shown in Table I. BCA protein assay kit[®] was obtained from Pierce Chemical Co. (Illinois, U.S.A.). All other chemicals used were reagent grade and obtained from commercial sources.

Preparation of Rat Small Intestinal Brush-Border Membrane Vesicles Male Wistar rats weighing 220–250 g were fasted for 18 h with free access to water, and killed by cutting the cervical artery under pentobarbital (Nembutal[®]) anesthesia. The membrane vesicles were prepared by the Mg^{2+} precipitation method.¹²⁾ The final brush-border membrane vesicles were suspended at a concentration of 20 mg protein/ml in 5 mM Tris-HCl buffer, pH 7.4 (buffer A) containing 50 mM D-mannitol at 4 °C, unless otherwise specified.

The quality of the preparation was determined by assay of the marker enzyme activities, *i.e.*, alkaline phosphatase and sucrase. The activities of alkaline phosphatase and sucrase were enriched about 15 and 10 fold compared to those of the original mucosa, respectively. The membrane vesicles were used for uptake studies within 8 h of preparation.

Enzyme and Protein Assay The activities of alkaline phosphatase and sucrase were assayed by the methods reported by Murer *et al.*¹³⁾ and Takesue and Sato,¹⁴⁾ respectively. Protein concentration was assayed with

Table 1. HLB Number and cmc Value for Nonionic Detergents

Detergent	Commercial name	HLB	cmc (mg/l)
Sorbitan monolaurate	Span 20	8.6	12.6
Sorbitan sesquioleate	Span 30	3.7	11.9
Sorbitan monooleate	Span 80	4.3	11.3
Sorbitan trioleate	Span 85	1.8	10.9
Polyoxyethylene (20) sorbitan monolaurate	Tween 20	16.7	27.0
Polyoxyethylene (20) sorbitan monopalmitate	Tween 40	15.6	17.0
Polyoxyethylene (20) sorbitan monostearate	Tween 60	14.9	15.9
Polyoxyethylene (20) sorbitan monooleate	Tween 80	15.0	15.6
Polyoxyethylene (20) sorbitan trioleate	Tween 85	11.0	11.5
Polyoxyethylene (6) sorbitan monolaurate	Tween 21	13.3	15.8
Polyoxyethylene (6) sorbitan monooleate	Tween 81	10.0	13.6
Tetraglycerol monooleate	MO-310	10.2	19.0
Hexaglycerol monooleate	MO-500	12.2	19.9
Decaglycerol monooleate	MO-750	14.5	21.6
Tetraglycerol monolaurate	ML-310	11.9	19.7
Hexaglycerol monolaurate	ML-500	13.8	23.1
Decaglycerol monolaurate	ML-750	15.7	26.6

HLB number of each detergent was calculated from Griffin's equation and cmc value of each one was determined by measurement of pyrene fluorescence intensity at 25 °C using a Hitachi spectrofluorometer 850.

a BCA assay kit[®] using bovine serum albumin as a standard.

Measurement of Turbidity of the Membrane Suspension The membrane vesicles (0.7 mg protein/ml) were incubated with various concentrations (0–0.5% (w/v)) of nonionic detergents in buffer A for 10 min at 25 °C. The turbidity of the membrane suspension was measured by monitoring the absorbance at 450 nm.¹⁵⁾

Uptake Experiments The experiment of disopyramide uptake was carried out in the presence and absence of outward H⁺-gradient condition by the method described by Iseki *et al.*¹⁶⁾ Measurement of nonspecific binding of the drug by the vesicles was then made as a function of osmolarity of the extravesicular medium.¹¹⁾ The membrane vesicles prepared were preincubated with 20 mM MES (2-(*N*-morpholino)ethanesulfonic acid)–Tris buffer, pH 5.5 (buffer B) or 20 mM HEPES–Tris buffer, pH 7.5, (buffer C) containing 100 mM D-mannitol and 100 mM KCl for 10 min at 25 °C. The membrane vesicles (0.4 mg protein/ml as a final concentration) were incubated with 60 μM disopyramide and one of the nonionic detergents (0–0.05% (w/v)) in buffer C for 30–600 s at 25 °C. After the incubation, the reaction mixture was withdrawn at different intervals, the sample was trapped in a millipore filter (HAWP, 0.45 μm pore size, 2.5 cm diameter, Japan Millipore, Ltd., Tokyo, Japan). The extent of disopyramide incorporated into the membrane vesicles was measured by the modified method described by Ito *et al.*¹⁷⁾ as follows; the millipore filter which trapped the membrane vesicles was washed with 3 ml of ice-cold buffer C, and dried at room temperature. The dried filter was then placed in a solution containing 0.2 ml of 0.05 N NaOH, 0.5 ml of distilled water, 0.2 ml of 2 μg/ml *p*-chlorodisopyramide (an internal standard) and 6 ml of diethylether. After the mixture was shaken for 30 min at room temperature, the diethylether phase was separated and evaporated. The residue was dissolved in 0.04 ml of a mixture containing methanol and 50 mM KH₂PO₄ (1:1, v/v). The amount of disopyramide in the mixture was measured using a HPLC (LC-9A, Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a UV detector C-R6A at a wavelength of 214 nm. Separation was carried out on a reversed phase column (Cosmosil 5C8, 4.6 mm i.d. × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) using a mobile phase [methanol–50 mM KH₂PO₄ (1:1, v/v)] at a flow rate of 0.6 ml·min⁻¹.

Preparation of DPH- or TMA-DPH-Labeled Membrane Vesicles The membrane vesicles (1 mg protein/ml) were incubated with 1 μM DPH or 1 μM TMA-DPH (dissolved in tetrahydrofuran) for 10 min at 37 °C. The mixture was then centrifuged at 70000 × *g* for 30 min, and the pellets obtained were resuspended in buffer A. The final concentration of tetrahydrofuran in the reaction mixture was less than 0.3% (v/v).

Measurement of DPH or TMA-DPH Shifted from Membrane Vesicles to Detergent Micelles One milliliter aliquots of the DPH- or TMA-DPH-labeled membrane vesicles were dissolved in 0.1 N NaOH (Total-A, 0.2 mg protein/ml as a final concentration). The residue membrane vesicles were incubated in the presence and absence of the detergents for 5 min at 25 °C. The mixture (0.6 mg protein/ml) was immediately filtered using a millipore filter (HAWP, 0.45 μm pore size, 2.5 cm diameter) pretreated with 0.3% (w/v) polyethyleneimine to avoid nonspecific binding to the filter. One milliliter aliquots of the filtered solution were also mixed with 2 ml of 0.1 N NaOH. The fluorescence intensity of the final solution with the detergent (*I_b*) was compared to that of the final solution without the detergents (*I_c*), and the ratio for the fluorescence intensity of total-A, *i.e.*, (*I_b*–*I_c*)/*I_{Total-A}*, was regarded as the shifted fraction of the fluorescence probes.

Fluorescence Measurements Fluorescence measurements were carried out using a Hitachi spectrofluorometer 850 equipped with a rhodamine B quantum counter (Hitachi Seisakusho Co., Tokyo) at 25 °C. The excitation and emission wavelengths used for DPH and TMA-DPH fluorescence measurement were 363 and 428 nm, 365 and 428 nm, respectively. The fluorescence anisotropy (*γ*) of DPH embedded in the membrane lipids was calculated using the following equation, $\gamma = (I_V - I_H) / (I_V + 2I_H)$, where *I_V* and *I_H* represent the fluorescence intensities of the vertically and horizontally polarized excitation, respectively.¹⁸⁾

Measurement of cmc of Detergents Determination of critical micelle concentration (cmc) value of detergents used was carried out by measurement of pyrene fluorescence intensity at 25 °C using a Hitachi spectrofluorometer 850.^{19,20)}

Statistical Analysis Statistical analysis was conducted using a multiple comparison procedure.²¹⁾ Correlation coefficients between variables were calculated by the method reported by Pearson and Lee.²²⁾

RESULTS

Effects of Detergents on Disopyramide Uptake Table 2 shows the effect of the detergents on disopyramide uptake by the membrane vesicles under the condition of an outward H^+ -gradient ($pH_{in}=5.5$; $pH_{out}=7.5$) and in the absence of the H^+ -gradient condition. An overshoot phenomenon (maximum uptake) of the drug uptake was observed after 30 s of incubation of the membrane vesicles with the drug in the absence of the detergents as reported by Takahashi *et al.*,¹¹⁾ and the drug uptake reached a constant level after 1 min of incubation of the membrane vesicles regardless of the absence or presence of the H^+ -gradient (Fig. 1). From these results, extents of the drug uptake with the detergents were assayed at 30 s of incubation of the vesicles.

As shown in Table 2, Span-, Tween-type detergents and glycerol esters significantly suppressed the drug uptake by the vesicles in the presence of the H^+ -gradient, while

Fig. 1. Time Course Profiles of Disopyramide Uptake by Rat Intestinal Brush-Border Membranes

The procedures and conditions of disopyramide uptake are described under "Materials and Methods." Values are expressed as the means $\pm$ S.D. of 3 independent determinations. Symbols: $\circ$, in the presence of H^+ -gradient condition ($pH_{in}=5.5$, $pH_{out}=7.5$); $\triangle$, in the absence of H^+ -gradient condition ($pH_{in}=7.5$, $pH_{out}=7.5$).

Table 2. Extent of Disopyramide Uptake by the Membrane Vesicles at 25 $^{\circ}C$

Agent	Disopyramide uptake (nmol/mg protein/30s)	
	$pH_{in}=5.5$, $pH_{out}=7.5$	$pH_{in}=7.5$, $pH_{out}=7.5$
No addition	0.665 ± 0.089	0.344 ± 0.068
Span 20	$0.385 \pm 0.046^{**}$	0.349 ± 0.058
Span 30	$0.451 \pm 0.042^{**}$	0.359 ± 0.044
Span 80	$0.445 \pm 0.059^{**}$	0.320 ± 0.051
Span 85	$0.542 \pm 0.074^{**}$	0.316 ± 0.066
Tween 20	$0.342 \pm 0.053^{**}$	0.292 ± 0.029
Tween 40	$0.330 \pm 0.103^{**}$	0.284 ± 0.031
Tween 60	$0.401 \pm 0.066^{**}$	0.271 ± 0.087
Tween 80	$0.380 \pm 0.046^{**}$	0.274 ± 0.062
Tween 85	$0.390 \pm 0.080^{**}$	0.296 ± 0.061
Tween 21	$0.367 \pm 0.088^{**}$	0.254 ± 0.048
Tween 81	$0.425 \pm 0.051^{**}$	0.368 ± 0.055
MO-310	$0.352 \pm 0.051^{**}$	0.321 ± 0.056
MO-500	$0.287 \pm 0.042^{**}$	0.314 ± 0.049
MO-750	$0.264 \pm 0.048^{**}$	0.304 ± 0.064
ML-310	$0.259 \pm 0.062^{**}$	0.286 ± 0.057
ML-500	$0.239 \pm 0.040^{**}$	0.252 ± 0.043
ML-750	$0.228 \pm 0.055^{**}$	0.240 ± 0.059

The concentration of detergent is 0.05% (w/v). Values are expressed as the means $\pm$ S.D. of 5 determinations. Statistical significance between the systems with and without detergents is indicated by $** p < 0.01$.

they had no effect on the extent of the drug uptaken by the vesicles in the absence of the gradient.

Effects of Fluorescence Probes Shifted to Detergent Micelles on Fluorescence Anisotropy of Membrane Vesicles The shifted fraction ratio of DPH and TMA-DPH was stable in the range of 0.176 to 0.086 and 0.128 to 0.066, respectively, under the condition of 0.05% (w/v) of the detergents used. Therefore, it seemed that the fluorescence anisotropy of DPH- or TMA-DPH-labeled membrane vesicles was little affected by the fluorescence probe shifted into the micelle of the detergents.

Contribution of Membrane Lipid Fluidity to Disopyramide Uptake Figure 2 shows the effects of the detergents on the fluorescence anisotropy of DPH-labeled membrane vesicles. The addition of Span-, Tween-type detergents or glycerol esters to the membrane vesicles resulted in a decrease in the fluorescence anisotropy in a dose-dependent manner. In addition, the extent of the decreasing anisotropy of DPH-labeled membrane vesicles differed greatly among Tween-type detergents.

The fluorescence anisotropy of TMA-DPH embedded membrane vesicles was different from that of DPH-labeled membrane vesicles (Fig. 3), *i.e.*, the addition of Span-type detergents, Tween 81 and MO-310 to the vesicles resulted in a dose-dependent decrease in the anisotropy. Tween 85 and ML-310 had no effect on the fluorescence anisotropy of TMA-DPH embedded in the vesicles, while this anisotropy increased with the addition

Fig. 2. Effects of Nonionic Detergents on Fluorescence Anisotropy of DPH-Labeled Membranes

The procedures and conditions of the fluorescence measurements are described under "Materials and Methods." Values are expressed as the means of 3 independent determinations.

Fig. 3. Effects of Nonionic Detergents on Fluorescence Anisotropy of TMA-DPH-Labeled Membranes

The procedures and conditions of the fluorescence measurements are described under "Materials and Methods." Values are expressed as the means of 3 independent determinations.

of other detergents (Tween 20, Tween 40, Tween 60, Tween 80, Tween 21, MO-500, MO-750, ML-500 or ML-750). The correlation between the fluorescence anisotropy of DPH or TMA-DPH labeled membranes and the drug uptake by the vesicles was then estimated (Fig. 4), and it was learned that the extent of the drug uptake by the vesicles was affected by change in the membrane lipid fluidity. Especially, there was a good correlation between changes in membrane outer lipid layer of the vesicles as assessed by TMA-DPH and the extent of drug uptake.

Effects of cmc Values and (Hydrophile-Lipophile Balance) (HLB) Number of Detergents on Disopyramide Uptake and Membrane Lipid Fluidity Next, we examined the effects of physicochemical parameters of the detergents, *i.e.*, cmc values and HLB number, on the membrane lipid fluidity and the drug uptake (Table 1). Concentrations of the detergents tested (0.01–0.05% (w/v)) were all above the cmc value of each detergent, therefore, reaction solutions containing the detergents formed micelles under the experimental conditions. There was no significant correlation between the cmc values of these detergents and the fluorescence anisotropy of DPH- or TMA-DPH-labeled membranes or the extent of the drug uptake by the vesicles (data not shown).

The relationship between the HLB number of the detergents and the extent of drug uptake by the membrane vesicles is shown in Fig. 5. Drug uptake in the presence

Fig. 4. Relationship between Fluorescence Anisotropy of DPH (A) or TMA-DPH (B) Labeled Membrane Vesicles and Disopyramide Uptake by the Membrane Vesicles

Disopyramide uptake were carried out in the presence of outward H^+ -gradient condition. Symbols: ○, Span-type detergents; △, Tween-type detergents; □, glycerol esters.

Fig. 5. Relationship between HLB Number of Nonionic Detergents and Disopyramide Uptake by Membrane Vesicles in the Presence (A) and Absence (B) of Outward H^+ -Gradient Condition

The conditions of disopyramide uptake are as described in the legend to Table 2. Symbols: ○, Span-type detergents; △, Tween-type detergents; □, glycerol esters.

Fig. 6. Relationship between HLB Number of Nonionic Detergents and Fluorescence Anisotropy of DPH-Labeled Membranes (A) or TMA-DPH-Labeled Membranes (B) by Membrane Vesicles

The conditions of fluorescence measurements are as described in the legends to Figs. 2 and 3, respectively. Symbols: ○, Span-type detergents; △, Tween-type detergents; □, glycerol esters.

of the H^+ -gradient was decreased with increase in the HLB number of all detergents used, while this uptake by the vesicles was suppressed when detergents were added compared to when they were absent (Table 2 and Fig. 5A). In contrast, the extent of drug uptake in the absence of the H^+ -gradient was not affected by the HLB number of the detergents (Fig. 5B).

The relationship between detergent HLB number and

the fluorescence anisotropy of DPH- or TMA-DPH embedded membranes is shown in Figs. 6A and B. HLB number had no impact on the fluorescence anisotropy of DPH-labeled membranes at the range of $1 < \text{HLB} < 10$, but the anisotropy increased with increase in this number at $10 < \text{HLB} < 20$. There was, however, a strong correlation between HLB number and the fluorescence anisotropy of TMA-DPH-labeled membranes ($r = 0.926$) (Fig. 6B).

DISCUSSION

We found in this study that the maximum uptake of disopyramide by membrane vesicles was significantly decreased by the addition of all detergents used (Table 2). This suppression effect was observed when the H^+ -gradient was present. This phenomenon appears to be different from that where an increase in membrane lipid fluidity by mixed micelles containing unsaturated fatty acid enhances membrane permeability of a drug.²³⁾ There are several explanations for the suppression of the drug uptake by the detergents; (a) solubilization of the membrane vesicles associated with the detergent parameters, such as detergent cmc value and HLB number, and (b) interaction between the detergent and the membrane lipid or the membrane protein. Similar phenomena were reported for the relation of membrane lipid fluidity and uptake of drugs or eluates in the presence of a pharmaceutical adjuvant such as fatty acid by Fernandez *et al.*,²⁴⁾ Carriere and LeGrimellec²⁵⁾ and Wagner *et al.*⁴⁾ In these communications, they reported that a detergent-induced increase in the membrane lipid fluidity causes an inhibition of D-glucose uptake in rat intestinal brush-border membranes²⁴⁾ and rat kidney brush-border membranes.²⁵⁾ Wagner *et al.* also recently reported that the addition of oleic acid to rat renal brush-border membranes causes an increase in the membrane lipid fluidity and an inhibition of D-glucose uptake.⁴⁾

We examined the involvement of solubilization of the membrane vesicles by the detergents for the former hypothesis by measuring the detergent cmc values, because it was reported that solubilization and perturbation of the membrane lipid layer by nonionic detergents are closely related to these values and detergent HLB number.^{26,27)} As shown in Table 1, all detergents used formed the micelles under the present experimental conditions. Furthermore, the turbidity of the membrane vesicles did not change in the concentration range of the detergents in the uptake experiments, *i.e.*, up to 0.05% (w/v) (data not shown). These results suggest that modulation of the drug uptake by the detergents is not directly related to their micelle formation. Suppression of the drug uptake under the H^+ -gradient condition by the detergents was dependent on detergent HLB number (Fig. 5). These results suggest that the efficiency of the drug uptake by the membrane vesicles is dependent on the hydrophobicity of the detergent, because HLB number of a detergent is an empirical measure of its relative hydrophobicity.²⁸⁾

To examine this latter hypothesis, we investigated the effects of detergents on the fluidity in inner and outer

lipid layers of the membrane vesicles using DPH and TMA-DPH, respectively. The fluorescence anisotropy of DPH-labeled membranes decreased in parallel with detergent concentration (Fig. 2). This suggests that there is a correlation between an increase in fluidity of the membrane inner lipid and suppression of the drug uptake by vesicles. The extent of fluorescence anisotropy of TMA-DPH-labeled membranes, however, differed with the type of detergent (Fig. 3). Especially, changes in anisotropy by both the fluorescence probes differed depending on the increase in concentration of Tween-type detergents and glycerol esters. Thus, suppression of the drug uptake by the vesicles seems not to be correlated with the changes in the membrane outer lipid fluidity. As shown in Fig. 4, however, there was a strong correlation between changes in this fluidity and drug uptake by the vesicles. It appears that the interaction between the detergents and the membrane outer lipid may be evaluated by physicochemical characteristics of the detergents, *i.e.*, their hydrophobicity such as HLB number, because modulation of the membrane lipid fluidity using micelles containing fatty acid results from interaction of the polar head group of phospholipids.²³⁾ Since the drug uptake by the vesicles was not suppressed in the absence of H^+ -gradient condition, it seems that the detergents compensate the outward H^+ -gradient condition. The interaction between the detergents and the uptake mechanism of a drug was undetermined in this study, however.

Taken together, these findings suggest that the modulation of disopyramide uptake by detergents in the H^+ -gradient condition was affected by changes in the membrane lipid fluidity of the outer lipid layer rather than by that in the membrane inner lipid of the vesicles, and the changes in lipid fluidity were closely related to the hydrophobicity indicated by the HLB number of detergents. Therefore, we conclude that alteration in the lipid outer layer by the detergents at non-solubilizing concentration is an important mechanism in the modulation of drug uptake.

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